Myeloid Antigen Presenting Cell Populations in the Murine Uterus

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Abstract

The uterus is unique amongst mucosal organs in that as well as generating protective immunity against pathogens, it must respond immunologically to antigens present in semen and on the conceptus in a manner which will allow pregnancy to ensue. Emerging evidence in other mucosal tissues indicates the capacity to discriminate between and respond appropriately to antigens of different types rests primarily with resident antigen presenting cells (APCs). Although the uterus is known to contain populations of myeloid APCs including macrophages (Mϕs) and cells with some features characteristic of dendritic cells, the precise phenotypes and functional potential of those cells to initiate immune responses have not been characterised.

The purpose of this study was therefore to undertake a detailed characterisation of the molecular phenotypes of the various APC populations present in the uterus, with a particular focus on those cells present at estrus, following insemination and at the time of embryo implantation (day 4 of pregnancy).

The first aim was to identify whether the APCs present in the murine uterus express cell surface phenotypic markers known to be associated with the events of antigen processing and presentation in other populations of interstitial Mϕs and dendritic cells. By immunohistochemistry, it was shown that cells with a dendriform morphology typical of APCs are present in the uteri of cycling virgin mice, and that these cells express F4/80 antigen, class A scavenger receptor, macrosialin, sialoadhesin, Ia and B7-2 at different relative abundancies.

The development of methods for dual-colour flow cytometric analysis of uterine cells established that at least three lineages of APCs are present within the uteri of estrous mice. On the basis of their side and forward scatter profiles and their cell surface phenotypes, the cells were designated 'undifferentiated Mϕs', 'differentiated Mϕs' and 'dendritic cells'. Both differentiated Mϕs and dendritic cells had a cell membrane profile consistent with their participation in antigen uptake, processing and presentation in the non-pregnant uterus. It was postulated that the undifferentiated Mϕs, which had a relatively simple cell surface phenotype, represented a precursor cell for activated Mϕs and possibly also dendritic cells.

That the majority of uterine APCs were sensitive in morphology and in their location within the endometrial stroma to ovarian steroid hormones was suggested by additional immunohisto-chemical and flow cytometric analysis of uteri from diestrous and ovariectomised mice. Notably, after ovariectomy the cells expressing each phenotypic marker were diminished in number, although a small population of APCs with a distinct, dendriform morphology was still evident. These cells were identified as dendritic cells, since the majority expressed Ia and

macrosialin very intensely, some expressed sialoadhesin and F4/80 but very few expressed scavenger receptor or B7-2.

The cytokine and leukocytic response of the murine uterus to mating is reminiscent of a classical inflammatory response. Upon exposure to seminal plasma, uterine epithelial cells secrete a diverse array of pro-inflammatory cytokines and chemokines, most notably GM-CSF, which is known to target myeloid leukocytes, affecting their recruitment and activation status. In accordance with this, the studies presented here show that in addition to Mϕs expressing F4/80 antigen, APCs expressing macrosialin, class A scavenger receptors, sialoadhesin, and B7-2 are all recruited into the day 1 pregnant uterus, where they accumulate in the superficial endometrial tissue in close proximity to luminal epithelial cells. Dual colour flow cytometry showed that mating affected the expression of activation markers by the APCs, and evidence of a more naïve phenotype in undifferentiated Mϕs suggested an accumulation of recently recruited cells. Cells expressing antigen presentation molecules Ia and CD1 were also present on day 1. Unexpectedly, a possible role for uterine epithelial cells in processing and presenting antigens within the uterine milieu was also evident since they too expressed macrosialin and CD1 on day 1 of pregnancy.

An examination of the APCs resident within the uteri of genetically GM-CSF-deficient mice suggested that although the recruitment of F4/80$^+$ Mϕs can occur normally in the absence of GM-CSF, this cytokine was requisite for normal activation and/or trafficking of Mϕs and dendritic cells within the uterine compartments before and after insemination. Most notably, differentiated APCs from GM-CSF-deficient mice were reduced in number and the intensity of their expression of activation markers was relatively low.

APCs contained within the day 4 pregnant uterus were found to be significantly reduced in number compared to day 1 of pregnancy, and were preferentially located in the deep endometrial tissue. A clustering of APCs in a formation reminiscent of organised lymphoid tissue was also noted in the deep endometrium of many uteri at day 4 of pregnancy, although the exact cellular composition of these structures was not determined. FACS analysis suggested that the majority of the APCs that were retained within uteri of day 4 pregnant mice were highly differentiated, since they expressed all of the activation markers at relatively high levels.

In order to further investigate the phenotype of uterine Mϕs by *in vitro* analysis, F4/80$^+$ cells were purified with the use of immunomagnetic cell selection techniques from single cell suspensions released by enzymatic digestion of uteri. These cells exhibited features common to most *ex vivo* Mϕ populations, being adherent to tissue-culture grade plastic and rapidly phagocytic of small latex beads. When assessed for their immunoaccessory function in a spleen

cell mitogenesis assay, the Mϕs were found to be potently immunosuppressive due to the synthesis of a soluble inhibitory molecule which was determined not to be a prostaglandin nor nitric oxide. The immunoinhibitory phenotype of uterine Mϕs was found to be most potent at estrus and day 4 of pregnancy, but was moderated after insemination, and particularly after ovariectomy when the Mϕs were found to be immunostimulatory in nature. Thus a role for steroid hormone- and insemination-regulated cytokines in regulating the secretory phenotype of uterine Mϕs seems likely. However, the highly complex nature of the regulation of uterine Mϕs was illustrated by experiments showing that this inhibitory phenotype could not be induced by steroid hormone-replacement of ovariectomised mice, nor could it be removed by culture in GM-CSF.

In summary, these studies suggest that myeloid APCs present within the murine uterus are similar to APC populations found in other mucosal tissues such as the lung, where they play a role in mediating tissue remodeling, immune homeostasis and the initiation of antigen-specific immunity. During the estrous cycle, the recruitment and *in situ* differentiation of uterine Mϕs and dendritic cells appears to be regulated in an ovarian steroid hormone-dependent manner, presumably as a result of the release of cytokines from uterine stromal and/or epithelial cells. The post-mating recruitment into the endometrium of increased numbers of APCs expressing molecules suggestive of an activated phenotype indicates an enhanced capacity for antigen uptake and processing at this time of exposure to paternal and other antigens. The pleiotrophic cytokine GM-CSF seems particularly important in mediating the activation of uterine Mϕs and particularly dendritic cells at day 1. By the fourth day of pregnancy, the uterine APCs are markedly reduced in number, are of a relatively differentiated phenotype and exhibit an altered pattern of distribution in the tissue.

To conclude, these studies have identified and characterised abundant and heterogeneous populations of Mϕs and dendritic cells within uterine tissues which express a large number of molecules known to be associated with antigen processing and presentation. The maturation and activation of these cells appears to be acutely responsive to the diverse microenvironments induced in the uterus by ovarian steroid hormones and the events of early pregnancy. The precise roles of APCs within the uterine milieu are yet to be identified. However, based on the current study and other reports of mucosal organs some speculations can be made. The initiation of antigen-specific immunity by uterine APCs would be vital in the generation of protective immunity against opportunistic pathogens. However, in order to accommodate insemination and pregnancy, the APCs would also need to be able to generate immune responses which mediate tolerance of innocuous antigens encountered in semen and on the semi-allogeneic conceptus.

The mechanisms underpinning such a diverse array of immune responses to antigens encountered within the uterus are yet to be elucidated, but would almost certainly be determined principally by cytokine-regulated events of antigen uptake, processing and presentation by uterine Mϕs, dendritic cells and perhaps even epithelial cells. Future studies directed at a more detailed analysis of the cell membrane and secretory phenotypes of uterine APCs in the uterus and its draining lymph nodes will address these issues.

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University and to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis. The author consents to this thesis being made available for photocopying and loan, if applicable and if accepted for the award of the degree.

Sarah Hudson
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Abbreviations

A	adenine
ATCC	American Type Tissue Culture
bp	base or nucleotide pair
BSA	bovine serum albumin
C	cytosine
°C	degrees Celsius
CC	chemokine molecule with adjacent cysteines
CCR	CC chemokine receptor
CD40L	CD40 ligand
cm	centimetre
CSF	colony stimulating factor
CSF-1	colony stimulating factor-1 (or M-CSF)
d	day (s)
DAB	diaminobenzidine
DC	dendritic cell
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTPs	2'-deoxynucleotide 5'-triphosphate
dpm	disintegrations per minute
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EtBr	ethidium bromide
FACS	fluorescence activated cell scanning
FCS	fetal calf serum
FITC	fluoroscein isothiocyanate
FSC	forward scatter
g	force of gravity
G	guanine
GM-CSF	granulocyte-macrophage colony stimulating factor
h	hour (s)
^{3}H-thymidine	tritiated thymidine
H_2O	water
HBSS	Hanks buffered saline solution
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HI	heat inactivated
HRP	horseradish peroxidase
IEL	intra-epithelial lymphocyte
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IU	international units
kb	kilobase pair (s) or 1000 bp
kDa	kilodalton
LC	Langerhans cell
LCA	leukocyte common antigen
LFA-1	leukocyte functional antigen-1
LIF	leukaemia inhibitory factor
LPS	lipopolysaccharide

M	molar
mAb	monoclonal antibody
MCP	monocyte chemoattractant protein
Mϕ	macrophage
MHC	major histocompatability complex
min	minute (s)
MIP	macrophage inflammatory protein
ml	millilitre
MQ	Milli Q
mRNA	messenger RNA
MTS	mouse thymic stroma
ng	nanogram (s)
NK	natural killer
NKT	natural killer T cell
nm	nanometres
NMS	normal mouse serum
NO	nitric oxide
O/N	overnight
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PGE_2	prostaglandin E2
PHA	phytohaemagglutinin
RANTES	regulated upon activation, normal T expressed and secreted
RBC	red blood cells
RNA	ribonucleic acid
RPE	phycoerythrin
rpm	revolutions per minute
RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
SR	scavenger receptor (class A)
SRBC	sheep red blood cells
SSC	side scatter
T	thymine
TE	thymic epithelium
TNF	tumour necrosis factor
uNK	uterine natural killer cells
UV	ultraviolet
v/v	volume per volume
VIA	video image analysis
w/v	weight per volume

Chapter 1

Review of the literature

1.1 Introduction

The immunological requirements of the uterus are unique in that as well as providing protective immunity against pathogens, the leukocyte populations resident therein must also generate an immune environment which allows implantation and growth of the semi-allogeneic conceptus for the duration of pregnancy. The mechanisms which govern the capacity of the uterus to discriminate between and respond accordingly to these different antigenic stimuli are not yet elucidated.

In the last decade, compelling evidence has emerged to suggest that the nature and quality of antigen-specific immune responses are controlled by myeloid antigen presenting cells (APCs). This is particularly evident in mucosal organs, where features of local populations of APCs such as macrophages (Mϕs) and dendritic cells can be critical in determining whether an antigen elicits an immune response of a rejective or a tolerant nature. The local microenvironment prevailing at the site of antigen exposure, particularly the presence of cytokines synthesised by local stromal cells or by the APCs themselves, can be key determinants in this regard. These cytokines appear to act by inducing specific phenotypes in the responder APCs and subsequently in the T cell populations with which they interact.

Although the uterus is similar to other mucosal tissues in that it contains many APCs and is a rich source of cytokines of relevance to these cells, the precise roles of myeloid APCs within the uterus have not been delineated. In particular, it is not known whether the regulation of APC phenotype and initiation of antigen-specific immunity to antigens encountered in the uterus is similar to that which occurs in other organs.

This discussion will focus on the current understanding of the roles of myeloid APCs in the uterus and other mucosal organs, with a particular focus on the actions of specific cytokines in their differentiation and phenotypic maturation to become effector cells for the induction of immunity.

1.2 Myeloid Antigen Presenting Cells

Myeloid APCs (Mϕs and dendritic cells) are bone marrow derived cells which have a primary role in initiation of immune responses through interaction with T lymphocytes. APCs are found throughout all tissues of the body in various developmental and phenotypic forms, each showing independent cell membrane and secretory characteristics consistent with their role within that tissue. The following section will review the general features of the Mϕ and dendritic cell lineages, and the evidence of their roles in innate immune surveillance, maintenance of tissue homeostasis and initiation of antigen-specific immunity. Also provided is a summary of some of the better characterised membrane markers on APCs and their distribution in different cell populations.

1.2.1 Macrophages (Mϕs)

a. the Mϕ lineage

Mϕs are derived from pluripotent stem cells which form within the bone marrow. During their development, a range of growth factors act to regulate the division and subsequent differentiation of stem cells into committed progenitor cells, including myeloid precursors which ultimately give rise to monocytes (Rutherford *et al.* 1993, Stumbles *et al.* 1999). In mice, monocytes remain within the bone marrow for approximately 24 h before entering the blood stream where their half life is about 17 hours (Naito *et al.* 1996). While circulating monocytes do exhibit heterogeneity in terms of cyotoplasmic density, size, morphology and surface antigen expression, whether they are destined for any particular tissue when they leave the marrow is not known (Johnston 1988, Gordon 1995). The migration of the cells into tissue compartments is precisely regulated by specific cytokines and chemokines (Rollins 1997), which trigger enhanced expression of integrins and other molecules that mediate monocyte binding to selectins and intercellular adhesion molecules on endothelial cells (Carlos and Harlan 1994). Through enhanced expression of these adhesion molecules and their receptors, the number of monocytes arriving in a peripheral tissue can be upregulated depending on fluctuations in tissue requirements, for example during inflammatory events associated with infection or tissue damage (van Furth *et al.* 1973).

In tissues, the differentiation of Mϕs from monocytes occurs due to their exposure to the prevailing cytokines and other features of the microenvironment, including hormones, other soluble mediators, extracellular matrix molecules and products of bacteria (Rutherford *et al.* 1993, Naito *et al.* 1996, Gordon 1998). This process means that the features of a resident Mϕ

population are 'fine-tuned' according to the requirements of that tissue, and therefore, a remarkable diversity (even in the absence of overt inflammatory stimuli) within tissue Mϕ populations can be generated. If a sterile or pathogenic inflammatory stimulus is delivered, additional 'elicited' Mϕs are recruited after a period of several days, and these cells can be distinguished from the resident cells by their relatively activated membrane phenotype and their enhanced abilities to engage in activities such as phagocytosis and release of cytotoxic molecules (Yoshikai *et al.* 1983, Gordon 1995).

The fate of Mϕs once they have differentiated is less clear, but presumably varies from tissue to tissue and according to perturbations in homeostasis. Inflammatory Mϕs in the peritoneal cavity, lung and kidney are known to traffic to the marginal sinus (Rosen and Gordon 1990, Lan *et al.* 1993, Bellingan *et al.* 1996) and paracortical T cell area (Thepen *et al.* 1993) of the draining lymph node, and are replaced in the tissue by the arrival of new, undifferentiated cells (Johnston 1988, Gordon 1995, Naito *et al.* 1996). In contrast, populations of Mϕs in non-inflamed organs may be retained for periods of weeks (Bellingan *et al.* 1996) or even months (Stumbles *et al.* 1999).

b. role of Mϕs in innate immunity

The innate immune system provides antigen non-specific mechanisms of surveillance at epithelial and mucosal surfaces, and provides immediate, short term protection against pathogens and other sources of potential danger. Mϕs form one of the key components of the innate immune system through their ability to engage in a number of protective behaviours. Firstly, they secrete bioactive substances, including lysosomal enzymes, oxygen metabolites, proteases and cytokines which can have a direct toxic effect on invading pathogens (Johnston *et al.* 1978, Berton and Gordon 1983, Johnston 1988). Secondly, Mϕs express a diverse range of membrane receptors which mediate binding and then internalisation (through processes such as endocytosis and phagocytosis) of microorganisms and other particulate matter (reviewed in Aderem and Underhill 1999). The discrimination of 'self' from 'non-self', or perhaps more correctly 'danger' from non-threatening insults (Matzinger 1994) is now believed to be achieved through the expression of 'pattern recognition receptors' which bind highly conserved motifs present on the surface of pathogens but absent in higher eukaryotes (Aderem and Underhill 1999). Examples include the mannose receptor, scavenger receptors, macrosialin, selectins, integrins and C-type lectins (see section 1.2.4). Complement and Fc receptors mediate highly efficient phagocytosis if the microorganism has been opsonised with complement or immunoglobulin respectively (Harding *et al.* 1988). Subsequently, the phagosome containing the internalised material matures

via a series of fusion events with components of the endocytic and exocytic pathways, culminating in the formation of the mature phagolysosome in which the pathogen is degraded by increasing acidity (Mukherjee *et al.* 1997). Phagocytosis also triggers a range of changes in Mϕ gene expression, inducing the release of secretory products including mediators of inflammation, growth factors, cytokines and cytocidal molecules (Gordon 1995).

c. role of Mϕs in tissue homeostasis and remodeling

Mϕs have been described as "a major cellular organ responsible for tissue homeostasis" (Gordon 1995). These remarkable cells have earned this reputation by virtue of their unique ability to respond to a variety of stimuli, and their impressive battery of secreted bioactive molecules, including polypeptide hormones, complement components, coagulation factors, enzymes, enzyme inhibitors, cell adhesion molecules, bioactive lipids, and reactive oxygen and nitrogen intermediates (Nathan 1987). In addition, their inherent phagocytic ability and their abundance in most peripheral tissues means that Mϕs are well placed to have actions in tissue breakdown, reorganisation and repair. Mϕs also provide an abundant source of growth factors and cytokines, which act to recruit other inflammatory cells and influence cell proliferation (Gordon *et al.* 1995).

One of the more important homeostatic roles of Mϕs in steady-state mucosal organs is believed to be their down-regulation of local populations of dendritic cells and lymphocytes in order to prevent the initiation of immune responses to inert antigens, for example pollens and dust inhaled into the lung (Holt 1986, Thepen *et al.* 1994, Stumbles *et al.* 1999). This suppression is accomplished by Mϕ secretion of inhibitory molecules such as nitric oxide (Holt *et al.* 1993, Strickland *et al.* 1996), prostaglandins (Stevens *et al.* 1989), inhibitory cytokines (Arend 1993, Colotta *et al.* 1994, Sakurai *et al.* 1996) and enzymes (Munn *et al.* 1999).

1.2.2 Dendritic cells

Dendritic cells, first identified by Steinman and Cohn over 25 years ago (Steinman and Cohn 1973), are characterised by their potent ability to immunologically activate naive T cells and their high constitutive expression of major histocompatability complex (MHC) class II molecules. These features, combined with their location at mucosal and other epithelial sites, plus their capacity to capture and retain antigen then home via the lymph and blood to T-dependent areas of lymphoid organs, form stable conjugates with resting, antigen-specific T cells and induce lymphoblast formation, makes dendritic cells the most specialised of the APCs

(reviewed in Steinman 1991, Stingl and Bergstresser 1995, Hart 1997, Banchereau and Steinman 1998).

The existence of a dendritic cell developmental lineage has been formulated as a result of many studies which show that cells with features of mature myeloid dendritic cells can be derived *in vitro* (by exposure to cytokines and other ligands) from bone-marrow-derived proliferating progenitors (Inaba *et al.* 1993, Banyer and Hapel 1999), non-proliferating precursors, such as monocytes (Randolph *et al.* 1998), and 'immature' dendritic cells in peripheral organs (Romani *et al.* 1989, Streilein and Grammer 1989, McWilliam *et al.* 1994). *In vivo*, the most likely sequence of events is that $CD34^+$ myeloid progenitor cells are released from bone marrow and can develop, via blood monocytes and other precursor cells, into dendritic cells following exposure to appropriate cytokines and interactions with endothelial adhesion molecules and tissue matrix components (Randolph *et al.* 1998, Dieu *et al.* 1999, Steinman and Inaba 1999). The resulting dendritic cell is thought to be closely related to the Mϕ, and in certain circumstances the two cell types can be induced to interconvert (Banyer and Hapel 1999, Rezzani *et al.* 1999, Vandenabeele and Wu 1999).

Dendritic cells are resident in virtually all mucosal and peripheral organs studied so far (reviewed in Steinman 1991, Hart 1997). They can be observed in skin (Streilein and Grammer 1989), lung (Stumbles *et al.* 1999), gut (Mayrhofer *et al.* 1986) and genitourinary tract (Parr and Parr 1991) as a tightly meshed network of MHC class II^+ cells. In keeping with their primary role as cells specialised for antigen uptake and processing, the cells also commonly express high levels of endocytic receptors and endosomal glycoproteins (Banchereau and Steinman 1998).

In a manner similar to that already described for Mϕs, dendritic cell precursors are believed to be recruited to organs as a result of their receptor-mediated sensitivity to a specific array of chemokines (Dieu *et al.* 1999). The turnover of dendritic cells varies from organ to organ and according to inflammatory events, such that the recruitment of dendritic cell precursors in peripheral organs increases markedly upon exposure to inflammatory stimuli (McWilliam *et al.* 1994). The resident and newly arrived cells then have the capacity to undergo phenotypic maturation and exodus from the tissue to give rise to 'veiled' cells in the afferent lymphatics which migrate to lymph nodes draining the tissue (Mayrhofer *et al.* 1986, MacPherson *et al.* 1995).

Several different populations of dendritic cells exist in lymphoid organs. Myeloid dendritic cells, thought to be responsible for the initiation of specific immunity to antigens collected in the periphery, can be found in the afferent lymph that accesses T cell areas and within the T cell areas themselves. These cells are believed to be derived from veiled cells arriving via the afferent lymphatics, and after presenting antigens their fate is probably death

(Banchereau and Steinman 1998). Lymphoid organs also contain a lineage of lymphoid dendritic cells, which are thought to derive from a non-bone marrow-derived precursor (Hart 1997). These cells are non-mobile, and are located in the T cell areas of lymphoid follicles and in the thymic medulla. Lymphoid dendritic cells are implicated in regulatory roles and in the maintenance of immunological rather than the initiation of new immune responses (Banyer and Hapel 1999, Steinman and Inaba 1999) by virtue of their capacity to tolerise T cells by the production of cytokines, or to delete them by the action of molecules such as Fas ligand. Lymphoid dendritic cells can be distinguished from myeloid dendritic cells due to their lack of CD11b expression and their expression of CD8 (Steinman and Inaba 1999).

1.2.3 Roles of Mϕs and dendritic cells in antigen-specific immunity

In contrast to innate immunity, antigen-specific immunity or acquired immunity generates immune responses targeted at specific antigens. The initiation and amplification of antigen-specific immune responses occurs when $CD4^+$ T cells become activated through ligation of their T cell receptor (TCR) with the cognate antigen-MHC class II complex on APCs. However, most intact extracellular protein antigens do not associate with MHC molecules, and require processing and coupling with MHC before recognition by cognate T cells (Unanue 1984, Harding *et al.* 1988). Thus to initiate antigen-specific immunity, professional APCs including Mϕs and dendritic cells must internalise intact extracellular proteins, process them to generate antigenic fragments, and then display these on the cell surface bound to Class II MHC. The interaction of the TCR with the antigen-MHC class II complex is further stabilised by interactions between cognate accessory molecules, and is accompanied by delivery of additional soluble signals to the T cell.

a. antigen uptake

Mϕs and dendritic cells utilise similar mechanisms to acquire proteins from the environments which they survey. Phagocytosis is an actin-dependent mechanism which mediates internalisation of large (> 0.5 μm), cell surface receptor-bound particulate matter (Aderem and Underhill 1999) and has until recently been considered a behaviour largely limited to Mϕs for clearance and killing of pathogens (see section 1.3.1c). However it is now recognised that dendritic cells are also actively phagocytic cells *in vivo* and *in vitro* (Mayrhofer *et al.* 1986, Moll *et al.* 1993, Rescigno *et al.* 1999).

Smaller receptor-bound ligands as well as fluid and solutes are internalised by Mϕs and dendritic cells via endocytosis which occurs via clathrin-dependent and clathrin-independent coated pits on the cell surface (Hewlett *et al.* 1994, Mukherjee *et al.* 1997). Receptors involved in mediating endocytosis include mannose receptor and DEC-205, which when bound by ligand are delivered to endosomal compartments and then recycled to the cell surface (Jiang *et al.* 1995, Sallusto *et al.* 1995). Other receptors involved in endocytosis and phagocytosis by Mϕs and dendritic cells include Fc receptors, CD11b/CD18 (Mac-1), CD14 (LPS receptor) and a relatively new family of 'danger' receptors called Toll-like receptors (Rescigno *et al.* 1999).

Macropinocytosis is a separate process by which relatively large and heterogeneous fluid-containing vesicles are internalised at the leading edge of APCs, often in association with membrane 'ruffling' (Rabinowitz *et al.* 1992, Hewlett *et al.* 1994). Constitutive macropinocytosis confers dendritic cells with a high capacity, non-saturable mechanism for the capture of solutes which become concentrated in intracellular compartments containing proteases and MHC class II molecules (Sallusto *et al.* 1995). There is some evidence that dendritic cells can also acquire antigen from extracellular proteolysis following protease release during an inflammatory response in skin, from other cells such as Mϕs (Romani *et al.* 1989), or alternatively through phagocytosis of endocytic, antigen-containing epithelial cells (MacPherson and Liu 1999).

b. antigen processing

The fate of internalised proteins is complex and involves a process of sorting through discrete intracellular compartments to mediate peptide fragmentation and loading onto MHC molecules. As reviewed (Harding 1996), Mϕs appear preferentially equipped to accumulate and rapidly deliver large amounts of particulate matter to lysosomes for degradation by the action of proteases and increasing acidity generated by proton pumps. For protein fragments to be presented on the cell surface, empty MHC class II molecules are directed from the endoplasmic reticulum to lysosomal compartments through their association with invariant chain, which prevents peptide binding until it is proteolytically removed in peptide loading compartments. The antigen-MHC class II is then directed to the cell surface.

By comparison, in dendritic cells the processes which mediate antigen loading to MHC molecules appear much more efficient (Inaba *et al.* 1990, Hart 1997). Material internalised by interstitial and other relatively immature populations of dendritic cells is delivered rapidly (within 30 minutes) to large, intracellular, MHC class II-rich compartments containing other components required for efficient antigen processing, including invariant chain, cathepsin D,

lysosomal-associated membrane protein and macrosialin (Hewlett *et al.* 1994, Nijman *et al.* 1995, Sallusto *et al.* 1995).

c. phenotypic maturation and trafficking to lymphoid organs

After exposure to antigen or an inflammatory stimulus at a peripheral site, both Mϕs and dendritic cells move rapidly out of the tissue into lymphatic vessels and from there into draining lymph nodes (Lan *et al.* 1993, Bellingan *et al.* 1996, Banchereau and Steinman 1998). This emigration is accompanied by a marked shift in the APC phenotype, a process which has been well characterised in dendritic cells, but not so thoroughly in Mϕs although similar trends have been observed (Yoshikai *et al.* 1983). Trafficking dendritic cells exhibit a dramatically downregulated capacity to internalise and process antigen, conferring 'fidelity' to the antigen acquired under inflammatory conditions (Streilein and Grammer 1989, Inaba *et al.* 1990, Sornasse *et al.* 1992). The decreased accumulation and processing of antigens is mediated by reduced cell membrane expression of endocytic receptors (Sallusto *et al.* 1995) and the transformation of the MHC class II-rich compartments within the dendritic cell cytoplasm to vesicles that discharge their contents to the cell surface where the antigen-MHC class II complex remains stable on the cell surface for several days (Nijman *et al.* 1995, Pierre *et al.* 1997).

In dendritic cells the stimulus inducing the change from the 'immature' or sentinel dendritic cell to a 'mature', trafficking dendritic cell is likely to include multiple signals provided within the inflammatory site. Application of bacteria to lung, skin or gut induces rapid exodus of dendritic cells to the draining lymph (Mayrhofer *et al.* 1986, Moll *et al.* 1993, McWilliam *et al.* 1994) but antigen-binding alone is not sufficient to generate significant exodus (van Wilsem *et al.* 1994). Additional signals probably include bacterial products such as LPS (MacPherson *et al.* 1995), increased local synthesis of pro-inflammatory cytokines (MacPherson *et al.* 1995, Sallusto *et al.* 1995, Cumberbatch *et al.* 1997) and appropriate concentration gradients of certain chemokines (Dieu *et al.* 1998, Dieu *et al.* 1999).

d. antigen presentation and T cell activation

APCs appear to activate T cells by the delivery of two types of signal (reviewed in Croft and Dubey 1997, Greenfield *et al.* 1998). The first signal is delivered through the TCR by binding to the antigen-MHC class II complex and thus is antigen-specific. The second signal is non-specific, and occurs when adhesion and costimulatory molecules on the APC membrane bind receptors on the T cell membrane. If both signals are provided, the T cell undergoes a number of phenotypic changes which include the synthesis and secretion of IL-2 and

proliferation (evident about 12h later). Additional immune-deviating signals may be provided to the T cell in the form of cytokines secreted by the APC (see section 1.3.4c).

Delivery of the first signal to T cells is limited by the absolute level of MHC class II expression on APCs (Unanue 1984). Constitutive expression of MHC class II molecules is restricted to Mϕs, dendritic cells and other professional APCs (such as B cells and thymic epithelium), but the absolute levels of expression on these cells does vary according to their stage of differentiation and environmental cues, such as cytokine signals, which alter the rate of gene transcription (Rohn *et al.* 1996). Mature dendritic cells (those within lymphoid tissue) are renowned for their very high expression of MHC class II (Steinman 1991, Stingl and Bergstresser 1995, Hart 1997), which can be 10-100 times higher than on Mϕs and B cells (Inaba *et al.* 1997). Under inflammatory conditions, other cell types (so-called 'non-professional APCs') such as epithelial cells may also express MHC class II (Mayer 1997).

For the second signal, APCs express a range of cell surface molecules that bind counter-receptors on T cells (reviewed in Croft and Dubey 1997). In the past, these have been divided into 2 categories : those that simply mediate adhesion between the APC and the T cell, and those that provide costimulation to the T cell. However, it now appears that virtually all of these, including the intercellular adhesion molecules, induce intracellular signals within the T cell which eventually lead to the production of IL-2 and proliferation. Multiple interactions probably also increase the affinity and duration of both the 1^{st} and 2^{nd} signal, and accessory molecule co-receptors also induce additional costimulatory signals to further amplify the signal.

The B7 family are probably the best described costimulatory molecules (reviewed in Croft and Dubey 1997, Greenfield *et al.* 1998). CD80 (B7-1) and CD86 (B7-2) are both members of the immunoglobulin supergene family, and are expressed by professional APCs and some T cells. *In vitro* studies with neutralising antibodies suggest that surface expression of B7-2 increases rapidly after APC activation. B7-2 binding to CD28 on T cells is probably the principal and immediate provider of 2^{nd} signal during an APC-T cell interaction, as B7-1 does not appear on the APC surface until 24-48 h after TCR binding. CTLA4, which appears on the T cell surface 48-72 h after T cell activation, is another receptor for the B7 ligands and is thought to deliver a negative, perhaps even anergy-inducing signal to T cells (Constant and Bottomly 1997). The interaction between CD40 on the APC with CD40 ligand (CD40L) on the T cell membrane is thought to be another of the important molecules for T cell activation (Laman *et al.* 1996).

As well as being influenced by the expression on APCs of MHC class II and costimulatory molecules, the outcome of antigen presentation and costimulation varies according to the pre-existing activation and differentiation state of the T cell (reviewed in Croft and Dubey 1997). Naïve T cells, which have not previously encountered their cognate antigen, appear to

rely absolutely on accessory molecule interactions to become activated. Thus, mature dendritic cells, which express high constitutive levels of many costimulatory molecules (including B7-1, B7-2 and CD40) are most likely to be the main participant in activating naïve T cells *in vivo*. The location of dendritic cells within the cortical region of secondary lymphoid tissues also place them in the ideal position to encounter naïve T cells. In contrast, the lower expression of both MHC class II and costimulatory molecules on Mϕs probably results in their relatively poor ability to activate naïve T cells. Indeed, a failure to deliver appropriate co-stimulation to naïve T cells at the time of the TCR-MHC class II interaction may even result in antigen-specific anergy (Greenfield *et al.* 1998).

In contrast to naïve T cells, both memory and particularly effector T cells can respond to some extent to TCR stimulation alone and show less dependency on costimulatory signals (Croft and Dubey 1997). Presumably, these T cells do not rely only on dendritic cells to generate activation, but can utilise other APCs such as Mϕs or B cells, or even non-professional APCs. Furthermore, the trafficking patterns of memory and effector T cells mean that the secondary activation of these cells can occur in the periphery as well as in lymphoid organs.

1.2.4 Cell membrane markers on Mϕs and dendritic cells

Mϕs and dendritic cells are remarkable for their expression of a large array of cell surface molecules. These include receptors for antigen uptake, homing molecules, receptors for cytokines, chemokines and other ligands, antigen presentation molecules, adhesion and costimulation molecules, and a number of molecules specific to the cell lineages (often referred to as 'markers') to which a defined function is yet to be ascribed. Despite this abundance, a single molecule which can be used consistently and in isolation to distinguish between Mϕs and dendritic cells has not yet been identified, attesting to their shared features and their remarkable heterogeneity. The salient features of a number of the better characterised cell membrane markers on murine Mϕs and dendritic cells are summarised below.

a. F4/80

F4/80 is a rat monoclonal antibody (mAb) reactive with an extracellular glycoprotein of molecular weight 150 kDa (Starkey *et al.* 1987) which is found on monocytes, Mϕs, Langerhans cells and some dendritic cells. Although a role of F4/80 antigen peculiar to myeloid APCs seems likely given its restricted expression pattern, knowledge of the exact function of this molecule has remained elusive since its first description (Austyn and Gordon 1981). Currently, a role in

cell:stroma (Haidl and Jefferies 1996) or cell:cell interactions (Warschkau and Kiderlen 1999) is proposed. Ligands for F4/80 have also not yet been defined, but it could be expected that ligation of the F4/80 molecule would trigger signal transduction via G-proteins since its primary amino-acid sequence shows homology to the epidermal growth factor-like family and to the seven transmembrane hormone receptor family (McKnight *et al.* 1996, McKnight and Gordon 1998). Expression of F4/80 varies on different Mϕ populations, being relatively low on immature Mϕs, on cultured Mϕs and following exposure to cytokines such as IFNγ (Gordon *et al.* 1992). The pattern of distribution of F4/80$^+$ Mϕs, Langerhans cells and dendritic cells within a diverse range of murine tissues has been described in detail (Hume and Gordon 1983, Hume *et al.* 1983, Hume *et al.* 1984a, Hume *et al.* 1984b, Hume *et al.* 1984c).

b. macrosialin

Macrosialin, a member of the lamp/lpg family of lysosomal/endosomal glycoproteins, is a molecule more widely expressed by tissue Mϕs than is F4/80, but also found on dendritic cell populations (Rabinowitz and Gordon 1991, Gordon *et al.* 1992). Detected with the rat mAb FA/11, it is expressed predominantly on intracellular phagolysosomal and vacuolar membranes but also on the cell surface (Rabinowitz and Gordon 1991, Holness *et al.* 1993, Ramprasad *et al.* 1996). Of the total molecular mass (87-115 kDa), the polypeptide backbone of macrosialin contributes only 35 kDa: O-linked and N-linked sugars comprise approximately 40% and 20-25% of the total mass respectively (Rabinowitz and Gordon 1991, Holness *et al.* 1993).

The role of such an abundant glycosylated molecule in prelysomes, phagolysosomes and on the plasma membrane of professional phagocytic cells is almost certain to include binding and chaperoning internalised antigens though intracellular compartments during processing (Rabinowitz and Gordon 1991, Holness *et al.* 1993). Upon Mϕ activation, the expression of internal but particularly cell surface macrosialin increases significantly, and its O-linked and N-linked sugars show extensive remodeling, including the acquisition of numerous terminal sialic acid residues and polylactosaminoglycans which confer new lectin binding capabilities. Macrosialin and other heavily sialyated molecules on Mϕs may also be involved in selectin-binding events on epithelium or in the uptake of oxidised low density lipoproteins (Ramprasad *et al.* 1996, Van Velzen *et al.* 1997).

c. class A scavenger receptor

Class A scavenger receptor is a trimeric integral membrane protein which may exist in two forms, Type I and Type II, generated by alternative splicing of a single gene product (Hughes

et al. 1995). Both forms are recognised by the rat mAb 2F8, which labels tissue Mϕs in thymus, lymph nodes, solid organs including skin and 'free' Mϕs in lung alveoli and the peritoneal cavity (Fraser *et al.* 1993). However, some populations of Mϕs are clearly unreactive with 2F8, such as metallophillic Mϕs in the spleen (Hughes *et al.* 1995). Although Langerhans cells in mice and other species are known not to express class A scavenger receptor (Naito *et al.* 1991, Hughes *et al.* 1995), other interstitial dendritic cells can do so (Pearson 1996).

Expression of class A scavenger receptor correlates with an ability of Mϕs to phagocytose colloidal carbon (Hughes *et al.* 1995), and the molecule is implicated in the uptake of oxidised lipoproteins and other polyanionic moieties in atherosclerotic plaques (de Villiers *et al.* 1994, Gordon *et al.* 1995, Hughes *et al.* 1995). However, there is also some evidence that the adhesion of Mϕs within lymphoid and non-lymphoid organs is mediated via an interaction between class A scavenger receptor and an endogenous tissue ligand (Fraser *et al.* 1993, Hughes *et al.* 1995). Class A scavenger receptor is also implicated in mediating phagocytosis of apoptotic cells by binding ligands on the dying cell (Aderem and Underhill 1999). Expression of Class A scavenger receptor is regulated by CSF-1 (de Villiers *et al.* 1994).

d. sialoadhesin

Sialoadhesin (originally named sheep erythrocyte receptor, or SER) is a 185 kDa member of the immunoglobulin superfamily detected with rat mAbs 3D6 and SER-4 (van den Berg *et al.* 1992). Sialoadhesin is not expressed by all Mϕs, but is largely limited to populations interacting with other cells or stromal elements within tissue compartments such as bone marrow, lymphoid organs and liver and not in 'spaces' such as the peritoneal cavity (Crocker and Gordon 1986, van den Berg *et al.* 1992, Muerkoster *et al.* 1999). Sialoadhesin is also expressed on some dendritic cell populations (Berney *et al.* 1999, Muerkoster *et al.* 1999).

Sialoadhesin is thought to mediate sialic acid-dependent binding of Mϕs to granulocytes, lymphocytes and other sources of sialyated moieties (van den Berg *et al.* 1992, Crocker *et al.* 1994, Crocker *et al.* 1995). In bone marrow, sialoadhesin is likely to be one of a range of molecules mediating Mϕ adhesion to dead or dying cells requiring phagocytic clearance (Crocker and Gordon 1986, Crocker *et al.* 1994). In peripheral organs, the role of sialoadhesin is less clear, but may include binding to neutrophils for phagocytic uptake (Crocker *et al.* 1995) or adhesive interactions with $CD4^+$ and $CD8^+$ lymphocytes to stabilise antigen presentation and T cell activation (Muerkoster *et al.* 1999). Correspondingly, sialoadhesin can be induced on Mϕs recruited to a site of inflammation (Crocker *et al.* 1995). Sialoadhesin could also play a role in

binding B lymphocytes (van den Berg *et al.* 1992). The precise ligand(s) for sialoadhesin in each of these interactions remains unknown.

e. *β_2 integrins*

Adherence of Mϕs to cells and extracellular matrices is mediated by three families of cell surface proteins: the immunoglobulin-like molecules, selectins and integrins (reviewed in Carlos and Harlan 1994). Of these, β_2 integrins are particularly relevant to the function of myeloid APCs. In each of the molecules comprising this family, the β chain CD18 combines with one of 3 α chains to form 3 separate integrin molecules containing the determinants CD11a (LFA-1), CD11b (Mac-1 or CR3) or CD11c (p150,95) respectively.

Each of the β_2 integrins can be found on cells of the monocyte/Mϕ lineage, including myeloid dendritic cells, and each has an individual profile of cell surface and extracellular ligands (Rosen and Gordon 1989). By binding to selectins and intracellular adhesion molecules, Mac-1 and CD11a/CD18 are thought to mediate monocyte-endothelial cell interaction during rolling and extravasation (Carlos and Harlan 1994). Mac-1 is also implicated in inducing endocytosis through binding complement-coated pathogens and in binding other cells via ligands such as fibrinogen, factor X and LPS, and in adhesive interactions with extracellular stromal components (Rosen and Gordon 1989, Fraser *et al.* 1993, Gessani *et al.* 1993). Thus Mϕ expression of Mac-1 reflects recent trafficking behaviour and/or activation for cell mediated immunity (Springer *et al.* 1979). Compared to Mac-1, CD11c/CD18 seems to be less important in adhesion to endothelial cells (Beekhuizen *et al.* 1990) and other roles still remain uncertain although expression is thought to correlate directly with differentiation along the monocyte-Mϕ pathway (Dudley *et al.* 1989).

f. *mannose receptor*

The Mϕ mannose receptor is a 175 kDa phagocytic receptor which mediates Mϕ and dendritic cell binding and ingestion of microorganisms with surface mannose residues and soluble mannose-containing glycoproteins (Wileman *et al.* 1986). The three-domain structure of the mannose receptor, one arm of which confers its carbohydrate recognition domains, is also common to the antigen-binding receptor DEC-205 (Martinez Pomares *et al.* 1996). Once mannose receptor-bound ligands are delivered to the intracellular antigen-processing compartments, the molecule is rapidly recycled to the cell surface (Sallusto *et al.* 1995). There is

some evidence that free mannose receptor may also play a role in transporting antigen through lymphoid organs to T and B cell rich areas (Martinez Pomares *et al.* 1996).

g. 33D1

The rat mAb 33D1 identifies a low-density antigen of unknown functional significance on mouse marginal zone spleen dendritic cells (Nussenzweig *et al.* 1982, Crowley *et al.* 1990, Girolomoni *et al.* 1990), which are thought to originate from highly mobile dendritic cells of peripheral tissues and interstitial spaces (Crowley *et al.* 1989). The antigen is also found on *in vitro*-derived myeloid dendritic cells (Lu *et al.* 1995, Gao *et al.* 1997, Cao *et al.* 1998, Masurier *et al.* 1999).

h. DEC-205

NLDC-145 rat mAb detects the dendritic cell surface antigen DEC-205, which is expressed at high levels on dendritic cell progenitors, on Langerhans cells and on dendritic cells in lymphoid organs and spleen (Inaba *et al.* 1995, Swiggard *et al.* 1995, Witmer Pack *et al.* 1995). The antigen is also expressed on thymic, intestinal and lung epithelium and elicited peritoneal Mϕs and is thought to act as an endocytic receptor to mediate antigen uptake (Inaba *et al.* 1995, Jiang *et al.* 1995, Witmer Pack *et al.* 1995).

i. CD1

The CD1 family of proteins are novel antigen-presenting molecules encoded by genes outside of the major histocompatability complex (reviewed in Porcelli and Modlin 1999). Group I CD1 proteins (CD1a, -b and –c) are the classic CD1 antigens, but these are not expressed in murine cells; instead, the two group II CD1 genes produce extremely similar proteins known as murine CD1d. In this thesis, the term 'CD1' refers to murine CD1d.

CD1 is widely expressed on haematopoietic cells in mice (Brossay *et al.* 1997), and in mature cells is found constitutively and at high levels on APCs such as splenic dendritic cells, Mϕs and B cells (Roark *et al.* 1998). In interstitial dendritic cells, such as Langerhans cells, its expression is less intense (Porcelli and Modlin 1999). CD1 is also expressed by a small subset of intestinal epithelial cells in mice (Brossay *et al.* 1997), rats and humans (Porcelli and Modlin 1999).

CD1 appears to have a unique role as an antigen presentation molecule, both in terms of the ligands it binds and the T cells it activates (reviewed in Porcelli and Modlin 1999). Although

exhibiting some capacity to bind protein antigens, the majority of antigens bound and presented by CD1 are glycolipids. Although CD1-reactive T cells can be located within the $CD4^+$ population in mice, they are also common amongst an unusual T cell population known as NKT cells, so-called because they express cell-surface proteins previously associated with the natural killer (NK) cell lineage. NKT cells express an invariant TCRα chain and a limited TCRβ chain repertoire, endowing a receptor repertoire of limited diversity. Furthermore, these cells rapidly secrete large amounts of inhibitory and other cytokines upon TCR engagement, suggesting they play a role in deviating immune responses in peripheral organs (Bendelac *et al.* 1997).

1.3 Cytokines

1.3.1 Cytokines and their receptors : an overview

Often named for their first identified biological effects, cytokines are small (usually < 80 kDa) glycoproteins that serve to regulate the production, migration and functional activation of haematopoietic and other cell types (reviewed in Robertson, 1998). Apart from their small size, cytokines are an extremely heterogeneous group of molecules whose actions are usually mediated in a local sphere through receptor-specific interactions with target cells. Cytokines do not act as effector molecules directly, but instead bind to specific, high affinity receptors in the cytoplasmic membrane of the target cell. The binding of a specific cytokine to a specific receptor initiates a cascade of intracellular signaling events which culminate in altered mRNA and protein synthesis. However, the outcome of a particular binding event varies greatly according to a number of features of the interaction, including the lineage and phenotype of the target cell, the receptor type and the presence or absence of other biological signals (including other cytokines) acting on the target cell at the time. Furthermore, cytokines are renowned for functional redundancy and their pleiotrophy of actions, which have become particularly evident by analysis of mice with null mutations in cytokine or cytokine receptor genes (cytokine 'knockout' mice).

1.3.2 Cytokines as regulators of APC haemopoiesis and early differentiation

The processes by which progenitor and precursor cells in the Mϕ and dendritic cell lineages are derived in the bone marrow are precisely regulated by a large group of cytokines known as the haematopoietins, which include interleukin (IL)-3 and IL-6, and the colony stimulating factors (CSFs) CSF-1 and granulocyte-macrophage-CSF (GM-CSF) (Socolovsky *et al.* 1998). These cytokines act in a variety of combinations at each of the developmental stages in a highly complex manner which has not yet been fully elucidated. However, some of the

individual roles of each of the cytokines have been identified by *in vitro* and *in vivo* analysis of cells in cytokine knockout and replete mice, with the haematopoietins IL-3, CSF-1 and GM-CSF demonstrating surprising redundancy in myeloid cell growth and differentiation (Johnston 1988, Rutherford *et al.* 1993, Nishinakamura *et al.* 1996, Burdach *et al.* 1998). Whether in the bone marrow Mϕ and dendritic cell precursors develop separately is not known, but could be accomplished through the acquisition of differential sensitivities to the different haematopoietins (Banyer and Hapel 1999, Santiago Schwarz 1999).

A role for specific cytokines in the differentiation of Mϕs and dendritic cells becomes more apparent once the precursor cells leave the bone marrow and encounter differentiation signals during trafficking and upon becoming resident within tissues. Thus, precursors collected from the blood of humans and mice can be directed along various differentiation pathways by *in vitro* culture under specific conditions. At this stage of APC development, the divergent actions of CSF-1 and GM-CSF on differentiation of precursor cells become particularly apparent. Thus, exposure of precursor cells to CSF-1-rich environments tends to promote the differentiation of a characteristic tissue Mϕ, a cell which exhibits cell membrane and behavioural phenotypes consistent with its roles in tissue remodeling, phagocytosis and destruction of unwanted debris and the suppression of local immune responses. By contrast, culture in GM-CSF +/- IL-4 [particularly following exposure to tumour necrosis factor (TNF)α and interferon (IFN)γ] preferentially stimulates dendritic cell development (Banyer and Hapel 1999, Santiago Schwarz 1999, Steinman and Inaba 1999). Transforming growth factor (TGF)-β appears to be crucial for the development of Langerhans cells from monocyte-like precursor cells (Zhang *et al.* 1999).

1.3.3 Cytokines and chemokines as regulators of APC chemotaxis

The efficacy of Mϕs and dendritic cells as APCs hinges, amongst other behaviours, on their ability to traffic to and from inflamed tissues and subsequently into lymphoid organs. The capacity to perform such precisely controlled movement is accomplished by virtue of their ability to express different cell adhesion molecules in response to a specific subset of cytokines known as chemokines. Chemokines are small (molecular weights in the range of 8-12 kDa), highly conserved proteins which are released in a regulated manner into the tissues, where either as free protein or bound to proteoglycans they interact with specific G-protein coupled receptors on target cells (Dieu *et al.* 1999). The chemokine family is also highly redundant and pleiotrophic, with each of the many constituent molecules able to act on a number of receptors to produce different outcomes depending on the context and target cell type (Rollins 1997, Kelso, 1998).

The principal group of chemokines active on Mϕs and dendritic cells are the 'CC' chemokines, so-called due to their common structural feature of two adjacent cysteines near the N-terminus of the protein (reviewed in Rollins 1997). CC chemokines are synthesised by a wide variety of cell types, including lymphocytes and cells of mesenchymal origin, and examples active in the recruitment of monocytes and Mϕs include monocyte chemoattractant protein (MCP)-1, RANTES (**r**egulated upon **a**ctivation, **n**ormal **T** **e**xpressed and **s**ecreted) and macrophage inflammatory protein (MIP)-1α and MIP-1β. Mϕs can also secrete most of these chemokines themselves in response to inflammatory stimuli. The key monocyte chemokines act through binding to receptors CCR1-CCR5 on the Mϕ cell membrane, the expression of which may also be regulated by other cytokines.

The control of dendritic cell movement between blood, peripheral tissues and lymphoid tissues is also thought to be primarily regulated by CC chemokines, although other chemokines may also be involved (Dieu *et al.* 1998, Dieu *et al.* 1999). Recent immunohistochemical and flow cytometric studies provide evidence that dendritic cell trafficking between peripheral and lymphoid tissues occurs as a result of highly organised and segregated patterns of expression of specific CC chemokines and the differential expression of specific CC receptors on the immature and mature cells. A similar mechanism probably also exists for the control of Mϕ recruitment to lymphoid organs.

1.3.4 Cytokines and immunity

Once Mϕs and dendritic cell precursors become resident within peripheral organs, their subsequent differentiation is determined largely by the prevailing cytokine environment. Thus features of the cells including their expression of cell membrane markers, their collection and processing of antigens and their ability to initiate antigen-specific immunity all hinge on the presence or absence of certain combinations of cytokines. Furthermore, the outcome of the interaction between APCs and T cells is also dependent on the production of cytokines by both of these cells types. Thus cytokines are implicated as key regulators in the control of immunity. Below are summarised some of the key cytokines believed to be involved in the phenotypic regulation of Mϕs and dendritic cells in resting and inflamed peripheral organs, and the cytokines involved in the initiation and effector stages of antigen-specific immunity.

a. regulation of APC phenotype in steady-state peripheral organs

Probably one of the best examples of the role of cytokine environment in determining APC phenotype is seen in the anterior chamber of the eye. Due to its synthesis by local stromal cells, this organ contains high concentrations of TGFβ, a cytokine recognised for its many actions, including control of the differentiation, proliferation and state of activation of many leukocyte lineages (Letterio and Roberts 1998). Mϕs and dendritic cells collected from the anterior chamber of the eye possess unique immune properties which have been coined 'anterior chamber-associated immune deviation' (ACAID) since they are unable to generate antigen-specific delayed-type hypersensitivity (Wilbanks and Streilein 1992, Streilein 1993). That Mϕs from other sites can be induced to assume a similar phenotype by culture in anterior chamber fluid or TGFβ highlights the universal ability of this cytokine to have a potent effect on APC phenotype.

Apart from unique organs such as the eye, the specific roles of individual cytokines in determining the phenotype of steady state dendritic cells is still speculative and largely based on *in vitro* data. A key candidate for the phenotypic regulation of immature dendritic cells is GM-CSF (Kaplan *et al.* 1992, O'Sullivan *et al.* 1996), a role for which in the periphery is supported by evidence of its production by steady state stromal cells such as the keratinocytes of the skin (Kupper *et al.* 1988) and epithelial cells in the lung (Christensen *et al.* 1995) and other organs (Robertson *et al.* 1992). IL-4 (Stoppacciaro *et al.* 1997) and TGFβ (Yamaguchi 1998) may be similarly involved in the maintenance of relatively immature dendritic cells subsets within peripheral organ environments. Indeed, dendritic cells which exhibit one of the key features of peripheral organ dendritic cells (namely poor costimulatory function but efficient antigen collection behaviour) can be generated *in vitro* by culture in GM-CSF + IL-4 (Cella *et al.* 1997). The stromal cell transmembrane proteins c-kit ligand and FLt3 ligand may have a similar effect through binding with dendritic cell tyrosine kinase receptors (Banchereau and Steinman 1998).

