MATERNAL IMMUNE CHANGES DURING BOVINE PREGNANCY: A FOCUS ON THE ENDOMETRIAL MACROPHAGE

By

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LIST OF ABBREVIATIONS

C1QA	Complement component
C1R	Complement C1r subcomponent
C1S	Complement C1s subcomponent
CALCB	Calcitonin-related polypeptide beta
CCL	Chemokine (C-C motif) legend
CCL17	C-C motif chemokine 17
CCL18	Chemokine (C-C motif) legend 18
CCL22	C-C motif chemokine 22
CCL22	Chemokine (C-C motif) ligand 22
CCL24	Chemokine (C-C motif) ligand 22
CCL3	C-C motif chemokine 3
CCL8	Chemokine (C-C motif) ligand 8
CCL8	Chemokine (C-C motif) ligand 8
CCR	C-C chemokine receptor
CCR3	Chemokine (c-c motif) receptor 3
CD	Cluster of differentiation
CD163	CD163 molecule
CD21	Complement component (3d/Epstein Barr virus) receptor 2,
CD335	Natural cytotoxicity triggering receptor 1
CD63	CD63 antigen
CD80	CD80 antigen
CD86	CD86 molecule
CD94/KLRD1	Killer cell lectin-like receptor subfamily D, member 1
CdC42	Cell division control protein 42
CDH1	E-cadherin
CFB	Complement factor B
CXCR4	Chemokine (CXC motif) receptor 4
CIDEB	Cell death-inducing DFF45-like effector-B
CLDN1	Claudin-1
CLDN1	Claudin 1
CLDN3	Claudin 3
CLDN8	Claudin-8
CLU	Clusterin
CRLF2	Cytokine receptor like factor 2
CRLF2	Cytokine receptor-like factor 2
CSF2	Colony stimulating factor 2
CSF3	Colony stimulating factor 3
CTGF	Connective tissue growth factor
CXCL10	Chemokine (C-X-C motif) ligand 10

CXCL11	Chemokine (C-X-C motif) ligand 11
CXCL14	Chemokine (C-X-C motif) ligand 14
CXCL9	Chemokine (C-X-C motif) ligand 9
DIAPH1	Diaphanous homolog 1
ESM-1	Endothelial cell-specific molecule 1
FCGR3A	Fc fragment of IgG, low affinity IIIa, receptor (CD16a)
FGF	Fibroblast growth factor
FGF1	Fibroblast growth factor 1
FIZZ1	Resistin like beta
FN1	Fibronectin-1
FOLR2	Folate receptor 2 (fetal)
FOXP3	Forkhead box P3
GAS6	Growth arrest-specific 6
GATM	L-arginine:glycine amidinotransferase
GRLF1	Glucocorticoid receptor DNA binding factor-1
GZMA	Granzyme A (granzyme 1, cytotoxic T-lymphocyte-associated serine esterase 3)
gzmA	Granzyme A
GZMB	Granzyme B
HLA	Human leukocyte antigen
HLAC	Major histocompatability complex, class I, C
HLADP	Major histocompatability complex, class II, DP
HLADQ	Major histocompatability complex, class II, DQ
HLADR	Major histocompatability complex, class II, DR
HLAE	Major histocompatability complex, class I, E
HLAG	Major histocompatability complex, class I, G
HMOX1	Heme oxygenase (decycling) 1
IDO	Indoleamine 2,3- dioxygenase
IFNAR1	Interferon receptor 1
IFNAR1	Interferon, alpha; receptor
IFNG	Interferon gamma
IFNT	interferon tau
IGF1	Insulin-like growth factor 1
IGFBP-4	Insulin-like growth factor binding protein 4
IGFBP6	Insulin-like growth factor binding protein-6
IL10	Interleukin 10
IL12	Interleukin 12
IL15	Interleukin 15
IL15RA	IL15 receptor alpha chain
IL17	Interleukin 17
IL17RA	IL17 receptor alpha

IL17RA	Interleukin 17 receptor A
IL18R1	Interleukin 18 receptor 1
IL18RAP	Interleukin 18 receptor accessory protein
IL1B	Interleukin 1, beta
IL1B	Interleukin 1 beta
IL1R	IL1 receptor
IL1R2	IL1 receptor
IL23	Interleukin 23
IL2RA	IL2 receptor alpha chain
IL4	Interleukin 4
IL6	Interleukin 6
IL8RA	Interleukin 8 receptor alpha
IL8RB	Interleukin 8 receptor beta
IL8RB	Interleukin 8 receptor
IL8RB	Interleukin 8 receptor, beta
INOS	Inducible nitric oxide synthase
ITGA4	integrin alpha 4
IL1A	Interleukin 1 alpha
ISG15	Interferon stimulated gene 15
JAM2	Junctional adhesion molecule 2
Kdm6b	KDM1 lysine (K)-specific demethylase 6B
KIR2DL1	Killer cell immunoglobulin-like receptor 2DL1
KRT7	Keratin 7
LILR	Leukocyte Ig-like receptor
LPS	Lipopolysaccharide
MAP1S	Microtubule-associated protein-1S
MAP4K2	Mitogen-activated protein kinase kinase kinase kinase
MCP1	Monocyte chemotactic protein-1
MHC	Major histocompatability complex
MHC class II	Major histocompatability complex II
MIF	Macrophage migration inhibitory factor
MMP1	Matrix metalloproteinase 1
MMP12	Macrophage-specific metalloproteinases 12
MMP12	Matrix metalloproteinase 12
MMP9	Matrix metallopeptidase 9
MPS	Mononuclear phagocyte system
MR	Mannose receptor
MRC1	Mannose receptor C type 1
MUC1	mucin 1
Mx1	Myxovirus resistance 1
MyD88	Myeloid differentiation primary response gene (88)

MYH10	Myosin, heavy chain 10, non-muscle
MYL2	Myosin, light chain 2, regulatory, cardiac, slow
MYLPF	Myosin light chain, phosphorylatable, fast skeletal muscle
NRP1	Neurophilin-1
NFKB	Nuclear factor kappa beta
NK	Natural killer cell
NO	Nitric oxide
PAF	Platelet-activating factor
PAG	Pregnancy-associated glycoprotein
PAMPS	Pathogen-associated molecular patterns
PDGF	Platelet derived growth factor B
PDGFB	Platelet derived growth factor, B polypeptide
PGE	Prostaglandin E
PGE2	Prostaglandin E2
PGF2A	Prostaglandin F2 alpha
POLB	Polymerase (DNA directed) beta
PPARG	Peroxisome proliferator-activated receptor gamma
PRP	Prolactin related protein
PTX3	Pentraxin 3
PVRL3	Nectin-3
PXN	Paxillin
RAC	Rho-GTPase (rac) gene
RNASE6	Ribonuclease, RNase A family, k6
ROS	Reactive oxigen species
SERPINA14	Uterine serpin
SERPINB2	Serpin 2
SLCO2B1	Solute carrier organic anion transporter family, member 2B1
SPP1	Secreted phosphoprotein 1
STAB1	Stabilin-1
STAT1	Signal transducer & activator of transcription 1
Stat6	Signal transducer and activator of transcription 6
SDF1	Stromal cell-derived factor-1
TGFB	Transforming growth factor-beta
TGFB1	Transforming growth factor beta 1
TGFBI	Transforming growth factor beta-induced
TIMP2	TIMP metallopeptidase inhibitor 2
TLR	Toll-like receptor
TLR 4	Toll like receptor 4
TLR 6	Toll like receptor 6
TLR 7	Toll like receptor 7
TLR 9	Toll like receptor 9

TLR1	Toll like receptor 1
TLR10	Toll like receptor 10
TLR2	Toll-like receptor 2
TMSB4X	thymosin beta 4
TNF	Tumor necrosis factor alpha
TNFRS1B	Tumor necrosis factor receptor superfamily, member 1B
TNFRSF6	Fas (TNF receptor superfamily, member 6)
TNFRSF8	Tumor necrosis factor receptor superfamily, member 8
TRAIL	Tumor necrosis factor ligand superfamily member 2
Tregs	Regulatory T cells
TREM2	Triggering receptor expressed in myeloid cells-2
TSGA14	Testis specific, 14
VEGFA	Vascular endothelial growth factor A
VCAM-1	Vascular cell adhesion molecule 1
VSIG4	V-set and immunglobulin domain-containing 4
VTCN1	v-set domain containing T cell activation inhibitor 1
Ym1	Chitinase 3-like 3
γδT	Gamma-delta T cell

CLUSTER OF DIFFERENTIATION ANTIGENS USED TO IDENTIFY CELLS

Name	Description
CD4	Co-receptor for MHC class II molecules; expressed on thymocytes, Th1 and Th2 T cells, monocytes and macrophages
CD8	Co-receptor for MHC class I molecules; expressed on thymocytes, cytotoxic T cells.
CD25	Il-2 receptor alpha chain; expressed on activated T and B cells, and monocytes.
CD68	Also called macrosialin; expressed on monocytes, macrophages, neutrophils, basophils and large lymphocytes.
CD14	Receptor for LPS and LPS binding protein; expressed on myelomonocitic cell.
CD11b	Alpha M subunit of integrin CR3 associated with CD18; expressed on myeloid and NK cells.

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The presence of conceptus alloantigens necessitates changes in maternal immune function. Here, using the cow as a model, we evaluated these changes. In early pregnancy, there was no effect of pregnancy status on number of cells positive for CD8, CD4, γδT cell receptor, or the monocyte marker CD68 in the peripheral blood mononuclear cell (PBMC) population compared to nonpregnant cows. However, there was an increase in the proportion of $CD4^+$ cells that were positive for CD25 at day 33-34 of pregnancy in the cow. Periparturient cows had an increased percent of PBMC that were γδ T cells and that co-expressed $CD4^+CD25^+$. Moreover, there was a tendency for a lower percentage of circulating $CD68^+$ cells. The increase in cells co-expressing $CD4^+CD25^+$ reflects an increase in the proportion of cells that were $CD4^+$ rather than in the proportion of $CD4^+$ cells that also were $CD25^+$. In the endometrium, there were increased numbers of $CD68^+$ cells in interplacentomal regions in pregnant cows as compared to non-pregnant cows and these cells were also abundant in caruncular septa of the placentome. However, $CD68^+$ cells were not present in the in fetal villi of the placentomes or in the interplacentomal chorion. Regardless of location, the majority

of $CD68^+$ cells also expressed CD14, another monocyte macrophage marker. In the interplacentomal endometrium, $CD68^+$ cells were present in deeper areas of the endometrial stroma co-expressed $CD11b^+$, but not in shallow region. In caruncular septa of the placentome, $CD68^+$ cells were negative for CD11b. $CD68^+$ cells in the interplacentomal endometrium were negative for MHC class II while most $CD68^+$ cells in caruncular septa were positive for MHC class II. Macrophages co-expressing $CD68^+CD14^+$ present in the stroma of the interplacentomal endometrium and in the caruncular septa of the placentome were regionally differentiated with regards to expression of CD11b and MHC class II. Moreover the macrophages present in the pregnant endometrium are regionally differentiated perhaps as a result of the different maternal and fetal interaction between the interplacentomal and placentome region in the cow. Transcriptomal analysis of endometrial $CD14^+$ cells showed that these cells express genes characteristic of macrophages; the endometrial macrophages over-expressed genes related to alternative (M2) activation pathway. Gene ontology analysis comparing differentially-regulated genes between endometrium and blood $CD14^+$ cells showed an enrichment of ontologies related to proteolysis activity which suggests a tissue remodeling role for the endometrial macrophages. Despite differences in the types of placentation between human and bovine, many genes preferentially regulated in decidual macrophages were also differentially regulated between blood and endometrial macrophage in the cow. Around 17% of the genes found to be differentially expressed between decidual and blood human monocytes were also differentially expressed in the same direction for the cow. Presence of macrophages in the endometrium during late pregnancy suggests a role for these cells

in parturition, expulsion of fetal membranes, and the process of uterine involution. Better understanding of the complex role of the endometrial macrophages may help researchers develop new practical techniques to positively affect the ability of cows to establish pregnancy following parturition.

CHAPTER 1
INTRODUCTION

Physiological cross-talk between the mother and the developing fetus allows successful birth of a neonatal animal in a manner that does not imperil the mother's life or potential for producing future offspring. Among the maternal adjustments to support pregnancy is the establishment of an immunological relationship with the conceptus that allows the allogeneic fetus to evade immunological rejection while allowing the uterus to remain in a sterile condition. The immunological problems posed by the allogeneic nature of the conceptus were first identified by the transplantation biologist, Sir Peter Medawar in 1953 (Billigham et al. 1953). At that time, the immune system was thought of largely as a system to protect the body against invasion by foreign microorganisms. Today, however, it is recognized that the immune system plays a much more global role to promote homeostasis of the body beyond the prevention of microorganism invasion, such as the role of natural killer (NK) cells in placental vascularization (Bilinsk et al. 2008)

Key to the survival of the fetal allograft is modulation of the immune response at the fetal-maternal interface by locally acting immunosuppressive cells and their cytokine secretions (Hill 1995; van Nieuwenhoven et al. 2003; Guleria and Sayegh 2007; Mold et al. 2008). Activation of a local immune response, such as when infection takes place in the uterus, can result in abortion of the fetus (Krishnan et al. 1996; Robertson et al. 2007; Thaxton et al. 2009). Development of an immunosuppressive environment depends upon expression of non-classical major histocompatability complex (MHC) molecules on trophoblast cells (King et al. 2000; Ishitani et al. 2003; Hunt et al. 2005; Lightner et al. 2008), tryptophan catabolism by the enzyme indoleamine 2,3-

dioxygenase (IDO) to starve T cells and generate immunosuppressive products (Munn et al. 1998), T cell apoptosis (Hunt et al. 1997; Guleria et al. 2005), inhibition of the complement system (Holmes et al. 1992; Nishikori et al. 1993;Bulla et al. 2003), and the migration of the specific leukocyte populations to the uterus including regulatory T cells (Tregs) (Aluvihare et al. 2004; Heikkinen et al. 2004; Sommerset et al. 2004; Tilburgs et al. 2006;Tilburgs et al. 2008), $\gamma\delta$T-cells (Heyborne et al. 1992; Mincheva-Nillsson et al. 1992, 1997) and NK cells (Bilinski et al. 2008; Shigeru et al. 2008).

Another cell that accumulates in the uterus during pregnancy is the macrophage. The recruitment of macrophages to the pregnant uterus has been described in many species including humans (Heikkinen et al. 2003; Cupurdija et al. 2004; Kim et al. 2007), mice (Hunt et al. 1985), pigs (Kaeoket et al. 2001); cynomologus and vervet monkeys, (Dambaeva et al. 2008) and sheep (Tekin and Hansen 2004). Macrophages are important immune effectors that bridge the innate and adaptive immune responses. They recognize, process and present antigens to initiate an immune response specific to a given antigen and secrete cytokines to prime and support immune responses (Janeway et al. 2004; Wu et al. 2009). They also have a role in the wound-healing process by the secretion of cytokines and growth factors (Chung et al. 2009; Chung and Kao 2009; Mosser and Edwards 2009; Barrientos et al. 2008), removal of apoptotic cells (Gregory and Devitt 1999; Chang et al. 2004; Krysko et al. 2006; Xu et al. 2006), and angiogenesis (Pollard 2009).

Given the involvement of macrophages in antigen processing, regulation of immune responses, tumor growth and angiogenesis, these cells are likely to play a key role in ensuring the proper immunological environment for development of the

conceptus as well as preparing the uterus for the post-parturient period when parturition is likely to result in contamination of the reproductive tract with microorganisms.

Little is known about the particular role of macrophages in pregnancy. The purpose of this dissertation is to use the cow as a model to identify possible roles for uterine macrophages during pregnancy. This literature review will focus on describing briefly the function of the macrophage and what is known about its role during pregnancy in species other than the cow. In addition, knowledge about immunological function in the uterus of the pregnant cow will be reviewed to provide a background for understanding original studies on the number, location and possible function of endometrial macrophages described in later chapters of this dissertation.

CHAPTER 2
LITERATURE REVIEW

Origin, Development, and Function of Macrophages

Macrophages are part of the mononuclear phagocyte system (MPS), which includes the polymorphonuclear phagocytes (granulocytes) and the mononuclear phagocytes as originally proposed in 1924 by Aschoff (van Furth et al. 1972). Cells of the MPS are derived from the hematopoietic cell lineage in the bone marrow; myeloid progenitor cells differentiate into circulating monocytes and then migrate to the tissue to become macrophages (Hume et al. 2002). The macrophages are widely distributed in the body, in part because of their wide distribution but vary in function and activation.

In 1908, Metchnikoff won the Nobel Prize for his description of amoeboid cells in lower animals such as sponges and their function to engulf foreign antigens (http://nobelprize.org/nobel_prizes/medicine/laureates/1908/mechnikov-lecture.html). Metchnikoff demonstrated the presence of amoeboid cells by inserting a thorn in a starfish and viewing recruitment of amoeboid cells towards the injury. Later, Metchnikoff called the amoeboid cells macrophages to distinguish them from the so-called microphages previously described by him and now identified as polymorphomononucleated cells, i.e neutrophils. Since Metchnikoff's early studies, macrophages have been recognized as being one of the most ancient cells in the immune system. They are present in lower organisms such as coelomocytes, corals and annelids (Anastassova-Kristeva 2003).

Development

In the mouse, the first hematopoietic cells arise in the yolk sac around day 7.5 of embryonic development (Sánchez et al. 1996), and macrophages can be detected in

the yolk sac at day 9 (Takahashi et al. 1988). By day 10 of development, hematopoietic cells arise in the intraembryonic aortic-gonadal-mesonephros region (Sánchez et al. 1996; Nobuhisa et al. 2007) and migrate towards to the fetal liver at day 11 (Sánchez et al. 1996). Later in embryonic life and adult life, hematopoietic cells in the bone marrow comprise the main hematopoietic stem cell pool in the body (Papayannopoulou et al. 2003).

The idea of the existence of a multipotent progenitor cell lineage arose from the fact that irradiated mouse and guinea pigs were able to recover hematopoiesis after bone marrow transplantation (Til et al. 1961; Becker et al. 1963). The transplanted bone marrow cells from previous transplanted animal were also able to form a new colony of hematopoietic stem cell after being retransplantated to a new animal (Juraskova et al.1965). This experiment demonstrates ability of hematopoietic stem cells for self-renewal.

The most accepted model for differentiation of hematopoietic stem cells is called the myeloid-lymphoid dogma. In this model, the pluripotent hematopoietic stem cell commits itself to an increasingly specialized cell lineage at each branch point. The first differentiation event is lineage commitment to either myeloid or lymphoid cells. Exposure to colony stimulating factor 2 (CSF2) and interleukin 3 (IL3) results in differentiation of hematopoietic stem cells to myeloid stem cells whereas exposure to IL3 alone results in differentiation into lymphoid stem cells. Ordinarily, myeloid progenitors subsequently differentiate into eosinophils, megakaryocytes, mast cells, granulocytes and macrophages while lymphoid progenitors are the source of T, B and NK lymphocytes (Metcalf 2007). Each stage in the differentiation process is under

control of cytokines. For example, granuclocyte/macrophage progenitors are formed from myeloid stem cells under the influence of IL3, CSF2 and interleukin 6 (*IL6*). Subsequently, the stimulation with colony stimulating factor 1(CSF1) and CSF2 leads to formation of a progenitor of monocytes and neutrophils. The migration of monocytes from periphery to the tissues can be induced by a variety of factors that change the expression and activation of both monocyte and endothelial adhesion molecules. Usually, transendothelial migration is influenced by soluble factors such as chemokines expressed on the surface of endothelial cells and cytokines that are released into the bloodstream (Imhof and Aurrand-Lions 2004).

Chemokines are ligand-specific. The interaction of a specific chemokine with its ligand promotes the adhesion of the circulating monocyte to the endothelial cell surface; the strength of this interaction is specific to the type of chemokine-ligand interaction (Maslin et al. 2005). For example, interleukin 8 receptor alpha (IL8RA) expressed by the monocytes strongly interacts with the interleukin 8 (IL8) expressed on the surface of endothelial cells, however the IL8 is not strong on attractor of cells to the site where they are expressed (Luscinskas et al. 2000). On the other hand, the interaction of the C-C chemokine receptor type 1(CCR1) on the monocytes to the chemokine (C-C motif) ligand 5 (CCL5) on the endothelium promotes a strong attraction to the site of inflammation (Wang et al. 2009).

Soluble factors such as colony stimulating factor 1 (CSF1) can change gene expression of monocytes and induce their migration into the tissues (Jones 2000). The proposed model for that states that the gradient of CSF1 would progressively change monocyte gene expression to induce the expression of genes related to filopodia

formation [cell division control protein 42 (*CDC42*) and rho-GTPase (rac) (*RAC*)]; cellular spreading via extension of lamellipodia and polarization towards the focus of expression of CSF1, and further active migration by the expression of Rho and RAC proteins (Jones 2000). Once the monocytes reside in the tissues, many signals can induce their maturation into macrophages; one example is by CSF2 stimulation (Metacalf 2007).

The myeloid–lymphoid dogma of hematopoisis has been revisited. Adolfsson et al. (2005) proposed a new composite model of blood cell differentiation in which granulocytes and monocytes can originate from either lymphoid or myeloid precursors. Thymic progenitor cells so-called DN1 cells are characterized to be $CD4^{-}CD8^{-}$c-Kit^{+} $CD25^{-}$ and they lack B-progenitor capability (Benz and Bleul 2005). Indeed, in clonal studies using DN1 cells from green fluorescent protein (GFP) transgenic mice macrophages were formed by the co-culture of DN1 cells with with PA6 stromal cell line (bone marrow-derived stromal cell line) in the presence of colony stimulating factor 3 (CSF3). Moreover, DN1 cells from GFP-transgenic mice showed to give a rise of macrophages after single cell intra-thymic transplantation (Wada et al. 2008).

Moreover, there is evidence that monocyte/macrophages can arise in tissues other than those derived from the bone marrow. Local proliferation of macrophages has been described in lungs (Sawyer 1986; Wesselius and Kimler 1989; Lohmann-Matthes et al. 1994), kidneys (Lan et al. 1995), and testis (Schlatt et al. 1999). Repopulation of tissue macrophages in lungs and peritoneum can occur in mice subjected to total body irradiation to deplete blood monocytes (Sawyer et al.1982).

Macrophage Activation and Differentiation

Under the influence of specific locally-produced mediators, macrophages are capable of displaying different functional phenotypes, many of which are antagonistic to each other. An additional cause for variability in function of macrophages is heterogeneity in the circulating pool of monocytes. Subtypes of monocytes circulating can respond differently once they differentiate into macrophages in the tissues (Auffray et al. 2009). For example, the majority of blood monocytes are represented by $CD14^{+}CD16^{+}$ cells, and express low levels of proinflammatory cytokines and high levels of IL10 after lipopolysaccharide (LPS) stimulation (Passlick et al. 1989). However, a small number of cells are $CD14^{+}CD16^{-}$ and they produce high levels of proinflammatory cytokines after LPS stimulation (Passlick et al.1989).

The classical functions of macrophages are to phagocytose microorganisms, promote an acute inflammatory response by production of proinflammatory factors such as tumor necrosis factor alpha (TNF), IL1 and IL6, and antigen presentation from phagocytosed microorganisms to T lymphocytes thus initiating an acquired immune response (Stout and Suttles 2005). For a long time, macrophages were thought to be a less efficient type of cell in regards to antigen presenting function as compared with dendritic cells (DC) (Ueno et al. 2007). Nevertheless, macrophages have been shown to possess many characteristics in common with DC such as the expression of surface markers and the ability to initiate an immune response by the stimulation of naïve lymphocytes (Hume 2008). In addition, macrophages are involved in tissue remodeling (clearance of damaged or apoptotic cells, matrix and necrotic debris; inducing cell proliferation; matrix regeneration), apoptosis, and angiogenesis (Pollard 2009).

Immune responses are usually characterized by a changing cytokine milieu during the progression from activation of inflammation, resolution of the immune response, wound healing and removal of inflammatory cells. Changes in macrophage function are an important part of this progression. Two main patterns of macrophage activation are recognized - classical (M1) and alternative (M2). M1 macrophages, which are typically produced during inflammation, elicit pro-inflammatory immune responses and the killing of intracellular pathogens. The major activation signals are INFG and TNF. M2 macrophages, which are often produced during resolution of the immune response and wound healing, can develop several specific phenotypes depending upon the cytokine environment. They are involved in regulation of Th2-like immune responses, tissue remodeling, angiogenesis and immunosuppression (Gordon 2003). The M2 activation pathway can be divided into three subpathways. The M2a pathway represents macrophages that are stimulated by IL13 and IL4; M2b pathway involves macrophage differentiation under the influence of immune complexes via toll-like receptor (TLR) and IL1 receptor (IL1R); M2c are macrophages that differentiate under the influence of IL10 and transforming growth factor-beta (TGFB) (Mantovani et al. 2002; Gordon 2003) or glucocorticoid hormones (Mantovani 2006) (Figure 2-1).

The M1 activation pathway occurs in response to IFNG and TNF inducers or TNF alone (Natan 1991; Gordon 2003). Activated NK cells usually are the main source of IFGN at the earlier stages of the immune response. As the proinflammatory immune response progressess; the components of the acquired immune system such as activated T helper 1 (Th1) cells become the main source of IFNG (Mosser and Edwards 2008). The Th1 activated cells produce IFNG in sufficient quantities to support a large

population of activated macrophages (Mosser and Edwards 2008). The autocrine and paracrine production of TNF by macrophages can be upregulated via TLR activation in a MyD88-dependent manner (Mosser and Edwards 2008).

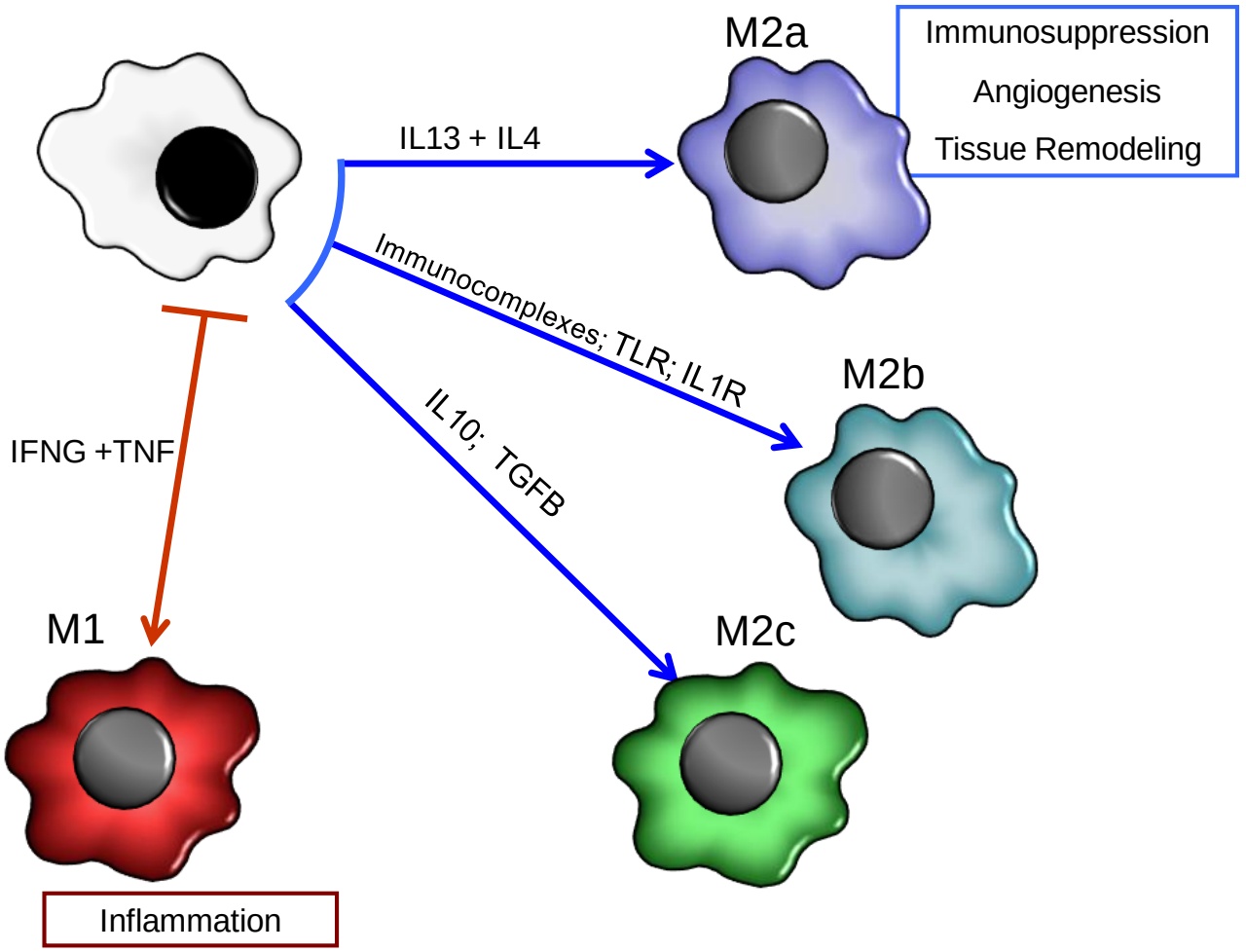

Figure 2-1 Pathways of macrophage activation. Classically activated or M1 macrophages typically promote immune responses. They differentiate under the influence of TNFA, IFNG and/or LPS. Examples of characteristic functions of M1 macrophages are shown in the red box. Alternatively activated or M2 macrophages are involved in immunosuppression, tissure remodeling and angiogenesis. The type of differentiation can be divided into M2a (differentiate under control of IL4 and/or IL13), M2b immunocomplexes, TLR and/or IL-1R) and M2c (IL10 and/or TGFB). Examples of functions for all three subtypes of M2 macrophages are shown in the blue box.

M1activated macrophages promote inflammation by releasing high amounts of proinflammatory cytokines such as IL12 and IL23 (O'Shea and Murray 2008). Antigen presenting capacity is also increased as a result of up-regulation of expression of MHC class II (Pai et al. 2002) and CD86 (Mosser 2003). Other effector functions are also increase, for example, increased production of nitric oxide (NO) and reactive oxygen species (ROS) (Mosser 2003; Mantovani et al. 2009).

M1 activated macrophages express high levels of genes for inducible nitric oxide synthase (*INOS*), IL1 beta (*IL1B*), *IL6, IL12, IL15, IL23, IL17, TNF*, chemokine (C-X-C motif) ligand 9 (*CXCL9*), *CXCL10*, *CXCL11*, IL2 receptor α chain (*IL2RA*), IL15 receptor α chain (*IL15RA*), tumor necrosis factor ligand superfamily, member 2 (*TRAIL*), tumor necrosis factor receptor superfamily, member 6 (Fas), pentraxin 3 (*PTX3*), and insulin-like growth factor binding protein 4 (*IGFBP-4*) (Martinez et al. 2006; Mantovani et al. 2009) to induce and sustain an inflammatory immune response.

Alternative or M2 activation was first described as a response to interleukin 4 (IL4) (Gordon 2003). Other inducers of alternative activation have now been described including IL13, immunocomplexes, IL10 and glucocorticoid hormones (Mantovani 2006). The M2 activated macrophages possess various functions including regulation of tissue remodeling processes, angiogenesis and antibody-mediated inflammatory responses (Hebert et al. 2005; Mosser and Edwards 2008; Barrientos et al. 2008; Martinez et al. 2009). Because of the trophic functions such as angiogenesis, the M2 macrophages are associated with tumor progression (Sica et al. 2008; Allavena et al. 2008; Mantovani et al. 2009) and parasite resistance (encapsulation) (Anthony et al. 2006; Reece et al. 2006; Ishii et al. 2009).

Among M2 macrophages subsets, there is a tendency for downregulation of CD86/80 and inducible nitric oxide synthase (*INOS*) while mannose receptor (MR), arginase 1(*ARG1*) and IL10 are frequently upregulated in all three subtypes of M2 activation pathways (Gordon 2003). Other markers have been related to M2 activation include chitinase 3-like 3 (*Ym1*), resistin like beta (*FIZZ1*), *CCL22*, *CCL17*, IL1 receptor, type II (*IL1R2*), *IL10* and galactose receptors (Mantovani et al. 2009). Martinez et al. (2006) found a total of 52 genes upregulated in M2 activated macrophages including insulin-like growth factor 1 (*IGF1*), fibronectin 1 (*FN1*), growth arrest-specific 6 (*GAS6*), *CCL18* and mannose receptor C type 1 (*MRC1*).

The molecular mechanisms for M1 and M2 polarization are being elucidated. For example, it has been shown that M2 differentiation ensues when p50 homodimers inhibit activation of nuclear factor kappa B (NFKB)-driven M1 activation. Moreover, peroxisome proliferator-activated receptor gamma (PPARG) agonists have been shown to induce M2-like differentiation in blood monocytes but not in macrophages already committed to M1 activation (Bouhlel et al. 2007). A molecular pathway involved in the epigenetic regulation of expression of key M2-associated genes in the mouse has been proposed (Ishii et al. 2009). This model involves IL4 upregulating the KDM1 lysine (K)-specific demethylase 6B (*Kdm6b*) via signal transducer and activator of transcription 6 (Stat6) to activate promoters of M2 marker genes such as mannose receptor.

Regulation of Immune Responses

Macrophages induced to differentiate along the M2 pathway experience a decrease in all functions characteristic of M1 macrophages including antigen presentation, proinflammatory cytokine secretion and pathogen killing through secretion of NO and ROS (Edwards et al. 2006). M2 macrophages can also exert regulatory

effects on the immune response because of their increased arginase activity into ornithine, which is a precursor of polyamines such as bis-naphthalimidopropyl putrescine and collagen; components of the extra cellular matrix (ECM). Also the aginine metabolism deviated to L-onithine instead NO production can suppress the clonal expansion of activated neighboring lymphocytes by interfering cell proliferation in vitro (Cordeiro-da-Silva et al. 2004). Recently, M2 cells were shown to induce differentiation of regulatory T-cells in vitro using the human leukocyte antigen (HLA) mismatch model (Savage et al. 2008). Regulatory T-cells, in turn, can induce alternative activation of human mononuclear phagocytes (Tiemessen et al. 2007). The co-culture of Treg cells and monocytes induced the upregulation of M2 markers (MCR1, CD163 and CCL18) and downregulation of M1 marker HLADR. Moreover, the Treg cells inhibit the proinflammatory immune response to LPS, by decreasing the secretion of proinflammatory cytokines such as TNF, IL6, interleukin 1 beta (IL1B), IL8 and C-C motif chemokine 3 (CCL3). Indeed, the induction of M2 macrophage differentiation by Treg cells is a partially cytokine-dependent event, but also depends on cell-to-cell contact (Tiemessen et al. 2007).

A subpopulation of M2 macrophages known as regulatory macrophage can be induced by immune complexes, prostaglandins, glucocorticoids, apoptotic cells and IL10 (Pollard 2009). The production of prostaglandins by endometrial cells (Cheung et al. 1990), glucorticoids (Alfaidy et al. 2003) and the presence of apoptotic trophoblastic cells (Von Rango et al. 2003) indicate the potential for macrophages to be activated along the M2 activation pathway during pregnancy.

The regulation of immune response by M2 activated macrophages relies mainly on their cytokine secretion and their poor ability to present antigens to T-cells (Hume 2009). The M2 activated macrophage can release large amounts of cytokines such as transforming growth factor beta (TGFB) and suppress T-cell activation as shown in the mouse model where the injection of a TLR2 ligand –zymozan increased the secretion of TGFB by the macrophages (Dillon et al. 2006). Furthermore, when TLR2 ligand – zymozan injected animals were challenged by vaccination against ovalbumin, they showed an increased secretion of TGFB and IL10 accompanied by decreased secretion of IL6 (Dillon et al. 2006).

Phagocytosis of apoptotic cells also increases the expression of TGFB in macrophages (Dillon et al. 2006), induces downregulation of proinflammatory cytokines (IL1B, IL8, IL10 and CSF2), leukotriene C4 and tromboxane B2, an increase of prostaglandin E2 (PGE2), and platelet-activating factor (PAF) so that local inflammation is reduced (Fadok et al.1998). In the bovine placenta, apoptotic cells were detected, especially at the edges of the placentomes and in the interplacentomal region (Facciotti et al. 2009). Also, apoptosis is one mechanism by which extravillous trophoblast cells are eliminated in the human placenta (Von Rango et al. 2003).

Wound Healing and Tissue Remodeling

One function of M2 macrophages is to promote tissue remodeling (Allavena et al. 2008). Wound healing macrophages are generated under influence of IL4 released from resident basophils, mast cells and granulocytes in response to tissue injury (Loke et al. 2005). Additionally, IL4 can be produced in response to biopolymers such as chitin found in fungi and parasites (Reese et al. 2000). Moreover, IL4 and IL13 are

considered the most abundant cytokines in the Th2 immune response (promotes antibody-mediated immunity) (Reese et al. 2000; Loke et al. 2007).

IL-4 activation of human macrophages induces upregulation of extracellular matrix proteins fibronectin, tenascin-C, and transforming growth factor, beta-induced (TGFBI) (Gratchev et al. 2001). The expression of enzymes such as matrix metalloproteinase 1 (MMP1) and matrix metallopeptidase 12 (macrophage elastase) (MMP12) (Gratchev et al. 2005), which degrade extracellular matrix components such as elastin type IV collagen and fibronectin (Chen 2004; Djonov et al. 2001) and granzyme B (GZMB) that cleaves extracellular matrix components such as vitronectin, fibronectin and laminin (Buzza et al. 2005). IL4 also stimulates the production of extracellular matrix by macrophages through upregulation of ARG1 expression (Kreider et al. 2007). Another tissue remodeling factor produced by macrophages is platelet derived growth factor B (PDGF). This growth factor stimulates proliferation and regulates differentiation of fibroblasts (Barrientos et al. 2008).

Angiogenesis

Macrophages can contribute to the process of angiogenesis in the tumor by the secretion of angiogenic factors including VEGF, fibroblast growth factor (FGF), TGFB and chemokines (Dace and Ape 2007). They also induce degradation and remodeling of the matrix by expression of MMPs such as matrix metalloproteinase 9 (*MMP9*) (Dace and Ape 2007). Moreover, tumor associated macrophage (TAM) is classified as a distinct M2 activated population and can induce angionesis in the growing tumor (Sica et al. 2006). Macrophages have been implicated in the regulation of angiogenesis during wound healing (Lighnen et al. 2001; Bergmann et al. 2007). Also, M2 macrophages can promote angiogenesis through expression of chemokine (CXC motif)

receptor 4 (*CXCR4*) via VEGF signaling. CXCR4 is a ligand for macrophage recruitment which is expressed by pericytes (Song et al. 2009), where they secrete angiogenic molecules such as VEGF (Grunewald et al. 2006) in response to stromal cell-derived factor-1 (SDF1) signaling (Shiba et al. 2009). A similar mechanism was reported during organogenesis where $CXCR4^{+}$ bone-marrow progenitor cells are guided to the site of vascular expansion in embryo via *SDF1* signaling (Ceradini et al. 2004). Moreover, *CXCR4* and *SDF1* were upregulated in response to hypoxia which resulted in the recruitment of similar myeloid cell (Ceradini et al. 2004). Furthermore, during embryo implantation, the absence of monocyte-derived dendritic cells compromises decidualization because of a defective angiogenesis which causes the pregnancy to terminate (Plaks et al. 2008). Dendritic cells (DC) has been shown to produce a variety of both pro-angiogenic and anti-angiogenic factor such as vascular endothelial growth factor A (*VEGFA*) and pentraxin 3 (*PTX3*) respectively (Barientos et al. 2008).

Tumor Progression

In established tumors, tumor associated macrophages (TAMs) are the principal leukocyte subset. In the some cases of breast cancer, leukocytes can account for almost 50% of the tumor mass (Lewis and Pollard 2006).

The TAM possesses a phenotype that resembles M2 activation (Sica et al. 2006) and therefore inflammatory responses are inhibited through secretion of M2 cytokines. Tumor cells can release many cytokines that induce an immunosuppressive environment (Sica et al. 2008). CSF1 promotes the recruitment of monocytes and lymphocytes to the tumor mass, whereas IL10 promotes monocyte differentiation into macrophages but not into DC (Sica et al. 2008). Moreover, IL10, TGFB, PGE2 and IL6

have the potential to promote the polarization of M2 macrophages (Mantovani et al. 2004).

IL-10 itself or together with IL-6 induce upregulation of macrophage v-set domain containing T cell activation inhibitor 1 (*VTCN1*) expression, is a molecule implicated in the suppression of antigen-specific anti-tumor immunity (Kryczek et al. 2006) and *VTCN1* is expressed by a subset of decidual macrophages in humans (Galazka et al. 2009). Moreover, TAMs secrete TGFB that can stimulate development of tumor cells (Byrne et al. 2008).

The amino acid metabolism seems to be important to macrophage activation and polarization. The shift in amino acid catabolism also promotes tumor progression due to the unbalanced activity of iNOS and ARG1 (Grohmann et al. 2003). *ARG1* expression by TAM is suggested to be an adaptor mechanism to avoid formation of cytotoxic *NO* in consequence to the immunosuppressive tumor environment and low expression of nuclear factor Kappa B (*NFKB*) by tumor resident immune cells (Weigerta and Brüne 2008). The expression of antiinflammatory cytokines such as TGFB and IL10 is probably due to the phagocytosis of apoptotic cellular debris in the tumor (Johann et al. 2007). The proangiogenic role of M2 macrophages results in a further stimulation of tumor growth. The maternal fetal interface as in the tumor microenvironment possess an For example, TAMs accumulate in the hypoxic regions of the tumor (Du et al. 2008) and produces angiogenic factors such as VEGF (Lewis et al. 2006).

Macrophages in the Pregnant Endometrium

Accumulation of macrophages is one of the characteristic changes in the endometrium during pregnancy. The recruitment of macrophages to the pregnant endometrium occurs in a wide range of mammalian species including mice (Hunt et al.

1985), humans (Heikkinen et al. 2003; Cupurdija et al. 2004), cynomologus and vervet monkeys (Dambaeva et al. 2009) and sheep (Tekin and Hansen 2004). The majority of what is known about the role of macrophages during pregnancy is based on human and mouse models.

In the mouse, distribution of macrophages in the uterus fluctuates during the estrous cycle. Estrogen induces influx of macrophages into the endometrium whereas progesterone antagonizes the effect of estrogen (De et al. 1990; Tibetts et al. 1999). During pregnancy, the presence of macrophages can be observed from day 7 of pregnancy. Endometrial macrophages are associated closely with the luminal epithelium in the mouse and some macrophages were observed in the uterine lumen (Hunt et al. 1985). Moreover, there is an accumulation of macrophages in the term placenta of the mouse where they are observed as a thick layer between decidua and trophoblast (Lagadari et al. 2004). The increased expression of chemokine ligands such as *CCL2*, a leukocyte chemoattractant factor (Carr at al. 1994), and *CSF1* by the uterus of pregnant mice suggests that these molecules regulate macrophage recruitment and differentiation to the uterus (Kyaw et al. 1998).

An essential role for macrophages in pregnancy can be inferred through indirect evidence from the $Csf1^{op-/op-}$ mouse where expression of *CSF1* is absent. Homozygous females show compromised recruitment of macrophages to the uterus and are infertile (Pollard et al. 1991). Pregnancies are possible, although with reduced litter size, when homozygous females were bred with heterozygous males, probably because of CSF1 from the fetal tissue or seminal fluid.

In humans, the number of macrophages in the endometrium is essentially constant throughout the menstrual cycle and 7 to 9 weeks of pregnancy (Rieger et al. 2004). After about 9 to 12 weeks of gestation, there is an accumulation of macrophages. Macrophages account for about 20% of the immune cells in the decidua during first trimester of gestation, and they are present throughout the first and second trimester of pregnancy (Bulmer et al. 1995; Trundley and Moffett 2004). In the third trimester of pregnancy, the number of macrophages decreases in the human placenta (Williams et al. 2009). There is an increase in number of $CD68^+$ macrophages in the basal plate and myometrium during labor, and macrophage number is higher in pregnancies with clinical complications such as premature labor (Leong et al. 2008) suggesting that labor is a proinflammatory event, and macrophages are involved in this process.

The signals for accumulation of macrophages in the endometrium remain unclear. Glandular epithelium and stromal cells of the endometrium produce macrophage migration inhibitory factor (MIF) (Acuri et al. 2001) as do trophoblast cells, and this chemokine could promote accumulation of macrophages in the decidua. Moreover, human trophoblast can cause migration of blood monocytes in vitro (Fest et al. 2007).

Most of the decidual macrophages originate from the mother (Sutton et al. 1999). Decidual macrophages express CD14 (Bulmer et al. 1984) and HLADR, HLADP and HLADQ which are MHC class II proteins (Bulmer et al. 1988; Heikkinen et al. 2003). There is also low expression of CD80 and CD86 costimulatory molecules (Heikkinen et al. 2003).

Macrophages in the human decidua can present alloantigen *in vitro* (Mizuno et al.1994), and are therefore able to arm an adaptative immune response. They also

express an array of enzymes such as acid phosphatase, non-specific esterase, alpha1-anti-protease and alpha1-anti-chymotrypsin that are involved in the phagocyte activity of these cells (Bulmer et al. 1984). Furthermore, pathogen recognition and clearance activity of macrophages are preserved in the decidua as suggested by production of superoxide radicals and TNF upon stimulation with bacteria (Singh et al. 2005). Thus, decidual macrophages can participate in host defense during pregnancy. In addition, decidual macrophages are associated intimately with the extravillous trophoblast (Bulmer et al. 1988; Bulmer et al. 1995) and may have a role on the maternal control of trophoblast invasion.

The human maternal-fetal interface is an immunosuppressive environment characterized by the high levels of cytokines that can cause M2 activation such as IL10 (Chaouat et al. 1999; Hanna et al. 2000; Sacks et al. 2001), IL4 (Chaouat et al. 1999; Sacks et al. 2001) and IL13 (Dealtry et al. 1998; Rieger et al. 2002; Brown et al. 2004). During pregnancy, the activation of macrophage in the decidua can be modulated by interaction with trophoblast HLAG molecules (Li et al. 2009). HLAG is a nonclassic MHC protein (Le Gal et al. 1999), conversely other HLA genes; *HLAG* is a nonpolymorphic gene (Huddleston and Schust 2004). Alternate splicing provides two forms of HLAG, one that is membrane-bound protein, and one that is a soluble protein (O'Callaghan and Bell 1998). The soluble form of HLAG can occur in a monomeric form or a homodimeric form (Kovats et al. 1990). The recognition of the leukocyte Ig-like receptor (LILR) by HLA-G dimers seems to have a pivotal role in the immune suppression at the maternal-fetal interface (Apps et al. 1999). The soluble form of HLA-G (sHLA-G) is shed from the trophoblast cell surface and it can inhibit immune

responses by binding to LILRB1/2-expressing cells in the decidua (O'Callaghan and Bell 1998).

HLAE usually is expressed concomitant to HLAG (Wei et al. 1990) and is involved in the inhibition of NK cell activation. HLAE binding to the inhibitory receptor CD94/NKG2A (Braud et al. 1998) triggers disruption of the actin network at the immunological synapse and consequently inhibits NKG2A-expressing NK and $CD8^+$ and γδ-T (Masilamani et al. 2006). HLAC has been shown to interact with killer cell immunoglobulin-like receptor 2DL1 (KIR2DL1) present in the surface of NK cells and some subsets of T-cell (Colonna et al. 1995). In HLAC-mismatched pregnancies, there is an increased number of activated $CD4^+CD25^+$ ($CD4^+CD25^{dim}$ and $CD4^+CD25^{bright}$) T cells in decidua compared to HLAC matched pregnancies. The true specificity of decidual $CD4^+CD25^{dim}$ and $CD4^+CD25^{bright}$ T cells remains unclear (Tilburgs et al. 2009). Macrophages undergo a M2 polarization in the presence of Treg cells (Tiemessen et al. 2007).

Decidual macrophages display an immunosuppressive profile. First trimester decidual macrophages in humans express markers related to M2 activation such as expression of stabilin-1 (*MS1*) and coagulation factor XIIIa (Curpudija et al. 2004). Decidual macrophages also secrete high levels of IL10 (Heikkinen et al. 2003; Cupurdija et al. 2004). In addition, there is expression of the lymphocyte inhibitory enzyme, *indoleamine 2,3 dioxygenase* (IDO), in the third trimester of pregnancy (Heikkinen et al. 2003) but not in the first trimester (Cupurdija et al. 2004), suggesting that there are adjustments of the immunosuppression mechanisms during pregnancy.

Recently, Repnik et al. (2008) studied the macrophage phenotype from human decidua in early/mid pregnancy and at term by flow cytometry. They tested whether stage of pregnancy or location had an effect on the state of activation of the uterine macrophage. They found higher expression of CD80, CD86 and human leukocyte antigen HLADR in early/mid pregnancy than at term (Heikinen et al. 2003), suggesting that the phenotype of decidual macrophages changes during pregnancy. They also found higher expression of M2 markers on cells from the decidua basalis compared to cells from decidua parietalis. This result suggests macrophages closer to the implantation site become more activated along the M2 activation pathway. Decidual macrophages from normal pregnant women secrete lower levels of interleukin 1 alpha (IL1A) and IL1B when compared to women who subsequently spontaneously aborted their pregnancy (Laird et al. 2003), suggesting that an immunosuppressive environment is necessary to sustain pregnancy.

Recently, comparative analysis of gene expression in blood monocytes and human decidual macrophages revealed 120 genes that were differentially regulated (Gustafsson et al. 2008). A total of 100 of these differentially expressed genes were in four major clusters: immunomodulation (30 genes), tissue remodeling (20 genes), cell cycle (20 genes) and cell metabolism and transport (20 genes). Some markers of M2 activation were expressed in higher levles in the decidual macrophages compared to blood monocytes such as: neurophilin-1 (*NRP1*), which is associated with T-regulatory cell synapses (Bruder et al. 2004); triggering receptor expressed in myeloid cells-2 (*TREM2*), which is downregulated by IFNG and upregulated by IL4 (Turnbull et al. 2006); and monocyte chemotactic protein-1 (*MCP1*), which is upregulated by IL4 and

IL-3 (Bouchelouche et al. 2006). Also decidual macrophages had increased expression of genes involved in tissue remodeling such as fibronectin-1 (*FN1*), and MMP9 (Gustafsson et al. 2008).

Immunology of the Uterus in the Cow

Anatomy of the Placenta. The bovine placenta is classified as epitheliochorial and is characterized by apposition and interdigitation of chorionic epithelium with endometrial epithelium. Evolutionary studies on eutherian mammals showed that epitheliochorial placentation arose as a specialization from an ancestral hemotropic placenta (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 and Carter 2007). The epitheliochorial placenta might also have evolved as a strategy for immunological defense of the conceptus by limiting the degree of contact between the tissues (Moffett and Loke 2006).

The bovine placenta is cotyledonary and there is no decidualization of the endometrium. There are two areas of interaction between maternal and fetal tissues - the placentomal and interplacentomal regions. In the placentomal region, the fetal cotyledon interacts with maternal caruncles to form the placentome. Both cotyledons and caruncules are richly vascularized tissues and the placentome is the predominant site of gas and nutrient exchange between mother and fetus (Miglino and DiDio 1992, Miglino et al. 2007). Maternal and fetal blood flow to the placenta is initially multivillous and later in gestation the blood flow becomes counter-current characterized by the maternal and fetal blood flux running in opposite directions (Leiser and Kaufmann, 1994). Counter-current blood flow is a very efficient method of nutrient transfer and

allows the mother more control of placental blood flow than is the case for endotheliochorial placentae. The efficiency of placental transfer, as measured by the grams of fetus supported per gram of placenta, is higher for ruminants than humans (Leiser et al. 1997).

At the interplacentomal region, the chorionic epithelium is apposed to the luminal epithelium. In addition, binucleated cells from the trophoblast migrate into the luminal epithelium of the endometrium where they fuse with epithelial cells to form multinucleated syncytium (Leiser and Kaufmann 1994). This form of placenta is often referred to as a synepithelialchorial placenta. Migration of fetal cells begins between days 18-19 of pregnancy and is a constant process during gestation (Wooding and Wathes 1980, Kaufman and Leiser 2004) and continuous throughout the pregnancy. The function of the syncytium is not known. It is proposed that the fusion of fetal binucleated cells with maternal epithelial cells represents a mechanism to transport substances that are not easily permeable in the maternal fetal interface (Wooding and Wathes 1980).

In the ruminant, the binucleate cell (BNC) plays a central role in forming the structures and secretions at the fetomaternal interface that are crucial in establishing and maintaining pregnancy. BNCs comprise aorund 20% of the total trophoblast cell population (Wooding et al. 1996). They continuously migrate from the time of the implantation and throughout gestation across chorionic tight junctions to fuse with maternal luminal epithelial cells and feto-maternal syncytial plaques (Wooding and Flint 1994). The BNCs are source of an array of factors including bovine placental lactogens (bPL), prolactin-related protein-1 (bPRP-1) and pregnancy-associated glycoproteins

(bPAG) (Wooding and Flint 1994). They are also a source of steroid hormones such as progesterone, prostanoids, peptides and other protein hormones (Wooding and Flint 1994).

MHC Proteins Expression by the Trophoblast

The expression of paternal antigens by the trophoblast is generally suppressed in all species examined including the mice (Hunt et al. 1985), sheep (Gogolin-Ewens et al. 1989) and mares (except endometrial cups) (Donaldson et al. 1990; Kydd et al. 1991) The lack of expression of MHC proteins on the placenta reduces exposure of the maternal system to paternal antigens (Moffett-King 2002) but also makes trophoblast susceptible to NK cells since these cells lyse target cells without MHC expression (Heemskerk et al. 2005). Accordingly, non-classical MHC antigens that are largely monomorphic are expressed on trophoblast (Bulmer and Johnson 1990). For example, in humans, trophoblast expresses HLAC (Tilburgs et al. 2009), HLAE and HLAG (Hunt et al. 2000; Ishitani et al. 2003; Hviid 2006).

In the cow, too, chorionic tissue in the placentome is negative for MHC class I expression throughout pregnancy. However, chorionic tissue in the interplacentomal regions can express MHC class I in the last four months of pregnancy (Low et al. 1990; Davies et al. 2000; Davies et al. 2004). Interestingly, in pregnancies established by transfer of somatic cell nuclear transferred cloned embryos, expression of MHC class I was detected as early as day 36 of pregnancy (Davies et al. 2004), suggesting that abnormal expression of MHC class I genes may be correlated with the high embryo wastage in cloned cattle. MHC class I proteins on the chorion are predominately encoded by non-classical MHC class I genes (Davies et al. 2006). These non-classical

MHC class I proteins are possibly analogous to HLAG in humans that can regulate NK cell and macrophage function (Li et al. 2009).

Uterine Immune System

Little is known about the immunology of the bovine uterus. A functional immune system is a requirement since the uterus becomes exposed to microorganisms at various times. The parturition process, in particular, is characterized by influx of bacteria so that almost all cows have bacteria present in the uterine lumen shortly after calving (Foldi et al. 2006). The uterus of the cow is an immunocompetent site which can generate an immune response against infections by recruiting leukocytes and production of cytokines (Rosbottom et al. 2008). In the sheep, a closely related animal, allografts expressing classical histocompatability proteins are promptly rejected when placed in the uterus (Hansen et al. 1989).

Toll-like receptors (TLRs) is a family of pattern recognition receptors characterized by an extracellular leucine-rich domain and a cytoplasmic domain that share homology with the drosophila toll protein. TLRs belong to a large family of receptors on cells of the innate immune response that recognize specific antigens on viruses and bacteria called pathogen-associated molecular patterns (PAMPS) and which cause activation of cells to pathogen (O'Neil et al. 2007). The bovine endometrium expresses TLR1 to TLR10. Purified populations of epithelial cells expressed TLRs 1 to 7 and 9, and stromal cells expressed TLRs 1 to 4, 6, 7, 9 and 10 (Petzl et al. 2008, Davies et al. 2008).

During pregnancy, the expression of TLR2, -3, -4, -6 and -9 was greater in the interplacentomal endometrium than in the placentome (Petzl 2007). Endometrial epithelial cells stimulated in vitro with LPS also express acute phase proteins and secrete the proinflammatory eicosanoid prostaglandin E2 (Davies et al. 2008). The

endometrium also expresses other proteins involved in the innate immunity such as β-defensins (Cormican et al. 2008) and mucin 1 (MUC1) (Davies et al. 2008).

The distribution of T-lymphocytes depends upon the location in the uterus. $CD5^+$ cells are widely distributed throughout the endometrium, being present in the stroma, and in the luminal and glandular epithelium (Cobb and Watson 1995). $CD8^+$ cells are present mainly within the glandular and luminal epithelia and some scattered cells are located in the shallow stroma immediately beneath the luminal epithelium (Cobb and Watson 1995). $CD4^+$ cells are present almost exclusively in the deep stroma; few $CD4^+$ cells are present in the shallow stroma and none within the epithelium (Cobb and Watson 1995). The number of $CD4^+$ cells increases in the later stages of the luteal phase so that the ratio of CD4:CD8 cells increases from 3:1 (mid-luteal phase) to 7:1 (late luteal phase) (Cobb and Watson 1995).

B-cells are very rare in the noninfected uterus (Cobb and Watson 1995), however infection can lead a production of antibodies (Corbaeil et al. 1974) and the presence of lymphocytic foci in endometritis probably contain B-cells (Hartigan et al. 1972).

In the cyclic cow, MHC class II positive cells are widely distributed in the endometrial stroma (Cobb and Watson 1995). The MHC class II cells are more abundant in stroma closer to luminal epithelium than in deeper areas of the endometrium. It was concluded in this study that the MHC class II^+ cells were mainly lymphocytes and macrophages. However, no dual-color immnunostaining was performed and conclusions were made based on morphology and location of the MHC class II^+ cells (Cobb and Watson 1995). Macrophages are limited to the endometrial stroma (both deep and shallow endometrium). They are absent in the luminal or

glandular epithelia and accumulation of macrophages around the uterine glands is rare (Cobb and Watson 1995). Around day 20 of pregnancy, the number of intraepithelial $CD5^+$ cells decreases in the bovine endometrium (Vander and King 1984). In mid and late pregnancy, the bovine endometrium experiences an increase of MHC class II^+ cells in the interplacentomal compared to placentomal endometrium (Low et al. 1990).

In sheep, macrophages are very abundant in endometrial stroma during mid-to-late pregnancy (Tekin et al. 2004). In a unilateral pregnancy model, in which the conceptus was surgically restricted to one uterine horn, accumulation of macrophages occurred in both pregnant and nonpregnant uterine horns (Tekin and Hansen, 2004). This result is interpreted to mean that systemic signals are involved in macrophage recruitment to the uterus. However the number of macrophages in the nonpregnant horn was lower than in the pregnant horn indicating that local signals are also important as well. Progesterone alone is not a signal for accumulation of macrophages because progesterone treatment of ovariectomized ewes did not cause macrophage accumulation in the endometrium (Tekin and Hansen 2004).

There are no reports on the presence of $\gamma\delta$ T-cells in the bovine endometrium. In sheep, these cells are major components of the immune cell population in the uterus during pregnancy. There is an increase in number of $\gamma\delta$-T-cells in the endometrium after day 50 of pregnancy with cells being mainly localized within the luminal epithelium (Leslie and Hansen 1991, Lee et al. 1992; Meuseen et al. 1993). It has been speculated that $\gamma\delta$ cells promote a tolerogenic microenvironment by secretion of cytokines (Heyborne et al. 1994) or directly regulating cells activated against fetal antigens (Suzuki et al. 1995).

The presence of endometrial masT-cells has been reported in cows and heifers (Galleotti et al. 1997). They are present in all uterine layers, and they are higher in number in the shallow stroma of the endometrium than elsewhere (Galleotti et al. 1997). MasT-cells and eosinophils are present immediately beneath the uterine epithelium during diestrus in heifers (Galleotti et al. 1997) and increase in number at estrus (Tibbits et al. 1989). They may be there to protect the uterus against infection during mating.

It is not known if the bovine endometrium experiences an influx of NK cells during normal pregnancy as shown to occur in mice and humans (Croy et al. 2006; Moffett and Loke 2006). In fact, the discovery of bovine NK cells was only made a few years ago, by Storset et al. (2004) when they used a human antibody against natural cytotoxicity triggering receptor 1 also called NKp46 or CD335. The bovine NKp46^{+} cells has typical, large granular lymphocyte morphology, is able to proliferate in response to bovine IL2, and develops lytic activity after IL2 stimulation in vitro (Storset et al. 2004). The presence of NK cells (as indicated by detection of the NKp46 antigen) was described in the caruncular septa of the placentome after experimental infection of pregnant cows with Neospora canis (Rosbottom et al. 2008). In the uterus of ruminants, the only functional evidence for the presence of NK cells comes from an experiment by Tekin and Hansen (2004); where they found lymphokine-activated killer function in epithelial uterine lymphocytes after stimulation with IFNG and interferon tau (IFNT)

Regulatory Molecules Affecting Endometrial Immune Function

Pregnancy is marked by the production of systemic and locally-acting factors that can affect the maternal immune response. Whether there are systemic effects of pregnancy on the maternal immune system in the cow is not clear. Reports showing the decreased lymphocyte proliferation in vitro from cows during mid-pregnancy (Manak

1982) are in contradiction with a variety of experiments showing no difference in lymphocyte proliferation comparing pregnant and nonpregnant cows (Winter et al. 1986). The expression of IFNT by the embryo at the early implantation period modulates expression of interferon stimulated genes (ISGs) such as ISG15 in peripheral blood (Han et al. 2006). Nonetheless, most regulation of immune responses during pregnancy is probably at the maternal-fetal interface by locally acting factors.

Progesterone

Progesterone is essential for maintenance of pregnancy in mammals. In cattle, the major source is the corpus luteum with some placental synthesis late in pregnancy (Senger 2003). The importance of progesterone as an immunomodulatory molecule is indicated by studies where rejection of allografts placed into the uterus of the sheep can be delayed by treatment with progesterone (Hansen et al. 1989).

Among its many actions, progesterone is immunomodulatory and can directly inhibit lymphocyte proliferation (Low and Hansen 1988; Monteroso and Hansen 1993). The concentrations required for inhibition are in the micromolar range and it is likely, therefore, that progesterone synthesized locally by the placenta could reach such high concentrations but that effects on peripheral blood leukocytes would be absent because concentrations of progesterone in the blood are 100-fold less than needed for inhibition.

In addition to its direct effects on lymphocytes, progesterone can also induce synthesis of endometrial molecules that act locally to inhibit lymphocyte proliferation. Treatment of ovariectomized cows with progesterone increased lymphocyte inhibitory activity in uterine flushes as compared with uterine flushes from control cows (Lander et al. 1990). One progesterone induced protein is the uterine serpin or SERPINA14 (Leslie and Hansen 1991). SERPINA14 is secreted by the endometrial glands and, in

sheep, showed antiproliferative properties on lymphocytes and cancer cells (Padua and Hansen 2008).

Prostaglandins

In the cow, the sources of prostaglandin E_2 (PGE2) are the endometrium, trophoblast and, in early pregnancy, embryo (Shemesh et al. 1979; Scodras et al. 1990). Moreover, PGE2 can enhance the inhibitory capacity of human $CD4^+CD25^+$ Treg cells and induce a regulator phenotype in $CD4^+CD25^-$ T-cells (Baratelli et al. 2005). PGE2 inhibits lymphocyte proliferation in vitro (Low and Hansen 1988). Furthermore, PGE2 contributes to the suppression of dendritc cells differentiation and function through its capacity to inhibit IL-12 synthesis (Kalinski et al. 1999; Sombroek et al. 2002). Treatment of bovine peripheral blood leukocytes (PBL) with PGE2 downregulates *IL2* and *CSF2* mRNA levels (Emond et al. 1998). However, PGE2 stimulates CSF2 gene expression in PBL when the PBL is preconditioned with IFNT (Emond et al. 2000). The supplementation of CSF2 in the culture of preimplantation bovine embryo has been shown to increase posttranfer embryo survival (Loureiro et al. 2009).

Interferon-tau

Interferon-tau (IFNT) is an embryo-derived cytokine in the type I interferon family secreted by the trophoblast from day 15-25 of pregnancy that acts on the endometrium to inhibit endometrial synthesis of the luteolytic signal prostaglandin F2 alpha (PGF2A) and allow continued survival of the corpus luteum (reviewed by Spencer et al. 2004). In addition, IFNT inhibits lymphocyte proliferation in vitro (Skopets et al. 1992) and could contribute to maternal tolerance towards the conceptus in early pregnancy. However, IFNT does not affect the endometrial immune cell populations because there are no

differences in number of CD4 (T helper cell), CD21 (B-cells) and CD14 (monocyte/macrophages) cells in the endometrium of pregnant cows as compared to nonpregnant cows at Day 16 after artificial insemination (Leung et al. 2000). Nevertheless, analysis of the global transcriptome of bovine endometrium during the pre-attachment period demonstrated the upregulation of many interferon-stimulated genes that may be involved in embryo-maternal immune modulation including complement C1s subcomponent (*C1S*), complement C1r subcomponent (*C1R*), *SERPINA14* and bone marrow stromal antigen 2 (*BST2*) (Bauersachs et al. 2008).

Systemic effects on circulating leukocytes can be detected in response to IFNT. In particular, interferon stimulated gene 15 (*ISG15)* and myxovirus resistance 1, interferon-inducible protein p78 (*Mx1)* genes are upregulated in pregnant cows around day 20 after insemination compared to nonpregnant cows (Gifford et al. 2007), suggesting that are effects of IFNT.

Parturition

There is evidence that the periparturient dairy cow is immunosuppressed with a decline in numbers of $CD4^+$, $CD8^+$ and γδ-T-cells in peripheral blood (Van Kampen and Mallard 1997; Kimura et al. 1999; 2002) and reduced proliferation and interferon-gamma (IFNG) secretion by mitogen-stimulated lymphocytes (Detilleux et al. 1995; Nonnecke et al. 2003). These findings have not always been replicated. Karcher et al. (2008), for example, found a tendency for an increase in the proportion of γδ-T-cells as parturition approached.

In the peripartum period, there is also impairment of function of circulating neutrophils with respect to phagocytosis and oxidative function (Kehrli et al. 1989; Kehrli and Goff 1989; Cai et al. 1994; Detilleux et al. 1995).

Evidence for immunological participation in the parturition process is indicated by the increased incidence of retained placenta in cows which share major histocompatability class I antigens with their conceptus (Joosten et al. 1981). Also, chemotaxis and myeloperoxidase activities are reduced in circulating neutrophils from cows that had retained fetal membranes (Kimura et al. 2002). This reduced neutrophil activity was detected before parturition and it lasted until 1 to 2 weeks after parturition (Kimura et al. 2002). Moreover, retained placenta has been correlated with decreased activity of macrophages in the caruncular area (Miyoshi et al. 2002).

Synopses and Objectives

The goal of the research described in this dissertation is to test the thesis that endometrial macrophages accumulate during pregnancy in the cow, and possess an M2 phenotype to promote a microenvironment at the maternal-fetal interface that inhibits maternal immune responses against the conceptus and supports fetal development and growth. To address this objective, three working hypotheses were tested:

1. There is accumulation of macrophages in the endometrium during pregnancy.
2. Endometrial macrophages in the pregnant cow possess macrophage markers that include those indicative of M2 activation (i.e., an immunosuppressive or tolerogenic function) rather than characteristic markers of activation via the classical pathway that promotes cytotoxic immune responses.
3. Residency of macrophages in the uterine endometrium of the pregnant cow results in upregulation of genes related to M2 activation.

CHAPTER 3
DEVIATIONS IN POPULATIONS OF PERIPHERAL BLOOD MONONUCLEAR CELLS AND ENDOMETRIAL MACROPHAGES IN THE COW DURING PREGNANCY

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, hemotropic placenta in which trophoblast invades the endometrium. Among the changes in immune function during pregnancy are an increase in circulating regulatory T cells (Treg), defined as $CD4^+CD25^+FoxP3^+$ cells, which act to downregulate T lymphocyte function (Aluvihare et al. 2004; Somerset et al. 2004; Yang et al. 2007), a temporary anergy of maternal lymphocytes to conceptus MHC class I antigens (Tafuri et al. 1995), and synthesis of immunosuppressive proteins at the maternal-fetal interface including IL10 (Hanna et al. 2000; Murphy et al. 2005a) and TGFB (Suzuki et al. 1995; Gorivodsky et al. 1999; Simpson et al. 2002). In addition, pregnancy is characterized by an increase in numbers of specific leukocyte populations within the uterus including macrophages (Hunt et al. 1985; Heikkinin et al. 2003 Cupurdija et al. 2004; Kim et al. 2007), $\gamma\delta$T cells (Heyborne et al. 1992; Mincheva-Nillson et al. 1992, 1997) and 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 TGFB ($\gamma\delta$T cells - Suzuki et al. 1995;

Nagaeva et al. 2002; NK cells - Murphy et al. 2005b) 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 hemotropic 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 and 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 likened to the immunological relationship between commensal bacteria and host organisms – with little immunological recognition occurring unless the epithelial barrier is breached (Moffett and 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 numbers of macrophages in stroma (Tekin and 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 activity towards paternal antigens in pregnant mares (Baker et al. 1999) and there is accumulation of cells with non-cytotoxic 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 changes in immune function during pregnancy with a view to test whether 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 numbers of the latter two cell types reflects recruitment to the uterus. Identification of 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 altered immune function in the periparturient period when the female is susceptible to uterine infection and other immune challenges.

Materials and Methods

Materials

Tissue Culture Medium-199 (TCM-199), normal goat serum, bovine serum albumin (BSA) Fraction-V, Dulbecco's phosphate buffered saline (DPBS) and Hoescht 33342 were purchased from Sigma-Aldrich (St. Louis, MO, USA). 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 647mouse IgG2a, labeling kit and the mounting medium (Prolong® Antifade Kit) were obtained from Invitrogen Molecular Probes (Eugene, OR, USA). 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 γδT (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 μg /mL) was obtained from Biomeda (Foster City, CA, USA), mouse anti-bovine CD14 (clone MM61A, clarified ascites fluid, 10 μg /mL), mouse anti-bovine CD4 (clone CATC 138A; clarified ascites fluid, 10 μg /mL), and mouse anti-bovine bovine CD25 (clone CATC 108A; clarified ascites fluid, 10 μg /mL) were from VMRD (Pullman, WA, USA). Control mouse ascites fluid (clarified, clone NS1) was from Sigma-Aldrich (St Louis, MO, USA). Normal goat serum was purchased from Pel-Freez Biologicals (Rogers, AR, USA). The Histoscan Monoclonal Detector kit and mounting medium were obtained from Biomeda. Tissue freezing medium was obtained from Biotech Medical Corporation (Kuala Lampur, Malaysia). Lab-Tek® Glass Chamber Slides™ were obtained from Electron Microscopy Sciences (Hatfield, PA, USA).

Flow Cytometric Analysis of Peripheral Blood Mononuclear Cells

Animals. Cows were maintained at the University of Florida Dairy Research Unit at Hague, Florida. The first experiment was designed to determine differences in peripheral blood lymphocyte populations between nonpregnant and pregnant cows at Day 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 days after calving (range = 127-389). Cows

were examined for pregnancy using transrectal ultrasound at Day 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 non-pregnant and 15 pregnant cows.

The second experiment was designed to determine differences between nonpregnant and preparturient cows. A coccygeal blood sample was collected from a total of 8 nonpregnant cows that were non-lactating and at random stages of the estrous cycle and from 8 pregnant cows that were nonlactating 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 Peripheral Blood Mononuclear Cells (PBMC)

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

Flow cytometry analysis. 5×10^6 cells were placed into 13 x 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. Cells were 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 at 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 R-phycoerythrin and Alexa 647 using the Zenon® Mouse Labeling IgG kits as per manufacturer's instructions. The labeled antibody complex was then diluted in antibody staining buffer to a final concentration of 10 μg/mL at room temperature for 30 min. After incubation, samples were washed with 2 mL staining buffer, and resuspended with DPBS containing 4% (w/v) paraformaldehyde for fixation. Before analysis, cells were washed once with 1 mL of staining buffer and resuspended in 300 μL 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.

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 fetal age (Noden and Lahunta, 1985). 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 54-240 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 manufacturer's instructions. Briefly, sections were sequentially incubated with peroxidase blocking buffer [histochemistry buffer with 0.3% (v/v) H_2O_2] 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 30min, and hematoxylin (as supplied in the kit) for 2 min. Slides were washed under tap water, cover slips were mounted, and 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 reproductive stage.

Two-color Immunofluorescence for CD68 and CD14

Uterine sections were prepared as described above. Sections were incubated with blocking buffer [DPBS supplemented with 20% (v/v) goat serum] for 20 min followed by incubation overnight at 4^{o}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 before. 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, coverslips mounted using Prolong® Antifade reagent and slides examined using a Zeiss Axioplan 2 epifluorescence microscope (Zeiss, Gottingen, Germany) with a 40x 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 Adherent Cells from Endometrium

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 at a local abattoir and transported to the lab 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 1h 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 cell number was determined using a hemacytometer. The cell suspension was placed into 8-well sterile chamber slides (Lab-Tek® Glass Chamber Slides™; Electron Microscopy Sciences, Hatfield, PA, USA) 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. Wells were washed three times in DPBS and two-color immunoflourescence staining was performed as described above.

Statistical Analysis

Data were analyzed by least square analysis of variance using the General Linear Models procedure of SAS (SAS for Windows, version 9.3, SAS Institute Inc., Cary, NC, USA). For the flow cytometry studies, the model included effect of physiological status (pregnant vs nonpregnant), date samples were collected (i.e, replicate) and the interaction. For intensity of CD68 staining in endometrium, cows were grouped into 4 groups based on stage of pregnancy (nonpregnant, n=7; Day 54-100 of pregnancy, n=7; Day 101-200 of pregnancy, n=8 and Days 201-240 of pregnancy, n=5). The mathematical model included the effect of stage of pregnancy.

Results

Representative Flow Cytometry Patterns

Typical dot plots are shown in Figure 3-1. Analysis of PBMC by side scatter and forward scatter resolved cells into two populations (Figure 3-1A). The first, more abundant population was of small size and little granularity, and represents lymphocytes

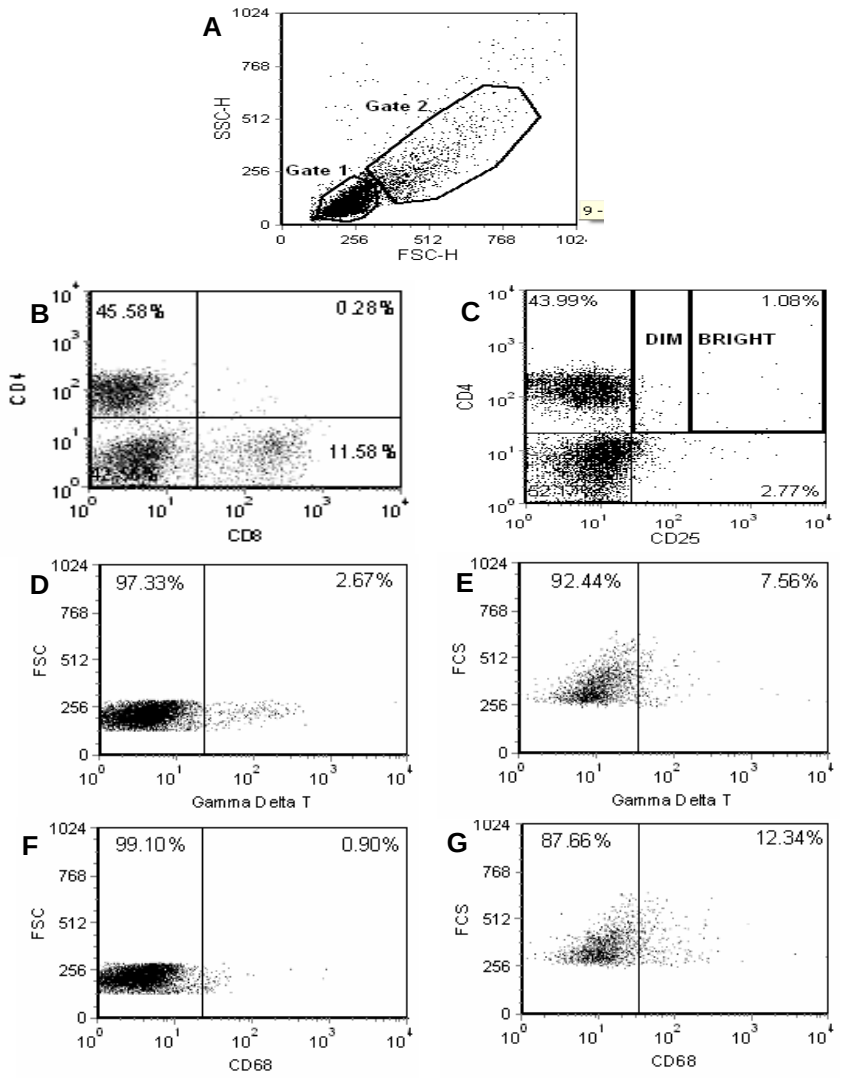

Figure 3-1. Representative acquisition dot plots to analyze subpopulations of bovine peripheral blood mononuclear cells. (A) Forward (x-axis) and side (y-axis) scatter plots to analyze cells on the basis of size (x-axis) and granularity (y-axis). The lymphocyte (gate 1) and monocyte (gate 2) gates are 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 γδT 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 second population, which represents primarily monocytes, was of large size and – a $CD25^{bright}$ and a $CD25^{dim}$ population (Figure 1C). There were, however, no treatment differences in the percent of $CD25^+$ cells that were bright and dim. Therefore, data were analyzed after pooling both populations.

Differences between Pregnant and Nonpregnant Cows at Day 33-34 of Gestation in Subpopulations of PBMC

There were no differences between pregnant and nonpregnant cows in the proportions of PBMC in the lymphocyte gate that were positive for CD4 (Fig 3-2A) and CD8 (Fig 3-2A). Pregnant cows had, however, a higher ($P<0.06$) percent of PBMC that were $CD25^+$ (Fig 2A; $P<0.06$). Moreover, the percent of $CD4^+$ cells that were also positive for CD25 was higher ($P<0.05$) for pregnant cows (Fig 3-2C).

There was no effect of pregnancy status on the proportion of PBMC in the lymphocyte gate and monocyte gate that were $\gamma\delta T^+$ and $CD68^+$ cells (Fig 3-2D and 3-2E).

Differences between Preparturient and Nonpregnant Cows in Subpopulations of PBMC

There was no effect of pregnancy status on the percent of cells in the lymphocyte gate that were positive for CD8 (Fig 3-3A). However, the percent 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 3-3A and 3-3B). The increase in number of $CD4^+CD25^+$ cells represents an increase in $CD4^+$ cells rather than an increase in the proportion of $CD4^+$ cells that were $CD25^+$ because the number of $CD4^+$ that were $CD25^+$ cells did not change with pregnancy status (Fig 3-3C). Preparturient cows also had a greater proportion of cells in the lymphocyte gate that were $\gamma\delta T^+$

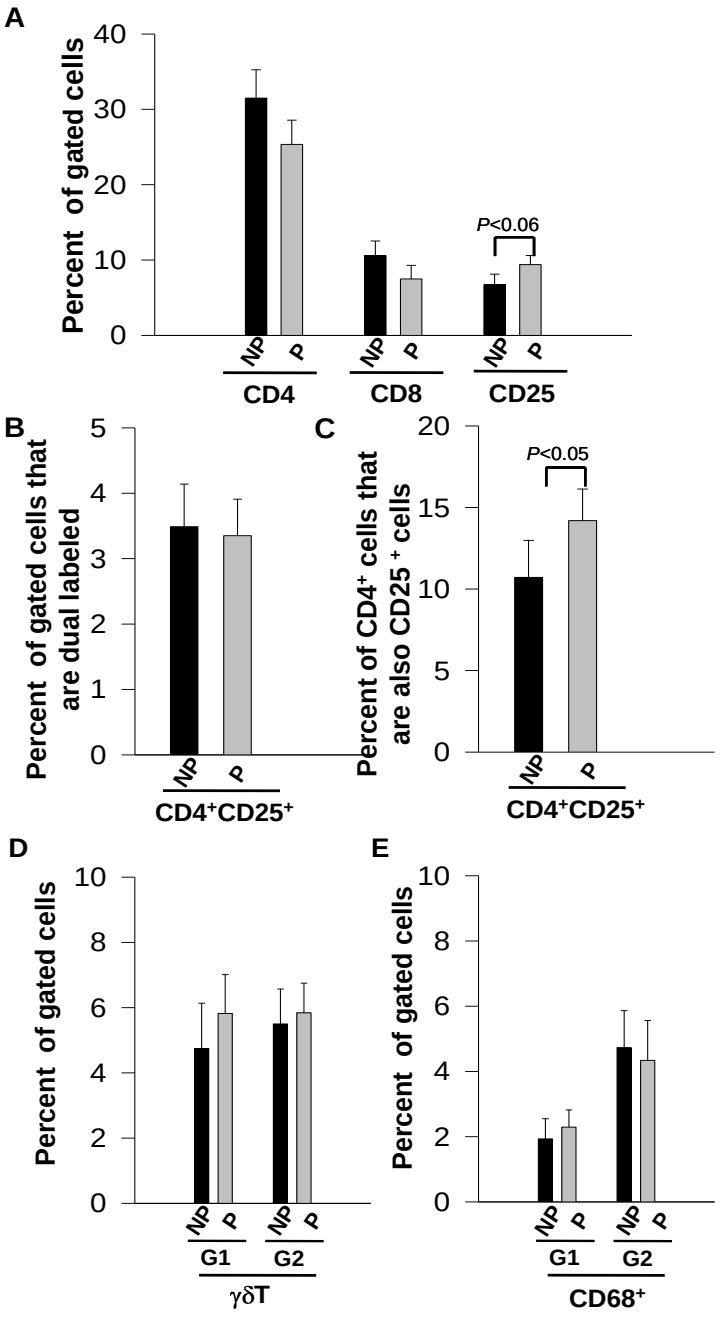

Figure 3-2. Effect of pregnancy status at days 33–34 after insemination on populations of peripheral blood mononuclear cells as determined by flow cytometry. Data are least-squares means±S.E.M. for results for nonpregnant (NP) and pregnant cows (P). (A) Percentage of cells in the lymphocyte gate positive 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 T. (E) Percentage of cells in the lymphocyte gate (G1) and monocyte gate (G2) that are positive for CD68.