In contrast to dendritic cells, and as summarised by Gordon and co-workers (Gordon 1995, Gordon 1998), the phenotype of Mϕs in steady state peripheral organs tends to be much more heterogeneous, varying from the large, cytokine producing, highly phagocytic Kupffer cells of the liver to the small, immunosuppressive alveolar Mϕs of the lung. There are many cytokines implicated in generating such diversity, including IL-4, IL-13 and IL-10 (Gordon *et al.* 1995). CSF-1 remains a cytokine of central importance to Mϕs in the later stages of maturation, particularly for those Mϕs thought to be involved in tissue remodeling and morphogenesis (Stanley *et al.* 1997). Thus, culture of *ex-vivo* Mϕs in CSF-1 has been found to be a potent and selective upregulator of their expression of class A scavenger receptors, conferring the cells with

the capacity to endocytose polyanionic ligands such as modified lipoproteins and lipoteichoic acid on gram positive bacteria (de Villiers *et al.* 1994). CSF-1 also upregulates expression of Fc receptors (Magee *et al.* 1987) and the endosomal and cell surface glycoprotein macrosialin, also thought to play a role in antigen uptake as well as intracellular antigen transport or processing (Li *et al.* 1998). Furthermore, CSF-1 is implicated in the differentiation of Mϕs which play a homeostatic role in mucosal organs by inducing their synthesis of immunosuppressive molecules including the tryptophan catabolising enzyme IDO (Mellor and Munn 1999), IL-1 inhibitors (Matsushime *et al.* 1991, Strassmann *et al.* 1991) and other immunosuppressive molecules (Sakurai *et al.* 1996). The central role of CSF-1 for many peripheral Mϕ populations has been highlighted by analysis of CSF-1-deficient mice (see section 1.3.5b). Similarly, that GM-CSF is crucial for the maintenance of normal alveolar Mϕ populations is evident in GM-CSF-deficient mice (see section 1.3.5a).

Thus it appears that both Mϕ and dendritic cell precursors arriving in peripheral organs acquire the features of typical fixed tissue cells due to their exposure to cytokines and other molecules. The duration of exposure, the concentration of each of these molecules and the expression of receptors on the Mϕ and dendritic cell membranes would be just some of the factors governing these interactions.

b. initiation of antigen-specific immunity during inflammatory responses

Mϕs and dendritic cells in steady state tissues provide innate immune surveillance and maintain homeostasis. However, upon invasion by a pathogen or another source of perceived danger, the tissue must be able to generate rapid defence mechanisms in order to minimise tissue damage. The emerging picture, derived by analysis of the cellular and molecular networks existing in inflamed organs *in vivo* and in *in vitro* systems which replicate these, is that upon introduction of danger or 'alarm' signals such as LPS or other conserved pathogen products to tissues, epithelial cells and fibroblasts become transiently activated to synthesise a diverse array of chemokines and cytokines. The chemokines synthesised target many cell lineages including Mϕs and dendritic cells (Lo *et al.* 1998) and cytokines produced include GM-CSF, TNFα and IL-8 (Xu *et al.* 1989, Nonaka *et al.* 1996).

The consequences of this sudden increase in local chemokine and cytokine concentrations are almost immediate. For example, within an hour of aerosol treatment of rats with bacteria, new cohorts of dendritic cells and neutrophils can be observed in the lung parenchyma followed later by Mϕs (McWilliam *et al.* 1994). *Ex vivo* cellular analysis and *in vitro* experiments replicating such conditions suggest that due to their exposure to elevated concentrations of GM-

CSF, TNFα and TGFβ, Mϕs arriving in inflamed organs cease their synthesis of immunoinhibitory molecules (Bilyk and Holt 1993, Bilyk and Holt 1995) and produce stimulatory cytokines including IL-1 (Kato *et al.* 1990) and TNFα (Hart *et al.* 1988) and upregulate their expression of Class II MHC (Kato *et al.* 1990). The cells may even proliferate (Chodakewitz *et al.* 1988). Other cytokines such as IL-1 and IL-6 may also be implicated in the transformation of a resting Mϕ to one activated for immunostimulation (Gordon *et al.* 1995).

Dendritic cells exposed *in vivo* or *in vitro* to high concentrations of GM-CSF (Stoppacciaro *et al.* 1997), TNFα, CD40L (Cella *et al.* 1997), bacterial products (MacPherson *et al.* 1995) or contact sensitisers (Cumberbatch *et al.* 1991) cease capturing antigens and instead undergo phenotypic transformation to become potent antigen presenting and T cell stimulator cells as they begin to traffic to local lymph nodes (Holt *et al.* 1999). The dendritic cells also start to produce cytokines for the initiation of cell mediated immune responses (Stumbles *et al.* 1998). Thus, following exposure of tissues to danger signals, an opportunity for the induction of antigen-specific immune responses is provided by the cytokine-induced phenotypic transformation of Mϕs and dendritic cells.

c. activation and polarisation of T lymphocytes

Upon trafficking to draining lymph nodes, the main role of Mϕs and particularly dendritic cells is that of antigen presentation and activation of T cells. As already discussed (see section 1.2.3d), the activation of T cells involves their reception of two 'signals' from the APC. The first signal is antigen-specific and is delivered through the TCR after binding with MHC class II + antigen. The second signal is antigen independent, and relies on the binding of ligands on the T cell membrane with co-stimulatory molecules on the APC. However, within the last 15 years it has become apparent that this is a simplified view of the process, and in fact that the quality and duration of the APC: T cell interaction can induce the T helper cells to assume one of two or more alternative phenotypes (Mosmann *et al.* 1986, Cher and Mosmann 1987).

The division of T helper cells into alternative phenotypes is based on their functional capabilities and the profile of cytokines they produce. T helper 1 (Th1) cells, which secrete and respond to cytokines such as IL-12, IFNγ and TNFα, are implicated in cell-mediated inflammatory responses such as delayed-type hypersensitivity. In contrast, T helper 2 (Th2) cells promote humoral immunity by secretion of cytokines such as IL-4 and IL-5. The two cell populations are thought to be counter-regulatory, with Th1 cytokines having a negative regulatory effect on Th2 cells and vice versa (Mosmann and Sad 1996). T lymphocytes which are thought to play distinct regulatory and 'bystander suppressive' roles in peripheral organs may

also be activated, including TGFβ-secreting T helper 3 (Th3) cells (Weiner 1997), IL-10-secreting T regulatory 1 (Tr1) cells (Groux and Powrie 1999) and CD1-restricted NKT cells which synthesise large amounts of IL-4 (Bendelac *et al.* 1997).

The polarisation of naïve T lymphocytes to activated, cytokine-secreting cells occurs at the time of sensitisation and is influenced by a number of factors, including the physiochemical properties of the antigen and the presence or absence of certain cell membrane costimulatory molecules (see section 1.4.4). One of the most potent forces driving Th cell phenotype appears to be the synthesis of cytokines, in particular IL-12, by the dendritic cell at the time of priming. This has been referred to as the 'third signal' during T cell activation (Kalinski *et al.* 1999). According to this model, the dendritic cell is the key element in the polarisation of T cell phenotypes, with the nature of the polarising signal varying according to the features of the microenvironment in which the dendritic cell undergoes its final stages of differentiation. For example, dendritic cells cultured in microenvironments rich in IFNγ form a Th1-inducing dendritic cell subset (DC-1), whereas those differentiated under prostaglandin-rich conditions lead to cells with Th2-polarising capacity (DC-2). Factors such as IL-10 and TGFβ are thought to give rise to DC-3 dendritic cells. Therefore, according to this model, the profile of cytokines induced in a peripheral organ at the time of antigen introduction can 'inform' the immune system of the type of T helper cell immune response required, through controlling the final stages of dendritic cell maturation.

1.3.5 Cytokine deficient mice

a. GM-CSF deficient mice

The advent of molecular technology has provided a unique means by which to investigate the role of specific cytokines in physiology, in the form of cytokine knockout mice. The current list of cytokines and other biological mediators which have been depleted by null mutation is very large, and is still growing rapidly. Interestingly, there are very few cytokines whose depletion has proven to be fatal [$TGF\beta_1$ being one exception (Shull *et al.* 1994)] and for many cytokines the effects of knockout are minor, highlighting the redundant and pleiotrophic nature of the cytokine family.

GM-CSF is a cytokine of remarkable pleiotrophy, exhibiting diverse effects on immune and some non-immune cells (Ruef and Coleman 1990, Baldwin 1992). During haemopoiesis, it induces proliferation and differentiation of granulocyte, Mϕ and eosinophil precursor cells, and in the periphery it can induce proliferation and differentiation of these and many other cell lineages. GM-CSF is thought to have a particular role in mediating the phenotypic maturation of myeloid

APCs (eg Mϕs and dendritic cells) for initiation of antigen-specific immunity during inflammatory responses.

Mice genetically deficient in GM-CSF were generated by gene-targeting techniques in two separate laboratories in 1994 (Dranoff and Mulligan 1994, Stanley *et al.* 1994). Surprisingly, the mice showed no major perturbation in haemopoiesis, but instead exhibited an abnormality in their lung physiology featuring lymphocytic infiltration of the stromal tissue and accumulation of surfactant and granular material in the alveoli, with an accompanying accumulation of foam-like Mϕs containing phagocytosed lipids and surfactant material. These findings clearly indicate a central role for GM-CSF in the maintenance of lung homeostasis, but suggest that other cytokines such as IL-3 can compensate for the role of GM-CSF in steady state haemopoiesis. In the resting state, the knockout mice also appear to contain normal levels of both myeloid and lymphoid dendritic cells in the major lymphoid organs (Vremec *et al.* 1997).

Despite these initial observations, it has now become apparent that the response of GM-CSF-deficient mice to respond to some pathogenic and immune challenges is impaired. Thus, *Listeria* and *Streptococcal* infections are prolonged in GM-CSF-deficient mice due to a failure of emergency haemopoiesis (Zhan *et al.* 1998) and poor production of cytotoxic molecules due to inadequate activation of the Mϕs at peripheral sites (although ability to phagocytose was apparently normal) (LeVine *et al.* 1999, Zhan *et al.* 1999). Similarly, APCs in GM-CSF-deficient mice exhibit an impaired ability to generate Th1-polarised immune responses to type II collagen in a model of collagen-induced arthritis (Campbell *et al.* 1998) and to endotoxin after injection into the peripheral circulation (Basu *et al.* 1997). The underlying lesion leading to this impairment in Th1-inducing immunity appears to lie with dendritic cells (Basu *et al.* 1997, Wada *et al.* 1997, Noguchi *et al.* 1998).

More recently, it has also become apparent that GM-CSF-deficient mice exhibit abnormalities in reproductive performance (Robertson *et al.* 1999). Homozygous GM-CSF-deficient mating pairs generate fewer live-born pups, due to significant increases in the frequency of late resorptions and morphologically abnormal conceptuses. In live-born pups, fewer survive to weaning (largely due to a selective loss of male pups) leading to approximately 25% smaller litter sizes at weaning. Further, in viable implantation sites collected in the late stages of gestation, fetal weights are significantly reduced (a perturbation which persists to the age of 3 weeks, even to 7 weeks in male offspring) and placentae exhibit an altered structure suggestive of an impaired capacity for nutrient exchange. Interestingly, preliminary studies of mice deficient in the signal-transcribing β-subunit of the GM-CSF receptor suggest that similar deficiencies may also exist in these mice (L. Robb, unpublished).

b. CSF-1 deficient mice

Mice lacking CSF-1 (*csfm^{op}/csfm^{op}*) due to a naturally occurring null mutation in the CSF-1 gene (Yoshida *et al.* 1990) have been studied extensively. As reviewed (Stanley *et al.* 1997), the main phenotypic characteristic of these mice is severe osteoporosis due to a significant deficiency in the generation of osteoclasts, the cells of the mononuclear phagocytic lineage primarily responsible for bone remodeling. Other populations of Mϕs that are severely depleted in *csfm^{op}/csfm^{op}* mice include those of the peritoneal cavity, uterus, spleen marginal zone (the metallophils) and lymph node subcapsular sinus, whereas Mϕs in thymic cortex, the red pulp of the spleen, lymph node medulla, intestinal lamina propria, liver, lung and brain are relatively spared (Witmer Pack *et al.* 1993, Stanley *et al.* 1997).

Apart from their skeletal abnormalities, *csfm^{op}/csfm^{op}* male and particularly female mice demonstrate severely compromised fertility apparently due to perturbations in the neuronal pathways involved in mate selection or libido, disrupted ovarian and testicular function, as well as compromised blastocyst, placental and mammary gland development (Pollard 1997). Whereas in the placenta a direct role for CSF-1 is implicated for normal function (Regenstreif and Rossant 1989), in many of the other sites CSF-1 may be vital through maintaining stromal Mϕ populations (Pollard 1997).

1.4 Immunoregulation in mucosal tissues

The roles of leukocytes in mucosal tissues such as the lung, the gastrointestinal tract, the eye and the genitourinary system are complex in that these tissues must be able to maintain homeostasis without generating immune responses to innocuous and commensal antigens, but still maintain the capacity to generate rapid antigen-specific immunity when pathogens invade. The mechanism by which this is achieved centres largely on Mϕs and dendritic cells, since these are the cells which initiate and direct antigen-specific immune responses, and determine the phenotype of the lymphocytes which are subsequently recruited to a site of secondary antigen exposure. The following discussion will focus on the roles of Mϕs and dendritic cells in immune regulation within steady state and inflamed mucosal organs, using the lung, gastrointestinal tract and anterior chamber of the eye as examples.

1.4.1 Immunoregulatory actions of mucosal Mϕs

Mϕs are abundant in mucosal organs. Of all the mucosal Mϕ populations, those located in the lumen of pulmonary tissues (the airways and alveolar Mϕs), are the best characterised due

to their ease of isolation (Stumbles *et al.* 1999), and are found to exhibit a characteristic surface phenotype reminiscent of Mϕs contained within lymphoid organs; that is, they express little or no F4/80 antigen, no Mac-1, but high levels of DEC-205 and sialoadhesin (Bilyk and Holt 1991). Abundant Mϕs expressing a range of cell surface markers are also found in the lamina propria of the small and the large intestine (Pavli *et al.* 1990, Pavli *et al.* 1996), and in the connective tissue surrounding the eye (Steptoe *et al.* 1995).

The immunoaccessory phenotype of Mϕs derived from steady-state mucosal organs has been found to be markedly different to that of Mϕs derived from other body sites such as the peritoneal cavity. Specifically, Mϕs from lung alveoli (Holt 1979, Holt 1980, Bilyk and Holt 1993), gastrointestinal tract (Pavli *et al.* 1990) and anterior chamber of the eye (Steptoe *et al.* 1995) function very poorly as stimulator cells in mitogen- and allo-antigen-induced proliferation assays due to their constitutive synthesis of nitric oxide (NO) (Holt *et al.* 1993, Strickland *et al.* 1996), prostaglandins (Pavli *et al.* 1990) or inhibitory cytokines (Huaux *et al.* 1998). These molecules are thought to have a number of important actions *in vivo*, including the inhibition of local $CD4^+$ T cell populations involved in the generation of T-memory cell-dependent secondary immune responses to commonly encountered antigens (Holt 1986, Strickland *et al.* 1993). Secondly, NO and other inhibitory molecules also appear to have the effect of maintaining a relatively 'immature' phenotype in local mucosal dendritic cells populations (Holt *et al.* 1988).

1.4.2 Mucosal dendritic cells and immune surveillance

Like Mϕs, dendritic cells also form a significant cellular component of all mucosal organs studied to date. In the lung (Holt *et al.* 1988, Holt *et al.* 1994, McWilliam *et al.* 1994), gastrointestinal tract (Pavli *et al.* 1996) and anterior chamber if the eye (Steptoe *et al.* 1995), dendritic cells can be detected as an intricate network distributed throughout the lining epithelium. A proportion of the cells also express other cell membrane markers characteristic of their lineage, including CD11c/CD18 (LFA-1), Mac-1, intracellular adhesion molecules (Stumbles *et al.* 1998), DEC-205 (Witmer Pack *et al.* 1995) and CD68 [an endosomal glycoprotein closely related to macrosialin (Pavli *et al.* 1996)]. As mentioned, the phenotype of mucosal dendritic cells in the steady state appears to be tightly regulated by the local Mϕ population. Thus dendritic cells freshly isolated from lung or anterior chamber of the eye in the steady state show low to moderate APC activity (Holt *et al.* 1993), express relatively little surface MHC class II and tend to synthesise inhibitory cytokines such as IL-10 (Stumbles *et al.* 1998).

Despite their apparent immaturity in terms of costimulatory function and pro-inflammatory cytokine production, there is compelling evidence that the resident populations of dendritic cells in the non-inflamed lung and gastrointestinal tract are turned over into the draining lymph quite rapidly, with a half life of the order of 2 days (Mayrhofer *et al.* 1986, Holt *et al.* 1994). This is in contrast to other interstitial dendritic cell populations, whose half life can be as long as 15 days (Holt *et al.* 1994). It also appears that cells trafficking from mucosal organs are not 'empty' but rather contain antigens acquired within the mucosal tissue by virtue of their highly endocytic nature (Mayrhofer *et al.* 1986, Stumbles *et al.* 1998). This phenomenon of continual delivery of antigen-loaded immature dendritic cells to the draining lymph nodes is believed to be one of the unique features of immunoregulation in mucosal organs. Studies in the lung suggest that in the steady-state, trafficking and IL-10-synthesising dendritic cells preferentially stimulate Th2-polarised as opposed to Th-1 polarised immune responses (Stumbles *et al.* 1998). This would presumably favour a functional tolerance of self or ubiquitous antigens.

1.4.3 Discriminating 'danger' from 'non-danger'

Clearly mucosal organs need to maintain some capacity to mount protective, Th-1 deviated immune responses should the need arise. Studies in the rodent lung suggest that the opportunity to generate such responses is provided by a 'window' (Bilyk and Holt 1993, Stumbles *et al.* 1999) arising as a consequence of events initiated by pro-inflammatory cytokine and chemokine synthesis in response to reception of 'danger signals' by the stromal cells (Lo *et al.* 1998).

A role for proinflammatory cytokines in inducing a shift in the phenotype of Mϕs has already been discussed (see section 1.3.4b), and that similar mechanisms operate in mucosal organs is suggested by *in vitro* studies showing that culture of alveolar Mϕs in cytokines such as GM-CSF and TNFα induces a transient abrogation of their inhibitory phenotype by downregulating NO production (Bilyk and Holt 1993, Holt *et al.* 1993, Bilyk and Holt 1995). A similar role for local inflammatory cytokines in inducing a phenotypic shift in resident Mϕs has been postulated to occur in the anterior chamber of the eye (Steptoe *et al.* 1995). Furthermore, it appears that the monocytes recruited to the lung in response to an inflammatory stimulus do not display the immunosuppressive phenotype of resident cells (which presumably develops slowly in response to steady state cytokine signals) (Bilyk and Holt 1995). In combination, these inflammation-induced changes in the populations of Mϕs would release the inhibition of local leukocytes, a postulate supported by evidence of relatively activated phenotypes in T cells (Strickland *et al.* 1993) and dendritic cells (Holt *et al.* 1993) collected from the lungs of rodents

following alveolar Mϕs depletion by aerosol administration of cytotoxic dichloromethylene diphosphonate-containing liposomes.

As well as being phenotypically 'released' by phenotypic changes in mucosal Mϕs during inflammation, a direct role for proinflammatory cytokines in inducing maturation of mucosal dendritic cells is also indicated. Thus, dendritic cells harvested from steady state lung (Holt and Thomas 1997, Stumbles *et al.* 1998, Holt *et al.* 1999), intestine (MacPherson 1989) and anterior chamber of the eye (Steptoe *et al.* 1995) can be phenotypically 'matured' by culture in GM-CSF (+/- TNFα or CD40L), and show all of the characteristic changes in phenotype typical of GM-CSF-exposed dendritic cells from other sites (Heufler *et al.* 1988, Cella *et al.* 1997). Importantly, as well as exhibiting changes in their cell membrane phenotype and antigen uptake and processing behaviours, the dendritic cells become primed to release enhanced levels of IL-12, and when transferred to naïve rats, these DC-1-like dendritic cells can stimulate the production of Th1-polarised immune responses (Stumbles *et al.* 1998). The rapid recruitment of new dendritic cells into inflamed tissues (McWilliam *et al.* 1994) would also provide an additional means for the generation of antigen-specific immunity, since these cells would undergo their final stages of differentiation in the context of inflammatory cytokines and thus would be more likely to generate Th1-deviated immunity (Kalinski *et al.* 1999).

1.4.4 Immune deviation

The inverse relationship between the cellular (Th1) and humoral (Th2) arms of immunity and the factors influencing the two outcomes has been referred to as 'immune deviation' (Streilein 1993). The discussion above (see section 1.3.4c) has suggested that one of the features of antigen presentation which can 'deviate' the default Th2 immune responses at mucosal surfaces to Th-1 polarity is the delivery of specific cytokine signals to T cells at the time of their activation. Other determinants of immune deviation are briefly summarised below, and a more detailed review of these can be found elsewhere (Constant and Bottomly 1997).

The evidence that differences in costimulation through B7 molecules and their ligands might also provide an alternate mechanism for skewing immunity is accumulating. *In vitro* and *in vivo* experiments in which the delivery of costimulation to T cells by APCs is blocked by mAbs suggest that the generation of Th2-polarised immunity relies on the interaction between B7-2 and CD28, whereas B7-1 interacting with CD28 tends to produce Th1 T cells. In contrast, ligation of CTLA4 on T cells by either of the B7 molecules tends not to polarise them but instead downregulates all T cell activation.

The concentration of antigen in an environment can also be a determinate in immune deviation. There are no clear cut conclusions regarding whether 'high' or 'low' doses of antigen are best suited to induce each type of immune response, but in general it appears that high doses of complex bacterial or parasitic antigens induce Th2 responses and high doses of peptide antigens induce Th1 cells, and vice versa. That this is in some part due to differences in antigen processing is highly likely, since the ability of different APCs to take up and process antigens varies according to the initial form of the antigen and the extent of its glycosylation. For example, maleyation of antigens targets them for uptake by scavenger receptors and enhances their immunogenicity (Abraham *et al.* 1995). Immune deviation may also result from the capacity of different antigens to induce different cytokines in the APC (D'Orazio and Niederkorn 1998).

Finally, the differential induction of Th1 and Th2 can also be influenced by the binding affinity of a particular peptide antigen for the TCR (Constant and Bottomly 1997). Thus it appears that immune deviation can be induced by differences in each one of the 1st, 2nd and 3rd signals delivered to T cells during their activation. *In vivo*, differences in each of these three signals in each individual T lymphocyte activation events probably contribute to the outcome of an immune response.

1.4.5 Tolerance

During fetal life, T cells expressing TCRs reactive with self proteins are eliminated via clonal deletion in the thymus, ensuring immunological tolerance specific to most 'self' antigens (reviewed in Mondino *et al.* 1996). In the periphery, mechanisms for tolerance induction also exist, and these have been proposed to provide a means for protection against self-reactive T cells which escape from the thymus. The processes by which peripheral tolerance is maintained may be diverse, and have been speculated to include clonal deletion, clonal unresponsiveness or anergy, downregulation of TCR or co-receptors on peripheral T cells or polarisation of T cell responses to induce T cells with a suppressive phenotype (Miller and Morahan 1992, Bendelac *et al.* 1997, Weiner 1997, Groux and Powrie 1999). Peripheral tolerance can also be induced to non-self antigens, indeed, in the gastrointestinal tract, the immune response to innocuous antigens encountered in food is tolerance (Brandtzaeg 1996).

Peripheral tolerance is initiated during the APC : T lymphocyte interaction. For example, deletion of T cells can be induced by the delivery of a cell death signal such as Fas-L at the time of activation (Nagata and Golstein 1995) or by a failure of appropriate costimulatory signal delivery (Brandtzaeg 1996). Therefore, the initiation of tolerance has been shown to be

remarkably sensitive to the presence of specific cytokine signals which may control the level of expression of molecules such as Fas L or costimulatory molecules such as B7. For example, the potent Th1-deviating cytokine IL-12 can prevent the induction of mucosal tolerance when delivered at the site of sensitisation (Claessen *et al.* 1996), presumably by activation of local APC populations. Conversely, environments at which tolerance induction is routine, such as the anterior chamber of the eye (Wilbanks and Streilein 1992) and the gastrointestinal tract (Brandtzaeg 1996), are rich sources of cytokines such as TGFβ and IL-10, which are believed to downregulate the accessory function of local APC populations. Such regulatory cytokines may be synthesised by stromal cells, or by local populations of activated Th3 or Tr1 cells which mediate antigen non-specific bystander suppression (Weiner 1997, Groux and Powrie 1999). The non-classical MHC molecule CD1 may be involved in tolerance induction through activation of local populations of IL-4- or IL-10-secreting NKT cells (Porcelli and Modlin 1999). Another mechanism by which tolerance is believed to be induced involves a phenomenon known as cross-presentation, in which antigens derived from the extracellular environment are processed in the MHC class I pathway (Harding and Song 1994) and thus fail to elicit T helper cell immunity (Brandtzaeg 1996).

1.5 Uterine leukocyte populations

Similar to the mucosal organs already considered in this discussion, the cycling and pregnant murine uterus contains many diverse leukocyte populations, including Mϕs, lymphocytes, granulocytes and a lineage of putative dendritic cells. The features of these populations relevant to the maintenance of homeostasis and the induction of immune responses in the uterus are presented below.

1.5.1 Mϕs

Mϕs form an abundant but heterogeneous leukocyte population within the murine uterus (Hunt *et al.* 1985, Hunt and Pollard 1992). In the virgin uterus, abundant F4/80$^+$ Mϕs are evident throughout the myometrium, the mesometrial triangle and the endometrium (De and Wood 1990, Stewart and Mitchell 1991, Pollard *et al.* 1998). The number and distribution of Mϕs within the endometrium and their morphology is acutely sensitive to the stage of the estrous cycle, with proestrus and estrus promoting the accumulation of large cells in the superficial endometrium immediately subjacent to luminal and glandular epithelial cells. Estimates made by cell counting in tissue sections or after enzyme-induced release from whole uteri show that Mϕs comprise

approximately 10-18% of the total stromal cells at estrus (De and Wood 1990, De and Wood 1991, Pollard *et al.* 1998). In contrast, at diestrus the cells seem less abundant (comprising only 5-7% of total cells in sections or released from digested uteri) and exhibit a more rounded appearance (De and Wood 1990, Pollard *et al.* 1998).

The steroid-hormone dependency of uterine Mϕs is further illustrated by their rapid loss following ovariectomy, such that 6 days following surgery they comprise only 2% of cells enzymatically released from uteri (De and Wood 1990). Furthermore, the Mϕs can be restored by administration of physiological doses of ovarian steroid hormones, with each of progesterone or estrogen rapidly (within 24 h) inducing a sub-epithelial accumulation of Mϕs. Both hormones administered together have an additive effect, returning the number of endometrial Mϕs to even greater numbers than seen during the cycle (De and Wood 1990). The administration of cytokines CSF-1 (Wood *et al.* 1992) and GM-CSF (Robertson *et al.* 2000) directly to the uterine lumen in ovariectomised mice can also induce recruitment of uterine Mϕs suggesting that the effect of ovarian steroid hormones on uterine Mϕ numbers may be mediated at least in part indirectly through steroid-hormone regulation of uterine epithelial cell cytokine synthesis (see section 1.6.3).

Following insemination, the number of Mϕs within the endometrium increases markedly such that over twice as many F4/80$^+$ cells are released from digests of uteri at day 1 of pregnancy than at estrus (De and Wood 1991, Wood *et al.* 1997). The number of F4/80$^+$ Mϕs within endometrial sections (Brandon 1993) and released from enzymatically digested uterine tissues remains high to the second day of pregnancy, and then a decline in numbers occurs such that on days 3 and 4 of pregnancy, F4/80$^+$ cells comprise < 20% of released cells. Mϕs then increase in number in certain locations within the reproductive tract (see below), comprising 20-30% of cells released from digested uteri on days 5-17 of pregnancy (Hunt *et al.* 1985, De and Wood 1991, Wood *et al.* 1997).

The distribution of uterine Mϕs also fluxes according to day of pregnancy. At days 1 and 2, the F4/80$^+$ cells can be seen concentrated in the superficial endometrium adjacent to but not within the luminal epithelium (De and Wood 1991, Brandon 1993). By the third day of pregnancy, the Mϕs are distributed more evenly throughout the endometrium and myometrium, before again preferentially associating with luminal and glandular epithelial cells on day 4, a pattern which continues through day 5 (De and Wood 1991, Brandon 1995). As the primary decidua begins to form at around the sixth day of pregnancy, F4/80$^+$ Mϕs appear to be excluded from the peri-implantation zone (De and Wood 1991, Brandon 1993, Brandon 1995). For the remainder of pregnancy the cells are largely concentrated in the deep endometrium, the

myometrium, the mesometrial triangle and the inter-implantation sites of the uterus (De and Wood 1991), where they appear to fulfill largely scavenger and tissue remodeling roles (Stewart and Mitchell 1992, Brandon 1995, Kyaw *et al.* 1998). Mϕs also persist in the post-partum wound from 1-90 days after parturition (Brandon 1994).

1.5.2 Dendritic cells

That a lineage of dendritic cells exists in the rodent uterus was first suggested by Head and colleagues, who found that dendriform cells which have potent antigen-presenting capacity but lack non-specific esterase can be harvested from rat uteri. In tissue sections the MHC class II^+ putative dendritic cells were found throughout the endometrium, particularly in association with glands, and their accumulation could be replicated in ovariectomised mice by estrogen supplementation (Head and Gaede 1986, Head *et al.* 1987). A lineage of $F4/80^-Ia^+$ putative dendritic cells has also been identified in murine uterus (Robertson *et al.* 1998) and these cells appear to have potent immunostimulatory activity in T cell proliferation assays (S. Robertson, unpublished). A population of Langerhans-like cells has been identified in murine vaginal and cervical epithelium (Hume *et al.* 1984c, Parr *et al.* 1991).

1.5.3 Lymphocytes

$CD3^+$ T cells, most of which also express CD8, first appear in the murine uterus at the age of 2-3 weeks, and remain a consistent but small and heterogeneous population of cells distributed throughout the endometrium regardless of exposure to microbial flora. Approximately 50% have αβ TCR [these are probably largely memory cells (Ibraghimov *et al.* 1995)] and the remainder express γδ TCR (Croy *et al.* 1993). The γδ T cells are very homogeneous (Itohara *et al.* 1990), and during pregnancy this population appears to expand within the uterine compartment (total numbers can increase approximately 100-fold). Approximately one third of these lymphocytes become activated (Heyborne *et al.* 1992) and have been shown to secrete a novel TGFβ2-related suppressor factor (Clark *et al.* 1997). Other populations of suppressor lymphocytes also exist within the pregnant murine uterus (Thomas and Erickson 1986, Brierly and Clark 1987), and these may yet prove to be related to bystander suppressive Th3, Tr1 or NKT cell lineages (Robertson 2000).

B cells are also present in the uteri of mice, where their number and secretion of immunoglobulin is believed to be influenced by ovarian steroid hormones and pregnancy (Rachman *et al.* 1983, Parr and Parr 1985, Parr and Parr 1986).

As well as forming a significant cellular component of the cycling mouse uterus (Chen *et al.* 1994), uterine natural killer (uNK) cells become a prominent cell population in the decidua basalis of the rodent uterus during early pregnancy, and during this time adopt a heavily granulated phenotype. If harvested from pregnant uteri, uNK exhibit the capacity to lyse various cellular targets, including trophoblast cells, suggesting a relatively activated phenotype *in vivo* which may act to limit trophoblast invasion (Liu *et al.* 1994). Furthermore, the cells are a potent source of many cytokines, reactive oxygen intermediates and bioactive lipids which could regulate the function of other cells within the pregnant uterus (Chen *et al.* 1994, Hunt 1994, Hunt *et al.* 1997), and a key role in regulating placental development appears likely (Croy *et al.* 1996-97).

1.5.4 Granulocytes

Neutrophils and eosinophils comprise approximately 10% and 4% respectively of endometrial cells counted in sections of uteri at estrus (Robertson *et al.* 1996a), whereas after mating their numbers increase dramatically (De and Wood 1991, Robertson *et al.* 1996a, Wood *et al.* 1997). In tissue sections of inseminated uteri, both lineages of cells are distributed densely in the superficial endometrium, and neutrophils can also be observed trafficking through luminal epithelium into the uterine lumen where they phagocytose sperm and other seminal debris (Austin 1960, Ball and Mitchinson 1977, Robertson *et al.* 1996a). The number of granulocytes released from digested pregnant uteri decreases rapidly after day 1, and the cells comprise < 5% of total uterine cells from days 3-17 of pregnancy (De and Wood 1991, Wood *et al.* 1997).

Mast cells are also found in murine uteri, where their secretion of mediators such as NO and histamine may play a role in uterine cyclicity and preparation for pregnancy (Huang *et al.* 1995, Hunt *et al.* 1997b).

1.6 Cytokines in the cycling and peri-implantation uterus

The synthesis of cytokines by uterine tissues was first suggested by studies showing that extracts from pregnant mouse uterus, placenta and embryo have the capability to stimulate growth of granulocyte-Mϕ colony forming cells *in vitro* (Bull and Rosendaal 1978, Hodgson and Bradley 1979). It is now realised that a diverse array of cytokines is synthesised by pregnant and non-pregnant uterine tissues, and that these molecules form an important component of the dynamic network of intercellular communication in this organ (reviewed in Robertson *et al.* 1994). Many cytokines, in particular leukaemia inhibitory factor (LIF) (Stewart 1994), CSF-1

(Bartocci *et al.* 1986, Pollard *et al.* 1987, Arceci *et al.* 1989) and GM-CSF (Robertson *et al.* 1999, Sjöblom *et al.* 1999), have been proposed to specifically target the cells of the blastocyst and placental trophoblast to maintain their viability and promote implantation and differentiation during pregnancy. However, for the purposes of the current studies, the discussion below will focus on CSF-1, GM-CSF and other cytokines synthesised by uterine stromal cells which are believed to be of primary relevance to the function of myeloid APCs during the estrous cycle and pre- and peri-implantation period of pregnancy. The cytokines synthesised by uterine Mϕs and other leukocytes during these times are also discussed.

1.6.1 Cytokines synthesised by epithelial and stromal cells

a. CSF-1

CSF-1 is synthesised by uterine and glandular epithelial cells during the estrous cycle and in pregnancy (Pollard *et al.* 1987, Arceci *et al.* 1989, Sanford *et al.* 1992, Wood *et al.* 1992). During the estrous cycle, CSF-1 synthesis seems to be closely regulated by ovarian steroid hormones, since although both isoforms of CSF-1 mRNA levels can be detected at all stages of the cycle, bioactive CSF-1 can only be detected at proestrus, and following ovariectomy, both CSF-1 mRNA and protein are depleted (Wood *et al.* 1992). Uterine CSF-1 concentrations can be restored in ovariectomised mice by administration of either estrogen or progesterone, which when administered together have a synergistic effect (Pollard *et al.* 1987, Wood *et al.* 1992).

Following mating, mRNA and bioactive CSF-1 are transiently up-regulated, and then decline before a further surge at the time of implantation on day 4 (Sanford *et al.* 1992, De *et al.* 1993a). Later in pregnancy, the levels of bioactive CSF-1 rise to attain levels 1000-fold higher than are observed in the non-pregnant state (Bartocci *et al.* 1986, De *et al.* 1993a). Although luminal and glandular epithelial cells also appear to be the main source of uterine CSF-1 during pregnancy (Pollard *et al.* 1987, Arceci *et al.* 1989), other sources may include decidualised stromal cells (Hatayama *et al.* 1994) and lymphocytes (Parker and Metcalf 1974, Bulmer 1994).

b. GM-CSF

Uterine luminal and glandular epithelial cells are a potent source of GM-CSF during the estrous cycle and in pregnancy (Robertson *et al.* 1992). During the estrous cycle, expression of GM-CSF is thought to be regulated primarily by estrogen, since expression of bioactive protein and mRNA by epithelial cells is highest at estrus and can be induced in ovariectomised mice by

administration of estrogen. In contrast, progesterone has a moderate inhibitory effect on GM-CSF expression by uterine epithelium (Robertson *et al.* 1996b).

GM-CSF synthesis by uterine epithelium is markedly upregulated following insemination, a phenomenon which can be detected by analysis of mRNA and protein at day 1 of pregnancy (Robertson and Seamark 1990, Robertson *et al.* 1992, Robertson and Seamark 1992, Sanford *et al.* 1992, Robertson *et al.* 1996, Tremellen *et al.* 1998). The elevated synthesis at this time is thought to be primarily induced by seminal vesicle-derived $TGF\beta_1$ present in the ejaculate, since the response can be abrogated *in vivo* by mating females with seminal vesicle-deficient males (Robertson and Seamark 1990, Robertson *et al.* 1996) or *in vitro* by administration of α-$TGF\beta_1$ antibodies to seminal-plasma treated epithelial cells (Tremellen *et al.* 1998). Furthermore, the elevated synthesis of uterine GM-CSF which occurs at day 1 can be replicated by instillation of recombinant $TGF\beta_1$ into the uterine lumen of estrous mice (Tremellen *et al.* 1998). By the third and fourth days of pregnancy, uterine epithelial cell GM-CSF synthesis declines rapidly, a response which is thought to result from the rising levels of circulating progesterone (Robertson and Seamark 1990, Robertson *et al.* 1996a, Robertson *et al.* 1996b). Epithelial cells harvested from mid-gestation uteri also secrete GM-CSF (Robertson *et al.* 1992), as do decidual T cells (Athanassakis 1993) and NK cells (Jokhi *et al.* 1994b).

c. TNFα, IFNγ and IL-6

Expression of TNFα and IFNγ by epithelial and other cells in the murine uterus have been thoroughly examined by Hunt and colleagues (reviewed in Hunt 1993, Hunt *et al.* 1996). Expression of both cytokines within the uterus has been localised to luminal and glandular epithelial cells (Yelavarthi *et al.* 1991, Hunt *et al.* 1993, Platt and Hunt 1998). TNFα expression is thought to be regulated by ovarian steroid hormones, since levels of immunoreactive and bioactive TNFα in uterine homogenates peak at the proestrus and estrus stages of the cycle, and are diminished following ovariectomy but can be reinstated at both the mRNA and protein level by estrogen and/or progesterone replacement (De *et al.* 1992). Uterine epithelial cell synthesis of IFNγ is also believed to be regulated by ovarian steroid hormones (Platt and Hunt 1998).

IL-6 is synthesised by uterine epithelial and stromal cells during the estrous cycle (Robertson *et al.* 1992), and again a role for ovarian steroids in regulation of its expression seems likely given that mRNA levels in uterine homogenates and bioactivity in uterine fluids peak during proestrus and estrus, and after administration of both estrogen and progesterone to ovariectomised mice (De *et al.* 1992, Jacobs *et al.* 1992, Sanford *et al.* 1992).

Following mating, mRNA transcripts for TNFα and IL-6 are transiently upregulated (Sanford *et al.* 1992), and a 250-fold elevation of IL-6 activity in uterine luminal fluid can also be detected at this time (Robertson *et al.* 1992). Whereas IL-6 mRNA and bioactivity decrease slightly at day 2 of pregnancy, and then attain relatively high levels on day 3 and beyond (De *et al.* 1993b), TNFα mRNA and bioactivity declines by day 3 (Sanford *et al.* 1992). Other cytokines produced by epithelial and stromal cells within the cycling and pre-implantation uterus include LIF, TGFβ, IL-5, TGFα and type I interferons (Robertson *et al.* 1994).

d. chemokines

As well as the cytokines already discussed, the expression of a number of chemokines of relevance to Mϕs and dendritic cells has been examined in the murine uterus. mRNAs for the CC chemokines RANTES, MIP-1α, MIP-1β and MCP-1 have all been detected in homogenates of whole estrous uteri, and most appear to be upregulated after mating suggesting a role in recruitment of the elevated numbers of myeloid APCs noted at this time (Wood *et al.* 1997, Pollard *et al.* 1998, Robertson *et al.* 1998). On days 2, 3 and 4 of pregnancy, levels of RANTES and MIP-1α remain elevated, whereas those of MIP-1β and MCP-1 decline rapidly (Robertson *et al.* 1998).

1.6.2. Cytokines synthesised by Mϕs and other leukocytes

As well as epithelial and stromal cells, Mϕs themselves appear to be a potent source of cytokines in the uterus. During the estrous cycle, mRNA for IFNγ (Platt and Hunt 1998) and IL-1 (Takacs *et al.* 1988), and mRNA and protein for TNFα (Yelavarthi *et al.* 1991, Hunt *et al.* 1997a) have all been localised to Mϕs within the endometrium. An inhibitory role for progesterone in regulating uterine Mϕ expression of TNFα appears likely (Miller and Hunt 1998). mRNAs for IL-1α, IL-1β and TNFα are all elevated in uterine Mϕs and other leukocytes comprising the inflammatory infiltrate following mating, but these abate by day 4 of pregnancy (McMaster *et al.* 1992). Bioactive IL-6 is also secreted by Mϕs isolated from mated uteri (Robertson *et al.* 1992).

In addition to Mϕs, NK cells appear to be particularly active in terms of expression of IFNγ and TNFα in the cycling and particularly in the pregnant uterus (Hume *et al.* 1984, Hunt *et al.* 1996, Hunt *et al.* 1997), and uterine mast cells may similarly be involved in cytokine secretion (Hunt *et al.* 1997a).

1.6.3. Uterine Mϕs and dendritic cells as cytokine targets

In several different laboratories, the role of specific cytokines in regulating the number and phenotype of uterine Mϕs has been investigated by analysis of the numbers and morphology of F4/80$^+$ cells in (1) the uteri of cytokine-deficient mice and (2) the uteri of ovariectomised mice which had been administered recombinant cytokines directly into the luminal cavity *in vivo*. Also, the expression of receptors for specific cytokines on uterine Mϕs has been examined. However, investigation of the role of specific cytokines in regulation of uterine dendritic cells has proven more difficult due to the lack of a specific marker for these cells, and thus putative uterine dendritic cells have been identified on the basis of their size, morphology and their membrane phenotype (Robertson *et al.* 2000).

There is compelling evidence that uterine epithelial cell-derived CSF-1 has a direct role in regulating uterine Mϕ numbers and phenotype during the estrous cycle. Congenitally CSF-1 deficient c*sfm*^{op}/*csfm*^{op} mice exhibit very few F4/80$^+$ uterine Mϕs (Pollard *et al.* 1991), and those that are present are rounded and fail to exhibit the morphology typical of uterine Mϕs in cycling wild-type mice (Pollard *et al.* 1998). Although this deficit may be in part due to systemic factors, a direct role for locally synthesised CSF-1 in maintaining endometrial Mϕ numbers is suggested by two lines of evidence. Firstly, administration of recombinant CSF-1 directly into the lumen of ovariectomised mice induces an accumulation of endometrial Mϕs comparable to that observed during the estrous cycle (Wood *et al.* 1992). Secondly, systemic administration of CSF-1 to c*sfm*^{op}/*csfm*^{op} mice from birth does not affect uterine endometrial density (Pollard *et al.* 1998). Interestingly however, this treatment does restore a population of Mϕs to the mesometrial triangle, indicating a degree of heterogeneity between the cytokine responsiveness of these two populations of uterine Mϕs. Also, during conditions of peak uterine epithelial cell synthesis of CSF-1 (at proestrus and estrus, or after administration of estrogen and progesterone to ovariectomised mice), large endometrial Mϕs accumulate in the superficial endometrium in close proximity to epithelial cells (Wood *et al.* 1992, De *et al.* 1993a, Pollard *et al.* 1998). Interestingly, the number of F4/80$^+$ Mϕs recruited into the uterus of c*sfm*^{op}/*csfm*^{op} mice after mating is similar to the number observed in mated wild-type mice; presumably other cytokines such as GM-CSF (Robertson *et al.* 2000) or chemokines (Pollard *et al.* 1998) can compensate at this time. Indeed, direct application of the CC chemokine RANTES to the lumen of ovariectomised mice recruits large numbers of Mϕs with an activated appearance to the endometrium (Wood *et al.* 1997).

Instillation of recombinant GM-CSF into the uterine lumen of ovariectomised mice also triggers significant recruitment of F4/80$^+$ Mϕs into the endometrium, and a similar effect is seen

on Mac-1$^+$ and Ia$^+$ cells (which would include Mϕs but also neutrophils and putative dendritic cells respectively) (Robertson *et al.* 2000). However, it would appear that cytokines and chemokines other than GM-CSF can compensate for its potential chemotactic and activating effects on Mϕs during the estrous cycle, since the number of endometrial F4/80$^+$ Mϕs is not depleted in the uteri of GM-CSF-deficient mice (Robertson *et al.* 1998, Robertson *et al.* 1999). Instead, an obligatory role for GM-CSF in recruiting or activating uterine Mϕs and/or dendritic cells during the inflammatory response to insemination is more likely, since the uteri of GM-CSF-deficient mice at day 1 of pregnancy contain slightly fewer Ia$^+$ endometrial cells than wild-type animals (Robertson *et al.* 1998). Furthermore, dual colour flow cytometric analyses of cells released by enzymatic digestion of day 1 pregnant uteri has shown that F4/80$^+$, Ia$^+$ and macrosialin$^+$ cells (Mϕs +/- putative dendritic cells) do indeed express both α- and β-subunits of the GM-CSF receptor at this time (Robertson *et al.* 2000).

IFNγ and TNFα are also implicated in regulating uterine Mϕs. The mRNA for IFNγ receptor has been localised to uterine Mϕs in the cycling mouse uterus, and Northern blot analysis of total mRNA obtained from uterine tissue homogenates suggests this may be hormonally regulated (Chen *et al.* 1994). Similarly, TNFα receptors are expressed in cycling and pregnant murine uteri, although the cellular source has not been identified (Hunt *et al.* 1996).

1.7 Immunological features of mating, implantation and pregnancy

As already highlighted, the uterus is similar to other mucosal organs in that it contains abundant and diverse populations of Mϕs, putative dendritic cells and other leukocytes of relevance to the initiation and generation of innate and antigen-specific immunity. Following the exposure of the uterus to seminal plasma at the time of mating, the numbers of Mϕs and other myeloid cells present within the endometrium increases markedly in response to upregulated concentrations of cytokines and chemokines, and the cells show evidence of immune activation, expressing Ia antigens and synthesising a number of pro-inflammatory cytokines. This has been referred to as the 'post-mating inflammatory response', and is only transient since the numbers of inflammatory cells and the local synthesis of pro-inflammatory cytokines begins to decline by day 2 of pregnancy. However, APCs do remain in the uterine stroma throughout the peri-implantation period.

The roles of uterine APCs in the mated uterus and for the duration of an ensuing pregnancy have not been established, yet based on studies in other mucosal organs could be expected to include the generation of immune responses to antigens present within the uterine

milieu at these times. The following discussion focuses on relevant features of the ejaculate and the conceptus, as well as pathogens which may elicit immune responses in the uterus, and a summary of some of the mechanisms believed to contribute to prevention of maternal immune rejection of the semi-allogeneic conceptus is also provided.

1.7.1 Features of the ejaculate

a. paternal antigens in the ejaculate

Sperm and seminal fluid contain many antigens of paternal origin, including MHC antigens and minor histocompatability antigens such as H-Y, blood group antigens, trophoblast-lymphocyte cross-reactive antigen (CD56) and sperm specific autoantigens, as well as leukocytes (Clarke 1984, Alexander and Anderson 1987, Kajino *et al.* 1988, Naz and Mehta 1989). It would appear that the antigens on sperm are truly immunogenic, since a single immunisation of washed spermatozoa into the footpad of mice consistently induces cell mediated immunity specific for the sperm alloantigens (Rutherford and Searle 1984). Furthermore, washed allogeneic epididymal sperm inoculated directly into the uterine lumen of rats induces local lymph node hypertrophy and transplantation immunity (Beer and Billingham 1974) and purified sperm antigens sensitise spleen cells for antigen-specific immunity *in vitro* (Naz and Mehta 1989). In addition, there is evidence for uptake of seminal antigens by uterine cells, since neutrophils in the lumen of inseminated uteri can be observed actively phagocytosing whole sperm (Austin 1960, Ball and Mitchinson 1977, Robertson *et al.* 1996a), and *in vitro* assays suggest a similar function for uterine Mϕs (Peluso *et al.* 1994). A role for uterine epithelial cells in mediating uptake of uterine antigens is suggested by findings that seminal plasma proteins can be detected in uterine epithelial cells after insemination in rats (Carballada and Esponda 1997) and in mice, HRP administered into the uterine lumen on days 1-5 of pregnancy accumulates in endocytic vesicles of luminal and glandular epithelial cells (Tung *et al.* 1988). Furthermore, 24-48 h after artificial insemination of mice with ^{3}H-thymidine-labeled spermatozoa, radioactivity is detectable in the endometrium and in lymph nodes which drain the uterus (Watson *et al.* 1983) and sperm-derived nucleic acids are detectable in the cytoplasm and nucleus of epithelial cells of the reproductive tract after artificial insemination in rats, and in phagocytes in the endometrium and the draining lymph nodes (Reid 1965, Reid 1966). In bats, whole sperm heads can be visualised embedded in uterine epithelial cells following natural mating (Racey et al, 1987).

b. cytokines in seminal plasma

As well as sperm and other cells derived from the male reproductive tract, semen contains an array of proteins, enzymes and prostaglandins (Alexander and Anderson 1987). It is now recognised that semen also contains a number of cytokines, including IL-6, IFNγ and TGFβ (Naz and Kaplan 1994, Nocera and Chu 1995). $TGF\beta_1$ of seminal vesicle origin is found at particularly high concentrations in murine semen (Robertson *et al.* 1996, Tremellen *et al.* 1998), where it might at least partially be responsible for regulating male and female immune responses to sperm antigens (Alexander and Anderson 1987, Nocera and Chu 1993).

1.7.2 Pathogens introduced at mating

The cycling uterus is believed to be a sterile environment, with minimal evidence of fungal or bacterial colonisation during the estrous cycle (Robertson *et al.* 1999), although the clearance of pathogens artificially administered to reproductive tissues is sensitive to ovarian steroid hormones (Prabhala and Wira 1995). After mating, several lineages of gram-negative and –positive cocci and rods can be identified in the uterine lumen in bacterial/sperm aggregations (Parr and Parr 1985) and *Pasteurella pneumotropica* and *Proteus vulgaris* can be recovered from uterine fluids and tissue on day 1 of pregnancy (Robertson *et al.* 1999). That none of these bacteria can be isolated from the uteri of mice at day 2 or 3 of pregnancy suggests that the uterus is immunologically capable of clearing these microorganisms (Robertson *et al.* 1999).

1.7.3 The conceptus as an allograft

From the time of implantation to the establishment of an interstitial and haemochorial placenta and throughout the remainder of pregnancy, cells of the conceptus are in direct physical contact with cells of the uterus. In this sense, the conceptus can be considered an allograft, since it expresses paternal transplantation antigens as early as the 8-cell stage (Warner *et al.* 1988), in the ectoplacental cone of the recently implanted blastocyst (Hedley *et al.* 1989) and on spongiotrophoblast cells of the mid-gestation placenta (Redline and Lu 1989). The presence of such an immunologically disparate tissue within an apparently immune-competent organ such as the uterus would be expected to initiate rapid rejective immune responses, culminating in the destruction and demise of the conceptus. However, such a phenomenon occurs rarely in placental mammals. Indeed, quite to the contrary, in murine pregnancy the greater the genetic disparity between the paternal and maternal strains the greater the success of pregnancy (measured in terms of numbers of implanting and full-term conceptuses, and offspring weight at

delivery), a response which can be enhanced by immunological priming of a female mouse to paternal cells (Beer *et al.* 1975). Furthermore, since expansion and phenotypic changes in paternal antigen-specific T cells occur during pregnancy (Tafuri *et al.* 1995, Jiang and Vacchio 1998, Zhou and Mellor 1998), it would appear that rather than being a state of immunological ignorance, during pregnancy there is an active process of immune priming towards paternal antigens.

1.7.4 Mechanisms contributing to prevention of maternal immune rejection of the semi-allogeneic conceptus

Since the maternal immune system is immunologically 'aware' of paternal transplantation antigens during pregnancy, there must be mechanisms in place to ensure that the conceptual tissues are not destroyed by rejective immune responses. During the last 25 years, a number of mechanisms which operate in the afferent and efferent immune pathways during pregnancy to facilitate evasion of conceptus rejection have been proposed, and these are summarised below. Also reviewed is more recent literature supporting the theory that a state of immune deviation or antigen-specific tolerance is generated towards the conceptus during pregnancy.

a. afferent and efferent suppressive mechanisms operating in the uterus

There are several factors which may limit maternal recognition of antigens on the conceptus during pregnancy. Although MHC class I antigens are expressed on some cells of the murine placenta, in other species expression on placental cells may be highly regulated or even absent (Head *et al.* 1987), or non-polymorphic and non-classical class I genes (such as HLA-G) may be preferentially expressed (Hunt and Orr 1992). Antigen uptake and processing of trophoblast antigens might also be limited by inaccessibility of uterine APCs to the placental tissues (since uterine Mϕs and other leukocytes appear to be excluded from the decidualised tissue from early in pregnancy, see section 1.5). Furthermore, the access of uterine APCs to draining lymph nodes during pregnancy may be limited (Head and Billingham 1986) and their antigen-presenting function may be inhibited due to the local synthesis of immunosuppressive cytokines (Prabhala *et al.* 1998), prostaglandins (Boraschi *et al.* 1984) or progesterone (Siiteri *et al.* 1977).