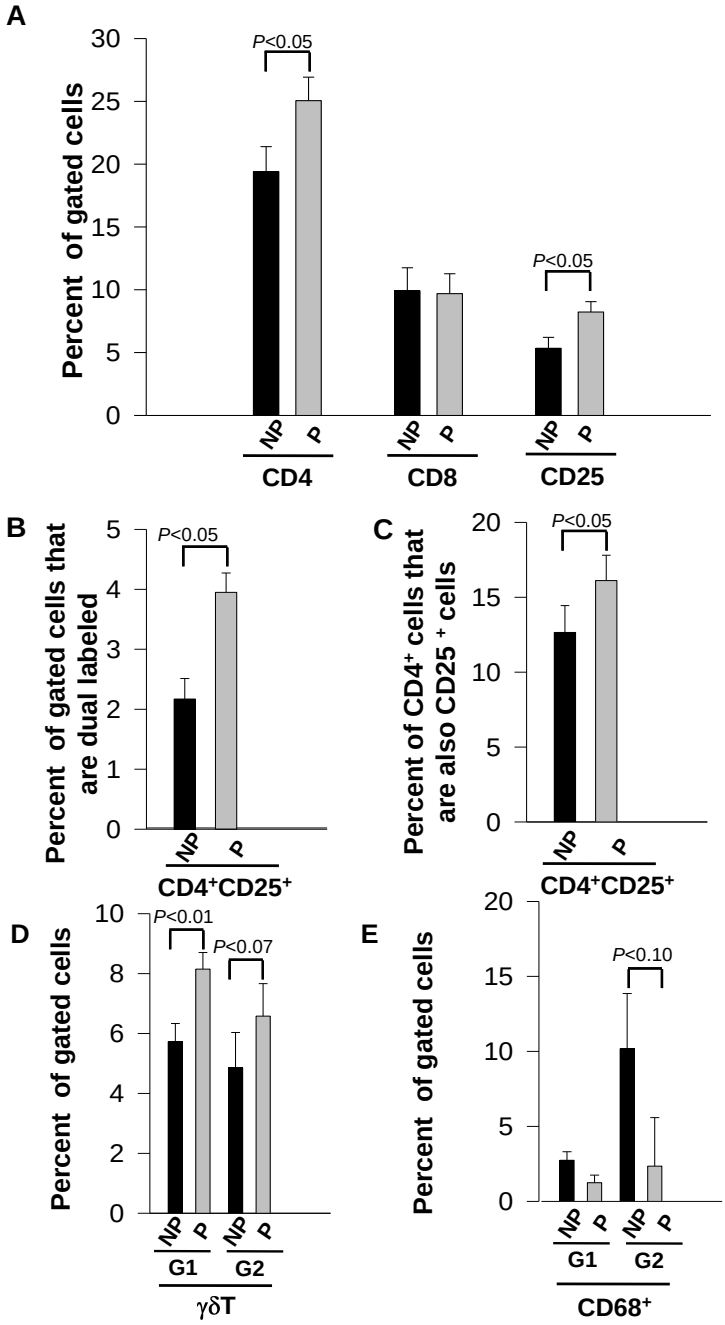

Figure 3-3. Differences in peripheral blood mononuclear cell populations between nonpregnant (NP) and preparturient cows (P) as determined by flow cytometry. Data are least-squares means ± SEM. A) Percent of cells in the lymphocyte gate positive for CD4, CD8, and CD25. B) Percent of cells in the lymphocyte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

(P<0.01) and a tendency for a greater proportion of cells in the monocyte gate that were γδT cells (P<0.07) (Fig 3-3D). 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 3-3E).

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 3-4B and 3-4C). A fewer number of positive cells were present in the submucosa. There were no $CD68^+$ cells in luminal or glandular epithelia. In contrast to the pattern in pregnancy, there were very few $CD68^+$ cells in endometrium from nonpregnant cows (Fig 3-4A). The pattern of $CD14^+$ cells was very similar to that for CD68 (figure 3-4D).

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 pregnancy status effects on numbers 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 3-5A) and submucosa (Fig 3-5B).

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 3-6). In the second experiment, two color immunofluorescent labeling of single cell preparations of adherent endometrial cells revealed that cells that labeled with CD14 were also labeled with CD68 (Fig 3-7).

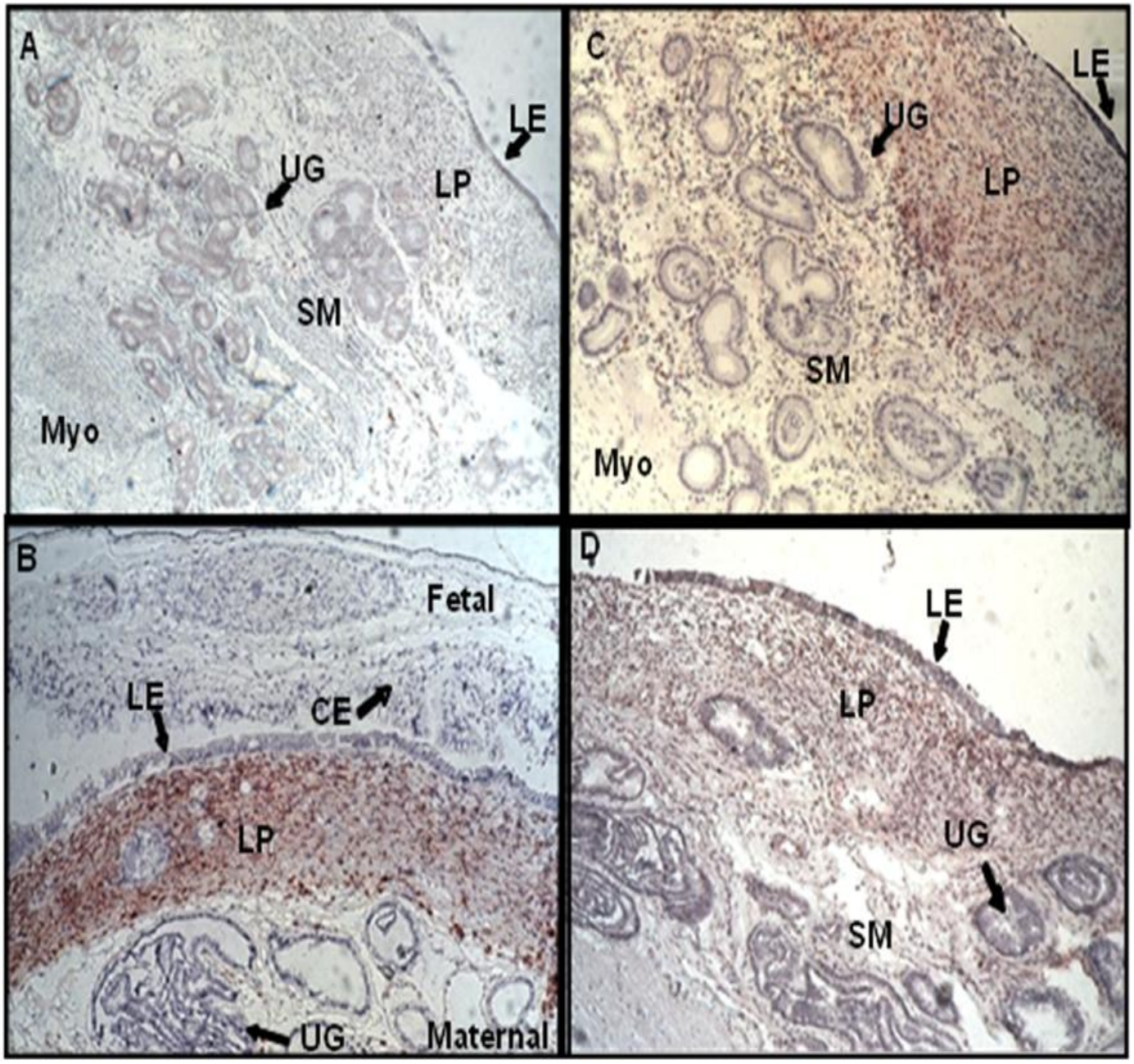

Figure 3-4. Immunolocalization of cells positive for (A–C) CD68 and (D) CD14 in bovine endometrium. Represented are sections of endometrium from a nonpregnant cow (A) and pregnant cows at days 75 (C) and 209 (B and D) of pregnancy. Note that fetal chorion has been retained for panel B only. Brown, immune reaction product; blue, hematoxylin counterstain. LE, luminal epithelium; LP, lamina propria; Myo, myometrium; UG, uterine gland; SM, submucosa; CE, chorionic epithelium.

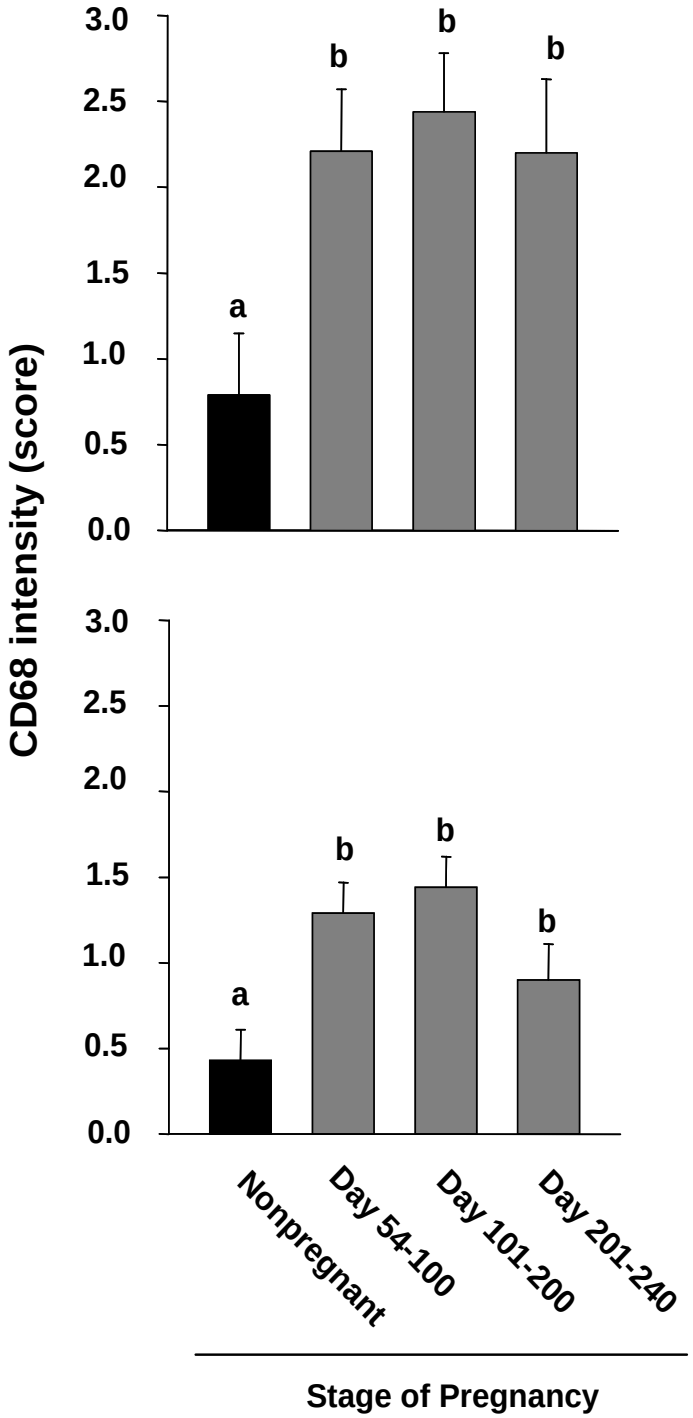

Figure 3-5. Effect of pregnancy status on the intensity of staining for CD68 in the lamina propria (A) and submucosa (B) of the endometrium. Shown are data for nonpregnant cows (n=7) and pregnant cows at days 54–100 (n=7), 101–200 (n=8), and 201–240 of pregnancy (n=5). Intensity was estimated subjectively using a scoring system from 0 to 5. Data are least-squares means±S.E.M. Bars with different letters are different from each other ($P<0.05$).

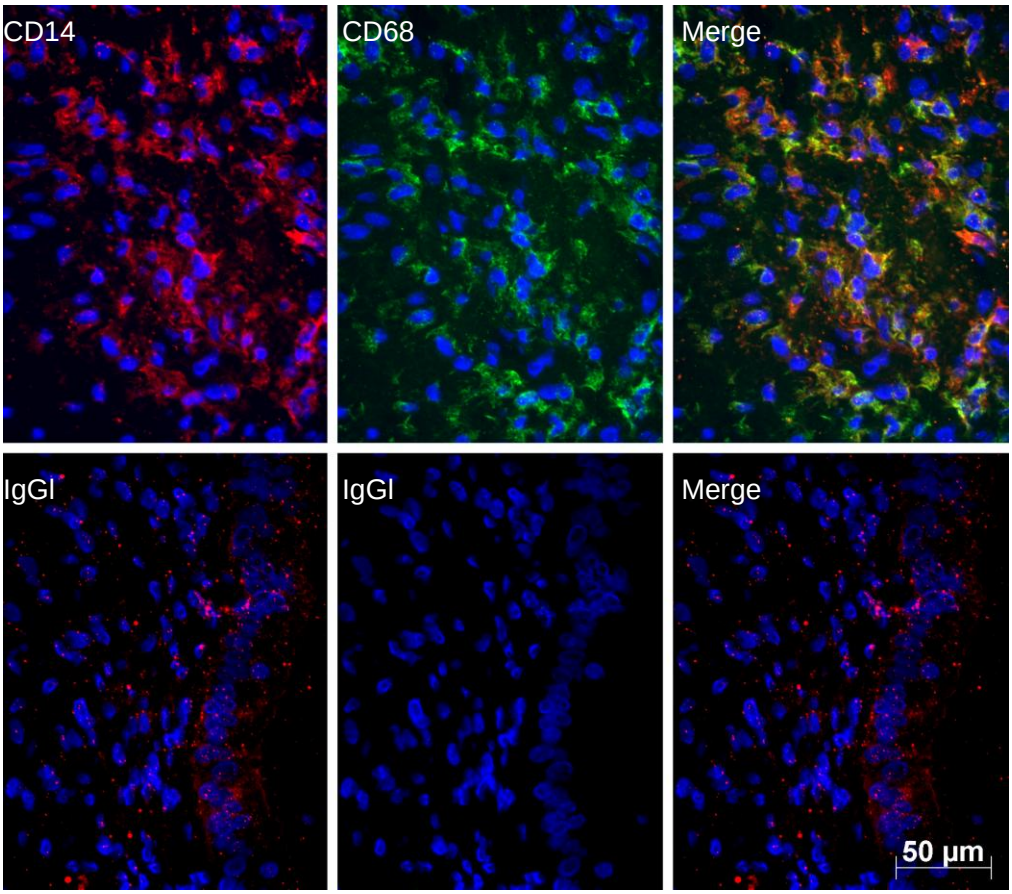

Figure 3-6. Co-localization of CD68 and CD14 in endometrium at day 209 of pregnancy. The top panels represent sections that were dual labeled with anti-CD14 (red) and anti-CD68 (green). Blue indicates nuclear stain. The bottom panels represent sections incubated with red- and green-labeled isotype controls.

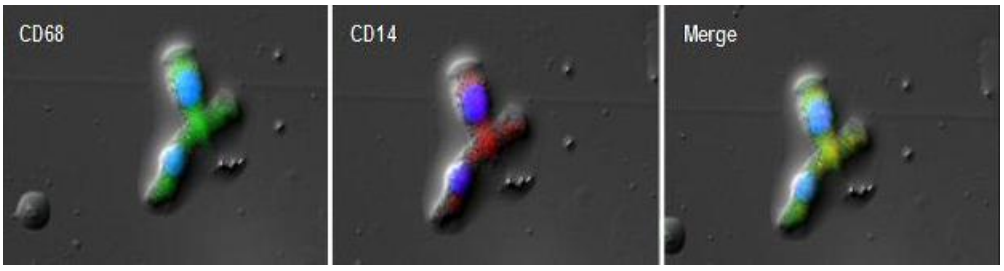

Figure 3-7. Co-localization of CD68 and CD14 in adherent cells isolated from dispersed endometrial cells from a pregnant cow at day 166 of pregnancy. Cells were labeled with anti-CD14 (red), anti-CD68 (green), and Hoescht 33342 (blue) for nuclei. Photomicrographs represent images obtained using differential 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).

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 Day 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 Treg cells described as increasing during pregnancy in mice and humans (Somerset et al. 2004; Aluvihare et al. 2004), were also present in higher amounts in preparturient cows as compared to 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 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 (Figure 3-4).

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 (Moffatt and Loke 2006), but one characterized by immunological adaptation.

At both Day 33-34 and late pregnancy, there were changes in the population of $CD4^+CD25^+$ cells in periheral blood. At Day 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 relative numbers of $CD4^+$ in the periphery rather than in 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 forkhead box P3 (*FOXP3)* is increased in peripheral blood during pregnancy (Somerset et al. 2004; Aluvihare et al. 2004). These cells have been identified as Treg cells that can secrete cytokines such as IL4 that inhibit activation of cytotoxic T cells against alloantigens (Mjösberg et al. 2007). The importance of Treg cells for pregnancy success is indicated by the observation that the percent of $CD4^+CD25^+$ cells in PBMC is reduced in women with unexplained recurrent spontaneous abortion (Yang et al. 2007). In mice, depletion of $CD25^+$ cells decreased the ability 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 Treg 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 tau (IFNT) as the trophoblast signal that inhibits endometrial prostaglandin F2α synthesis and allows prolonged lifespan of the corpus luteum (Roberts et al. 2003). IFNT has retained its immunomodulatory properties and changes in expression of interferon-stimulated genes have been described in PBMC in cattle as early as Day 16 of gestation (Han et al. 2006, Gifford et al. 2007). By Day 32 of pregnancy, expression of interferon stimulated gene 15 in blood

cells was similar to that for non-pregnant cows (Han et al. 2006) but it cannot be ruled out that changes in PBMC seen at Day 33-34 in the present study were caused by IFNT secretion by the conceptus earlier in pregnancy.

It was hypothesized that the relative numbers of γδT cells in peripheral blood would decline during pregnancy, especially in the preparturient period, because of recruitment to the uterus. Indeed, accumulation of γδT cells to 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; Meussen et al. 1993; Majewski et al. 2001). In contrast to our hypothesis, the relative numbers of γδT cells in peripheral blood was not affected by pregnancy status at Day 33-34 after insemination and was higher for preparturient cows than for non-pregnant cows. An increase in relative numbers of γδT cells in peripheral blood 3 weeks before parturition followed by a decrease to initial values at the time of parturition has been seen by others (Van Kampen and Mallard 1997).

The recruitment of macrophages to the pregnant uterus has been described in many species such as in humans (Heikkinen et al. 2003; Cupurdija et al. 2004; Kim et al. 2007), mice (Hunt et al. 1985) and sheep (Tekin and Hansen 2004). Present results indicate that this process occurs in the cow, also, with numbers increasing by Day 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 local differentiation once resident in the endometrium is not known. Mouse trophoblast can cause migration of blood monocytes in vitro (Fest et al. 2007) and change monocyte cytokine prolife and activation in response to lipopolysaccharide.

The accumulation of large numbers 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 placenta in cows which share major histocompatibility class I antigens with their conceptus (Joosten et al. 1981). 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 numbers of $CD4^+$, $CD8^+$ and $\gamma\delta$T cells in peripheral blood (Van Kampen and Mallard 1997; Kimura et al. 1999; 2002) and reduced proliferation and IFNG 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 compared with nonpregnant cows and numbers of $CD4^+$ and $\gamma\delta$T cells were increased. Karcher et al. (2008) also did not observe declines in $CD8^+$ cells as parturition approached and 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 numbers of $CD68^+$ cells. It is possible, however, that the increase in numbers 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 Treg cells.

Moffett and Loke (2006) have likened 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 Day 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 during pregnancy, 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 et al. 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 and Carter 2007) rather than to change the fundamental characteristics of the maternal-fetal immunological relationship.

CHAPTER 4
PHENOTYPIC CHARACTERIZATION OF MACROPHAGES IN THE ENDOMETRIUM OF THE PREGNANT COW

Introduction

Recruitment of macrophages to the endometrium is a characteristic of pregnancy in many species including the mouse (Hunt et al. 1985); human (Heikkinen et al. 2003; Cupurdija et al. 2004; Kim et al. 2007) cynomologus and vervet monkeys, (Dambaeva et al. 2008), sheep (Tekin and Hansen 2004) and cattle (Oliveira and Hansen 2008). One function of endometrial macrophages may be to serve as sentinels to prevent pathogens from establishing infection or to clear dead cells. Endometrial macrophages increase in number in caruncular septa after experimental infection with Neospora caninum in the pregnant cow (Rosbottom et al. 2008). Endometrial macrophages could also be key regulators of the immunological interaction between mother and conceptus. Human decidual $CD14^+$ cells express low levels of the costimulatory molecule CD86 as compared to cells in blood (Heikkinen et al. 2003) and spontaneously express alternative activation markers interleukin -10 (IL10), stabilin-1 and coagulation factor XIIIa. (Heikkinen et al. 2003; Kim et al. 2007). The comparison of the global transcriptome of circulating $CD14^+$ cells to decidual $CD14^+$ cells in human revealed that decidual $CD14^+$ have a distinct phenotype and express genes for regulatory proteins such as DC-SIGN (Gustafsson et al. 2008). A population of DC-SIGN positive macrophages also is present near the site of placental attachment in early pregnancy is also present in cynomologous and vervet monkeys (Dambaeva et al. 2008). It also has been suggested that endometrial macrophages regulate the concentrations of placental lactogen at the uteroplacental interface by binding of placental lactogen to macrophage stabilin-1 and subsequent processing of the protein (Kzhyshkowska et al. 2008). A role

in parturition is also possible because macrophages accumulate in the uterus near parturition (Mackler et al. 2000; Thomson et al. 1999).

The signal or signals that drive the accumulation and differentiation of macrophages in the pregnant endometrium remain unclear. Using the unilaterally-pregnant sheep as a model, there was evidence for both local and systemic signals for macrophage accumulation in the interplacentomal endometrium during pregnancy (Tekin and Hansen 2004). There is a variety of cytokines produced by the uterus or placenta that can regulate monocyte or macrophage differentiation. In the cow, these include transforming growth factor-β (Ravelich et al. 2006*)*, *IL-10*, *IL-12*, *IL-18*, *TNF* and *IFNG* (Rosbottom et al. 2008).

In ruminants, the nature of the relationship between mother and fetus varies regionally within the placenta. Most gas and nutrient exchange occurs at specialized structures called placentomes where fetal villi are interdigitated with maternal caruncular septa. In the intervening interplacentomal areas, the chorioallantois is apposed to the endometrial luminal epithelium. A synctium forms in the endometrial epithelium due to migration of fetal binucleated cells and their fusion with maternal epithelium (Wooding 1982). The immunogenicity of the placenta differs between placentomal and interplacentomal sites. Chorionic tissue in the placentome is negative for MHC class I expression throughout pregnancy while chorionic tissue in the interplacentomal regions can express MHC class I in the third trimester of pregnancy (Low et al. 1990; Davies et al. 2004). MHC class I proteins on the chorion are predominately encoded by non-classical MHC class I genes (Davies et al. 2006). These non-classical MHC class I

proteins are possibly analogous to human leukocyte antigen HLAG that can regulate natural killer cell and macrophage function (Li et al. 2009)

In this study, we hypothesized that the endometrial macrophage in the placentome has a different pattern of differentiation than the endometrial macrophage in the interplacentomal region. To identify macrophages, we used antibody to CD68, a lysosomal-associated protein that is expressed on monocytes, macrophages and dendritic cells (Ferenbach 2008). Expression of three markers by $CD68^+$ cells in the endometrium were evaluated to determine whether regional differentiation of endometrial macrophages occurs. The first marker examined was CD14, a co-receptor for bacterial lipopolysaccharide expressed on monocytes, macrophages and neutrophils (Paape et al. 1996; Triantafilou et al. 2002). CD14 is expressed on most interplacentomal $CD68^+$ cells in the pregnant cow (Chapter 3). The second was CD11b (β2 integrin) that, with CD18, composes the macrophage complex -1 (Mac-1) that is involved in leukocyte-endothelial adhesion (Fagerholm et al. 2006). The third molecule was MHC class II, which is involved in antigen presentation, and can be upregulated in activated macrophages and downregulated in inactivated macrophages (Gordon 2003).

Materials and Methods

Materials

Mouse anti-human CD68 (clone EBM11; ascites, 2.3 μg /mL) was obtained from Dako (Carpinteria, CA, USA) (Tekin and Hansen 2004), mouse anti-bovine CD14 (clone MM61A, ascites, 10 μg /mL) (Miyoshi et al. 2002), mouse anti-bovine MHC class II [a mixture of and equal mass of anti-DR alpha (clone TH14B) and an anti-DQ (clone TH81A5); ascites, 1 mg /mL] (Cannon and Pate 2003) and mouse anti-human CD11b

(clone MM12A; ascites, 1 mg /mL) (souza et al. 2008) were from VMRD (Pullman, WA, USA). IgG controls matching the isotype of each antibody used were obtained as control mouse ascites fluid (Sigma-Aldrich, St Louis, MO, USA). The Zenon Alexa Fluor labeling kit and the Prolong® Antifade mounting medium were obtained from Invitrogen (Eugene, OR, USA).

The antibodies, including the IgG control, were tagged with fluorescently-labeled fragment antigen-binding (Fab) fragments against mouse IgG conjugated using the Zenon® Mouse Labeling IgG kits as per manufacturer's instructions. The anti-CD68 was labeled with Alexa Fluor 488, the anti-CD14 was labeled with Alexa Fluor 488 or Alexa Fluor 594 for immunofluorescence and with Alexa Fluor R-Phycoerythrin for flow cytometry, and anti-bovine MHC class II and anti-CD11b were labeled with Alexa Fluor 594.

Normal goat serum and fetal bovine serum were purchased from Sigma-Aldrich or Pel-Freez Biologicals (Rogers, AR, USA). The tissue freezing medium (Tissue-Tek OCT) was purchased from Sakura Finetek USA, Inc. (Torrance, CA).

Tissue Culture Medium-199 (TCM-199), bovine serum albumin (BSA) Fraction-V, Dulbecco's phosphate buffered saline (DPBS), collagenase type I and Hoescht 33342 were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Tissues

Uteri were obtained from pregnant cows of various breeds at a local abattoir. The uteri were transported to the lab on ice within 1.5 h after slaughter. Fetal crown-rump length was measured to estimate fetal age (Noden and Lahunta 1990). Tissues from a total of 20 pregnant cows (estimated fetal age 137 - 242 days; mean $\pm$ SD = 182.0 $\pm$ 37 days) were collected. Samples of interplacentomal endometrium and placentome

ipsilateral to the corpus luteum were snap-frozen in Tissue-Tek OCT embedding compound (see Noden and de Lahunta 1990; Davies et al. 2000) for representation of the anatomy of the bovine placenta highlighting features of interplacentomal and placentomal endometrium). Samples of lymph node, thymus and spleen were also collected from cows at Day 17 of pregnancy for use as positive controls.

Two-color Immunofluorescence

Tissue was processed for dual-color immunofluorescence by preparing 4 μm sections with a cryostat microtome. Sections were placed onto precleaned poly-L-lysine coated glass slides, fixed in ice-cold acetone for 10 min and air dried. The sections were rehydrated in phosphate buffered solution (PBS) at 4°C [0.01 M Na_2HPO_4, pH 7.4 containing 0.85% (w/v) NaCl].

Sections were incubated with blocking buffer [PBS containing 10% (v/v) goat serum] for 1 h followed by incubation overnight at 4°C with labeled anti-CD68 (2.3 μg/mL) as a pan-macrophage marker concomitant with either labeled anti-CD14 (10 μg/mL), anti-CD11b (10 μg/mL) or anti-MHC class II (10 μg/mL). Other sections were incubated with Zenon-labeled isotype controls (10 μg/mL). Sections were then washed three times for 5 min using PBS and incubated with Hoescht 33342 reagent [2.3 μg/mL in PBS] for 15 min. Sections were washed an additional three times for 5 min each, coverslips mounted using Prolong® Antifade reagent, and slides examined using a Zeiss Axioplan 2 epifluorescence microscope (Zeiss, Gottingen, Germany) with a 40x 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.

Dual- Color Flow Cytometric Analysis for CD68 and CD14 in Dispersed Endometrial cells

Two-color immunofluorescence was performed using preparations of dispersed endometrial cells from the interplacentomal endometrium of one pregnant cow at an estimated Day 166 of pregnancy. The uterus was obtained at a local abattoir and transported to the lab on ice. Interplacentomal endometrium was obtained by dissection. Tissue fragments were collected into a 50 mL sterile culture tube containing 30 mL Tissue Culture Medium (TCM)-199 supplemented with type I collagenase at 150 U/mL and 10% (v/v) fetal bovine serum. Cells were incubated at 37°C for 1 h under gentle rotation. Dispersed cells were then filtered through a sterile 200 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 cell number was determined using a hemacytometer.

5 x 10^6 cells were placed into 13 x 100 mm polyethylene tubes in staining buffer [Dulbecco's PBS 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. Cells were stained for dual-color flow cytometry analysis using Zenon-labeled anti-CD68 (2.3 μg/mL; tagged with Alexa Fluor 488) and anti-CD14 (10 μg/mL; tagged with Alexa Fluor R-phycoerythrin) diluted in antibody staining buffer. After incubation, samples were washed with 2 mL staining buffer, and resuspended with Dulbecco's PBS containing 4% (w/v) paraformaldehyde for fixation. Before analysis, cells were washed once with 1 mL of staining buffer and resuspended in 300 μl 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).

Gates for identification of positive cells were established by analyzing other aliquots of cells that were incubated with control IgG (tagged with Alexa Fluor 488 and Alexa Fluor R-phycoerythrin) at the same dilution as the primary antibodies described above.

Results

Localization of Cells Positive for CD68 and CD14

In interplacentomal regions, $CD68^+$ cells were abundant in the endometrial stroma (Figure 3-1b). Density of $CD68^+$ cells was lowest in stroma closest to the luminal epithelium and in the deepest regions of the stroma near glands (Figure 4-3b). Regardless of location, the majority of $CD68^+$ cells also expressed CD14 (Figure 4-1c and d). There were also many cells in the stroma, most prominently in the areas closest to luminal epithelium, that were positive for CD14 but either negative or weakly staining for CD68 (Figure 4-1c and d).

In the placentomal areas, $CD68^+$ cells and $CD14^+$ cells were present in the caruncular septa (maternal tissue) but were absent in fetal villi (Figure 4-1e and f). The majority of $CD68^+$ cells also expressed CD14 (Figure 4-1g and h).

Analysis by flow cytometry confirmed that the majority of $CD68^+$ cells in interplacentomal endometrium expressed CD14 and vice versa (Figure 4-2).

Expression of CD11b on Cells Positive for CD68

In the stroma of the interplacentomal endometrium, cells dual-labeled for CD68 and CD11b were present in deep stroma but most $CD68^+$ cells closer to the luminal

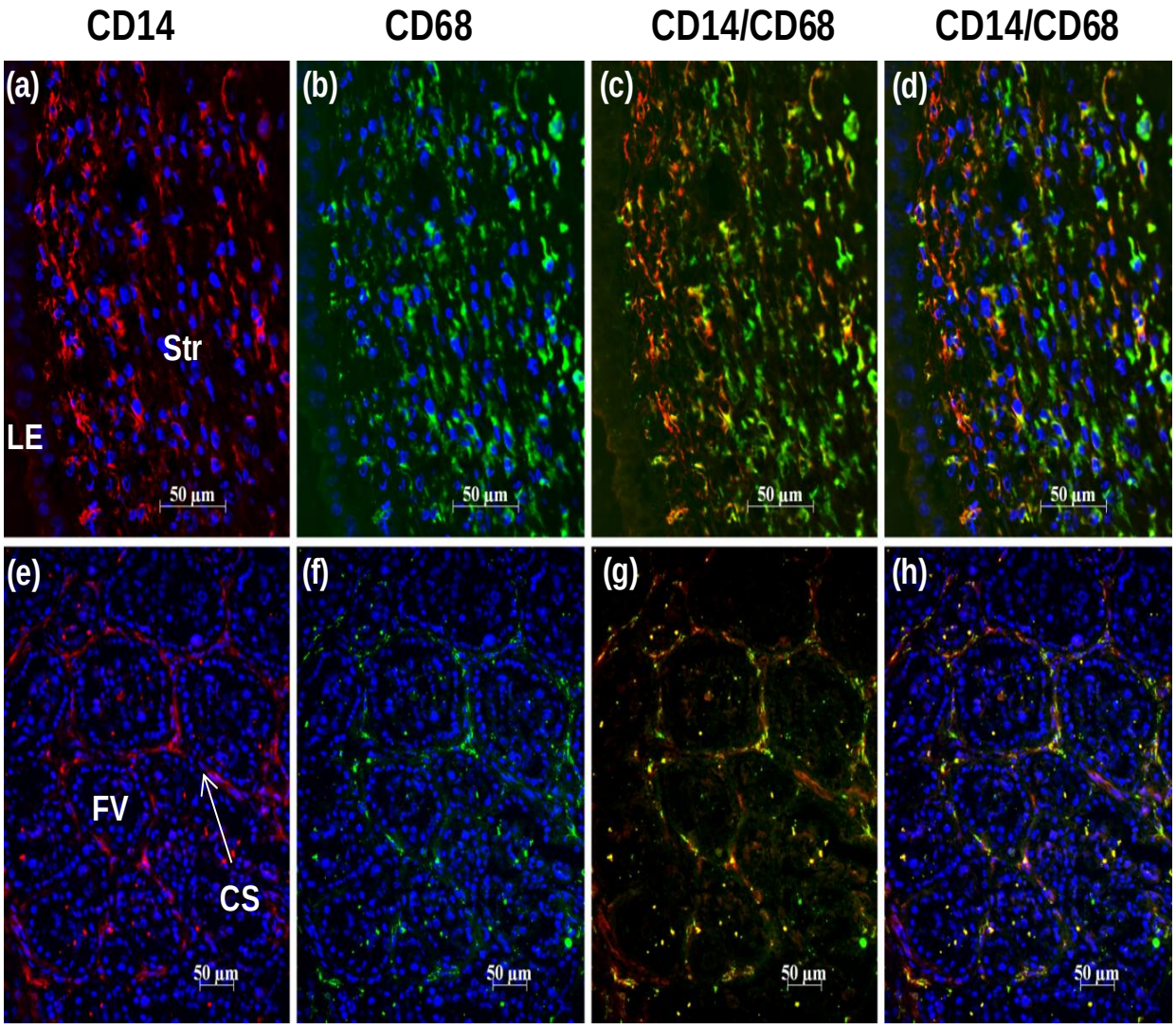

Figure 4-1. Immunofluorescent labeling of cells for CD68 (green) and CD14 (red) in endometrium from a cow at ~Day 145 of pregnancy. The top panels represent sections of interplacentomal endometrium (a-d) and the bottom panels represent sections of placentome (e-h). Nuclei were labeled with Hoescht 33342 (blue). Merged images are in panels c, d, g and h. CS = caruncular septum, FV = fetal villi,.LE= luminal epithelium, and Str=endometrial stroma.

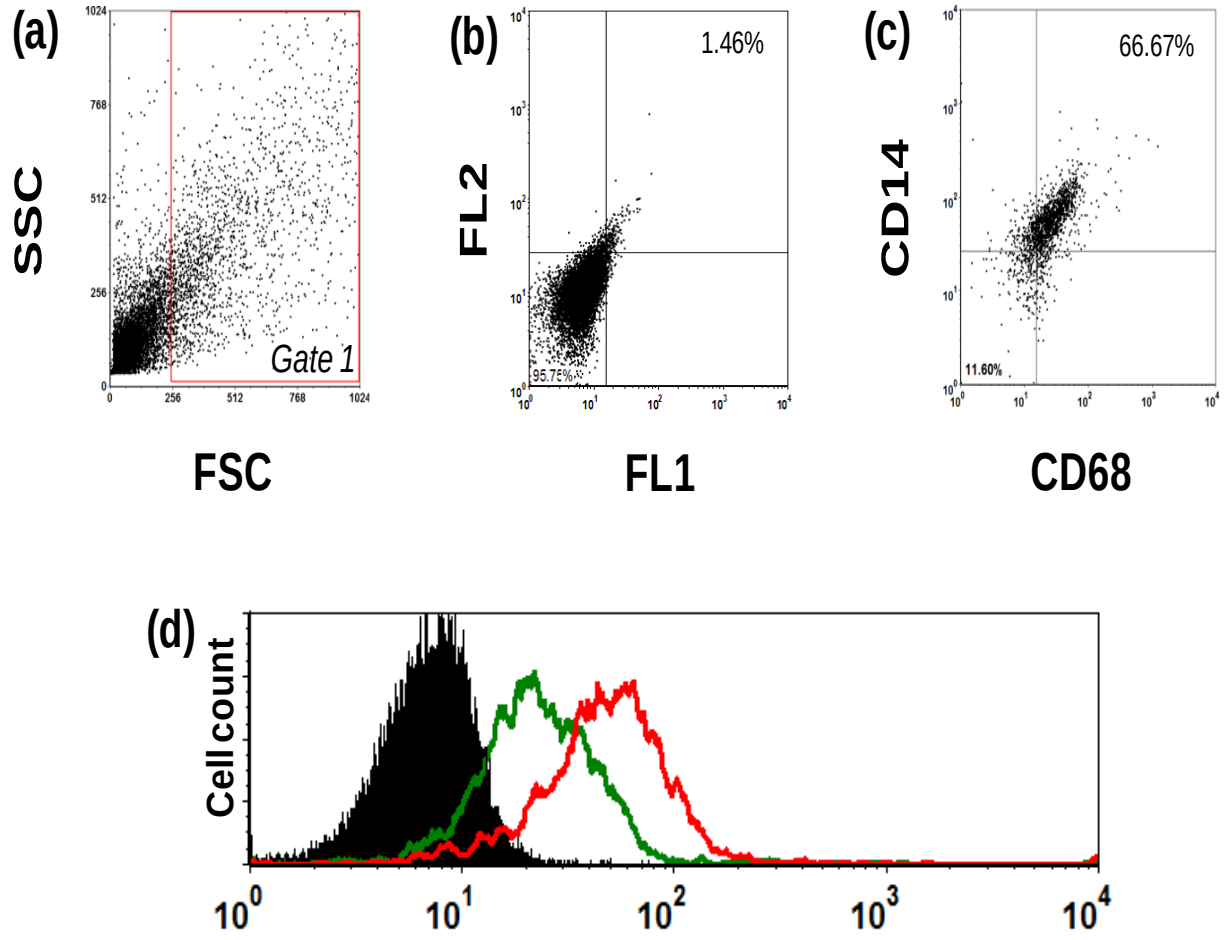

Figure 4-2. Acquisition dot-plots from flow cytometric analysis of the proportion of $CD68^{+}$ cells in dispersed endometrial cells from the interplacentomal endometrium of a cow at ~Day 166 of pregnancy that were $CD14^{+}$. Plots represent: (a) analysis of cells on the basis of size (FSC, forward scatter characteristics; x axis) and granularity (SSC, side scatter characteristics; y axis), with the gate subjected to further analysis (Gate 1) shown as the red rectangle, (b) fluorescence for cells incubated with isotype controls, (c) dual-labeling of cells in Gate 1 for CD14 (x axis) and CD68 (y axis) and (d) representative histogram showing cells labeled with isotope control in FL1 (black), anti-CD68 (green) and anti-CD14 (red).

epithelium did not express CD11b (Figure 4-3c). In the placentome, $CD11b^+$ cells were rare (Figure 4-3e) and none of the $CD68^+$ cells were positive for CD11b. (Figure 4-3g and h).

Localization of Cells Positive for MHC class II

In the interplacentomal endometrium, there were three major populations of MHC class II^+ cells. The first two were in the stroma immediately adjacent to the basement membrane of the luminal epithelium (Fig. 4a,e). These cells were negative or weakly positive for CD68 (Fig. 4c,d,g,h). One population of MHC class II^+ cells near the luminal epithelium was also positive for CD14 (Fig. 5c,d) whereas the other, more abundant, population, was negative for CD14 (Fig. 5c,d). The third population was in the stroma immediately adjacent to the basement membrane of the endometrial glands. These cells were negative for CD68 (Fig. 4c,d) and CD14 (not shown).

In the caruncular septa of placentomes, there were numerous MHC class II^+ cells (Figure 4i and m). Some were $CD68^+$ and others were $CD68^-$, Most $CD68^+$ cells were also MHC class II (Figure 4-4k, l, o and p). No MHC class II^+ cells were found in fetal villi (Figure 4-4i, k, l, m, o and p).

Discussion

Results indicate that the endometrial accumulation of $CD68^+$ cells during pregnancy in the cow seen previously for the interplacentomal endometrium (Chapter 3) also occurs in the caruncular septa of the placentome. That these $CD68^+$ cells originate from the myeloid hematopoietic lineage is likely because they also express CD14. CD68 is expressed on monocytes, macrophages and dendritic cells (Ferenbach

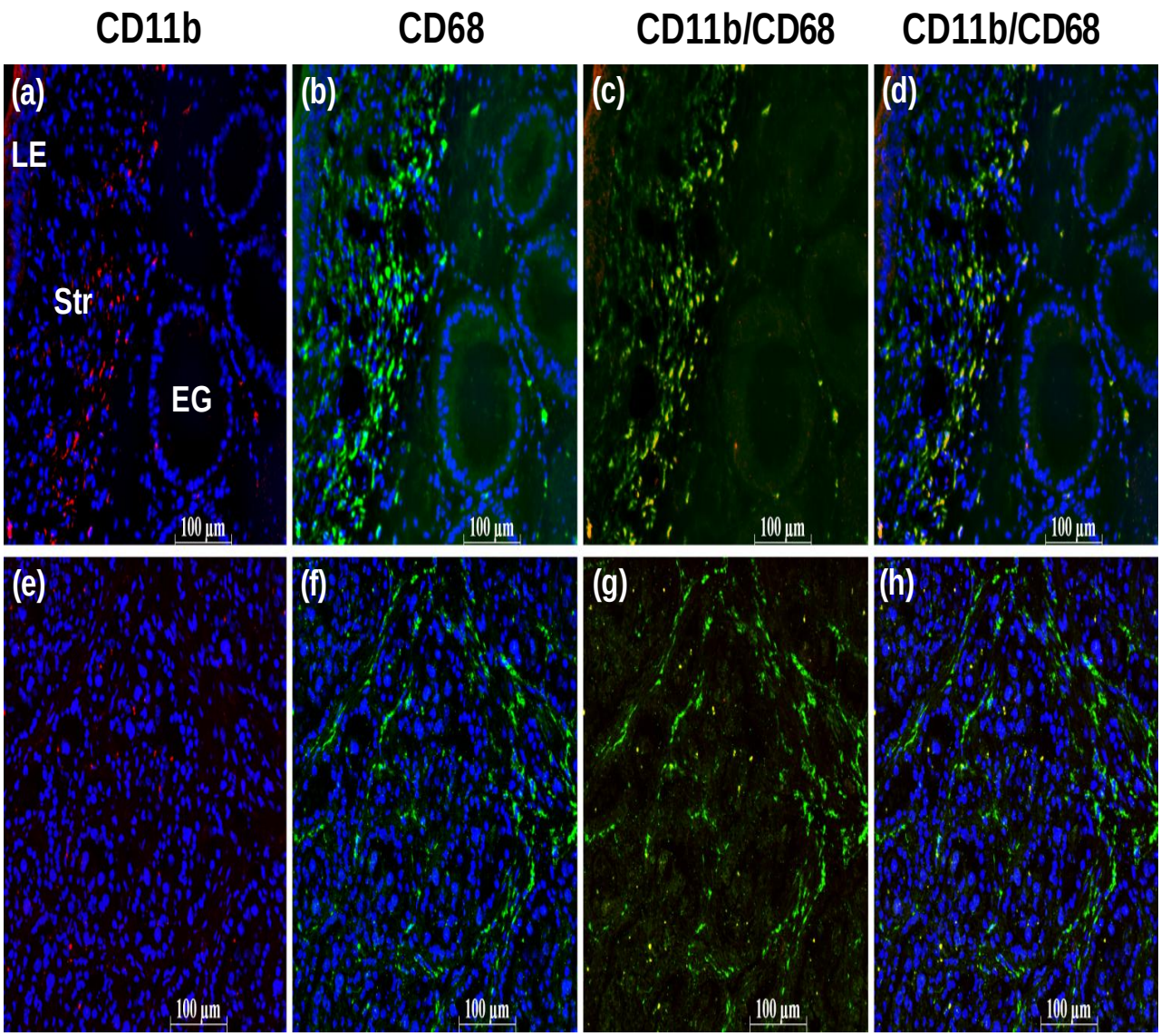

Figure 4-3. Immunofluorescent labeling of cells for CD68 (green) and CD11b (red) in endometrium from a cow at ~Day 145 of pregnancy. The top panels represent sections of interplacentomal endometrium (a-d) and the bottom panels represent sections of placentome (e-h). Nuclei were labeled with Hoescht 33342 (blue). Merged images are in panels c, d, g and h. CS = caruncular septum and FV = fetal villi.

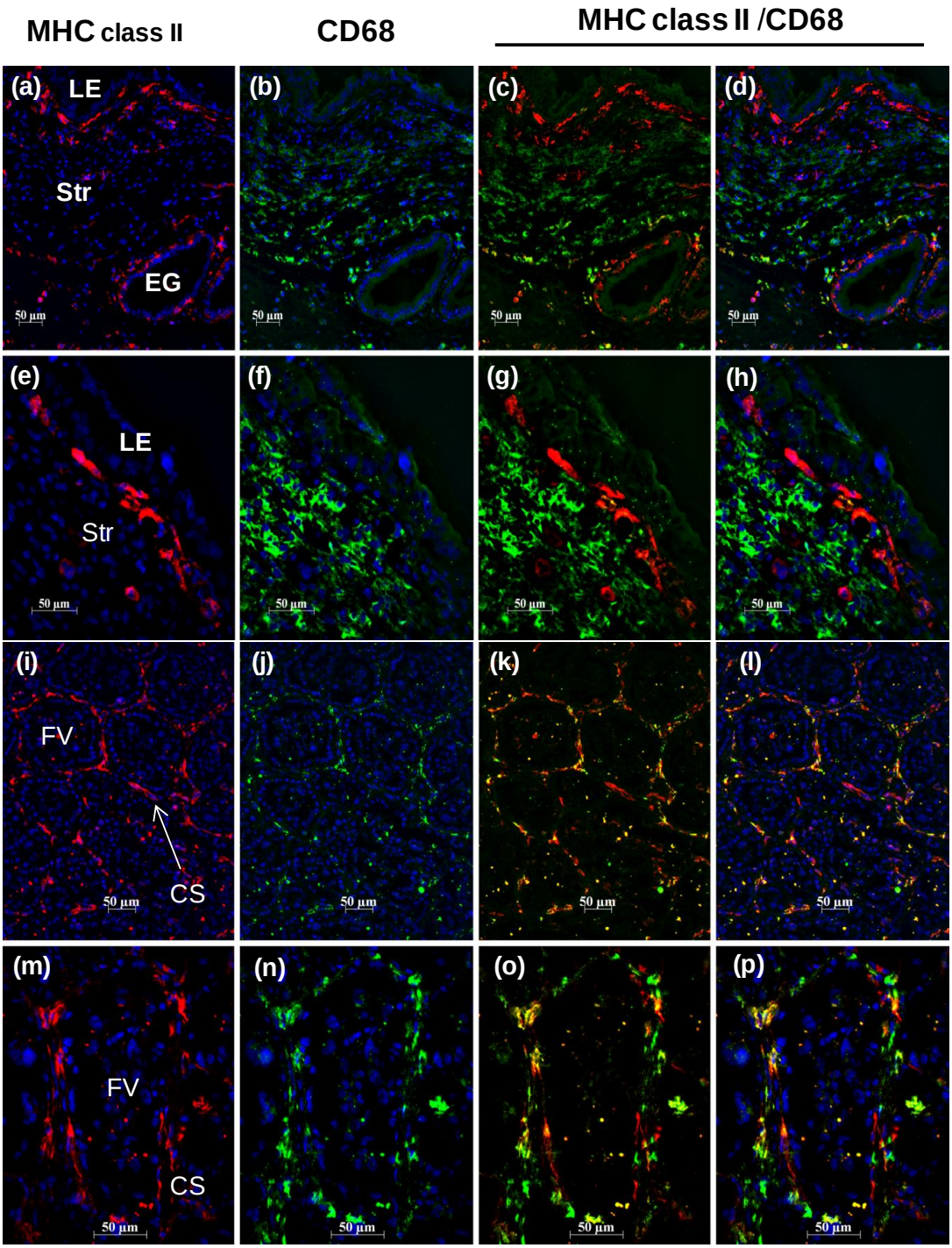

Figure 4-4 Immunofluorescent labeling of cells for CD68 (green) and MHC class II (red) in endometrium from a cow at ~Day 216 of pregnancy. The top panels represent sections of interplacentomal endometrium (a-h) and the bottom panels represent sections of placentome (i-q). Nuclei were labeled with Hoescht 33342 (blue). Merged images are in panels c, d, g, h, k, l, o, and p. CS = caruncular septum, EG=endometrial gland, FV = fetal villi,LE= luminal epithelium, and Str=endometrial stroma.

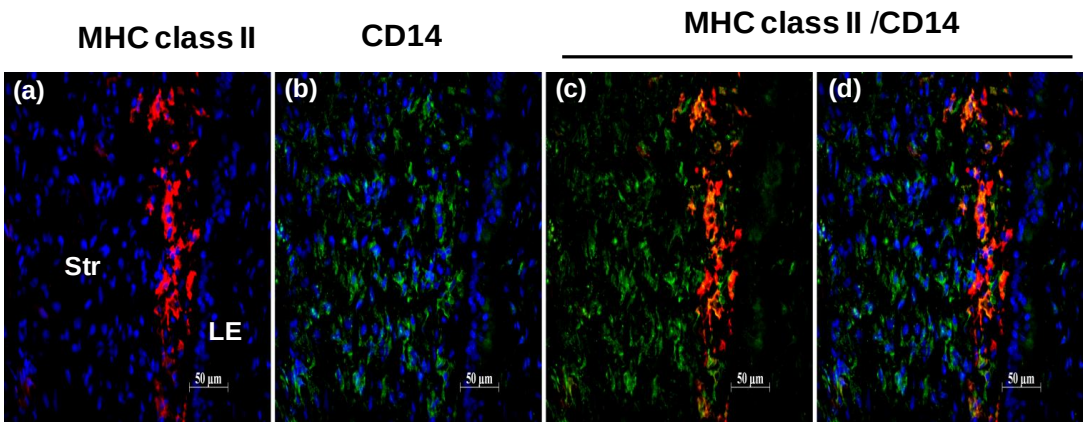

Figure 4-5 Immunofluorescent labeling of cells for MHC class II (red) and CD14 (green) in interplacentomal endometrium from a cow at ~Day 243 of pregnancy. Nuclei were labeled with Hoescht 33342 (blue). Merged images are in panels c and d. LE=luminal epithelium and Str =endometrial stroma.

et al.2008; Micklem et al. 1989; Ackermann et al. 1994) and CD14 is expressed on monocytes, macrophages and neutrophils (Paape et al. 1996).

It is also apparent from this study that endometrial macrophages are regionally differentiated. $CD68^+$ cells in the interplacentomal endometrium were negative for MHC class II and sometimes positive for CD11b, while $CD68^+$ cells in the placentomal endometrium were MHC class II^+ and negative for CD11b. This regional differentiation likely reflects different trafficking patterns and regulatory signals in the two placental regions.

The most striking difference between $CD68^+$ cells in interplacentomal and placentomal regions was in the expression of MHC class II. Presence of MHC class II on $CD68^+$ cells in the caruncular septa of the placentomal endometrium is consistent with the activation of these cells by the M1 or M2 pathway (Godon 2003). In contrast, the absence of detectable MHC class II in interplacentomal $CD68^+$ cells could indicate that the cells are inactivated macrophages (Godon 2003) or monocytes newly recruited

to the endometrium that have defective antigen-presentation function. Further evidence suggesting that at least some of the interplacentomal $CD68^+$ cells are newly arrived in the endometrium is the staining pattern for CD11b. This protein, which is involved in monocyte movement through the endothelium (Fagerholm et al. 2006) was only expressed on a population of $CD68^+$ cells in the deeper stromal areas of interplacentomal endometrium. The lack of $CD11b^+$ cells in shallow stroma and the placentome could reflect loss of CD11b expression as macrophages migrate towards the placenta and differentiate in response to changes in the endometrial microenvironment. Further evidence for a role of CD11b in monocyte trafficking to the endometrium is the finding in humans that expression of CD11b by circulating blood cells was higher in pregnant women compared with nonpregnant women (Luppi et al. 2004).

It is also possible that $CD68^+$ cells in the interplacentomal endometrium are negative for MHC class II because they are more distant from trophoblast-derived activation signals than $CD68^+$ cells in the placentomes or that a molecule secreted by the interplacentomal trophoblast or endometrium inhibits macrophages. Non-classical MHC class I antigens are expressed on the interplacentomal trophoblast of the cow (Davies et al. 2006). In the human, trophoblast non-classical MHC class I proteins can regulate endometrial macrophage function (Li et al. 2009). The interplacentomal endometrium produces uterine serpin in glandular epithelium (Peltier and Hansen 2000) and, at least in sheep, this protein can inhibit proliferation of peripheral mononuclear cells in vitro (Leslie and Hansen 1991; Padua and Hansen 2008).

Another possibility is that some or all of the $CD68^+$ cells in the interplacentomal endometrium are not derived from a myeloid mononuclear phagocyte lineage but instead represent stromal cells that have differentiated in a manner associated with upregulation of certain genes characteristically expressed by macrophages. In species where endometrium undergoes a decidual response, decidualized stroma express cytokines and chemokines characteristically secreted by immune cells (Salamonsen et al. 2007; Chen et al. 2009). Perhaps similar differentiation pathways exist in species like cattle that do not undergo decidualization.

In the interplacentomal endometrium, there were numerous MHC class II^+ cells that were not positive for CD68. These included cells in stromal tissue beneath the luminal epithelium, some of which were $CD14^+$, and cells in the glandular epithelium that were negative for CD14. A similar pattern of MHC class II^+ cells exists in sheep (Gottshall and Hansen 1992; Leung et al. 2000). The $CD14^+$ MHC class II^+ cells could be mononuclear phagocytic cells derived from $CD68^+$ macrophages in deep stroma or another other cell type. Presence of $CD14^+$ cells in the subepithelium stroma has been previously reported at Day 16 after estrus in cyclic and pregnant cattle (Leung et al. 2000). Some MHC class II^+ cells in the subepithelial stroma could also be B cells because cells staining for the B cell marker CD21 were localized mostly to this region (Leung et al. 2000).

As mentioned in the Introduction, recruitment of macrophages to the endometrium is a characteristic of pregnancy in many species. What sets the sheep and cow apart from the other species examined is the magnitude of infiltration, particularly into the

interplacentomal endometrium. The consequences of this infiltration for the course of pregnancy and immune function in the post-parturient period remain to be defined..

CHAPTER 5
TRANSCRIPTOMAL PROPERTIES OF THE ENDOMETRIAL MACROPHAGE IN THE PREGNANT COW

Introduction

Pregnancy leads to immunological adjustments in the mother that facilitate survival of the conceptus. One of these adjustments, recruitment of macrophages to the pregnant endometrium, occurs in a wide range of mammalian species including the mice (Hunt et al. 1985), humans (Heikkinen et al. 2003; Cupurdija et al. 2004; Kim et al. 2007), cynomologus and vervet monkeys (Dambaeva et al. 2008), sheep (Tekin and Hansen 2004) and bovids (Chapters 3 and 4). The role of endometrial macrophages is not known and may vary depending upon anatomical location. In cattle, for example, endometrial macrophages are regionally differentiated, with macrophages in the interplacentomal endometrium being largely $CD68^{+}CD14^{+}$MHC class II^{-} and often positive for CD11b while the less-numerous macrophages in the caruncular septa of placentomes are largely $CD68^{+}CD14^{+}$MHC class II^{+} and have little or no expression of CD11b (Chapter 4).

One possible function for endometrial macrophages is to participate in host defense. Indeed, endometrial macrophages increase in number in caruncular septa of the pregnant cow after experimental infection with Neospora caninum. (Rosbottom et al. 2008). Since accumulation can increase with impending parturition (Thompson et al., 1999; Mackler et al., 2000), a role for macrophages in clearing bacteria after parturition or in the expulsion of the conceptus is a possibility. There is evidence in the human that endometrial macrophages participate in vascular remodeling since they produce VEGF (Li et al. 2009) and macrophage accumulation in the uterus is abnormal in women with pre-eclampsia (Williams et al. 2009). Macrophages can also clear placental lactogen

(Kzhyshkowska et al. 2008) and may participate in regulation of this hormone at the fetal-maternal interface. One important function of macrophages is regulation of cells participating in the humoral and cellular arms of the immune response.

Given the potential antigenicity of the conceptus by virtue of inheritance of paternal antigens and, at least in cows, the expression of classical MHC proteins on the placenta (Davies et al. 2004; Davies et al. 2006), a role for macrophages in limiting activation of anti-conceptus immune responses is possible. Macrophages can follow different differentiation pathways when activated by cytokines. Exposure to interferon gamma (IFNG), tumor necrosis factor alpha (TNFA) and/or lipopolysaccharide (LPS) leads to activation along the classical or M1 activation pathway to produce a macrophage that promotes inflammation and cytotoxicity while exposure to interleukin 14 (IL4) and interleukin 13 (IL13) leads to activation via the alternative or M2 activation pathway to produce a macrophage that directs immunosuppression and wound healing (Mantovani et al. 2002; Gordon, 2003). Endometrial macrophages in humans and mice have been reported to have characteristics of M2 activation (Heikkinen et al. 2003; Curpudja et al. 2004; Gustafsson et al. 2008) although it is possible that endometrial macrophages possess a unique activation status.

In the experiment reported herein, likely functions of macrophages residing in the interplacentomal endometrium of pregnant cows were determined through use of global analysis of the macrophage transcriptome to characterize the pattern of gene expression and identify functional pathways that are activated or inhibited in endometrial macrophages compared with monocytes in blood. We hypothesized that macrophages in the pregnant endometrium will express genes that support placental growth

(angiogenesis and tissue remodeling) and that are indicative of a M2 phenotype that signifies a role in modulation of immune responses towards fetal antigens.

Materials and Methods

Materials

Mouse anti-bovine CD14 (clone MM61A, clarified ascites fluid, 10 μg /mL; (Miyoshi et al. 2002) was obtained from VMRD, Inc. (Pullman, WA, USA). The antibody was tagged with labeled Fab fragments against mouse IgG conjugated to Alexa Flour 488 using the Zenon® Mouse Labeling IgG kit from Invitrogen Molecular Probes (Eugene, OR, USA) as per manufacturer's instructions.

Fico/Lite LymphoH was purchased from Atlanta Biologicals (Norcross, GA, USA). Fetal bovine serum was purchased from Sigma-Aldrich (St. Louis, MO, USA) or Pel-Freez Biologicals (Rogers, AR, USA). Tissue Culture Medium-199 (TCM-199), bovine serum albumin (BSA) Fraction-V, Dulbecco's phosphate buffered saline (DPBS), and collagenase type I was purchased from Sigma-Aldrich (St. Louis, MO, USA). Other reagents were from Sigma-Aldrich or Fischer (Pittsburgh, PA, USA).

Collection of Endometrium and Blood

For microarray analysis, the uterus and cardiac blood were obtained at the time of euthanasia at a local abattoir from four cows at an estimated 164, 239, 242 and 248 days of pregnancy, respectively [i.e., dating based on fetal crown-rump length; Noden and Lahunta, 1985]. Blood was collected into heparinized tubes. Blood and the uterus were transported to the lab on ice within 1.5 h after slaughter. The uterus and cardiac blood from three additional cows at an estimated 156, 169 and 176 days of pregnancy were collected as described above for RNA collection to be used for quantitative real time PCR (qPCR) analysis.

Isolation of Mononuclear Leukocytes in Blood

Peripheral blood mononuclear cells (PBMC) were obtained from blood as follows. 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 LymphoH, centrifuged at 600 g for 10 min, resuspended in DPBS, and evaluated with a hemacytometer to determine cell concentration and viability (trypan blue exclusion). Cells were resuspended to a final concentration of $5x10^7$/mL in staining buffer (DPBS supplemented with 0.1% (w/v) BSA).

Preparation of Cell Suspension from Endometrium

A suspension of cells was prepared from interplacentomal endometrium dissected from the uterine horn containing the fetus. Dissected endometrium was finely minced in DPBS using a sterile scalpel blade and a petri dish and then placed into a 50 mL sterile culture tube containing 25 mL digestion medium (TCM-199 supplemented with 150 U/mL type I collagenase). Tissue was incubated at 37^{o}C under gentle rotation for 15 min. Undigested tissue was allowed to settle for 5 min; the supernatant fraction was then collected by aspiration, and 25 mL of fresh digestion medium was added to the tissue fraction. The remaining tissue was incubated for an additional 15 min under rotation, and the supernatant fraction produced after settling was pooled with the previous supernatant fraction. Cells in suspension in the supernatant fraction were collected by filtration through a sterile 100 mm cell strainer into 50 mL sterile culture tubes. Cells were centrifuged at 110x g for 5 min, resuspended in 5 mL TCM-199 supplemented with 10% (v/v) fetal bovine serum, analyzed for cell number using a

hemacytometer, and diluted to a final concentration of $5x10^5$/mL in TCM-199. A 10 mL aliquot of the dispersed endometrial cell suspension was layered onto 5 mL Fico/Lite LymphoH in a 15 mL conical tube. Cells were centrifuged at 600 g for 30 min. The interface containing mononuclear cells was collected and resuspended in 10 mL of staining buffer, washed once, resuspended in staining buffer, and evaluated for cell concentration and viability (trypan blue exclusion) using a hemacytometer. Cells were then resuspended to a final concentration of $5x10^6$/mL.

Purification of $CD14^+$ Cells

Endometrial cells and PBMC were separately subjected to flow cytometry and cell sorting to prepare $CD14^+$ cells. The entire pool of cells obtained as described above was placed into a 25 mL conical tube containing staining buffer, washed twice with 5 mL staining buffer, and resuspended in the smallest volume possible with staining buffer. Cells were then labeled by incubation with Zenon-labeled anti-CD14 by adding 0.1 µg of labeled antibody per $5x10^6$ cells. A Zenon-labeled mouse IgG1 at the same concentration as the primary antibody was incubated with another aliquant of cells to determine nonspecific labeling. Cells and labeled antibody were incubated at room temperature for 30 min. After incubation, cells were diluted to 5 mL with staining buffer at a rate of $5x10^6$ cells/mL and subjected to cell sorting using flow cytometry with a FACSCAria Cell Sorter supported by DiVa software (Becton-Dickinson, Franklin Lakes, NJ, USA). Sorting was performed with a 100 micron nozzle and pressure of 20 psi. Cells were gated on the basis of forward and side scatter to analyze the monocyte region. Two rounds of cell sorting were performed. The first round of sorting, at a high flow rate, was performed to maximize yield of $CD14^+$ cells while the second step was designed to maximize purity of the $CD14^+$ cells at low flow rate.

RNA Purification

Sorted $CD14^+$ cells were centrifuged at 600g for 5 min and the total cellular RNA was extracted using the using the RNeasy Micro kit (Qiagen Inc, Valencia, CA, USA). Concentration of total cellular RNA was determined using the Nanodrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA) and integrity determined by Agilent 2100 Bioanalyzer with RNA 6000 Pico LabChip kit (Agilent Technologies, Santa Clara CA, USA). Only samples that showed high RNA integrity (RIN > 7) were used for the microarray hybridization and quantitative PCR analysis. Extracted RNA was stored at -80^{o}C until microarray analysis.

Microarray Hybridization

Four pairs of samples of blood and endometrial $CD14^+$ cells were subjected to transcriptional profiling using the Bovine Oligo Microarray Chip (Bovine 4X44K G2519F) from Agilent (Santa Clara CA, USA). Each pair was collected from the same animal. The array contains 43,803 bovine probes representing 20,767 unique 60-mer probes and approximately 19,500 distinct bovine genes arranged on a slide as 4 arrays in a 4 x 44K format. The probes were developed by clustering more than 450,000 mRNA and EST sequences of the bovine genome (btau 2.1). All microarray protocols were carried out by Mogene LLC (St. Louis, MO, USA), an Agilent Certified Service Provider.

Prior to microarray hybridization, 10-50 ng of total RNA was amplified using the WT-Ovation Pico RNA Amplification Kit (NuGen Technologies, Inc, San Carlos, CA, USA) according to the manufacturer's protocol. A total of 2.0 µg of the amplified material was labeled with Cy3 or Cy5 using Agilent Genomic DNA Enzymatic Labeling Kit (Agilent Technologies, Santa Clara CA, USA) and 1.5 µg of each labeled sample were co-hybridized to the array for 17 h at 65^{o}C at 10 rpm in a rotating oven in an

ozone-free room using sureHyb chambers from Agilent (Agilent Technologies, Santa Clara CA, USA). Wash conditions were as outlined in the Agilent processing manual and the arrays were scanned using an Agilent G2505B scanner (Agilent Technologies, Santa Clara CA, USA). Two technical replicates were performed. For the first replicate, RNA samples from blood were labeled with Cy3 and RNA from endometrium was labeled with Cy5. For the second technical replication, 3 samples of RNA from blood were labeled with Cy5 and their matching pairs of RNA from endometrial were labeled with Cy3 while the fourth pair used Cy3 for blood and Cy5 for endometrium.

Analysis of Microarray Data

The microarray image extraction and data pre-processing were performed using Agilent's Feature Extraction software v 9.5 (Agilent Technologies, Santa Clara CA, USA). The intensity of each spot was summarized as the median pixel intensity followed by log transformation. The lowess normalization was done within-array lowess on median of the intensities of the spots. The JMP® Genomics 3.1 for SAS® 9.1.3 software (SAS Inst., Inc., Cary, NC) was used. The quantile normalization method for data global normalization was performed. Differentially regulated genes were identified by least-squares analysis of variance (ANOVA) by the PROC ANOVA procedure of JMP® Genomics 3.1 for SAS® 9.1.3. The model included animal, replicate, and tissue source (blood or endometrium). Fixed effects included tissue and replicate and random effects included animal. Tissue x animal was the error term for tissue. Correction for false discovery rate was performed by the Benjamini and Hochberg method (1995) with a maximum false discovery rate (FDR) of 0.01. Genes with a minimum intensity of 16 were considered to be expressed. Only genes with at least 2-fold difference and a probability of $P \leq 0.05$ for a tissue difference were considered differentially expressed.

Gene Ontology Analysis

An analysis was performed using FatiGo (a web-based program for functional profiling; http://www.babelomics.org) to indentify functional enrichment by comparing the two lists of differentially regulated genes from endometrium and blood by means of a Fisher´s exact test (Al-Shahrour et al. 2007). Furthermore, Pathway Express (http://vortex.cs.wayne.edu/ontoexpress) was used to identify relevant pathways represented by the differentially regulated gene set. Pathway Express calculates a probability (P) value, based on the density of genes that belong to a determined pathway using the Kyoto Encyclopedia of Gene and Genome database (Draghici et al., 2007). It also calculates a gamma P value using the perturbation factor for each input gene in the pathway that accounts for the normalized fold change of the gene and the number and the amount of perturbation of genes at downstream positions on the pathway. Both P- and gamma P-values are combined to generate the impact factor which was used to rank the pathways according to their biological significance. A pathway was considered to be significantly affected by tissue source if the gamma P-value ≤ 0.05.

Quantitative Real-Time PCR

Quantitative PCR was performed on 12 genes found to be differentially expressed by microarray hybridization as well as a housekeeping gene used as an internal control (RPL19 – bovine ribosomal protein L19). Samples from a total of five pairs were analyzed by qPCR. These included 2 pairs of samples that were also used for the microarray analysis and 3 pairs of new samples. We could not use samples from all 4 pairs used in the microarray analysis for the qPCR validation due to insufficient quantities of RNA.

Primers and probes are shown in Table 5-1. Quantitative real time PCR experiments were carried out by Mogene LLC (St. Louis, MO, USA). The hydrolysis probe based system was designed using the online tool “PrimeTime qPCR Assays” by IDT DNA (www.idtdna.com) and the primers and probes were purchased from the same company. The final primer and probe concentration was 200 nM. All probes had a 5' 6-Fam label and a 3' IA-Black Quencher. The reaction was designed for a total reaction volume of 25.0 µL. The template cDNA (25 ng per reaction) was added to each reaction in 5.0 µl. The Applied Biosystems Taqman Gene Expression Mastermix (Foster City, CA, USA) was used. Cycling conditions were 95^{o}C for 10 min, and 40 cycles at a 95^{o}C melting temperature for 15 sec. and 60^{o}C for 1min. Each sample was assayed in 3 replicates.

The level of expression of each tested gene was calculated using the ΔCt method with normalization to the RPL19 housekeeping gene. Treatment effects were analyzed by least square analysis of variance using the General Linear Models procedure of SAS (SAS for Windows, version 9.3, SAS Institute Inc., Cary, NC, USA). Results are presented as least-squares means ± standard errors. Animal and tissue intereaction was used as the error term for tissue.

Table 5-1. Primer/probe sets used for qRT-PCR

Gene Name	Accession	Primer/ Probe	Sequence (5'-3')
CD163 molecule (*CD163*)	NM_001077402	Forward[a]	AAT TTC GTG GAC AGA GTT CTC
		Probe[b]	/56-FAM/AGA CAG CGG CTT GCA GTT TCC /3IABlk_FQ
		Reverse[C]	AAT TTC GTG GAC AGA GTT CTC
Mannose receptor C1 (*MRC1*)	XM_001252128	Forward[a]	TCG AGT TGA GCC ACT TCA
		Probe[b]	/56-FAM/CCA CAC CCA CAT TCC TTC AAC ATT TCT /3IABlk_FQ/
		Reverse[C]	TCT TTC ACC AGA GGG ATC AC
Granzyme A (*GZMA*)	NM_001001142	Forward[a]	AAG ACG CTA CAT GGC TCT
		Probe[b]	/56-FAM/CGC TCA TTG TGA CCT GAA GGG C/3IABlk_FQ/
		Reverse[C]	ATG GGA TGT AGA GTG GGC
Heme oxygenase (decyclizing) 1 (*HMOX1*)	NM_001014912	Forward[a]	CGG AGA ATG CAG AGT TCA
		Probe[b]	/56-FAM/AAG GTT TTA AGC TGG TGA TGG CGT C/3IABlk_FQ/
		Reverse[C]	TGT TGC GTT CGA TCT CCT
Chemokine (C-C motif) ligand 2; CCL2 (*MCP1*)	XM_614752	Forward[a]	AGA GGC TGT GAT TTT CAA GAC
		Probe[b]	/56-FAM/AGT TAT GTG CAG ACC CCA AGC AGA /3IABlk_FQ/
		Reverse[C]	GGT TGT GGA GTG AGT GCT
Fc fragment of IgG, low affinity IIIa, receptor (*CD16A*)	NM_001077402	Forward[a]	GAA TGG AGG GAT GGC AAA
		Probe[b]	/56-FAM/TTA GGA CAA ATG GAG GCA TCT CTG GG/3IABlk_FQ/
		Reverse[C]	ACA GAG TTG GGT GAA GGA TC
Ribonuclease k6 (*RNASE6*)	NM_174594	Forward[a]	CCC CTT ATC ATT TGG TTC CTG
		Probe[b]	/56-FAM/AGG TTG TTT AAA TCA CTT TGC TTC TCG CT/3IABlk_FQ/
		Reverse[C]	AAA TCA ATA AAA GAC AAG AAA ATC AGA G
Testis specific, 14 (*TSGA14*)	XM_583431	Forward[a]	AGA ATC CCC GCT CAC TG
		Probe[b]	/56-FAM/CAA GGT CCT TTA CTT CCA GGG TTT GC/3IABlk_FQ/
		Reverse[C]	GAA AAC ATT TAT TTG ACT AAG GCA G
Pregnancy-associated glycoprotein 10 (*PAG10*)	NM_176621	Forward[a]	GCC CAA GCT TAC ATC CAA AG
		Probe[b]	/56-FAM/TCT CCG ACT CGT TCA CAC GCT /3IABlk_FQ/
		Reverse[C]	GAG AAA TAC AGC CTC AGG AAG
Solute carrier organic anion transporter family, member 2B1 (*SLCO2B1*)	NM_174843	Forward[a]	ATC CAG AGT GTG AGC TGT
		Probe[b]	/56-FAM/CCC AAT ACC CAA TTC CAG GCA AAG G/3IABlk_FQ/
		Reverse[C]	CAA GTT AGC GGA AGA CTC AC
Ribosomal protein L19 (*RPL19*)	NM_001040516	Forward[a]	CTG AAG GTG AAG GGT AAC G
		Probe[b]	/56-FAM/AGG CAG ACA AGG CTC GCA AGA /3IABlk_FQ/
		Reverse[C]	GGG CTT CCT TGG TCT TAG A

[a]Forward=sense (5') primer
[b]Each probe was synthesized with a 5' 6-FAM reporter dye and 3' IA-Black quencher.
[c]Reverse=antisense (3') primer.

Results

Purification of Endometrial and Blood $CD14^+$ Cells

The final preparation of endometrial $CD14^+$ cells had a purity of 90-95% based on flow cytometry while blood $CD14^+$ cells were 98-99% pure (Figure 5-1).

Expression of Genes in Blood and Endometrial $CD14^+$ Cells

The transcriptomes of blood and endometrial $CD14^+$ cells were compared using a bovine whole genome array that covers ~19,500 bovine genes. As shown from results of hierarchical cluster analysis (Figure 5-2A), the pattern of gene expression was largely similar for $CD14^+$ cells from both sources, suggesting that cells from both tissues are from the monocyte/macrophage lineage. Figure 5-2B shows a Venn diagram of expressed genes. A total of 14,808 genes were expressed in blood $CD14^+$ cells. Of these, 1,386 were not expressed in endometrial $CD14^+$ cells. A total of 13,872 genes were expressed in endometrial $CD14^+$ cells and 450 of these were not expressed by blood $CD14^+$ cells.

Identification of Differentially Expressed Genes

Criteria for identifying differentially expressed genes was a level of significance of $P \leq 0.05$, a fold change > 2, and a maximum false discovery rate of 0.01. A total of 1,364 unique genes were differentially expressed, with 680 genes upregulated in endometrial $CD14^+$ cells as compared to blood $CD14^+$ cells and with 674 genes downregulated in endometrial $CD14^+$ cells as compared to blood $CD14^+$ cells (i.e, upregulated in blood). Of these differentially expressed genes, 647 of the 685 upregulated genes in the endometrium and 581 of the 679 upregulated in the blood could be annotated.

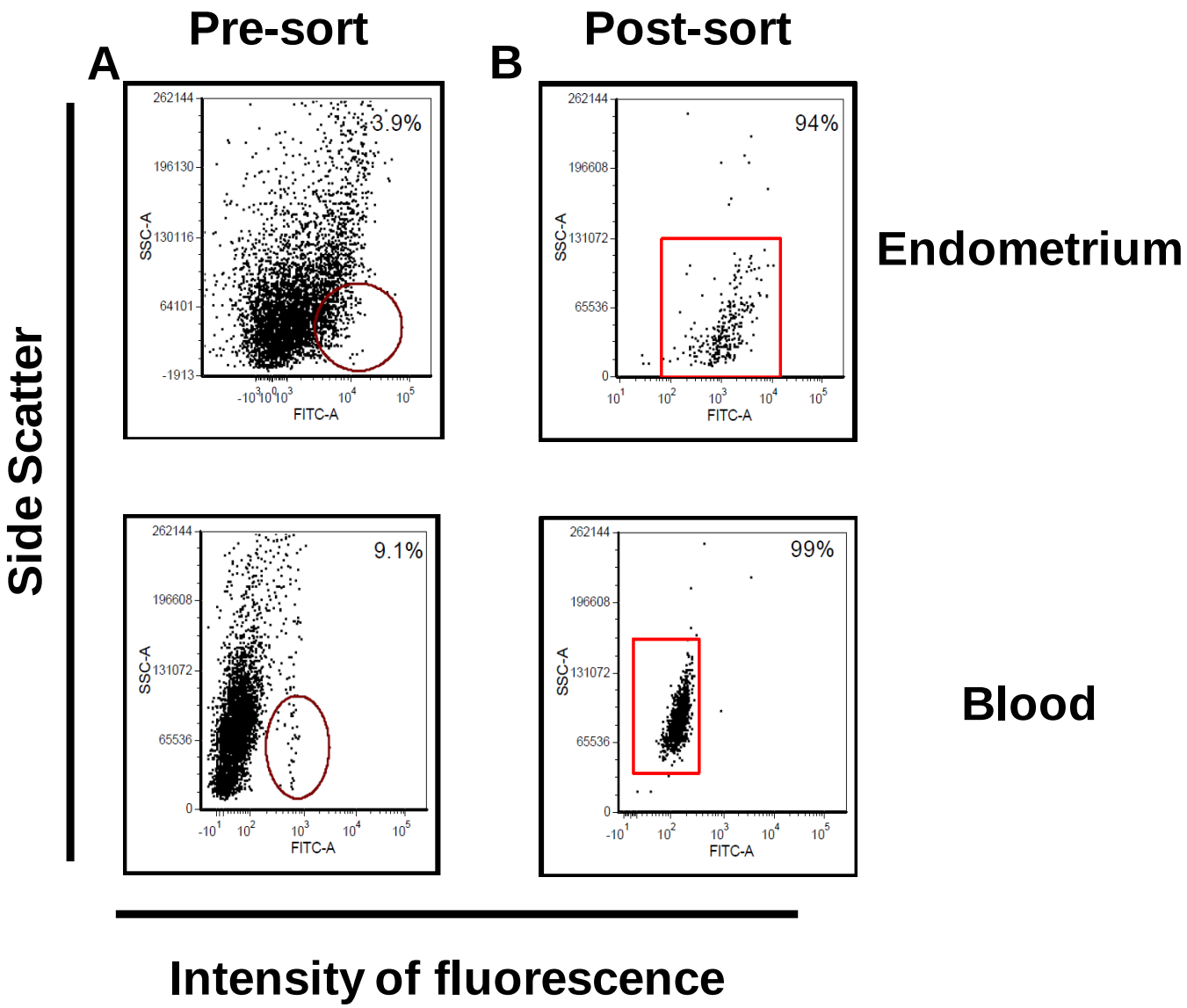

Figure 5-1 Representative acquisition dot plots for CD14$^+$ cells in endometrium and peripheral blood as determined by flow cytometry. Shown are analyses for dispersed endometrial cells and peripheral blood mononuclear cells from a cow at ~Day 230 of pregnancy before (A) and after two rounds of cell sorting (B). The y-axis depicts side scatter characteristics (SSC) to analyze cells on the basis of granularity and the x-axis depicts intensity of fluorescence (FL1) associated with antiCD14. The circles and rectangles in the dot plots identify CD14$^+$ cells targeted for sorting.

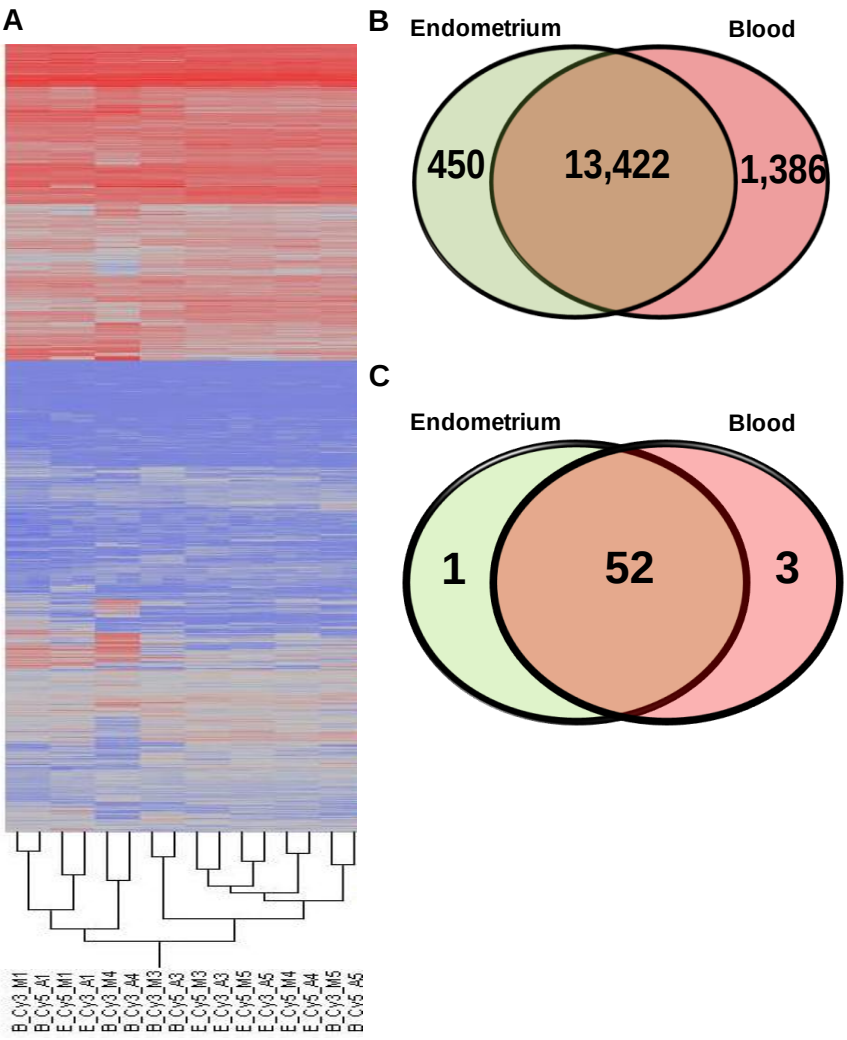

Figure 5-2. Graphical representation of differentially regulated genes in endometrial and blood CD14$^+$ cells. (A) Hierarchical cluster representing the global gene expression of endometrial and blood CD14$^+$ cells. Each sample is identified by tissue source [endometrium (E) and blood (B)], dye (Cy3 vs Cy5), replicate (A vs M), and cow number (1 to 5). (B) Venn diagram characterizing expressed genes in endometrium and blood. Circles represent the genes expressed in both cell types (brown; 13,422 genes), genes exclusively expressed by endometrial CD14$^+$ cells (green; 450 genes) and genes expressed exclusively by blood CD14$^+$ cells (red; 1386 genes). (C) Venn diagram characterizing the expression of immune genes reported by Jensen et al. (2006) to be expressed by the bovine macrophages. Circles represent the genes expressed in both cell types (brown; 52 genes), genes exclusively expressed by endometrial CD14$^+$ cells (green; 1 gene) and genes expressed exclusively by blood CD14$^+$ cells (red; 3 genes). An additional 4 genes reported by Jensen et al. (2006) were not identified in either cell population.