Systemic suppression of effector immunity towards paternal or other antigens does not occur since during pregnancy humoral and cellular immune responses to paternal major and minor paternal histocompatibility antigens administered ectopically can be generated without apparent detriment to fetal survival (Bell and Billington 1983, Hamilton 1983) and prior

immunisation with paternal leukocytes does not compromise pregnancy (Beer and Billingham 1974). However, locally active suppressive mechanisms may exist, evident as a blockage of T cell infiltration into the implantation site (Noun *et al.* 1989) and a limited capacity of the maternal immune system to lyse trophoblast cells due to their diminished MHC class I expression (Head *et al.* 1987) or complement-inhibiting factors in the milieu (Holmes and Simpson 1992).

b. deviation of immune responses to uterine antigens

With the concept of immune deviation (Mosmann and Sad 1996), it has been proposed that deviation of the maternal immune response to pregnancy may be one of the factors which prevents rejection of the conceptus. Accordingly, it could be expected that Th1, or cell-mediated immune responses would be harmful to pregnancy, whereas immune responses skewed towards Th2, or humoral immunity would be less detrimental. Some early support for this hypothesis was provided by studies which showed that the administration of Th1-skewing cytokines (eg TNF and IFNγ) to pregnant mice can lead to fetal demise (Chaouat *et al.* 1988) or preterm delivery (Romero *et al.* 1991) and conversely neutral or Th-2-deviating cytokines such as GM-CSF or IL-3 can enhance fetal survival in mice that are prone to fetal loss (Athanassakis *et al.* 1987, Armstrong and Chaouat 1989). Furthermore, clinical reports suggest that cell-mediated immune diseases such as rheumatoid arthritis undergo temporary remission during human pregnancy whereas those diseases believed to have a humoral basis (such as lupus erythematosis) are potentiated (Wegmann *et al.* 1993). Other investigations into this phenomenon have shown that placental tissues tend to secrete Th2-deviating cytokines during pregnancy (Wegmann *et al.* 1993) and that pregnancy can abrogate Th1-mediated clearance of parasitic infection (Krishnan *et al.* 1996a). Conversely, a curative Th1 response mounted against parasitic infection can compromise pregnancy (Krishnan *et al.* 1996b). Currently there is no evidence to suggest that Th2-deviation during pregnancy is paternal antigen-specific, and the majority of the Th2 cytokines synthesised during pregnancy appear to be produced by non-T cells of the placenta (Wegmann *et al.* 1993, Krishnan *et al.* 1996). Thus maternal-fetal cytokine regulation may involve constitutive production of Th2 and other suppressive cytokines to dampen any Th1 immune responses induced by paternal or other antigens during pregnancy.

c. paternal antigen-specific tolerance

The previous sections have discussed the evidence for the existence of suppressive and deviated maternal immune responses during pregnancy. In general terms, these data suggest that

during pregnancy there is limited opportunity for the generation of Th1-deviated immune responses to paternal transplantation antigens and that the consequences of any response initiated in the implantation site are minimised by suppressive mechanisms operating within the uterine milieu. However, an explanation for the phenomenon of maternal 'awareness' of paternal transplantation antigens during pregnancy has not been formulated, and the time during pregnancy at which this awareness is initiated is not known. Furthermore, a phenotypic analysis of the T cells activated during pregnancy has proven difficult in the past due to the low frequency of T cells specific for paternal transplantation antigens. However, with the advent of transgenic technology, examination of the phenotypes and fate of antigen-specific T cells during the generation and effector phases of immune responses has become possible. To investigate paternal antigen-specific immune responses during pregnancy, mice transgenic for T cell receptors (TCRs) reactive with paternal MHC molecules (Tafuri *et al.* 1995, Robertson *et al.* 1997), the H-Y antigen (Jiang and Vacchio 1998) or transgenes (Zhou and Mellor 1998) have been employed.

The first study to suggest that a state of functional tolerance specific for paternal antigens is established during pregnancy was published by Tafuri (Tafuri *et al.* 1995). Using Des-TCR mice, which harbour a T cell repertoire skewed towards the H-2K^b haplotype, these authors showed that in contrast to syngeneic and third party allogeneic pregnancies, mice bearing a K^b-positive conceptus had reduced numbers of K^b-reactive T cells, and those that were retained exhibited reduced expression of TCR, CD4 and CD8. T cell phenotype and functional responsiveness to paternal antigens was restored after delivery. The systemic nature of the tolerance was demonstrated by the acceptance of K^b-positive tumour grafts by the mice bearing a K^b-positive conceptus. Interestingly, these tumours were tolerated from as early as day 3 of pregnancy, suggesting that tolerance of paternal antigens was established prior to implantation (Tafuri *et al.* 1995). Employing congenic mice in the Balb series, Robertson and co-workers (Robertson *et al.* 1997) have examined the timing of induction of pregnancy-associated tolerance more thoroughly, and confirmed that a single mating with a Balb/c male, even in the absence of conception, is sufficient to inhibit rejection of Balb/c tumour cells injected on the 4th day following mating. Furthermore, the state of tolerance was found to be specific to paternal and not third party alloantigens.

Other groups (Jiang and Vacchio 1998, Zhou and Mellor 1998) have also provided evidence of deletion and functional anergy of paternal antigen-specific T cells during pregnancy. The time at which such responses are initiated and the APC responsible for this was not examined by these authors.

Thus there is accumulating evidence that a state of immunological tolerance specific for paternal antigens is established during pregnancy. The deletion and inactivation of paternal antigen-specific T cells which is evident in current models of pregnancy-associated tolerance would be expected to rely on the presentation of paternal antigens to maternal T cells, a feat which may be accomplished during pregnancy by cells of the conceptus or maternal APCs, or both (Jiang and Vacchio 1998). However, emerging evidence that pregnancy-associated tolerance does not depend on the presence of an embryo, and can be initiated with one exposure to semen, suggests that events of antigen presentation following exposure to paternal antigens after mating may be sufficient for this response (Robertson *et al.* 1997).

1.8 Summary

Myeloid APCs are extraordinarily diverse cells which play a variety of critical roles in maintaining homeostasis in mucosal organs. During the steady state, Mϕs provide innate immune protection and participate in tissue remodeling by virtue of their highly phagocytic nature and their secretion of cytotoxic molecules and enzymes. Although Mϕs may also participate in the generation of antigen-specific immunity, dendritic cells are believed to be largely responsible for this role *in vivo*, as evidenced by their highly efficient ability to collect, process and present antigens, as well as their propensity to traffic to local lymph nodes and their capacity to activate naïve T cells. The ability of given Mϕ and dendritic cell populations to participate in these processes can be assessed by examining their expression of specific molecules in the membrane or cytoplasm of the cell.

The development and differentiation of Mϕs and dendritic cells are precisely regulated by cytokines, which act through binding specific signal transducing receptors on the APC cell surface. The colony stimulating factors CSF-1 and GM-CSF as well as an array of pro-inflammatory chemokines and cytokines are believed to play central roles in the recruitment, differentiation and activation of APCs in the periphery, and the relative abundance of these molecules can have a dramatic influence on the number and phenotype of Mϕs and dendritic cells found within a tissue. The role for particular cytokines in APC regulation has been illustrated by *in vitro* data and analysis of cytokine deficient mice, with CSF-1 believed to be an important cytokine for normal Mϕ development and GM-CSF of particular relevance to dendritic cells. Cytokines are also critical for the initiation of antigen-specific immunity, through their immunoactivating and immune-deviating effects on APCs and T cells.

Mϕs and dendritic cells are particularly abundant in mucosal organs, which require well-coordinated homeostatic mechanisms to manage their constant exposure to many and diverse antigens. To this end, Mϕs in steady-state mucosal organs tend to be potent secretors of immunoinhibitory molecules, which regulate local dendritic and T cell populations and prevent the generation of Th1-polarised immunity in the absence of overt inflammation. Abrogation of the inhibitory effect of mucosal Mϕs only occurs following the 'danger'-induced cytokine/chemokine cascade, when the resident and newly arrived dendritic cells are mobilised to lymph nodes where they can induce Th1 immunity. Another outcome of antigen-presentation to T cells may be tolerance, the initiation of which is also controlled by specific features of the APC.

The murine uterus is typical of many mucosal organs, consisting of stromal and epithelial cell components and diverse populations of leukocytes. Mϕs are particularly abundant in the uterus, and in the non-pregnant state the number and position of these cells within the endometrium and related structures fluctuates in accordance with the secretion of ovarian steroid hormones. Although steroid hormones may have a direct action on uterine Mϕs, these changes are thought to be regulated primarily by the hormone-regulated synthesis of lymphohaemopoietic cytokines by the uterine luminal and glandular epithelial cells. Epithelial cell-derived CSF-1 appears particularly critical for maintaining normal uterine Mϕs during the estrous cycle, and other cytokines such as GM-CSF, TNFα and IFNγ may also play a role.

Following insemination, the cytokine and leukocytic response of the uterus is reminiscent of a typical inflammatory reaction. A predominant effect of insemination is a dramatic upregulation in synthesis of GM-CSF, IL-6 and CC chemokines by uterine epithelial cells, all of which appear to induce the recruitment and activation of uterine Mϕs and other leukocytes, including a putative lineage of uterine dendritic cells. This response subsides by the fourth day of pregnancy, when embryo implantation occurs, although APCs are still retained by the uterus at this time and for the duration of pregnancy.

There are a number of potential immunological consequences of insemination. Semen is a rich source of antigens, containing sperm and other cells which express a diverse array of proteins foreign to the maternal immune system. Other antigens potentially contained within semen include soluble macromolecules and those associated with bacterial or viral microorganisms. Presumably the uterus must have mechanisms to recognise and respond appropriately to these diverse antigenic stimuli, in order to maintain uterine sterility and to ensure pregnancy success. However, the precise nature of the myeloid APC populations present in the

uterus, and their potential responsiveness to the temporal fluctuations in uterine cytokine synthesis which accompanies the cycle and early pregnancy have not been examined.

1.9 Hypotheses

Based on the review of the literature presented above, the following hypotheses were formulated:

1. The populations of myeloid APCs contained within the murine uterus are similar in composition, in phenotypic heterogeneity and in function to myeloid APCs present within other mucosal organs.

2. Fluctuations in the relative abundance of uterine APCs and in their activation phenotypes occurs in parallel with changes in the ovarian steroid hormone and cytokine environment associated with the cycle, insemination and early pregnancy.

3. The cytokine GM-CSF is a key determinant of the recruitment and/or the activation phenotype of uterine APCs, especially during the inflammatory response to insemination.

Chapter 2

Materials and Methods

2.1 Chemicals and reagents

2.1.1 Antibodies

The rat and hamster monoclonal antibodies (mAbs) used in these studies are listed in Table 2.1, and the majority of these were prepared in house from hybridoma cell lines which were grown to confluence when mAb-containing supernatants were collected. Rat α-mouse thymic stroma #24 (MTS#24) mAb (Godfrey *et al.* 1990) was kindly provided as hybridoma supernatant by R. Boyd (Monash University, Victoria). Rat α-mouse macrosialin (FA/11), sialoadhesin (3D6) and scavenger receptor Class A (2F8) mAbs were generously provided as hybridoma supernatants by S. Gordon (Oxford, England). Biotin-conjugation of TIB 120 mAb was performed by M. Allanson (University of Adelaide, SA). Other mAbs were purchased from the sources indicated in Table 2.1.

Horseradish peroxidase (HRP)-conjugated goat α-rat immunoglobulin (Dako, Copenhagen, Denmark) or biotinylated rabbit α-rat (Dako) and HRP-conjugated streptavidin (Dako) were used to detect rat immunoglobulin in immunohistochemistry. For detection of unconjugated rat immunoglobulin for immunofluorescence and FACS analysis, fluorescein isothiocyanate (FITC)-conjugated sheep α-rat immunoglobulin (Silenus, Hawthorn, Vic.) or biotinylated rabbit α-rat (Dako) followed by phycoerythrin (RPE)-conjugated streptavidin (Dako) were used. For detection of biotinylated rat immunoglobulins for FACS analysis, RPE-conjugated streptavidin (Dako) was used.

2.1.2 Recombinant cytokines

Recombinant *E. coli* derived murine granulocyte-macrophage colony-stimulating factor (GM-CSF) and yeast-derived colony stimulating factor (CSF)-1 were kindly provided by N. Nicola (The Walter and Eliza Hall Institute of Medical Research, Victoria). Recombinant murine IFNγ was obtained from Genetech (Palto Alto, CA, USA).

Table 2.1 The species of origin, isotypes, antigenic specificities, cell lineage reactivities, dilutions for use and sources of mAbs used in these studies. Details are provided for unconjugated, biotin-conjugated and FITC-conjugated mAbs used for immunohistochemistry, FACS analysis, immunomagnetic cell selection, immunofluorescence analysis and complement-mediated cell depletion. Mϕ = macrophage, DC = dendritic cell, LCA = leukocyte common antigen, SR = scavenger receptor, LC = Langerhans cell, TE = thymic epithelium, UE = uterine epithelium, IEL = intraepithelial lymphocyte, NK cell = natural killer cell.

mAb	species (isotype)	antigenic specificity	reactive lineages	dilution	source
unconjugated mAbs					
TIB 128	rat (IgG2b)	CD11b/CD18 (Mac-1)	Mϕs, neutrophils	neat	ATCC[1]
TIB 122	rat (IgG2a)	CD45 (LCA)	pan leukocytes	neat	ATCC[1]
GL-1	rat (IgG2a)	CD86 (B7-2)	DCs, Mϕs & B cells	1:50	Pharmingen[2]
TIB 120	rat (IgG2b)	Class II MHC (Ia)	activated Mϕs, DCs	neat	ATCC[1]
FA11	rat (IgG2a)	macrosialin	Mϕs, some DCs	1:400	S. Gordon[3]
2F8	rat (IgG2b)	class A SR	Mϕs, some DCs	1:5	S. Gordon[3]
3D6	rat (IgG2b)	sialoadhesin	Mϕs, some DCs	1:2	S. Gordon[3]
NLDC-145	rat (IgG2a)	DEC-205	DCs, TE	1:5	Serotec[4]
F4/80	rat (IgG2b)	ND	Mϕs, LCs	neat	P. Kenny[5]
MIDC-8	rat (IgG2a)	ND	DCs	1:10	Serotec[4]
MTS #24	rat (Ig)	ND	TE, UE	1:4	R. Boyd[6]
RB6-8C5	rat (IgG)	Gr-1	neutrophils	neat	S.McColl[7]
biotin-conjugated mAbs					
1B1	rat (IgG2b)	CD1d (murine CD1)	Mϕs, DCs, leukocytes	1:50	Pharmingen[2]
145-2C11	hamster (IgG)	CD3	T cells	1:50	Pharmingen[2]
HL3	hamster (IgG)	CD11c/CD18	DCs, IEL	1:50	Pharmingen[2]
TIB 120	rat (IgG2b)	Class II MHC (Ia)	activated Mϕs, DCs	1:25	M. Allanson[8]
FITC-conjugated mAbs					
3123	rat (IgG2b)	CD40	B cells, DCs, TE	1:50	Pharmingen[2]
TM-β1	rat (IgG2b)	CD122	some T, NK, B cells	1:50	Pharmingen[2]
F4/80	rat (IgG2b)	ND	Mϕs, LCs	1:50	Serotec[4]

[1]American Type Tissue Culture, Rockville, Maryland, USA; [2]CA, USA; [3,4]Oxford, England; [5]Flinders University, South Australia; [6]Monash University, Victoria; [7]Microbiology and Immunology, University of Adelaide, South Australia; [8]Obstetrics and Gynaecology, University of Adelaide, South Australia

2.1.3 Sundry chemicals and reagents

All other chemicals and reagents were obtained from BDH chemicals (Dorset, UK) or Sigma Chemical Co. (MO, USA) unless specified in the text.

2.2 Mice

2.2.1 General

Adult (6-12 week old) virgin Balb/c x C57Bl/6 (Balb/c F1, H-2^{db}) females and adult (10-52 week old) Balb/k (H-2^{k}) males were used in all experiments unless specified. All mice were obtained from the University of Adelaide Central Animal House, and were maintained in minimal security barrier, specific pathogen-free facilities on a 12/12 hour (h) light/dark cycle with *ad libitum* food and water.

2.2.2 Diagnosis of stage of estrous cycle by vaginal cytology

Vaginal cells were sampled by gently flushing the external portion of the vagina with 20-40 µl of phosphate buffered saline (PBS). The resulting suspension of cells was viewed as an unstained, cover-slipped wet mount preparation under an inverted light microscope (Nikon TMS, Japan) and staged as pro-estrous, estrous, metestrous or diestrous according to previously outlined criteria (Snell 1941). In some experiments, the Whitten effect was employed to induce female mice into estrus by placing a small cage containing a stud male into a larger cage containing the female mice (Whitten 1956). The majority of females were found to exhibit estrus on the third day after caging in this manner.

2.2.3 Natural mating

For natural mating, female mice were placed 1-4 per cage with individual stud males of proven fertility. The day of sighting of a vaginal plug (checked between 0800-0900 h) was designated day 1 of pregnancy. Pregnant females were separated from males on day 1.

2.2.4 Ovariectomy

Surgical instruments were sterilised by autoclaving and kept in 70% ethanol. Mice were anaesthetised by intra-peritoneal injection of avertin [1 mg/ml tribromoethyl alcohol in tertiary amyl alcohol diluted to 2.5% (v/v)] at a dosage of 15 µl/g body weight. Mice were bilaterally ovariectomised by severing the oviduct close to the uterine-oviduct junction though a single small dorsal incision as previously described (Hogan *et al.* 1996). The incision was closed with a wound clip and animals were allowed to recover for 2-3 weeks prior to use.

2.2.5 Hormone replacement

For hormone replacement of ovariectomised mice, a regimen was employed that had been previously used to successfully prime recipient mice for embryo transfer experiments (Finn and Martin 1969, Finn and Martin 1972). 17β-estrodiol and progesterone were dissolved in 100% ethanol and diluted to the required concentration in olive oil for subcutaneous injection of 100 µl doses. Mice received 2 priming doses of 100 ng 17β-estrodiol on consecutive days, were rested for 2 days and then were maintained on daily doses of 25 ng 17β-oestrodiol or 500 µg progesterone or 25 ng 17β-estrodiol + 100 ng progesterone or vehicle for 6 days. On the seventh day the mice were sacrificed and uteri were collected, weighed and digested for isolation of macrophages (Mϕs).

2.2.6 GM-CSF deficient mice

Mice deficient in GM-CSF were generated using gene targeting techniques in 129/OLA-derived E14 embryonic stem cells, and were propagated from founder mice by mating with C57BL/6J mice as previously described (Stanley *et al.* 1994). GM-CSF-deficient breeding pairs were kindly provided by A. Dunn (Ludwig Institute for Cancer Research, Victoria). GM-CSF replete mice were derived from F2 offspring of GM-CSF-deficient females crossed with wild type 129/Sv males obtained from the Animal Resources Centre. The genotype of GM-CSF-deficient and -replete lines of mice were confirmed by polymerase chain reaction (PCR) of DNA extracted from blood or tail tissue of breeding pairs and offspring. Neither spleen nor uterine conditioned media derived from cells obtained from GM-CSF-deficient mice contained detectable bioactive GM-CSF (Stanley *et al.* 1994, Robertson *et al.* 1999).

2.3 Cell culture

2.3.1 General

All harvesting and culture of tissues and cells was performed under aseptic and pyrogen-free conditions. Solutions and media were prepared from stocks reserved for cell culture using water purified in a Milli-Q Reagent Grade Water System (Millipore, MA, USA) (MQ H_2O), and sterilised by filtration though 0.22 µm filters (Millex, Millipore) or by autoclaving. Disposable plastic culture-ware and pipettes were gas-sterilised prior to use, and glassware was washed in 7x detergent (ICN, Seven Hills, NSW) and rinsed extensively in MQ H_2O and autoclaved prior to use. Cell cultures were viewed daily with a phase contrast inverted microscope (Nikon TMS).

2.3.2 *Media and saline solutions*

Media used for hybridoma and primary cell culture was RPMI-1640 supplemented with 20 mM Hepes, 10% heat inactivated (HI) fetal calf serum (FCS) (Commonwealth Serum Laboratories, Australia), $5x10^{-5}$ M β-mercaptoethanol and antibiotics (penicillin and streptomycin) [RPMI-FCS].

Phosphate buffered saline (PBS) was made at 10x concentration and diluted for use in MQ H_2O to form a solution containing 1.4 M sodium chloride, 0.08 M sodium phosphate, 0.01 M potassium dihydrogen orthophosphate and 0.03 M potassium chloride. Hanks buffered saline solution (HBSS) was made from powdered stock with 5 mM Hepes.

The pH and osmolarity of media and saline solutions was adjusted to 7.4 and 280 mOsm/kg with the use of a pH meter (Activon 209, USA) and an osmometer (Wescor, Utah, USA) respectively.

2.3.3 *Hybridomas*

Cell lines were obtained from the source specified in Table 2.1 and were cultured in RPMI-FCS in 200 ml culture flasks (Costar, MA, USA) at 37°C, 10% CO_2 in air in a humidified incubator. Frozen cell stocks were maintained in 10% dimethyl sulphoxide in liquid nitrogen.

2.3.4 *Cell quantitation*

The density of cells in suspension was assessed using a Neubauer hemocytometer (Assistent, Germany). Viable cells (those that excluded Trypan Blue) within an area corresponding to 10^{-4} ml were counted using a phase contrast light microscope (Olympus BH-2). In some experiments, the numbers of adherent Mϕs in culture were counted at 20x magnification on an inverted phase contrast microscope (Nikon TMS).

2.4 Peritoneal, uterine and spleen cell collection

2.4.1 *Peritoneal lavage*

Freshly euthanased mice were placed on their back, bathed in 70% ethanol and abdominal skin was pierced and peeled back to expose the abdominal cavity enclosed by serosa. The serosa was grasped and lifted gently with forceps and 8-10 ml of HBSS with 5% HI FCS (HBSS-FCS) or RPMI-FCS injected into the peritoneal cavity with an 18-21 gauge needle fitted to a 10 ml syringe (both from Becton Dickinson, Singapore). The peritoneal cavity was gently massaged

for 2-3 min and 80-100% of injected solution was then recovered into the syringe. Mϕs were referred to as 'elicited peritoneal Mϕs' when mice were primed 4 days prior to peritoneal lavage by intra-peritoneal injection of proteose peptone (1% w/v in PBS), or 'resident Mϕs' when no priming was performed. Cells were washed (12 min, x1200 g) in RPMI-FCS and stored on ice until required.

2.4.2 Enzymatic digestion of uteri

a. digestion in collagenase/DNase

To digest uteri for preferential release of Mϕs, a modification of a method described by Hunt and co-workers (Hunt *et al.* 1984) was used. Uteri were removed aseptically, washed in sterile cold PBS and mesentery and fat were removed. Uteri were then slit longitudinally with the use of a dissecting microscope (Olympus SZ-PT, Japan) and minced extensively using fine scissors. The tissue fragments of individual or pooled uteri were stirred gently at room temperature (RT) for 2 h in 10-20 ml collagenase/DNase [1 mg/ml Type I collagenase (Clostridiopetidase A, from Clostridium histolyticum) and 2.5 μg/ml DNase I (Type II from bovine pancreas) in RPMI-FCS]. A 5 ml plastic pipette and pasteur pipette was used to mix the suspension periodically throughout the digestion. After 2 h, an equal volume of ice cold 5 mM ethylenediaminetetraacetic acid (EDTA) in Ca^{++}-, Mg^{++}-free HBSS with 5% FCS and 0.01% sodium azide [HBSS-EDTA] was added and stirred for a further 20 min. The digestion mixture was then filtered though a sterile stainless steel tea strainer followed by a sterile 70 μm nylon cell strainer (Falcon, Becton Dickinson Labware, NJ, USA) with HBSS-EDTA. The cell suspension, comprising approximately 10^7 cells per mouse, was washed 2x in RPMI-FCS before use in cell culture or FACS analysis.

b. digestion in trypsin/pancreatin

To digest uteri for preferential release of epithelial cells, a modified version of a procedure initially described by Sherman (Sherman 1978) was used. Uteri were excised, placed in ice cold PBS, trimmed of fat and mesentery and slit lengthwise. After a second rinse in PBS, uterine tissue was incubated in 1 ml of 0.5% trypsin plus 2.5% pancreatin (bovine pancreatic, Type III) in PBS for 45 min at 4°C, followed by 45 min at 37°C. One ml of RPMI-FCS was added and the uteri gently agitated by passage 3-4 times up and down a wide bore plastic pasteur pipette. The released cells, comprising approximately 75% epithelial cells (Robertson *et al.* 1996b), were collected and washed in RPMI-FCS. Harvested cells were used for

immunomagnetic selection (see section 2.5.4) or to prepare cytosmears for immunohisto-chemistry (see section 2.6.1).

2.4.3 Spleen cells

a. preparation of single spleen cell suspensions

Spleens were removed aseptically and rinsed in cold PBS or HBSS and cut into small pieces before being homogenised in a sterile, manually operated glass homogeniser (Kontes Glass Co., NJ, USA) in 10 ml PBS or HBBS-FCS. Tissue debris was removed by filtration though a sterile stainless steel tea strainer and subsequently a 70 μm nylon cell strainer (Falcon, Becton Dickinson Labware). Red blood cells were depleted by flash lysis (see section 2.5).

b. spleen cell culture

To generate activated leukocytes for FACS experiments, spleen cells were cultured overnight in 50 ml culture flasks (Falcon, Becton Dickinson Labware) with 10^3 U/ml IFNγ (Genetech, Palto Alto). Adherent cells and non-adherent cells were collected by gently scraping the bottom of the flask with a sterile glass probe, and the cells were subsequently labeled for FACS analysis.

c. enrichment of lymphocytes for mitogenesis assays

Mixed spleen cells were resuspended at 10^5-10^6/ml with F4/80 and TIB 120 mAb supernatants (see Table 2.1) diluted 80% (v/v) in RPMI and incubated for 1 h at room temperature (RT). Cells were washed 1x and resuspended at 10^5-10^6 cells/ml in RPMI with complement-rich guinea pig serum (diluted 1:20) and incubated at 37°C for 1 h in a 10 cm petri dish (Sarstedt, SA). Remaining non-adherent, viable cells (referred to as 'purified spleen cells') were collected, filtered through a sterile 70 μm membrane (Falcon, Becton Dickinson Labware) and washed 3x in RPMI-FCS. Cell density was adjusted for use in mitogenesis assays.

2.5 Flash lysis of red blood cells

For removal of red blood cells (RBC) from peritoneal, uterine or spleen cell preparations, cells were resuspended in 0.9 ml sterile MQ H_2O and gently swirled for several seconds before addition of 100 μl of sterile 10x PBS. Cells were washed 2x and resuspended in RPMI-FCS for

use in selection procedures. The flash lysis procedure was repeated if RBC were still visible in cell pellets.

2.6 Enrichment of Mϕs and epithelial cells from peritoneal lavages and uterine digests

2.6.1 Adherence

Cell suspensions (10^5-10^7 cells/ml) in RPMI-FCS were cultured in multi-well glass chamber slides (Lab-Tek, Nunc, Illinois, USA) for 2-24 h. Non-adherent cells were removed by repeated washing and forceful pipetting. Fresh RPMI-FCS was added for subsequent analyses.

2.6.2 Rosetting

Fresh whole sheep blood (collected in heparin, 1000 U/ml) was centrifuged (RT, x600 g) through 10 ml Ficoll-Paque and the serum-free, leukocyte-free sheep red blood cells (SRBC) were washed 2x and suspended at 5% (v/v) in HBSS-FCS. A 4 ml aliquot of 5% SRBC was incubated with rabbit α-sheep immunoglobulin (10 μg/ml, 20 min, 37°C), washed 4x and resuspended in 2 ml HBSS-FCS. The antibody coated-SRBC were incubated with 1 ml of mixed uterine cells (20 min, 4°C) then diluted further to 10 ml in HBSS-FCS and centrifuged (x 1200g, 12 min). The resulting pellet was resuspended in 2 ml, incubated with goat α-rabbit immunoglobulin (Silenus) (10 μg/ml, 20 min, 4°C) and then washed 2x in 10 ml HBSS-FCS and the final pellet resuspended in 5 ml HBSS-FCS. Rosetted cells were collected by centrifugation (RT, x 300g, 15 min) through 5 ml Ficoll-Paque (non-rosetted uterine cells remained at the Ficoll-Paque/buffer interface). SRBC in the positive fraction were removed by flash lysis (see section 2.5), the selected cells were washed, resuspended in 1 ml RPMI-FCS and counted, and then analysed for Mϕ purity or used in mitogenesis assays.

2.6.3 Immunomagnetic selection

Mixed peritoneal cell or uterine cell suspensions were incubated in RPMI-FCS at 10^5-10^6 cells/ml with neat or diluted mAb supernatant (see Table 2.1) containing 10% HI NMS (1 h, 4°C). After 1 wash, cells were incubated (20 min, 4°C) in RPMI-FCS (10^7 cells/ml) with 10% HI NMS and 20% (v/v) miniMACs goat α-rat immunoglobulin-coated microbeads (Miltenyi Biotech, Germany). FITC-conjugated sheep α-rat immunoglobulin (Silenus) was added (1:40 dilution) and incubated for a further 15 min (4°C). Cells were washed 2x and resuspended at 10^6-10^7 cells/ml, and sorted magnetically with miniMACS separation column (Miltenyi Biotech)

at 4°C into 'negative' and 'positive' fractions according to the manufacturers instructions. Fraction purity was determined by immunofluorescence, by immunohistochemical staining of cytosmears or by assessment of phagocytic activity (see section 2.7).

2.7 Determination of purity of crude and enriched cell preparations

2.7.1 Immunohistochemical analysis of cytosmears

Purified or crude cells were washed in 1 ml RPMI-FCS and resuspended at 10^8-10^9 cells/ml in neat HI FCS. Drops of 10 µl were smeared onto glass slides and air-dried. Slides were stored in sealed boxes with silica gel beads at RT for 2-12 h and cells were immunohistochemically labeled as specified in section 2.12. The proportion of labeled cells was determined by counting immunoperoxidase-labeled and total haematoxylin-labeled cells in 4 random fields by light microscopy (Olympus BH-2) at 20x magnification.

2.7.2 Immunofluorescence of adherent and suspended cells

To label adherent cells, cultured cells in multi-well slide chambers (Lab-Tek, Nunc) were incubated in RPMI-FCS with neat or diluted mAbs (see Table 2.1) and 10% NMS for 1 h at 37°C in 5% CO_2 in air. After 3 washes in RPMI-FCS, cells were incubated in RPMI-FCS with FITC-conjugated sheep α-rat immunoglobulin (1:40, Silenus) for 1 h at 37°C in 5% CO_2 in air. The entire slide was then washed 3x in PBS with 5% FCS and the chamber unit and glue were removed. Slides were wet mounted with glass coverslips and sealed with nail-polish. To label suspended cells, cell suspensions were incubated in RPMI-FCS with neat or diluted mAbs (see Table 2.1) and 10% HI NMS for 1 h at 4°C , washed 2x by centrifugation and then incubated in RPMI-FCS with 1:40 FITC-conjugated sheep α-rat immunoglobulin (Silenus) for 1 h at 4°C and washed 3x by centrifugation. Cells were adjusted to 10^8 cells/ml, and 10-20 µl drops were placed on glass slides and mounted with glass cover slips. Preparations were viewed immediately by phase contrast and UV microscopy (Olympus BH-2 with a UV light source), whereby the labeled cells were visible as bright apple green in colour (peak emission wavelength of 525 nm).

2.8 Assessment of phagocytosis by adherent cells

For phagocytosis, adherent cells in multi-well slide chambers (Lab-Tek, Nunc) were cultured in RMPI-FCS with 0.5% (v/v) fluorescent latex beads (Fluoresbrite™ plain YG

microspheres, diameter = 1.8 μm, Polysciences, Inc. PA, USA) for 2 h at 37°C in 5% CO_2 in air. Slides were prepared for viewing as described in section 2.7.2. The cells were viewed immediately by phase contrast and UV microscopy, where individual fluorescent latex beads were evident within the adherent cells.

2.9 Spleen cell mitogenesis assay

In spleen cell mitogenesis assays, 10^3-10^6 Mϕs derived from peritoneal lavages or uteri were incubated for 72-96 h in RPMI-FCS in 96 well tissue culture plates (Costar) with 10^4-10^6 purified spleen cells (see section 2.4.3c) and 10 μg/ml phytohaemagglutinin (PHA). In some assays, Mϕs were incubated in RPMI-FCS containing cytokines for 24 h before addition of PHA and spleen cells. To quantify proliferation, cell cultures were pulsed with 1 μCi/ml of ^{3}H thymidine (Amersham, IL, USA) for the final 24 h of culture and then harvested onto discs of glass fibre paper (Enzo Diagnostics, NY, USA) using an automated cell harvester (PHD, Cambridge, England). The discs, corresponding to individual wells, were allowed to air dry before 1 ml of scintillant (Ready-Safe, Beckman Instruments Inc, CA, USA) was added to each vial and radioactivity was measured as disintegrations per minute (dpm) in a liquid scintillation beta counter (Beckman Instruments Inc, CA, USA).

For collection of supernatants for transfer to fresh mitogenesis assays or for assays requiring containment of Mϕs within well inserts (Millicell-CM, 0.4 μm culture plate insert, diameter 12 mm, Millipore Products Division, MA, USA) mitogenesis assays were performed in 1 ml in 4 well multiwell trays (Nunc), and all cells were subsequently transferred to 96 well plates if quantitation of proliferation was required. Supernatants collected from mitogenesis assays were cleared of cells and debris by centrifugation (x2000 g, 5 min) and used immediately for cell culture or stored at –80°C for measurement of nitrite content.

2.10 Nitrite/nitrate assay of culture supernatants

Nitrite and nitrate, the metabolic products of nitric oxide (NO), were detected in culture supernatants by conversion of nitrate to nitrite and analysis of total nitrite in a colorimetric assay. Fifty or 100 μl of Greiss Reagent (1 part 0.1% napthylethylene dihydrochloride in MQ H_2O plus 1 part 1% sulfanilamide in 5% concentrated H_3P0_4) was added to wells containing an equal volume of sample or standard, wells were incubated for 10 minutes at RT and absorbance at 490 nm was read in an ELISA plate reader (Bio-tek Instruments, Australia). A standard curve

ranging from 2.5-160 μM nitrite was generated using $NaNO_2$. The intra-assay coefficient of variation was calculated to be < 6%.

2.11 Fluorescence activated cell scanning (FACS)

2.11.1 Labeling and storage of cells

For cell washing and labeling of cells for fluorescence activated cell scanning (FACS) analysis, ice cold FACS buffer (Ca^{++}, Mg^{++}-free HBSS with 5 mM EDTA, 5% HI FCS and 0.01% sodium azide) was used. Aliquots of $5x10^5$ uterine or spleen cells (obtained from mice as detailed in sections 2.4.2a and 2.4.3b respectively) were incubated in neat mAb supernatant with 10% HI NMS or in FACS buffer containing diluted unconjugated, FITC-conjugated or biotin-conjugated mAbs with 10% HI NMS (50 μl, 4°C, 45 min). The species of origin, isotypes and dilutions of mAbs used for FACS are specified in Table 2.1. Unconjugated rat immunoglobulins were subsequently labeled by incubation in FITC-conjugated goat α-rat immunoglobulin (Silenus) [1:40, 4°C, 45 min], or in biotinylated rabbit α-rat immunoglobulin (Dako) [1:300, 4°C, 45 min] followed by incubation in RPE-conjugated streptavidin (Dako) [1:10, 4°C, 45 min]. Biotin-conjugated mAbs were labeled by incubation in RPE-conjugated streptavidin (Dako) [1:10, 4°C, 45 min]. Unbound antibodies were removed by 3x washes in 2 ml ice cold FACS buffer after each labeling step. All labeling was performed in 6 ml gas-sterilised plastic tubes (Falcon). Aliquots of cells were also labeled using negative control mAbs for each of the labeling steps in all experiments. The control mAbs (MIDC-8, FITC-conjugated CD122 and biotin-conjugated CD3) were confirmed not to react with uterine cells in preliminary experiments. In some experiments, an additional control of no mAb was included. For dual fluorescence analysis, cells were labeled in sequence to detect two cell surface antigens as detailed in results chapters. After the final washes, cells were gently resuspended in 100 μl FACS buffer and stored in the dark at 4°C in 0.5-1 ml fresh 1% (w/v) buffered paraformaldehyde until analysis. Cells were stored for a maximum of one week before data acquisition.

2.11.2 Data acquisition

The FACScan (Becton Dickinson Immunocytometry Systems, CA, USA) was employed to acquire data from FITC- and RPE-labeled cells, whereby cells were forced in a moving fluid stream past a fixed laser beam (488 nm) and scattered light and fluorescent emissions from the cells were collected and analysed using Cellquest™ software (Becton Dickinson, CA, USA). FACScan detectors, amplifiers, threshold and compensation were optimised prior to data

acquisition by viewing a live display of data from the samples to be analysed. A dot plot showing forward scatter (FSC, proportional to cell size) and side scatter (SSC, proportional to intracellular complexity) was used to ensure that all cell populations of interest were on scale for those parameters. Fluorescence plots of positive and negative control cells were used to alter photomultiplier tube voltages and compensation as required. The peak emission wavelengths from laser-excited FITC (530 nm) and RPE (570 nm) were collected in channels 1 and 2 respectively. All data from cells labeled in a single experiment was acquired in one session. Comparable instrument settings were used in all experiments.

2.11.3 Data analysis

Quantification of the number of labeled cells and intensity of FITC- and RPE-labeling on uterine and spleen cells was analysed after the establishment of regions and quadrants defining background fluorescence with the use of negative control cells. More than 97% of control cells were excluded by regions and quadrants defining background fluorescence.

2.12 Immunohistochemistry

2.12.1 Tissue collection, preparation of sections and labeling

Tissues were excised from ovariectomised, cycling and pregnant mice, cleaned of fat and mesentery and embedded vertically in OCT compound (Tissue Tex, USA). The tissues were frozen immediately by immersion in liquid nitrogen-cooled isopentane and transferred to sealed containers at –80°C. Tissue blocks were equilibrated in an OTF cryostat (Bright, Huntington, UK) for 1 h, after which sections (7 μm thick) were cut, melted onto glass slides (Sail brand, China) and air-dried for 2 h. Slides were used immediately or stored at –80°C in sealed boxes with silica gel beads. Boxes of slides were equilibrated to room temperature prior to opening for use.

The panel of mAbs given in Table 2.1 was used to determine the numbers and distribution of reactive cells in duplicate sections of tissues or of cytosmears (see section 2.7.1). Sections and cytosmears were fixed in 96% ethanol (4°C, 10 min), washed 3x in PBS and incubated in mAbs (dilutions outlined in Table 2.1) in PBS with 1% bovine serum albumin (BSA) and 10% NMS (PBS-NMS) (2 h, 4°C). Prior to the application of each antibody and reagent, slides were washed 3x in PBS and incubated in 1% BSA in PBS (2 min) to minimise non-specific binding. For detection of MHC class II (Ia) and RB6-8C5, slides were incubated in HRP-conjugated rabbit α-rat immunoglobulins (Dako) [1:400, 2 h, 4°C]. For detection of other

mAbs slides were incubated with biotin-conjugated rabbit α-rat immunoglobulins (Dako) [1:300, 2 h, 4°C] followed by avidin-conjugated HRP (Dako) [1:400, 45 min, 4°C]. Control sections were incubated in PBS-NMS or irrelevant, isotype-matched mAbs. All incubations were performed in humidified chambers. HRP was localised with diaminobenzidine tetrachloride (DAB). After counterstaining in Gill's haematoxylin, sections were dehydrated in 2 changes of absolute ethanol, cleared in 2 changes of Safsolvent (Ajax Chemicals, Auburn, NSW), mounted in Depex (BDH) and viewed, quantified and photographed using an Olympus BH-2 light microscope and Kodak Ektachrome 64T (light microscopy) and Kodak Ektachrome 400x (UV microscopy) film (Eastman Kodak Company, Rochester, NY).

2.12.2 Quantification of labeling

A video image analysis system (VIA) was employed to quantify the density of positive (DAB) staining in the uterine tissues. The system comprised an Olympus BH-2 microscope bearing a Panasonic WV-CP610/A colour video camera connected to an IBM compatible computer, on which Video Pro 32 software (Leading Edge Pty. Ltd., Blackwood, Australia) was installed. The VIA system was calibrated to a standard field prior to acquisition of data. The intra-assay and inter-assay coefficients of variation were determined as 2% and 7% respectively. Percent positive stain (% positivity) was determined as the area of positive stain / (area of positive stain + area haematoxylin stain) x 100. For each tissue, 80-100% of the total endometrial area (approximately 10-15 fields) and 100% of the mesometrial triangle area (approximately 2-3 fields) was counted. In some experiments, 50% of the myometrial area was counted.

2.12.3 Compensation for uterine eosinophils

Uterine eosinophils were detected in control sections due to their endogenous peroxidase activity. To account for the presence of eosinophils in immunohistochemically-labeled tissues, the % positivity of eosinophils in individual control sections was determined by VIA and then subtracted from the densities obtained by VIA of matched immunohistochemically-labeled tissues.

2.13 Molecular biology

2.13.1 General procedures for RNAse-free conditions

Ribonuclease (RNAse)-free conditions were maintained for materials, chemicals and solutions used in the generation and handling of all materials used in RNA analysis. This included the use of disposable plastic pipettes and tubes where possible, baking of glassware and stainless steel-ware for 3 h at 160°C prior to use, and autoclaving of all solutions including MQ H_2O. Disposable plastic gloves were used throughout, and were replaced periodically throughout experimental procedures.

2.13.2 Preparation and storage of cells for RNA extraction

Crude and purified aliquots of 10^5-10^8 uterine, peritoneal or spleen cells were pelleted by centrifugation (x2000 g, 5 mins) in 1.5 ml tubes (Eppendorf, Germany), the supernatant removed by a sterile pasteur pipette and the cells immediately snap frozen in liquid nitrogen. Cells were stored at –80°C until RNA extraction.

2.13.3 Isolation of total cellular RNA

RNA was extracted from cells using an adaptation of the method described by Chomczynski and Sacchi (Chomczynski and Sacchi 1987). Cell pellets were rapidly denatured by the addition of 1 ml of 'solution D' [4 M guanidine thiocynate (Fluka Biochemika, Switzerland), 0.5% sarkosyl, 0.1 M β-mercaptoethanol, 25 mM sodium citrate pH 7.0]. Sequentially, 100 µl of 2 M sodium acetate pH 4.0, 1 ml of phenol (H_2O saturated) and 200 µl of chloroform-isoamyl alcohol mixture were added, and the mixture was vortexed and incubated at 4°C for 10 min. Samples were centrifuged (x10^4 g, 20 min, 4°C) and the RNA precipitated from the aqueous phase by the addition of an equal volume of isopropanol (-20°C, 12 h). The RNA was then pelleted (x10^4 g, 20 min, 4°C), washed 2x in ice cold 70% ethanol (x10^4 g, 20 min, 4°C), and the pellet was dried *in vacuo* and resuspended in 100 µl MQ H_2O.

2.13.4 DNase treatment of RNA

Total cellular RNA was deoxyribonuclease (DNase) treated as a precautionary measure against contamination by genomic DNA. The reaction solution of 25 µl 5x DNA buffer, 1 µl 40 U/µl RNase inhibitor and 5 µl 10 U/µl DNase I (all from Boehringer Mannheim, Germany) was incubated with 100 µl of cellular RNA (90-100µg) for 1 h at 37°C and the RNA was

subsequently extracted by the addition of 250 µl of H_2O saturated phenol, chloroform and isoamyl alcohol (25 : 24 : 1). The solution was vortexed and centrifuged at $x10^4$ g for 10 min at RT and the aqueous RNA phase was removed. The RNA was precipitated from the aqueous phase by the addition of 2.5x volume 100% ethanol and 0.1x volume sodium acetate (pH 5.2) at –20°C overnight. The RNA precipitate was pelleted at $x10^4$ g for 20 min at 4°C and then washed twice in ice cold 70% ethanol. The pellet was dried *in vacuo* and resuspended in 10-50 µl MQ H_2O.

2.13.5 Quantitation of RNA

RNA was quantified by measuring the absorption of light at a wavelength of 260 nm with the use of a spectrophotometer (Beckman DU-50, CA, USA). Since one unit of optical density (OD) is equivalent to 40 µg/ml RNA, the concentration of RNA was calculated from the equation OD_{260} x 40_{RNA} x dilution factor. An estimate of contamination by cellular protein was calculated by measuring OD_{280} (OD_{260}:OD_{280} ratio of 1.7-2 indicated sufficiently pure RNA). RNA was subsequently stored in 2.5x volume of 100% ethanol and 0.1x volume 2 M sodium acetate (pH 5.2) overnight at –20°C.

2.13.6 cDNA generation from RNA by reverse transcription

First strand cDNA synthesis of RNA by reverse transcription was performed employing a Superscript RNase H-reverse transcription kit (Gibco BRL, MD, USA). RNA (1 µg/10µl in MQ H_2O) was initially primed with 1 ml of 500 mg/ml random hexamer (Bresatec, SA) at 70°C for 10 min, and then chilled on ice for 5 min. The solution was spun to collect RNA and incubated with 4 µl 5x reverse transcription buffer (Gibco BRL), 2 µl 0.1 M dithiothreitol (DTT) (Gibco BRL) and 2 µl 10 mM 2'-deoxynucleotide 5'-triphosphate (dNTPs) (Pharmacia Biotech, Sweden) at 43°C for 2 min. Following the addition of 1 µl RT Superscript enzyme (Gibco BRL), the reaction proceeded for 90 min at 43°C. RNA-cDNA denaturation and enzyme inactivation was induced at 94°C for 5 min followed by an incubation on ice for 5 min. The solution was diluted to a final volume of 100 µl by the addition of MQ H_2O and stored at –20°C.

2.13.7 Oligonucleotide primers and design

Primer pairs specific for cDNAs were designed using published cDNA sequences and Primer Designer version 2 (Scientific and Educational Software). For consideration, primers

were required to meet strict criteria, including 50-60% GC content, melting temperature range between 55°C and 80°C and low primer-dimer potential either individually or in combination. For primers designed to detect β-actin, macrosialin and LCA cDNA, the target cDNA position and primer lengths, sequences, melting temperatures and generated product sizes are given in Table 2.2. The primers were synthesised using phosphoramidite chemistry (Gibco-BRL), purified by size exclusion chromatography to remove salts and organics and diluted to a concentration of 100 μM in MQ H_2O and stored at –20°C.

Table 2.2 Details of the primer pairs for macrosialin, LCA and β-actin.
B* = complementary strands; LCA = leukocyte common antigen

primer	target cDNA position	primer length (bp)	primer sequence (5'-3')	melting T (°C)	product size (bp)
macrosialin-A	55-72	18	CCAACAGTGGAGGATCTT	65	243
macrosialin-B*	297-279	18	TACTCTCCTGCCATCCTT	65	
LCA-A	330-347	18	GGCTCTTCAGAGACCACA	62	298
LCA-B*	627-610	18	GTGCTGACATTGGAGGTG	62	
β-actin-A	48-67	20	TGTGATGGTGGGTATGGGTC	60	372
β-actin-B*	419-400	20	TAGATGGGCACAGTGTGGGT	60	

2.13.8 Polymerase chain reaction (PCR)

The PCR amplification employed reagents supplied in a *Taq* DNA polymerase kit (Biotech International, TAQ-1, source). Each reaction volume (total of 25 μl) consisted of 67 mM Tris-HCl (pH 8.8), 16.6 mM $(NH_4)_2SO_4$, 0.2 mg/ml gelatin, 0.45% Triton X-100, 2.5 mM $MgCl_2$, 0.2 mM dNTPs (Pharmacia Biotech), 2 μM 3' and 5' primer, 2 μl of cDNA, over-layed with paraffin oil. *Taq* DNA polymerase (0.55 U) in 10 μl MQ H_2O was added at 94°C to initiate the reaction. After an initial incubation at 94°C for 5 min, samples were amplified in a PCR machine (Hybaid Omni Gene, UK) by multiple 1 min cycles of denaturation (94°C), annealing (58-61°C) and extension (72°C), followed by a final 7 min incubation at 72°C. cDNA obtained from peritoneal and spleen cells was used as a positive control and to optimise reaction conditions for each primer pair. The negative control included in each reaction consisted of MQ H_2O substituted for cDNA. PCR reaction products were analysed by gel electrophoresis.

2.13.9 Gel electrophoresis

Reaction products were analysed by gel electrophoresis through a 2% agarose (Promega, WI, USA), 0.5 mg/ml ethidium bromide (Eastman Kodak, city) gel in TAE buffer (0.06 M

EDTA, 1 M glacial acetic acid and 2 M Tris base) at a voltage of 90-100 V for 45-60 min and visualised over an ultraviolet light box (UVP Inc, Australia). PCR product (20 µl PCR product in 4 µl 6x loading dye, Biotech International, WA) size was verified by comparison to molecular weight marker *Hpa II*-digested pUC 19 DNA (Biotech International).

Chapter 3

Populations of antigen presenting cells in the uteri of cycling and pregnant mice

3.1 Introduction

During the last 20 years, it has become apparent that diverse lineages of bone marrow-derived leukocytes form a significant proportion of cells in the cycling and pregnant mammalian uterus. Of particular interest, especially in the mouse, has been the large population of F4/80-positive ($F4/80^+$) macrophages (Mϕs) located within the mucosal tissues (the endometrium) and in the muscular layers (the myometrium) of the uterus and the mesometrial triangle (De and Wood 1990, De and Wood 1991, Pollard *et al.* 1998). Fluctuations in the numbers, proximity to the luminal epithelium and phenotypic appearance of $F4/80^+$ uterine Mϕs occur in close temporal association with the local production of various immunoregulatory molecules. Most notably, a diverse array of cytokines and chemokines are synthesised by luminal and glandular uterine epithelial cells in response to ovarian steroid hormones and other biological influences such as the introduction of seminal plasma and bacterial products (Hunt and Robertson 1996). Neutrophils, eosinophils and smaller populations of lymphocytes are also contained within the endometrium (Hunt and Pollard 1992, Brandon 1993, Hunt and Robertson 1996, Robertson *et al.* 1998), and a population of F4/80-negative ($F4/80^-$), Ia^+ dendritic cells has also been identified in the murine endometrium (Robertson *et al.* 1998).

The uterus clearly requires mechanisms by which to eliminate pathogens introduced into the luminal cavity at mating or opportunistically during the estrous cycle, and the innate phagocytic and degradative capacity of neutrophils and Mϕs are ideally suited to this purpose. In addition, these cells would be expected to clear debris and extraneous cellular material generated during the massive structural changes which occur during the estrous cycle and pregnancy. However, Mϕs and particularly dendritic cells also comprise the inductive arm of the specific, or acquired immune system, and thus can prime lymphocytes to the antigens encountered in the uterine milieu. The ability of such professional antigen presenting cells (APCs) to perform these roles relies on their capability to (1) internalise proteins from the extra-cellular milieu, (2) process internalised proteins to form antigenic peptide fragments, and (3) traffic to lymphoid organs and present the antigens on the cell surface in the context of cell-surface class II MHC molecules with appropriate co-stimulation to cognate T cells. Studies in other mucosal tissues show that the capacity of Mϕs and dendritic cells to perform each of these roles is associated with

their assumption of distinct and diverse phenotypes which are reflected in the patterns of expression of specific molecules on the cell surface. The occurrence in the uterus of Mϕs and dendritic cells with phenotypes consistent with antigen presenting function, and the possibility of diversity within these phenotypes has not previously been examined.

In the studies described in this chapter, Mϕs and related cells were localised using indirect immunoperoxidase techniques in uteri from cycling (estrous and diestrous stages of the cycle), ovariectomised, and pregnant (day 1 and day 4) mice. A panel of six mAbs was selected to detect cell surface molecules implicated in a diverse range of behaviours, including antigen uptake, processing and presentation, and co-stimulation of T cells (for mAb specificities and reactive cell lineages see Table 2.1).