A list of the 100 differentially-expressed genes showing the highest fold increase in endometrium is presented in Table 5-2. The gene with the greatest fold-increase in the endometrium was granzyme A (*GZMA*), a serine proteinase enzyme produced by cytotoxic T cells and natural killer cells, which induces apoptosis of the target cells. There were other genes characteristically expressed in immune cells that were among the top 100 overexpressed genes such as stanniocalcin-1 (*STC1*), heat shock protein70 B (*HSPA6*) and macrophage scavenger receptor A (*MSR1*). There were several growth factor and cytokine genes that were upregulated in endometrium. These include connective tissue growth factor (*CTGF*), insulin-like growth factor binding protein-6 (*IGFBP6*), endothelial cell-specific molecule 1 (*ESM1*), chemokine (C-X-C motif) ligand 14 (*CXCL14*), and chemokine (C-C motif) ligand 22 (*CCL22*). Additionally, there were molecules involved in the complement system [V-set and immunglobulin domain-containing 4 (*VSIG4*), clusterin (*CLU*), and complement component (*C1QA*)], and genes related to cell adhesion [nectin-3 (*PVRL3*), claudin-1 (*CLDN1*), and claudin-8 (*CLDN8*)], and in responses to hypoxia [aryl-hydrocarbon receptor nuclear translocator 2 (*ARNT2*), angiogenin (*ANG*) and L-arginine:glycine amidinotransferase (*GATM*)].

Several genes characteristically produced by the placenta were in the group of genes upregulated in endometrial $CD14^{+}$ cells. These genes include several members of the pregnancy-associated glycoprotein family (*PAG17, PAG3, PAG6, PAG15, PAG5, PAG10, PAG7*), placental lactogen, a group of genes encoding prolactin-related proteins (*PRP3, PRP6, PRP15, PRP1, PRP8*), a folate receptor gene associated with the placenta and fetus (*FOLR2*) and keratin-7 (*KRT7*). Uterine milk protein precursor, a product of uterine epithelium also called uterine serpin (*SERPINA14*), was also highly

expressed in endometrial $CD14^+$ cells. Thus, it is likely that some cells in the endometrial $CD14^+$ cell preparation were of placental and endometrial epithelial origin.

A list of the 100 differentially-expressed genes showing the highest fold increase in the blood is shown in Table 5-3. Calcitonin-related polypeptide beta, transcript variant 2 (*CALCB*) was the gene most upregulated in blood $CD14^+$ cells. This gene encodes for a hormone involved in vascular homeostasis. Other clusters of genes are involved in inflammatory and immune response process such as pentraxin-related gene (*PTX3*), cytokine-cytokine interaction, chemokine (C-C motif) receptor 3 (*CCR3*), interleukin 8 receptor (*IL8RB*) and interleukin 18 receptor 1 (*IL18R1*). Moreover, B-lymphocyte antigen CD20 and B-cell receptor CD22 precursor were also upregulated in the blood. Other genes upregulated in the blood were involved in signal transduction including, among others, mitogen-activated protein kinase kinase kinase kinase (*MAP4K2*) and microtubule-associated protein-1S (*MAP1S*). There were also upregulated genes involved in apoptosis including serpin 2 (*SERPINB2*), polymerase (DNA directed), beta (*POLB*), and cell death-inducing DFF45-like effector-B (*CIDEB*).

To address the question of whether $CD14^+$ endometrial cells express genes that are characteristic of bovine macrophages, we compared the transcriptome of endometrial and blood $CD14^+$ cells to the list of genes identified by Jensen et al. (2006) using DNA microarray hybridization as being expressed by bovine macrophages. Of the 56 immune genes reported by Jensen et al. (2006) to be expressed in bovine macrophages, 52 were expressed by both endometrial and blood $CD14^+$ cells. Of these 52 genes, 5 were differentially expressed (Figure 5-2C). Two genes were upregulated in endometrium [CD63 antigen (*CD63*) and chemokine (C-C motif) ligand 8 (*CCL8*)] and

Table 5-2. Top 100 upregulated genes based on fold change in endometrial $CD14^+$ cells as compared to $CD14^+$ cells in blood

Description	Accession	Intensity		Fold Change	P
		Endometrium	Blood		
Granzyme A (*GZMA*)	NM_001001142	33196	276	120	0.00
Stanniocalcin 1 (*STC1*)	NM_176669	1795	16	110	0.01
Unknown	AU278548	6233	67	98	0.00
P antigen family, member 4 (prostate associated) (*PAGE4*)	NM_001081580	7230	75	97	0.00
Uterine milk protein precursor (*LOC286871*)	NM_174797	70190	759	92	0.00
Meprin A, beta (*MEP1B*)	NM_001144098	4271	52	81	0.00
Predicted: multiple EGF-like-domains 10 (*MEGF10*)	XM_001788482	3282	47	70	0.00
Hypothetical protein LOC614841 (*LOC614841*)	NM_001076350	55302	955	57	0.00
Heat shock 70kDa protein 6 (*HSPA6*)	XM_589747	4634	83	55	0.00
Prolactin-related protein VI (*PRP6*)	NM_205778	7527	141	53	0.00
Aspartoacylase (Canavan disease)(*ASPA*)	NM_001046033	939	18	51	0.00
Retinoic acid receptor responder- 1 (*RARRES1*)	NM_001075430	15631	305	51	0.00
Organic anion transporting polypeptide 2b1 (*SLCO2B1*)	NM_174843	8667	174	50	0.00
Cathepsin L (*CTSL*)	NM_174032	25931	526	49	0.00
Unknown	BM433723	1273	28	47	0.01
Endothelial cell-specific molecule 1 (*ESM1*)	NM_001098101	2174	46	46	0.00
Claudin 1 (*CLDN1*)	NM_001001854	2357	51	46	0.00
Ribonuclease (*RNASE1*)	NM_001014386	5671	124	45	0.01
Membrane metallo-endopeptidase (*MME*)	XM_616885	12095	269	45	0.00
Claudin 8 (*CLDN8*)	NM_001098096	962	22	45	0.00
Placental lactogen (*CSH1*)	NM_181007	5865	134	44	0.00
Synaptosomal-associated protein, 91kDa homolog (*SNAP91*)	NM_001105378	1575	36	43	0.00
Chromosome 1 open reading frame 210 ortholog (*C3H1ORF210*)	NM_001081517	1815	43	43	0.00
Unknown	AU279033	15008	363	41	0.00
Predicted: Folate receptor alpha (*FOLR1*)	XM_589186	3733	91	41	0.01
Solute carrier family 4, sodium bicarbonate cotransporter, member 4 (*SLC4A4*)	NM_174605	2264	56	40	0.01
Connective tissue growth factor (*CTGF*)	NM_174030	13225	337	39	0.04
DCN protein (*DCN*)	NM_173906	1608	42	38	0.02
SMAD family member 1 (*SMAD1*)	NM_001076223	652	17	38	0.00
Prolactin-related protein 1 (*PRP1*)	NM_174159	8462	222	38	0.00
Angiogenin (*ANG*)	NM_173891	18115	476	38	0.02
Carboxylesterase 2 (intestine, liver) (*CES2*)	NM_001034260	2088	55	37	0.01

Table 5-2. Continued.

Description	Accession	Intensity		Fold Change	P
		Endometrium	Blood		
Pregnancy-associated glycoprotein 1 *(PAG1B*)	NM_174411	1109	30	37	0.00
chemokine (C-X-C motif) ligand 14 (*CXCL14*)	NM_001034410	3374	92	38	0.00
Pregnancy-associated glycoprotein 4 (*PAG4*)	NM_176615	2662	73	36	0.00
Ovo-like 2 (Drosophila) (*OVOL2*)	XM_611927	825	23	36	0.01
Cadherin 1, type 1, E-cadherin (epithelial) (*CDH1*)	NM_001002763	3184	92	34	0.00
Alpha-2-glycoprotein 1, zinc-binding (*AZGP1*)	NM_001034331	982	29	34	0.00
RAB13, member RAS oncogene family (*RAB13*)	NM_001024540	33728	991	34	0.01
Rho guanine nucleotide exchange factor 5 (*ARHGEF5*)	NM_001110075	7470	224	33	0.00
Angiotensinogen (serpin peptidase inhibitor, clade A, member 8)(*AGT*)	NM_001035369	710	21	33	0.01
Gamma-aminobutyric acid (GABA) A receptor, pi (*GABRP*)	NM_001015618	2656	82	32	0.00
Natriuretic peptide precursor C (*NPPC*)	NM_174125	556	17	32	0.00
Secreted phosphoprotein 1 (*SPP1*)	NM_174187	4939	154	32	0.00
5' nucleotidase, ecto (*NT5E*)	NM_174129	5145	165	31	0.00
Oxytocin receptor (*OXTR*)	NM_174134	4207	136	31	0.00
Glycine amidinotransferase (L-arginine:glycine amidinotransferase) (*GATM*)	NM_001045878	7387	240	30	0.00
Calbindin 3, (vitamin D-dependent calcium binding protein) (*CALB3*)	NM_174257	17680	576	30	0.00
Sorbin and SH3 domain containing 2 (*SORBS2*)	NM_001079787	555	18	30	0.01
Prolactin-related protein VIII (*PRP8*)	NM_001038200	1779	58	30	0.00
Microfibril-associated glycoprotein-2 (*MAGP2*)	NM_174386	2365	78	30	0.00
T-cell immunoglobulin and mucin domain containing 4 (*TIMD4*)	NM_001075320	3946	130	30	0.00
Eetinol binding protein 4, plasma (*RBP4*)	NM_001040475	5918	196	30	0.00
sorbin and SH3 domain containing 2 (*SORBS2*)	NM_001079787	988	33	30	0.00
Pregnancy-associated glycoprotein 10 (*PAG10*)	NM_176621	33870	1148	29	0.00
Chemokine (C-C motif) ligand 22 (*CCL22*)	NM_001099162	897	31	29	0.00
Claudin 1 (*CLDN1*)	NM_001001854	5622	200	28	0.00
Ring finger protein 217 (*RNF217*)	XM_615503	25454	92	28	0.04
Unknown	CB224956	13099	479	27	0.00
Prolactin receptor (*PRLR*), transcript variant 1	NM_174155	4211	154	27	0.03
TBC1 domain family, member 12 (*TBC1D12*)	XM_585982	822	30	27	0.04
Clusterin (*CLU*)	NM_173902	18183	672	27	0.02
Fibroblast growth factor-binding protein (*FGF-BP*)	NM_174337	5672	210	27	0.00
Solute carrier family 27 (fatty acid transporter), member 2 (*SLC27A2*)	XM_615837	773	29	27	0.01
BARX homeobox 2(*BARX2*)	NM_001101266	2221	85	26	0.00
Unknown	CB436038	1383	53	26	0.02
Unknown	BI849183	722	28	26	0.01
Mal, T-cell differentiation protein 2 (*MAL2*)	NM_001081719	2141	84	26	0.00
Phosphatidylethanolamine-binding protein 4 (*PEBP4*)	NM_001079584	1600	63	26	0.01
Unknown	EE981171	3904	154	25	0.00
Hydroxyprostaglandin dehydrogenase 15-(NAD) (*PGDH*)	NM_001034419	1733	69	25	0.00

Table 5-2. Continued

Description	Accession	Intensity		Fold Change	P
		Endometrium	Blood		
Phosphotyrosine interaction domain containing 1 (*PID1*)	NM_001079584	21218	853	24	0.01
Pregnancy-associated glycoprotein 7 (*PAG7*)	NM_176618	8995	362	24	0.00
Unknown	CB457576	540	22	24	0.04
Macrophage scavenger receptor 1 (*MSR1*)	NM_174113	5072	208	24	0.00
Thy-1 cell surface antigen (*THY1*)	NM_001034765	418	17	24	0.02
Mannose receptor, C type 1-like 1 (*MRC1*)	XM_001252128	2246	93	24	0.00
Folate receptor 2 (fetal) (*FOLR2*)	NM_001075325	1486	63	24	0.00
Peptidylprolyl isomerase C (cyclophilin C) (*PPIC*)	NM_001076910	1165	50	24	0.01
Ribosomal modification protein rimK-like family member B (*RIMKLB*)	NM_001076113	1078	46	23	0.01
Rhomboid, veinlet-like 2 (Drosophila) (RHBDL2)	NM_001102014	5718	245	23	0.00
Protein S (alpha) (*PROS1*)	NM_174438	1670	72	23	0.00
Procollagen C-endopeptidase enhancer 2 (PCOLCE2)	NM_001075629	3717	160	23	0.05
Granzyme B (*GZMB*)	XM_585453	58478	2603	23	0.01
Unknown	AW429981	431	19	22	0.00
Tissue factor pathway inhibitor (*TFPI*)	XM_585593	4573	207	22	0.00
Cytochrome P450, family 4, subfamily A, polypeptide 11 (*CYP4A11*)	NM_001077908	3785	175	21	0.00
Claudin 3 (*CLDN3*)	NM_205801	4521	210	21	0.00
Epithelial cell adhesion molecule (*EPCAM*)	NM_001035290	3549	167	21	0.01
Fibrinogen beta chain (*FGB*)	NM_001142917	732	36	20	0.04
Hypothetical LOC614490 (*LOC614490*)	XM_866023	1510	74	20	0.00
V-set and immunoglobulin domain containing 4 (*VSIG4*)	NM_001046529	590	28	20	0.01
Lysophosphatidic acid receptor 1 (*LPAR1*)	NM_174047	393	19	20	0.02
Calcium-dependent phospholipase A2 (*PLA2G2D1*)	NM_001009572	1393	71	19	0.02
Nuclear factor I/B (*NFIB*)	NM_001076104	796	40	19	0.01
Complement component 1, q subcomponent, A chain (*C1QA*)	NM_001014945	15126	793	19	0.00
Predicted: Myosin-VI (Unconventional myosin VI) (*MYO6*)	XM_615128	367	19	19	0.02
Creatine kinase, mitochondrial 1 (ubiquitous) (*CKMT1*)	NM_174275	3257	174	18	0.00
v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian) (*ERBB3*)	NM_001103105	883	47	18	0.00
Phospholipase D1, phosphatidylcholine-specific (*PLD1*)	XM_592425	359	19	18	0.03

Table 5-3. Top 100 upregulated genes based on fold change in blood $CD14^+$ cells as compared to $CD14^+$ cells in the endometrium.

Description	Accession	Intensity		Fold change	P
		Endometrium	Blood		
Calcitonin-related polypeptide beta (*CALCB*)	NM_001001149	192	4564	24	0.02
Fc receptor-like 3 (*FCRL3*)	XM_593037	8	181	23	0.00
Unknown	NM_001077854	304	6759	22	0.04
POU domain, class 2, associating factor 1 (*POU2AF1*)	NM_001075915	22	465	21	0.01
Chemokine (C-C motif) receptor 3, transcript variant 2 (*CCR3*)	XM_869148	8	151	20	0.01
Tachykinin 3, neurokinin beta (*TAC3*)	NM_181017	11	201	18	0.05
Pentraxin-related gene, rapidly induced by IL-1 beta (*PTX3*)	NM_001076259	933	16716	18	0.05
Transcription factor Spi-B *(SPIB*)	XM_001252761	57	973	17	0.04
Membrane-spanning 4-domains, subfamily A, member 1 (*MS4A1*)	NM_001077854	56	933	17	0.04
Myosin, light polypeptide 2, regulatory, cardiac, slow (*MYL2*)	NM_001035025	20	323	16	0.02
Unknown	CB430318	312	4764	15	0.01
Chromosome 16 open reading frame 65 ortholog (*C25H16orf65*)	NM_001076094	12	179	15	0.01
Unknown	CB450328	207	3048	15	0.00
Interleukin 8 receptor, beta (*IL8RB*)	NM_174360	17	245	14	0.00
Patatin-like phospholipase domain containing 1 (*PNPLA1*)	NM_001099200	39	546	14	0.01
Immunoglobulin heavy constant gamma 3 (*IGHG3*)	U63639	15	198	13	0.03
Unknown	BE684495	11	137	13	0.00
Unknown	CB427006	71	895	13	0.02
similar to AT rich interactive domain 5B (MRF1-like) (*LOC783538*)	XR_027785	37	441	12	0.03
EGF-like module-containing mucin-like hormone receptor-like 3 precursor (*EMR3*)	XR_042808	44	513	12	0.01
Similar to Ki antigen (*LOC520216*)	XM_598451	37	405	11	0.02
Kelch-like 10 (Drosophila) (*KLHL10*)	NM_001078033	25	275	11	0.02
Triggering receptor expressed on myeloid cells-like 2 (*TREML2*)	XM_593583	109	1164	11	0.00
B lymphoid tyrosine kinase (*BLK*)	NM_001075968	65	667	10	0.00
Abl-interactor 1 (*ABI1*)	NM_001076241	28	275	10	0.01
KH and NYN domain containing (*KHNYN*)	NM_001102551	14	137	10	0.03
Cyclin E1 transcript variant 1 (*CCNE1*)	XM_612960	26	251	10	0.05
Creatine kinase, mitochondrial 2 (sarcomeric) nuclear gene encoding mitochondrial protein (*CKMT2*)	NM_001034656	7	65	9	0.01
B-cell CLL/lymphoma 11A (zinc finger protein) (*BCL11A*)	NM_001076121	18	169	9	0.03
Hypothetical protein LOC782848, transcript variant 1 (*KIAA1429*)	XM_001250788	110	1014	9	0.01
Xylosyltransferase I (*XYLT1*)	NW_001494295	141	1282	9	0.02
Kelch-like protein 3 (*KLHL3*)	XM_612749	43	388	9	0.04
UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 5 (*B3GNT5*)	NM_001076979	171	1512	9	0.02

Table 5-3. Continued.

Description	Accession	Intensity		Fold change	P
		Endometrium	Blood		
Misc_RNA (*LOC517126*)	XR_028030	11	94	9	0.00
Similar to hCG2042749 (*ZFP91*)	XM_607444	36	310	9	0.02
Unknown	EE971150	73	635	9	0.01
EGF-like module containing, mucin-like, hormone receptor-like sequence 1 (*LOC788835*)	XM_001789147	72	621	9	0.03
Zinc finger protein 295 (*ZNF295*)	NM_001083659	605	5167	9	0.04
Phosphoglucomutase 2 (*PGM2*)	XM_583514	23	191	8	0.00
CD200 molecule (*CD200*)	NM_001034620	73	570	8	0.02
Uncharacterized protein C13orf18 homolog (*MGC165939*)	NM_001102041	41	322	8	0.03
Similar to stromal antigen 3 (*STAG3*)	XM_593416	116	888	8	0.02
v-myb myeloblastosis viral oncogene homolog (avian) (*MYB*)	NM_175050	13	101	8	0.03
Phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma) (*PLA2G7*)	NM_174578	3417	25693	8	0.05
Casein kinase 1, alpha 1 (*CSNK1A1*)	NM_174711	62	464	7	0.02
Unknown	CB461815	74	557	7	0.00
Eukaryotic translation initiation factor 2C, 2 (*EIF2C2*)	NM_205794	1182	8812	7	0.04
Unknown	BP108872	57	426	7	0.02
Exocyst complex component Sec8 (*LOC537690*)	NM_001102207	11	84	7	0.00
Unknown	CB422154	115	854	7	0.04
Cell division cycle 27 homolog (S. cerevisiae) (*CDC27*)	NM_001105428	30	223	7	0.02
Unknown	BI84934	18	130	7	0.02
Serpin peptidase inhibitor, clade B (ovalbumin), member 2 (*SERPINB2*)	XM_582291	197	1455	7	0.03
Unknown	CB440027	215	1590	7	0.04
Polymerase (DNA directed), beta (*POLB*)	NM_001034764	28	205	7	0.00
Unknown	EE895728	29	214	7	0. 204
G protein-coupled receptor 114 (*GPR114*)	XM_587049	2337	16994	7	0.04
Regulatory factor X3 (*RFX3*)	XM_618263	21	150	7	0.02
Unknown	EE943006	9	66	7	0.04
Misc_RNA (*LOC532845*)	XR_027911	15	104	7	0.01
Calcium binding tyrosine-(Y)-phosphorylation regulated (*CABYR*)	NM_001038067	461	3283	7	0.03
TP53 regulating kinase (*TP53RK*)	NM_001099180	11	79	7	0.04
Unknown	EE894024	13	91	7	0.01
RAB11 family interacting protein 1 (class I) transcript variant 1 (*RAB11FIP1*)	XM_597040	641	4374	7	0.04
Spire homolog 2 (*SPIRE2*)	XM_001787738	227	1538	7	0.04
Unknown	EE903724	18	119	7	0.02
Carbonic anhydrase IV (*CA4)*	NM_173897	25	166	7	0.01
Unknown	CB457397	123	817	7	0.05

Table 5-3. Continued.

Description	Accession	Intensity		Fold change	P
		Endometrium	Blood		
Unknown	CB462630	12	79	7	0.04
Vacuolar protein sorting 37 homolog B (S. cerevisiae) (*VPS37B*)	XM_615672	328	2165	7	0.01
Chromatin assembly factor 1, subunit B (p60) (*CHAF1B*)	NM_001098014	148	976	7	0.04
Unknown	CB534202	9	60	7	0.02
Zinc finger, MYM-type 6 (*ZMYM6*)	XM_870563	962	6258	7	0.03
Gametocyte specific factor 1 (*GTSF1*)	NM_001034273	25	163	6	0.02
COX15 homolog, cytochrome c oxidase assembly protein (yeast), mitochondrial protein (*COX15*)	NM_001076861	55	353	6	0.02
E3 ubiquitin-protein ligase HECW2 (HECT, C2 and WW domain-containing protein 2) (NEDD4-like E3 ubiquitin-protein ligase 2) (*LOC531691*)	XM_001787494	17	110	6	0.03
Zinc finger CCCH-type containing 12A (*ZC3H12A*)	NM_001102187	2142	13562	6	0.03
Microtubule-associated protein 1S (*MAP1S*)	NM_001102114	10	64	6	0.01
Interleukin 18 receptor 1 (*IL18R1*)	XM_590497	46	290	6	0.04
Similar to NHS-like 2 (*NHSL2*)	XM_591407	336	2093	6	0.02
Unknown	CB172491	205	1276	6	0.02
Unknown	AV615490	38	232	6	0.02
Slingshot homolog 2 (Drosophila) (*SSH2*)	XM_590201	13698	84007	6	0.04
Special AT-rich sequence binding Protein SATB homeobox 11 (binds to nuclear matrix/scaffold-associating DNA's) (*SATB1*)	NM_001102040	2188	13269	6	0.04
TERF1 (TRF1)-interacting nuclear factor 2 (*TINF2*)	NM_001098114	83	497	6	0.03
Cell death-inducing DFFA-like effector b (*CIDEB*)	NM_001034567	84	503	6	0.01
Ropporin 1-like (*ROPN1L*)	NM_001075717	18	110	6	0.02
PPDE peptidase domain containing 1 (*PPPDE1*)	NM_001100384	132	790	6	0.03
Interleukin 18 receptor accessory protein	XM_001255518	15	89	6	0.02
Mitogen-activated protein kinase kinase kinase kinase 2 (*MAP4K2*)	XM_869702	366	2175	6	0.00
CD22 molecule (*CD22*)	NW_001493607	628	3723	6	0.05
Phosphoprotein associated with glycosphingolipid microdomains 1 (*PAG1*)	XM_865981	1650	9771	6	0.05
Unknown	EE935096	38	225	6	0.02
H2A histone family, member Y2 (*H2AFY2*)	NM_001076086	58	343	6	0.03
Unknown	EE906578	80	469	6	0.05
Synaptogyrin 3 (*SYNGR3*)	NM_001082464	100	573	6	0.04
Torsin family 1, member A (torsin A) (*TOR1A*)	NM_001099058	931	5310	6	0.04
Mannosyl (alpha-1,3-)-glycoprotein beta-1,4-N-acetylglucosaminyltransferase, isoenzyme A (*MGAT4A*)	NM_177520	256	1448	6	0.02
Similar to hCG22803 (*LOC516576*)	XM_594731	10	56	6	0.03
Unknown	AW428380	25	139	2	0.02

three were upregulated in blood [CD80 antigen (CD80); signal transducer and activator of transcription 1 (*STAT1*); apoptosis (APO1) antigen 1 (FAS), member 6 (*TNFRSF6*)].

Gene Ontology Analysis of Differentially Expressed Genes

The FatiGo (fatigo.org) web tool was used to perform a functional enrichment analysis to determine ontologies in which differentially regulated genes were more frequent in either endometrium or blood (Table 5-4.) A total of 10 ontology classes were significant. In 4 cases, there was over-representation of differentially-expressed genes in endometrium (peptidase activity, endopeptidase activity, proteolysis, and extracellular region part) while in 6 cases there was over-representation of differentially-expressed genes in blood (zinc ion binding, phosphotransferase activity - alcohol group as acceptor, biopolymer metabolic process, signal transduction, nucleus, and cell communication).

Differentially Expressed Pathways

Pathway Express software (http://vortex.cs.wayne.edu/ontoexpress) was used to identify pathways that were preferentially represented in the group of differentially expressed genes. A total of 33 pathways were significantly affected by the genes that were differentially regulated in our data set (Table 5-5). Among these were 6 pathways from the immune system group. Two pathways (complement and coagulation cascades, hematopoietic cell lineage) contained more upregulated genes in the endometrium than in the blood whereas three (toll-like receptor signaling, Fc epsilon RI signaling, and B cell receptor signaling) contained more upregulated genes in the blood. The pathway with the greatest impact factor, leuokocyte transendothelial migration, was represented by 6 genes upregulated in endometrium and 6 upregulated in blood.

There were 3 pathways of the signal molecules and interactions group (cell adhesion molecules, ECM-receptor interaction, and cytokine-cytokine receptor interaction) that were differentially regulated. There were more upregulated genes in the endometrium than in the blood in the first two pathways whereas, for the cytokine-cytokine receptor interaction pathway, there were 12 genes upregulated in endometrium and 9 upregulated in blood. Another group in which several pathways were differentially regulated was the signal transduction network. A total of 7 pathways were differentially regulated including those with a preponderance of genes upregulated in endometrium (calcium signaling), those with a preponderance of genes upregulated in blood (phosphatidylinositol signaling, Jak-STAT signaling and ErbB signaling) and those with large numbers of upregulated genes in both tissues (TGFB signaling, MAPK signaling and wnt signaling).

Table 5-4. Ontologies where the incidence of frequency of overexpressed genes in the endometrium was different than the incidence of overexpressed genes in the blood.[a]

Ontology term	Genes differentially expressed in the endometrium (E) and blood (B)	Percentage of genes in the ontology		P
		Endometrium	Blood	
Molecular function				
Peptidase activity (GO:0008233)	E: *CTSK; CTS; DPP4; GZMA; PAG3; PAG4; PAG5; PAG6; PAG10; PAG11; PAG15; PAG17; PAG18; PAG1B; PAG21; PLAT; PSEN1; ST14*	16.1	0.0	0.003
	B: none			
Endopeptidase activity (GO:0004175)	E: *CTSB; CTSK; CTSL; CTSZ; DPP4; GZMA; GZMA; PAG10; PAG11; PAG15; PAG17; PAG18; PAG1B; PAG21; PAG3; PAG4; PAG5; PAG6; PAG9; PLAT; ST14*	15.3	0.0	0.016
	B:none			
Zinc ion binding (GO:0008270)	E: *FBLIM1;egr1;RNF128;NT5E ;NR1H3*	5.6	26.9	0.048
	B: CA4; FGD6; MYNN; NR1H2; PCGF1; PCGF4; PHF17; PPARD; PRKCB1; RABEX5; RARA; SEC23B; ZFX; ZNF410			
Phosphotransferase activity; alcohol group as acceptor (GO:0016773)	E: *IHPK3; STK25*	1.5	13.2	0.052
	B: CSNK1A1; CSNK1E; DGKH; FYN; IPMK; LOC538702; PDIK1L; PRKCB1; RIOK3			
Biological process				
proteolysis (GO:0006508)	E: C1QA; CTSB; CTSK; CTSL; CTSZ; DPP4; gzmA; GZMA ; PAG10; PAG11; PAG15; PAG17; PAG18; PAG1B; PAG21; PAG3; PAG4; PAG5; PAG6; PAG9; PLAT; PSEN1; RNF128; ST14;	25.0	0.0	0.001
	B: none			
signal transduction (GO:0007165)	E: *ANG; EDG2; EDNRB; EPAS1; FZD3; GRIM19; ITGB6; OXTR; PGDH; PRLR; PSCD4; PSEN1; RAB13; RRAS;*	11.3	34.3	0.013
	B: *ARNT; ASB6; CDK5R1; CNIH4; CSNK1A1; CSNK1E; DGKH; FGD6; ITGA4; MS4A1; OPN1SW; PPP2CA; PRKCB1; RAB11A; RAB3A; RABIF; RASGRP4; STAT3; STAT5A; STAT5B; TAC3; TIMAP; TNFRSF1B; TNFRSF6; UPK1A;*			

Table 5-4. Continued.

Ontology Term	Genes differentially expressed in the endometrium (E) and in the blood (B)	Percentage (%) of genes in the ontology		P
		Endometrium	Blood	
biopolymer metabolic process (GO:0043283)	E: *ANG; AR; CBX6; CTGF; EGR1; ELF5; EPAS1; ERCC5; FOS; FOXA3; GGCX; GRIM19; HAND1; JUN; MSX1; NR1H3; PSEN1; RNF128; ST3GALIV; STK25; TFB1M;* B: *ARNT;B3GNT5; BAT3; CBX7; CSNK1A1; CSNK1E; DUSP12; IRF1; L41691;MYB; MYNN; NR1H2; OPN1SW; PDIK1L; PHF17; POP4; PPARD; PPIL1; PPP2CA; PRKCB1; RARA; RPP38; SNURF; STAT3; STAT5A; STAT5B; TREX1; UBE2B; UFM1; ZFX;*	16.9	41.1	0.013
cell communication (GO:0007154)	E: *ANG; APOE; EDG2; EDNRB; EPAS1; FZD3; GJB1; GRIM19; ITGB6; OXTR; PGDH; PRLR; PSCD4; PSEN1; RAB13; RRAS*; B: *ARNT; ASB6; CDK5R1; CNIH4; CSNK1A1; CSNK1E; DGKH; FGD6; ITGA4; MS4A1; OPN1SW; PPP2CA; PRKCB1; RAB11A; RAB3A; RABIF; RASGRP4; STAT3; STAT5A; STAT5B; TAC3; TIMAP; TNFRSF1B; TNFRSF6; UPK1A;*	12.7	33.3	0.042
Cellular component				
nucleus (GO:0005634)	E: *ANG; AR; BANF1; CBX6; egr1; ELF5; EPAS1; ERCC5; FOS; FOXA3; GRIM19; HAND1; JUN; MSX1; NR1H3; PSEN1; UACA;* B: *ARNT; CBX7; CCNE2; CDK5R1; IRF1; L41691; MYB; MYNN; NR1H2; PHF17; POLR2J2; POP4; PPARD; PPIL1; PPP2CA; RARA; RBM15B; RPP38; SNURF; STAT3; STAT5A; STAT5B; TAF11; TREX1; UBE2B; UFM1; ZFX;*	29.82	64.29	0.019
extracellular region part (GO:0044421)	E: *ANG; APOE; CCL2; CCL8; COL1A2; CTGF; DCN; GJB1; MAGP2; SPP1;* B: none	8.85	0.00	0.079

[a]Analysis was performed using FatiGo—a web tool for finding significant associations of Gene Ontology terms with groups of genes (Al-Shahrour et al., 2007).

Table 5-5. Pathways subject to differential regulation.[a]

Pathway Name	IF[b]	Genes overexpressed in endometrium (E) and blood (B)	IG/GP[b]	P[b]	γP[b]
Immune system					
Leukocyte transendothelial migration	895	E: *PXN; VCAM1; THY1; CLDN3; CLDN1; CLDN8*	12/119	0.02	0.00
		B: *GRLF1; MYL2; ITGA4; PIK3R5; PIK3R3; MYLPF*			
Toll-like receptor signaling pathway	11	E: *MAPK3; FOS; JUN; TLR3; SPP1*	16/102	0.00	0.00
		B: *MAP2K4; RELA; MAP2K3; PIK3R3; STAT2; TIRAP; MAPK3K8; IFNAR1; TRAF3; CD80; PIK3R5*			
Complement and coagulation cascades	11	E: *C1QA; C1QB; C1QC; C3AR1; CD59; CFB; PLAT; PROS1; SERPINA5; TFPI; FGB*	11/69	0.00	0.00
		B: none			
Hematopoietic cell lineage	5	E*: CD59; TFRC; ITGA6; CD7; CSFR1; MME*	9/87	0.03	0.03
		B: *MS4A1; CD22; ITGA4*			
Fc epsilon RI signaling pathway	5	E: *MAPK3; RAC3; PLA2G3*	9/78	0.01	0.04
		B: *PIK3R5; PIK3R3; GRB2; MAP2K3; MAP2K4; FYN*			
B cell receptor signaling pathway	5	E*: RAC3; JUN; FOS*	8/65	0.04	0.04
		B: *CD79A; RELA; PIK3R5; PIK3R3; CD22*			
Signaling molecules and interaction					
Cell adhesion molecules (CAMs)	632	E: *ALCAM; SIGLEC1; ITGA6; VCAM1; PVRL3; MPZL1; CDH1; CLDN8; CLDN1; CLDN3; SDC3*	14/134	0.01	0.00
		B: *CD80; CD22; ITGA4*			
ECM-receptor interaction	6	E: *AGRN; SDC3; ITGA6; ITGB6; LAMC1; COL1A2; COL3A1; SPP1*	9/84	0.02	0.02
		B: *ITGA4*			
Cytokine-cytokine receptor interaction	6	E: *CXCL14; CCL22; PRLR; INHBB; CCL24; CSF1R; PRL; LTBR; TNFRSF9; CCL8; CCL2; PDGFB*	21/263	0.04	0.02
		B: *CCR3; IL8RB; IL18RAP; CRLF2; IFNAR1; TNFRSF1B; IL17RA; TNFRSF8; CLCF1*			

Table 5-5. Continued.

Pathway Name	IF[b]	Genes overexpressed in endometrium (E) and blood (B)	IG/GP[b]	P[b]	γP[b]
Signal transduction					
Phosphatidylinositol signaling system	17	E:*CLML5*	7/76	0.08	0.00
		B: *CDS2; DGKH; DGKZ; INPP5K; PIK3R5; PIK3R3*			
TGF-beta signaling pathway	7	E: *MAPK3; INHBB; ID4; DCN*	7/87	0.14	0.01
		B: *PPP2CA; CREBBP; E2F5*			
Jak-STAT signaling pathway	7	E: *BCL1L1; PRPL; CSH1; PRL*	16/155	0.00	0.01
		B: *PIK3R5; CRFL2; IFNAR1; CLCF1; PIAS2; STAT5B; STAT1; PIK3R3; GRB2; CREBBP; STAT3; STAT5A*			
ErbB signaling pathway	7	E: *MAPK3; JUN; ERBB3*	11/87	0.00	0.01
		B: *CDKN1A; MAP2K4; STAT5B; PIK3R5; PIK3R3; GRB2; STA5A; NCK1*			
Calcium signaling pathway	7	E: *CALM5; EDNRB; ERBB3; OXTR; SLC8A3*	5/182	0.95	0.01
		B: none			
MAPK signaling pathway	6	E: *MAPK3; FOS; JUN; RAC3; PDGFB; NF1; RRAS; PLA2G3; FGF1; HSPA1B; HSPA1A; HSPA6*	21/272	0.05	0.02
		B: *DUSP1; MAP4K2; MAP3K5; MAP3K8; GRB2; MAP2K3; RELA; MAP2K4; RASGRP4*			
Wnt signaling pathway	5	E: *PSEN1; FZD3; JUN; RAC3; WNT10B; DKK1*	14/152	0.02	0.03
		B: *CSNK1A1; PPARD; CSNK1A1L; PPP2R5B; DAAM1; CREBBP; CSNK1E; PPP2CA*			
Cancers					
Pathways in cancer	16	E: *CDH1; ARNT2; PLD1; CSF1R; AR; CCNA1; FGF1; LAMC1; ITGA6; BCL2L1; WNT10B; PDGFD; RAC3; JUN; FOS; FZD3; EPAS1; MAPK3*	39/330	0.00	0.00
		B: *CCNE1; PIK3R5; TRAF3; RARA; PIAS2; PML; STAT5B; STAT1; PIK3R3; PPARD; GRB2; RET; CDKN1A; RELA; CCNE2; CREBBP; STAT3; ARNT; RUNX1; STAT5A; TCEB1*			
Acute myeloid leukemia	13	E: *CCNA1; MAPK3*	13/59	0.00	0.00
		B: *STAT5A; RUNX1; STAT3; RELA; GRB2; PPARD; PIK3R3; STAT5B; PML; RARA; PIK3R5*			
Renal cell carcinoma	9	E*: MAPK3; JUN; PDGFB; EPAS1; ARNT2*	11/69	0.00	0.00
		B: *PIK3R3; PIK3R5; GRB2; CREBBP; ARNT; TCEB1*			

Table 5-5. Continued.

Pathway Name	IF[b]	Genes overexpressed in endometrium (E) and blood (B)	IG/GP[b]	P[b]	γP[b]
Cancers					
Prostate cancer	9	E: *MAPK3; CREB5; PDGFB; CRE3L2; AR*	13/90	0.00	0.00
		B: *PIK3R3; GRB2; CDKN1A; RELA; CCNE2; CREBBP*			
Chronic myeloid leukemia	6	E: *MAPK3; BCL2L1*	10/75	0.00	0.01
		B: *PIK3R5; STAT5B; PIK3R3; GRB2; CDKN1A; RELA; RUNX1; STAT5A*			
Small cell lung cancer	6	E: *BCL2L1; ITGA6; LMAC1*	10/86	0.01	0.02
		B: *CCNE1; PIK3R5; PIK3R3; TRAF3; PIAS2; RELA; CCNE2*			
Pancreatic cancer	5	E: *MAPK3; RAC3; BCL2L1; PLD1*	9/72	0.01	0.03
		B: *PIK3R3; PIK3R5; STAT1; RELA; STAT3*			
Melanoma	5	E: *MAPK3; PDGFB; FGF-1; CDH1*	7/71	0.05	0.05
		B: *PIK3R5; PIKR3R3; CDKN1A*			
Cell communication					
Adherens junction	15	E: *RAC3; LMO7; PVRL4; PVRL3; CDH1; MAPK3; PARD3;*	10/78	0.01	0.00
		B: *CREBBP; FYN; SNAI1*			
Tight junction	13	E: *RRAS; AMOTL1; RAB13; CLDN3; CLDN1; CLDN8; CGN; PARD3*	16/135	0.00	0.00
		B: *MYL2; EXOC4; ASHL1; MYLPF; MYH10; PPP2CA; CASK; PRKCI*			
Focal adhesion	7	E: *MAPK3; RAC3; PDGFB; JUN; ITGB6; ITGA6; LAMC1; COL1A2; COL3A1; SPP1*	20/203	0.00	0.00
		B: *MYL2; PIK3R5; ITGA4; MYLPF; GRLF1; PXN; DIAPH1; GRB2; PIK3R3; FYN*			
Endocrine system					
PPAR signaling pathway	8	E: *NR1H3; PCK1; SCD; CYP27A1; CYP4A11; SLC27A2*	11/70	0.00	0.00
		B: *CPT1A; CPT1C; OLR1; PPARD; GK*			
Renin-angiotensin system	6	E: *AGT; CTSA; MME*	3/17	0.04	0.01
		B: none			

Table 5-5. Continued.

Pathway Name	IF[b]	Genes overexpressed in endometrium (E) and blood (B)	IG/GP[b]	P[b]	γP[b]
Behavior					
Circadian rhythm		E: none			
	10	B: *CRY2; CSNK1E; PER1*	3/13	0.02	0.00
Infectious diseases					
Pathogenic Escherichia coli infection	6	E: *TUBB6; TUBA3C; KRT18; CLDN1; CDH1*	7/54	0.02	0.02
		B: *FYN; NCK1*			
Transcription					
Basal transcription factors	5	E: *GTF2A1; TAF1; GTF2I*	6/37	0.01	0.03
		B: *TAF4B; TAF11; GTF2B*			
Cell motility					
Regulation of actin cytoskeleton	6	E: *MAPK3; TMSB4X; RAC3; PDGFB; RRAS; ITGA6; ITGB6; ENAH; FGF1*	19/217	0.01	0.02
		B*: MYL2; SSH2; PIK3R5; PIK3R3; ITGA4; GRLF1; MYLPF; MYH10; DIAPH1; PXN*			

[a]Pathway express was used to determine pathways that are differentially regulated (Draghici et al. 2007)

[b]Abbreviations are as follows: IF, Impact Factor; IG/GP, number of differentially regulated genes/ total number of genes involved in the pathway; P, probability value by hypergeometric distribution analysis; γP: gamma P value, which is the P value corrected by impact factor.