3.2 Numbers and distribution of APCs in the endometrium, mesometrial triangle and myometrium

To determine the number and distribution of APCs in the endometrium, mesometrial triangle and myometrium, sections of uteri collected from cycling, ovariectomised and pregnant Balb/c F1 mice were analysed immunohistochemically. For collection of uteri from cycling mice, mice were monitored daily by vaginal smear analysis and sacrificed at estrous or diestrous. For collection of uteri from ovariectomised mice, ovaries were removed by a single dorsal incision under anaesthesia (Hogan *et al.* 1996) three weeks prior to collection of uteri. For collection of uteri from pregnant mice, mice were caged with Balb/k studs of proven fertility and sacrificed on the day of sighting of a vaginal plug (day 1) or on the 4th day after sighting of a vaginal plug (day 4). Fresh frozen 7μm sections of uteri were fixed in ethanol and leukocytes were detected using a range of mAbs and an indirect immunoperoxidase technique (see section 2.12). Optimal concentrations of mAbs were determined and specificity of staining was confirmed as consistent with that observed by other authors in preliminary experiments on sections of murine spleen (data not shown). Slides were counter-stained in haematoxylin, cleared, fixed and mounted. Slides stained with a given mAb were processed in a single experiment and quantified under the same conditions.

The morphologies of APCs expressing each of the target antigens were assessed by light microscopy. The density of APCs in endometrium and mesometrial triangle was quantified by video image analysis (see section 2.12). The distribution of APCs in superficial and deep endometrium, and the number of APCs in myometrium was assessed with the use of a semi-quantitative method of analysis (see Table 3.1 and Table 3.2).

3.2.1 *Macroscopic and histological features of uteri from cycling, ovariectomised and pregnant mice*

A schematic representation of the macroscopic and histological features of the murine uterus is provided in Figure 3.1. By macroscopic examination, uteri collected from mice in estrus were large, oedematous and pink in appearance due to abundant vascularisation. Histologically the uteri had a thick endometrial layer, a large, convoluted lumen and numerous endometrial glands. Uteri from diestrous mice were < 2-fold smaller in diameter than those from estrous mice, and were comparatively pale in appearance, presumably due to less abundant vascularisation. The endometrial layer was relatively thin upon histological examination, and the lumen was smaller and much simpler in structure, and endometrial glands were not as large. Uteri obtained from ovariectomised mice were very narrow (diameter approximately < 2 mm), with a very pale, unvascularised appearance. By histological analysis, the nuclei were very densely arranged, and there was a narrow luminal cavity and few glandular structures.

Uteri collected from day 1 pregnant mice were long and of large diameter, and very swollen due to the presence of fluid in the lumen. In some instances the uteri were frozen with the fluid intact, resulting in the histological appearance of a very large, distended lumen surrounded by a compressed endometrium. In most cases however, the fluid escaped before embedding and the resultant histological picture consisted of a highly convoluted, branched lumen and a thick endometrium containing many glands. Uteri obtained from day 4 pregnant mice macroscopically and histologically resembled uteri of diestrous mice, being pale and non-oedematous with small, narrow luminae and densely arranged stromal cell nuclei.

3.2.2 *Expression of F4/80 antigen in uterine APCs*

APCs expressing F4/80 antigen were detected in cycling, ovariectomised and pregnant uteri by indirect immunohistochemical analysis using the F4/80 mAb (Austyn and Gordon 1981). This mAb detects nearly all populations of Mϕs identified in lymphoid (Hume *et al.* 1983), endocrine and other organs (Hume and Gordon 1983, Hume *et al.* 1984a, Hume *et al.* 1984b) and those in association with epithelia, including Langerhans cells (Hume *et al.* 1984c).

a. effect of estrous cycle and ovariectomy

At estrus, F4/80$^+$ cells were evident as abundant, large, rounded cells which exhibited short processes in the cytoplasmic membrane, and which were distributed evenly throughout the superficial and deeper layers of the endometrium (Figure 3.2A and Figure 3.2G, Table 3.1).

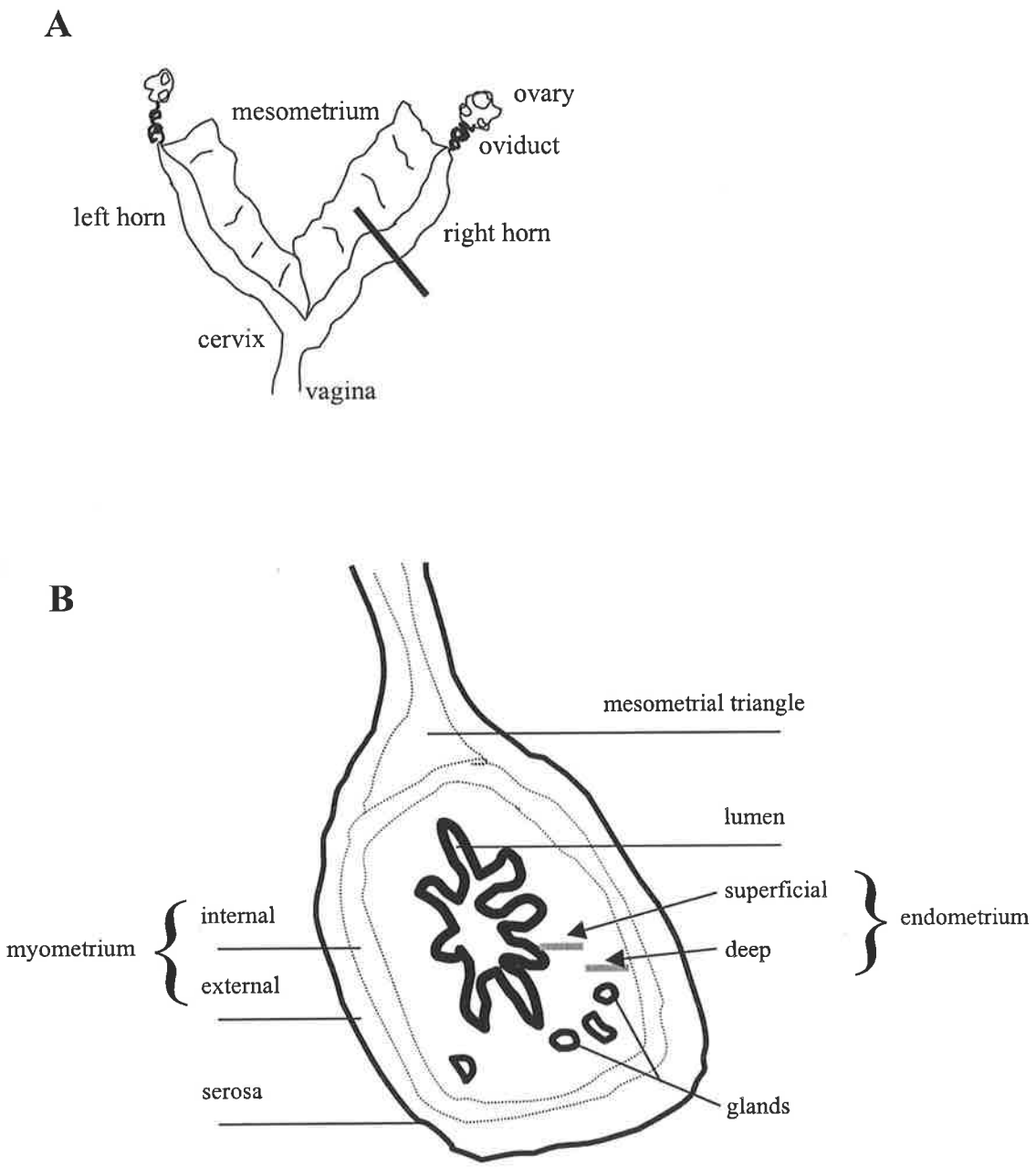


Figure 3.1 Schematic representation of the features of the murine uterus. The major macroscopic (A) and histological (B) features of the murine uterus are shown schematically. The approximate plane of transverse sections taken for immunohistochemical analysis of uteri is shown with a bold line in (A). A typical transverse section taken through a uterus at estrus is shown in (B).

F4/80$^+$ cells with a distinctly flattened and spread morphology were also evident in the endometrium immediately subjacent to the basal aspect of luminal and glandular epithelial cells. Large dendriform F4/80$^+$ cells were also present in the mesometrial triangle where they could occasionally be seen in intimate association with the basement membranes of blood vessels (Figure 3.4A). F4/80$^+$ cells were also evident but were relatively sparse in the circular and longitudinal layers of myometrium and within the serosal border of the uteri (Figure 3.2A).

In the endometria of uteri from diestrous mice, F4/80$^+$ cells were smaller and more compact than those from estrous mice (Figure 3.2B and Figure 3.2H). The density of F4/80$^+$ cells at diestrus was significantly greater than that observed at estrus (median value at diestrus was 1.7-fold higher than that at estrus, $p = 0.05$, Figure 3.3A). The cells were found to be similarly distributed in both the superficial and deep layers of the endometrium (Table 3.1). The mesometrial triangles in uteri from diestrous mice contained similar densities of F4/80$^+$ cells as those from estrous mice ($p = 0.34$, Figure 3.5A) but these were difficult to distinguish morphologically from eosinophils (Figure 3.4B). There were slightly fewer F4/80$^+$ cells in the myometrium at the diestrous phase of the cycle compared to the number observed at estrus (Table 3.2).

After ovariectomy, F4/80$^+$ cells were evident as highly dendriform cells which were sparsely distributed throughout the endometrium (Table 3.1) and the mesometrial triangle (Figure 3.6A). The densities of F4/80$^+$ cells in endometrium and mesometrial triangle of ovariectomised mice were significantly (> 90% reduction in density, $p < 0.009$) lower than those observed in uteri of cycling mice (Figure 3.3A and Figure 3.5A). Some dendriform F4/80$^+$ cells were also retained in the myometrium of uteri from ovariectomised mice (Figure 3.6A and Table 3.2).

b. effect of early pregnancy

After mating, the median density of F4/80$^+$ APCs in the endometrium increased by 1.4-fold compared to that seen at estrus ($p = 0.05$, Figure 3.3A). The cells were large and often elongated and distributed densely throughout the superficial and deep layers of the endometrium (Figure 3.7A and Table 3.1). The density of F4/80$^+$ cells in the mesometrial triangle also increased after mating (median value increased by 1.6-fold), although this was not significant ($p = 0.06$, Figure 3.5A) and these too were large, elongated cells (Figure 3.4C). At day 1 of pregnancy, the number of F4/80$^+$ cells in the myometrium was also increased relative to the numbers observed during the estrous cycle (Figure 3.7A and Table 3.2).

Table 3.1 The effect of ovariectomy, stage of estrous cycle and day of early pregnancy on the distribution of APCs and eosinophils in the endometrium. Fresh frozen sections of uteri from ovariectomised (ovx), diestrous, estrous, day 1 and day 4 pregnant mice were analysed for distribution of immunohisto-chemically-labeled F4/80$^+$, macrosialin$^+$, scavenger receptor$^+$, sialoadhesin$^+$, Ia$^+$ and B7-2$^+$ APCs and endogenous peroxidase$^+$ eosinophils within the superficial (s/f) and deep compartments of the endometrium. The number of reactive cells in each compartment was estimated by counting in 4-6 uterine fields at 20x magnification and scores are given. - = zero cells/field, +/- = 1-5 cells/field, + = 6-10 cells/field, ++ = 11-20 cells/field, +++ = > 21 cells/field.

	stage of estrous cycle / day of pregnancy									
	ovx		*diestrus*		*estrus*		*day 1*		*day 4*	
compartment:	s/f	deep	s/f	deep	s/f	deep	s/f	deep	s/f	deep
F4/80$^+$	+	+	++	++	++	++	+++	+++	+	++
macrosialin$^+$	+	+	++	+	++	++	+++	+++	+	++
scavenger receptor$^+$	+/-	+/-	++	+	++	++	+++	+++	+	+
sialoadhesin$^+$	+	+	++	++	++	++	+++	+++	+	+
Ia$^+$	++	++	+	++	+	+	++	+	+	+
B7-2$^+$	-	-	-	-	-	-	+	+/-	-	-
eosinophils	-	+/-	+/-	+	+/++	+/++	+++	++	+/-	+
n/group	*5*		*8*		*8*		*6*		*8*	

By day 4 of pregnancy, the density of F4/80$^+$ cells in the endometrium had declined to levels similar to those observed at estrus (p = 0.49 compared to estrus, Figure 3.3A) and the cells were of a small, compact dendriform morphology and were preferentially distributed in the deep endometrium (Figure 3.7B, Table 3.1). In 1/8 of the uteri from day 4 pregnant mice, a small cluster of F4/80$^+$ cells was observed in the deep endometrium. F4/80$^+$ cells in the mesometrial triangle at day 4 were difficult to morphologically distinguish from eosinophils (Figure 3.4D), and their density had not altered from day 1 (p = 0.30, Figure 3.5A). The number of F4/80$^+$ cells in the myometrium had also declined by day 4 compared to day 1 (Figure 3.7B and Table 3.2).

3.2.3 Expression of macrosialin in uterine APCs

APCs expressing macrosialin were detected in cycling, ovariectomised and pregnant uteri by indirect immunohistochemical analysis using the FA11 mAb (Rabinowitz and Gordon 1991). This mAb detects all cells of the mononuclear lineage, including monocytes, Mϕs and some dendritic cells (Rabinowitz and Gordon 1991, Li *et al.* 1998).

a. effect of estrous cycle and ovariectomy

APCs expressing macrosialin were evident in the uteri of estrous mice in two forms. The majority were large, round cells with a characteristic spread morphology and the remainder were smaller and more dendriform. Together, the macrosialin$^+$ cells were abundant and were distributed throughout the superficial and deep layers of endometrium (Figure 3.9A, Table 3.1). Many macrosialin$^+$ APCs of a large, round phenotype were also evident in the mesometrial triangle of uteri from estrous mice (not shown) and some were also present in the myometrium (Figure 3.9A).

In the endometria of uteri from diestrous mice, macrosialin$^+$ APCs were slightly smaller cells with a 'foam-like' appearance due to the presence of multiple rounded internal macrosialin$^+$ structures which resembled lysosomes (Figure 3.9B). The morphology of these cells was reminiscent of that of tingible-body Mϕs found in splenic white pulp (Rabinowitz and Gordon 1991). The cells were very densely distributed in the superficial endometrial layer but were also found in deep endometrium (Table 3.1). Overall, the density of endometrial macrosialin$^+$ APCs was significantly higher in the uteri of diestrous mice compared to the uteri of estrous mice (median % positivity was 1.9-fold higher at diestrus, $p = 0.009$, Figure 3.3B). In the mesometrial triangle, the density of macrosialin$^+$ APCs was similar in diestrous uteri to that seen in estrous uteri ($p = 0.95$, Figure 3.5B). The number of macrosialin$^+$ APCs in the myometrium was slightly diminished at diestrus compared to estrus (Table 3.2).

After ovariectomy, macrosialin$^+$ APCs were intensely stained cells of a delicately branched morphology which were sparsely distributed in the superficial and deep endometrium (Table 3.1), in the mesometrial triangle and in the myometrium (Figure 3.6B). Compared to densities in uteri obtained from estrous and diestrous mice, significant reductions in the density of macrosialin$^+$ cells in the endometrium (>60% reduction in median density, p <0.004, Figure 3.3B) and the mesometrial triangle (>90% reduction in median density, p <0.008, Figure 3.5B) were observed. The number of macrosialin$^+$ APCs in the myometrium of uteri from ovariectomised mice was similar to the number observed at diestrus (Table 3.2).

b. effect of early pregnancy

On day 1 of pregnancy, the density of macrosialin$^+$ APCs in the endometrium increased significantly compared to that at estrus (median % positivity was 1.7-fold higher at day 1, p = 0.028 compared to estrous values, Figure 3.3B). The cells were large and elongated and were distributed throughout the superficial and deep endometrium (Figure 3.9C, Table 3.1). In the mesometrial triangle the median % positivity of macrosialin$^+$ APCs at day 1 had increased by

nearly 1.5-fold relative to estrus although this was not significant ($p = 0.12$, Figure 3.5B). The macrosialin$^+$ cells in mesometrial triangle at day 1 had a rounded morphology (not shown). The number of macrosialin$^+$ APCs in the myometrium also increased at day 1 (Figure 3.9A and Table 3.2).

Table 3.2 The effect of ovariectomy, stage of estrous cycle and day of early pregnancy on the number of APCs in the myometrium. Fresh frozen sections of uteri from ovariectomised (ovx), diestrous, estrous, day 1 pregnant and day 4 pregnant mice were analysed for number of immunohistochemically-labeled F4/80$^+$, macrosialin$^+$, scavenger receptor$^+$, sialoadhesin$^+$, Ia$^+$ and B7-2$^+$ APCs and endogenous peroxidase$^+$ eosinophils within the myometrium. The number of reactive cells in each compartment was estimated by counting in 4-6 uterine fields at 20x magnification and scores are given. - = zero cells/field, +/- = 1-5 cells/field, + = 6-10 cells/field, ++ = 11-20 cells/field, +++ = > 21 cells/field.

	stage of cycle or day of pregnancy				
	ovx	*diestrus*	*estrus*	*day 1*	*day 4*
F4/80$^+$	+	+	+/++	+++	++
macrosialin$^+$	+	+	+/++	+++	+
scavenger receptor$^+$	+/-	+	+	++	+
sialoadhesin$^+$	+	+/++	+/++	++	+
Ia$^+$	+	+	+	+/++	+
B7-2$^+$	+/-	+/-	+/-	+/-	+/-
eosinophils	+/-	++	+	++	++
n/group	*6*	*8*	*8*	*6*	*8*

By day 4 of pregnancy, the density of macrosialin$^+$ APCs in the endometrium had decreased significantly ($p = 0.005$ compared to day 1 values) to become of similar magnitude to that observed at estrus ($p = 0.92$ compared to estrous values, Figure 3.3B). The macrosialin$^+$ cells were preferentially located in the deep endometrium (Table 3.1) and in 3/8 uteri discrete clusters of these cells were evident in the proximity of glands and in the myometrium (Figure 3.9E). The cells were relatively small and either round or dendriform, with distinct internal lysosome-like structures visible in many cells, although this was not as pronounced as was observed at diestrus (Figure 3.9E). In the mesometrial triangle at day 4, macrosialin$^+$ cells were of a dendriform morphology (not shown) and were decreased in density relative to day 1 ($p = 0.014$, Figure 3.5B). The number of macrosialin$^+$ cells in the myometrium at day 4 was also reduced considerably compared to day 1 (Table 3.2).

3.2.4 Expression of class A scavenger receptor in uterine APCs

APCs expressing class A scavenger receptor (scavenger receptor) were detected in cycling, ovariectomised and pregnant uteri by indirect immunohistochemical analysis using the mAb 2F8 (Fraser *et al.* 1993). This mAb detects phagocytically-competent Mϕs in lymphoid and other organs (Hughes *et al.* 1995) and in atheromatous plaques (de Villiers *et al.* 1994).

a. effect of estrous cycle and ovariectomy

In estrous uteri, scavenger receptor$^+$ APCs appeared less abundant than those expressing F4/80 and macrosialin, and were of a rounded appearance (Figure 3.2C) and were distributed throughout the superficial and deep endometrium (Table 3.1). There were very few scavenger receptor$^+$ APCs in the mesometrial triangle at estrus (Figure 3.4E) although some cells were evident in the myometrium at this time (Figure 3.2C).

At diestrus, there was a similar density of scavenger receptor$^+$ APCs in the endometrium as was seen at estrus ($p = 0.6$ compared to estrous values, Figure 3.3C). The scavenger receptor$^+$ cells were preferentially located in the superficial endometrium (Table 3.1), and were often positioned so closely together that it was difficult to delineate one cell from another (Figure 3.2D). Internal cellular structures in scavenger receptor$^+$ cells were visible as intensely stained compartments. There were significantly fewer scavenger receptor$^+$ APCs in the mesometrial triangle of diestrous mice compared to those of estrous mice ($p = 0.028$, Figures 3.4F and 3.5C). A similar number of scavenger receptor$^+$ cells was observed in the myometrium of uteri from diestrous and estrous mice (Figure 3.2 and Table 3.2).

After ovariectomy, there were very few scavenger receptor$^+$ cells in the endometrium (Figure 3.6C). The median % positivity at this time was reduced to zero, and overall the densities were significantly lower than those in uteri of both diestrous and estrous mice ($p = 0.003$, Figure 3.3C). Similarly, scavenger receptor$^+$ cells were undetectable in the mesometrial triangles in uteri of ovariectomised mice (Figure 3.6C), and thus their density was significantly lower than that density at estrus ($p = 0.05$) but similar to the density at diestrus ($p = 0.46$, Figure 3.5C). Consistent with this pattern, the number of scavenger receptor$^+$ cells also appeared reduced in the myometrium after ovariectomy (Figure 3.6C and Table 3.2).

b. effect of early pregnancy

Compared to estrous values, the density of scavenger receptor$^+$ APCs in the endometrium increased significantly after mating (median % positivity increased by 2.8-fold, $p = 0.038$, Figure

3.3C). Labeled cells were large and elongated, and contained intensely labeled internal features (Figure 3.7C), were found distributed densely throughout the endometrium (Table 3.1) and occasionally could be seen extending processes between epithelial cells towards the luminal cavity. The number of scavenger receptor$^+$ cells in the mesometrial triangle also increased significantly after mating (median % positivity increased by 3-fold compared to estrus, $p = 0.028$, Figure 3.5C) and the cells were large with an elongated morphology (Figure 3.4G). The number of scavenger receptor$^+$ APCs was increased in the myometrium of uteri of day 1 pregnant mice compared to the number observed at estrus (Figure 3.7C and Table 3.2).

By day 4 of pregnancy, the densities of scavenger receptor$^+$ APCs in the endometrium were very significantly reduced compared to day 1 ($p = 0.002$) and had indeed returned to levels similar to those observed during the estrous cycle ($p > 0.17$ compared to diestrous and estrous values, Figures 3.3C and 3.7D). There were effectively no scavenger receptor$^+$ APCs in the mesometrial triangles of day 4 pregnant mice ($p = 0.002$ compared to day 1, Figures 3.4H and 3.5C) and similarly the number of myometrial scavenger receptor$^+$ cells was diminished at day 4 compared to day 1 (Figure 3.7D and Table 3.2).

3.2.5 Expression of sialoadhesin in uterine APCs

APCs expressing sialoadhesin were detected in cycling, ovariectomised and pregnant uteri by indirect immunohistochemical analysis using the 3D6 mAb (van den Berg *et al.* 1992). This mAb detects bone marrow Mϕs and interstitial Mϕs, but not monocytes or Mϕs contained within tissue spaces such as the alveoli of the lung or the peritoneal cavity (Crocker and Gordon 1986).

a. effect of estrous cycle and ovariectomy

At estrus, APCs expressing sialoadhesin were abundant in the endometrium, and were of a large, rounded morphology with occasional fine dendrites, and labeled internal structures were also visible (Figure 3.2E). The cells were found distributed throughout superficial and deep endometrial tissue (Table 3.1). In the mesometrial triangle of uteri from estrous mice, sialoadhesin$^+$ APCs were of a similar morphology to those in the endometrium (not shown), and were present at a relatively low density (Figure 3.5D). Some sialoadhesin$^+$ APCs were also found in the myometrium at estrus (not shown).

The density of sialoadhesin$^+$ APCs in the endometrium was significantly higher at diestrus compared to estrus (compared to estrus, median % positivity was 2-fold higher at diestrus, $p = 0.04$, Figure 3.3D). The cells were small and compact with fine dendritic processes

and labeled internal structures (not shown), and were distributed densely throughout the superficial and deep endometrium (Table 3.1). Sialoadhesin$^+$ APCs were present at high densities in the mesometrial triangles of 2/6 diestrous mice, but in the majority labeled cells were rare ($p = 0.42$ compared to the mesometrial triangle of estrous mice, Figure 3.5D). In the myometrium of uteri from diestrous mice, sialoadhesin$^+$ APCs were observed at similar numbers to those seen at estrus (Table 3.2).

After ovariectomy, the density of sialoadhesin$^+$ cells in the endometrium was significantly reduced compared to cycling densities (median % positivity decreased by >9-fold, $p < 0.011$, Figure 3.3D). The cells were small and exhibited fine dendritic processes of varied labeling intensities (Figure 3.6D). In the mesometrial triangle following ovariectomy, sialoadhesin$^+$ cells had a similar morphology (Figure 3.6D) and were significantly less dense than at estrus (median % positivity was >70% lower than at estrus, $p = 0.025$) but of similar density to that at diestrus ($p = 0.519$, Figure 3.5D). Sialoadhesin$^+$ cells in the myometrium after ovariectomy had a similar morphology to those in the endometrium, and the numbers were reduced compared to the numbers in the myometrium observed during the estrous cycle (Figure 3.6D and Table 3.2).

b. effect of early pregnancy

In the endometrium, the density of sialoadhesin$^+$ APCs increased significantly ($p = 0.045$) from estrus to day 1 (2-fold increase in median % positivity, Figure 3.3D). The cells were large, elongated and dendriform in appearance, with visibly labeled internal structures (not shown), and were densely distributed throughout the superficial and deep endometrium (Table 3.1). In the mesometrial triangle the density of sialoadhesin$^+$ APCs was similar at day 1 as was observed at estrus ($p = 0.521$, Figure 3.5D). The number of sialoadhesin$^+$ APCs in the myometrium was increased at day 1 compared to during the estrous cycle (Table 3.2).

By day 4 of pregnancy, the density of sialoadhesin$^+$ APCs in the endometrium had decreased significantly compared to day 1 (median % positivity decreased by approximately 50%, $p = 0.015$) to levels similar to those seen at estrus ($p = 0.57$, Figure 3.3D). The cells were small with dendrites visible extending between the nuclei of neighbouring cells (not shown) and were evenly distributed throughout the superficial and deep endometrial tissue (Table 3.1). Small clusters of sialoadhesin$^+$ cells were observed in the deep endometrium of 2/8 day 4 uteri (not shown). The density of sialoadhesin$^+$ APCs in the mesometrial triangle was reduced to a median % positivity of zero at day 4 ($p = 0.002$ compared to day 1, Figure 3.5D). The number of sialoadhesin$^+$ cells in the myometrium of day 4 pregnant mice had similarly diminished compared to day 1.

3.2.6 Expression of MHC class II (Ia) in uterine APCs

APCs expressing Ia were detected in cycling, ovariectomised and pregnant uteri by indirect immunohistochemical analysis using the TIB 120 mAb (Bhattacharya *et al.* 1981). This mAb detects the MHC class II antigen presentation molecule Ia, which is expressed in other tissues on dendritic cells, activated Mϕs and B cells (Inaba *et al.* 1997).

a. effect of estrous cycle and ovariectomy

At estrus, Ia^+ APCs in the endometrium were relatively sparse and had a small, rounded morphology (Figure 3.2E). They were distributed evenly in the superficial and deep endometrium (Table 3.1). In the mesometrial triangle of estrous mice, Ia^+ cells had a similar morphology (not shown) and were present at a similar density to those in the endometrium (Figure 3.5E). Some Ia^+ cells were also observed in the myometrium at estrus (Figure 3.2E).

At diestrus, Ia^+ cells were present in both the endometrium and the mesometrial triangle at a density which was similar to that observed at estrus (Figures 3.3E and 3.5E, $p > 0.3$). In the endometrium the cells were small and round, and the majority were located in the deep endometrium just under the myometrial interface (Figure 3.2F, Table 3.1). The cells in the mesometrial triangle of uteri collected from diestrous mice were difficult to visually distinguish from eosinophils (not shown). A similar number of Ia^+ cells was observed in the myometrium at diestrus as was observed at estrus (Figure 3.2F and Table 3.2).

After ovariectomy, Ia^+ cells were retained within the superficial and deep endometrium (Table 3.1) and mesometrial triangle, in densities that were not significantly different from those observed during the estrous cycle ($p > 0.3$, Figures 3.3E and 3.5E). Similarly, the number of Ia^+ cells in myometrium was also retained after ovariectomy (Table 3.2). In all three locations the cells were very intensely labeled and had an elongated, dendriform morphology (Figure 3.6E and F).

b. effect of early pregnancy

The density of Ia^+ cells in the endometrium was similar on day 1 to that observed at estrus (Figure 3.3E, $p = 0.309$) although the cells were of a larger morphology and were often found in clusters in close proximity to luminal epithelial cells (Figure 3.7E). In the mesometrial triangle of uteri from day 1 pregnant mice, the density of Ia^+ cells was slightly lower than that observed at estrus ($p = 0.070$ compared to estrus, Figure 3.5E). The number of myometrial Ia^+ cells appeared to be moderately enhanced after mating (Figure 3.7E and Table 3.2), although this was not quantified.

By day 4 of pregnancy, Ia^+ cells were retained within the endometrium at a similar density as was observed at estrus and day 1 ($p = 0.309$, Figure 3.3E). However, morphologically the cells were different, having a smaller, more rounded appearance and exhibited a comparably weak intensity of labeling. Clusters of Ia^+ cells were present in the deep endometrium of 6/8 uteri at day 4 of pregnancy (Figure 3.7F), although the cells were also found in the superficial endometrium (Table 3.1). In mesometrial triangle at day 4 of pregnancy, the density of Ia^+ cells was significantly reduced compared to the density at estrus and after ovariectomy (80-90% decrease in median % positivity, $p < 0.004$) and reduced slightly but not significantly compared to the density at day 1 ($p = 0.098$, Figure 3.5E). The Ia^+ cells were difficult to distinguish from eosinophils in the mesometrial triangle at day 4 (not shown). At day 4, the number of Ia^+ cells in the myometrium was maintained from day 1 of pregnancy (Figure 3.7F and Table 3.2).

3.2.7 Expression of B7-2 in uterine APCs

APCs expressing B7-2 were detected in cycling, ovariectomised and pregnant uteri by indirect immunohistochemical analysis using the mAb GL-1 (Freeman *et al.* 1993). B7-2 is expressed by activated dendritic cells and Mϕs in mucosal tissues, particularly after immune or other stimuli (Croft and Dubey 1997).

a. effect of estrous cycle and ovariectomy

None of the uteri collected from estrous mice showed any detectable B7-2^+ cells in the endometrium or the mesometrial triangle (median % positivity in both compartments was zero, Figures 3.3F and 3.5F). Very occasional B7-2^+ cells were observed in superficial endometria of 3/8 diestrous mice (not shown) but overall the densities remained similar to those seen at estrus ($p = 0.062$, Figure 3.5F). In the mesometrial triangle, the density of B7-2^+ cells was significantly higher at diestrus than at estrus ($p = 0.019$, Figure 3.5F). At diestrus, B7-2^+ cells in the endometrium and the mesometrial triangle were very weakly labeled and of a compact, dendriform morphology (not shown). B7-2^+ cells were also very sparse in the myometrium at both estrus and diestrus (Table 3.2).

After ovariectomy, only 1/5 mice had B7-2^+ cells detectable in the endometrium and thus the density was similar to that at estrus and diestrus ($p > 0.24$, Figure 3.3F). Of the mesometrial triangles of uteri collected from ovariectomised mice, 5/5 contained B7-2^+ cells, and the density of these cells was significantly greater than at estrus ($p = 0.005$, Figure 3.5F). The myometrium after ovariectomy also contained few B7-2^+ cells (Table 3.2) and in all three compartments the

few B7-2$^+$ cells that were identifiable were very weakly stained and of a dendriform morphology (not shown).

b. effect of early pregnancy

All uteri collected from day 1 pregnant mice had detectable B7-2$^+$ APCs present in the endometrium, and the density of these cells was significantly higher than the densities seen at all other times ($0.001 < p < 0.005$, Figure 3.3F). The B7-2$^+$ cells were weakly stained, elongated cells (Figure 3.7G) and were preferentially distributed in the superficial endometrium (Table 3.1). There were B7-2$^+$ cells detectable in the mesometrial triangles of only 2/5 uteri collected from day 1 pregnant mice (not shown), and thus the densities were not significantly different from those at estrus ($p = 0.131$, Figure 3.5F). In the myometrium of day 1 pregnant mice, B7-2$^+$ cells were rare (Figure 3.7G and Table 3.2).

On day 4 of pregnancy, only 1/7 uteri contained B7-2$^+$ cells in the endometrium (Figure 3.7H) and thus the density of these cells was significantly lower than that seen at day 1 ($p = 0.004$, Figure 3.3F). In the mesometrial triangle on day 4, 5/6 uteri showed B7-2$^+$ cells (not shown) but their density was not significantly greater than that seen in the mesometrial triangle of day 1 pregnant mice ($p = 0.131$, Figure 3.5F). B7-2$^+$ cells were only occasionally observed in the myometrium of uteri of day 4 pregnant mice (Figure 3.7H and Table 3.2).

3.2.8 Endogenous peroxidase activity in uterine eosinophils

Eosinophils in sections of cycling, ovariectomised and pregnant uteri were detected by virtue of their endogenous peroxidase activity.

a. effect of estrous cycle and ovariectomy

Ovarian steroid hormones had significant impact on the number and distribution of eosinophils in the endometrium and the mesometrial triangle. At estrus, eosinophils were distributed throughout the endometrium (Table 3.1) but very few were contained in the mesometrial triangle or the myometrium (Figure 3.8C). Compared to estrus, the density of eosinophils was significantly lower in the endometrium of uteri from diestrous mice ($p = 0.001$, Table 3.3), and significantly higher in the mesometrial triangle ($p = 0.001$, Table 3.3). The number of eosinophils in the myometrium of diestrous mice was also increased compared to the myometrium of estrous mice (Figure 3.8B and Table 3.2). Uteri of ovariectomised mice contained significantly fewer eosinophils in the endometrium and mesometrial triangle compared

to diestrous and estrous uteri ($p < 0.03$, Table 3.3). Similarly, eosinophils were also diminished in the myometrium after ovariectomy (Figure 3.8A and Table 3.2).

Table 3.3 The effect of ovariectomy, stage of estrous cycle and day of early pregnancy on the density of eosinophils in the endometrium and the mesometrial triangle. The densities of endogenous peroxidase$^+$ eosinophils in the endometrium (endo) and mesometrial triangle (mt) in fresh frozen sections of uteri collected from estrous, diestrous, ovariectomised (ovx), day 1 and day 4 pregnant mice were quantified by video image analysis, and are shown as median (range). Data were compared by Kruskal-Wallis one-way ANOVA and Mann-Whitney Rank Sum Test. Groups annotated with different lower case letters are significantly different for the given mAb within the endometrium or the mesometrial triangle ($p < 0.04$).

	stage of estrous cycle or day of pregnancy				
	ovx	*diestrus*	*estrus*	*day 1*	*day 4*
endo	[a]0 (0-1.8)	[b]4.5 (2.2-6.1)	[c]17.1 (7.1-23.0)	[d]22.1 (13.8-30.1)	[b]4.0 (3.1-5.1
mt	[a]2.0 (1.2-3.1)	[b]27.1 (13.0-42.1)	[c]3.2 (1.6-5.1)	[c]6.1 (2.3-8.9)	[b]19.1 (17.2-33.5)
n / group	*6*	*8*	*8*	*6*	*8*

b. effect of early pregnancy

Relative to estrus, the density of endometrial eosinophils increased significantly after mating ($p = 0.033$, Table 3.3), and the cells became preferentially distributed in the superficial endometrium (Table 3.1). There was a small, non-significant increase in the density of eosinophils in the mesometrial triangle from estrus to day 1 ($p = 0.071$, Table 3.3). The number of eosinophils in the myometrium also increased at day 1 compared to estrus (Figure 3.8 D and Table 3.2). By day 4 of pregnancy, the distribution and number of eosinophils in the endometrium, the mesometrial triangle and the myometrium was similar to that observed in diestrous uteri (Figure 3.8 E, Table 3.3 and Table 3.2).

3.3 Macrosialin expression in uterine epithelial cells

The well characterised mAb FA11 is specific for the Mϕ-lineage surface and endosomal glycoprotein macrosialin, and it detects the majority of Mϕs in lymphoid and peripheral organs (Rabinowitz and Gordon 1991). A peculiar feature of FA11 detected in the current studies is that it also bound to uterine epithelial cells. This unexpected labeling occurred at a concentration of FA11 optimised to detect Mϕs in spleen and uterus: any further dilution resulted in a dose-dependent decrease in the intensity of labeling on both Mϕs and epithelial cells in the uterus (not shown). In order to determine the effect of ovarian steroid hormones and day of early pregnancy

on FA11 labeling of uterine epithelial cells, the slides already prepared for detection of macrosialin$^+$ APCs were examined.

3.3.1 Effect of estrous cycle, ovariectomy and early pregnancy

The reactivity of uterine epithelial cells with the mAb FA11 was evident in the uteri of estrous mice as a uniform, granular staining (Figure 3.9A). The staining of epithelial cells in uteri from diestrous (Figure 3.9B) and ovariectomised (Figure 3.6B) mice was considerably less intense.

Luminal epithelial cells in uteri from day 1 pregnant mice were labeled intensely, and indeed were comparable in intensity to endometrial Mϕs in the same sections (Figure 3.9C). The labeling was most evident in the cell membrane but also within the cytoplasm at the basal and luminal extremities of the epithelial cells, and was granular in appearance. It did not co-localise with neutrophils (identified with the mAb RB6-8C5) which traffic through the epithelial cells at this time (Figure 3.9D). By day 4 of pregnancy, the FA11-reactivity in uterine epithelial cells was very weak or undetectable even though macrosialin$^+$ endometrial Mϕs were still evident (Figure 3.9E).

3.3.2 Expression patterns in other epithelial surfaces

In order to confirm the specificity of the FA11 mAb to the Mϕ lineage, the indirect immunoperoxidase technique using the mAb FA11 was applied to a range of mucosal and epithelial tissues collected from estrous (n = 2) and day 1 mated (n = 2) mice. FA11 mAb did not label epithelial cells in skin, large intestine, lung alveoli, trachea, ovary or vagina, yet it did detect stromal Mϕ-like cells in each of these tissues (large intestine shown in Figure 3.9F). Some weak labeling similar to that observed in uterine epithelia was seen in epithelial cells of the isthmus of the oviduct (not shown).

3.3.3 Expression by trypsin/pancreatin harvested uterine epithelial cells

The propensity of the FA11 mAb to bind to the luminal aspect of uterine luminal epithelial cells was suggestive of binding to the glycocalyx, an extra-cellular layer of glycoproteins and glycolipids secreted by the epithelial cells (Chavez and Anderson 1985). In order to investigate this hypothesis, epithelial cells were stripped of their glyocalyx by trypsin/pancreatin digestion of day 1 uteri (see section 2.4.2b), and cytosmears of the mixed uterine cell preparations were labeled with FA11 using the indirect immunoperoxidase technique.

Even after this rather harsh enzymatic treatment, the sheets of released epithelial cells were still labeled, as were individual Mϕ-like cells evident amongst the epithelial cells (Figure 3.9G). The finding also suggested that epithelial cell expression of macrosialin occurs on the surface of the cytoplasmic membrane, since the cytosmeared cells were not permeabilised prior to staining.

3.3.4 Expression of macrosialin mRNA by uterine epithelial cells

In order to investigate whether epithelial cells might were not sequester cell surface macrosialin after synthesis by endometrial Mϕs, reverse transcriptase polymerase chain reaction (RT-PCR) analysis was used to measure the macrosialin mRNA content of uterine epithelial cells.

Primers for murine macrosialin and leukocyte common antigen (LCA) were designed with the aid of 'Primer Designer' software (see section 2.13.7). Primers for actin cDNA were also used to confirm successful RNA extraction and first strand synthesis by reverse transcription.

RNA was prepared (see section 2.13.3) from > 98% pure preparations of MTS#24$^+$ uterine epithelial cells collected by miniMACS purification (see section 2.6.3) after collagenase/DNAse digestion (see section 2.4.2a) of 5 pooled uteri recovered from day 1 pregnant mice. Complimentary DNA (cDNA) was prepared by reverse transcription using oligo-dT primers. cDNA from peritoneal and spleen cells was also prepared to determine the optimal PCR conditions for the primer pairs, and to act as a positive control.

Epithelial cells collected from the uteri of day 1 pregnant mice were found to contain macrosialin mRNA, since PCR of the epithelial cell cDNA preparation using the macrosialin primers yielded an amplicon of the expected size of 243 base pairs. This band was indistinguishable from the product obtained from PCR using the same primers with cDNA preparations derived from mRNA of peritoneal and spleen cells (both rich sources macrosialin$^+$ Mϕs). PCR of a replicate sample of the uterine epithelial cell cDNA using primers specific to the leukocyte-specific protein LCA confirmed that leukocytes did not contaminate the epithelial cell preparation (Figure 3.10). In preliminary experiments using comparable PCR parameters these primers were found to detect <1:1000 lymphocytes spiked into fibroblast cell suspensions (data not shown).

3.4 Conclusions and discussion

The studies presented in this chapter confirm and extend previous studies describing the existence of dynamic populations of cells expressing APC markers in the murine uterus. Abundant and heterogeneous populations of uterine stromal cells were found to express cell membrane markers suggestive of their functional capability as APCs in the uterine milieu. The numbers, morphologies and distributions of these APCs were found to fluctuate differentially over the estrous cycle and during pregnancy. Furthermore, a steroid-hormone independent population of APCs was identified within the uteri of ovariectomised mice. Together, these findings indicate that Mϕs and/or other leukocytes in the uterus are likely to have APC function and suggest that different populations of uterine APCs are differentially susceptible to changes in the uterine microenvironment. The studies also provide some preliminary evidence of a role for uterine epithelial cells in antigen uptake and/or processing.

As well as the commonly employed pan-Mϕ marker F4/80, the panel of mAbs selected for use in these experiments was deliberately chosen to target molecules involved in phagocytosis and endocytosis, antigen processing and antigen presentation. Abundant populations of cells expressing scavenger receptor and macrosialin were detected, and this is consistent with data showing that *in vivo* uterine Mϕs possess endocytic capacity during the estrous cycle (Stewart and Mitchell 1991). Other authors have detected cells expressing these antigens in the uterus during mid-late pregnancy (Brandon 1995, Kyaw *et al.* 1998), but their presence during the estrous cycle and pre-implantation stages of pregnancy has not been described previously. Cells expressing sialoadhesin, a cell:cell adhesion molecule (van den Berg *et al.* 1992, Muerkoster *et al.* 1999) were also abundant in the uterus. Expression of Ia and the costimulatory molecule B7-2 (the latter of which was more limited than the other antigens) suggests that uterine APCs might have the capacity to participate in events of antigen presentation and T-cell activation.

During the estrous cycle, the recruitment and maintenance of F4/80$^+$ Mϕs within the uterine stroma appears to be controlled largely by uterine epithelial cell-derived CSF-1 (De and Wood 1990, Wood *et al.* 1992), although an array of other cytokines synthesised within the uterus may play a similar or synergistic role (Robertson *et al.* 1994, Hunt and Robertson 1996). The peak expression of most of these molecules at the proestrous and estrous phases of the cycle due to the stimulatory actions of estrogen (Wood *et al.* 1992) correlates temporally with the accumulation of large, dendriform F4/80$^+$ cells in the endometrium immediately subjacent to epithelial cells, as has been described in the current and previous studies (Wood *et al.* 1992, Pollard *et al.* 1998). It would appear from the current study that uterine APCs expressing

macrosialin, scavenger receptor and sialoadhesin are similarly regulated in terms of their abundance and location within the endometrium, as is a smaller population of cells expressing Ia. Cells expressing B7-2 were very rare in cycling uteri and their intensity of antigen expression comparably weak.

The quantitative data shown here did not accord with the diminution of total endometrial Mϕs at the diestrous phase of the cycle reported previously by others (Wood *et al.* 1992). This apparent discrepancy may be attributed to strain differences (Griffith *et al.* 1997), differences in diagnosis of stages of the estrous cycle or differences in the method by which quantification was performed. Our studies controlled for the presence of the dynamic population of eosinophils in the uterus by subtracting the densities of endogenous peroxidase$^+$ cells from the densities of immunohistochemically-labeled cells. This method may have inadvertently diminished the real density of APCs at estrus since eosinophils and APCs tend to co-localise and physically overlap in the superficial endometrium at this time. In addition, the higher densities observed at diestrus are likely to be a consequence of the relatively diminished cellularity of the uterus at this stage of the cycle, and might not reflect actual increases in the absolute number of cells. Other authors use point counting techniques (Pollard *et al.* 1998) which rely on the ability of the operator to visually discriminate individual labeled APCs from each other and from eosinophils.

Of less contention is the flux in morphological phenotype of Mϕs across the different stages of the estrous cycle. In contrast to the large, dendriform cells seen at estrus, the current study and others (Wood *et al.* 1997, Pollard *et al.* 1998) show that at diestrus the uterine APCs are often smaller and rounded. This too is likely to be attributable to steroid hormone-regulated fluctuations in local concentrations of CSF-1 (Wood *et al.* 1992), GM-CSF (Robertson *et al.* 1996b), IL-1, IL-6, TNFα (De *et al.* 1992, Hunt *et al.* 1996) and IFNγ (Platt and Hunt 1998), the peak expression of which at proestrus and estrus could be expected to promote Mϕ differentiation and activation (Rutherford *et al.* 1993, Gordon *et al.* 1995). Steroid hormones may also act directly on Mϕs (Hunt and Robertson 1996, Miller and Hunt 1996), and a particular role for progesterone as a powerful 'negative' regulator of these cells appears likely (Miller *et al.* 1996, Hunt *et al.* 1998). Interestingly, macrosialin$^+$ APCs at diestrus contained internal structures which were reminiscent of tingible-body Mϕs of the spleen which are implicated in the removal of dead and dying cells (Rabinowitz and Gordon 1991). Perhaps this reflects a role for Mϕs in phagocytosis of debris generated during tissue remodeling at the diestrous phase of the cycle. In particular, the location of these cells suggests a role in ingestion of epithelial cells.

Insemination in the mouse results in the luminal cavity becoming filled with the ejaculate. Seminal vesicle derived TGFβ$_1$ (Robertson *et al.* 1997) has been shown to trigger the synthesis

of large quantities of GM-CSF and IL-6 by steroid hormone-primed uterine epithelial cells (Robertson and Seamark 1990, Robertson *et al.* 1992), and dramatically upregulated synthesis of chemokines RANTES, MIP-1α, MIP-1β and MCP-1 also occurs at this time (Robertson *et al.* 1997, Wood *et al.* 1997, Pollard *et al.* 1998). An immediate consequence is the recruitment into the sub-epithelial endometrial stroma of large numbers of neutrophils (De and Wood 1991, Choudhuri and Wood 1992, Pollard *et al.* 1998) and eosinophils (McMaster *et al.* 1992, Robertson *et al.* 1996, Robertson *et al.* 1998). Of particular interest in the current study was the strikingly high content of APCs, in particular F4/80$^+$ Mϕs in the day 1 pregnant uterus. The quantitative data presented here confirms similar observations of F4/80-reactive cells reported previously (De and Wood 1991, Choudhuri and Wood 1992, De *et al.* 1993, Robertson *et al.* 2000).

The roles of the abundant populations of Mϕs in the day 1 pregnant uterus are not known, but the studies presented here allow some speculations to be made. For example, the marked and significant increase in the density of scavenger receptor$^+$ and macrosialin$^+$ cells seen at day 1 of pregnancy would suggest that at this time uterine APCs are capable of binding, internalising and processing ligands with polyanionic (Fraser *et al.* 1993, Hughes *et al.* 1995) and lectin moieties (Rabinowitz and Gordon 1991), which might include bacteria or indeed sperm cells (Eksittikul and Chulavatnatol 1980, Kaneko *et al.* 1984). Scavenger receptor could also play a role in mediating binding between APCs and extra-cellular matrix molecules within the local environment (Fraser *et al.* 1993, Hughes *et al.* 1995). A role for locally synthesised CSF-1 and GM-CSF in regulating the expression of macrosialin and scavenger receptor expression on uterine Mϕs would seem likely given these regulatory mechanisms have been shown to exist *in vitro* (Guidez *et al.* 1998, Li *et al.* 1998). Other cytokines synthesised in abundance in the day 1 pregnant uterus which might activate uterine Mϕ populations include IL-6, IL-1 and TNFα (Robertson *et al.* 1992, Sanford *et al.* 1992).

Unexpectedly, uterine epithelial cells were also found to express macrosialin, the intensity of which peaked during the cycle at estrus and then was further upregulated after mating. Expression of macrosialin in uterine or other epithelia has not been reported previously, and clearly this requires further investigation. However, the concept of epithelial cell involvement in behaviours usually limited to professional APCs is not novel. Under inflammatory conditions, epithelia of the murine and human intestine can express classical (MHC class II) and non-classical (CD1) molecules of antigen-presentation, as well as a host of co-stimulatory molecules (Mayer 1997). Thus epithelial cells might also have the capacity to bind and internalise ligands in the uterine lumen. Indeed, rodent uterine epithelium has

previously been shown to be capable of endocytic activity (Tung *et al.* 1988, Carballada and Esponda 1997). Furthermore, preliminary data from co-culture experiments in our laboratory suggest that, as in other mammals (Racey *et al.* 1987) murine sperm can bind to uterine epithelial cells (data not shown). Whether uterine epithelium could then process and present sperm antigens, or transfer them to professional APCs in the endometrium is unknown.

As well as antigen uptake and processing, the APCs in day 1 uteri appear well endowed to perform antigen presentation and co-stimulation by virtue of their expression of Ia, B7-2 and sialoadhesin. Cell surface expression of Ia with bound antigen generates the specific ligand required for APC interaction with the TCR on $CD4^+$ T lymphocytes, and B7-2 is one of many costimulatory molecules thought to stabilise this interaction and provide activation signals to the T cell (Croft and Dubey 1997, Greenfield *et al.* 1998). Although the exact role of sialoadhesin in the APC:T cell interaction is not known, recent evidence suggests that high expression of sialoadhesin on Kupffer cells of the liver correlates with the ability of these cells to process and present exogenous antigens via class I MHC to $CD8^+$ T cells, and via class II MHC to $CD4^+$ T cells through stable, "clustering" interactions with these lymphocytes (Muerkoster *et al.* 1999).

By day 4 of pregnancy, the microenvironmental features of the uterus are markedly different to those seen on day 1, largely due to the influence of rising levels of serum progesterone. Compared to the day 1 uterus, the profile of molecules produced by epithelial cells appears considerably less inflammatory in nature, with lower expression of GM-CSF (Robertson *et al.* 1996a), CSF-1, IL-1α, IL-1β, TNFα (McMaster *et al.* 1992, Sanford *et al.* 1992) and chemokines (Robertson *et al.* 1998) on days 3 and 4 of pregnancy. In parallel, the number of granulocytes and $F4/80^+$ Mϕs in the endometrium are significantly reduced in number (De and Wood 1991, Stewart and Mitchell 1991, Brandon 1993, De *et al.* 1993a, Wood *et al.* 1997, Pollard *et al.* 1998). The current study extends these observations, and shows that uterine APCs as measured by a range of activation markers are significantly reduced in number by day 4 of pregnancy, and those that are retained are small and preferentially located in the deep endometrial tissues.

Interestingly, small clusters of cells expressing APC markers were observed deep in the endometrium on day 4 of pregnancy. Other authors have described aggregates of a similar appearance comprised of leukocytes and T lymphocytes in the uteri of day 4 pregnant mice and rats (Lobel *et al.* 1967, Brandon 1993). Whether these cells are involved in active antigen presentation or restimulation of memory lymphocytes has not been examined. Similarly, aggregates of $CD8^+$ T lymphocytes, B cells and Mϕs are present in the endometrium of cycling women (Yeaman *et al.* 1997, Yeaman *et al.* 1998).

The cells labeled with the mAbs employed in these studies have been referred to as uterine APCs since although markers such as F4/80, macrosialin, scavenger receptor and sialoadhesin were originally described as expressed by Mϕs, it is well recognised that these molecules can also be expressed by dendritic cells. Indeed, Ia and B7-2 are associated predominantly with dendritic cells in many mucosal organs. The issue of precisely whether Mϕs or dendritic cells or both were detected in these studies is difficult to ascertain on the basis of immunohistochemical data alone since only single labeling protocols were employed. While F4/80, macrosialin and sialoadhesin appeared to be expressed by similar numbers of cells, APCs expressing scavenger receptor and Ia during the estrous cycle were clearly lower in density than $F4/80^+$ cells, suggesting that at least some of the $F4/80^+$ cells did not express these latter markers. Interestingly, after mating the density of scavenger $receptor^+$ cells became similar to that of F4/80, allowing for the proposal that there is a degree of up-regulation of scavenger receptor on $F4/80^+$ cells on day 1 of pregnancy. This could be achieved through the recruitment of scavenger $receptor^+$ cells at day 1, or upregulation of scavenger receptor expression on resident $F4/80^+$ cells, perhaps in response to increases in local concentrations of GM-CSF (Guidez *et al.* 1998) or CSF-1 (de Villiers *et al.* 1994).

Better evidence for the existence of sub-populations of uterine APCs was provided by analysis of ovariectomised uteri. Consistent with other reports (De and Wood 1990, Wood *et al.* 1992), the density of cells expressing all of the antigens examined was very significantly diminished by ovariectomy, presumably as a result of a cease in the synthesis of chemotactic molecules by uterine epithelial cells (Wood *et al.* 1997). However, steroid hormone-resistant populations of finely branched, dendriform cells expressing high levels of Ia and macrosialin still persisted in uteri from ovariectomised mice. Smaller populations of $F4/80^+$ and $sialoadhesin^+$ cells were also still present. In contrast, there was a paucity of $scavenger\ receptor^+$ and $B7\text{-}2^+$ cells in ovariectomised uteri.

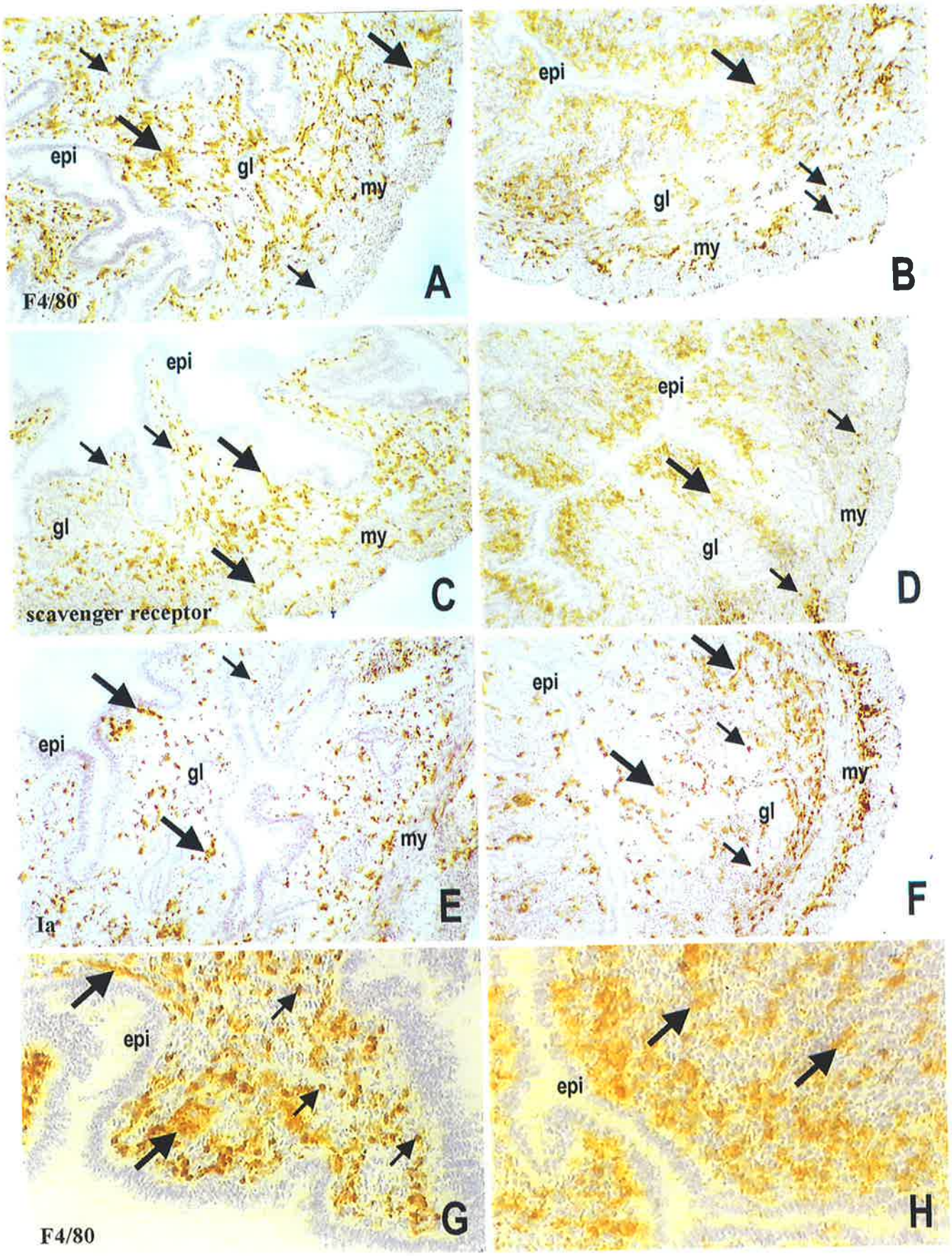
The morphological features and labeling profile of an APC population expressing high levels of Ia and macrosialin, lower levels of sialoadhesin and F4/80 and no scavenger receptor or B7-2 in ovariectomised uteri is consistent with the presence of a lineage of dendritic cells. Ia expression is constitutive and high on all populations of dendritic cells described thus far (Banchereau and Steinman 1998), and a population of $F4/80^-$, Ia^+ dendritic cells has been noted in the murine uterus (Robertson *et al.* 1998), with a similar population being found in the rat uterus (Head and Gaede 1986, Head *et al.* 1987). F4/80, macrosialin and sialoadhesin, while initially thought of as specific Mϕ markers, can also label sub-populations of dendritic cells (Rabinowitz and Gordon 1991, Hart 1997, Strobl *et al.* 1998, Berney *et al.* 1999). Thus it may

be that the steroid hormone-resistant APCs retained after ovariectomy represent a population of unique dendritic cell-like APCs. However, the abundance of these cells within the cycling and pregnant uterus is difficult to ascertain conclusively using single-colour immunohistochemistry without a dendritic cell-specific marker.

Further evidence of sub-populations of APCs within the uterus was provided by examination of the mesometrial triangle, since the current experiments support previous observations (De and Wood 1991, Pollard *et al.* 1991, Stewart and Mitchell 1992, Brandon 1995) of differential regulation of mesometrial and endometrial APC populations. In the current studies, abundant and stable population of F4/80$^+$ and macrosialin$^+$ APCs were evident in the mesometrial triangle of cycling mice, whereas cells expressing sialoadhesin, scavenger receptor, Ia and B7-2 were relatively scarce. After insemination, cells expressing F4/80, scavenger receptor, macrosialin and sialoadhesin were most abundant, perhaps due to activation of the resident F4/80$^+$ cells and/or trafficking of APCs to draining lymph nodes through lymphatic vessels in the region of the mesometrial triangle (Head and Lande 1983). Trafficking of Mϕs to draining lymph nodes from other inflamed sites is clearly evident (Lan *et al.* 1993, Thepen *et al.* 1993), and occurs within 48 h of an inflammatory stimulus (Bellingan *et al.* 1996). The paucity of Ia$^+$ and B7-2$^+$ cells at day 1 might reflect a delay in trafficking of dendritic cells since the trafficking of dendritic cells from the bacterially challenged lung is delayed for at least 48 hours from the time of immune challenge (McWilliam *et al.* 1994). Consistent with this speculation, some B7-2$^+$, macrosialin$^+$ and Ia$^+$ cells were evident in mesometrial triangle on day 4. Analysis of the expression of these markers in mesometrial triangle and draining lymph nodes on days 1, 2, 3 and 4 of pregnancy would clarify this issue.

In conclusion, the studies presented in this chapter illustrate that dynamic and diverse populations of APCs are present within the cycling and pregnant murine uterus. In terms of their expression of many phenotypic markers, these cells appear to possess the capacity to perform many of the roles which confer professional APCs with their potency in generating immune responses to antigens at epithelial surfaces. In the uterus, this capacity appears to be maximal at day 1, when introduction of the ejaculate poses a considerable antigenic challenge to the uterine environment. Heterogeneity in the populations of uterine APCs expressing the phenotypic markers was evident, suggesting that both Mϕs and dendritic cells are contained within the cycling and pregnant uterus.

Figure 3.2 APCs in the endometrium and myometrium at estrus and diestrus. Fresh frozen sections of uteri collected from estrous (A, C, E, G) and diestrous (B, D, F, H) mice were labeled with an indirect immunoperoxidase technique using mAbs specific for F4/80 antigen (A, B, G, H), class A scavenger receptor (C, D) and Ia (E, F). Immunoperoxidase-labeled APCs are evident at low (x25; A-F) or high (x50; G, H) magnification in the endometrium, and in the myometrium (my) as large-medium sized dendriform cells (large arrows). Endogenous peroxidase$^+$ eosinophils are evident as small, dark brown cells in the endometrium and myometrium (small arrows). epi = luminal epithelium, gl = glandular epithelium.



estrus

diestrus

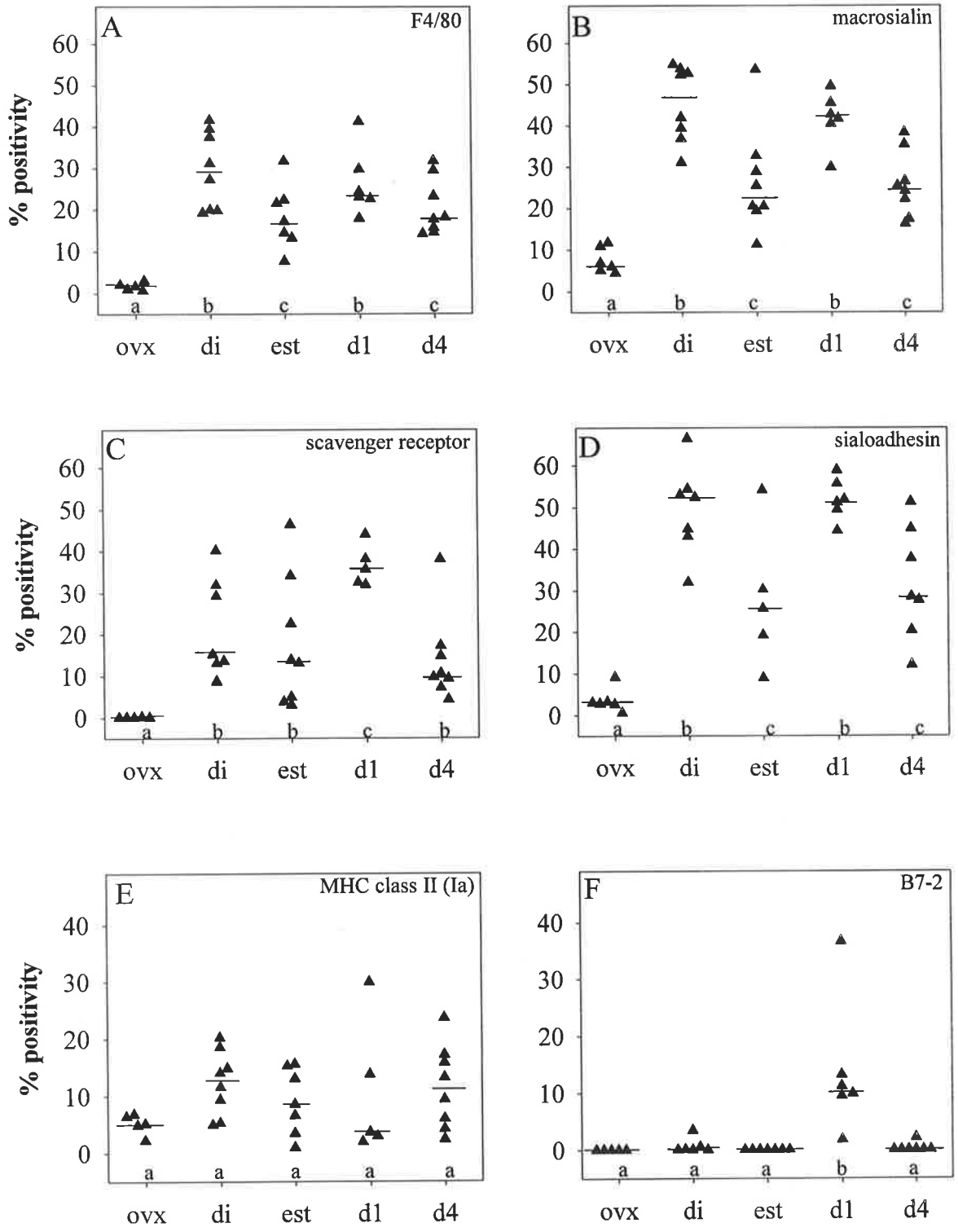
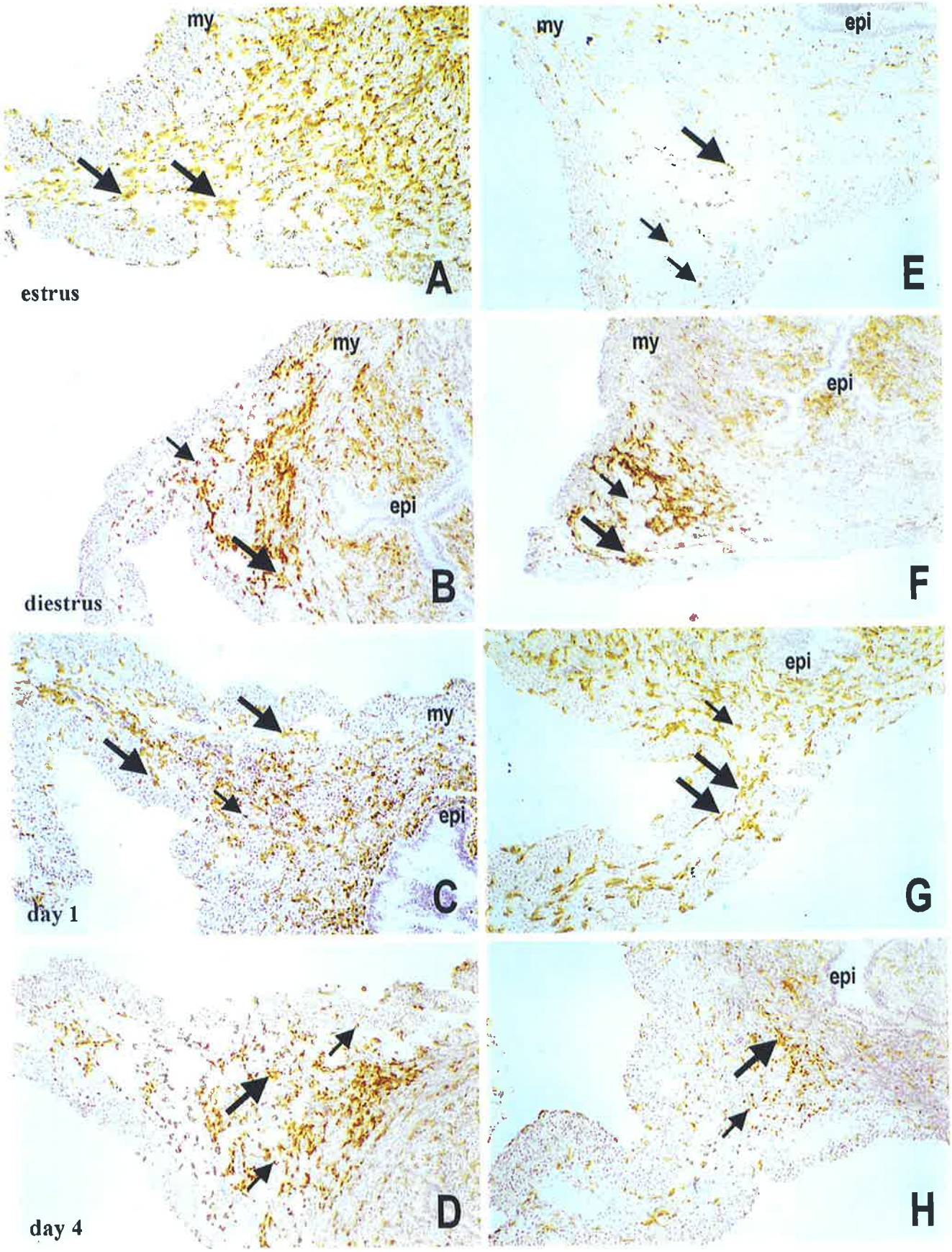


Figure 3.3 The effect of stage of estrous cycle, ovariectomy and day of early pregnancy on the density of APCs in the endometrium. Fresh frozen sections of uteri collected from estrous (est), diestrous (di), ovariectomised (ovx), day 1 pregnant (d1) and day 4 pregnant (d4) mice were labeled with an indirect immunoperoxidase technique using mAbs specific for F4/80 antigen (A), macrosialin (B), class A scavenger receptor (C), sialoadhesin (D), Ia (E) and B7-2 (F). The density of immunoreactive cells in the endometrium was quantified by video image analysis. Symbols represent the mean density (% positivity) of immunoreactive cells in individual uteri, and median values for each group are scored. Data were compared by Kruskal-Wallis one-way ANOVA and Mann-Whitney Rank Sum Test. Groups with different lower case letters on the x axis are significantly different for the given mAb ($p < 0.05$).

Figure 3.4 F4/80$^+$ and class A scavenger receptor$^+$ APCs in the mesometrial triangle during the estrous cycle and in early pregnancy. Fresh frozen sections of uteri collected from estrous (A, E), diestrous (B, F), day 1 pregnant (C, G) and day 4 pregnant (D, H) mice were labeled with an indirect immunoperoxidase technique using mAbs specific for F4/80 antigen (A-D) and scavenger receptor (E-H). Immunoperoxidase-labeled APCs are evident in the mesometrial triangle at estrus and day 1 as large, dendriform cells (large arrows). APCs were partially obscured in the mesometrial triangle at diestrus and day 4 due to the presence of abundant endogenous peroxidase$^+$ eosinophils (small arrows). epi = luminal epithelium, my = myometrium. x25 magnification.



F4/80

scavenger receptor

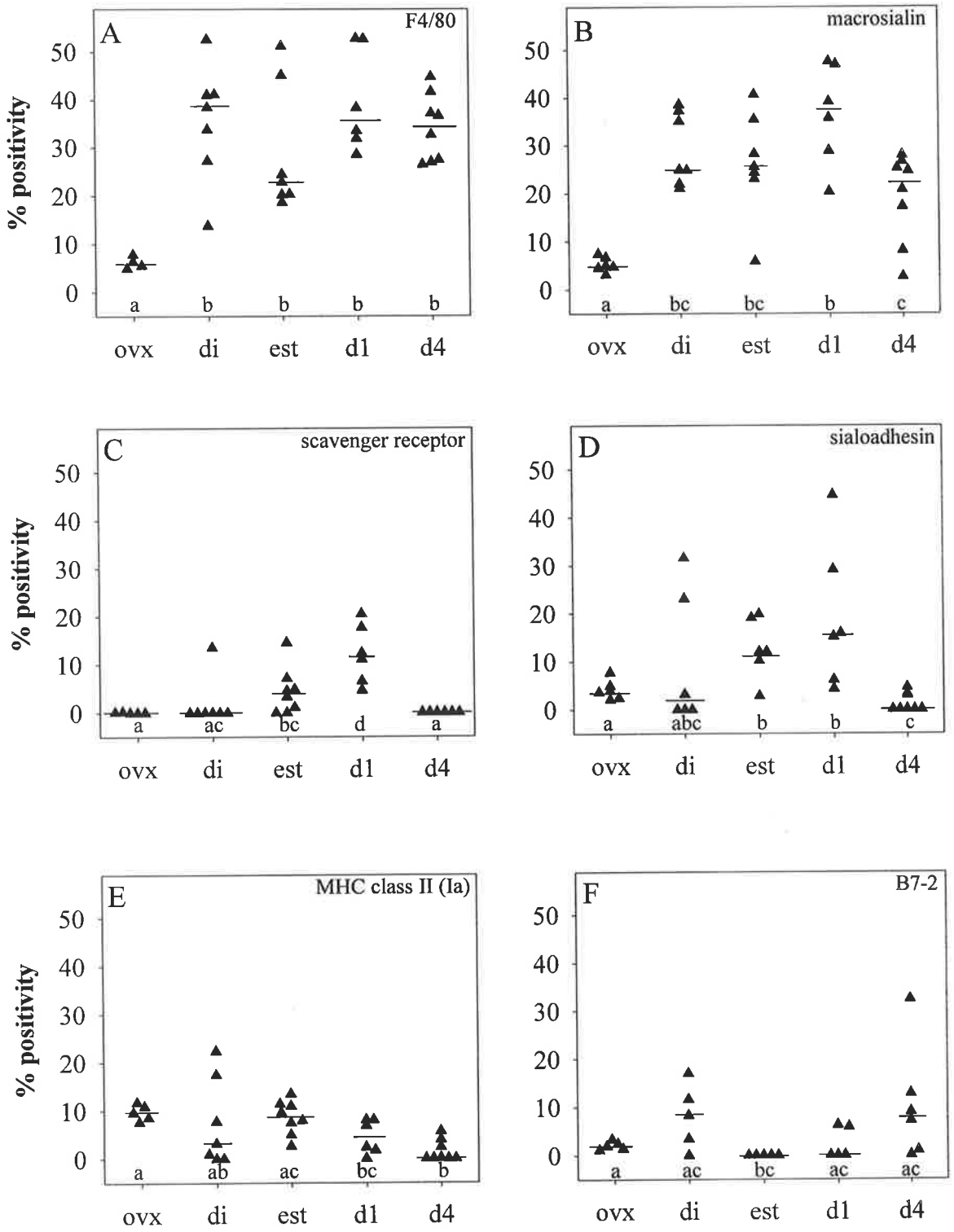


Figure 3.5 The effect of stage of estrous cycle, ovariectomy and day of early pregnancy on the density of APCs in the mesometrial triangle. Fresh frozen sections of uteri collected from estrous (est), diestrous (di), ovariectomised (ovx), day 1 pregnant (d1) and day 4 pregnant (d4) mice were labeled with an indirect immunoperoxidase technique using mAbs specific for F4/80 antigen (A), macrosialin (B), class A scavenger receptor (C), sialoadhesin (D), Ia (E) and B7-2 (F). The density of immunoreactive cells in the mesometrial triangle was quantified by video image analysis. Symbols represent the mean density (% positivity) of immunoreactive cells in individual uteri, and median values for each group are scored. Data were compared by Kruskal-Wallis one-way ANOVA and Mann-Whitney Rank Sum Test. Groups with different lower case letters on the x axis are significantly different for the given mAb ($p < 0.05$).

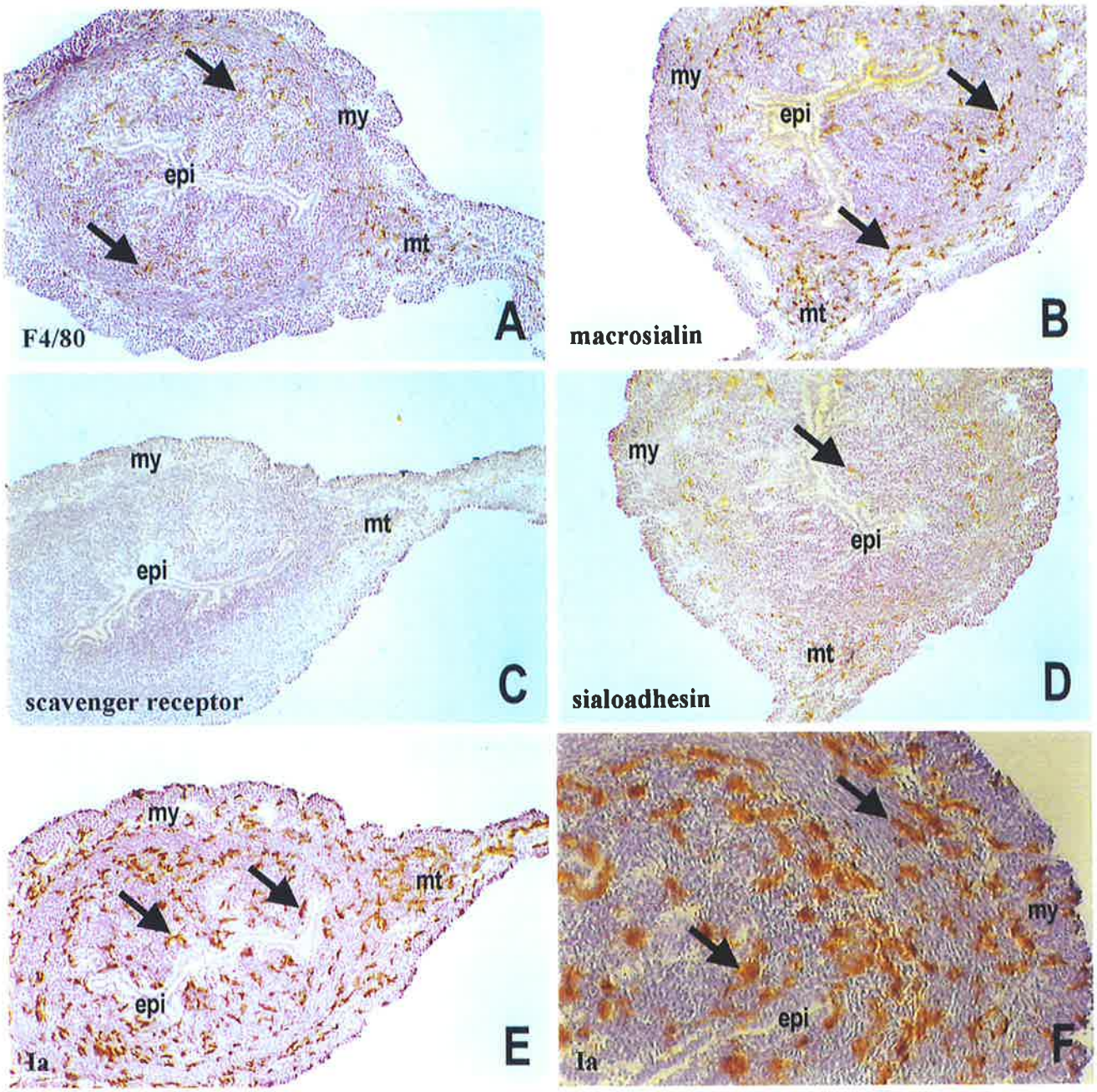
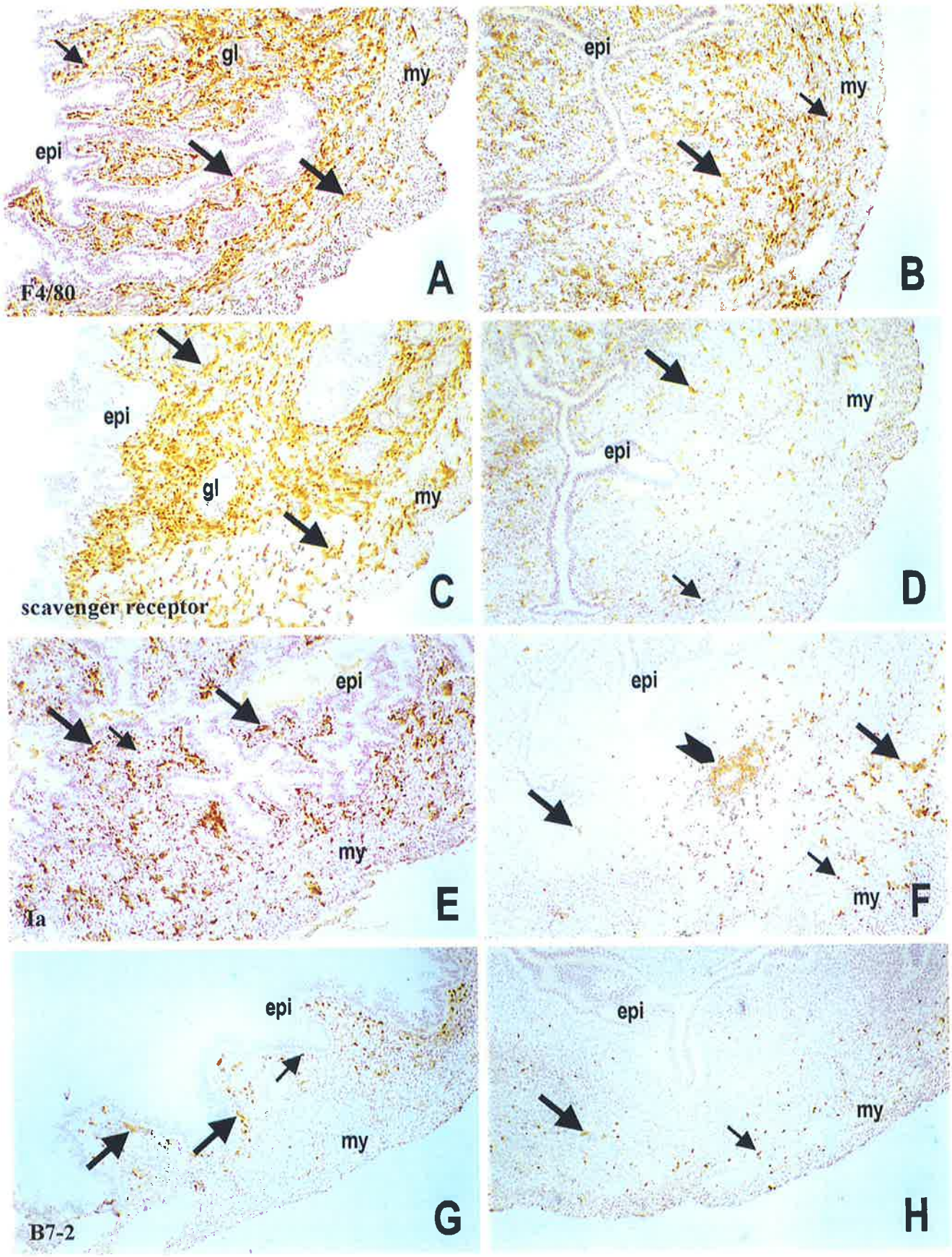


Figure 3.6 APCs in the endometrium, mesometrial triangle and myometrium following ovariectomy. Fresh frozen sections of uteri collected from mice which had been ovariectomised three weeks prior to sacrifice were labeled with an indirect immunoperoxidase technique using mAbs specific for F4/80 antigen (A), macrosialin (B), class A scavenger receptor (C), sialoadhesin (D) and Ia (E, F). Immunoperoxidase-labeled APCs are evident in some sections (A-E at x25 magnification; F at x50 magnification) as finely branched, dendriform cells (arrows). epi = luminal epithelium, my = myometrium, mt = mesometrial triangle.

Figure 3.7 APCs in the endometrium and myometrium at days 1 and 4 of pregnancy. Fresh frozen sections of uteri collected from day 1 pregnant (A, C, E, G) and day 4 pregnant (B, D, F, H) mice were labeled with an indirect immunoperoxidase technique using mAbs specific for F4/80 antigen (A, B), class A scavenger receptor (C, D), Ia (E, F) and B7-2 (G, H). Immunoperoxidase-labeled APCs are evident in the endometrium and myometrium (my) as medium-large sized rounded and dendriform cells (large arrows). An aggregation of Ia^+ APCs is present in the deep endometrium in F (block arrow). Endogenous $peroxidase^+$ eosinophils are evident as small, dark brown cells in the endometrium and myometrium (small arrows). epi = luminal epithelium, gl = glandular epithelium. x25 magnification.


gl
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B7-2
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day 1

day 4

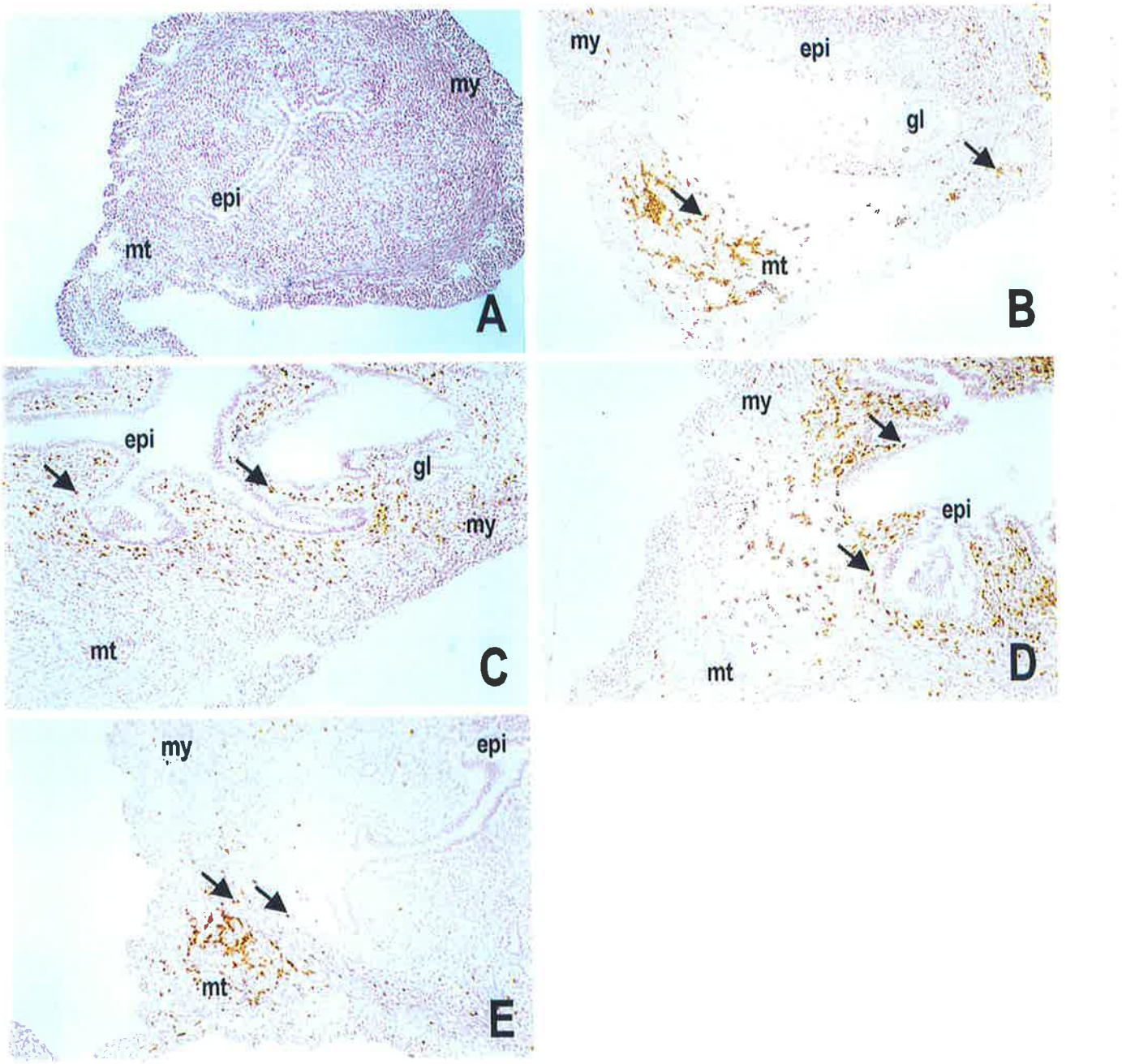
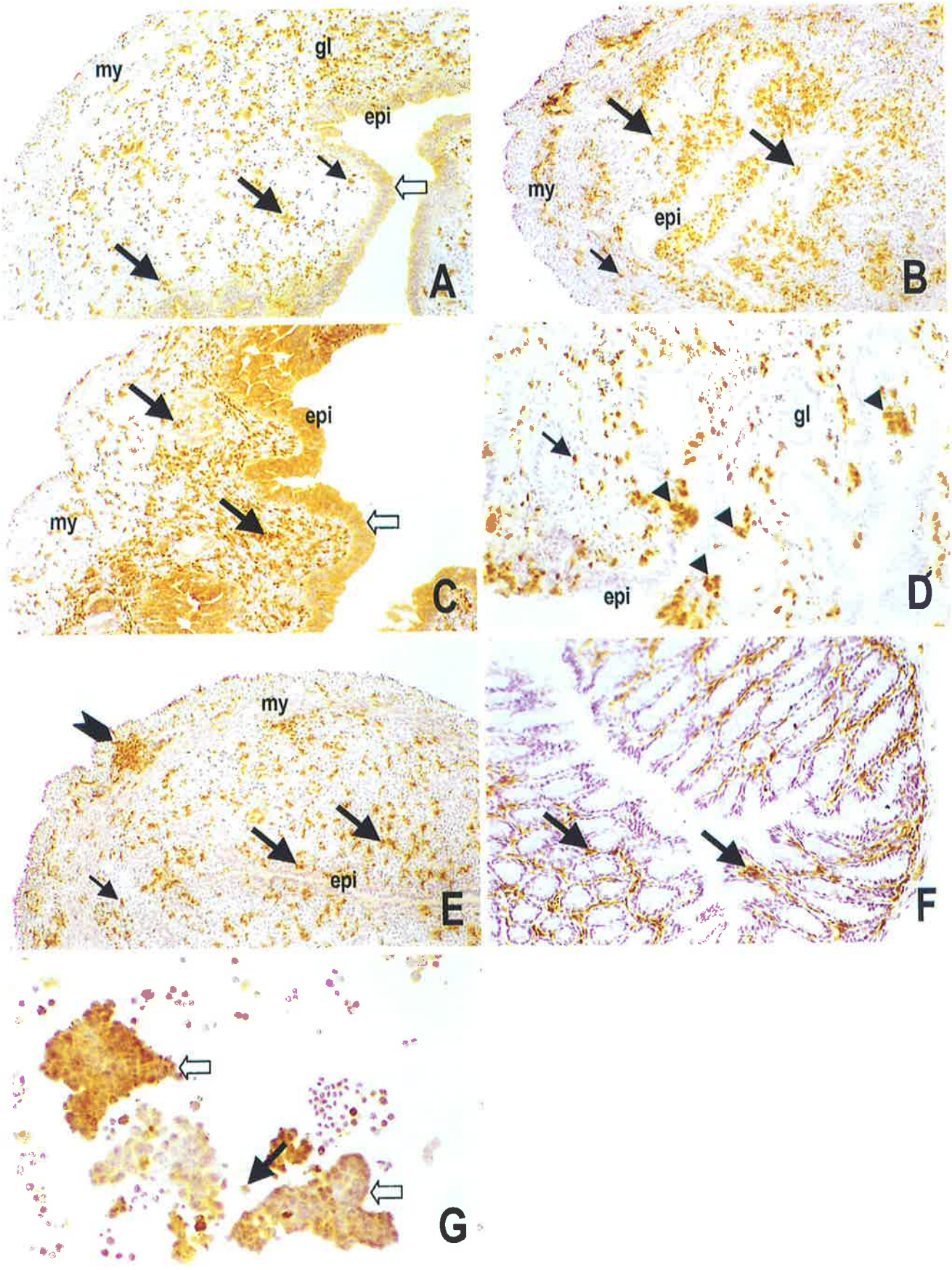


Figure 3.8 Eosinophils in the endometrium, mesometrial triangle and myometrium during the estrous cycle, following ovariectomy and on days 1 and 4 of pregnancy. Fresh frozen sections of uteri collected from ovariectomised (A), diestrous (B), estrous (C), day 1 pregnant (D) and day 4 pregnant (E) mice were labeled to detect endogenous peroxidase activity. Endogenous peroxidase^{+} eosinophils are evident as small, dark brown cells (arrows) in the endometrium, mesometrial triangle (mt) and myometrium (my). epi = luminal epithelium. gl = glandular epithelium. x25 magnification.

Figure 3.9 Expression of macrosialin by uterine epithelial cells during the estrous cycle and in early pregnancy. Fresh frozen sections of uteri (A-C, E) and large intestine (F), and cytosmears of enzymatically digested uteri (G) prepared from tissues collected from estrous (A), diestrous (B), day 1 pregnant (C, D, F, G) or day 4 pregnant (E) mice were labeled with an indirect immunoperoxidase technique using a mAb specific for macrosialin (A-C, E-G) or RB6-8C5 antigen (D). Immunoperoxidase-labeled epithelial cells are evident in sections of uteri and in the cytosmear of uterine cells (unfilled arrows). Immunoperoxidase-labeled stromal antigen presenting cells (A-C, E-G, large arrows) and neutrophils (D, arrow heads) are also indicated. In E, an aggregation of macrosialin$^+$ antigen presenting cells is evident in the myometrium (block arrow). epi = luminal epithelium, gl = glandular epithelium, my = myometrium. A-C, E, F at x25 magnification; D, G at x50 magnification.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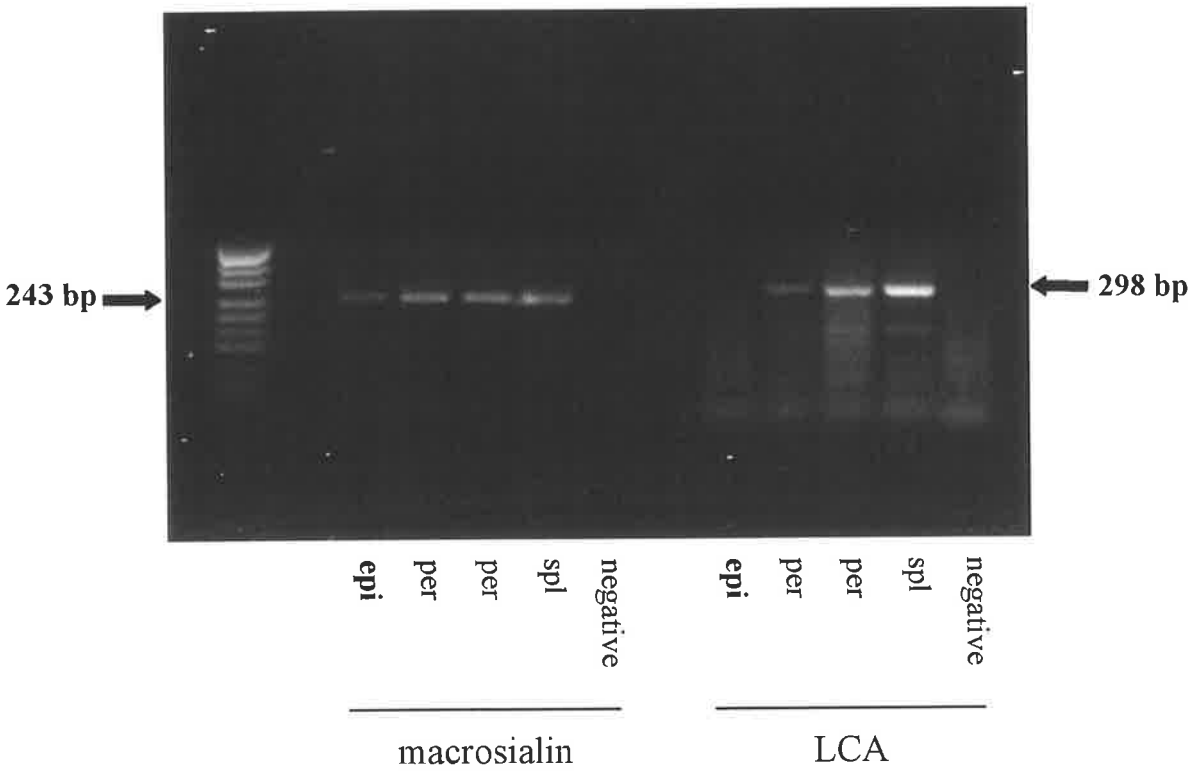


Figure 3.10 RT-PCR analysis of macrosialin mRNA expression by uterine epithelial cells. Messenger RNAs isolated from uterine epithelial cells purified by miniMACs using MTS#24 (epi), or peritoneal (per) or splenic (spl) leukocytes were reverse transcribed to cDNAs using oligo-dT primers and then amplified using primers specific for macrosialin and leukocyte common antigen (LCA). Reaction products (20 µl aliquots) were analysed by electrophoresis through a 1% agarose gel containing 50 pg/ml of ethidium bromide, and photographed with UV illumination. The amplicons generated from the primers for macrosialin (243 bp) and primers for LCA (298 bp) are arrowed.

Chapter 4

In vitro and flow cytometric analysis of uterine antigen presenting cells at estrus

4.1 Introduction

The studies presented in Chapter 3 described an analysis by single-colour immunohistochemistry of the large populations of professional antigen presenting cells (APCs) residing in the murine uterus. However, since Mϕs and dendritic cells can express many of the same markers, and have a similar morphological appearance *in situ*, this technical approach did not permit clear distinction of one cell lineage from the other.

In many previous studies Mϕs have been distinguished from other APCs, such as dendritic cells, by phenotypic analysis of *ex-vivo* live cells, using methods which exploit their innate behavioural characteristics. Mϕs derived from peripheral organs and peritoneal cavity are large, often heterogeneous cells which rapidly adhere to tissue culture grade plastic, and will readily phagocytose latex beads and other particulate matter at 37°C (Girolomoni *et al.* 1990, Pollard and Lipscomb 1990, Gordon 1995). By comparison, interstitial and lymphoid organ dendritic cells may initially be loosely adherent, but often become detached during prolonged culture (Pollard and Lipscomb 1990, Hart 1997), and they show far less capacity for phagocytic behaviour although they will endocytose fluid phase material (Sallusto *et al.* 1995).

The extraordinary diversity of Mϕs and related cells perhaps is most apparent when considering their complex and varied patterns of expression of cell surface antigens. Monocytes, Mϕs and dendritic cells express a large array of cell surface molecules involved in cell adhesion and chemotaxis, endocytosis and phagocytosis, cytotoxicity, antigen presentation and costimulation, ligation of chemokines and cytokines and other still unidentified roles (Banchereau and Steinman 1998, Gordon 1999). In combination, the patterns of expression of markers combined with analysis of the location within a tissue and morphological features of these cells can be used to make certain predictions regarding their current roles.

Fluorescent activated cell scanning (FACS) is a highly sensitive means by which to analyse the cell membrane phenotype of leukocytes. FACS involves the forcing of fluorochrome-labeled cells in a continuous stream past a fixed laser beam and their phenotypic analysis according to the manner in which they scatter the incident laser light and emit fluorescence. The quality of the scattered light pertains to the size and internal complexity (an index of the density of organelles within the cytoplasm) of the cells, whereas the intensity of

fluorescence is proportional to the amount of fluorochrome-labeled antigen present on the cell surface membrane.

Although FACs has not previously been applied to the analysis of murine uterine APCs, the literature indicates that enzymatic digestion of virgin and pregnant murine uteri can be achieved and that semi-purified populations of uterine leukocytes can be obtained for morphometric analysis. Following enzymatic digestion of virgin uteri in collagenase and DNase, $F4/80^+$ Mϕs have been reported to comprise approximately 10% of the cells released (Hunt *et al.* 1985, De and Wood 1990, De and Wood 1991, Wood *et al.* 1997). On day 1 of pregnancy, 20-30% of released cells are $F4/80^+$ (De and Wood 1991, Wood *et al.* 1997) and in the late stages of gestation up to 50% of cells released are $F4/80^+$ (Hunt *et al.* 1985, Wood *et al.* 1997). These cells have a morphology typical of Mϕs, and can be further purified on the basis of Fc receptor (FcR) expression by rosetting with sheep red blood cells (Hunt *et al.* 1984, Hunt *et al.* 1985).

The aim of experiments presented in this chapter was to develop techniques for the preparation of uterine APCs by enzymatic digestion, to characterise the cells according to their *in vitro* properties and then to use flow cytometry to analyse their surface phenotype. The data show that viable populations of adherent FcR^+, $F4/80^+$ and Ia^+ cells of differential phagocytic abilities were released from digested uteri. Furthermore, the use of flow cytometry enabled the identification of three distinct populations of uterine APCs, which were classified as 'non-differentiated Mϕs', 'differentiated Mϕs' and 'dendritic cells' on the basis of their forward and side scatter characteristics and their expression of F4/80, Ia and other cell surface markers.

4.2 Identification of APCs in uterine single cell suspensions

In order to obtain suspensions of uterine cells, uteri were digested with collagenase and DNase using a modified version of a protocol previously described (Hunt *et al.* 1984). Mixed and semi-purified preparations of uterine cells were assessed for expression of Mϕ and other leukocyte markers by immunofluorescence analysis of suspended or adherent cells, by immunohistochemical analysis of cytosmears and for phagocytic activity *in vitro.*

Resident and elicited Mϕs obtained from the peritoneal cavity were used for protocol work-up and to act as a comparative 'positive control'. Peritoneal cells collected from virgin mice were comprised of > 85% large, adherent dendriform cells which were classified as Mϕs based on their expression of F4/80 and Mac-1, and their innate phagocytic ability.

As shown in Table 4.1, of the total cells released from pooled, randomly cycling virgin uteri, 35% were leukocytes (identified by their expression of LCA) and 23% were $F4/80^+$ Mϕs.

Adherence alone did not enrich for Mϕs or other leukocytes since only 10-20% of cells enriched by adherence expressed F4/80, Ia or Mac-1, and a similar proportion were phagocytic.

Uterine cells selected on the basis of FcR expression (by rosetting) followed by adherence were enriched for Mϕs with >80% of these cells exhibiting phagocytic activity. Uterine cells selected for F4/80 expression by affinity purification using miniMACS magnetic beads were confirmed as >95% F4/80$^+$ cells by immunofluorescence. After adherence to plastic, up to 90% of these cells were also phagocytic (Figure 4.1). In contrast, only 39% of cells selected for Ia expression followed by adherence to plastic were phagocytic (not shown).

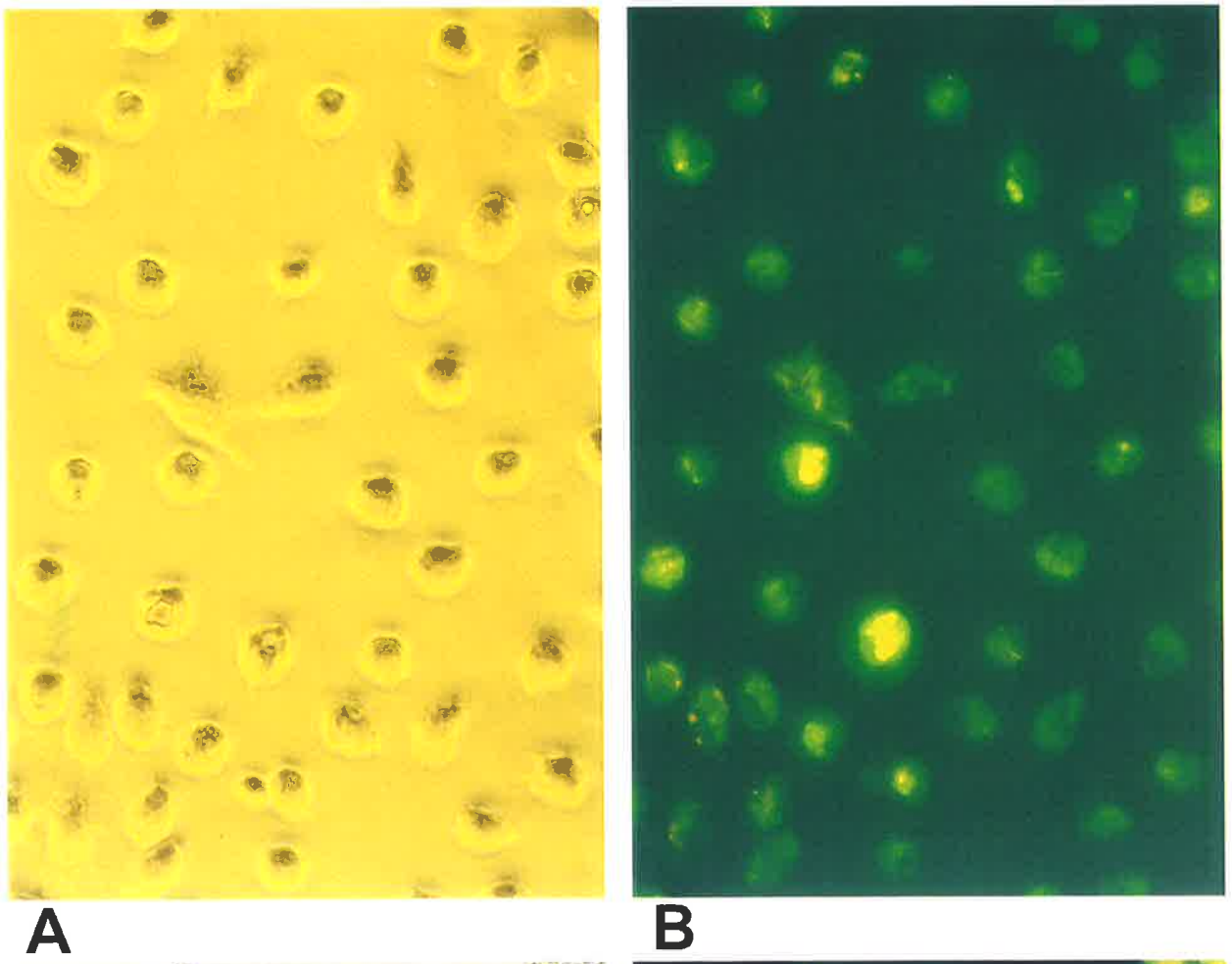
Table 4.1 Characteristics of uterine cells released by enzymatic digestion. Crude or purified cells obtained by collagenase/DNase digestion of pooled uteri obtained from randomly cycling or estrous virgin mice were assessed for phagocytosis of fluorescent beads and expression of leukocyte and Mϕ surface antigens by immunofluorescence of suspended or adherent cells, or immunohistochemistry (IHC) of cytosmear preparations. Purification was on the basis of adherence (Ad.) to tissue culture-grade plastic, FcR expression by rosetting or F4/80 or Ia expression by miniMACS immunomagnetic selection. The cellular characteristics of each preparation of cells are given as % purified cells. ND = not determined. Stage of estrous cycle, number of pooled uteri per experiment (n pooled) and number of experiments (n expts) performed for each set of experimental conditions are given.

				cellular characteristics (% purified)		
				phagocytosis	***surface phenotype***	
cycle stage	n pooled	n expts	purification		*fluorescence*	*IHC*
random	15	1	none	ND	ND	LCA$^+$:35 F4/80$^+$:23
random	4	3	Ad.	10-30	F4/80$^+$:20 Ia$^+$:10 Mac-1$^+$:10	ND
random	5-12	3	FcR + Ad.	>80	ND	ND
estrus	1	8	F4/80	ND	F4/80$^+$:>95	ND
estrus	5	3	F4/80 + Ad.	73-90	ND	ND
estrus	5	1	Ia + Ad.	39	ND	ND

4.3 Flow cytometric analysis of uterine cells

The experiments described in section 4.2 established that viable populations of Mϕs and other leukocytes could be obtained by enzymatic digestion of murine uteri, and so a method for flow cytometric analysis of uterine cells was developed.