Differentially Expressed Genes Characteristically Regulated during Macrophage Differentiation

To determine the maturational state of endometrial macrophages as compared to blood monocytes, we evaluated which genes that are regulated during macrophage differentiation are differentially regulated between endometrial and blood $CD14^+$ cells. The data set used as reference was developed by Martinez et al. (2006). In that study, human blood monocytes were cultured with colony stimulating factor 1 (CSF1) for 7 days to drive differentiation of monocytes to macrophages. Subsequently, CSF1 treated cells were cultured with either interferon gamma (*IFNG*) and LPS to cause differentiation to the classically-activated or M1 phenotype or interleukin-4 (IL4) to cause differentiation to the alternatively-activated or M2 phenotype (Figure 5-3).

Martinez et al. (2006) identified 143 genes that were differentially regulated in monocytes after three days of culture in CSF1 medium (i.e., intermediate differentiation). A total of 122 of these 143 differentially-expressed genes were expressed by endometrial and blood $CD14^+$ cells and 11 were differentially expressed between endometrium and blood (Figure 5-3). Of the 73 genes shown by Martinez et al. (2006) to be upregulated in differentiating macrophages, expression of 7 were higher in the endometrium and 1 was higher in the blood (see Table 5-6). Of the 55 genes shown by Martinez et al. (2006) to be downregulated in the differentiating macrophage, 2 were expressed in higher amounts in endometrium and 2 were expressed in higher amounts in blood (Table 5-6).

Table 5-6. Differentially regulated genes in endometrial and blood CD14+ cells that are differentially regulated in the intermediately-differentiated macrophage.[a]

Description	Accession	Intensity		Fold change	P	Tissue[b]
		Endometrium	Blood			
Upregulated genes during monocyte to macrophage differentiation						
Fc fragment of IgG, high affinity I, receptor for (*FCGR1*)	NM_174538	306	11	28	0.05	Endo
Chemokine (C-C motif) ligand 2 (*CCL2*)	NM_174006	18786	3526	5	0.01	Endo
Early growth response 1 (*EGR1*)	NM_001045875	64632	17902	4	0.02	Endo
Centromere protein A transcript variant 1 (*CENPA*)	XM_864817	11731	5692	2	0.02	Endo
Chromosome 6 open reading frame 115 (*C6orf115*)	XM_582278	10895	6280	2	0.02	Endo
Lectin, mannose-binding 2 (*LMAN2*)	NM_001101309	34142	17438	2	0.03	Endo
24-dehydrocholesterol reductase (*DHCR24*)	XM_613218	101952	47891	2	0.05	Endo
Opa interacting protein 5 (*OIP5*)	XM_588370	204	630	3	0.00	Blood
Downregulated genes during monocyte to macrophage differentiation						
Predicted: Palladin, cytoskeletal associated protein (*PALLD*)	XM_869983	753	48	16	0.00	Endo
BCL2-like 1, nuclear gene encoding mitochondrial protein (*BCL2L1*)	NM_001077486	2017	342	6	0.00	Endo
Inhibitor of growth family, member 3, transcript variant 2 (*ING3*)	XM_863966	4923	15733	3	0.05	Endo

[a] The list of genes that are upregulated or downregulated during the monocyte to macrophage differentiation process was obtained from Martinez et al. (2007). See Figure 5-3 for details of conditions for macrophage differentiation used by Martinez et al. (2007). [b]Tissue with higher expression in the current experiment. Endo signifies that expression was higher for endometrial $CD14^+$ cells whereas Blood signifies higher expression in blood $CD14^+$ cells.

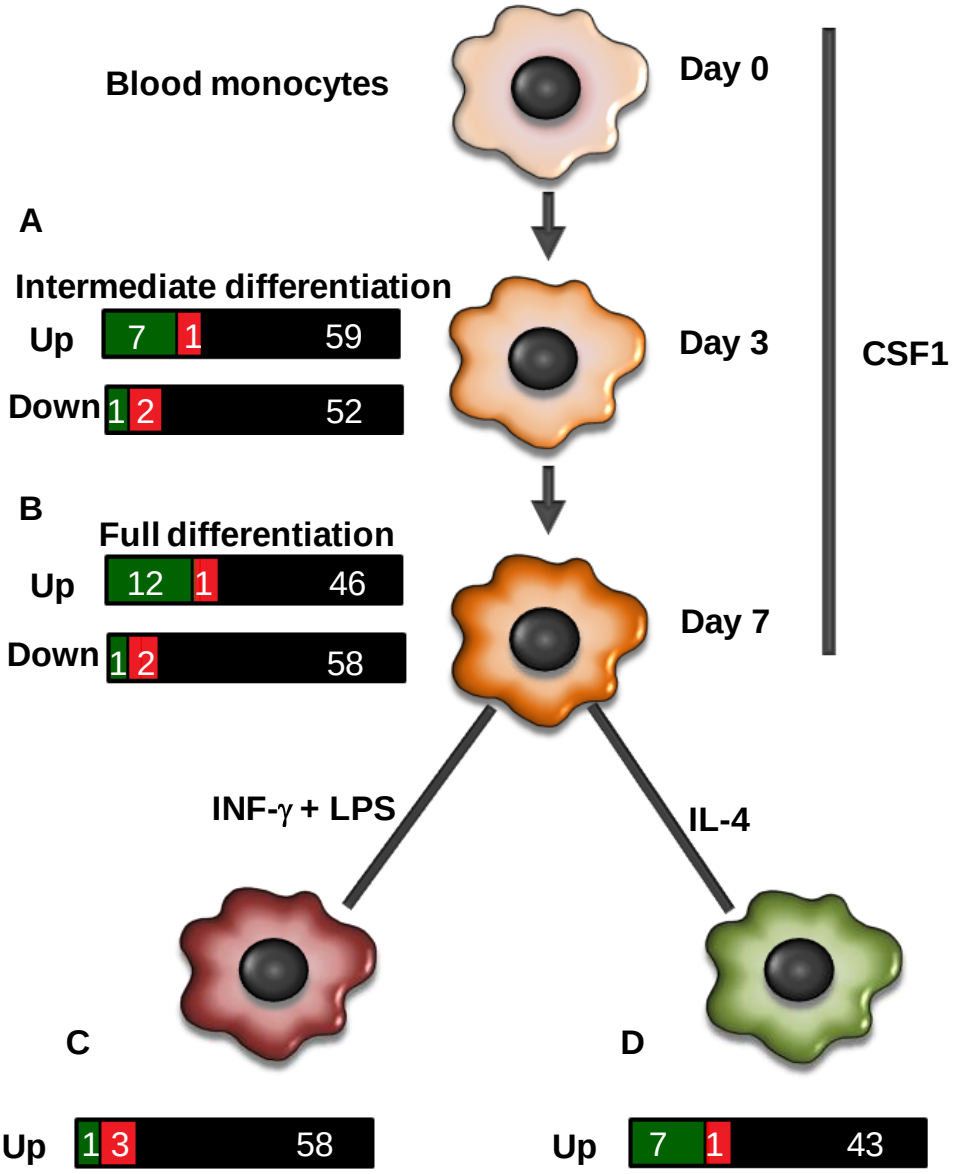

Figure 5-3. Graphical representation of differentially regulated genes in endometrial and blood CD14$^+$ cells that have been reported to be differentially regulated during macrophage differentiation. Martinez et al. (2006) identified genes that are differentially expressed in monocytes after 3 (A) or 7 days (B) of culture with CSF1 to cause differentiation into macrophages and after macrophages were polarized towards the classical activation (M1) pathway (C, achieved by culture with interferon-γ and lipopolyssacharide) or alternative activation (M2) pathway (D, achieved by culture with interleukin 4). For each stage of differentiation, bars represent the number of genes in the Martinez et al. (2006) data set that were found in the present data set of endometrial and blood CD14$^+$ cells. Genes that are upregulated in endometrium are in green, genes upregulated in blood are in red, and genes that were not significantly upregulated are in black.

Martinez et al. (2006) identified 120 genes that were differentially regulated in monocytes after 7 days of culture with CSF1 (i.e., full differentiation). All of these genes were expressed in endometrial and blood $CD14^+$ cells. Of the 59 genes that were shown by Martinez et al. (2006) to be upregulated after macrophage differentiation, 12 were upregulated in the endometrium and none were upregulated in blood (Figure 5-3). Of the 61 genes that were reported to be downregulated, 9 were upregulated in endometrium and 3 were upregulated in the blood. A list of these genes is in Table 5-7.

Martinez et al. (2006) identified 62 genes upregulated in M1 activated macrophages and 52 genes upregulated in M2 activated macrophages. Of the 62 genes identified to be upregulated in M1 macrophages, 1 gene was upregulated in the endometrium and 3 were higher in the blood (Figure 5-3). Of the 52 upregulated in M2 macrophages, 7 genes were significantly higher in the endometrium and 1 gene was upregulated in the blood. The list of these genes is in Table 5-8.

Comparison to Genes Expressed by Human Decidual Macrophages

In the human, decidual macrophages possess a unique pattern of gene expression as compared to monocytes (Gustafson et al. 2008). We asked the question if the genes that are upregulated or downregulated in human decidual macrophages are also differentially regulated in bovine endometrial macrophages. A total of 90% (107 of 120) of the genes found by Gustafson et al. (2008) to be differentially-expressed in human decidual macrophages were present in our dataset. Of the 80 genes identified to be upregulated in decidual macrophages, 19 genes were significantly higher in the endometrium and 2 were higher in the blood. Of the 52 upregulated in blood monocytes, 3 genes were significantly higher in the blood and none were higher in the endometrium. The list of these genes is in Table 5-9.

Table 5-7. Differentially regulated genes in endometrial and blood CD14$^+$ cells that are differentially regulated in fully differentiated macrophages.[a]

Description	Accession	Intensity		Fold change	P	Tissue[b]
		Endometrium	Blood			
Upregulated genes in differentiated macrophages						
Ribonuclease (*RNASE1*)	NM_001014386	5671	124	46	0.01	Endo
Folate receptor 2 (fetal) (*FOLR2*)	NM_001075325	1486	63	24	0.00	Endo
Component 1, q subcomponent, alpha polypeptide (*C1QA*)	NM_001014945	15126	793	19	0.00	Endo
Apolipoprotein E (*APOE*)	NM_173991	3576	208	17	0.00	Endo
Complement component 1, q subcomponent, B chain (*C1QB*)	NM_001046599	29681	2122	14	0.00	Endo
Transcobalamin II; macrocytic anemia (*TCN2*)	NM_174195	6560	811	8	0.03	Endo
Myloid beta (A4) precursor protein (*APP*)	NM_001076796	18442	2748	7	0.04	Endo
Sialoadhesin (*SIGLEC1*)	XM_870818	277	50	6	0.05	Endo
Hypothetical LOC539693 (*AK3L1*)	XM_588793	163	49	3	0.04	Endo
Phospholipase D family, member 3 (*PLD3*)	NM_001078041	3318	1003	3	0.03	Endo
Zinc finger, FYVE domain containing 26 (*ZFYVE26*)	XM_592247	6979	3169	2	0.02	Endo
Nuclear receptor subfamily 1, group H, member 3 (*NR1H3*)	NM_001014861	10998	5265	2	0.01	Endo
Scm-like with four MBT domains protein 2 (*SFMBT2*)	XM_607062	63	211	3	0.05	Blood
Downregulated genes in differentiated macrophages						
Desmoglein 2 preproprotein (*DSG2*)	XM_584890	201	7	30	0.05	Endo
Cystatin E/M (*CST6*)	NM_001012764	17864	1411	13	0.01	Endo
Ankycorbin (Retinoic acid-induced protein 14) (*RAI14*)	XM_604226	1960	160	12	0.00	Endo
Regulator of G-protein signaling 2, 24kDa (*RGS2*)	NM_001075596	57900	6307	9	0.05	Endo
Cytochrome P450, family 27, subfamily A, polypeptide 1 (*CYP27A1*)	NM_001083413	2018	366	6	0.04	Endo
FBJ murine osteosarcoma viral oncogene homolog (*FOS*)	NM_182786	34786	8393	4	0.02	Endo
Zinc finger, MYND domain containing 15 (predicted) (*ZMYND15*)	XM_589053	2419	868	3	0.02	Endo
DEAD (Asp-Glu-Ala-Asp) box polypeptide 17 (*DDX17*)	NM_001101993	41773	22419	2	0.03	Endo
Interleukin 27 receptor, alpha (*IL27RA*)	NM_001098028	28729	12407	2	0.05	Endo
Predicted: cytokine receptor-like factor 2 (*CRLF2*)	XM_608251	188	880	5	0.04	Blood
Low density lipoprotein (lectin-like) receptor 1 (*OLR1*)	NM_174132	564	2330	4	0.03	Blood
FYN oncogene related to SRC, FGR, YES (*FYN*)	NM_001077972	4343	7566	2	0.05	Blood

[a] The list of genes that are upregulated or downregulated in fully differentiated macrophages as compared to blood monocytes was obtained from Martinez et al. (2007). See Figure 5-3 for details of conditions for macrophage differentiation used by Martinez et al. (2007).

[b] Tissue with higher expression in the current experiment. Endo signifies that expression was higher for endometrial CD14$^+$ cells whereas Blood signifies higher expression in blood CD14$^+$ cells.

Table 5-8. Differentially regulated genes in endometrial and blood CD14$^+$ cells that are differentially regulated in M1 and M2 macrophages.[a]

Description	Accession	Intensity		Fold change	P	Tissue[b]
		Endometrium	Blood			
Overexpressed genes in M1 macrophages						
Complement factor B (*CFB*)	NM_001040526	18183	1647	11	0.03	Endo
Pentraxin-related gene, rapidly induced by IL-1 beta (*PTX3*)	NM_001076259	933	16716	18	0.05	Blood
Fas (TNF receptor superfamily, member 6) (*FAS*)	NM_174662	360	1971	5	0.03	Blood
Indoleamine 2,3-dioxygenase 1 (*IDO1*)	NM_001101866	380	1549	4	0.05	Blood
Overexpressed genes in M2 macrophages						
Solute carrier organic anion transporting polypeptide 2b1 (*SLCO2B1*)	NM_174843	8667	173	50	0.00	Endo
Glycine amidinotransferase (L-arginine:glycine amidinotransferase) (*GATM*)	NM_001045878	7387	240	31	0.00	Endo
Predicted: mannose receptor C1 (MRC1)	XM_001252128	2246	93	24	0.00	Endo
Aldehyde dehydrogenase 1 family, member A1 (*ALDH1A1*)	NM_174239	3722	210	18	0.01	Endo
Prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase 1) (*PTGS1*)	NM_001105323	2102	242	9	0.03	Endo
Ribonuclease, RNase A family, k6 (*RNASE6*)	NM_174594	33082	4824	7	0.04	Endo
C-type lectin domain family 7, member A (*CLEC7A*)	NM_001031852	50	22	2	0.03	Endo
Predicted: dipeptidase 2 (*DPEP2*)	XM_586714	140	328	2	0.05	Blood

[a]The list of genes that are differentially regulated in M1 as compared to M2 was obtained from Martinez et al. (2007). See Figure 5-3 for details of conditions for macrophage differentiation used by Martinez et al. (2007).

[b]Tissue with higher expression in the current experiment. Endo signifies that expression was higher for endometrial CD14$^+$ cells whereas Blood signifies higher expression in blood CD14$^+$ cells.

Table 5-9. Differentially expressed genes in endometrial and blood CD14$^+$ cells that are also differentially expressed in human decidual macrophages vs human blood monocytes.[a]

Description	Accession	Intensity		Fold change	P	Tissue[b]
		Endometrium	Blood			
Upregulated genes in decidual macrophages						
Granzyme A (*GZMA*)	NM_001001142	33196	276	120	0.00	Endo
Solute carrier organic anion transporter family, member 2B1 (*SLCO2B1*)	NM_174843	8667	173	50	0.00	Endo
Ribonuclease, RNase A family, 1 (*RNASE1*)	NM_001014386	5671	124	46	0.01	Endo
Secreted phosphoprotein 1 (*SPP1*)	NM_174187	4939	154	32	0.00	Endo
Collagen, type III, alpha 1 (*COL3A1*)	XM_588040	430	15	29	0.02	Endo
Mannose receptor, C type 1 (*MRC1*)	NM_002438	2246	93	24	0.00	Endo
Protein S (alpha) (*PROS1*)	NM_174438	1670	72	23	0.00	Endo
V-set and immunoglobulin domain containing 4 (*VSIG4*)	XM_865690	590	29	20	0.01	Endo
Complement component 1, q subcomponent, alpha polypeptide (*C1QA*)	NM_001014945	15126	793	19	0.00	Endo
Apolipoprotein E (*APOE*)	NM_173991	3576	208	17	0.00	Endo
Leprecan-like 1 (*LEPREL1*)	XM_589206	1776	125	14	0.03	Endo
Complement component 1, q subcomponent, beta polypeptide (*C1QB*)	XM_869694	29681	2122	14	0.00	Endo
Collagen, type I, alpha 2 (*COL1A2*)	NM_174520	892	72	12	0.04	Endo
Chemokine (C-C motif) ligand 8 (*CCL8*)	NM_174007	9546	1735	6	0.03	Endo
Chemokine (C-C motif) ligand 2 (*CCL2*)	NM_174006	18786	3526	5	0.01	Endo
NKG2C protein (*NKG2C*)	NM_001098163	665	139	5	0.03	Endo
Nerve growth factor receptor (TNFRSF16) associated protein 1 (*NGFRAP1*)	NM_014380	6512	1418	5	0.03	Endo
Endothelial PAS domain protein 1 (*EPAS1*)	NM_174725	7981	2114	4	0.05	Endo
Transmembrane protein 97 (*TMEM97*)	NM_001034403	6653	3454	2	0.05	Endo
Stathmin 1/oncoprotein 18 (*STMN1*)	XM_589478	384	2113	6	0.05	Blood
Nicotinamide riboside kinase 1 (*LOC510456*)	XM_587597	166	422	3	0.05	Blood
Upregulated genes in blood monocytes						
Selenoprotein X, 1 (*SEPX1*)	NM_001034810	7361	19546	3	0.03	Blood
Dipeptidase 2 (*DPEP2*)	XM_586714	140	328	2	0.05	Blood
Benzodiazapine receptor (peripheral) (*BZRP*)	NM_175776	2367	5508	2	0.04	Blood

[a]The list of genes that are differentially regulated in human tissues are obtained from Gustafsson et al. (2008).

[b]Tissue with higher expression in the current experiment. Endo signifies that expression was higher for endometrial CD14$^+$ cells whereas Blood signifies higher expression in blood CD14$^+$ cells.

Quantitative RT-PCR

To validate the findings of the microarray analysis, qPCR was performed for 12 differentially expressed genes and a housekeeping gene (RPL19 – bovine ribosomal protein L19). Analyses were performed on two sets of samples of mRNA. The first set was composed of mRNA from two pairs of endometrial and blood $CD14^+$ cells that were used in the microarray. The second set was composed of mRNA from an additional three pairs of endometrial and blood $CD14^+$ cells that were not used for the microarray analysis.

In the first analysis (Table 5-10), with samples used in the microarray, 7 of 12 genes were significantly affected by tissue type [*PAG10; GZMA; HMOX1; MRC1; RNASE6; TSGA14; FCGR3A*]. In each case, expression was higher in endometrium, in agreement with results of the microarray analysis. In addition, an additional gene, SLCO2B1, tended to be higher in the endometrium (7.2-fold; P = 0.09). This gene was also more highly expressed in endometrium in the microarray analysis. Amounts of mRNA as determined by qPCR were not affected by tissue type for two other genes (*PTX3* and *MCP1*). In both cases, the fold-change was numerically higher for endometrium whereas, in the microarray analysis, expression was higher in blood.

For the group of independent samples, 4 genes were significantly upregulated in endometrium compared with the blood (*PAG10; GZMA, TSGA14 and HMOX1*) and all of these genes were higher in the endometrium in the microarray analysis. An additional 5 genes show differences in the same direction as for the microarray although differences were not significant (*MCP1, RNASE6, SLCO2B1, PTX3 and MCP1*). Two genes were not significantly affected by tissue but differences were in the opposite direction as for the microarray analysis (*CD163, CD16*).

Table 5-10. qPCR validation of 12 genes that were differentially regulated in the microarray analysis

		RNA samples submitted to microarray and qPCR analysis							RNA samples submitted only to qPCR analysis						
		ΔCt[a]							ΔCt[a]						
Gene	Accession	Endometrium	Blood	Ste	ΔΔCt[b]	Fold change	P	MC[c]	Endometrium	Blood	Ste	ΔΔCt[b]	Fold change	P	MC[c]
PAG10	NM_176621	8.2	18.7	0.1	-10.4	1389	0.0003	Confirmed	7.5	19.1	1.2	-11.5	2964.0	0.002	Confirmed
GZMA	NM_001001142	6.8	16.5	0.9	-9.8	867	0.0112	Confirmed	7.7	16.1	1.5	-8.3	322.5	0.016	Confirmed
HMOX1	NM_001014912	12.4	16.8	0.1	-4.4	22	0.0002	Confirmed	11.9	16.0	1.3	-4.1	17.5	0.000	Confirmed
MRC1	XM_001252128	15.7	18.4	0.5	-2.7	6.7	0.0009	Confirmed	15.7	18.6	0.6	-3.0	7.8	0.065	Same direction
TSGA14	XM_583431	13.0	14.5	0.1	-1.5	2.9	0.0064	Confirmed	13.1	14.2	1.1	-1.1	2.1	0.523	Confirmed
CD16a	NM_001077402	10.1	11.0	0.2	-0.9	1.9	0.0115	Confirmed	11.4	11.1	1.4	0.3	1.2	0.889	Opposite direction
CD163	XM_613380	12.4	13.1	0.2	-0.7	1.6	0.0115	Confirmed	12.1	12.0	1.4	0.2	1.1	0.938	Opposite direction
PTX3	XM_614752	16.4	16.9	0.2	-0.5	1.4	0.0156	Opposite direction	17.4	16.4	0.6	1.0	2.0	0.351	Same direction
MCP1	NM_174006	15.2	15.3	0.1	0.0	1.0	0.0002	Opposite direction	15.4	14.5	2.3	0.8	1.8	0.814	Same direction
RNASE6	NM_174594	14.5	16.1	0.5	-1.6	3.0	0.1567	Same direction	11.1	14.2	1.0	-3.2	9.0	0.079	Same direction
SLCO2B1	NM_174843	15.8	18.7	0.1	-2.8	7.2	0.0887	Same direction	14.1	19.1	3.4	-5.0	31.3	0.365	Same direction

[a]ΔCt was calculated by the subtraction of the Ct value for the target gene from the Ct value for the housekeeping gene
[b]ΔΔCt was calculated by the subtraction of the ΔCt value for the endometrium from the ΔCt value for the blood
[c]MC = Microarray confirmation.

Discussion

Accumulation of macrophages is one of the characteristic changes in the endometrium during pregnancy (Hunt et al. 1985; Heikkinen et al. 2003; Tekin and Hansen 2004, Cupurdija et al. 2004; Kim et al. 2007, Dambaeva et al. 2008; Chapters 3 and 4). The fact that accumulation of macrophages is widespread phylogenetically and of large magnitude are evidence that these immune cells play an important role in supporting the growth and survival of the conceptus. Using global transcriptomal analysis, it can be inferred that macrophages in the interplacentomal endometrium play important roles in immune regulation, tissue remodeling, and apoptosis. Moreover, this is likely to be a general function of endometrial macrophages and not a specific role of these cells in the cow because the pattern of genes that are differentially regulated in endometrial macrophages of the cow is similar to genes that are differentially regulated in decidual macrophages in the human (Gustafsson et al. 2008).

To assess gene expression, cells expressing CD14 were purified from endometrium and blood. CD14 is a co-receptor for bacterial lipopolysaccharide and is expressed on monocytes, macrophages and neutrophils (Paape et al. 1996; Triantafilou et al. 2002). Earlier (Chapters 3 and 4), $CD14^+$ cells in endometrium of pregnant cows were identified as macrophages because they also express CD68, a lysosomal-associated protein that is expressed on monocytes, macrophages and dendritic cells (Ferenbach and Hughes 2008). The classification of endometrial $CD14^+$ cells as macrophages is supported by additional evidence reported here. In particular, endometrial $CD14^+$ cells had a pattern of gene expression that was largely similar to that of blood $CD14^+$ cells (Figure 5-2A). Moreover, 53 of 56 genes reported as being characteristic of macrophages by Jensen et al. (2006) were identified in endometrial

$CD14^+$ cells. Given the similarity in gene expression between endometrial and blood $CD14^+$ cells, it is likely that the massive infiltration of $CD14^+$ cells in the pregnant endometrium is a result of the recruitment from blood monocytes. Interestingly, the proportion of PBMC that are monocytes decreases in late pregnancy in the cow (Chapter 3), possibly as a result of recruitment to the endometrium.

The pattern of differential gene expression is indicative of changes in cell migratory function as monocytes move from blood to endometrial stroma. Blood monocytes appear to have a high capacity for responsiveness to external signals that ensures activation of migration into tissues upon chemotactic signals from tissues. There was a preponderance of upregulated genes in blood for several differentially-regulated pathways involved in cell signaling including toll-like receptor signaling, Fc epsilon RI signaling, B cell receptor signaling, phosphatidylinositol signaling, JAK-STAT signaling and ErbB signaling (Table 5-5). There were also numerous genes in the MAPK, wnt and cytokine-cytokine receptor signaling pathways that were upregulated in blood (Table 5-5) as well as large numbers of genes in the signal transduction, cell communication and nuclear ontologies (Table 5-4). Among the upregulated genes that would promote activation of migration into tissues are various cytokine receptors including IL8 receptor beta (*IL8RB*), IL18 receptor accessory protein (*IL18RAP*), cytokine receptor like factor 2 (*CRLF2*), interferon receptor 1 (*IFNAR1*), tumor necrosis factor receptor superfamily, member 1B (*TNFRS1B*), IL17 receptor alpha (*IL17RA*), and tumor necrosis factor receptor superfamily, member 8 (*TNFRSF8*). Monocytes in blood also have increased expression of several genes involved in transendothelial migration. One pertinent gene is integrin alpha 4 (*ITGA4*), that encodes for the alpha 4 subunit of

the VLA-4 receptor and which promotes transendothelial migration by interacting with endothelial cell surface receptors VCAM-1 and JAM2 (Johnson-Léger et al 2002). Another is the gene for the nuclear repressor protein glucocorticoid receptor DNA binding factor-1 (*GRLF1*) that mediates effects of transforming growth factor beta 1 (*TGFB1*) on monocyte recruitment from the periphery (Kim et al. 2006). Several genes involved in cell motility were also upregulated in blood including several myosin genes (*MYL2*, *MYLPF*, and *MYH10*) and two regulators of the actin cytoskeleton, diaphanous homolog 1 (*DIAPH1*) and paxillin (*PXN*) would promote activation of migration into tissues are various cytokine receptors including IL8 receptor beta (*IL8RB*), IL18 receptor accessory protein (*IL18RAP*), cytokine receptor like factor 2 (*CRLF2*), interferon receptor 1 (*IFNAR1*), tumor necrosis factor receptor superfamily, member 1B (*TNFRS1B*), IL17 receptor alpha (*IL17RA*), and tumor necrosis factor receptor superfamily, member 8 (*TNFRSF8*). Monocytes in blood also have increased expression of several genes involved in transendothelial migration. One pertinent gene is integrin alpha 4 (*ITGA4*), that encodes for the alpha 4 subunit of the VLA-4 receptor and which promotes transendothelial migration by interacting with endothelial cell surface receptors VCAM-1 and JAM2 (Johnson-Léger et al. 2002). Another is the gene for the nuclear repressor protein glucocorticoid receptor DNA binding factor-1 (*GRLF1*) that mediates effects of transforming growth factor beta 1 (*TGFB1*) on monocyte recruitment from the periphery (Kim et al. 2006). Several genes involved in cell motility were also upregulated in blood including several myosin genes (*MYL2, MYLPF*, and *MYH10*) and two regulators of the actin cytoskeleton, diaphanous homolog 1 (*DIAPH1*) and paxillin (*PXN*).

Once the macrophages are in the endometrium, the above-mentioned genes involved in cell signaling and diapedesis become downregulated. Other genes involved in motility increase, including thymosin beta 4 (*TMSB4X*), which promotes cell motility through sequestration of G-actin monomers during actin-cytoskeletal organization (Safer et al. 1991; Bock-Marquette et al. 2004) and enabled homolog (*ENAH*), which blocks capping of actin filaments and promotes filopodia formation (Gomez and Robles 2004). These changes may indicate a different pattern of cell motility once monocytes enter the endometrium. At the same time, endometrial $CD14^+$ cells gain increased capacity for interacting with other cellular and extracellular components of the endometrium as indicated by increased expression of genes in the cell adhesion molecules and ECM-receptor interaction pathways (Table 5-5). Cell adhesion proteins on endometrial macrophages likely function to facilitate regulation of other immune cells. For example, e-cadherin (*CDH1*), a gene upregulated in endometrium, promotes macrophage – T cell interactions (Van den Bossche et al. 2009). Dendritic cells and macrophages can also form tight junctions with epithelial cells to allow uptake of antigens from the lumen without disruption of the epithelial barrier (Lehman et al. 2009) and upregulation of genes controlling formation of juctional complexes. Interestingly, $CD14^+$ cells in the interplacentomal endometrium of pregnant cows express MHC class II more intensely when near the luminal epithelium (Chapter 4). Perhaps fetal antigens shed by the trophoblast and taken up by macrophages interacting with endometrial epithelial cells leads to increased MHC class II expression.

Endometrial $CD14^+$ cells may themselves promote migration of monocytes and other leukocytes into the endometrium as indicated by upregulation of chemokine (C-X-

C motif) ligand 14 (*CXCL14*) and chemokine (C-C motif) ligands 8 (*CCL8*), 22 (*CCL22*) and 24 (*CCL24*). The platelet derived growth factor; B polypeptide (PDGFB) and inhibin beta B gene (*INHBB*) were also upregulated in endometrium. PDGF directs movement of neutrophils and macrophages (Barrientos et al. 2008). INHBB is a subunit for inhibin B and activin B. While the roles of these members of the TGFB family are unknown, activin A can induce recruitment of mast cells (Funabu et al. 2003).

Despite the identification of $CD14^+$ cells as macrophages, some of the upregulated genes in endometrium were placental and uterine genes. Examples include pregnancy associated glycoproteins (Green et al. 1998), prolactin related proteins (Hashizume 2007) and uterine serpin (Padua and Hansen 2009). Endometrial $CD14^+$ cells were 90-95% pure, and the presence of placental or endometrial genes could reflect contamination of the $CD14^+$ population with trophoblast, endometrial epithelium or endometrial stroma. Another possibility is that some of the $CD14^+$ cells in the interplacentomal endometrium are not derived from a myeloid mononuclear phagocyte lineage but instead represents trophoblast cells or cells from the endometrial stroma or epithelium. In species where endometrium undergoes a decidual response, decidualized stroma express cytokines and chemokines characteristically secreted by immune cells (Salamonsen et al. 2007; Chen et al. 2009). Perhaps similar differentiation pathways exist in species like cattle so that CD14 becomes upregulated in non-myeloid cells.

Once migrating into the endometrium, it is apparent that at least a subset of $CD14^+$ monocytes undergoes differentiation. This is so because many of the genes identified by Martinez et al. (2006) as being upregulated in macrophages during differentiation

were upregulated in endometrial $CD14^+$ cells as compared to $CD14^+$ cells in blood. Of the 128 genes shown by Martinez et al. (2006) to be upregulated in monocytes after 3 or 7 days of culture with CSF1, 19 were higher in the endometrium and 2 were higher in the blood. Of the 116 genes that were downregulated after 3 or 7 days of culture (full differentiation), 2 were upregulated in the endometrium and 3 in the blood. Absence of a more extensive list of differentially-regulated genes associated with macrophage differentiation may reflect the existence of multiple subpopulations of endometrial macrophages with some populations differentiated and others not.

Regulation of immune function is an important function of macrophages. In the reproductive tract of the pregnant female, immune responses against paternal antigens on the trophoblast must be limited to prevent immunological destruction of the conceptus. In the cow, trophoblast expresses classical and non-classical MHC antigens in the interplacentomal regions where accumulation of endometrial macrophages is most dense (Davies et al. 2004; Davies et al. 2006). At the same time, the reproductive tract is susceptible to infection, particularly at parturition when the cervix is relaxed and the placenta and fetus extend across the cervical barrier. Thus, it could be hypothesized that populations of macrophages in the endometrium serve to inhibit immune responses against conceptus antigens while other populations serve a surveillance role against pathogens to directing inflammatory responses against microorganisms. We hypothesized that, in the absence of infection, endometrial macrophages would be preferentially activated along the M2 activation pathway so that that differentiation results in macrophages that promote inhibition of inflammation, angiogenesis, and tissue remodeling (Sica et al. 2006; Benoit et al. 2008; Allaventa et

al. 2008). Indeed, as detailed in the next paragraphs, there is evidence for skewing of endometrial macrophages towards M2 activation. Nonetheless, it is likely that there is more than one subpopulation of endometrial macrophage resident in the endometrium of pregnant cows. In addition, it remains possible that the endometrial macrophage undergoes a differentiation process that is unique for that tissue.

Seven of the 52 genes found by Martinez et al. (2006) to be upregulated in M2 activated macrophages were upregulated in endometrial $CD14^+$ cells. One of these genes was glycine amidinotransferase (*GATM*) which competes with the inducible form of nitric oxide synthase for arginine and thereby reduces production of the proinflammatory molecule nitric oxide (Bronte and Zanovello 2005). Two C-type lectins characteristically activated in M2 macrophages, mannose receptor C1 (*MRC1*) and C-type lectin domain family 7, member A (*CLEC7A* or dectin-1) were also upregulated in endometrial $CD14^+$ cells. Both of these molecules recognize various pathogens as part of the innate immune response (Hsu et al. 2009). Activation of MRC1 can lead to inhibition of secretion of IL12 (stimulates NK cell and T-cells) and increased secretion of the anti-inflammatory cytokines IL8, IL10, IL1 receptor antagonist and IL1 receptor type II (Gazi and Martinez-Pomares 2009). In contrast, CLEC7A, which recognizes fungal carbohydrates, can participate in release of inflammatory cytokines (Underhill 2007). Another gene upregulated in endometrium was prostaglandin G/H synthase (*PTGS1* or cyclooxygenase 1) which is a key enzyme in prostaglandin, prostacyclin, and thromboxane A2 biosynthesis. These eicasonoids have been implicated in immunosuppression (prostaglandin E2) and angiogenesis (prostaglandins E2, I2, F2alpha, and thromboxane A2 (Fürstenberger et al. 2006).

The one upregulated gene in endometrium that could be classified as characteristic of M1 macrophages (complement factor B, *CFB*) also encodes for a protein that can have anti-inflammatory properties. Cleavage of CFB by complement factor D liberates the Ba fragment that inhibits proliferation of B cells (Ambrus et al. 1990).

In addition to the genes identified by Martinez et al. (2006), some other genes expressed in M2 macrophages were upregulated in endometrium including *CD163*, which is a scavenger receptor that, in its soluble form, exerts anti-inflammatory actions (Onofre et al. 2009) as well as the anti-inflammatory cytokines *CCL22* and *CCL24* that are produced by M2a macrophages (Benoit et al. 2008). *CCL2,* an M1 cytokine (Benoit et al. 2008), was also upregulated in endometrium. Another gene upregulated in M2 macrophages, E-cadherin (*CDH1*), was one of the top 100 upregulated genes in the endometrium. This cell adhesion protein promotes macrophage fusogenic activity and macrophage – T cell interactions (Van den Bossche et al. 2009). Two other genes with anti-inflammatory activity were upregulated in endometrium. TMSB4 can be oxidized by macrophages exposed to glucocorticoteroids to form thymosin beta 4 sulfoxide that has anti-inflammatory properties (Young et al. 1999). Fibroblast growth factor 1 (*FGF1)*, another gene upregulated in endometrium, inhibits expression of monocyte adhesion molecules on endothelial cells involved in transendothelial movement and may be anti-inflammatory (Zhang and Issekutz 2002).

One function of M2 macrophages is to promote tissue remodeling (Allavena et al. 2008) and analysis of the transcriptome of endometrial vs blood $CD14^+$ cells is consistent with endometrial $CD14^+$ cells being actively involved in this process. There

was over-representation of differentially-expressed genes in endometrium for three ontologies related to proteolysis (peptidase activity, endopeptidase activity, and proteolysis). Among these genes were granzyme B (GZMB), which cleaves extracellular matrix components such as vitronectin, fibronectin and laminin (Buzza et al. 2005), TMSBX4, which can increase metalloproteinase expression in macrophages (Philip et al. 2005) and macrophage-specific metalloproteinases 12 (MMP12) and 19 (MMP19) which degrade extracellular matrix components such as elastin type IV collagen, fibronectin, and promoting tissue remodeling (Chen 2004; Djonov et al. 2001). Another gene upregulated in endometrium was platelet derived growth factor, B polypeptide (*PDGFB*). PDGF could participate in remodeling of the endometrial stroma since macrophage –derived PDGF stimulates fibroblast proliferation and regulates fibroblast differentiation (Barrientos et al. 2008).

Tissue remodeling in the endometrium during pregnancy is important for angiogenesis, migration of trophoblast binucleate cells into the endometrial epithelium, development of endometrial glands and facilitation of endometrial growth necessary for expansion of uterine size. Inappropriate tissue remodeling may compromise pregnancy. There is increased expression of the metalloproteinase inhibitor *TIMP2* in the endometrium of cows carrying cloned fetuses (Kim et al. 2008). Upregulation of proteinase gene expression in the endometrium of pregnant cattle has been observed previously (Takagi et al. 2007; Bauersachs et al. 2008) and the present results implicate macrophages as one source of those enzymes.

The massive upregulation of GZMA and GZMB in endometrial $CD14^+$ cells is consistent with a role for these cells in induction of apoptosis. One possible target is the

trophoblast cell. Apoptotic cells are common in the placenta, especially at the edges of the placentomes and in the interplacentomal region (Facciotti et al. 2009). Apoptosis is one mechanism by which extravillous trophoblast cells are eliminated in the human placenta (Von Rango et al. 2003). While the bovine trophoblast is considered to be non-invasive and not capable of crossing the basement membrane of the luminal epithelium of the endometrium, it is possible that endometrial macrophages remove placental cells that stray past this barrier. Apoptosis could also be a signal for regulation of macrophage function. Abrahams et al. (2004) hypothesized that ingestion of apoptotic trophoblast cells causes decidual macrophages to develop an immunosuppressive phenotype.

Other signals for regulation of monocyte migration into the endometrium during pregnancy and for differentiation of these cells once in the endometrial stroma are not clear. In human, monocyte migration can be stimulated by trophoblast cells in culture (Fest et al. 2007). In sheep, there were more macrophages in the pregnant uterine horn of unilaterally-pregnant ewes than in the non-pregnant uterine horn (Tekin and Hansen 2004), suggesting that a placental signal could be involved. However, there were also large numbers of macrophages in the non-pregnant horn of the unilaterally-pregnant ewe than in endometrium of nonpregnant ewes (Tekin and Hansen 2004). Thus, it is likely that there are also systemic signals that control influx of monocytes into the endometrium during pregnancy. Progesterone, the characteristic hormone of pregnancy, is not that signal because progesterone treatment did not increase macrophage numbers in endometrium of sheep (Tekin and Hansen 2004). Progesterone may be involved in controlling macrophage differentiation in the

endometrium, however, because there is evidence using a wound healing model in mice that progesterone or estrogen can promote macrophage polarization along the M2 pathway (Routley and Ashcroft 2009).

Validation of microarray results with qPCR validation resulted in differential expression being confirmed for 8 of 11 genes evaluated. Examination of differences between endometrial and blood $CD14^+$ cells using qPCR of an independent data set indicated a smaller degree of agreement with microarray results (4 of 11 genes) but a total of 9 of 11 genes showed differences in the same direction as the results from microarray hybridization. The independent samples were from a slightly earlier stage of pregnancy and this fact could have resulted in some discrepancies with the microarray data set.

In summary, here we show that endometrial $CD14^+$ cells express genes characteristically found in macrophages. Moreover, analysis of the transcriptome of these cells indicates that at least a subpopulation of the cells differentiates along an M2 activation pathway and that the cells play roles in immune regulation, tissue remodeling, apoptosis, and angiogenesis. These roles are likely of broad relevance to mammalian reproduction rather than to be specific properties of pregnancy in the cow. Endometrial macrophages in the human also undergo M2 activation (Cupurdija et al. 2004; Gustafsson et al. 2008). In addition, 17% of the genes found by Gustafsson et al. (2008) to be differentially expressed between decidual and blood macrophages were also differentially expressed in the same direction in cow.