Figure 4.1 Morphology of uterine Mϕs in culture. Mϕs collected from enzymatically released uterine cells purified by miniMACs using F4/80 mAb were adherent to tissue culture plastic (A, shown in phase contrast) and >95% were F4/80$^+$ by immunofluorescence (B). Up to 90% of the adherent uterine Mϕs were also phagocytic of fluorescent latex beads (shown at high power in C & D under phase contrast and UV illumination respectively).


A
B
C
D

4.3.1 Forward and side scatter analysis of uterine cells

To assess the forward and side scatter characteristics of uterine cells, pooled or individual uteri obtained from estrous mice were digested, and with the FACs machine 10 000 events were acquired within a gate designed to exclude dead cells and cellular debris. Dot-plots showing forward- and side-scatter characteristics from both pooled (Figure 4.2A) and individual (Figure 4.2B) uteri were found to be similar, and showed that the cells released by enzymatic digestion were very diverse in terms of their size and their intra-cellular complexity. The profile was highly reproducible since similar results were obtained in more than 7 experiments involving analysis of pooled estrous uteri, and in 4 experiments in which a total of more than 15 individual estrous uteri were digested (results not shown). Identifiable within the dot-plots were sub-populations of cells which were clearly distinguishable on the basis of their similar size and intra-cellular characteristics.

4.3.2 Single colour fluorometric analysis of uterine cells

a. identification of uterine leukocytes by flow cytometry

In order to identify uterine leukocytes by single colour flow cytometry, aliquots of $5x10^5$ cells obtained by enzymatic digestion of pooled uteri from 10 estrous mice were directly or indirectly labeled with mAbs (LCA, MTS#24, F4/80 or Ia) tagged with either fluorescein isothiocyanate (FITC) or phyocoerythrin (RPE) (FITC- and RPE-conjugated reagents outlined in Table 4.2). Using the FACs machine, 10 000 events from each aliquot were acquired in a gate designed to exclude dead cells and cellular debris. Fluorescent signals at the peak emission wavelengths for FITC (525 nm) and RPE (570 nm) were collected in separate channels (channels 1 and 2 respectively).

As shown in Figure 4.3A, most cells (61%) released by the digestion protocol and captured within the gate defining live and viable cells were leukocytes, identified on the basis of their expression of LCA. Epithelial cells expressing the antigen detected by mAb MTS#24 comprised the majority of the remaining cells (36% of total cells, Figure 4.3B). Myeloid APCs, identified on the basis of their expression of F4/80 and Ia, comprised a significant proportion (24-27% and 8-9% of cells respectively) of these cell preparations (Figure 4.3C-G).

b. specificity of uterine cell lineage detection by flow cytometry

In order to confirm the specificity of the cell lineage detection by fluorometric analysis, aliquots of cells labeled with an irrelevant mAb (see section 2.11.1) and appropriate secondary

reagents were used in all experiments to define 'background' fluorescence. Control samples were labeled in parallel and acquired using the same instrument settings. For single colour fluorometric analysis, marker regions excluding > 98% of control cells were used to define background fluorescence and thereby to detect positive cells (Figure 4.3).

Table 4.2 Protocols for direct and indirect labeling of uterine cell surface antigens for flow cytometric analysis. For immunophenotyping of uterine leukocytes by flow cytometry, aliquots of $5x10^5$ uterine cells were labeled with mAbs and fluorochromes. Labeling protocols numbered 1-4 were direct, indirect in 2 steps with FITC, or indirect in 2 or 3 steps with RPE. Reagent dilutions and incubation volumes are given. All incubations contained 10% heat inactivated NMS. mAb = monoclonal antibody, -biotin = Ab conjugated to biotin, -FITC = Ab conjugated to FITC, -RPE = Ab conjugated to RPE

protocol	step A	step B	step C
1. direct	mAb-FITC (1:50, 50µl)	-	-
2. indirect 2-step FITC	mAb (neat or diluted, 50µl)	sheep α-rat-FITC (1:50, 50µl)	-
3. indirect 2-step RPE	mAb-biotin (1:25-1:50, 50µl)	streptavidin-RPE (1:10, 10µl)	-
4. indirect 3-step RPE	mAb (neat or diluted, 50µl)	rabbit α-rat Ig-biotin (1:300, 50µl)	streptavidin-RPE (1:10, 10µl)

c. sensitivity and assay variation of flow cytometric analysis of uterine cells

In order to measure the sensitivity and within- and between-assay variation of detection of uterine leukocytes by FACs, replicate aliquots of $5x10^5$ uterine cells were prepared using each of the different labeling protocols to be used in further experiments (outlined in Table 4.2) for analysis of $F4/80^+$ and Ia^+ cells. The proportion of total cells labeled by each protocol was calculated by flow cytometry as described in section 4.3.2.

Sensitivity of detection of $F4/80^+$ cells was similar using direct and 3-step indirect methods, but was slightly lower using an indirect 2-step FITC method. Sensitivity of Ia detection using indirect FITC and RPE methods was similar. Intra-assay coefficients of variation for the labeling protocols were 7% or less (Table 4.3). Inter-assay variation was < 10% when replicate aliquots from one pooled preparation of labeled uterine cells were analysed on different days (data not shown).

Table 4.3 Sensitivity and intra-assay variation of flow cytometric analysis of F4/80$^+$ and Ia$^+$ uterine cells. Aliquots of $5x10^5$ cells obtained by digestion of pooled uteri from estrous mice (n = 10) were labeled directly and indirectly to detect expression of F4/80 and Ia. The number of F4/80$^+$ and Ia$^+$ cells as a percentage of total cells (% positive cells, mean +/- SD) and intra-assay coefficient of variation (C.O.V.) are given. ND = not determined.

	labeling protocol	n	% positive cells (mean +/- SD)	C.O.V. (SEM/mean x 100)
F4/80	Direct, FITC	9	30 +/- 6	6%
	indirect 2-step, FITC	5	22 +/- 1	2%
	indirect 3-step, RPE	1	29	ND
Ia	indirect 2-step, FITC	2	9, 11	ND
	indirect 2-step, RPE	10	8 +/- 2	7%

4.4 Identification of uterine APC sub-populations by dual colour flow cytometry

The experiments described in sections 4.2 and 4.3 clearly demonstrate that F4/80$^+$ and Ia$^+$ APCs were released by enzymatic digestion of uterine tissue, and could be identified by single-colour flow cytometry. Both F4/80 and Ia can be expressed by several different populations of APCs, and the patterns of co-expression of these and other cell surface markers can be used as defining features for the classification of subsets of APCs. In order to more precisely define the populations of APCs within uterine tissue, a method for dual colour cytometric analysis of uterine cells was developed.

4.4.1 Sub-populations of uterine APCs expressing F4/80 and Ia

To perform dual colour cytometric analysis of F4/80 and Ia expression in uterine cells, five separate experiments were performed. In each experiment, suspensions of cells obtained by enzymatic digestion of pooled or individual uteri from estrous mice were labeled indirectly with F4/80-FITC and Ia-RPE using protocols 2 and 3 (Table 4.2) in sequence.

In these experiments and in all subsequent dual labeling FACS experiments, the primary and tertiary reagents for each labeling step were chosen carefully to ensure that different physiochemical and immunological binding characteristics would maintain the specificity of detection for each antigen. All aliquots for labeling contained $5x10^5$ cells, which were fixed in 1% paraformaldehyde following labeling. Channel 1 (energy emitted from FITC) and channel 2 (energy emitted from RPE) fluorescence data was acquired from $1\text{-}2x10^4$ events within a gate

designed to exclude dead cells and cellular debris and is presented in the form of dot plots (FITC and RPE fluorescence shown on x and y axes respectively). As with single colour fluorometric analysis, the specificity of each labeling protocol was confirmed with irrelevant mAbs in both channels 1 and 2 and to determine the thresholds defining background fluorescence (indicated in each plot by quadrant markers). Each of the upper left hand, upper right hand and lower right hand quadrants contained <1% of gated cells following staining with irrelevant isotype control mAbs.

Three distinct populations of uterine cells expressing F4/80 and/or Ia were identified in these experiments (Table 4.4). A median value of 6% of recovered cells expressed F4/80 but not Ia ($F4/80^+Ia^-$) (shown in right hand lower quadrant of FACS profile from experiment 1 in Figure 4.4B), whereas 3.7% of cells (median value) expressed both F4/80 and Ia ($F4/80^+Ia^+$) (shown in right hand upper quadrant of FACS profile from experiment 1 in Figure 4.4B). A median value of 3% of total cells expressed only Ia, and not F4/80 ($F4/80^-Ia^+$) (shown in left hand upper quadrant in FACS profile of experiment 1 in Figure 4.4B). In total, 9.7% of total cells released expressed F4/80: 38% of these expressed Ia. Expression of Ia was detected on 6.7% of total cells, and 55% of these cells also expressed F4/80.

4.4.2 Separation of uterine APC sub-populations by gates

In order to better define the physical characteristics of the three populations of $F4/80^+$ and Ia^+ uterine cells, the data from each of the five experiments described in section 4.4.1 were examined on the basis of side- and forward-scatter characteristics. The total events could be divided into three regions, or gates, encompassing cells of similar size and intra-cellular complexity (Figure 4.4A). Gate 1 contained a population of small, complex cells which formed approximately 10% of total events. Gate 2, encompassing approximately 20% of total events, was comprised of a heterogeneous group of complex, large cells. Gate 3 contained approximately 40% of total events, and these were much less complex than cells in the other two gates, and of intermediate size.

In all five experiments, the $F4/80^+Ia^-$, $F4/80^+Ia^+$ and $F4/80^-Ia^+$ populations of cells were enriched within gates 1, 2 and 3 respectively (Table 4.4). The discussion below refers to the median values presented in Table 4.4.

Of all the $F4/80^+Ia^-$ cells, 87% were confined to gate 1. Only 3.4% and 0.5% of gate 1 cells were $F4/80^+Ia^+$ cells and $F4/80^-Ia^+$ cells respectively. The majority (65%) of the cells with a $F4/80^+Ia^+$ phenotype were contained within gate 2, comprising 12% of cells in that gate. Gate 2 also contained smaller populations of $F4/80^+Ia^-$ cells (3%) and $F4/80^-Ia^+$ cells (0.5%). The

majority (70%) of the F4/80$^-$Ia$^+$ cells were confined within gate 3. Gate 3 was also comprised of 0.6% F4/80$^+$Ia$^-$ cells and 2.5% F4/80$^+$Ia$^+$ cells.

Table 4.4 Populations of uterine cells expressing F4/80 and Ia at estrus. Cells obtained by enzymatic digestion of pooled and individual uteri from estrous mice were analysed by dual colour fluorocytometry for expression of F4/80 and Ia. For the three identified APC phenotypes (F4/80$^+$Ia$^-$, F4/80$^+$Ia$^+$ and F4/80$^-$Ia$^+$) in gates 1, 2 and 3 (described in section 4.4.2), data is shown as % gated cells for each of five separate experiments (expts 1-5). [a]data from experiment shown in Figure 4.4; [b]data from further experiments using pooled (n = 5-10) uteri; [c]data [median (range)] from individual uteri (n = 4). Median values across the 5 experiments are also presented : [de]median % gated and % total cells in each of gates 1, 2 and 3; [f]all cells of each phenotype as median % total cells, [gh]all F4/80$^+$ and Ia$^+$ cells as median % gates 1, 2 and 3 and as median % total cells.

phenotype	expt.	Gate 1	Gate 2	Gate 3	[f]all
			(% gated cells)		*(% total)*
F4/80$^+$Ia$^-$	1[a]	50.0	3.2	0.8	
	2[b]	65.4	3.0	1.1	
	3[b]	82.2	0.2	0.1	
	4[b]	22.2	3.5	3.4	
	5[c]	43.7 (16.4-61.4)	2.9 (0.9-4.0)	0.3 (0.1-1.1)	
	[d]median % gate	*52.2*	*3.0*	*0.6*	-
	[e]median % total	*5.2*	*0.6*	*0.2*	*6.0*
F4/80$^+$Ia$^+$	1[a]	1.7	13.4	4.0	
	2[b]	5.0	8.1	8.8	
	3[b]	2.7	10.6	2.5	
	4[b]	4.1	6.2	2.5	
	5[c]	3.4 (2.1-4.5)	15.2 (7.0-20.9)	1.6 (0.7-4.5)	
	[d]median % gate	*3.4*	*12.0*	*2.5*	-
	[e]median % total	*0.3*	*2.4*	*1.0*	*3.7*
F4/80$^-$Ia$^+$	1[a]	0.8	3.5	11.2	
	2[b]	2.1	0.2	6.8	
	3[b]	0.1	2.1	1.5	
	4[b]	1.8	2.2	1.6	
	5[c]	0.1 (0-1.5)	5.9 (4.2-8.9)	5.2 (2.5-6.6)	
	[d]median % gate	*0.5*	*3.9*	*5.2*	-
	[e]median % total	*0.1*	*0.8*	*2.1*	*3.0*
[g]All F4/80$^+$	% gate	55.6	15.0	3.1	-
	% total	5.6	2.9	1.2	9.7
[h]All Ia$^+$	% gate	3.9	15.9	7.7	-
	% total	0.4	3.2	3.1	6.7

As evident in Figure 4.4, the quadrants used to define background fluorescence of uterine cells were slightly different in each of gates 1, 2 and 3. This was largely due to variation in the autofluorescence of cells in each of the gates (FACS analysis of cells not labeled with any reagents also exhibited comparable differences in autofluorescence in each of the gates, data not shown). Autofluorescence in both channel 1 and channel 2 was highest on cells in gate 2 and lowest on cells in gate 1.

4.4.3 Intensity of F4/80 and Ia expression on uterine APC sub-populations

In order to compare the intensity of F4/80 and Ia expression on each of the APC populations, the fluorescence intensity in channels 1 and 2 was compared for $F4/80^+Ia^-$, $F4/80^+Ia^+$ and $F4/80^-Ia^+$ cells from four individually digested uteri (experiment 5 in Table 4.4). As shown in Table 4.5, within gate 1 the intensity of F4/80 expression was relatively low on $F4/80^+Ia^-$ cells compared to $F4/80^+Ia^+$ cells. In gate 2, intensity of F4/80 and Ia expression was similar on all populations of APCs. In gate 3, intensity of F4/80 expression was highest on $F4/80^+Ia^-$ cells and intensity of Ia highest on $F4/80^+Ia^+$ cells. Across all gates, F4/80 expression was highest on $F4/80^+Ia^+$ cells in gate 2 and $F4/80^+Ia^-$ cells in gate 3, and Ia expression was highest on $F4/80^+Ia^+$ cells in gate 2. Similar results were obtained if the data was corrected for the different quadrant thresholds in each of the three gates (data not shown).

Table 4.5 The intensity of F4/80 and Ia expression on uterine APCs. APCs in mixed cell suspensions obtained by enzymatic digestion of individual uteri from estrous mice (n = 4) were analysed by dual colour flow cytometry. For $F4/80^+Ia^-$, $F4/80^+Ia^+$ and $F4/80^-Ia^+$ populations of APCs in gates 1, 2 and 3, the intensity of F4/80 and Ia expression (in arbitrary intensity units) is given as median (range).

		APC population		
		$F4/80^+Ia^-$	*$F4/80^+Ia^+$*	*$F4/80^-Ia^+$*
Gate 1	intensity F4/80:	21 (19-35)	88 (58-115)	-
	intensity Ia:	-	102 (86-174)	86 (76-110)
Gate 2	intensity F4/80:	421 (245-690)	472 (245-669)	-
	intensity Ia:	-	433 (399-497)	362 (282-434)
Gate 3	intensity F4/80:	476 (286-803)	272 (121-335)	-
	intensity Ia:	-	138 (97-304)	106 (68-124)

4.5 Phenotypic characterisation of uterine APCs

The previous experiments identified three separate populations of uterine APCs which could be distinguished on the basis of their expression of F4/80 and Ia. In order to further define the phenotypes of these APCs, aliquots of $5x10^5$ uterine cells were single or dual labeled with F4/80 or Ia and an array of other mAbs defining markers of interest. The selected panel of additional mAbs targeted Mϕ activation markers macrosialin, class A scavenger receptor (referred to from here on as 'scavenger receptor') and sialoadhesin, β2 integrins CD11b/CD18 (Mac-1) and CD11c/CD18, dendritic cell markers 33D1, DEC-205 and CD1 and costimulatory molecules B7-2 and CD40. $Mac\text{-}1^+$ cells were also dual labeled for CD11c/CD18 expression

since co-expression patterns of these β2 integrins can be useful for the identification of specific APC lineages such as dendritic cells, which often express CD11c/CD18 and not Mac-1. Details of mAb specificities and concentrations of reagents used for FACS labeling are shown in Table 2.1 and Table 4.2.

4.5.1 Phenotypic characteristics of F4/80$^+$ uterine cells

Since each of gates 1, 2 and 3 contained cells expressing F4/80, the phenotype of F4/80$^+$ cells in all 3 gates was assessed and data from each gate is discussed in sequence.

a. characteristics of F4/80$^+$ cells in gate 1

A summary of the phenotypic characteristics of F4/80$^+$ cells in gate 1 is provided in Table 4.6. F4/80$^+$Ia$^-$ cells were preferentially enriched in this gate.

i) expression of Mϕ activation markers

Only 8-11% of F4/80$^+$ cells in gate 1 expressed macrosialin, scavenger receptor or sialoadhesin, and the intensity of activation marker expression was quite heterogeneous on the few numbers of cells that were positive (Figure 4.5B-D and Table 4.6). A population of macrosialin$^+$ cells which was F4/80$^-$ was also apparent in gate 1. These latter cells comprised 12 % of gated cells, and exhibited a 2-fold lower intensity of macrosialin expression than the small population of cells expressing both F480 and macrosialin.

ii) expression of β2 integrins

A high proportion of F4/80$^+$ cells in gate 1 expressed β2 integrins (Figure 4.5E and F). 70% of F4/80$^+$ cells were Mac-1$^+$, and intensity of Mac-1 expression on these cells ranged from very low (just over the quadrant marker) to high (over one log higher than the quadrant marker). In contrast, the 82% of F4/80$^+$ cells which expressed CD11c/CD18 expressed homogeneous and comparatively low levels of CD11c/CD18 (Table 4.6). The intensity of F4/80 expression was similar on β2-integrin$^+$ cells and β2-integrin$^-$ cells (data not shown).

Table 4.6 Summary of the cell membrane phenotype of F4/80$^+$ uterine cells in gate 1. F4/80$^+$ uterine cells in gate 1 were immunophenotyped by 2-colour fluorometric analysis. The numbers of F4/80$^+$ cells co-expressing Mϕ activation markers, β2 integrins and dendritic cell markers are given as a % of all gate 1 F4/80$^+$ cells (% expressing). A measure of mean intensity of antigen expression on F4/80$^+$ cells is also provided (intensity score). + = < 60 intensity units, ++ = 60-110 intensity units, +++ = > 110 intensity units. For corresponding graphs see Figure 4.5. [*results are representative of 2 experiments]

		% expressing	intensity score
Mϕ activation markers	*macrosialin	11	++
	*scavenger receptor	6	++
	*sialoadhesin	8	++
β2 integrins	*Mac-1	70	+++
	CD11c/CD18	82	+
dendritic cell markers	33D1	5	+++
	DEC-205	8	+
	CD1	22	+++

iii) expression of dendritic cells markers

Expression of dendritic cell markers 33D1 and DEC-205 on F4/80$^+$ cells in gate 1 was minimal (<8% of F4/80$^+$ cells expressed either marker, Figure 4.5G and H). In contrast, 22% of gate 1 F4/80$^+$ cells expressed high levels of CD1 (Table 4.6), and many more cells appeared to be just under the threshold of positive expression. There was a small group of F4/80$^-$, DEC-205$^+$ cells in gate 1 (6% of gated cells).

b. characteristics of F4/80$^+$ cells in gate 2

A summary of the phenotypic characteristics of F4/80$^+$ cells in gate 2 is provided in Table 4.7. F4/80$^+$Ia$^+$ cells were preferentially enriched within this gate.

i) expression of Mϕ activation markers

Approximately 50% of F4/80$^+$ cells in gate 2 expressed macrosialin, scavenger receptor, and sialoadhesin (Figure 4.6B-D). The 'double positive' cells appeared reasonably homogeneous both in terms of activation marker expression intensity (Table 4.7) and F4/80 expression intensity. The mean F4/80 intensity on cells which did not express activation markers was

similar to those that did. There were no macrosialin$^+$, scavenger receptor$^+$ or sialoadhesin$^+$ cells which did not co-express F4/80 in gate 2.

ii) expression of β2 integrins

Of the F4/80$^+$ cells in gate 2, 65% were Mac-1$^+$ and 43% were CD11c/CD18$^+$ (Figure 4.6E and F). The intensities of Mac-1 and CD11c/CD18 expression were similar on F4/80$^+$ and F4/80$^-$ cells (intensity on F4/80$^+$ cells shown in Table 4.7). Mac-1 was also expressed on populations of cells which were F4/80$^-$ (these cells comprised 7% of gate 2). In contrast, only 2% of gate 2 cells were CD11c/CD18$^+$ and F4/80$^-$.

iii) expression of dendritic cell markers

Of the F4/80$^+$ cells in gate 2, 42% expressed 33D1 and 25% expressed DEC-205 (Figure 4.6G and H). Mean intensity of F4/80 expression was 2-fold lower on DEC-205$^-$ cells. All of the 33D1$^+$ cells and all of the DEC-205$^+$ cells in gate 2 co-expressed F4/80$^+$. Of the gate 2 F4/80$^+$ cells, 89% expressed CD1, and intensity of F4/80 expression was approximately 2-fold higher on CD1$^+$ cells than on CD1$^-$ cells. Of the CD1$^+$ cells in gate 2, 33% did not express F4/80. The intensity of dendritic cell marker expression on F4/80$^+$ cells is shown in Table 4.7.

Table 4.7 Summary of the cell membrane phenotype of F4/80$^+$ uterine cells in gate 2. F4/80$^+$ uterine cells in gate 2 were immunophenotyped by 2-colour fluorometric analysis. The numbers of F4/80$^+$ cells co-expressing Mϕ activation markers, β2 integrins and dendritic cell markers are given as a % of all gate 2 F4/80$^+$ cells (% expressing). A measure of mean intensity of antigen expression on F4/80$^+$ cells is also provided (intensity score). + = < 1000 intensity units, ++ = 1000-2000 intensity units, +++ = > 2000 intensity units. For corresponding graphs see Figure 4.6. [*Results are representative of 2 experiments]

		% expressing	intensity score
Mϕ activation markers	*macrosialin	46	++
	*scavenger receptor	50	++
	*sialoadhesin	42	++
β2 integrins	*Mac-1	65	+++
	CD11c/CD18	43	+
dendritic cell markers	33D1	42	++
	DEC-205	25	++
	CD1	89	++

c. *characteristics of F4/80$^+$ cells in gate 3*

A summary of the phenotypic characteristics of F4/80$^+$ cells in gate 3 is provided in Table 4.8. Although F4/80$^-$Ia$^+$ cells were preferentially enriched in this gate, a small population of F4/80$^+$Ia$^+$ cells was also present herein.

i) expression of Mϕ activation markers

43-66% of gate 3 F4/80$^+$ cells expressed sialoadhesin, scavenger receptor and macrosialin at relatively low intensities (Figure 4.7 and Table 4.8). There were small populations of cells in gate 3 expressing macrosialin (2.5% of gate), scavenger receptor (1.9% of gate) and sialoadhesin (0.9% of gate) which did not co-express F4/80.

ii) expression of β2 integrins

Of the F4/80$^+$ cells in gate 3, 33% expressed Mac-1 and 70% expressed CD11c/CD18. Intensity of Mac-1 was over 2-fold higher than that of CD11c/CD18 (Figure 4.7 and Table 4.8). There were substantial populations of Mac-1$^+$ and CD11c/CD18$^+$ cells in gate 3 which did not co-express F4/80 (9% and 18% of gated cells respectively).

iii) expression of dendritic cell markers

Of the F4/80$^+$ cells in gate 3, 43% expressed 33D1 and 29% expressed DEC-205. 100% expressed CD1. Intensity of CD1 expression was slightly higher than that of the other two dendritic cell markers (Figure 4.7 and Table 4.8). There were small populations of cells (2-3%) in gate 3 which expressed 33D1 and DEC-205 but not F4/80. Of the gate 3 cells, 24% expressed CD1 and not F4/80.

4.5.2 Phenotypic characteristics of Ia$^+$ uterine cells

Since only gate 2 and gate 3 contained significant populations of Ia$^+$ cells, the co-expression characteristics of gate 1 Ia$^+$ cells were not considered. Due to reagent limitations, phenotypic analysis of Ia$^+$ cells was limited to β2 integrins and dendritic cell markers.

Table 4.8 Summary of the cell membrane phenotype of F4/80$^+$ uterine cells in gate 3. F4/80$^+$ uterine cells in gate 3 were immunophenotyped by 2-colour fluorometric analysis. The numbers of F4/80$^+$ cells co-expressing Mϕ activation markers, β2 integrins and dendritic cell markers are given as a % of all gate 3 F4/80$^+$ cells (% expressing). A measure of mean intensity of antigen expression on F4/80$^+$ cells is also provided (intensity score). + = < 500 intensity units, ++ = 500-1000 intensity units, +++ = > 1000 intensity units. For corresponding graphs see Figure 4.7. [*Results are representative of 2 experiments]

		% expressing	intensity score
Mϕ activation markers	*macrosialin	50	+
	*scavenger receptor	66	+
	*sialoadhesin	43	+
β2 integrins	*Mac-1	33	+++
	CD11c/CD18	70	+
dendritic cell markers	33D1	43	+
	DEC-205	29	+
	CD1	100	++

a. characteristics of Ia$^+$ cells in gate 2

A summary of the phenotypic characteristics of F4/80$^+$ cells in gate 2 is provided in Table 4.9. F4/80$^+$Ia$^+$ cells were preferentially enriched within this gate.

i) expression of β2 integrins

Of the Ia$^+$ cells in gate 2, 85% and 57% expressed Mac-1 and CD11c/CD18 respectively. The intensity of CD11c/CD18 expression was slightly lower than that of Mac-1 on gate 2 Ia$^+$ cells (Figure 4.8A and B and Table 4.9). The intensity of both β2 integrins was approximately 2-fold higher on Ia$^+$ cells than on Ia$^-$ cells. Only 2% of gate 2 cells were Mac-1$^+$ or CD11c/CD18$^+$ and did not co-express Ia.

ii) expression of dendritic cell markers

Of the gate 2 Ia$^+$ cells, 55% expressed 33D1, 44% expressed DEC-205 and 77% expressed CD1 (Figure 4.8). Intensity of CD1 expression was slightly less intense than the other two dendritic cell markers (Figure 4.8C and E and Table 4.9). There were very few 33D1$^+$, DEC-205$^+$ and CD1$^+$ cells which did not co-express Ia.

b. characteristics of Ia$^+$ cells in gate 3

A summary of the phenotypic characteristics of F4/80$^+$ cells in gate 3 is provided in Table 4.9. F4/80$^-$Ia$^+$ cells were preferentially enriched within this gate.

i) expression of β2 integrins

Of the Ia$^+$ cells in gate 3, 73% and 56% expressed Mac-1 and CD11c/CD18 respectively and at similar intensities (Figure 4.8F and G and Table 4.9). The intensity of Ia expression was similar on Mac-1$^+$ and Mac-1$^-$ cells. In contrast, the intensity of Ia expression was 2-fold higher on CD11c/CD18$^+$ cells than on CD11c/CD18$^-$ cells. There were populations of Ia$^-$ cells in gate 3 which also expressed β2 integrins, and the intensity of CD11c/CD18 expression was over 3-fold lower on Ia$^-$ cells than Ia$^+$ cells.

ii) expression of dendritic cell markers

Of the Ia$^+$ cells in gate 3, 33% expressed 33D1 and 23% expressed DEC-205. Intensity of CD1 expression was slightly lower than the other two dendritic cell markers (Figure 4.8H-J and Table 4.9). In contrast, 95% expressed CD1. A small number of the Ia$^-$ cells expressed low intensity CD1.

Table 4.9 Summary of the cell membrane phenotype of Ia$^+$ uterine cells in gates 2 and 3. Ia$^+$ uterine cells in gates 2 and 3 were immunophenotyped by 2-colour fluorometric analysis. The numbers of Ia$^+$ cells co-expressing β2 integrins and dendritic cell markers are given as a % of gated Ia$^+$ cells (% expressing). A measure of mean intensity of antigen expression on Ia$^+$ cells is also provided (intensity score). + = < 200 intensity units, ++ = 200-500 intensity units, +++ = 500-1000 intensity units, ++++ = > 1000 intensity units. For corresponding graphs see Figure 4.8.

		gate 2		*gate 3*	
		% expressing	*intensity score*	*% expressing*	*intensity score*
β2 integrins	Mac-1	85	++++	73	++
	CD11c/CD18	57	+++	56	++
dendritic cell markers	33D1	55	++++	33	++
	DEC-205	44	++++	23	++
	CD1	77	+++	95	+

4.5.3 Co-expression of Mac-1 and CD11c/CD18 by uterine cells

Dual analysis of Mac-1 and CD11c/CD18 expression showed that nearly 100% of the cells which expressed CD11c/CD18 in gates 1 and gate 2 also expressed Mac-1 (Figure 4.9A and B). Thus the F4/80$^+$, CD11c/CD18$^+$ cells identified in Figures 4.5 and 4.6 are likely to have also been Mac-1$^+$. In contrast, in gate 3, 65% of CD11c/CD18$^+$ cells were Mac-1$^-$, indicating differential expression of the β2 integrins molecules amongst the APCs within that gate (Figure 4.9C). The intensity of CD11c/CD18 expression was similar on gate 3 Mac-1$^+$ and Mac-1$^-$ cells. In gate 1, 69% of Mac-1$^+$ cells also expressed CD11c/CD18. In gates 2 and 3, approximately 45% of Mac-1$^+$ cells also expressed CD11c/CD18. In all three gates, the intensity of Mac-1 expression tended to be lower on CD11c/CD18$^-$ cells than on CD11c/CD18$^+$ cells (Figure 4.9).

4.5.4 Expression of costimulatory molecules by uterine cells

Expression of costimulatory molecules B7-2 and CD40 on uterine cells was analysed by single-colour flow cytometry. B7-2 expression, although present on some cells in gates 1 and 2, was of a very low intensity. Only 1% of gate 1 cells and 0.5% of gate 2 cells expressed B7-2 at sufficient levels to be accurately quantified. In contrast, 25% of cells in gate 3 expressed moderate levels of B7-2. B7-2 expression was also detected on IFNγ-cultured spleen cells (Figure 4.10A-D).

CD40 was not expressed on any uterine cells in gates 1, 2 and 3, but was detectable on IFNγ-cultured spleen cells (Figure 4.10E-H).

4.6 Effect of ovariectomy on APCs in uteri

Immunohistochemical studies described in Chapter 1 demonstrated that there were sub-populations of APCs in the murine uterus which were sensitive to removal of ovarian steroid hormones by ovariectomy. In order to examine the populations of APCs in ovariectomised uteri by single and dual colour flow cytometry, pools of 12-15 uteri from mice which had been bilaterally ovariectomised 3 weeks previously were enzymatically digested and dual-labeled for F4/80 and Ia (using protocols 2 and 3 in Table 4.2), or single-labeled for macrosialin, scavenger receptor, sialoadhesin, DEC-205 and 33D1 (using protocol 4 in Table 4.2) for flow cytometric analysis.

4.6.1 Effect of ovariectomy on forward and side scatter analysis of uterine cells

Forward and side scatter analysis of cells from ovariectomised uteri produced dot plots which were different to those seen in studies of uterine cells from normally cycling mice. The populations of cells previously observed in gates 1, 2 and 3 were severely diminished in ovariectomised uteri. Each of the gates contained only 3.5%, 10.5% and 12.5% of the released cells respectively (the same gates in estrous uteri contained approximately 10%, 20% and 40% of total cells respectively). The remaining 73% of total cells from ovariectomised uteri were not contained by these gates : presumably these were stromal cells with different forward and side scatter characteristics (Figure 4.11A).

4.6.2 Effect of ovariectomy on numbers of uterine cells expressing F4/80 and Ia

The populations of cells expressing F4/80 and Ia were clearly different in ovariectomised uteri to those in estrous uteri (Figure 4.11B-D and Table 4.10). As a proportion of total counted cells, there were over 10-fold fewer cells of the $F4/80^{+}Ia^{-}$ phenotype following ovariectomy than at estrus. This was reflected in a > 17% reduction in the proportion of gate 1 cells comprising this phenotype. Interestingly, there was an increase in the number of $F4/80^{+}Ia^{+}$ cells and $F4/80^{-}Ia^{+}$ found in gate 1 in ovariectomised uteri.

$F4/80^{+}Ia^{+}$ and $F4/80^{-}Ia^{+}$ cells comprised similar proportions of total cells of uteri from ovariectomised mice as was observed at estrus. This was due to an increase in the proportion of cells of these phenotypes found in both gates 2 and 3 following ovariectomy. In total, as a proportion of total cells released by enzymatic digestion, the cells released from the uteri of ovariectomised mice contained approximately 50% less $F4/80^{+}$ cells but a similar proportion of Ia^{+} cells compared to cells released from the uteri of estrous mice.

4.6.3 Effect of ovariectomy on numbers of uterine cells expressing other APC markers

Cells expressing macrosialin, scavenger receptor, sialoadhesin, 33D1 and DEC-205 were all identified by single-colour flow cytometric analysis of cells from ovariectomised uteri (Figure 4.11E-I). $Macrosialin^{+}$ and $33D1^{+}$ cells were most abundant, being expressed by 12% and 13% of total cells. Cells expressing scavenger receptor and sialoadhesin comprised 9% of total cells, and those expressing DEC-205 comprised 5% of total cells. Also evident were populations of cells expressing low intensity macrosialin, 33D1 and DEC-205 which could not be quantified accurately.

Table 4.10 Populations of uterine cells expressing F4/80 and Ia following ovariectomy. Cells obtained by enzymatic digestion of pooled uteri (n=12-15) from mice which had been ovariectomised 3 weeks earlier were analysed by flow cytometry for expression of F4/80 and Ia. [a]For each of 2 experiments (ovx 1 and ovx 2), the number of cells expressing F4/80 alone ($F4/80^+Ia^-$), both F4/80 and Ia ($F4/80^+Ia^+$) or Ia alone ($F4/80^-Ia^+$) is shown as % gated cells and % total cells in gates 1, 2 and 3 (described in section 4.4.2). [b]The sum of all cells in each population as % total cells is also given. [c]For comparison, the median corresponding values obtained by analysis of uterine cells from estrous mice (est, data presented originally in Table 4.4) are also shown. [d]total $F4/80^+$ and Ia^+ cells in ovariectomised uteri as a % of gated and % of all cells is also shown as mean value from experiments ovx 1 and ovx 2.

		Gate 1		*Gate 2*		*Gate 3*		*[b]all*
population	[a]expt.	*%gate*	*%total*	*%gate*	*%total*	*%gate*	*%total*	*% total*
$F4/80^+Ia^-$	ovx 1	1.4	0.1	0.5	0.1	0.1	0.0	0.2
	ovx 2	3.4	0.1	5.0	0.5	0.2	0.0	0.6
	[c]est.	*52.2*	*5.2*	*3.0*	*0.6*	*0.6*	*0.2*	*6.0*
$F4/80^+Ia^+$	ovx 1	20.4	0.7	28.2	2.9	5.1	0.6	4.2
	ovx 2	8.9	0.3	30.3	3.2	5.9	0.7	4.2
	[c]est.	*3.4*	*0.3*	*12.0*	*2.4*	*2.5*	*1.0*	*3.7*
$F4/80^-Ia^+$	ovx 1	4.4	0.2	10.5	1.1	11.1	1.4	2.7
	ovx 2	9.7	0.3	7.9	0.8	20.5	2.6	3.7
	[c]est.	*0.5*	*0.1*	*3.9*	*0.8*	*5.2*	*2.1*	*3.0*
[d]all $F4/80^+$	ovx (mean)	17.1	0.6	32.0	3.4	5.7	0.7	4.6
	[c]est.	*55.6*	*5.6*	*15.0*	*3.0*	*3.1*	*1.2*	*9.8*
[d]all Ia^+	ovx (mean)	21.7	0.8	38.5	4.0	21.5	2.7	7.4
	[c]est.	*3.9*	*0.4*	*15.9*	*3.2*	*7.7*	*3.1*	*6.7*

4.7 Expression of CD1 by uterine epithelial cells

The CD1 family of proteins (comprised of group I and group II forms) has recently been identified as a novel group of antigen presenting molecules thought to have a role in activation of cell mediated immunity, particularly through their interactions with a sub-population of T cells known as natural killer (NK) T cells (reviewed in Porcelli and Modlin 1999). Although expression of CD1 is largely limited to professional APCs and some other leukocytes, several studies of humans and rodents have found expression of group II CD1 (murine CD1 and its homologues) by normal gastrointestinal epithelia, which is perhaps a reflection of unique modes of immune induction and regulation in this mucosal tissue. Whether epithelia in other mucosae in mice also express CD1 has not been established.

In order to assess the expression of CD1 by uterine epithelial cells by dual colour flow cytometry, cells obtained by enzymatic digest of pooled estrous uteri were labeled with mAbs MTS#24 and CD1 using protocols 2 and 3 (Table 4.2). In a gate which enclosed live cells, 23% of $MTS\#24^+$ uterine epithelial cells were found to co-express CD1 (Figure 4.12).

4.8 Conclusions and discussion

The experiments in this chapter describe the development of methods for phenotypic analysis of *ex-vivo* uterine APCs. The findings have established that flow cytometry can be used to immunophenotype leukocytes within relatively crude preparations of uterine cells and the data provides evidence of several distinct populations of APCs within the murine uterus.

Initially, it was established that viable F4/80 and Ia expressing cells could be harvested from cycling murine uteri. Consistent with data derived by other authors (Hunt *et al.* 1985, De and Wood 1990, De and Wood 1991, Wood *et al.* 1997), 10-20% of cells released by enzymatic digestion of uteri were identified as Mϕs based on their expression of FcR and F4/80, their adherence to tissue culture plastic and their ability to phagocytose latex beads. Interestingly, cells selected on the basis of their Ia expression were much less phagocytically competent than $F4/80^+$ cells.

Cells expressing F4/80 were divided into two populations on the basis of their flow cytometric features. The first population, which comprised 60% of total $F4/80^+$ cells at estrus, was Ia^- and these cells were easily phenotyped since they could be contained within gate 1 for analysis without significant 'contamination' by other $F4/80^+$ cells. In addition to their lack of Ia expression, these $F4/80^+$ cells appeared to be of a relatively unactivated phenotype since they were small in size, and expressed no or very low levels of cell-surface macrosialin, scavenger receptor, sialoadhesin or B7-2. The expression of both Mac-1 and CD11c/CD18 by the majority of these cells is suggestive of their recent interaction with endothelial cell adhesion molecules during recruitment from the blood, but also in their maintenance within the tissue by interacting with local ligands (Carlos and Harlan 1994, Prieto *et al.* 1994, Lundahl *et al.* 1996).

A second population of Mϕs, comprising approximately 40% of total $F4/80^+$ cells, was initially differentiated from the other Mϕs on the basis of their relatively large size and their expression of cell surface Ia. These cells were moderately enriched by enclosure within gate 2, but the presence of small numbers of $F4/80^+Ia^-$ and $F4/80^-Ia^+$ cells within that gate made phenotypic analysis in this case a little more complex. However, by analysis of both $F4/80^+$ and Ia^+ cells in gate 2, it was established that in comparison to the Mϕs in gate 1, gate 2 Mϕs were of a considerably more activated phenotype.

Up to 94% of the gate 2 Mϕs expressed CD1: concurrent Ia and CD1 expression on murine Mϕs has been reported previously (Roark *et al.* 1998), and together these characteristics are suggestive of a role for these cells in presentation of protein and lipid antigens to T cells (Hart 1997, Porcelli and Modlin 1999). Further evidence of the activated nature of these cells was provided by data showing that approximately 50% of them expressed cell surface scavenger

receptor, macrosialin and sialoadhesin, suggestive of their ability to internalise and process antigen, and to cluster T cells (Rabinowitz and Gordon 1991, de Villiers *et al.* 1994, Muerkoster *et al.* 1999). A higher proportion of these cells probably also expressed these antigens (particularly macrosialin) internally (Rabinowitz and Gordon 1991, de Villiers *et al.* 1994), but this was not assessed in these studies.

The high expression of Mac-1 found on the gate 2 Mϕs is a common feature of activated tissue Mϕs (Gessani *et al.* 1993, Duraiswamy *et al.* 1994), particularly those exposed to inflammatory and chemokine-rich milieux (Rogler *et al.* 1997). Expression of β2 integrins is thought to be reflective of the capacity of these cells to participate in ligand-binding for endocytosis or retention within a defined microenvironment. In particular, Mac-1 (also known as Complement Type 3 Receptor) can mediate uptake of complement-coated pathogens (Rosen and Gordon 1989). The less frequent and lower intensity of CD11c/CD18 expression may have been a result of the use of different labeling protocols for the two integrins or might be reflective of the more limited roles of the CD11c/CD18 molecule after recruitment and transendothelial migration (Rosen and Gordon 1989, Carlos and Harlan 1994).

Thus, two clearly distinct populations of F4/80$^+$ Mϕs exist in the uterus of cycling virgin mice. The relatively less activated population seems likely to represent a relatively homogeneous group of cells which henceforth be referred to as 'undifferentiated Mϕs'. In contrast, the second population of Mϕs ('differentiated Mϕs') exhibits heterogeneous features consistent with more activated phenotypes.

A third population of APCs identified by dual colour flow cytometry expressed Ia but not F4/80. This staining pattern is immediately suggestive of a lineage of dendritic cells, and similar cells in the murine uterus have been noted previously (Robertson *et al.* 1998). These cells (hereafter called 'dendritic cells') were able to be gated separately from the majority of differentiated Mϕs based on their relatively poor capacity to scatter light, an indication of their smaller size and lower density of intracellular organelles, particularly lysosomes. These are both characteristic features of dendritic cells from other mucosal tissues (Crowley *et al.* 1989, Girolomoni *et al.* 1990, Havenith *et al.* 1993). A proportion of the uterine dendritic cells appeared to express several cell surface markers (Mac-1, CD11c/CD18, 33D1 and DEC-205) known to be expressed on myeloid dendritic cells in other peripheral organs (Steinman 1991, Duraiswamy *et al.* 1994) and up to 95% expressed the antigen-presentation molecule CD1 which is found at high levels on splenic dendritic cells (Roark *et al.* 1998). However, a similar problem to that encountered in analysis of differentiated Mϕs was encountered in analysing uterine dendritic cells. A small population of F4/80$^+$Ia$^+$ cells which 'contaminated' gate 3 meant that

interpretation of the data describing the expression of phenotypic markers on Ia^+ cells in gate 3 was limited. Better characterisation of these cells could be performed if the contaminating $F4/80^+$ cells were removed prior to FACs analysis, or if a three colour FACS system was employed.

DEC-205 and 33D1 are two molecules thought to be restricted to quite distinct dendritic cell populations. DEC-205 is a cell-surface endocytic receptor (Jiang *et al.* 1995) expressed at very high levels on the sparse but long-lived populations of interdigitating dendritic cells of lymph node, spleen and thymus (Crowley *et al.* 1989, Crowley *et al.* 1990, Steinman 1991) and on interstitial dendritic cells such as the Langerhans cells of the skin (Inaba *et al.* 1995). In contrast, the mAb 33D1 is though to detect much more short-lived dendritic cells, such as the highly mobile population of marginal zone splenic dendritic cells and dendritic cells in corresponding areas of lymph node and Peyers Patch (Nussenzweig *et al.* 1982, Crowley *et al.* 1989, Crowley *et al.* 1990). Whether the same cells amongst the uterine APCs expressed both DEC-205 and 33D1 was not established, but is a possibility (Gao *et al.* 1997, Cao *et al.* 1998, Masurier *et al.* 1999). Alternatively, the markers could have defined cells derived from different locations within the uterus. One plausible scenario is that cells located near the lumen might express DEC-205, reflective of their role in luminal antigen sampling, whereas those in the mesometrial triangle would be more likely to express 33D1, in keeping with their presumed trafficking behaviour. Clearly these studies were not designed to assess the dynamics of within-tissue migration of uterine APCs, but such data might be obtained using tagged antigen or after passive transfer of labeled dendritic cell progenitors.

As recently reviewed (Porcelli and Modlin 1999), the expression of CD1 by uterine APCs is consistent with a role for these cells in generating antimicrobial immunity. Furthermore, although several T cell lineages can express TCRs specific for CD1, of particular interest for mucosal immunity is the apparent reliance of NKT cells on antigen presentation by CD1 molecules. NKT cells, which comprise a significant proportion of the T cells in mice, are though to play a role in the regulation and perhaps deviation of immune responses through their propensity to release large quantities of IL-4 and other polarising cytokines at sites of antigen presentation (Bendelac *et al.* 1997). If NKT cells were activated in the uterus, the nature of immune responses directed against antigens introduced into the uterine lumen either at the time of mating or subsequently during pregnancy could be markedly influenced by the secretion of such cytokines. Whether $CD1^+$ uterine epithelial cells could play a similar role requires further investigation.

Flow cytometric analysis of uterine cells from ovariectomised mice allowed the identification of steroid hormone-resistant populations of APCs. The undifferentiated Mϕs were

found to be very sensitive to removal of circulating steroid hormone levels by ovariectomy; it might be argued that these cells require active secretion of chemotactic molecules and growth factors from steroid hormone-primed uterine epithelial cells to be continually recruited or to remain viable (De and Wood 1990, Wood *et al.* 1992, Pollard *et al.* 1998). In contrast, populations of differentiated Mϕs and dendritic cells appeared to be more resistant to removal of steroid hormones, since they were comparable in number on a per cell level after ovariectomy. Thus it appears that the population of dendriform cells resident within uteri of ovariectomised mice identified by immunohistochemistry in Chapter 3 is comprised of differentiated Mϕs and dendritic cells. Presumably a proportion of these cells is remarkably long-lived or can be replenished independently of steroid-hormone regulated growth factor support from stromal cells. Alternatively, it might be that sufficient trophic signals, perhaps even originating from the Mϕs and dendritic cells themselves, are synthesised in the uterus despite ovariectomy.

In summary, the virgin cycling uterus appears to host at least three distinguishable populations of APCs : undifferentiated Mϕs, differentiated Mϕs and dendritic cells. The relationship between these cells remains to be explored, but based on findings in other mucosal tissues certain speculations are justified. The present data is consistent with a model whereby the small granular Mϕs represent the starting point of a differentiation continuum (Figure 4.13). In this model, it is postulated that undifferentiated monocytes arrive in the uterus after recruitment due to chemotactic gradients emanating from steroid hormone primed uterine epithelial cells (Wood *et al.* 1992, Pollard *et al.* 1998, Robertson *et al.* 1998, Robertson *et al.* 2000). The cells would enter the uterine tissue by virtue of their β2-integrin mediated interactions with adhesion molecules on endothelial cells. β2 integrins may also facilitate the binding of extra-cellular ligands and migration between stromal cells once Mϕs enter the endometrium (Carlos and Harlan 1994, Prieto *et al.* 1994, Lundahl *et al.* 1996). As the cells remain in the uterus, their cell surface antigen expression profile would be expected to change due to exposure to local cytokines and other differentiating factors. A key candidate is locally synthesised CSF-1 (Wood *et al.* 1992, Pollard 1997), which would act to maintain Mϕ viability, promote the acquisition of a larger morphology and stimulate expression of macrosialin and scavenger receptor (de Villiers *et al.* 1994, Li *et al.* 1998, Pollard *et al.* 1998). Enhanced expression of scavenger receptor would further promote retention of the cells in an extra-cellular matrix-rich tissue such as the uterus (de Villiers *et al.* 1994). Based on *in vitro* experiments, uterine epithelial cell derived GM-CSF (Robertson *et al.* 1992, Robertson *et al.* 1996b) would also be expected to promote Mϕ viability, proliferation and the acquisition of a large, spread morphology (Chodakewitz *et al.* 1988, Kato *et al.* 1990). Furthermore, GM-CSF may stimulate the cells to assume a more activated phenotype

by virtue of enhanced expression of Mac-1, macrosialin, Ia and B7-2 (Gordon *et al.* 1995, Rohn *et al.* 1996, Selgas *et al.* 1996, Li *et al.* 1998). IFNγ and TNFα derived from uterine cells (De *et al.* 1992, Platt and Hunt 1998) could have similar effects (Hathcock *et al.* 1994, Gordon *et al.* 1995, Rohn *et al.* 1996). Thus a range of differentiated Mϕ phenotypes might evolve depending on the precise cytokine milieu prevailing within the local microenvironment, with certain combinations of cytokines favouring the development of highly differentiated cells.

An alternative differentiation outcome for monocytes recruited to the uterus might be evolution into dendritic cells (Figure 4.13). *In vitro* studies suggest that cells with many features in common with peripheral tissue myeloid dendritic cells can develop from precursor cells such as monocytes under favourable cytokine and tissue matrix environments, such as those rich in GM-CSF and those which allow normal cell trafficking to occur (Inaba *et al.* 1993, Randolph *et al.* 1998, Steinman and Inaba 1999). Whether cells can progress directly from monocytes to dendritic cells, or alternatively from monocytes via differentiated Mϕs to dendritic cells is not known. The generation of dendritic cells from differentiated Mϕs within the uterus seems possible since the $F4/80^+$ and $F4/80^-$ APCs in gates 2 and 3 express many of the same markers, including those thought to be restricted to dendritic cells. Indeed, the small population of $F4/80^+$ cells in gate 3 may represent an intermediate cell between monocytes and dendritic cells, or between differentiated Mϕs and dendritic cells. Interestingly, similarly heterogeneous populations of Langerhans-like cells in murine vagina have been identified. Vaginal APCs were found to exhibit any of the following phenotypes : $Ia^+DEC\text{-}205^+$, $Ia^+DEC\text{-}205^-$, $F4/80^+Ia^+$, $F4/80^-Ia^+$ or $F4/80^+Ia^-$ (Parr and Parr 1991).

To better define the possible developmental pathways of monocytes, Mϕs and dendritic cells within the murine uterus, several experimental approaches could be taken. Firstly, the use of multi-colour FACS analysis would allow the identification of cells concurrently expressing more than 2 cell membrane markers. Secondly, an analysis of the APCs in uteri of mice following administration of Flt3 ligand could facilitate the identification of mature and precursor dendritic cell populations in the uterus by increasing the total numbers of cells present (Pulendran *et al.* 1997). Also, prolonged culture of uterine Mϕs and dendritic cells under defined cytokine conditions may provide some insight into their developmental potentials, and similarly an analysis of the uterine APCs in mice deficient in key APC cytokines (such as GM-CSF) may also prove illuminating. Finally, a flow cytometric analysis of uterine APCs during an inflammatory response, such as that which occurs following insemination, would be expected to provide some information pertaining to the phenotypes and relative proportions of recently recruited and highly activated uterine APCs.