CHAPTER 6
GENERAL DISCUSSION

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), pigs (Kaeoket et al. 2003), cynomologus and vervet monkeys (Dambaeva et al. 2008) and sheep (Tekin and Hansen 2004). To date, little is known about the macrophage role during pregnancy in the cow. There is evidence of defective macrophage activation in cases of retention of fetal membranes in the cow (Miyoshi et al. 2002). The goal of this dissertation was to develop a better understanding of the pregnancy- related changes in the maternal immune system in the cow. The central thesis of this research is that endometrial macrophages accumulate during pregnancy in the cow and possess an M2 phenotype that promotes a microenvironment at the maternal-fetal interface that inhibits maternal immune responses against the conceptus and supports fetal development and growth. In particular, this dissertation focused on three major questions as follows below:

1) Does the accumulation of macrophages in the endometrium during pregnancy as reported in other species also occur in the cow?

The analysis of selected immune cell populations was performed in the blood to identify changes in the peripheral blood that would reflect modulation of the maternal immune system in early pregnancy (Day 34 after artificial insemination) and close to parturition. The most intriguing result was the decreased number of circulating $CD68^+$ monocytes in periparturient cows compared with the nonpregnant cows. In addition, there was an accumulation of $CD68^+$ cells in the interplacentomal (Figure 3-3) and placentomal endometrium (Figure 4-1) as shown by immunohistochemistry. The

decreased number of circulating $CD68^+$ monocytes in blood of periparturient cows as compared to nonpregnant cows (Chapter 3) may reflect massive recruitment of blood monocytes to the pregnant endometrium.

2) Are the endometrial macrophages present in the interplacentomal endometrium differentially activated from those present in the placentomal endometrium with regards to the expression of activation markers in the pregnant cow?

The expression of three markers of activation was evaluated by dual-color immunofluorescence using the pan-macrophage marker CD68 protein. The CD68 is a lysosomal-associated protein that is expressed on monocytes, macrophages and dendritic cells (Ferenbach and Hughes 2008). The three markers analyzed were CD14, a co-receptor for bacterial lipopolysaccharide expressed on monocytes, macrophages and neutrophils (Paape et al 1996; Triantafilou et al 2002), CD11b (β2 integrin) that with CD18 composes the macrophage complex-1 (Mac-1) and is involved in leukocyte-endothelial adhesion (Fagerholm et al 2006) and the MHC class II which is involved in antigen presentation and can be upregulated in activated macrophages and downregulated in inactivated or deactivated macrophages. (Gordon 2003).

In the interplacentomal region, there were at least 3 distinct populations of endometrial macrophages. The first population of macrophages was present in the deeper regions of the stroma and were $CD68^+CD14^+$MHC classII^-CD11b^+. The second population, which accumulated in the shallow stroma, was $CD68^+CD14^+$MHC class II^+CD11b^-. This suggests that some of the endometrial macrophages are already activated and they may be functioning as antigen presenting cells because of the upregulation of the MHC class II molecules. The third population of macrophages,

which was the most abundant type of $CD68^+$ cell adjacent to the luminal epithelium, was $CD68^+CD14^+$MHC classII^-CD11b^-. In contrast, in the placentomal endometrium the macrophages were mainly $CD68^+CD14^+$MHC classII^+CD11b^-. There was a regional regulation of macrophages in the bovine placenta between regions (interplacentomal vs placentomal regions) and within the interplacentomal region with regards to CD11b expression by the cells in the deep endometrium stroma

3) Does the presence of macrophages in the uterine endometrium of the pregnant cow result in upregulation of genes related to M2 activation?

Using the tools for global transcriptomal analysis, we characterized the pattern of gene expression of interplacentomal endometrial macrophages compared to resting monocytes in blood. Endometrial $CD14^+$ cells had a very similar gene expression profile to their blood counterparts (Figure 5-2A) although over a 1000 genes were differentially regulated between endometrium and blood. More than 90% (53 of 56) of the genes characteristically expressed in macrophages were identified in endometrial $CD14^+$ cells (Jensen et al. 2006). Moreover, regardless of placenta type, the endometrial macrophages had a similar gene expression profile to decidual $CD14^+$ cells in humans (Gustafsson et al. 2008) where ~90% of the genes that were differentially regulated in the decidual macrophages were expressed by the endometrial macrophages.

The pattern of differential gene expression indicated that $CD14^+$ cells change their migratory function as the monocytes move from blood to endometrial stroma. The blood cells contain upregulated genes that promote migration from the periphery to the tissues and cell-endothelium interaction (*IL8RB; IL18RAP; CRLF2; IFNAR1; TNFRS1B;*

IL17RA and TNFRSF8), whereas the endometrial cells express genes that enhance cell adhesion and communication between neighboring cells (*CDH1*; *CLDN1; CLDN3; SPP1 and PDGFB*).

The upregulation of genes involved in the monocyte to macrophage activation process indicates that at least a subset of $CD14^+$ monocytes undergo differentiation (Martinez et al. 2006). Also, there were nine genes associated with macrophage activation upregulated in the endometrial $CD14^+$ cells. Eight of these upregulated genes were associated with the M2 macrophage activation pathway including *GATM*; *MRC1*; *CLEC7A*, *CD163* and chemokine ligands (*CCL22* and *CCL24*). In contrast, only one gene characteristic of the M1 activation pathway, *CFB*, was upregulated in the endometrium. Thus, endometrial macrophages in the pregnant cow are likely to be activated via M2 activation. Human decidual macrophages also possess a bias towards M2 activation, as reported by Gustafsson et al. (2008) by comparison of decidual macrophages ($CD14^+$ cells) to their blood counterparts.

One possibility is that endometrial macrophages possess a unique pattern of activation so that they do not totally fit into the M1 or M2 activation pathways. Such an occurrence could explain why only some of the genes characteristic of M2 activation were upregulated in endometrium.

Identification of such a large number of $CD68^+$ cells in the endometrium lead early on to the question of whether these cells are in fact macrophages or another cell type expressing CD68. One of the pieces of evidence that endometrial $CD68^+$ cells were macrophages was the co-expression of other characteristic markers of macrophages, as indicated by immunohistochemistry. Results from Chapters 3 and 4, indicated that

$CD68^+$ cells also expressed CD14, which is a macrophage marker in both interplacentomal and placentomal endometrium. Moreover, the expression of MHC class II by the $CD68^+CD14^+$ cells in the placentomal endometrium indicates that they are macrophages due to the restricted expression of MHC class II by immune cells. Additional evidence for the conclusion that most endometrial $CD68^+$ cells are macrophages comes from Chapter 5, in particular, the simillarity of the general transcriptomal profile between endometrial and blood $CD14^+$ cells and the expression of genes characteristic of bovine macrophages (Jensen et al. 2006).

Human and bovine placentation are characterized by distinct anatomical differences. Nonetheless, bovine endometrial macrophages have genes differentially regulated in a similar manner as that of decidual macrophages in humans (Gustafsson et al. 2008). What sets ruminants apart from other mammals such as mice (Hunt et al. 1985) is the magnitude of macrophage accumulation in the pregnant endometrium. Such a large-scale infiltration of macrophages may be the result of ruminant evolution. Analysis of the bovine genome indicates that evolution in this species has been accompanied by large-scale duplication of genes involved in innate immunity (Bovine Genome Sequencing and Analysis Consortium 2009). For example, there are ~106 β-defensin genes in cattle compared to 39 in human and 52 in mouse. It has been speculated that this change in the bovine genome was the consequence of evolution of the rumen where a large number of microorganisms are resident within the animal (Bovine Genome Sequencing and Analysis Consortium 2009). Perhaps, the ruminant immune system responds to pregnancy with a heightened macrophage accumulation in the uterus because of these evolutionary changes in the immunogenome. The

consequences of this infiltration of macrophages for the course of pregnancy and immune function in the post-parturient period remain to be defined.

Macrophages that accumulated in the interplacentomal region may be distinct in functional properties from macrophages present in the placentomal endometrium. The ruminant placenta is a unique type of placenta that possesses two sites of maternal fetal interaction, the interplacentomal and the placentomal regions (Wooding and Wathes 1980; Schlafer et al. 2004). In the interplacentomal region, the chorionic epithelium is apposed to the luminal epithelium and there is little invasion of fetal cells towards the maternal side. In contrast, the placentomal region is the site where most of the interaction between mother and fetus occurs. Chorionic villi are interdigitated with the maternal septa and migration of fetal cells to the maternal side leads to formation of a syncytium (Wooding and Wathes 1980; Schlafer et al. 2004). Evidence for regional differentiation between interplacentomal and placentomal regions is clear. $CD68^+$ cells in the interplacentomal endometrium were mainly negative for MHC class II while $CD68^+$ cells in the placentomal endometrium were MHC class II^+ (Chapter 4). The expression of MHC class II indicates that $CD68^+$ in the caruncular septa are activated by either the M1 or M2 pathway, whereas the $CD68^+$ cells in the interplacentomal stroma are likely inactivated macrophages (Gordon 2003) or monocytes that have a defective antigen-presentation function. The expression of CD11b was also regionally differentiated between placentomal and interplacentomal regions and within the interplacentomal region itself (Chapter 4). The $CD68^+$ cells in the caruncular septa were largely negative for CD11b. In the interplacentomal region, only $CD68^+$ cells present deep in the endometrial stroma were positive for CD11b. This regional differentiation

likely reflects different trafficking patterns and regulatory signals in the two placental regions.

Although the activation state of the macrophages previously mentioned is better understood, the signals for macrophage accumulation in the endometrium of the cow remain unclear. Systemic signals are involved in macrophage recruitment to the uterus as shown by the data from the unilateral pregnancy sheep model where the nonpregnant horn also showed macrophage accumulation (Tekin and Hansen 2004). Nevertheless, there is evidence that suggests the existence of an embryo-derived factor that induces the macrophage accumulation due to the higher density of macrophages in the pregnant horn compared to the nonpregnant one (Tekin and Hansen 2004). In addition, the treatment of ovariectomized sheep with progesterone did not trigger macrophage recruitment to the endometrium indicating that neither progesterone nor other progesterone-induced factors are primarily involved in the recruitment of macrophages to the endometrium during pregnancy (Tekin and Hansen 2004). Progesterone does not seem to be the most important signal for the trafficking of macrophages to the endometrium, but it may control macrophage differentiation in the endometrium, because of the evidence from the wound healing model in mice in which progesterone or estrogen can promote macrophage polarization along the M2 pathway (Routley and Ashcroft 2009).

Some embryo-derived factors such as interferon-tau (IFNT) and prolactin (PRL) may be involved in this recruitment of macrophages. To date, it is not known if the migration of macrophages begins at this time of pregnancy in the cow.

Interferon-tau (IFNT) is a ruminant specific cytokine (Spencer et al. 2004) secreted by the conceptus during the perimplantation period (from day 14 to day 25). The earliest date that macrophages were found in the endometrium of the pregnant cow in this study was at day 50 of the pregnancy. In humans and mice, macrophage accumulation was detected in the first trimester of pregnancy (Guleria et al. 2005; Hunt et al. 1985). It is not known if the macrophage accumulation in the cow endometrium begins at the same time that INFT peaks during pregnancy. Several attempts to understand the effects of IFNT secretion on the maternal immune system have been made, however there is no evidence that IFNT can trigger the migration of macrophages to the endometrium during pregnancy. Nevertheless, analysis of the global transcriptome of bovine endometrium during the pre-attachment period demonstrated the upregulation of many interferon-stimulated genes that may be involved in embryo-maternal immune modulation, including complement C1s subcomponent (*C1S*), complement C1r subcomponent (*C1R*), *SERPINA14* and bone marrow stromal antigen 2 (*BST2*) (Bauersachs et al. 2008). Perhaps IFNT primes the endometrium to help recruit immune cells to the endometrium to support pregnancy.

Prolactin is a hormone that is involved in both maintenance and lysis of corpus luteum (Morishige and Rothchild 1974). Prolactin has been shown to recruit macrophages to the corpus luteum during lutogenesis and luteolysis, and the inhibition of prolactin activity reduces this macrophage recruitment to the corpus luteum. Perhaps, prolactin is one of the signals for macrophages accumulation to the endometrium (Gaytan et al. 1997).

Knowing the phenotype and gene expression profile of the endometrial macrophages can lead to some ideas about their function during pregnancy. One function of M2 macrophages is to promote tissue remodeling (Allavena et al. 2008). The over-representation of differentially-expressed genes in endometrium for three ontologies related to proteolysis supports the idea that endometrial macrophages are involved in the tissue remodeling process in the cow. This over-representation of proteinase gene expression in the endometrium of pregnant cattle has been observed previously (Takagi et al. 2007; Bauersachs et al. 2008) and the present results implicate macrophages as one source of those enzymes.

The upregulated expression of genes such as granzymes (*GZMA, gzmA and GZMB*) and genes involved in metalloproteinase activity (*MMP12, MMP9* and *TMSBX4*) supports the tissue remodeling function of the endometrial macrophages in the cow. Moreover, *PDGFB* expression was higher in the endometrium and therefore could participate in remodeling of the endometrial stroma since macrophage –derived PDGF stimulates proliferation and fibroblast differentiation (Barrientos et al. 2008).

In the pregnant uterus, tissue remodeling is essential for the adjustment of the uterus to the growing fetus. In cows carrying fetuses derived from cloning, there is increased expression of the metalloproteinase inhibitor TIMP-2 in the endometrium (Kim et al. 2009). Moreover, placentome formation in cows carrying cloned fetuses is decreased (Miglino et al. 2007). This may be a result of inappropriate tissue remodeling.

The expression of GZMA and GZMB is consistent with a role for $CD14^+$ cells in the induction of apoptosis. Presence of apoptotic cells is common in the placenta,

especially at the edges of the placentomes and in the interplacentomal region (Facciotti et al 2009). In addition, extravillous trophoblast cells are eliminated in the human placenta through apoptosis (Von Rango et al. 2003). The bovine trophoblast is considered to be less-invasive and not capable of crossing the basement membrane of the luminal epithelium of the endometrium. It is possible that endometrial macrophages remove placental cells that stray past this barrier. Futhermore, apoptosis could also be a signal for regulation of macrophage function. Abrahams et al. (2004) hypothesized that ingestion of apoptotic trophoblast cells causes decidual macrophages to develop an immunosuppressive phenotype. Thus, it is likely that endometrial macrophages play a role in immune regulation, tissue remodeling, apoptosis, and angiogenesis.

A better understanding of the functions of endometrial macrophages implied by the global gene expression analysis requires confirmation of changes at the level of protein synthesis. Changes in the expression of a given gene do not always translate into similar changes in the level of the respective protein. Moreover, experiments to test the functional characteristics of endometrial macrophages in vitro are an essential step to understand the role of endometrial macrophages during pregnancy. For example, results of this dissertation suggest that endometrial macrophages play a role in the tissue remodeling process during pregnancy. To address this function, assays to evaluate ECM degradation or cell migration in vitro can be performed using purified preparations of endometrial macrophages. Moreover, it is important to test the thesis that endometrial macrophages are involved in modulation of the immune system to help prevent deleterious maternal responses against the fetus. Mixed lymphocyte reaction

using the purified endometrial macrophages as APCs can be done to evaluate their effects on clonal lymphocyte expansion and cytokine expression.

If the role of endometrial macrophages as immunomodulators is confirmed, it will be important to understand the mechanisms responsible. It will be instructive to determine whether the immunosuppressive effect requires cell-to-cell contact or is mediated via secreted factors. Proliferation assays in the presence of the purified endometrial macrophages or using the conditioned culture media from endometrial macrophages would answer this question.

The presence of macrophages in the pregnant endometrium of the cow leads to the question of the signals involved in immune cell migration to the endometrium. The study of the functional and gene expression assays of pregnancy-related factors such as IFNT on resting blood monocytes would help to understand how pregnancy modulates the trafficking of these cells and also how pregnancy-related factors can modulate differentiation and function of blood monocytes in vitro. Also, the large-scale accumulation of macrophages in the bovine endometrium may be a result of a decidual-like response in the intercaruncular regions as seen in other species that undergo a decidual response, decidualized stroma express cytokines and chemokines characteristically secreted by immune cells (Salamonsen et al. 2007; Chen et al. 2009). The expression of prolactin and members of the prolactin related family have been reported to be upregulated in the decidua reactions in vitro in rat (Matsumoto et al. 2009), these genes were also upregulated by the endometrial $CD14^{+}$ cells. Perhaps similar differentiation pathways exist in species like cattle so that CD14 becomes upregulated in non-myeloid cells.

The results of this dissertation help provide a better understanding of how the uterine immune system is involved in pregnancy and parturition in the cow. Involvement of macrophages in disturbances of the immune system in pathological conditions in the cow such as retained placenta (Myioshi et al 2002), post-partum endometritis and early embryo loss cannot be ruled out. Knowledge of the presence of macrophages and their state of activation in the endometrium during pregnancy may lead to practical benefits for dairy producers. For instance, new approaches for altering placental function, fetal growth, and the immunosuppressive state of the uterus around the time of parturition may help to reduce the incidence of retained placenta and also hasten uterine involution after parturition.

In summary, here we show that endometrial $CD14^+$ cells express genes characteristically found in macrophages. Moreover, analysis of the transcriptome of these cells indicates that at least a subpopulation of the cells differentiates along an M2 activation pathway and that the cells play a role in immune regulation, tissue remodeling, apoptosis, and angiogenesis. These roles are likely of broad relevance to mammalian reproduction rather than specific properties of pregnancy in the cow. Endometrial macrophages in the human also undergo M2 activation (Cupurdija et al. 2004; Gustafsson et al. 2008). In addition, 17% of the genes found by Gustafsson et al. (2008) to be differentially expressed between decidual and blood macrophages were also differentially expressed in the same direction for the cow. Macrophage recruitment during the peripartum period suggests they have a role in parturition, expulsion of fetal membranes and uterine involution. Malfunction of the endometrial macrophages may have a negative effect on the ability of cows to establish pregnancy after parturition.

LIST OF REFERENCES

Abrahams VM, Kim YM, Straszewski SL, Romero R, Mor G 2004: Macrophages and apoptotic cell clearance during pregnancy. Am J Reprod Immunol **51**, 275-282.

Ackermann MR, DeBey BM, Stabel TJ, Gold JH, Register KB, Meehan JT 1994: Distribution of anti-CD68 (EBM11) immunoreactivity in formalin-fixed, paraffin-embedded bovine tissues. Vet Pathol **31**, 340-348.

Adolfsson J, Månsson R, Buza-Vidas N, Hultquist A, Liuba K, Jensen CT, Bryder D, Yang L, Borge OJ, Thoren LA, Anderson K, Sitnicka E, Sasaki Y, Sigvardsson M, Jacobsen SE 2005: Identification of Flt3+ lympho-myeloid stem cells lacking erythro-megakaryocytic potential a revised road map for adult blood lineage commitment. Cell **121**, 295-306.

Alfaidy N, Gupta S, DeMarco C, Caniggia I, Challis JR 2002: Oxygen regulation of placental 11 beta-hydroxysteroid dehydrogenase 2: physiological and pathological implications. J Clin Endocrinol Metab **10**, 4797-805.

Allavena P, Sica A, Garlanda C, Mantovani A 2008: The Yin-Yang of tumor-associated macrophages in neoplastic progression and immune surveillance. Immunol Rev. **222**,155-161.

Al-Shahrour F, Minguez P, Tárraga J, Medina I, Alloza E, Montaner D, Dopazo J 2007: FatiGO: a functional profiling tool for genomic data. Integration of functional annotation, regulatory motifs and interaction data with microarray experiments. Nucleic Acids Res **35**, W91-W96.

Aluvihare VR, Kallikourdis M, Betz AG 2004: Regulatory T cells mediate maternal tolerance to the fetus. Nat Immun **5**, 266-271.

Ambrus Jr. JL, Peters MG, Fauci AS, Brown EJ 1990: The Ba fragment of complement factor B inhibits human B lymphocyte proliferation. J Immunol **144**,1549-1553.

Anastassova-Kristeva, M 2003: The origin and development of the immune system with a view to stem cell therapy. J Hematother Stem Cell Res **12**, 137-154.

Anthony RM, Urban JF, Alem F, Hamed HA, Rozo CT, Boucher JL, Rooijen NV, Gause WC 2006: Memory T(H)2 cells induce alternatively activated macrophages to mediate protection against nematode parasites. Nat Med 12, 955-960.

Apps R, Gardner L, Sharkey AM, Holmes N, Moffett A 2007: A homodimeric complex of HLA-G on normal trophoblast cells modulates antigen-presenting cells via LILRB1. Eur J Immunol **37**, 1924-1937.

Arcuri F, Ricci C, Ietta F, Cintorino M, Tripodi SA, Cetin I, Garzia E, Schatz F, Klemi P, Santopietro R, Paulesu L 2001: Macrophage migration inhibitory factor in the human endometrium: expression and localization during the menstrual cycle and early pregnancy. Biol Reprod **64,** 1200-1205.

Auffray C, Sieweke MH, Geissmann F 2009: Blood monocytes: development heterogeneity and relationship with dendritic cells. Annu Rev Immunol **27**, 669-692.

Baker JM, Bamford AI, Antczak DF 1999: Modulation of allospecific CTL responses during pregnancy in equids: an immunological barrier to interspecies matings? J Immunol **162**, 4496-4501.

Baratelli F, Lin Y, Zhu L, Yang SC, Heuzé-Vourc'h N, Zeng G, Reckamp K, Dohadwala M, Sharma S, Dubinett SM 2005: Prostaglandin E2 induces FOXP3 gene expression and T regulatory cell function in human CD4+ T cells. J Immunol **175**,1483-1490.

Barrientos S, Stojadinovic O, Golinko, MS, Brem H, Tomic-Canic M 2008: Growth factors and cytokines in wound healing. Wound Repair Regen **16**, 585-601.

Bauersachs S, Mitko K, Ulbrich SE, Blum H, Wolf E 2008: Transcriptome studies of bovine endometrium reveal molecular profiles characteristic for specific stages of estrous cycle and early pregnancy. Exp Clin Endocrinol Diabetes **116**,371-384.

Becker AJ, McCulloch CE, Till JE 1963: Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells. Nature **197**, 452–454.

Benoit M, Desnues B, Mege JL 2008: Macrophage polarization in bacterial infections.J Immunol **181**, 3733-3739.

Benz C, Bleul CC 2005: A multipotent precursor in the thymus maps to the branching point of the T versus B lineage decision. J Exp Med **202**, 21-31.

Bergmann CE, Hoefer IE, Meder B, Roth H, van Royen N, Breit SM, Jost MM, Aharinejad S, Hartmann S, Buschmann IR 2006: Arteriogenesis depends on circulating monocytes and macrophage accumulation and is severely depressed in op/op mice. J Leukoc Biol **80**, 59-65.

Bilinski MJ, Thorne JG, Oh MJ, Leonard S, Murrant C, Tayade C, Croy BA 2008: Uterine NK cells in murine pregnancy. Reprod Biomed Online **16**, 218-226.

Billigham RE, Brent L, Medawar PB 1953: Actively acquired tolerance of foreign cells. Nature. **1724**, 603-6.

Bock-Marquette I, Saxena A, White MD, Dimaio JM, Srivastava D 2004: Thymosin beta4 activates integrin-linked kinase and promotes cardiac cell migration, survival and cardiac repair. Nature **432**, 466-472.

Bouchelouche K, Andresen L, Alvarez S, Nordling J, Nielsen OH, Bouchelouche P 2006: Interleukin-4 and 13 induce the expression and release of monocyte chemoattractant protein 1, interleukin-6 and stem cell factor from human detrusor smooth muscle cells: synergy with interleukin-1beta and tumor necrosis factor-alpha. J Urol **175**, 760-765.

Bouhlel MA, Derudas B, Rigamonti E, Dièvart R, Brozek J, Haulon S, Zawadzki C, Jude B, Torpier G, Marx N, Staels B, Chinetti-Gbaguidi G 2007: PPARgamma activation primes human monocytes into alternative M2 macrophages with anti-inflammatory properties. Cell Metab **6**,137-143.

Braud VM, Allan DS, O'Callaghan CA, Söderström K, D'Andrea A, Ogg GS 1998: HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. Nature **391**, 795–799.

Bronte V, Zanovello P 2005: Regulation of immune responses by L-arginine metabolism. Nat Rev Immunol **5**,641–654.

Brown M, Gustafson M, Saldana S, Baradaran A, Miller H, Halonen M 2004: Correlation of human decidual and cord blood mononuclear cell cytokine production. Hum Immunol **65**, 1336-1343.

Bruder D, Probst-Kepper M, Westendorf AM, Geffers R, Beissert S, Loser K, von Boehmer H, Buer J, Hansen W 2004: Neuropilin-1: a surface marker of regulatory T cells. Eur J Immunol **34**, 623-630.

Brusveen DJ, Cunha AP, Silva CD, Cunha PM, Sterry RA, Silva EP, Guenther JN, Wiltbank MC 2008: Altering the time of the second gonadotropin-releasing hormone injection and artificial insemination (AI) during Ovsynch affects pregnancies per AI in lactating dairy cows. J Dairy Sci **91**, 1044-1052.

Bulla R, Bossi F, Radillo O, de Seta F, Tedesco F 2003: Placental trophoblast and endothelial cells as target of maternal immune response. Autoimmunity **36**, 11–18.

Bulmer JN, Billington WD, Johnson PM 1984: Immunohistologic identification of trophoblast populations in early human pregnancy with the use of monoclonal antibodies. Am J Obstet Gynecol **148**, 19-26.

Bulmer JN, Johnson PM 1985: Antigen expression by trophoblast populations in the human placenta and their possible immunobiological relevance. Placenta **6**, 127-140.

Bulmer JN, Rodeck C, Adinolfi M 1995: Immunohistochemical characterization of cells retrieved by transcervical sampling in early pregnancy. Prenat Diagn **15**, 1143-1153.

Bulmer JN, Smith J, Morrison L, Wells M 1998: Maternal and fetal cellular relationships in the human placental basal plate. Placenta **9**, 237-246.

Buzza MS, Zamurs L, Sun J, Bird CH, Smith AI, Trapani JA, Froelich CJ, Nice EC, Bird PI 2005: Extracellular matrix remodeling by human granzyme B via cleavage of vitronectin, fibronectin, and laminin. J Biol Chem **280**, 23549-23558.

Byrne SN, Knox MC, Halliday GM 2008: TGFbeta is responsible for skin tumour infiltration by macrophages enabling the tumours to escape immune destruction. Immunol Cell Biol **86**, 92-97.

Cai TQ, Weston PG, Lund LA, Brodie B, McKenna DJ, Wagner WC 1994: Association between neutrophil functions and periparturient disorders in cows. Am J Vet Res **55**, 934-943.

Cannon MJ, Pate JL 2003: The role of major histocompatibility complex molecules in luteal function. Reprod Biol Endocrinol **1**, 93.

Ceradini DJ, Kulkarni AR, Callaghan MJ, Tepper OM, Bastidas N, Kleinman ME, Capla JM, Galiano RD, Levine JP, Gurtner GC 2004: Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1. Nat Med **10**, 858-864.

Chang YC, Hsu TL, Lin HH, Chio CC, Chiu AW, Chen NJ, Lin CH, Hsieh SL 2004: Modulation of macrophage differentiation and activation by decoy receptor 3. J Leukoc Biol **75**, 486-494.

Chaouat G, Cayol V, Mairovitz V, Dubanchet S 1999: Localization of the Th2 cytokines IL-3, IL-4, IL-10 at the fetomaternal interface during human and murine pregnancy and lack of requirement for Fas/Fas ligand interaction for a successful allogeneic pregnancy. Am J Reprod Immunol **42**, 1-13.

Chen L, Belton RJ Jr, Nowak RA 2009: Basigin-mediated gene expression changes in mouse uterine stromal cells during implantation. Endocrinology **150**, 966-976.

Chen YE 2004: MMP-12, an old enzyme plays a new role in the pathogenesis of rheumatoid arthritis? Am J Pathol **165**, 1069-1070.

Cheung PY, Walton JC, Tai HH, Riley SC, Challis JR 1990: Immunocytochemical distribution and localization of 15-hydroxyprostaglandin dehydrogenase in human fetal membranes, decidua, and placenta. Am J Obstet Gynecol **163**,1445-1449.

Chung AS, Kao WJ 2009: Fibroblasts regulate monocyte response to ECM-derived matrix: the effects on monocyte adhesion and the production of inflammatory, matrix remodeling, and growth factor proteins. J Biomed Mater Res A **89**, 841-853.

Chung AS, Waldeck H, Schmidt DR, Kao WJ 2008: Monocyte inflammatory and matrix remodeling response modulated by grafted ECM-derived ligand concentration. J Biomed Mater Res A in press.

Cobb SP, Watson ED 1995: Immunohistochemical study of immune cells in the bovine endometrium at different stages of the oestrous cycle. Res Vet Sci **59**, 238-241.

Colonna M, Samaridis J 1995: Cloning of immunoglobulin-superfamily members associated with HLA-C and HLA-B recognition by human natural killer cells. Science **268**, 405-408.

Cordeiro-da-Silva A, Tavares J, Araújo N, Cerqueira F, Tomás A, Kong Thoo Lin P, Ouaissi A 2004: Immunological alterations induced by polyamine derivatives on murine splenocytes and human mononuclear cells. Int Immunopharmacol **4**, 547-556.

Croy BA, van den Heuvel MJ, Borzychowski AM, Tayade C 2006: Uterine natural killer cells: a specialized differentiation regulated by ovarian hormones. Immunol Rev **214**,161-185.

Cupurdija K, Azzola D, Hainz U, Gratchev A, Heitger A, Takikawa O, Goerdt, S, Wintersteiger R, Dohr G, Sedlmayr P 2004: Macrophages of human first trimester decidua express markers associated to alternative activation. Am J Reprod Immunol **51**, 117-122.

Dambaeva SV, Breburda EE, Durning M, Garthwaite MA, Golos TG 2009: Characterization of decidual leukocyte populations in cynomolgus and vervet monkeys. J Reprod Immunol **80**,57-69.

Davies CJ, Eldridge JA, Fisher PJ, Schlafer DH 2006: Evidence for expression of both classical and non-classical major histocompatibility complex class I genes in bovine trophoblast cells. Am J Reprod Immunol **55**, 188-200.

Davies CJ, Fisher PJ, Schlafer DH 2000: Temporal and regional regulation of major histocompatibility complex class I expression at the bovine uterine/placental interface. Placenta **21**, 194-202.

Davies CJ, Hill JR, Edwards JL, Schrick FN, Fisher PJ, Eldridge JA, Schlafer DH 2004: Major histocompatibility antigen expression on the bovine placenta: its relationship to abnormal pregnancies and retained placenta. Anim Reprod Sci **82-83**, 267-280.

Davies D, Meade KG, Herath S, Eckersall PD, Gonzalez D, White JO, Conlan RS, O'Farrelly C, Sheldon IM 2008: Toll-like receptor and antimicrobial peptide expression in the bovine endometrium. Reprod Biol Endocrinol **18**, 53.

De M, Choudhuri R, Wood GW 1991: Determination of the number and distribution of macrophages, lymphocytes, and granulocytes in the mouse uterus from mating through implantation. J Leukoc Biol **50**, 252-262.

Dealtry GB, Clark DE, Sharkey A, Charnock-Jones DS, Smith SK 1998: Expression and localization of the Th2-type cytokine interleukin-13 and its receptor in the placenta during human pregnancy. Am J Reprod Immunol **40**, 283-290.

Detilleux JC, Kehrli ME Jr, Stabel JR, Freeman AE, Kelley DH 1995: Study of immunological dysfunction in periparturient Holstein cattle selected for high and average milk production. Vet Immunol Immunopathol **44**, 251-267.

Dillon S, Agrawal S, Banerjee K, Letterio J, Denning TL, Oswald-Richter K, Kasprowicz DJ, Kellar K, Pare J, van Dyke T, Ziegler S, Unutmaz D, Pulendran B 2006: Yeast zymosan, a stimulus for TLR2 and dectin-1, induces regulatory antigen-presenting cells and immunological tolerance. J Clin Invest **116**, 916-928.

Djonov V, Högger K, Sedlacek R, Laissue J, Draeger A 2001: MMP-19: cellular localization of a novel metalloproteinase within normal breast tissue and mammary gland tumours. J Pathol **195**, 147-155.

Donaldson WL, Zhang CH, Oriol JG, Antczak DF 1990: Invasive equine trophoblast expresses conventional class I major histocompatibility complex antigens. Development **110**, 63-71.

Draghici S, Khatri P, Tarca AL, Amin K, Done A, Voichita C, Georgescu C, Romero R 2007: A systems biology approach for pathway level analysis. Genome Res **17**, 1537-1545.

Du R, Lu KV, Petritsch C, Liu P, Ganss R, Passegué E, Song H, Vandenberg S,Johnson RS, Werb Z, Bergers G 2008: HIF1alpha induces the recruitment of bone marrow-derived vascular modulatory cells to regulate tumor angiogenesis and invasion. Cancer Cell **13**, 206–220.

Edwards JP, Zhang X, Frauwirth KA, Mosser DM 2006: Biochemical and functional characterization of three activated macrophage populations. J Leukoc Biol **80**, 1298-1307.

Emond V, Asselin E, Fortier MA, Murphy BD, Lambert RD 2000: Interferon-tau stimulates granulocyte-macrophage colony-stimulating factor gene expression in bovine lymphocytes and endometrial stromal cells. Biol Reprod **62**, 1728-1737.

Emond V, Fortier MA, Murphy BD, Lambert RD 1998: Prostaglandin E2 regulates both interleukin-2 and granulocyte-macrophage colony-stimulating factor gene expression in bovine lymphocytes. Biol Reprod **58**, 143-151.

Facciotti PR, Rici RE, Maria DA, Bertolini M, Ambrósio CE, Miglino MA 2009: Patterns of cell proliferation and apoptosis by topographic region in normal Bos taurus vs. Bos indicus crossbreeds bovine placentae during pregnancy. Reprod Biol Endocrinol **7**, 25.

Fadok VA, Bratton DL, Frasch SC, Warner ML, Henson PM 1998: The role of phosphatidylserine in recognition of apoptotic cells by phagocytes. Cell Death Differ **5**, 551-562.

Fagerholm SC, Varis M, Stefanidakis M, Hilden TJ, Gahmberg CG, 2006: α-chain phosphorylation of the human leukocyte CD11b/CD18 (Mac-1) integrin is pivotal for integrin activation to bind ICAMs and leukocyte extravasation. Blood **108**, 3379–3386.

Ferenbach D, Hughes J, 2008: Macrophages and dendritic cells: what is the difference? Kidney Int **74**, 5-7.

Fest S, Aldo PB, Abrahams VM, Visintin I, Alvero A, Chen R, Chavez SL, Romero R, Mor G, 2007: Trophoblast-macrophage interactions: a regulatory network for the protection of pregnancy. Am J Reprod Immunol **57**, 55-66.

Földi J, Kulcsár M, Pécsi A, Huyghe B, de Sa C, Lohuis JA, Cox P, Huszenicza G 2006: Bacterial complications of postpartum uterine involution in cattle. Anim Reprod Sci **96**, 265-281.

Fürstenberger G, Krieg P, Müller-Decker K, Habenicht AJ 2006: What are cyclooxygenases and lipoxygenases doing in the driver's seat of carcinogenesis? Int J Cancer **19**, 2247-2254.

Galeotti M, Belluzzi S, Volpatti D, Bergonzoni ML, D'Agaro, E, and Volpelli, LA 1997: Evaluation of mast cells in calf and heifer uteri. Theriogenology **48**, 1301–1311.

Gaytan F, Morales C, Bellido C, Aguilar E, Sanchez-Criado JE. 1997: Role of prolactin in the regulation of macrophages and in the proliferative activity of vascular cells in newly formed and regressing rat corpora lutea. Biol Reprod **57**, 478-86.

Gazi U, Martinez-Pomares L 2009: Influence of the mannose receptor in host immune responses. Immunobiology **214**, 554-561.

Gifford CA, Racicot K, Clark DS, Austin KJ, Hansen TR, Lucy MC, Davies CJ, Ott TL 2007: Regulation of interferon-stimulated genes in peripheral blood leukocytes in pregnant and bred, nonpregnant dairy cows. J Dairy Sci **90**, 274-280.

Goff JP, Kehrli ME Jr, Horst RL 1989: Periparturient hypocalcemia in cows: prevention using intramuscular parathyroid hormone. J Dairy Sci **72**, 1182-1187.

Gogolin-Ewens KJ, Lee CS, Mercer WR, Brandon MR 1989: Site-directed differences in the immune response to the fetus. Immunology **66**, 312-317.

Gomez TM, Robles E 2004: The great escape phosphorylation of Ena/VASP by PKA promotes filopodial formation. Neuron **42**, 1-3.

Gordon S, 2003: Alternative activation of macrophages. Nat Rev Immunol **3**, 23-35.

Gorivodsky M, Torchinsky A, Zemliak I, Savion S, Fein A, Toder V, 1999: TGF α2 mRNA expression and pregnancy failure in mice. Am J Reprod Immunol **42**, 124-133.

Gottshall SL, Hansen PJ 1992: Regulation of leucocyte subpopulations in the sheep endometrium by progesterone. Immunology **76**, 636-641.

Gratchev A, Guillot P, Hakiy N, Politz O, Orfanos CE, Schledzewski K, Goerdt S 2001: Alternatively activated macrophages differentially express fibronectin and its splice variants and the extracellular matrix protein betaIG-H3. Scand J Immunol **53**, 386-392.

Gratchev A, Kzhyshkowska J, Utikal J, Goerdt S 2005: Interleukin-4 and dexamethasone counterregulate extracellular matrix remodelling and phagocytosis in type-2 macrophages. Scand J Immunol **61**, 10-17.

Gregory CD, Devitt A 1999: CD14 and apoptosis. Apoptosis **4**, 11-20.

Grohmann GP, Schirmacher P, Manzke O, Hanisch FG, Dienes HP, Baldus SE 2003: Modulation of MUC1 and blood group antigen expression in gastric adenocarcinoma cells by cytokines. Cytokine **23**, 86-93.

Grunewald M, Avraham I, Dor Y, Bachar-Lustig E, Itin A, Jung S, Chimenti S, Landsman L, Abramovitch R, Keshet E 2006: VEGF-induced adult neovascularization: recruitment, retention, and role of accessory cells. Cell **124**, 175–189.

Guleria I, Khosroshahi A, Ansari MJ, Habicht A, Azuma M, Yagita H, Noelle RJ, Coyle A, Mellor AL, Khoury SJ, Sayegh MH 2005: A critical role for the programmed death ligand 1 in fetomaternal tolerance. J Exp Med **202**, 231-237.

Guleria I, Sayegh MH 2007: Maternal acceptance of the fetus: true human tolerance. J Immunol **178**, 3345-3351.

Gustafsson C, Mjösberg J, Matussek A, Geffers R, Matthiesen L, Berg G, Sharma S, Buer J, Ernerudh J 2008: Gene expression profiling of human decidual macrophages: evidence for immunosuppressive phenotype. PLoS ONE **3**, e2078.

Han H, Austin KJ, Rempel LA, Hansen TR 2006: Low blood ISG15 mRNA and progesterone levels are predictive of non-pregnant dairy cows. J Endocrinol **191**, 505-512.

Hanna N, Hanna I, Hleb M, Wagner E, Dougherty J, Balkundi D, Padbury J, Sharma S 2000: Gestational age-dependent expression of IL-10 and its receptor in human placental tissues and isolated cytotrophoblasts. J Immunol **164**, 5721-5728.

Hansen PJ, Stephenson DC, Low BG, Newton GR 1989: Modification of immune function during pregnancy by products of the sheep uterus and conceptus. J Reprod Fertil Suppl **37**, 55-61.

Hartigan JM 1972: Great Plains Organization for Perinatal Health Care. Clin Pediatr **11**, 492.

Hashizume K 2007: Analysis of uteroplacental-specific molecules and their functions during implantation and placentation in the bovine. J Reprod Dev **53**, 1-11.

Hébert G, Mingam R, Arsaut J, Dantzer R, Demotes-Mainard J 2005: A role of IL-1 in MPTP-induced changes in striatal dopaminergic and serotoninergic transporter binding: clues from interleukin-1 type I receptor-deficient mice. Brain Res Mol Brain Res **136**, 267-270.

Heemskerk B, Lankester AC, van Vreeswijk T, Beersma MF, Claas EC, Veltrop-Duits LA, Kroes AC, Vossen JM, Schilham MW, van Tol MJ 2005: Immune reconstitution and clearance of human adenovirus viremia in pediatric stem-cell recipients. J Infect Dis **191**, 520-530.

Heikkinen J, Mottonen M, Komi J, Alanen A, Lassila O 2003: Phenotypic characterization of human decidual macrophages. Clin Exp Immunol **131**, 498-505.

Heyborne KD, Fu YX, Nelson A, Farr A, O'Brien R, Born W 1994: Recognition of trophoblasts by gamma delta T cells. J Immunol **153**, 2918-2926.

Heyborne KD, Cranfill RL, Carding SR, Born WK, O'Brien RL 1992: Characterization of γδ-T lymphocytes at the maternal-fetal interface. J Immunol **149**, 2872-2878.

Hill JA, 1995: T-helper 1-type immunity to trophoblast: evidence for a new immunological mechanism for recurrent abortion in women. Hum Reprod **10**, 114-120.