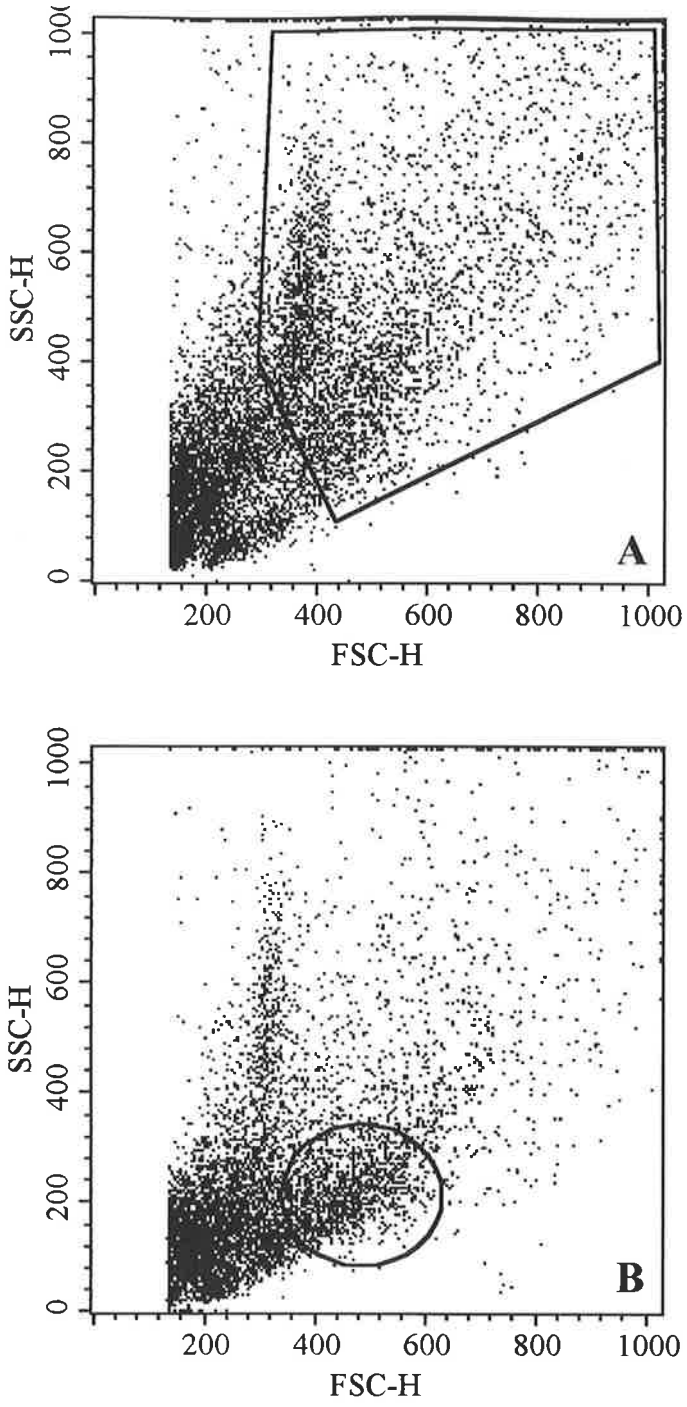


Figure 4.2 Forward and side scatter analysis of uterine cells at estrus. The light scattering characteristics of cells obtained by enzymatic digestion of pooled (n = 5; A) and individual (B) uteri of estrous mice were analysed by flow cytometry. The light scattered by the cells is presented in arbitrary units as forward scatter (FSC-H, a measure of cell size) and side scatter (SSC-H, a measure of intra-cellular complexity). The region outlined in (A) was used to define the live cells and exclude dead cells and debris in all experiments. The gate in (B) encloses a population of cells with similar forward and side scatter characteristics: in this instance, the gated cells are of medium size and low intra-cellular complexity. The data is representative of 7 and 4 experiments of pooled and individual estrous uteri respectively.

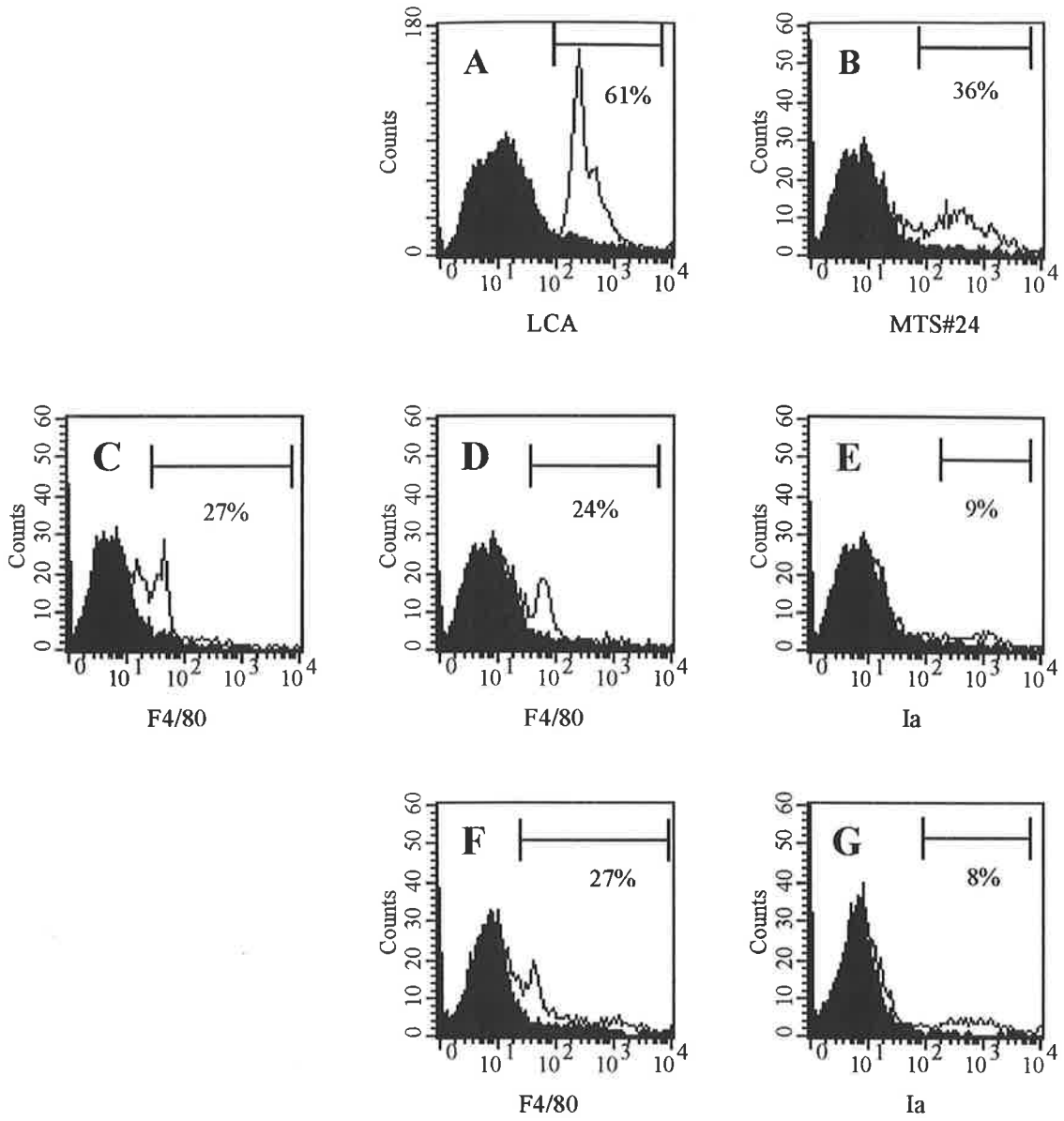
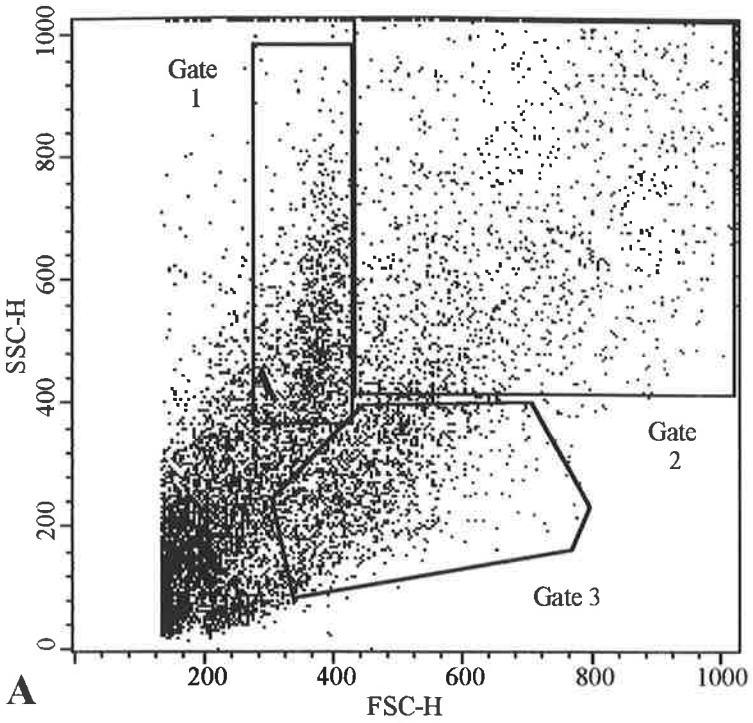
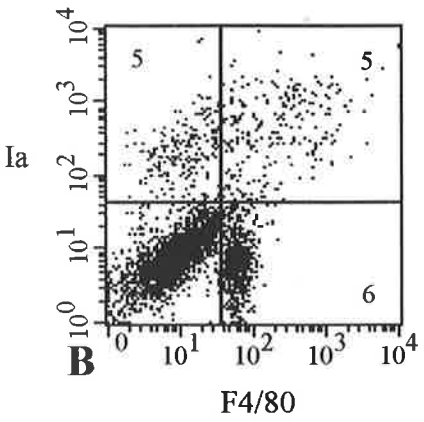
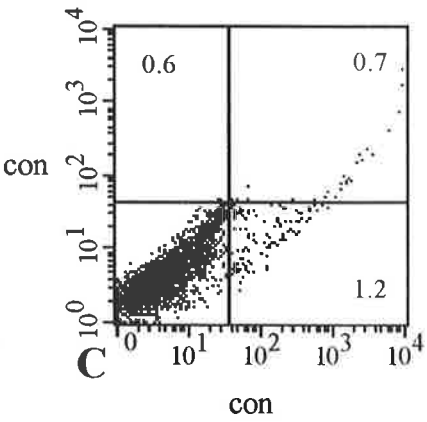


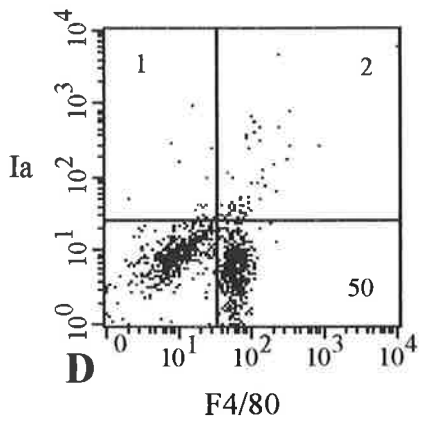
Figure 4.3 Single colour flow cytometric analysis of uterine cells at estrus. Aliquots of cells obtained by enzymatic digestion of uteri from estrous mice (n = 10) were indirectly labeled with mAbs and FITC or RPE and analysed by single colour flow cytometry. Data is presented as histograms showing fluorescence intensity in arbitrary units (x axis) for FITC or RPE as a function of cell frequency (Counts, y axis). Unfilled histograms show fluorescence of cells labeled directly (C) or indirectly (A, B, D-G) with FITC (A-E) or RPE (F, G) using mAbs specific for leukocyte common antigen (LCA, A), MTS#24 (B), F4/80 (C, D, F) and Ia (E, G). For each mAb, the number of FITC- or RPE-labeled cells as a percentage of total cells is given. These values were calculated using the indicated marker regions from which >98% of cells labeled with an irrelevant mAb (shown as a filled histogram in each graph) were excluded. Data is representative of 3-10 experiments.

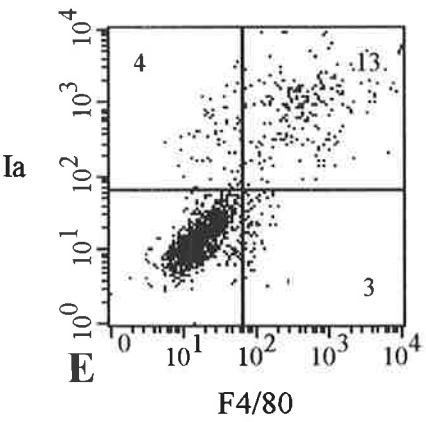
Figure 4.4 Dual colour flow cytometric analysis of F4/80$^+$ and Ia$^+$ uterine APCs at estrus. Cells obtained by enzymatic digestion of uteri from estrous mice (n = 7) were indirectly labeled with Ia-RPE and F4/80-FITC and analysed by dual colour flow cytometry. The light scattering characteristics of the cells are shown in (A), in which gates 1, 2 and 3 define small and granular cells, large and granular cells and cells of medium size and medium granularity respectively. Fluorescence intensity (in arbitrary units) attributed to FITC (x axis) and RPE (y axis) on non-gated uterine cells (B) and on cells contained within gates 1 (D), 2 (E) and 3 (F) is also shown, with the numbers of F4/80$^+$Ia$^-$, F4/80$^+$Ia$^+$ and F4/80$^-$ Ia$^+$ cells given as a proportion of total (B) or gated cells (D, E, F) in the lower right, upper right and upper left quadrants respectively. Each of these quadrants contained <1% of gated cells in irrelevant isotype control preparations (C). Data is representative of 4 experiments.

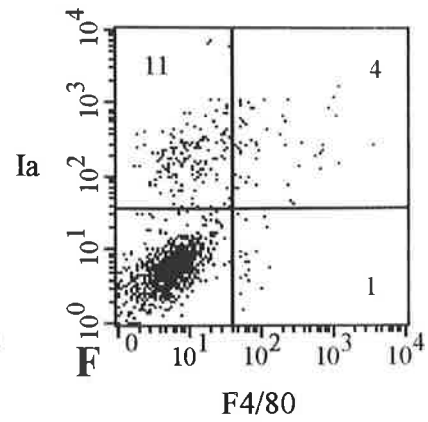

Gate 1
Gate 2
Gate 3
SSC-H
FSC-H
0
200
400
600
800
1000
A


Ia
5
5
6
F4/80
B


con
0.6
0.7
1.2
con
C


Ia
1
2
50
F4/80
D


Ia
4
13
3
F4/80
E


Ia
11
4
1
F4/80
F

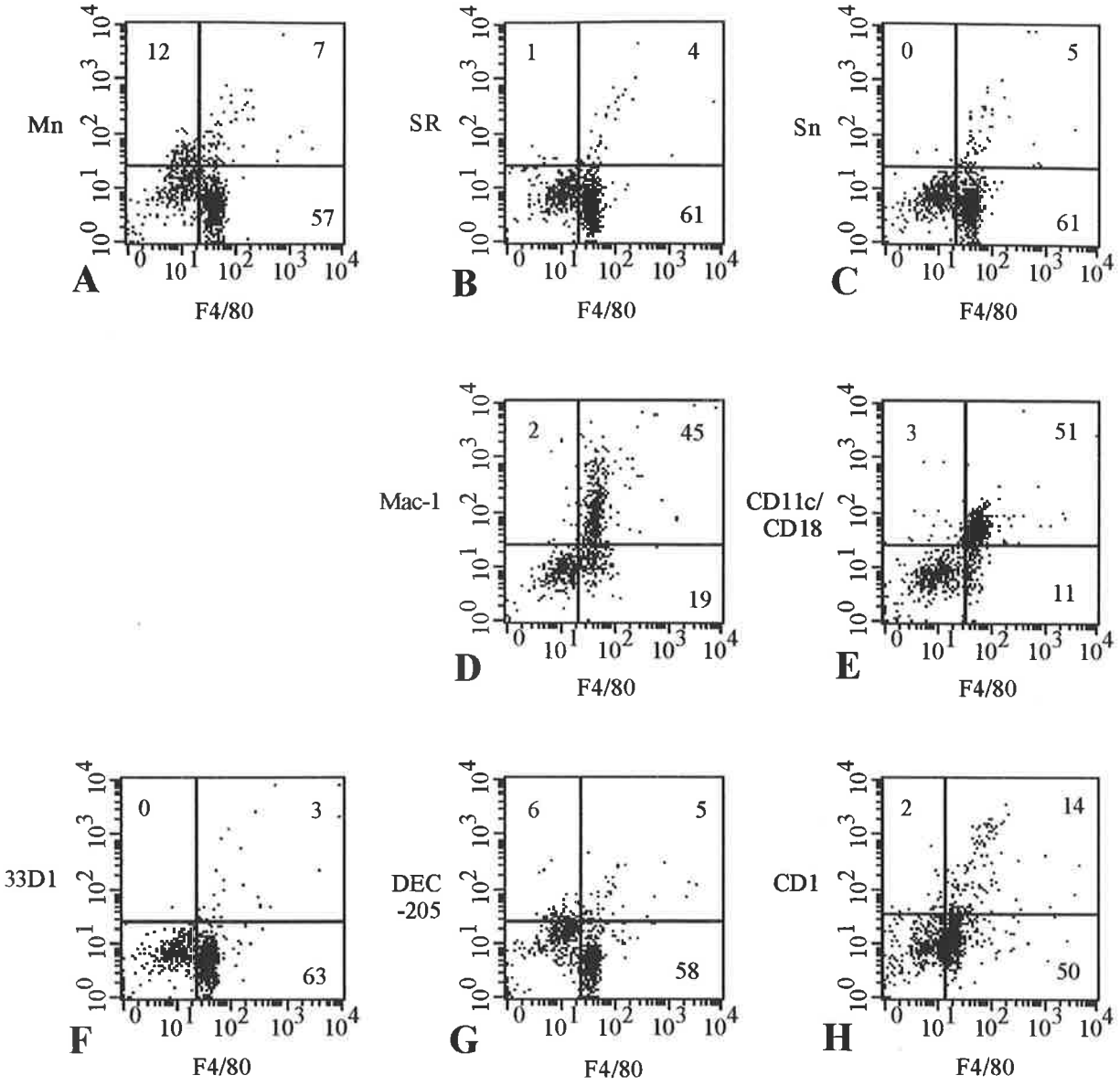


Figure 4.5 Phenotype of gate 1 F4/80$^+$ uterine cells at estrus. Aliquots of cells obtained by enzymatic digestion of uteri from estrous mice (n = 10) were indirectly labeled with RPE using mAbs specific for macrosialin (Mn, A), class A scavenger receptor (SR, B), sialoadhesin (Sn, C), Mac-1 (D), CD11c/CD18 (E), 33D1 (F), DEC-205 (G) and CD1 (H). The cells were then labeled directly with F4/80-FITC and analysed by dual colour flow cytometry. Fluorescence intensity (in arbitrary units) attributed to FITC (x axis) and RPE (y axis) on cells in gate 1 is shown, with the numbers of cells comprising each phenotype given as a proportion of gate 1 cells in the lower right, upper right and upper left quadrants respectively. Each of these quadrants contained <1% of gated cells in irrelevant isotype control preparations. In A-D, data is representative of 2 experiments.

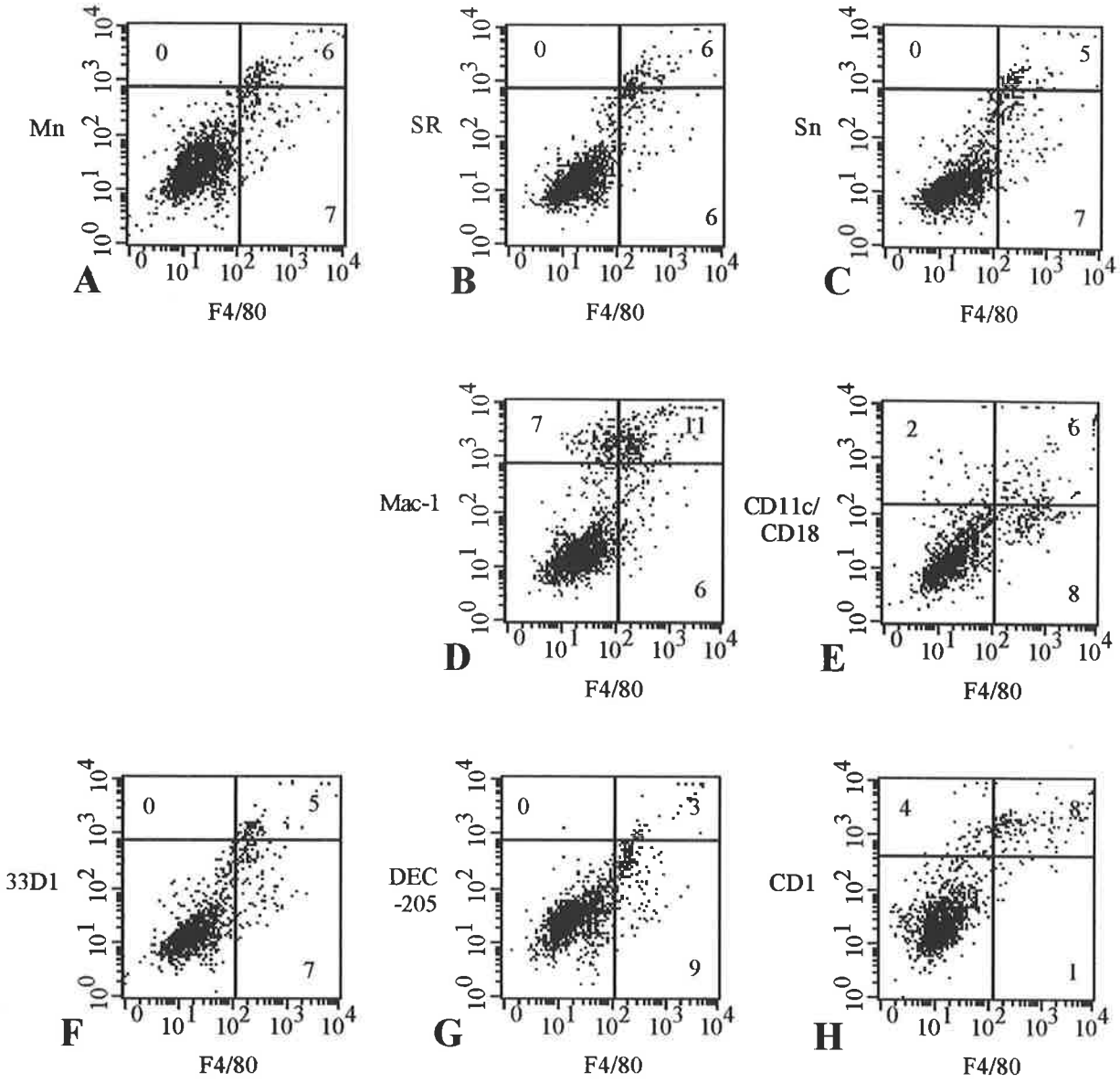


Figure 4.6 Phenotype of gate 2 F4/80$^+$ uterine cells at estrus. Aliquots of cells obtained by enzymatic digestion of uteri from estrous mice (n = 10) were indirectly labeled with RPE using mAbs specific for macrosialin (Mn, A), class A scavenger receptor (SR, B), sialoadhesin (Sn, C), Mac-1 (D), CD11c/CD18 (E), 33D1 (F), DEC-205 (G) and CD1 (H). The cells were then labeled with F4/80-FITC and analysed by dual colour flow cytometry. Fluorescence intensity (in arbitrary units) attributed to FITC (x axis) and RPE (y axis) on cells in gate 2 is shown, with the numbers of cells comprising each phenotype given as a proportion of gate 2 cells in the lower right, upper right and upper left quadrants respectively. Each of these quadrants contained <1% of gated cells in irrelevant isotype control preparations. In A-D, data is representative of 2 experiments.

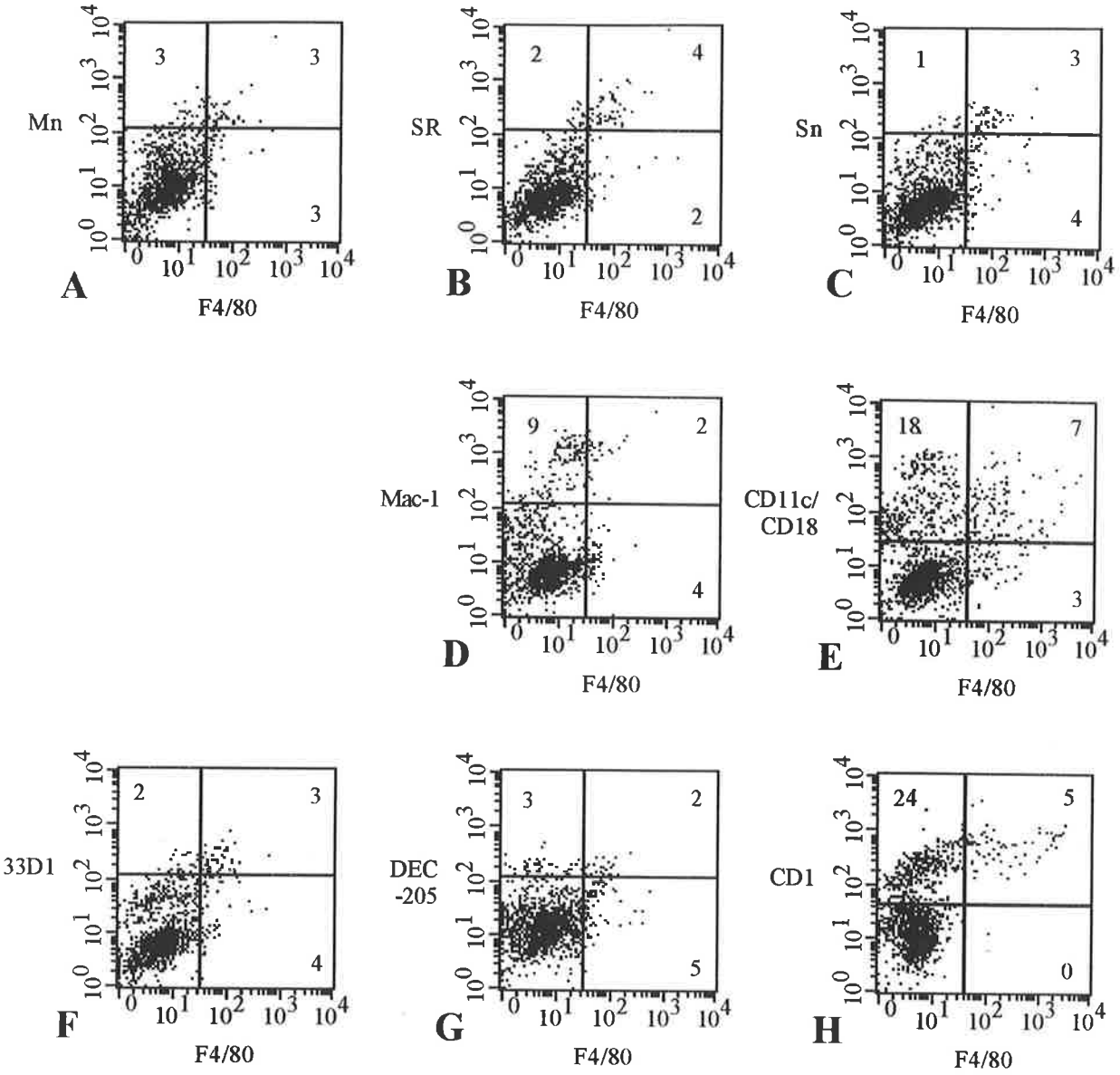
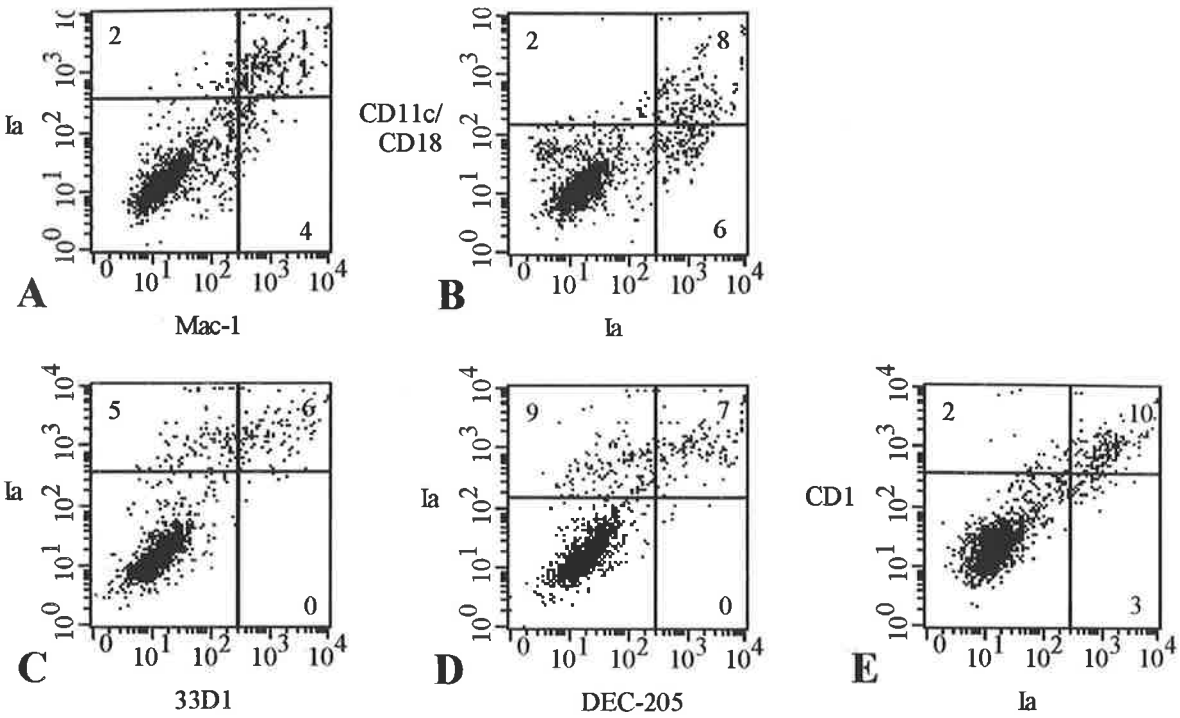
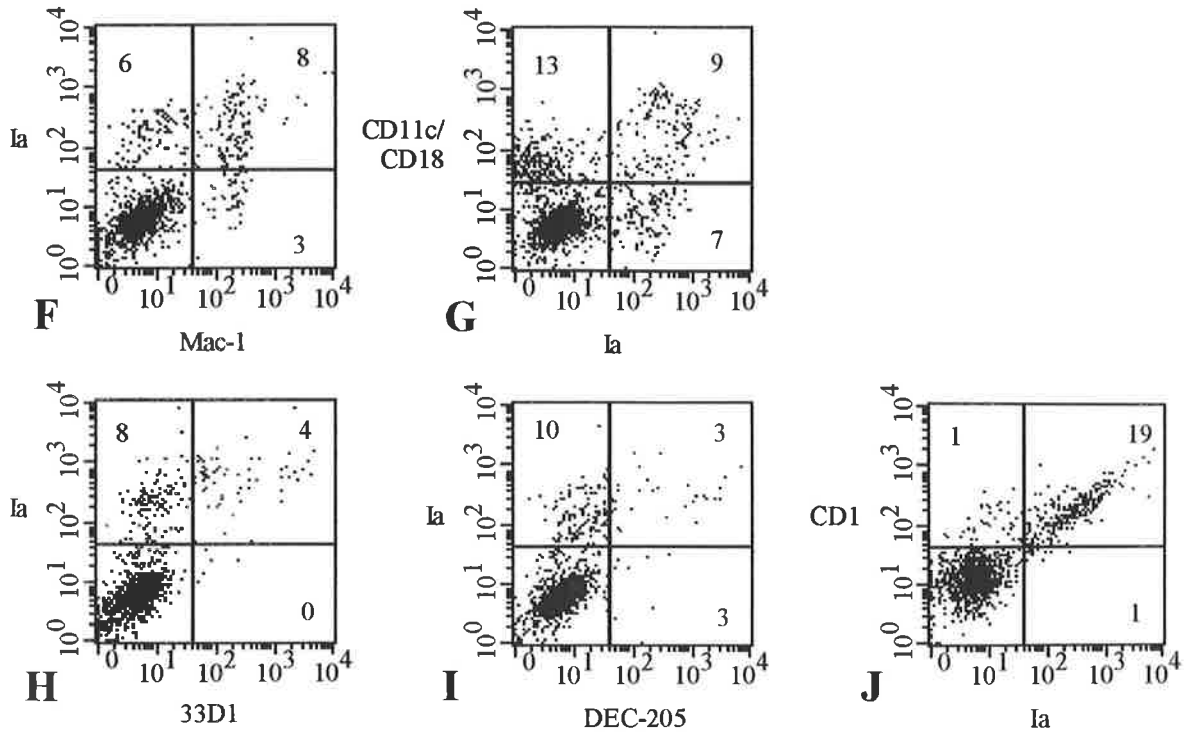


Figure 4.7 Phenotype of gate 3 F4/80$^+$ uterine cells at estrus. Replicate aliquots of cells obtained by enzymatic digestion of pooled estrous uteri (n = 10) were indirectly labeled with RPE using mAbs specific for macrosialin (Mn, A), class A scavenger receptor (SR, B), sialoadhesin (Sn, C), Mac-1 (D), CD11c/CD18 (E), 33D1 (F), DEC-205 (G) and CD1 (H). The cells were subsequently labeled with F4/80-FITC and analysed by dual colour flow cytometry. Fluorescence intensity (in arbitrary units) attributed to FITC (x axis) and RPE (y axis) on cells in gate 3 is shown, with the numbers of cells comprising each phenotype given as a proportion of gate 3 cells in the lower right, upper right and upper left quadrants respectively. Each of these quadrants contained <1% of gated cells in irrelevant isotype control preparations. In A-D, data is representative of 2 experiments.

Figure 4.8 Phenotype of gate 2 and gate 3 Ia^+ uterine cell at estrus. Aliquots of cells obtained by enzymatic digestion of pooled estrous uteri (n = 10) were indirectly labeled with FITC using mAbs specific for Mac-1 (A, F), 33D1 (C, H) or DEC-205 (D, I) followed by Ia-RPE, or alternatively with Ia-FITC and then RPE using mAbs specific for CD11c/CD18 (B, G) or CD1 (E, J). The cells were subsequently analysed by dual colour flow cytometry. Fluorescence intensity (in arbitrary units) attributed to FITC (x axis) and RPE (y axis) on cells in gate 2 (A-E) and gate 3 (F-J) are shown, with the numbers of cells comprising each phenotype given as a proportion of gated cells in the lower right, upper right and upper left quadrants respectively. Each of these quadrants contained <1% of gated cells in irrelevant isotype control preparations.


A
Ia
Mac-1
2
1
4
B
CD11c/
CD18
Ia
2
8
6
C
Ia
33D1
5
6
0
D
Ia
DEC-205
9
7
0
E
CD1
Ia
2
10
3

Gate 2


F
Ia
Mac-1
6
8
3
G
CD11c/
CD18
Ia
13
9
7
H
Ia
33D1
8
4
0
I
Ia
DEC-205
10
3
3
J
CD1
Ia
1
19
1

Gate 3

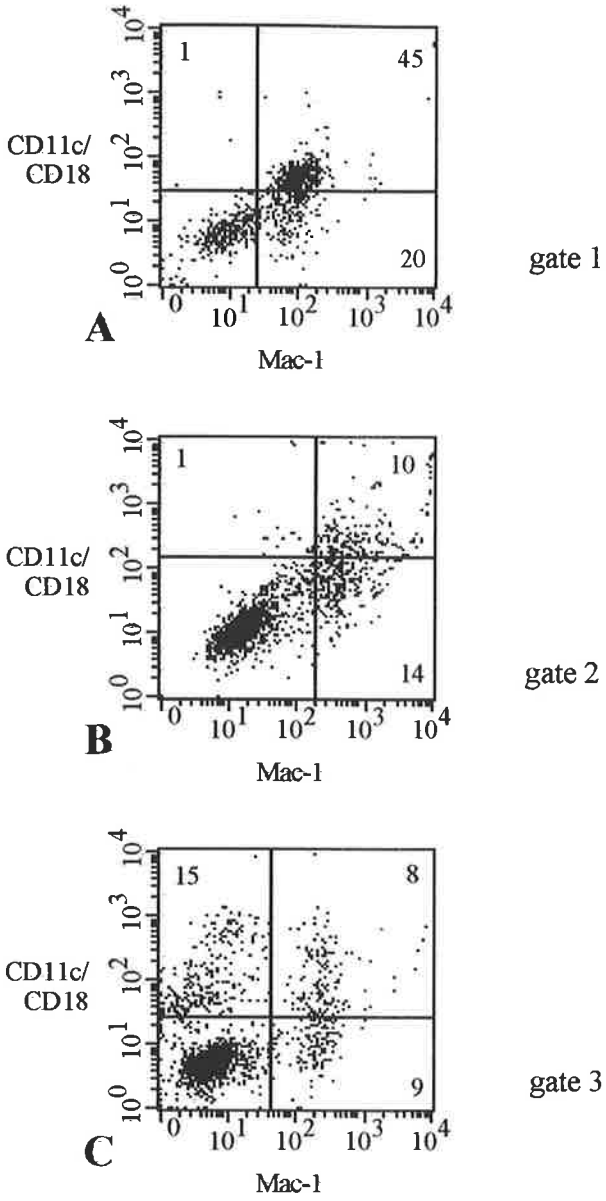


Figure 4.9 Co-expression of Mac-1 and CD11c/CD18 on uterine cells at estrus. Cells obtained by enzymatic digestion of pooled estrous uteri (n = 10) were indirectly labeled with Mac-1-FITC followed by CD11c/CD18-RPE and analysed by dual colour flow cytometry. Fluorescence intensity (in arbitrary units) attributed to FITC (x axis) and RPE (y axis) on cells in gate 1 (A), 2 (B) and 3 (C) is shown, with the numbers of Mac-1$^+$CD11c/CD18$^-$, Mac-1$^+$CD11c/CD18$^+$ and Mac-1$^-$ CD11c/CD18$^+$ cells given as a proportion of gated cells in the lower right, upper right and upper left quadrants respectively. Each of these quadrants contained <1% of gated cells in irrelevant isotype control preparations.

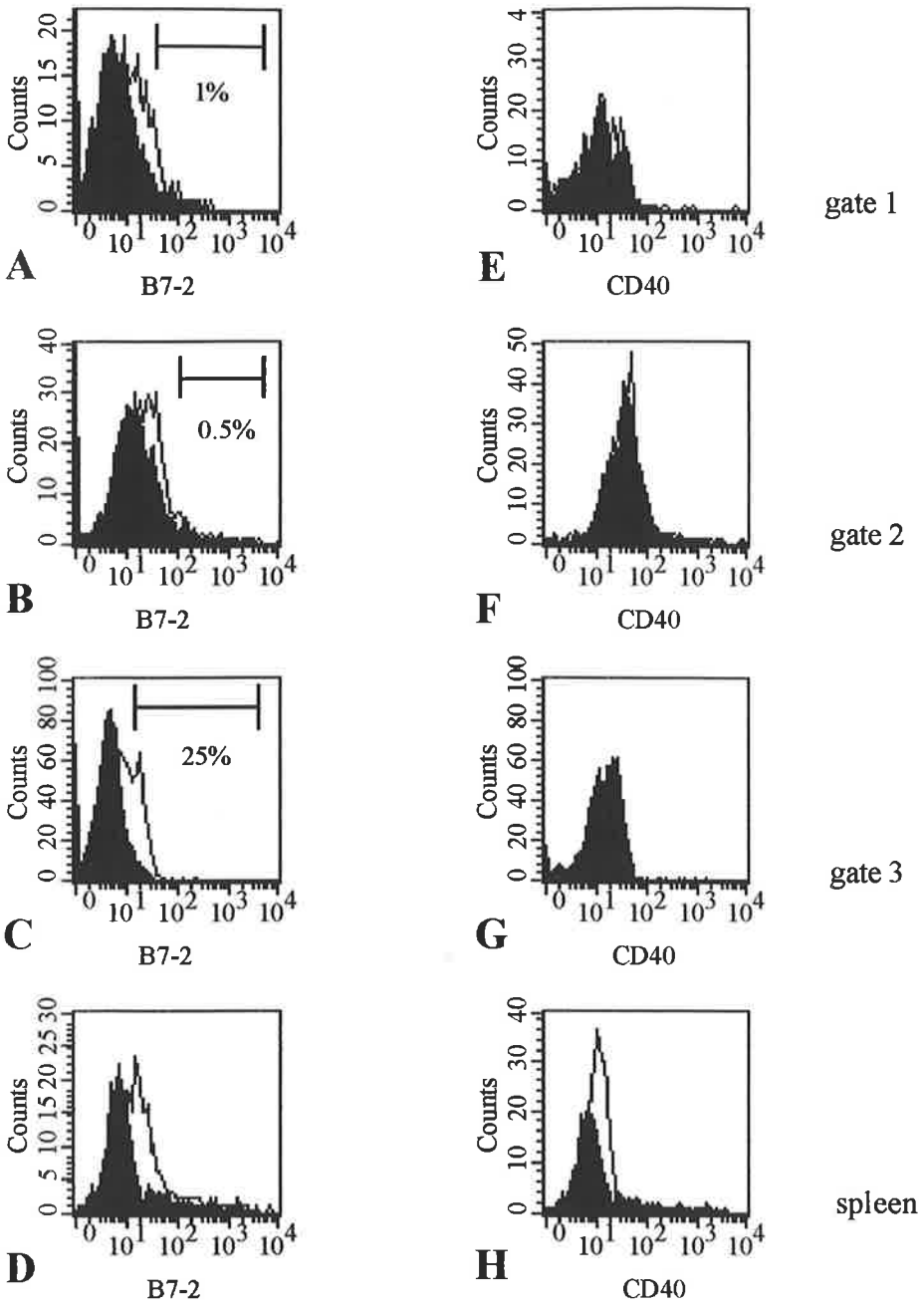
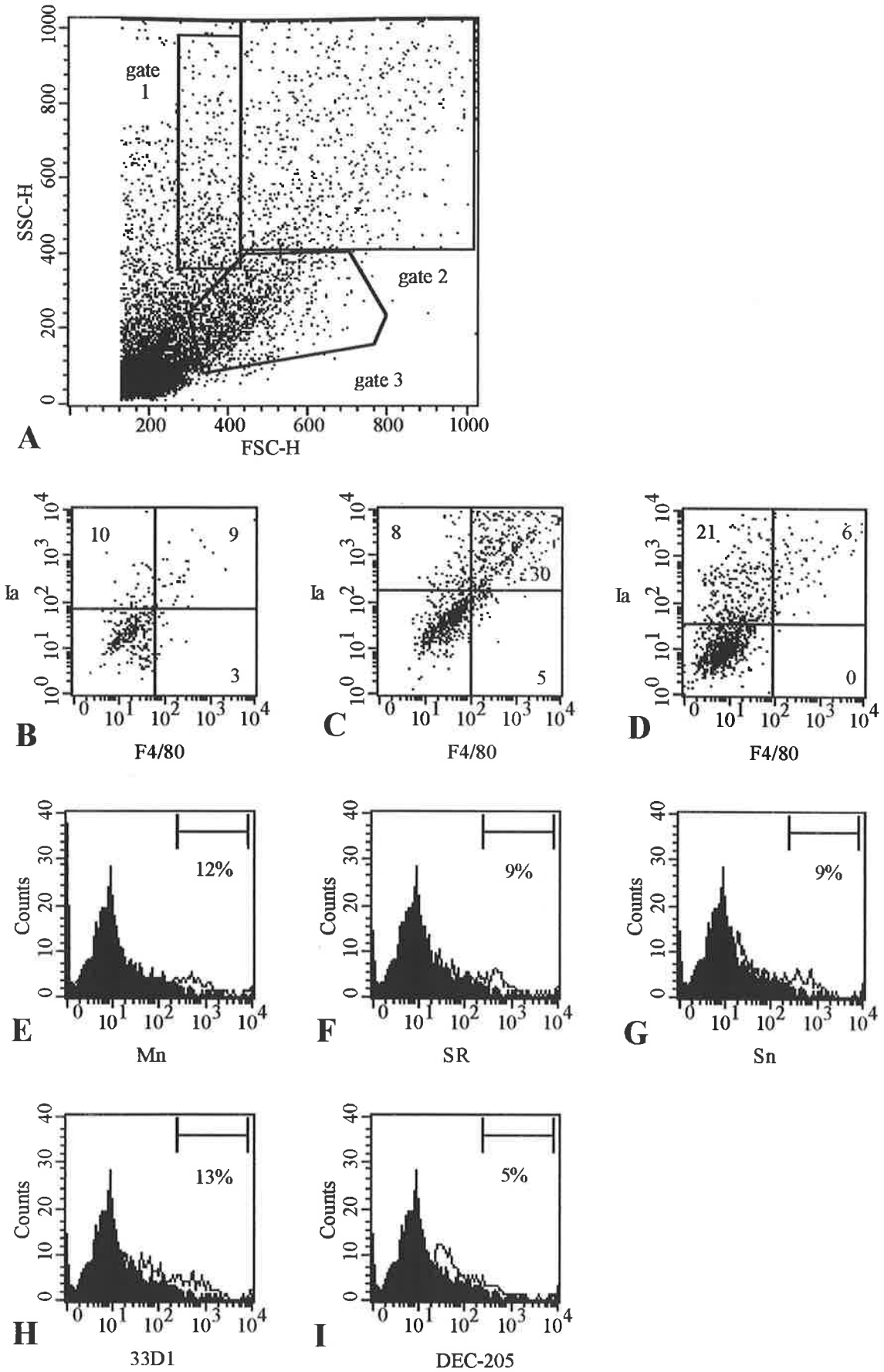


Figure 4.10 Expression of B7-2 and CD40 on uterine cells at estrus. Replicate aliquots of uterine cells (obtained by enzymatic digestion of a uterus from an estrous mouse, A-C, E-G) or IFNγ-cultured spleen cells (D, H) were indirectly labeled with B7-2-RPE (A-D) or CD40-RPE (E-H) and analysed by single colour flow cytometry. Data presented as unfilled histograms shows fluorescence intensity in arbitrary units (x axis) for B7-2-RPE and CD40-RPE as a function of cell frequency (Counts, y axis) in gates 1 (A, E), 2 (B, F) and 3 (C, G) for uterine cells and in all spleen cells (D, H). Fluorescence of cells labeled with an irrelevant mAb is shown as a filled histogram in each graph. The number of B7-2-RPE-labeled uterine cells is given as a percentage of gated cells in gate 1 (A), gate 2 (B) and gate 3 (C) respectively. These values were calculated with the use of the indicated regions from which >98% of cells labeled with the irrelevant mAb were excluded. For A-C and E-G, similar results were obtained from a further 3 individual uteri in the same experiment.

Figure 4.11 Flow cytometric analysis of uterine APCs following ovariectomy. Aliquots of cells obtained by enzymatic digestion of uteri from ovariectomised mice (n = 12) were indirectly labeled with Ia-RPE and F4/80-FITC and analysed by dual colour flow cytometry. The light scattering characteristics of the cells are shown in (A), in which gates 1, 2 and 3 define small and granular cells, large and granular cells and cells of medium size and medium granularity respectively. Fluorescence intensity (in arbitrary units) attributed to F4/80-FITC (x axis) and Ia-RPE (y axis) on cells contained within gates 1, 2 and 3 (B, C, D) is shown, with the numbers of $F4/80^{+}Ia^{-}$, $F4/80^{+}Ia^{+}$ and $F4/80^{-}Ia^{+}$ cells given as a proportion of gated cells in the lower right, upper right and upper left quadrants respectively. Each of these quadrants contained <1% of gated cells in irrelevant isotype control preparations. Data is representative of 2 experiments.

In the same experiment, further aliquots of uterine cells from ovariectomised mice were indirectly labeled with RPE using mAbs to macrosialin (Mn; E), class A scavenger receptor (SR; F), sialoadhesin (Sn; G), 33D1 (H) and DEC-205 (I) and analysed by single colour flow cytometry. Data presented as unfilled histograms shows fluorescence intensity in arbitrary units (x axis) for RPE as a function of cell frequency (Counts, y axis) in total uterine cells. The number of RPE-labeled cells is given as a percentage of total cells in E-I. These values were calculated with the use of the indicated marker regions from which >98% of cells labeled with the irrelevant mAb (shown as a filled histogram in each graph) were excluded.


A
gate 1
gate 2
gate 3
SSC-H
FSC-H
B
C
D
Ia
F4/80
10
9
3
8
30
5
21
6
0
E
F
G
Counts
Mn
SR
Sn
12%
9%
9%
H
I
33D1
DEC-205
13%
5%

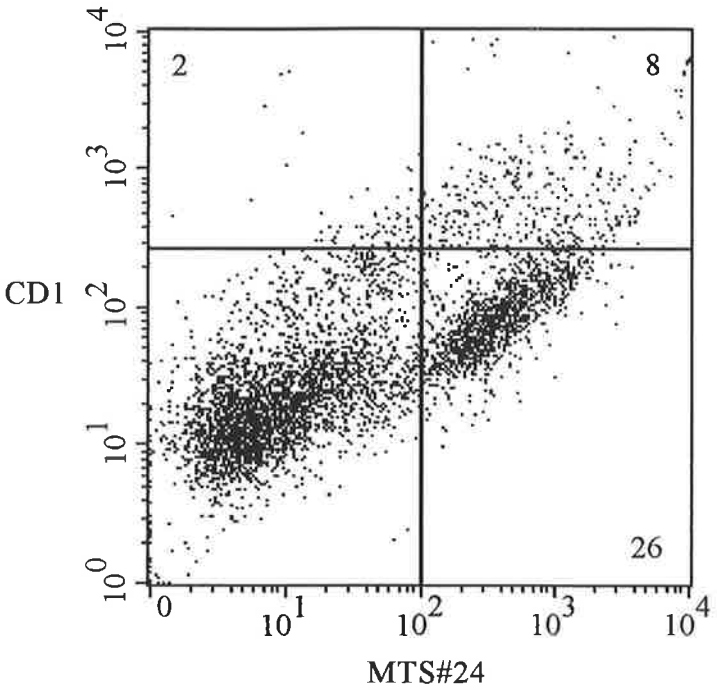


Figure 4.12 Expression of CD1 on uterine epithelial cells at estrus. Cells obtained by enzymatic digestion of uteri from estrous mice (n = 10) were indirectly labeled with MTS#24-FITC and CD1-RPE and analysed by dual colour flow cytometry. Fluorescence intensity (in arbitrary units) attributed to FITC (x axis) and RPE (y axis) on all viable uterine cells is shown, with the numbers of MTS#24$^+$CD1$^-$, MTS#24$^+$CD1$^+$ and MTS#24$^-$CD1$^+$ cells given as a proportion of total cells in the lower right, upper right and upper left quadrants respectively. Each of these quadrants contained <1% of gated cells in irrelevant isotype control preparations. Data is representative of 3 experiments.

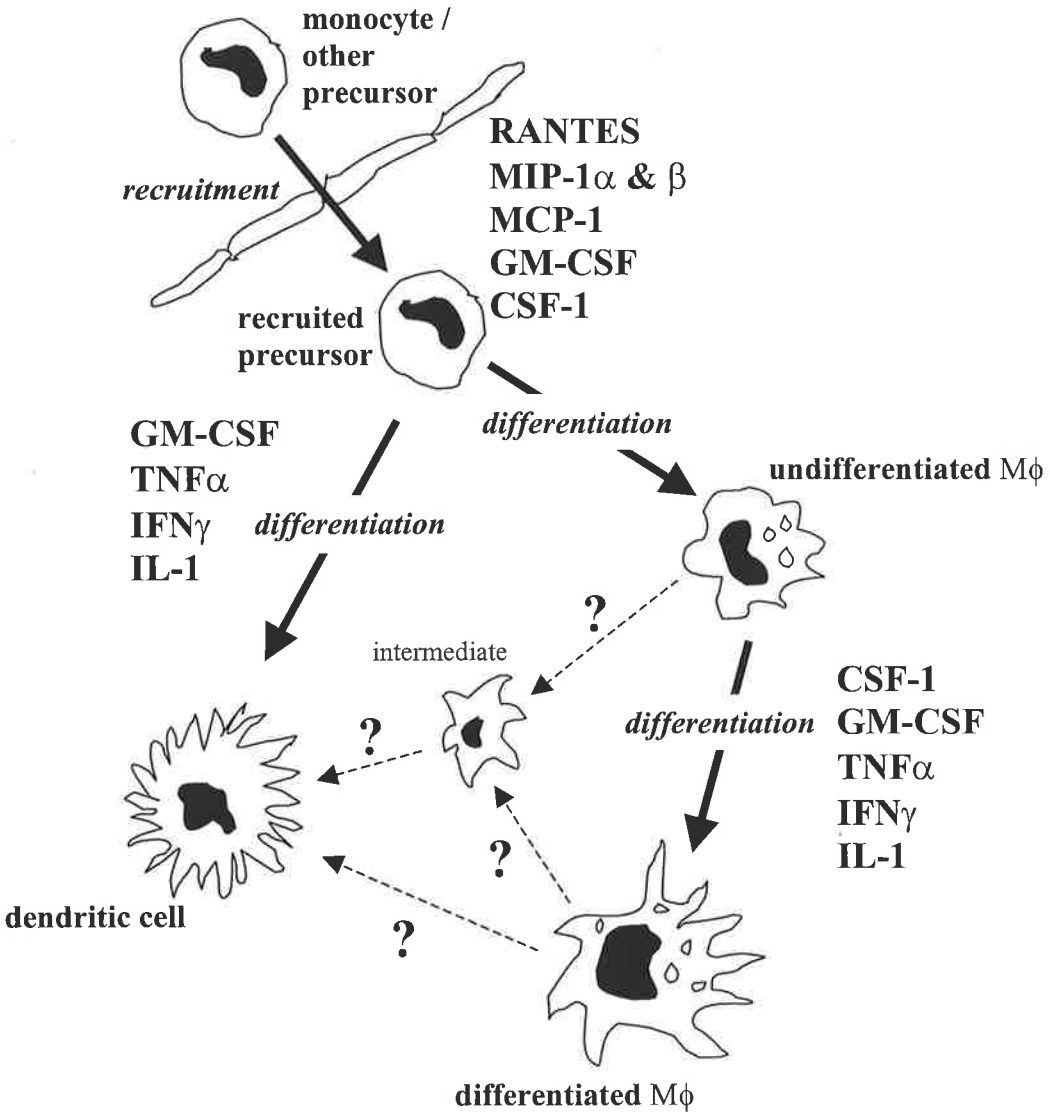


Figure 4.13 A model for differentiation of uterine APCs from precursor cells. Based on concepts emerging through the study of APCs in other mucosal tissues and on the data described herein, it is proposed that monocytes and other precursor cells in the blood are attracted to the uterus due to the expression of cytokines and CC chemokines by uterine epithelial and stromal cells. Due to the action of locally produced colony-stimulating factors and inflammatory cytokines, the precursor cells would be expected to differentiate along one of several possible pathways to form differentiated Mϕs and dendritic cells. The differentiation may occur directly, or indirectly through intermediate cell phenotypes.