Holmes CH, Simpson KL, Okada H, Okada N, Wainwright SD, Purcell DF, Houlihan JM 1992: Complement regulatory proteins at the feto-maternal interface during human placental development: distribution of CD59 by comparison with membrane cofactor protein (CD46) and decay accelerating factor (CD55). Eur J Immunol **22**, 1579–1585.

Huddleston H, Schust DJ 2004: Immune interactions at the maternal-fetal interface: a focus on antigen presentation. Am J Reprod Immunol **51**, 283-289.

Hume DA 2008a: Macrophages as APC and the dendritic cell myth. J Immunol **181**, 5829-5835.

Hume DA 2008b: Differentiation and heterogeneity in the mononuclear phagocyte system. Mucosal Immunol **1**, 432-441.

Hume DA, Underhill DM, Sweet MJ, Ozinsky AO, Liew FY, Aderem A 2001: Macrophages exposed continuously to lipopolysaccharide and other agonists that act via toll-like receptors exhibit a sustained and additive activation state. BMC Immunol **2**,11.

Hunt JS, Manning LS, Mitchell D, Selanders JR, Wood GW 1985: Localization and characterization of macrophages in murine uterus. J Leukoc Biol **38**, 255-265.

Hunt JS, Petroff MG, Burnett TG 2000: Uterine leukocytes: key players in pregnancy. Semin Cell Dev Biol **11**, 127-137.

Hunt JS, Petroff MG, McIntire RH, Ober C 2005: HLA-G and immune tolerance in pregnancy. FASEB J **19**, 681-693.

Hunt JS, Vassmer D, Ferguson TA, Miller L, 1997: Fas ligand is positioned in mouse uterus and placenta to prevent trafficking of activated leukocytes between the mother and the conceptus. J Immunol **158**, 4122-4128.

Hviid TV 2006: HLA-G in human reproduction: aspects of genetics, function and pregnancy complications. Hum Reprod Update **12**, 209-232.

Imhof BA, Aurrand-Lions M 2004: Adhesion mechanisms regulating the migration of monocytes. Nat Rev Immunol **4**, 432-444.

Ishii N, Matsumura T, Kinoshita H, Motoshima H, Kojima K, Tsutsumi A, Kawasaki S, Yano M, Senokuchi T, Asano T, Nishikawa T, Araki E 2009: Activation of AMP-activated protein kinase suppresses oxidized low-density lipoprotein-induced macrophage proliferation. J Biol Chem **284**, 34561-34569.

Ishitani A, Sageshima N, Lee N, Dorofeeva N, Hatake K, Marquardt H, Geraghty DE, 2003: Protein expression and peptide binding suggest unique and interacting functional roles for HLA-E, F, and G in maternal-placental immune recognition. J Immunol **171**, 1376-1384.

Jensen K, Talbot R, Paxton E, Waddington D, Glass EJ 2006: Development and validation of a bovine macrophage specific cDNA microarray. BMC Genomics **7**, 224.

Johann AM, Barra V, Kuhn AM, Weigert A, von Knethen A, Brüne B 2007: Apoptotic cells induce arginase II in macrophages, thereby attenuating NO production. FASEB J **21**, 2704-2712.

Johnson-Léger CA, Aurrand-Lions M, Beltraminelli N, Fasel N, Imhof BA. 2002: Junctional adhesion molecule-2 (JAM-2) promotes lymphocyte transendothelial migration. Blood. **100**, 2479-86

Jones GE 2000: Cellular signaling in macrophage migration and chemotaxis. J Leukoc Biol **68**, 593-602.

Joosten I, Sanders MF, Hensen EJ 1981: Involvement of major histocompatibility complex class I compatibility between dam and calf in the aetiology of bovine retained placenta. Anim Genet **22**, 455-463.

Juraskova V, Tkadlecek L 1965: Character of primary and secondary colonies of haematopoiesis in the spleen of irradiated mice. Nature **206**, 951–952.

Kaeoket K, Dalin AM, Magnusson U, Persson E 2001: The sow endometrium at different stages of the oestrous cycle: immunohistochemical study on the distribution of SWC3-expressing cells (granulocytes, monocytes and macrophages). J Vet Med A Physiol Pathol Clin Med **48**, 507-511.

Kaliński P, Hilkens CM, Wierenga EA, Kapsenberg ML 1999: T-cell priming by type-1 and type-2 polarized dendritic cells: the concept of a third signal. Immunol Today **20**, 561-567.

Karcher EL, Beitz DC, Stabel JR 2008: Parturition invokes changes in peripheral blood mononuclear cell populations in Holstein dairy cows naturally infected with Mycobacterium avium subsp. paratuberculosis. Vet Immunol Immunopathol **124**, 50-62.

Kehrli ME Jr, Goff JP 1989: Periparturient hypocalcemia in cows: effects on peripheral blood neutrophil and lymphocyte function. J Dairy Sci **72**, 1188-1196.

Kim HR, Naruse K, Lee HR, Han RX, Park CS, Jin DI 2009: Abnormal expression of TIMP-2, SOD, vimentin and PAI proteins in cloned bovine placentae. Reprod Domest Anim **44**, 714-717.

Kim JS, Romero R, Cushenberry E, Kim YM, Erez O, Nien JK, Yoon BH, Espinoza J, Kim CJ 2007: Distribution of $CD14^+$ and $CD68^+$ macrophages in the placental bed and basal plate of women with preeclampsia and preterm labor. Placenta **28**, 571-576.

Kimura K, Goff JP, Kehrli ME Jr, Harp JA 1999: Phenotype analysis of peripheral blood mononuclear cells in periparturient dairy cows. J Dairy Sci **82**, 315-319.

Kimura K, Goff JP, Kehrli ME Jr, Harp JA, Nonnecke BJ 2002: Effects of mastectomy on composition of peripheral blood mononuclear cell populations in periparturient dairy cows. J Dairy Sci **85**, 1437-1444.

King A, Hiby SE, Gardner L, Joseph S, Bowen JM, Verma S, Burrows TD, Loke YW 2000: Recognition of trophoblast HLA class I molecules by decidual NK cell receptors--a review. Placenta **21**, S81-S85.

Kovats S, Main EK, Librach C, Stubblebine M, Fisher SJ, DeMars R 1990: A class I antigen, HLA-G, expressed in human trophoblasts. Science 248, 220-223.

Krishnan L, Guilbert LJ, Wegmann TG, Belosevic M, Mosmann TR 1996: T helper 1 response against Leishmania major in pregnant C57BL/6 mice increases implantation failure and fetal resorptions. Correlation with increased IFN-gamma and TNF and reduced IL-10 production by placental cells. J Immunol **156**, 653-662.

Krysko DV, Denecker G, Festjens N, Gabriels S, Parthoens E, D'Herde K, Vandenabeele P 2006: Macrophages use different internalization mechanisms to clear apoptotic and necrotic cells. Cell Death Differ **13**, 2011-2022.

Kyaw Y, Hasegawa G, Takatsuka H, Shimada-Hiratsuka M, Umezu H, Arakawa M, Naito M 1998: Expression of macrophage colony-stimulating factor, scavenger receptors, and macrophage proliferation in the pregnant mouse uterus. Arch Histol Cytol **61**, 383-393.

Kydd JH, Butcher GW, Antczak DF, Allen WR 1991: Expression of major histocompatibility complex (MHC) class 1 molecules on early trophoblast. J Reprod Fertil Suppl **44**, 463-477.

Kzhyshkowska J, Gratchev A, Schmuttermaier C, Brundiers H, Krusell L, Mamidi S, Zhang J, Workman G, Sage EH, Anderle C, Sedlmayr P, Goerdt S 2008: Alternatively activated macrophages regulate extracellular levels of the hormone placental lactogen via receptor-mediated uptake and transcytosis. J Immunol **180**, 3028-3037.

Lan HY, Nikolic-Paterson DJ, Mu W, Atkins RC 1995: Local macrophage proliferation in the progression of glomerular and tubulointerstitial injury in rat anti-GBM glomerulonephritis. Kidney Int **48**, 753-760.

Lander MF, Hansen PJ, Drost M 1990: Antisperm antibodies in cows after subcutaneous and intra-uterine immunisation. Vet Rec **126**, 461-462.

Le Gal FA, Riteau B, Sedlik C, Khalil-Daher I, Menier C, Dausset J, Guillet JG, Carosella ED, Rouas-Freiss N 1999: HLA-G-mediated inhibition of antigen-specific cytotoxic T lymphocytes. Int Immunol **11**, 1351-1356.

Lee CS, Meeusen E, Gogolin-Ewens K, Brandon MR, 1992: Quantitative and qualitative changes in the intraepithelial lymphocyte population in the uterus of nonpregnant and pregnant sheep. Am J Reprod Immunol **28**, 90-96.

Leiser R, Kaufmann P 1994: Placental structure: in a comparative aspect. Exp Clin Endocrinol **102**, 122-134.

Leiser R, Krebs C, Ebert B, Dantzer V 1997: Placental vascular corrosion cast studies: a comparison between ruminants and humans. Microsc Res Tech **38**, 76-87.

Leong AS, Norman JE, Smith R 2008: Vascular and myometrial changes in the human uterus at term. Reprod Sci 15, 59-65.

Leslie MV, Hansen PJ 1991: Progesterone-regulated secretion of the serpin-like proteins of the ovine and bovine uterus. Steroids **56**, 589-597

Leung ST, Derecka K, Mann GE, Flint AP, Wathes DC 2000: Uterine lymphocyte distribution and interleukin expression during early pregnancy in cows. J Reprod Fertil **119**, 25-33.

Lewis CE, Pollard JW 2006: Distinct role of macrophages in different tumor microenvironments. Cancer Res **66**, 605–612.

Li C, Houser BL, Nicotra ML, Strominger JL 2009: HLA-G homodimer-induced cytokine secretion through HLA-G receptors on human decidual macrophages and natural killer cells. Proc Natl Acad Sci USA **106**, 5767-5772.

Lightner A, Schust DJ, Chen YBA, Barrier BF 2008: The fetal allograft revisited: does the study of an ancient invertebrate species shed light on the role of natural killer cells at the maternal-fetal interface? Clin Dev Immunol 2008, 6319-20.

Lohmann-Matthes ML, Steinmüller C, Franke-Ullmann G 1994: Pulmonary macrophages. Eur Respir J **7**, 1678-1689.

Loke P, Gallagher I, Nair MG, Zang X, Brombacher F, Mohrs M, Allison JP, Allen JE 2007: Alternative activation is an innate response to injury that requires CD4+ T cells to be sustained during chronic infection. J Immunol **179**, 3926-3936.

Loke P, Zang X, Hsuan L, Waitz R, Locksley RM, Allen JE, Allison JP 2005: Inducible costimulator is required for type 2 antibody isotype switching but not T helper cell type 2 responses in chronic nematode infection. Proc Natl Acad Sci USA **102**, 9872-9877.

Loureiro B, Bonilla L, Block J, Fear JM, Bonilla AQ, Hansen PJ 2009: Colony-stimulating factor 2 (CSF-2) improves development and posttransfer survival of bovine embryos produced in vitro. Endocrinology **150**, 5046-5054.

Low BG, Hansen PJ, Drost M, Gogolin-Ewens KJ 1990: Expression of major histocompatibility complex antigens on the bovine placenta. J Reprod Fertil **90**, 235-243.

Luppi P, Irwin TE, Simhan H, Deloia JA 2004: CD11b expression on circulating leukocytes increases in preparation for parturition. Am J Reprod Immunol **52**, 323-329.

Luscinskas FW, Gerszten RE, Garcia-Zepeda EA, Lim YC, Yoshida M, Ding HA, Gimbrone MA Jr, Luster AD, Rosenzweig A 2000: C-C and C-X-C chemokines trigger firm adhesion of monocytes to vascular endothelium under flow conditions. Ann NY Acad Sci **902**, 288-293.

Mackler AM, Green LM, McMillan PJ, Yellon SM 2000: Distribution and activation of uterine mononuclear phagocytes in peripartum endometrium and myometrium of the mouse. Biol Reprod **62**, 1193-1200.

Majewski AC, Tekin S, Hansen PJ 2001: Local versus systemic control of numbers of endometrial T cells during pregnancy in sheep. Immunology **102**, 317-322.

Manak RC 1982: Mitogenic responses of peripheral blood lymphocytes from pregnant and ovariectomized heifers and their modulation by serum. J Reprod Immunol **4,** 263-276.

Mantovani A, Allavena P, Sica A 2004: Tumour-associated macrophages as a prototypic type II polarised phagocyte population: role in tumour progression. Eur J Cancer **40**, 1660-1667.

Mantovani A, Garlanda C, Locati M 2009: Macrophage diversity and polarization in atherosclerosis: a question of balance. Arterioscler Thromb Vasc Biol **29**, 1419-1423.

Mantovani A, Sozzani S, Locati M, Allavena P, Sica A 2002: Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends Immunol **23**, 549-555

Martinez FO, Gordon S, Locati M, Mantovani A 2006: Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression. J Immunol **177**, 7303-7311.

Martinez FO, Helming L, Gordon S 2009: Alternative activation of macrophages: an immunologic functional perspective. Annu Rev Immunol **27**, 451-483.

Masilamani M, Nguyen C, Kabat J, Borrego F, Coligan JE 2006: CD94/NKG2A inhibits NK cell activation by disrupting the actin network at the immunological synapse. J Immunol **177**, 3590–3596.

Maslin CL, Kedzierska K, Webster NL, Muller WA, Crowe SM 2005: Transendothelial migration of monocytes: the underlying molecular mechanisms and consequences of HIV-1 infection. Curr HIV Res **3**, 303-317.

Mess A, Carter AM 2007: Evolution of the placenta during the early radiation of placental mammals. Comp Biochem Physiol A Mol Integr Physi **148**, 769-779.

Morishige WK, Rothchild I. 1974: Temporal aspects of the regulation of corpus luteum function by luteinizing hormone, prolactin and placental luteotrophin during the first half of pregnancy in the rat. Endocrinology **95**, 260-74.

Metcalf D 1997: Suppression or overexpression of genes encoding myeloid growth factors or their receptors. Hematol Cell Ther **39**, 98-101.

Micklem K, Rigney E, Cordell J, Simmons D, Stross P, Turley H, Seed B, Mason D 1989: A human macrophage-associated antigen (CD68) detected by six different monoclonal antibodies. Br J Haematol **73**, 6-11.

Miglino MA, DiDio LJ 1992: Vasculature of bovine placentas studied by scanning electron microscopy of corrosion casts. Ital J Anat Embryol **97**, 23-35.

Miglino MA, Pereira FTV, Visintin JA, Garcia JM, Meirelles FV, Rumpf R, Ambrósio CE, Papa PC, Santos TC, Carvalho AF, Leiser R, Carter AM 2007 Placentation in cloned cattle: structure and microvascular architecture. Theriogenology **68**, 604-617.

Mincheva-Nilsson L, Hammarström S, Hammarström ML 1992: Human decidual leukocytes from early pregnancy contain high numbers of γδ+ cells and show selective down-regulation of alloreactivity. J Immunol **149**, 2203-2211.

Mincheva-Nilsson L, Kling M, Hammarström S, Nagaeva O, Sundqvist KG, Hammarström ML, Baranov V 1997: γδ T cells of human early pregnancy decidua. Evidence for local proliferation, phenotypic heterogeneity, and extrathymic differentiation. J Immunol **159**, 3266-3277.

Miyoshi M, Sawamukai Y, Iwanaga T 2002: Reduced phagocytotic activity of macrophages in the bovine retained placenta. Reproduction in Domestic Animals **37**, 53-56.

Mizuno M, Aoki K, Kimbara T 1994: Functions of macrophages in human decidual tissue in early pregnancy. Am J Reprod Immunol **31**, 180-188.

Mjösberg J, Berg G, Ernerudh J, Ekerfelt C 2007: CD4+ CD25+ regulatory T cells in human pregnancy: development of a Treg-MLC-ELISPOT suppression assay and indications of paternal specific Tregs. Immunology **120**, 456-466.

Moffett A, Loke C 2006: Immunology of placentation in eutherian mammals. Nature Reviews Immunology **6**, 584-594.

Moffett-King A 2002: Natural killer cells and pregnancy. Nat Rev Immunol **2**, 656-663.

Mold JE, Michaëlsson J, Burt TD, Muench MO, Beckerman KP, Busch MP, Lee TH, Nixon DF, McCune JM 2008: Maternal alloantigens promote the development of tolerogenic fetal regulatory T cells in utero. Science **322**, 1562-1565.

Monterroso VH, Hansen PJ 1993: Regulation of bovine and ovine lymphocyte proliferation by progesterone: modulation by steroid receptor antagonists and physiological status. Acta Endocrinol (Copenh) **129**, 532-535.

Mor G, Straszewski-Chavez SL, Abrahams,VM 2006: Macrophage-trophoblast interactions. Methods Mol Med **122**, 149-163.

Mosser DM 2003: The many faces of macrophage activation. J Leukoc Biol **73**, 209-212.

Mosser DM, Edwards JP 2008: Exploring the full spectrum of macrophage activation. Nat Rev Immunol **8**, 958-969.

Munn DH, Zhou M, Attwood JT, Bondarev I, Conway SJ, Marshall B, Brown C, Mellor AL 1998: Prevention of allogeneic fetal rejection by tryptophan catabolism. Science **281**, 1191-1193.

Murphy KM, Travers P, Walport M 2005a: Janeway's Immunobiology, 6th Ed., Garland Science Publications, London.

Murphy SP, Fast LD, Hanna NN, Sharma S 2005b: Uterine NK cells mediate inflammation-induced fetal demise in IL-10-null mice. J Immunol **175**, 4084-4090.

Nagaeva O, Jonsson L, Mincheva-Nilsson L 2002: Dominant IL-10 and TGF-β mRNA expression in γδT cells of human early pregnancy decidua suggests immunoregulatory potential. Am J Reprod Immunol **48**, 9-17.

Nishikori K, Noma J, Hirakawa S, Amano T, Kudo T 1993: The change of membrane complement regulatory protein in chorion of early pregnancy. Clin Immunol Immunopathol **69**, 167-174.

Nobuhisa I, Ohtsu N, Okada S, Nakagata N, Taga T 2007: Identification of a population of cells with hematopoietic stem cell properties in mouse aorta-gonad-mesonephros cultures. Exp Cell Res **313**, 965-974.

Noden DM, de Lahunta A 1990: Extraembryonic membranes and placentation. In: Noden DM, de Lahunta A, The Embryology of Domestic Animals. Baltimore: Williams and Wilkins 47–69.

Nonnecke BJ, Kimura K, Goff JP, Kehrli ME Jr. 2003: Effects of the mammary gland on functional capacities of blood mononuclear leukocyte populations from periparturient cows. J Dairy Sci **86**, 2359-2368.

Onofre G, Kolácková M, Jankovicová K, Krejsek J 2009: Scavenger receptor CD163 and its biological functions. Acta Medica (Hradec Kralove) **52**, 57-61.

O'Shea JJ, Murray PJ 2008: Cytokine signaling modules in inflammatory responses. Immunity **28**, 477-487.

Paape MJ, Lilius EM, Wiitanen PA, Kontio MP, Miller RH 1996: Intramammary defense against infections induced by Escherichia coli in cows. Am J Vet Res **57**, 477-482.

Padua MB, Hansen PJ 2008: Regulation of DNA synthesis and the cell cycle in human prostate cancer cells and lymphocytes by ovine uterine serpin. BMC Cell Biol **9**, 5.

Padua, MB, Kowalski AA, Cañas MY, Hansen PJ 2009: The molecular phylogeny of uterine serpins and its relationship to evolution of placentation. FASEB J, 2009 Oct. 30.

Pai RK, Askew D, Boom WH, Harding CV 2002: Regulation of class II MHC expression in APCs: roles of types I, III, and IV class II transactivator. J Immunol **169**, 1326-1333.

Papayannopoulou T 2003: Bone marrow homing: the players, the playfield, and their evolving roles. Curr Opin Hematol **10**, 214-219.

Passlick B, Flieger D, Ziegler-Heitbrock HW 1989: Identification and characterization of a novel monocyte subpopulation in human peripheral blood. Blood **74**, 2527-2534.

Peltier MR, Liu WJ, Hansen PJ 2000: Regulation of lymphocyte proliferation by uterine serpin: interleukin-2 mRNA production, CD25 expression and responsiveness to interleukin-2. Proc Soc Exp Biol Med **223**, 75-81.

Petzl N 2007: Peripartale Expression von Toll-like-Rezeptoren und β-Defensinen im Endometrium des Rindes. Inaugural-dissertation - zur Erlangung des Grades einer DOKTORIN DER VETERINÄRMEDIZIN durch die Tierärztliche Hochschule Hannover

Petzl W, Zerbe H, Günther J, Yang W, Seyfert HM, Nürnberg G, Schuberth HJ 2008: Escherichia coli, but not Staphylococcus aureus triggers an early increased expression of factors contributing to the innate immune defense in the udder of the cow. Vet Res **39**, 18.

Philp D, Chen SS, Fitzgerald W, Orenstein J, Margolis L, Kleinman HK 2005: Complex extracellular matrices promote tissue-specific stem cell differentiation. Stem Cells **23**, 288-296

Plaks V, Birnberg T, Berkutzki T, Sela S, BenYashar A, Kalchenko V, Mor G, Keshet E, Dekel N, Neeman M, Jung S 2008: Uterine dendritic cells are crucial for decidua formation during embryo implantation J Clin Invest. **118**, 3954–3965.

Politz O, Gratchev A, McCourt PA, Schledzewski K, Guillot P, Johansson S, Svineng G, Franke P, Kannicht C, Kzhyshkowska J, Longati P, Velten FW, Johansson S, Goerdt S 2002: Stabilin-1 and -2 constitute a novel family of fasciclin-like hyaluronan receptor homologues. Biochem J **362**, 155-164.

Pollard JW 2009: Trophic macrophages in development and disease. Nat Rev Immunol **9**, 259-270.

Pollard JW, Hunt JS, Wiktor-Jedrzejczak W, Stanley ER 1991: A pregnancy defect in the osteopetrotic (op/op) mouse demonstrates the requirement for CSF-1 in female fertility. Dev Biol **148**, 273-283.

Ravelich SR, Shelling AN, Wells DN, Peterson AJ, Lee RSF, Ramachandran A, Keelan JA 2006: Expression of TGF-β1, TGF-β2, TGF-β3 and the receptors TGF-βRI and TGF-βRII in placentomes of artificially inseminated and nuclear transfer derived bovine pregnancies. Placenta **27**, 307-316.

Reece JJ, Siracusa MC, Scott AL 2006: Innate immune responses to lung-stage helminth infection induce alternatively activated alveolar macrophages. Infect Immun **74**, 4970-4981.

Repnik U, Tilburgs T, Roelen DL, van der Mast BJ, Kanhai HH, Scherjon S, Claas FH 2008: Comparison of macrophage phenotype between decidua basalis and decidua parietalis by flow cytometry. Placenta **29**, 405-412.

Rieger L, Hofmeister V, Probe C, Dietl J, Weiss EH, Steck T, Kammerer U 2002: Th1- and Th2-like cytokine production by first trimester decidual large granular lymphocytes is influenced by HLA-G and HLA-E. Mol Hum Reprod **8**, 255-261.

Rieger L, Honig A, Sütterlin M, Kapp M, Dietl J, Ruck P, Kämmerer U 2004: Antigen-presenting cells in human endometrium during the menstrual cycle compared to early pregnancy. J Soc Gynecol Investig **11**, 488-493.

Roberts RM, Ezashi T, Rosenfeld CS, Ealy AD, Kubisch HM 2003: Evolution of the interferon tau genes and their promoters, and maternal-trophoblast interactions in control of their expression. Reprod Suppl **61**, 239-251.

Robertson SA, Care AS, Skinner RJ 2007: Interleukin 10 regulates inflammatory cytokine synthesis to protect against lipopolysaccharide-induced abortion and fetal growth restriction in mice. Biol Reprod **76**, 738-748.

Rosbottom A, Gibney EH, Guy CS, Kipar A, Smith RF, Kaiser P, Trees AJ, Williams DJL 2008: Upregulation of cytokines is detected in the placentas of cattle infected with Neospora caninum and is more marked early in gestation when fetal death is observed. Infect Immun **76**, 2352-2361.

Routley CE, Ashcroft GS 2009: Effect of estrogen and progesterone on macrophage activation during wound healing. Wound Repair Regen **17**, 42-50.

Sacks GP, Clover LM, Bainbridge DR, Redman CW, Sargent IL 2001: Flow cytometric measurement of intracellular Th1 and Th2 cytokine production by human villous and extravillous cytotrophoblast. Placenta **22**, 550-559.

Safer D, Elzinga M, Nachmias, VT 1991: Thymosin beta 4 and Fx, an actin-sequestering peptide, are indistinguishable. J Biol Chem **266**, 4029-4032.

Salamonsen LA, Hannan NJ, Dimitriadis E 2007: Cytokines and chemokines during human embryo implantation: roles in implantation and early placentation. Semin Reprod Med **25**, 437-444.

Sánchez MJ, Holmes A, Miles C, Dzierzak E 1996: Characterization of the first definitive hematopoietic stem cells in the AGM and liver of the mouse embryo. Immunity **5**, 513-525.

Savage ND, de Boer T, Walburg KV, Joosten SA, van Meijgaarden K, Geluk A, Ottenhoff TH 2008: Human anti-inflammatory macrophages induce Foxp3+ GITR+ CD25+ regulatory T cells, which suppress via membrane-bound TGFbeta-1. J Immunol **181**, 2220-2226.

Sawyer RT 1986: The ontogeny of pulmonary alveolar macrophages in parabiotic mice. J Leukoc Biol **40**, 347-354.

Sawyer RT, Strausbauch PH, Volkman A 1982: Resident macrophage proliferation in mice depleted of blood monocytes by strontium-89. Lab Invest **46**, 165-170.

Schlafer DH, Fisher PJ, Davies CJ. 2000: The bovine placenta before and after birth: placental development and function in health and disease. Anim Reprod Sci. **60-61**,145-60.

Schlatt S, de Kretser DM, Hedger MP 1999: Mitosis of resident macrophages in the adult rat testis. J Reprod Fertil **116**, 223-228.

Scodras JM, Parhar RS, Kennedy TG, Lala PK 1990: Prostaglandin-mediated inactivation of natural killer cells in the murine decidua. Cell Immunol **127**, 352-367.

Senger PL 2003: Pathway of pregnancy and parturition. Cadmus Professional Communicatiosn, USA.Shah S, Smith H, Feng X, Rancourt DE, Riabowol K 2009: ING function in apoptosis in diverse model systems. Biochem Cell Biol **87**, 117-125.

Shemesh M, Milaguir F, Ayalon N, Hansel W 1979: Steroidogenesis and prostaglandin synthesis by cultured bovine blastocysts. J Reprod Fertil **56**, 181-185.

Shiba Y, Takahashi M, Hata T, Murayama H, Morimoto H, Ise H, Nagasawa T, Ikeda U 2009: Bone marrow CXCR4 induction by cultivation enhances therapeutic angiogenesis. Cardiovasc Res **81**, 169-177.

Shigeru S, Akitoshi N, Subaru MH, Shiozaki A 2008: The balance between cytotoxic NK cells and regulatory NK cells in human pregnancy. J Reprod Immunol **77**, 14-22.

Sica A, Larghi P, Mancino A, Rubino L, Porta C, Totaro MG, Rimoldi M, Biswas SK, Allavena P, Mantovani A 2008: Macrophage polarization in tumour progression. Semin Cancer Biol **18**, 349-355.

Sica A, Schioppa T, Mantovani A, Allavena P 2006: Tumour-associated macrophages area distinct M2 polarised population promoting tumour progression: potential targets of anti-cancer therapy. Eur J Cancer **42**, 717-727.

Simpson H, Robson SC, Bulmer JN, Barber A, Lyall F 2002: Transforming growth factor β expression in human placenta and placental bed during early pregnancy. Placenta **23**, 44-58.

Singh MP, Singh G, Singh SM 2005: Role of host's antitumor immunity in exercise-dependent regression of murine T-cell lymphoma. Comp Immunol Microbiol Infect Dis **28**, 231-248.

Skopets B, Li J, Thatcher WW, Roberts RM, Hansen PJ 1992: Inhibition of lymphocyte proliferation by bovine trophoblast protein-1 (type I trophoblast interferon) and bovine interferon-alpha I1. Vet Immunol Immunopathol **34**, 81-96.

Sombroek CC, Stam AG, Masterson AJ, Lougheed SM, Schakel MJ, Meijer CJ, Pinedo HM, van den Eertwegh AJ, Scheper RJ, de Gruijl TD 2002: Prostanoids play a major role in the primary tumor-induced inhibition of dendritic cell differentiation. J Immunol. **168,** 4333-4343.

Somerset DA, Zheng Y, Kilby MD, Sansom DM, Drayson MT 2004: Normal human pregnancy is associated with an elevation in the immune suppressive CD25+ CD4+ regulatory T-cell subset. Immunology **112**, 38-43.

Song N, Huang Y, Shi H, Yuan S, Ding Y, Song X, Fu Y, Luo Y. 2009: Overexpression of platelet-derived growth factor-BB increases tumor pericyte content via stromal-derived factor-1alpha/CXCR4 axis. Cancer Res. **69**, 6057-6064.

Souza CD, Evanson OA, Weiss DJ 2008: Role of cell membrane receptors in the suppression of monocyte anti-microbial activity against Mycobacterium avium subsp. paratuberculosis. Microb Pathogen **44**, 215-223.

Spencer TE, Johnson GA, Bazer FW, Burghardt RC 2004: Implantation mechanisms:insights from the sheep. Reproduction. **128**, 657-668.

Storset AK, Kulberg S, Berg I, Boysen P, Hope JC, Dissen E. 2004: NKp46 defines a subset of bovine leukocytes with natural killer cell characteristics. Eur J Immunol. **34**,669-76.

Stout RD, Suttles J 2005: Immunosenescence and macrophage functional plasticity: dysregulation of macrophage function by age-associated microenvironmental changes. Immunol Rev. **205** ,60-71.

Sutton LN, Mason DY, Redman CW. 1989: Isolation and characterization of human fetal macrophages from placenta Clin Exp Immunol. **78**, 437-443.

Suzuki T, Hiromatsu K, Ando Y, Okamoto T, Tomoda Y, Yoshikai Y 1995: Regulatory role of γδ T cells in uterine intraepithelial lymphocytes in maternal antifetal immune response. J Immunol **154**, 4476-4484.

Tafuri A, Alferink J, Moller P, Hammerling GJ, Arnold B 1995: T cell awareness of paternal alloantigens during pregnancy. Science **270**, 630-633.

Takagi M, Yamamoto D, Ohtani M, Miyamoto A 2007: Quantitative analysis of messenger RNA expression of matrix metalloproteinases (MMP-2 and MMP-9), tissue inhibitor-2 of matrix metalloproteinases (TIMP-2), and steroidogenic enzymes in bovine placentomes during gestation and postpartum. Mol Reprod Dev **74**, 801-807.

Takahashi K, Yamamura F, Naito M 1988: Differentiation, maturation, and proliferation of macrophages in the mouse yolk sac: A light-microscopic enzyme-cytochemical, immunohistochemical and ultrastructural study. J Leukoc Biol **45**, 87-96.

Tekin S, Hansen PJ 2004: Regulation of numbers of macrophages in the endometrium of the sheep by systemic effects of pregnancy, local presence of the conceptus, and progesterone. Am J Reprod Immunol **51**, 56-62.

Thatcher WW, Bilby TR, Bartolome JA, Silvestre F, Staples CR, Santos JE 2006: Strategies for improving fertility in the modern dairy cow. Theriogenology **65**, 30-44.

Thaxton JE, Romero R, Sharma S 2009: TLR9 activation coupled to IL-10 deficiency induces adverse pregnancy outcomes. J Immunol **183**, 1144-1154.

Thompson EG, Schaheen L, Dang H, Fares H 2007: Lysosomal trafficking functions of mucolipin-1 in murine macrophages. BMC Cell Biol **8**, 54.

Thomson AJ, Telfer JF, Young A, Campbell S, Stewart CJ, Cameron IT, Greer IA, Norman JE 1999: Leukocytes infiltrate the myometrium during human parturition: further evidence that labour is an inflammatory process. Hum Reprod **14**, 229-236.

Tibbetts TA, Conneely OM, O'Malley BW 1999: Progesterone via its receptor antagonizes the pro-inflammatory activity of estrogen in the mouse uterus. Biol Reprod. **60**, 1158-1165.

Tibbits FD, Formigli L, del Vecchio RP, Foote WD, Randel RD and Del Vecchio, RP 1989: A suggested role for mast cell heparin in prostaglandin synthesis in bovine endometrium. Proc. Western Section, Am. Soc. Anim. Sci. **40**, 337–338.

Tiemessen MM, Jagger AL, Evans HG, van Herwijnen MJ, John S, Taams LS 2007: CD4+CD25+Foxp3+ regulatory T cells induce alternative activation of human monocytes/macrophages. Proc Natl Acad Sci U S A. 104, 19446-51.

Tilburgs T, Roelen DL, van der Mast BJ, de Groot-Swings GM, Kleijburg C, Scherjon SA, Claas FH 2008: Evidence for a selective migration of fetus-specific CD4+CD25bright regulatory T cells from the peripheral blood to the decidua in human pregnancy. J Immunol. **180**, 5737-5745.

Tilburgs T, Roelen DL, van der Mast BJ, van Schip JJ, Kleijburg C, de Groot-Swings GM, Kanhai HH, Claas FH, Scherjon SA 2006: Differential distribution of CD4+ CD25 (bright) and CD8+CD28– T-cells in decidua and maternal blood during human pregnancy. Placenta **27**, S47-S53.

Tilburgs T, Scherjon SA, van der Mast BJ, Haasnoot GW, Versteeg-V D Voort-Maarschalk M, Roelen DL, van Rood JJ, Claas FH 2009: Fetal-maternal HLA-C mismatch is associated with decidual T cell activation and induction of functional T regulatory cells. J Reprod Immunol. **82**, 148-157.

Triantafilou M, Triantafilou K 2002: Lipopolysaccharide recognition: CD14, TLRs and the LPS-activation cluster. Trends Immunol **23**, 301-304.

Trundley A, Moffett A 2004: Human uterine leukocytes and pregnancy. Tissue Antigens **63**, 1-12.

Turnbull IR, Gilfillan S, Cella M, Aoshi T, Miller M, Piccio L, Hernandez M, Colonna M 2006: Cutting edge: TREM-2 attenuates macrophage activation. J Immunol **177**, 3520-3524.

Ueno H, Klechevsky E, Morita R, Aspord C, Cao T, Matsui T, Di Pucchio T, Connolly J, Fay JW, Pascual V, Palucka AK, Banchereau J 2007: Dendritic cell subsets in health and disease. Immunol Rev **219**, 118-142.

Underhill DM 2007: Collaboration between the innate immune receptors dectin-1, TLRs, and Nods. Immunol Rev **219**, 75-87.

Van den Bossche J, Bogaert P, van Hengel J, Guerin CJ, Berx G, Movahedi K, Van den Bergh R, Pereira-Fernandes A, Geuns JM, Pircher H, Dorny P, Grooten J, De Baetselier P, Van Ginderachter JA 2009: Alternatively activated macrophages engage in homotypic and heterotypic interactions through IL-4 and polyamine-induced E-cadherin/catenin complexes. Blood **114**, 4664-4674.

van Furth R, Cohn ZA, Hirsch JG, Humphrey JH, Spector, WG, Langevoort HL 1972: The mononuclear phagocyte system: a new classification of macrophages, monocytes, and their precursor cells. Bull World Health Organ **46**, 845-852.

Van Kampen C, Mallard BA 1997: Effects of peripartum stress and health on circulating bovine lymphocyte subsets. Vet Immunol Immunopathol **59**, 79-91.

van Nieuwenhoven ALV, Heineman MJ, Faas MM 2003: The immunology of successful pregnancy. Hum Reprod Update **9**, 347-357.

Vogel P 2005: The current molecular phylogeny of Eutherian mammals challenges previous interpretations of placental evolution. Placenta **26**, 591-596.

von Rango U, Krusche CA, Kertschanska S, Alfer J, Kaufmann P, Beier HM 2003: Apoptosis of extravillous trophoblast cells limits the trophoblast invasion in uterine but not in tubal pregnancy during first trimester. Placenta **24**, 929-940.

Wada H, Masuda K, Satoh R, Kakugawa K, Ikawa T, Katsura Y, Kawamoto H 2008: Adult T-cell progenitors retain myeloid potential. Nature **452**, 768–772. Wang D, Dubois RN, Richmond A 2009: The role of chemokines in intestinal inflammation and cancer. Curr Opin Pharmacol. 9,688-96.

Wei XH, Orr HT, 1990: DifferentWei XH, Orr HT 1990: Differential expression of HLA-E, HLA-F, and HLA-G transcripts in human tissue. Hum Immunol **29**, 131–142.

Weigert A, Brüne B 2008: Nitric oxide, apoptosis and macrophage polarization during tumor progression. Nitric Oxide **19**, 95-102.

Wesselius LJ, Kimler BF 1989: Alveolar macrophage proliferation in situ after thoracic irradiation of rats. Am Rev Respir Dis **139**, 221-225.

Williams PJ, Searle RF, Robson SC, Innes BA, Bulmer JN 2009: Decidual leucocyte populations in early to late gestation normal human pregnancy. J Reprod Immunol **82**, 24-31.

Winter AJ, Hall CE, Jacobson RH, Verstreate DR, Meredith MP, Castleman WL 1986: Effect of pregnancy on the immune response of cattle to a Brucella vaccine. J Reprod Immunol **9**, 313-325.

Wooding FB 1982: The role of the binucleate cell in ruminant placental structure. J Reprod Fertil Suppl **31**, 31-39.

Wooding FB, Wathes DC 1980: Binucleate cell migration in the bovine placentome. J Reprod Fertil **59**, 425-430.

Wooding FB, Flint AP, Heap RB, Morgan G, Buttle HL, Young IR. 1986: Control of binucleate cell migration in the placenta of sheep and goats. J Reprod Fertil. **76**:499-512.

Wooding FBP, Roberts RM, Green JA 2005: Light and electron microscope immunocytochemical studies of the distribution of pregnancy associated glycoproteins (PAGs) throughout pregnancy in the cow: possible functional implications. Placenta **26**, 807-827.

Wu TT, Chen TL, Chen RM 2009: Lipopolysaccharide triggers macrophage activation of inflammatory cytokine expression, chemotaxis, phagocytosis, and oxidative ability via a toll-like receptor 4-dependent pathway: Validated by RNA interference. Toxicol Lett in press.

Xu W, Roos A, Daha MR, van Kooten C 2006: Dendritic cell and macrophage subsets in the handling of dying cells. Immunobiology **211**, 567-575. b

Yang H, Qiu L, Chen G, Ye Z, Lu C, Lin Q 2008: Proportional change of CD4+CD25+ regulatory T cells in decidua and peripheral blood in unexplained recurrent spontaneous abortion patients. Fertil and Steril **89**, 656-661.

Yang Z, Carter CD, Miller MS, Bochsler PN 1995: CD14 and tissue factor expression by bacterial lipopolysaccharide-stimulated bovine alveolar macrophages in vitro. Infection and Immunity **63**, 51-56.

Young JD, Lawrence AJ, MacLean AG, Leung BP, McInnes IB, Canas B, Pappin DJ, Stevenson RD 1999: Thymosin beta 4 sulfoxide is an anti-inflammatory agent generated by monocytes in the presence of glucocorticoids. Nat Med **5**, 1424-1427.

Yu Z, Croy BA, Chapeau C, King GJ 1993: Elevated endometrial natural killer cell activity during early porcine pregnancy is conceptus-mediated. J Reprod Immunol **24**, 153-164.

Zhang H, Issekutz AC 2002: Down-modulation of monocyte transendothelial migration and endothelial adhesion molecule expression by fibroblast growth factor: reversal by the anti-angiogenic agent SU6668. Am J Pathol. **160**, 2219-30.

BIOGRAPHICAL SKETCH

Lilian de Jesus Oliveira was born in Sao Paulo, Sao Paulo, Brazil, to Luiz Manoel de Jesus and Maria Aparecida de Jesus in January 1st of 1975. Lilian received her veterinary medical degree from Universidade Sao Paulo–Sao Paulo, Brazil, in 2001. After receiving her degree, she was accepted as trainee in Immunology of Transplants at the Instituto do Coracao (Heart Institute) at the school of medicine at Universidade de Sao Paulo from December 2001 until May 2002. Later, Lilian was hired to be lab manager by the "Laboratorio de Tecnicas Immunologicas aplicadas a Mofologia" at the school of veterinary medicine at "Universidade de Sao Paulo." While Lilian was working as a lab manager, she enrolled to the Master of Science program of the sciences with the major in anatomy of domestic and wild animals, at the same school of veterinary medicine. She received her master's degree in 2005. At this time, Lilian was awarded in first place with a CAPES/Fulbright fellowship and enrolled to the University of Florida–Gainesville Graduate School to pursue her Doctor of Philosophy in animal molecular and cellular biology at the department of Animal Sciences under the supervision of Dr. Peter J. Hansen. She expects to graduate in May 2010 and to pursuit her career as scientist in the area of animal reproduction and food animal clinics.