Chapter 5

Flow cytometric analysis of uterine antigen presenting cells at day 1 and day 4 of pregnancy

5.1 Introduction

The studies presented in the preceding chapter described the identification and characterisation of Mϕs and dendritic cells amongst the cells present in the uteri of estrous mice. These APCs could have many roles, including innate immune surveillance, the generation of antigen-specific immune responses, maintenance of uterine homeostasis through secretion of bioactive molecules, and phagocytosis and degradation of cellular material generated during tissue remodeling (Gordon 1995, Gordon 1998). Perhaps the greatest challenge to uterine homeostasis would occur at mating, at which time large amounts of many and diverse antigens are introduced into the uterine lumen. These would include paternal antigens present in sperm and other cells derived from the male reproductive tract, but potentially also viral or bacterial microorganisms. Commensal organs normally resident in the lower reproductive tract presumably also infiltrate the uterus at this time (Robertson *et al.* 1999). Of utmost importance for uterine health would be the capacity of the uterus to respond to each of these challenges appropriately. The control of pathogens would require active and aggressive clearance, degradation and, for a lasting immune response, the generation of antigen-specific T cells. On the other hand, the immunological response to paternal antigens would need to be of a nature compatible with an ensuing pregnancy.

Many immunohistochemical studies, including those presented in Chapter 3, show that there is a dramatic, inflammatory-like response to insemination, characterised by the influx into the endometrium of APCs of an activated phenotype. Whether the majority of these cells are typical of the Mϕ lineage, or more similar to classical dendritic cells is not known. A greater knowledge of the phenotypes of APCs in the mated uterus is crucial in order to better understand the nature of immune responses generated at this time, since it is clear that the quality and character of an immune response is determined principally by the phenotype of the APCs active in the initiation events. Furthermore, an analysis of the smaller population of APCs retained within the day 4 pregnant uterus may also provide some insight into the regulation of immune responses in the female reproductive tract at the time of embryo implantation.

The experiments presented in this chapter were undertaken in order to investigate the effects of insemination and early pregnancy on the sub-populations and phenotypes of APCs

present within the pregnant uterus. Using the parameters already established for cells obtained from estrous uteri, cells released by enzymatic digestion of the uteri of day 1 and day 4 pregnant mice were analysed by flow cytometry for their forward- and side-scatter characteristics and for their expression of F4/80 and Ia. The populations of APCs identified were then examined further for their expression of activation markers known to be associated with antigen processing and presentation activity.

5.2 Effect of days 1 and 4 of pregnancy on the efficiency of enzyme-digestion of uteri

Prior to flow cytometric examination of the populations of APCs resident in uteri during pregnancy, two experiments were performed in order to investigate whether day of pregnancy has an effect on the efficiency of enzyme-induced release of cells from uteri. In each experiment, uteri from estrous, day 1 pregnant and day 4 pregnant mice (n = 3-5/group) were pooled and digested as previously (see section 2.4.2), and the numbers of total and $F4/80^+$ cells released (the latter assessed in suspension by indirect labeling with FITC) were assessed. The results showed that the release of total cells from uteri was approximately twice as efficient at day 4 compared to at estrus and day 1 (Table 5.1). Moreover, as a proportion of total cells, $F4/80^+$ cells were released at 3-10 fold higher rates at day 4 than at estrus or day 1.

5.3 Forward- and side-scatter analysis of uterine cells at days 1 and 4 of pregnancy

To assess the flow cytometric characteristics of cell suspensions from uteri of mice in early pregnancy, pooled or individual uteri obtained from day 1 and day 4 pregnant mice were digested in collagenase and DNase (see section 2.4.2). With the FACS machine, 10 000 or 20 000 events of the resultant cell suspensions were acquired in a gate designed to exclude dead cells and cellular debris. Dot plots showing forward- and side-scatter characteristics of cell suspensions from uteri of day 1 and day 4 pregnant mice were found to be similar to those of estrous mice (Figure 4.2) and to each other (Figure 5.1). As was seen at estrus, the released cells were very diverse in terms of their size and their intracellular complexity. Furthermore, and consistent with observations of cells recovered at estrus, the profiles at day 1 and day 4 were highly reproducible since similar results were obtained in more than 5 experiments involving analysis of pooled uteri from day 1 pregnant mice, and in 3 experiments involving analysis of more than 12 individual uteri from both day 1 pregnant and day 4 pregnant mice (data not shown).

Table 5.1 Numbers of cells recovered from the uteri of estrous, day 1 and day 4 pregnant mice. In two separate experiments (expts 1 & 2), pooled uteri from estrous, day 1 and day 4 pregnant mice (n = 3-5/group) were digested in collagenase/DNAse. The total number of cells and number of F4/80$^+$ cells (labeled indirectly with FITC) released is shown as number cells released / uterus. The number of total and F4/80$^+$ cells at day 1 and day 4 of pregnancy is also expressed as an index of the number at estrus (number of cells released at estrus was ascribed a value of 1).

		total cells per uterus	*F4/80$^+$ cells per uterus*	*F4/80$^+$ cells (% total cells)*	*index total cells*	*index F4/80$^+$ cells*
expt 1	estrus	1.3×10^6	2.6×10^5	20%	1	1
	day 1	1.5×10^6	1.5×10^5	10%	1.1	0.6
	day 4	2.3×10^6	9.5×10^5	41%	1.8	3.7
expt 2	estrus	NA	3×10^4	-	-	1
	day 1	NA	4.7×10^4	-	-	1.6
	day 4	NA	3×10^5	-	-	10

For further consideration, the side- and forward-scatter data from cell suspensions of uteri from day 1 and day 4 pregnant mice were divided into gates encompassing cells of similar size and intracellular complexity (Figure 5.1). The gates used were identical to those previously used to distinguish between distinct sub-populations of APCs at estrus (Figure 4.4).

5.4 Populations of F4/80$^+$ and Ia$^+$ uterine APCs at days 1 and 4 of pregnancy

5.4.1 F4/80$^+$ and Ia$^+$ APCs released from pooled uteri at days 1 and 4 of pregnancy

In order to initially examine the occurrence of cells expressing F4/80 and Ia at day 1 and day 4 of pregnancy, two experiments were performed in which aliquots of 5×10^5 cells obtained by enzymatic digestion of pooled uteri from day 1 and day 4 pregnant mice were labeled indirectly with F4/80-FITC and Ia-RPE using protocols 2 and 3 (Table 4.2) in sequence. Using the FACS machine, 10 000 events from each aliquot were acquired in a gate designed to exclude dead cells and cellular debris.

The same three populations of APCs expressing F4/80 and Ia were found at day 1 and day 4 of pregnancy was observed at estrus (Figure 5.2 and Table 5.2). In gate 1, F4/80$^+$Ia$^-$ cells were preferentially enriched, comprising 35-63% of gated cells at day 1 and 56-84% of gated cells at day 4. F4/80$^+$Ia$^+$ were largely contained by gate 2, and formed 6-8% of that gate at day 1 and 11-16% of that gate at day 4. The greatest enrichment of F4/80$^-$Ia$^+$ cells was found in gate 3, where they comprised 1-24% of gated cells at day 1 and 1-3% of gated cells at day 4. As had been

observed at estrus, the population enriched to the greatest extent by the gating process was that of the $F4/80^+Ia^-$ phenotype. Gates 2 and 3 contained small 'contaminating' populations of $F4/80^-Ia^+$ and $F4/80^+Ia^+$ cells respectively.

Table 5.2 Populations of uterine cells expressing F4/80 and Ia at day 1 and day 4 of pregnancy. Cells obtained by enzymatic digestion of pooled uteri from day 1 and day 4 pregnant mice were analysed by dual colour flow cytometry for expression of F4/80 and Ia. Data is shown as % cells in each gate expressing F4/80 alone ($F4/80^+Ia^-$), both F4/80 and Ia ($F4/80^+Ia^+$) or Ia alone ($F4/80^-Ia^+$) in gates 1, 2 and 3 (described in section 5.2.3) in each of 2 experiments. *The median (range) of corresponding data obtained by analysis of uterine cells at estrus is also presented for comparison (for original data at estrus see Table 4.4).

stage of pregnancy	*population*	*experiment*	*gate 1*	*gate 2*	*gate 3*
			(% gated cells)		
*non-pregnant (estrous)	$F4/80^+Ia^-$	median	**52.2**	3.0	0.6
		(range)	(16.4-82.2)	(0.2-4.0)	(0.1-3.4)
	$F4/80^+Ia^+$	median	3.4	**12.0**	2.5
		(range)	(1.7-5.0)	(6.2-20.9)	(1.6-8.8)
	$F4/80^-Ia^+$	median	0.5	3.9	**5.2**
		(range)	(0.1-1.8)	(0.2-8.9)	(1.5-11.2)
day 1	$F4/80^+Ia^-$	1	**35.0**	1.1	1.7
		2	**62.9**	0.2	0.2
	$F4/80^+Ia^+$	1	2.0	**8.2**	4.1
		2	3.0	**6.0**	3.0
	$F4/80^-Ia^+$	1	0.0	14.1	**24.5**
		2	0.2	0.6	**1.1**
day 4	$F4/80^+Ia^-$	1	**56.1**	0.9	0.6
		2	**84.4**	0.1	0.0
	$F4/80^+Ia^+$	1	5.3	**11.0**	2.2
		2	2.9	**16.5**	1.6
	$F4/80^-Ia^+$	1	3.3	0.8	**3.5**
		2	0.2	1.1	**1.4**

5.4.2 *Effect of early pregnancy on the number of $F4/80^+$ and Ia^+ APCs released from individual uteri*

In order to determine the effect of pregnancy on the number of APCs released from enzyme-digested uteri, an experiment was performed in which individual uteri from estrous (n = 4), day 1 pregnant (n = 4) and day 4 pregnant (n = 3) mice were digested, labeled with F4/80-FITC and Ia-RPE and analysed by flow cytometry as described in section 5.4.1.

As a proportion of total viable cells, there was no detectable effect of day of pregnancy on the number of $F4/80^+Ia^-$, $F4/80^+Ia^+$ or $F4/80^-Ia^+$ APCs released from uteri ($0.3 < p < 0.8$, Figure

5.3). Furthermore, at each day of pregnancy similar numbers of APCs of each phenotype were released ($0.2 < p < 0.7$, data not shown).

In this experiment, the enrichment of $F4/80^+Ia^-$, $F4/80^+Ia^+$ and $F4/80^-Ia^+$ cells within gates 1, 2 and 3 respectively was also maintained in each individual cell preparations from estrous, day 1 and day 4 pregnant mice (data not shown).

5.5 The effect of days 1 and 4 of pregnancy on the expression of activation markers by $F4/80^+$ uterine Mϕs

The previous experiments (see Chapter 4) described the identification of two separate populations of $F4/80^+$ Mϕs in cycling and pregnant uteri : those that did not express Ia and were small in size (predominantly in gate 1), and those that did express Ia, and were larger in size (predominantly in gate 2, with a smaller population found in gate 3). Dual colour phenotypic analysis of each of these Mϕ populations at estrus described their expression of Mϕ activation markers, β2 integrins and dendritic cell markers, and was consistent with their identification as relatively undifferentiated and differentiated phenotypes respectively.

Insemination induces a cascade of molecular and cellular events in the uterus which have been likened to an inflammatory response. The pro-inflammatory cytokines and chemokines released after insemination would be highly likely to induce activation of resident and newly recruited uterine Mϕs, which may be reflected in enhanced expression on the cell surface of molecules known to be involved in the uptake, processing and presentation of antigens for the generation of immune responses. Such molecules, including macrosialin, scavenger receptor, sialoadhesin, MHC class II (Ia) and B7-2 were indeed all found by immunohistochemistry to be expressed to varying degrees by the abundant population of Mϕs in uteri collected from day 1 pregnant mice (see Chapter 3). The relevance of expression of these activation markers in the uterus is a function of (1) the proportion of APCs expressing the marker and (2) the level of expression on a per cell basis, information not provided by single colour immunohistochemistry.

In order to examine the effect of pregnancy on the expression of activation markers by uterine Mϕs, a series of dual colour flow cytometric experiments were performed. The expression of MHC class II (Ia), macrosialin, scavenger receptor class A (scavenger receptor), CD11c/CD18 and CD1 were assessed in $F4/80^+$ uterine cells released by enzymatic digestion of individual uteri collected from estrous (n=4), day 1 pregnant (n=3-4) and day 4 pregnant (n=3) mice. Aliquots of $5x10^5$ cells from each uterus were labeled indirectly with RPE to detect activation markers (using protocols 3 or 4 in Table 4.2) and then labeled directly with F4/80-FITC (protocol 1 in Table 4.2). For detection of F4/80 on cells labeled with Ia-RPE, an indirect

protocol was used (protocol 2 in Table 4.2). Using the FACS machine, 20 000 events were collected in a gate designed to exclude dead cells and cellular debris.

5.5.1 Effect of day of pregnancy on the proportion of Mϕs expressing activation markers

To determine the effect of day of pregnancy on the proportion of Mϕs expressing MHC class II (Ia), macrosialin, scavenger receptor, CD11c/CD18 and CD1, the number of F4/80$^+$ cells expressing each marker was considered as a proportion of all F4/80$^+$ cells contained within each of gates 1, 2 and 3 at estrus, day 1 and day 4 of pregnancy. The discussions below refer to the median percentage of F4/80$^+$ cells expressing each of the activation markers at estrus, day 1 and day 4 and are presented in sequence for each of gates 1, 2 and 3.

a. proportion of Mϕs expressing Ia

Of the F4/80$^+$ cells in gate 1, 5% expressed Ia at estrus, and a similar proportion was observed at day 4. In contrast, at day 1 over 10% of gate 1 F4/80$^+$ cells expressed Ia, and this was a significantly greater proportion than at the other two time points ($p = 0.03$, Figure 5.4 A).

Approximately 80% of F4/80$^+$ cells in gate 2 expressed Ia at estrus, and this did not change at day 1 of pregnancy. Although the median proportion of F4/80$^+$ cells which expressed Ia at day 4 was 98%, this was not significantly different from the other two groups ($p = 0.1$ across all groups, Figure 5.5A). Similarly, in gate 3 the proportion of F4/80$^+$ cells which expressed Ia remained at approximately 85% at estrus, day 1 and day 4 ($p = 0.3$, data not shown).

b. proportion of Mϕs expressing macrosialin

In gate 1, only 7% of F4/80$^+$ cells expressed macrosialin at estrus, and similar if not lower proportions were observed at day 1 and day 4 of pregnancy ($p = 0.2$, Figure 5.4B).

In gate 2, approximately 40% of F4/80$^+$ cells co-expressed macrosialin at estrus, and this did not change at day 1 ($p = 0.9$). In contrast, 80% of gate 2 F4/80$^+$ cells at day 4 expressed macrosialin, and this was significantly greater than at both estrus and day 1 ($p = 0.03$, Figure 5.5B). In gate 3, 35-40% of F4/80$^+$ cells expressed macrosialin at estrus and day 1, although the median value increased to 50% at day 4, this was not significant ($p = 0.2$, data not shown).

c. proportion of Mϕs expressing scavenger receptor

Of gate 1 F4/80$^+$ cells, 3.5-5% expressed scavenger receptor at estrus, day 1 and day 4, with no significant variation between the groups ($p = 0.8$, Figure 5.4C).

In gate 2, 30-40% of F4/80$^+$ cells expressed scavenger receptor at estrus and day 4 of pregnancy. After mating, the median proportion of F4/80$^+$ cells which expressed scavenger receptor increased to 52% however this was not significant ($p = 0.4$ across all groups, Figure 5.5C). In gate 3 there was no effect of mating on the proportion of F4/80$^+$ cells which expressed scavenger receptor (approximately 40% expressed scavenger receptor at all times, $p = 0.3$, data not shown).

d. proportion of Mϕs expressing CD11c/CD18

Approximately 75% of gate 1 F4/80$^+$ cells expressed CD11c/CD18 at estrus. On day 1 of pregnancy, this proportion fell significantly to a median value of 15% ($p = 0.02$ compared to estrous values), but then rose again to 92% by day 4 of pregnancy ($p = 0.03$ compared to day 4, $p = 0.2$ compared to estrus, Figure 5.4D).

In gate 2, a similar pattern was observed. Here, the proportion of F4/80$^+$ cells which expressed CD11c/CD18 at estrus (60%) fell significantly at day 1 ($p = 0.04$ compared to estrus) and then increased again to 83% at day 4, which was significantly higher than both estrus and day 1 ($p = 0.03$, Figure 5.5D). The proportions of F4/80$^+$ cells expressing CD11c/CD18 in gate 3 were almost identical to those observed in gate 2 at estrus, day 1 and day 4 (data not shown).

Dot plots showing the expression of F4/80 and CD11c/CD18 by uterine cells are provided in Figure 5.6.

e. proportion of Mϕs expressing CD1

In gate 1 at estrus, approximately 25% of F4/80$^+$ cells expressed CD1. Although there was no significant effect of day of pregnancy on this proportion, it appeared that fewer F4/80$^+$ cells expressed CD1 at day 4 than at both estrus and day 1 ($p = 0.06$ across all groups, Figure 5.4E).

Approximately 50% of F4/80$^+$ cells in gate 2 expressed CD1 at estrus, and this did not change at day 1 of pregnancy. By day 4 of pregnancy, the proportion of gate 2 F4/80$^+$ cells which expressed CD1 increased to over 90%, and this was significantly greater than at day 1 but similar to that at estrus ($p = 0.03$ compared to day 1, $p = 0.3$ compared to estrus, Figure 5.5E). Similarly, in gate 3 the proportion of F4/80$^+$ cells which expressed CD1 fell from 50% at estrus to 35% at day 1 ($p = 0.02$) and then rose to 90% at day 4 ($p = 0.03$, data not shown).

5.5.2 *Effect of day of pregnancy on the intensity of activation marker expression on Mϕs*

In order to investigate the effect of day of pregnancy on intensity of activation marker expression on uterine Mϕs, the data described in section 5.4.1 was analysed to determine the intensity of MHC class II (Ia), macrosialin, scavenger receptor , CD11c/CD18 and CD1 on F4/80$^+$ cells at estrus, day 1 and day 4 of pregnancy. The discussions below refer to the median intensity of activation marker expression on F4/80$^+$ cells at estrus, day 1 and day 4 and are presented in sequence for each of gates 1, 2 and 3.

a. intensity of Ia expression on Mϕs

The intensity of Ia expression on F4/80$^+$ cells in gates 1, 2 and 3 was similar at estrus, day 1 and day 4 of pregnancy ($p = 0.6$, $p = 0.4$ and $p = 0.4$ respectively). Between gates, intensity of Ia expression was highest in gate 2 and similar in gates 1 and 3 (Table 5.3).

Table 5.3 The effect of day of pregnancy on intensity of activation marker expression on F4/80$^+$ uterine cells. The intensities of expression of MHC class II (Ia), macrosialin and scavenger receptor on F4/80$^+$ uterine cells expressing each of these markers were derived by FACS analysis of dual labeled aliquots of uterine cell suspensions from individual uteri obtained from estrous, day 1 and day 4 pregnant mice (number / group given below). Data (in arbitrary intensity units) is given as median (range) in each of gates 1, 2 and 3, and were compared within each gate according to day of pregnancy by Kruskal-Wallis one-way ANOVA (p values are given).

		day of pregnancy			
		estrus	*day 1*	*day 4*	*p* value
MHC class II (Ia)	*gate 1*	102 (86-174)	134 (31-145)	75 (42-139)	0.6
	gate 2	433 (399-497)	302 (166-564)	240 (224-494)	0.4
	gate 3	138 (97-304)	110 (79-157)	127 (126-214)	0.4
macrosialin	*gate 1*	333 (286-365)	357 (288-549)	333 (320-382)	0.7
	gate 2	954 (471-1178)	790 (688-905)	513 (465-770)	0.2
	gate 3	266 (177-400)	251 (237-370)	282 (262-355)	0.7
scavenger receptor	*gate 1*	294 (251-487)	414 (252-559)	270 (235-316)	0.3
	gate 2	607 (396-999)	540 (461-731)	577 (500-1375)	0.9
	gate 3	254 (170-324)	248 (218-361)	220 (208-355)	0.8
number of uteri / group		4	3-4	3	

b. intensity of macrosialin expression on Mϕs

There was no effect of stage of pregnancy on the intensity of macrosialin expression on F4/80$^+$ cells in gate 1 ($p = 0.7$), gate 2 ($p = 0.2$) or gate 3 ($p = 0.7$). Between gates, intensity of macrosialin expression was highest in gate 2 and similar in gates 1 and 3 (Table 5.3).

c. intensity of scavenger receptor expression on Mϕs

There was no effect of stage of pregnancy on the intensity of scavenger receptor expression on F4/80$^+$ cells in gate 1 ($p = 0.3$), gate 2 ($p = 0.9$) or gate 3 ($p = 0.8$). Between gates, intensity of macrosialin expression was highest in gate 2 and similar in gates 1 and 3 (Table 5.3).

d. intensity of CD11c/CD18 expression on Mϕs

The intensity of CD11c/CD18 expression on F4/80$^+$ cells in gate 1 fell significantly from estrus to day 1 ($p = 0.02$), but increased again by day 4 of pregnancy ($p = 0.03$ compared to day 1, $p = 0.7$ compared to estrus, Figure 5.7A).

In gate 2, the median intensity of CD11c/CD18 was similar at estrus and day 1, and significantly higher at day 4 of pregnancy ($p = 0.03$, day 4 compared to estrus and day 1, Figure 5.7B). There was no significant effect of day of pregnancy on intensity of CD11c/CD18 expression on F4/80$^+$ cells in gate 3, although a similar trend to that observed in gate 2 was observed ($p = 0.1$ across all groups, Figure 5.7C).

e. intensity of CD1 expression on Mϕs

The median intensity of CD1 expression on F4/80$^+$ cells in gate 1 decreased slightly from estrus to day 1 ($p = 0.08$), and then increased markedly at day 4 ($p = 0.03$ compared to both day 1 and estrus, Figure 5.7D).

In gate 2, there was no significant effect of day of pregnancy on intensity of CD1 expression on F4/80$^+$ cells, although a trend towards a similar pattern to that seen in gate 1 was observed ($p = 0.1$ across all groups, Figure 5.7E). In gate 3 the highest CD1 expression on F4/80$^+$ cells was also seen at day 4 ($p = 0.03$ compared to both estrus and day 1), and intensity at estrus and day 1 was similar ($p = 0.6$, Figure 5.7F).

f. intensity of F4/80 expression on Mϕs

The intensity of F4/80 expression on F4/80$^+$ cells was determined by FACS analysis of duplicate samples of $5x10^5$ uterine cells which were labeled directly with F4/80-FITC. The intensity of F4/80 on F4/80$^+$ cells in gates 1, 2 and 3 are presented in Figure 5.8. The data is representative of 2 experiments.

In gate 1, the intensity of F4/80 expression on $F4/80^+$ cells remained relatively low and similar from estrus to day 1 to day 4 ($p = 0.2$), although at day 1 the range of intensities was greater than at the other two times (Figure 5.8A).

F4/80 expression was approximately 5-fold higher at estrus in gate 2 cells than in gate 1 cells. At day 1, the intensity of expression rose significantly ($p = 0.02$) and by the fourth day of pregnancy had returned to levels similar to those seen at estrus ($p = 0.03$ compared to day 1, $p = 0.3$ compared to estrus, Figure 5.8B). In gate 3, a similar pattern was observed, whereby the intensity of F4/80 expression rose significantly from estrus to day 1 ($p = 0.03$), but then remained constant at day 4 ($p = 0.2$ compared to day 1, $p = 0.1$ compared to estrus). Overall, the intensity of F4/80 expression in gate 3 was between that of gate 1 and gate 2 (Figure 5.8C).

5.6 Conclusions and discussion

The experiments presented in this chapter examined the effect of mating and day 4 of pregnancy on the presence and phenotype of uterine APCs. As described in Chapter 4, at estrus three populations of uterine APCs were identified, and were designated 'undifferentiated Mϕs', 'differentiated Mϕs' and 'dendritic cells' on the basis of their forward- and side-scatter characteristics, their expression profiles of F4/80 and Ia and their expression of molecules associated with Mϕ activation, β2 integrins and dendritic cell markers. In cells obtained from the uteri of estrous mice, these populations were enriched in gates 1, 2 and 3 respectively. In the current experiments, identical gates were applied to profiles of cells from the uteri of pregnant mice, and were found to enrich the same populations of cells, namely $F4/80^+Ia^-$ cells in gate 1, $F4/80^+Ia^+$ cells in gate 2 and $F4/80^-Ia^+$ cells in gate 3. The applicability of these same gates to similar populations of cells allowed comparisons of the phenotypes of cells at each of the time points examined. Thus, the effect of pregnancy on undifferentiated Mϕs was examined by using gate 1, whereas more differentiated Mϕs and dendritic cells were tracked within gate 2 and gate 3.

In Chapter 3, it was established that the number of uterine APCs detected by immunohistochemistry peaked after insemination and was lowest on day 4 of pregnancy. By FACS analysis, the absolute numbers of APCs released from uteri at estrus, day 1 and day 4 of pregnancy were not found to reflect this dynamic. This phenomenon appears to be at least partially to be due to differences in the efficiency of the collagenase/DNase digestion in uteri at the different times.

Differences in the specificities of the detection methods could also provide some of the explanation for the inconsistencies between the data obtained by immunohistochemistry and by FACS. The APCs contained within uterine sections processed for immunohistochemical analysis showed the presence of antigens in both the internal and cell surface membranes of the cells, whereas the intact, live cells used for FACS analysis were not permeabilised and thus only cell surface antigens were accessible. It has previously been shown that the majority of macrosialin expression by murine Mϕs occurs intracellularly (Rabinowitz and Gordon 1991), and scavenger receptor, F4/80 and Ia have all found to be expressed intracellularly at levels which equal and often exceed that found on the cell surface (de Villiers *et al.* 1994). Therefore, it is perhaps not surprising that a greater number of cells expressing cell surface and internal antigens would be detected by immunohistochemistry than by FACS analysis. Further exploration of the relative amounts of internal and cell-surface antigen expression in uterine APCs could be examined by FACS analysis of permeabilised cells (de Villiers *et al.* 1994).

The second phase of the studies presented in this chapter involved analysing the effect of day 1 and day 4 of pregnancy on the expression of activation markers by undifferentiated and differentiated Mϕs. It was found that the proportions of undifferentiated Mϕs which expressed macrosialin, scavenger receptor and CD1 were comparable after insemination and at estrus. Similarly, the proportion of differentiated Mϕs expressing macrosialin, CD1 and Ia remained unchanged on day 1 as had been observed on differentiated Mϕs at estrus, and expression of scavenger receptor increased only slightly. The intensity of expression of all of these markers on both Mϕ populations did not change from estrus to day 1. Thus it would appear that in terms of expression of macrosialin, scavenger receptor and CD1, the Mϕs released by enzymatic digestion of uteri of day 1 pregnant mice were of a similar phenotype to those seen at estrus. This was somewhat unexpected given the abundance of pro-inflammatory cytokines released by seminal plasma-exposed uterine cells. However, it is certainly possible that the cells released from day 1 pregnant uteri were not truly representative of all of the Mϕs cells present at that time. The relatively poor efficacy of the digestive enzymes in uteri recovered on day 1 of pregnancy could have contributed to this outcome. In particular, the large size of the inseminated uteri might have meant that Mϕs present in the deep endometrium in the process of trafficking to lymph vessels were under-represented in the cells recovered. A better estimate of Mϕ and dendritic cell activation following insemination might be gained by analysis of the expression of cell surface costimulatory molecules such as B7-1 and B7-2, and their production of cytokines such as IL-12.

A hypothesised consequence of the cascade of inflammatory cytokines and chemokines induced in the inseminated uterus was the recruitment of new populations of APCs into the

uterine stroma (McWilliam *et al.* 1994, Bilyk and Holt 1995, Stumbles *et al.* 1999). An indication that the majority of the APCs released from uteri at day 1 were indeed relatively new arrivals and not highly differentiated is provided by analysis of their expression of CD11c/CD18. CD11c/CD18 is a β2-integrin which is widely expressed on cells of the monocyte/Mϕ lineage and on several other leukocytes (Carlos and Harlan 1994), and is thought to play rather limited roles in mediating cell:cell adherence compared to other β2 integrins such as Mac-1 (Beekhuizen *et al.* 1990, Peri *et al.* 1990). Instead, CD11c/CD18 expression tends to reflect the differentiation state of mononuclear cells, and is found at low levels on monocytes and precursor cells, and in much higher amounts on *in vitro* derived and *ex vivo* tissue resident Mϕs (Dudley *et al.* 1989, Rogler *et al.* 1997). Correspondingly, the proportion of gate 1 F4/80$^+$ Mϕs which expressed CD11c/CD18 was significantly reduced after mating, suggestive of the recent immigration of a new cohort of cells expressing little or no β2-integrin. Furthermore, the intensity of CD11c/CD18 expression on gate 1 Mϕs was diminished on a per cell level at day 1 compared to at estrus. Interestingly, the more highly differentiated Mϕs in gate 2 also expressed less CD11c/CD18 at day 1 than at estrus, suggesting that a proportion of them may also be derived from recently arrived cells.

Fluctuations in F4/80 expression in Mϕs at different stages of pregnancy may also be regarded as an index of cell recruitment. In the current study, the intensity of F4/80 expression on Mϕs was found to peak at day 1. While the exact role of the F4/80 molecule is not known, it has been postulated to be involved in mediating cell adhesion within certain tissues (McKnight *et al.* 1996). Accordingly, Langerhans cells trafficking from inflamed organs to draining lymph nodes express very low levels of F4/80. As observed in the current study, relatively undifferentiated Mϕs could thus be expected to exhibit relatively high expression of F4/80. This could act to mediate increased retention of freshly recruited cells within the uterus after insemination.

Thus, despite their similar cell surface expression of activation markers, it seems likely that the Mϕs present within the uterus at day 1 of pregnancy are indeed not the same cells as those seen at estrus. Instead, it appears that the secretion of inflammatory cytokines and chemokines (Robertson *et al.* 1992, Wood *et al.* 1992, Robertson *et al.* 1996b, Pollard *et al.* 1998, Robertson *et al.* 1998) by uterine epithelial cells following insemination triggers the recruitment of new populations of cells and possibly also the differentiation of these cells and existing resident APCs (Gordon 1999). Considering the increased exodus of uterine APCs to draining lymph nodes at day 1 of pregnancy noted in Chapter 3, the data might suggest an increased rate of uterine APC turnover within the inseminated uterus. These outcomes would

occur by virtue of uterine APC expression of receptors for GM-CSF (Robertson *et al.* 2000), and other receptors for cytokines known to be expressed in the uterus including c-*fms* (Arceci *et al.* 1989, Regenstreif and Rossant 1989), IL-1 receptor (Tabibzadeh *et al.* 1990) and IFNγ receptor (Chen *et al.* 1994).

Although the phenotype of differentiated Mϕs did not alter markedly after insemination, evidence for the relatively activated nature of undifferentiated uterine Mϕs at day 1 may be provided by the data showing a slightly enhanced proportion of gate 1 Mϕs expressing Ia at this time. More extensive phenotypic shifts in this group of cells could alter their light-scattering characteristics to such an extent that they would begin to scatter in the region of gate 2 and thus not be measured by analysis of the cells in gate 1. Hence some of the large, granular Mϕs expressing Ia, macrosialin, scavenger receptor and CD1 found in gate 2 at day 1 could be derived from a proportion of the undifferentiated Mϕs which were found in gate 1 at estrus.

As discussed previously, an alternative pathway for undifferentiated Mϕs could be their differentiation into dendritic cells (see Figure 4.13). Indeed, the likelihood for such an event is supported by the fact that cytokines such as GM-CSF and TNFα encountered by cells in the uterus after mating are similar in profile to those known to drive dendritic cell differentiation *in vitro* (Banchereau and Steinman 1998). The effect of mating on the phenotype of uterine dendritic cells was not examined here.

The phenotype of the Mϕs retained in the uteri of day 4 pregnant mice is intriguing. Both undifferentiated and differentiated Mϕs were detected at day 4, and the populations were remarkably homogeneous compared to those observed at estrus and day 1. Undifferentiated Mϕs at day 4 were nearly all negative for Ia, macrosialin, scavenger receptor and CD1, whereas differentiated Mϕs at day 4 were nearly 100% Ia^+, $macrosialin^+$ and $CD1^+$, and approximately 60% also expressed scavenger receptor. Thus the differentiated Mϕs appeared to be even more differentiated at day 4 compared to at estrus and day 1 of pregnancy. Interestingly, nearly 100% of both cell populations expressed CD11c/CD18 at relatively high levels, suggesting that despite their low expression of activation molecules, even the so-called undifferentiated Mϕs were indeed relatively differentiated compared to monocytes (Dudley *et al.* 1989).

In summary, the studies presented in this chapter have established that APCs of three distinct phenotypes can be detected in the uteri of cycling and pregnant murine uteri. The three cell populations have been referred to as undifferentiated Mϕs, differentiated Mϕs and dendritic cells on the basis of their size, intracellular complexity, and their expression of cell surface phenotypic markers including F4/80, Ia, macrosialin, scavenger receptor, CD11c/CD18 and CD1. At the population level, the proportion of Mϕs which expressed many of the activation markers

remained similar from estrus to day 1, although whether individual cells underwent processes of differentiation could not be established using the current experimental strategy. The data does suggest however that new Mϕs are recruited to the uterus at insemination. Thus it seems likely that the populations of uterine APCs are 'turned over' at the time of mating through the recruitment of monocytes and other precursor cells, the differentiation of Mϕs and dendritic cells *in situ* and perhaps also the exodus of differentiated cells in response to inflammatory signals and changing chemokine expression. A more precise determination of the fate of resident and newly recruited Mϕs and dendritic cells in the inseminated uterus could be achieved through confocal histochemical analysis of uterine tissue sections and multiple-colour FACS analysis of cells at estrus, day 1 and day 4 of pregnancy. This could allow a more accurate determination of the identity of the predominant APC sub-type present in the uterus at insemination.

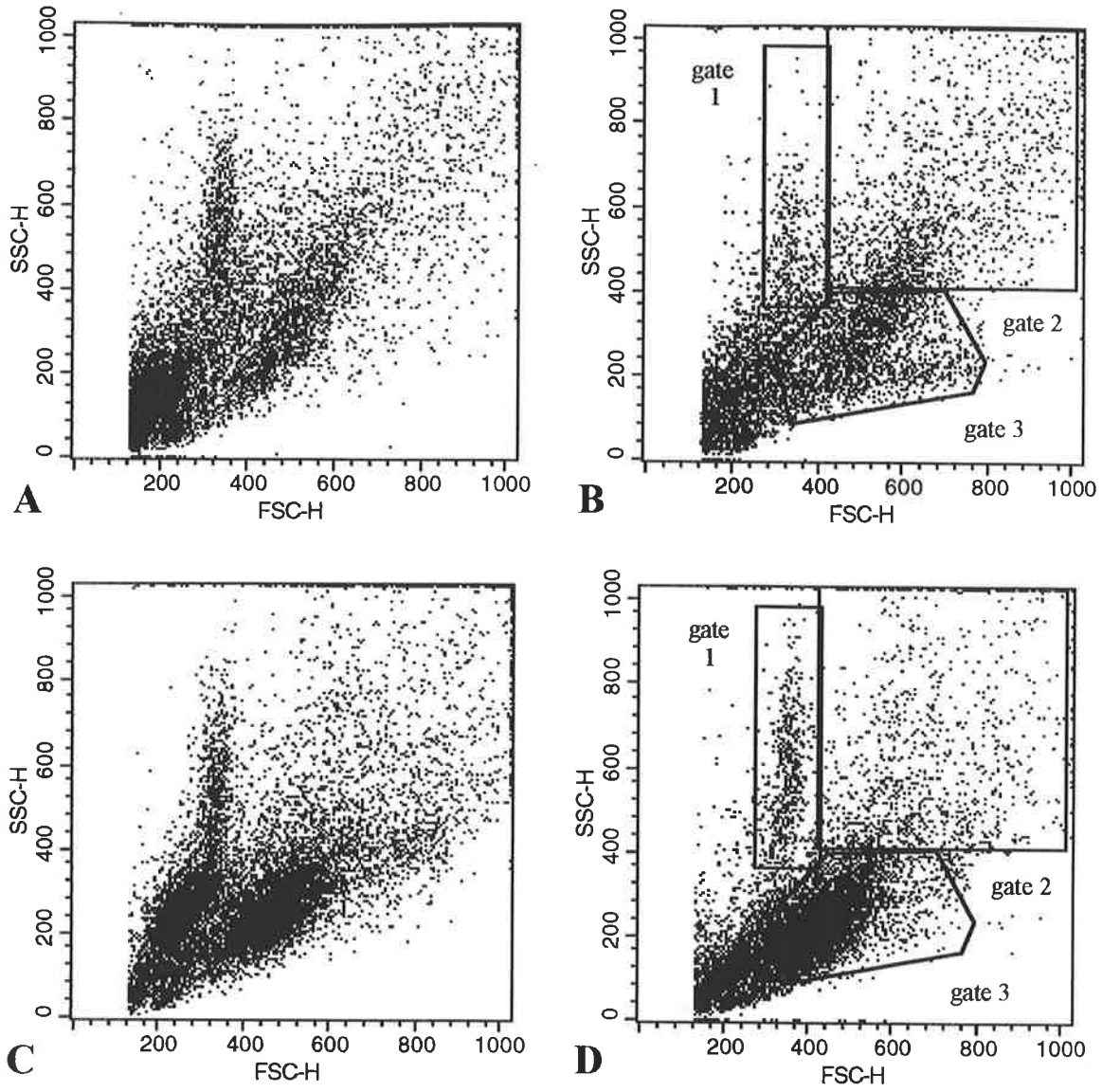


Figure 5.1 Forward and side scatter analysis of uterine cells at days 1 and 4 of pregnancy. The light scattering characteristics of cells obtained by enzymatic digestion of pooled (n = 5; A) and individual (B, C, D) uteri of day 1 pregnant (A, B) and day 4 pregnant (C, D) mice were analysed by flow cytometry. The light scattered by the cells is presented in arbitrary units as forward scatter (FSC-H, a measure of cell size) and side scatter (SSC-H, a measure of intra-cellular complexity). In (B and D), gates 1, 2 and 3 define small and granular cells, large and granular cells and cells of medium size and medium granularity respectively. The data is representative of 4 and 3 experiments of pooled and individual uteri respectively.

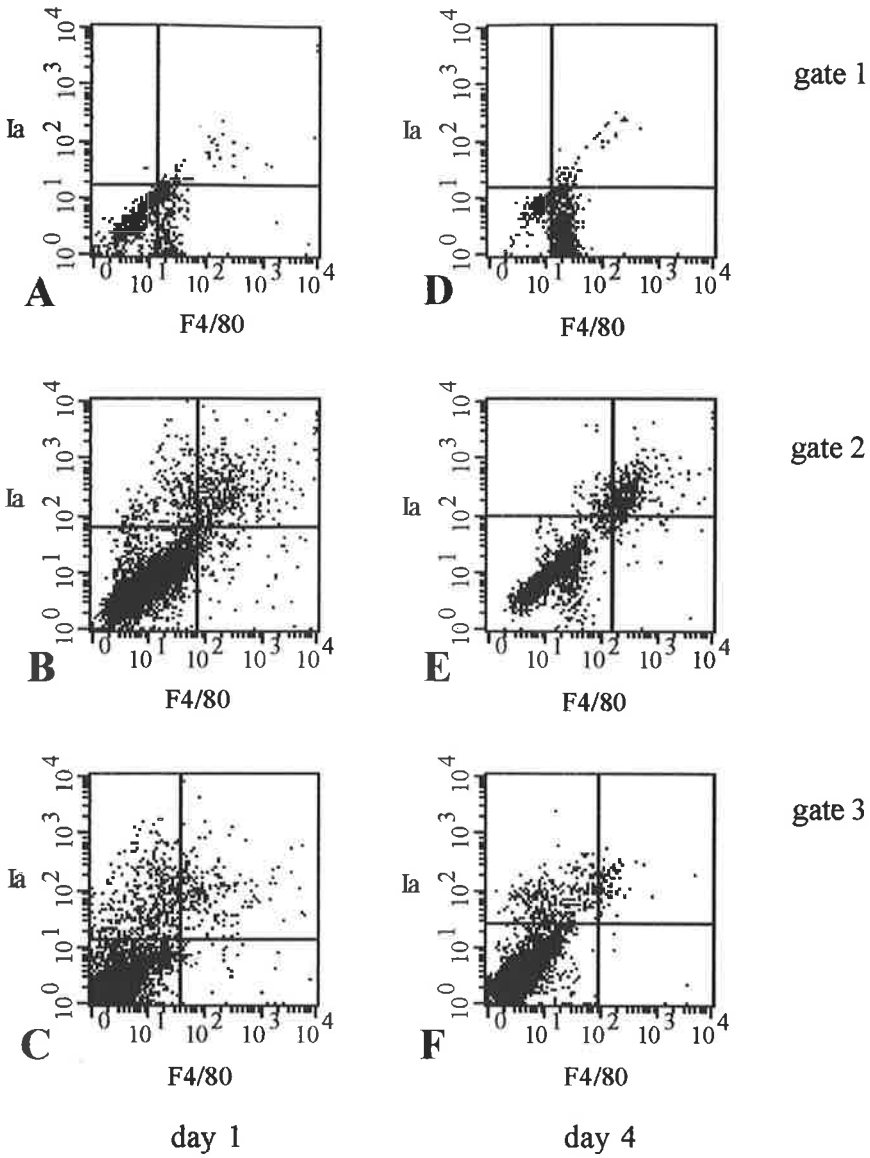


Figure 5.2 Dual colour flow cytometric analysis of F4/80$^+$ and Ia$^+$ uterine APCs at days 1 and 4 of pregnancy. Cells obtained by enzymatic digestion of individual uteri from a day 1 pregnant (A-C) or a day 4 pregnant (D-F) mouse were indirectly labeled with Ia-RPE and F4/80-FITC and analysed by dual colour flow cytometry. Fluorescence intensity (in arbitrary units) attributed to FITC (x axis) and RPE (y axis) on cells contained within gates 1 (A, D), 2 (B, E) and 3 (C, F) is also shown, with the numbers of F4/80$^+$Ia$^-$, F4/80$^+$Ia$^+$ and F4/80$^-$Ia$^+$ cells given as a proportion of gated cells in the lower right, upper right and upper left quadrants respectively (gates shown in Figure 5.1). Each of these quadrants contained <1% of gated cells in irrelevant isotype control preparations (F). Similar results were obtained for a further 3 uteri from day 1 pregnant mice and 2 uteri from day 4 pregnant mice.

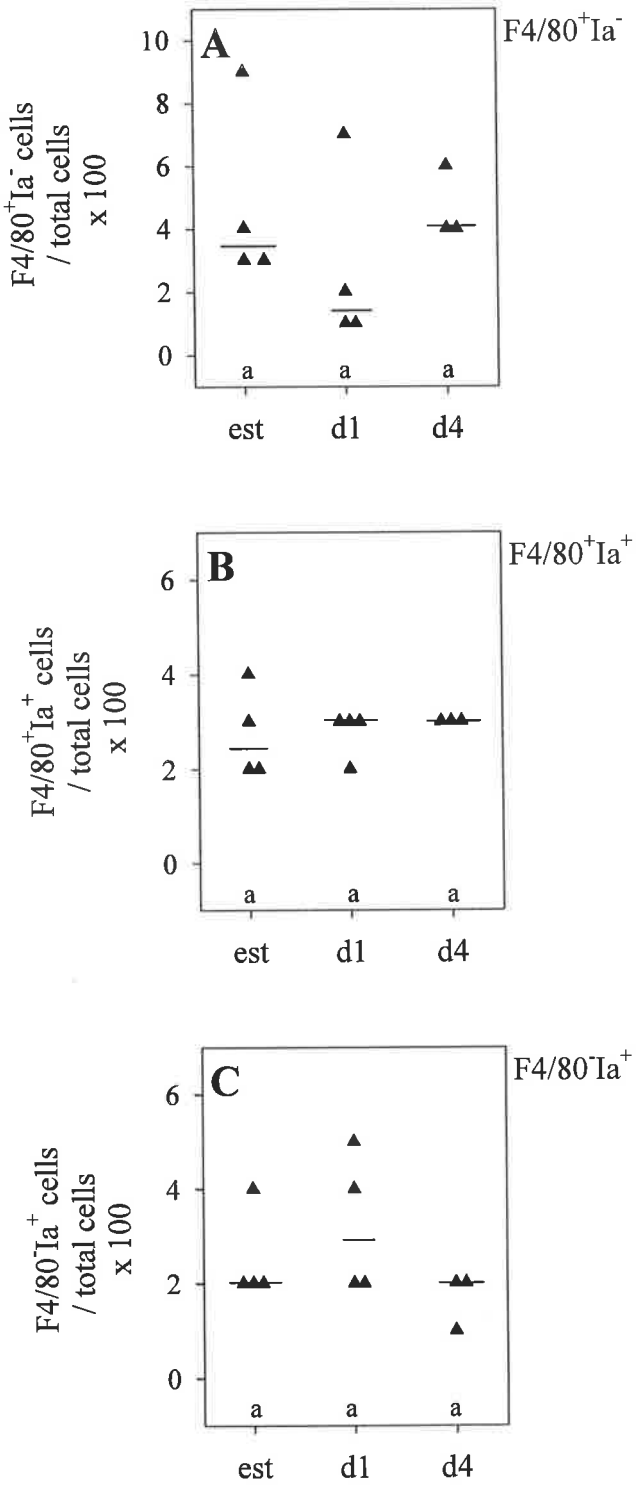


Figure 5.3 The effect of day of pregnancy on the numbers of F4/80$^+$ and Ia$^+$ APCs released from uteri. Cells obtained by enzymatic digestion of individual uteri from estrous (est), day 1 pregnant (d1) or day 4 pregnant (d4) mice were indirectly labeled with Ia-RPE and F4/80-FITC and analysed by dual colour flow cytometry. Symbols represent the numbers of F4/80$^+$Ia$^-$ cells (A), F4/80$^+$Ia$^+$ cells (B) and F4/80$^-$Ia$^+$ cells (C) as a proportion of total viable cells in individual uteri. Median values are scored. Data were compared by Kruskal-Wallis one-way ANOVA and Mann-Whitney Rank Sum Test. Groups assigned different lower case letters on the x axis are significantly different for each cell population.

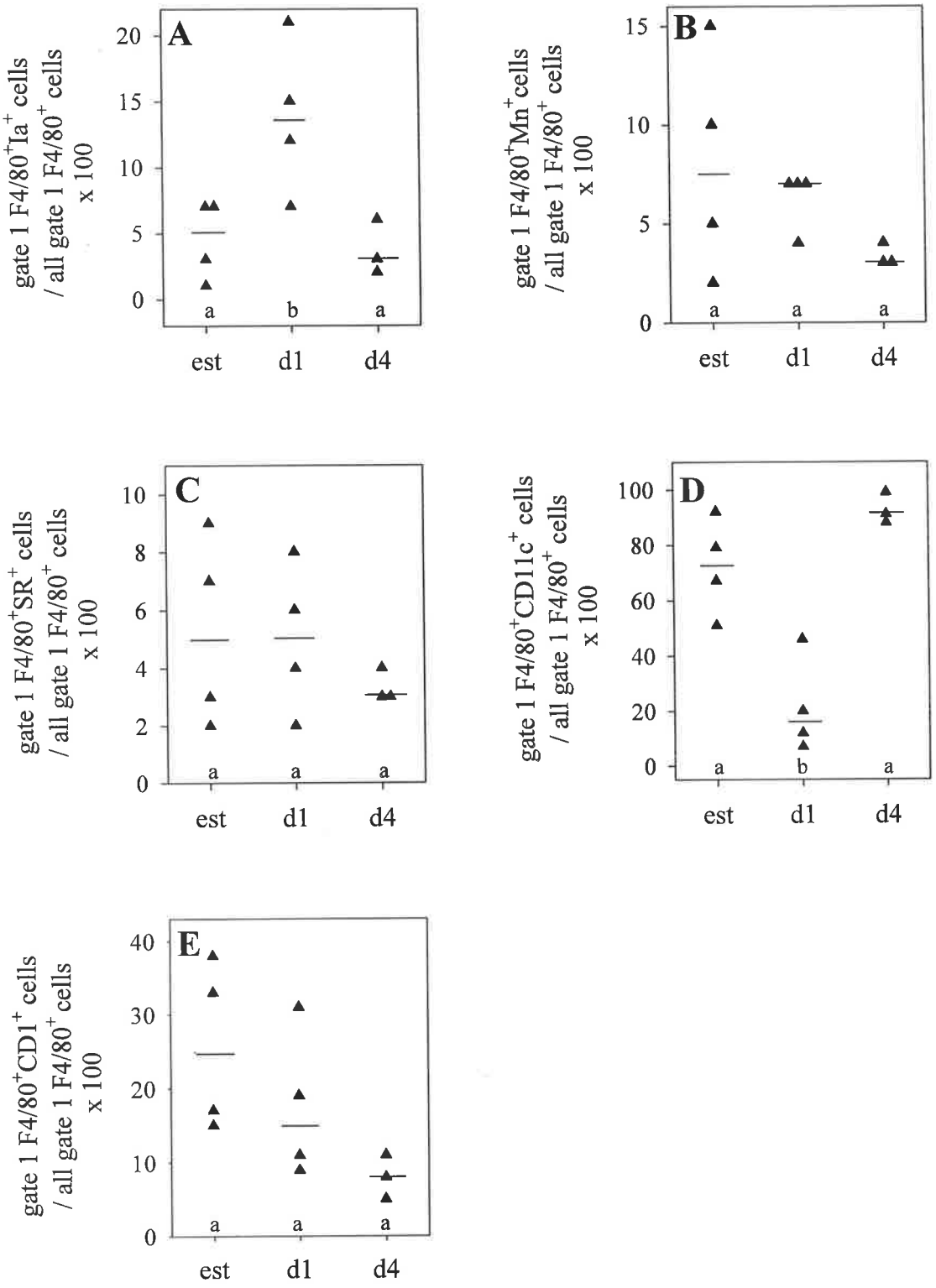


Figure 5.4 The effect of day of pregnancy on the proportion of gate 1 F4/80$^+$ cells expressing phenotypic markers. Cells obtained by enzymatic digestion of individual uteri from estrous (est), day 1 pregnant (d1) and day 4 pregnant (d4) mice were analysed by dual colour flow cytometry following labeling with F4/80-FITC and RPE using mAbs specific for the phenotypic markers Ia (A), macrosialin (Mn; B), class A scavenger receptor (SR; C), CD11c/CD18 (CD11c; D) and CD1 (E). Symbols represent the proportion of gate 1 F4/80$^+$ cells expressing each of the phenotypic markers in individual uteri. Median values are scored. Data were compared by Kruskal-Wallis one-way ANOVA and Mann-Whitney Rank Sum Test. Groups with different lower case letters on the x axis are significantly different for the given mAb ($p < 0.05$).

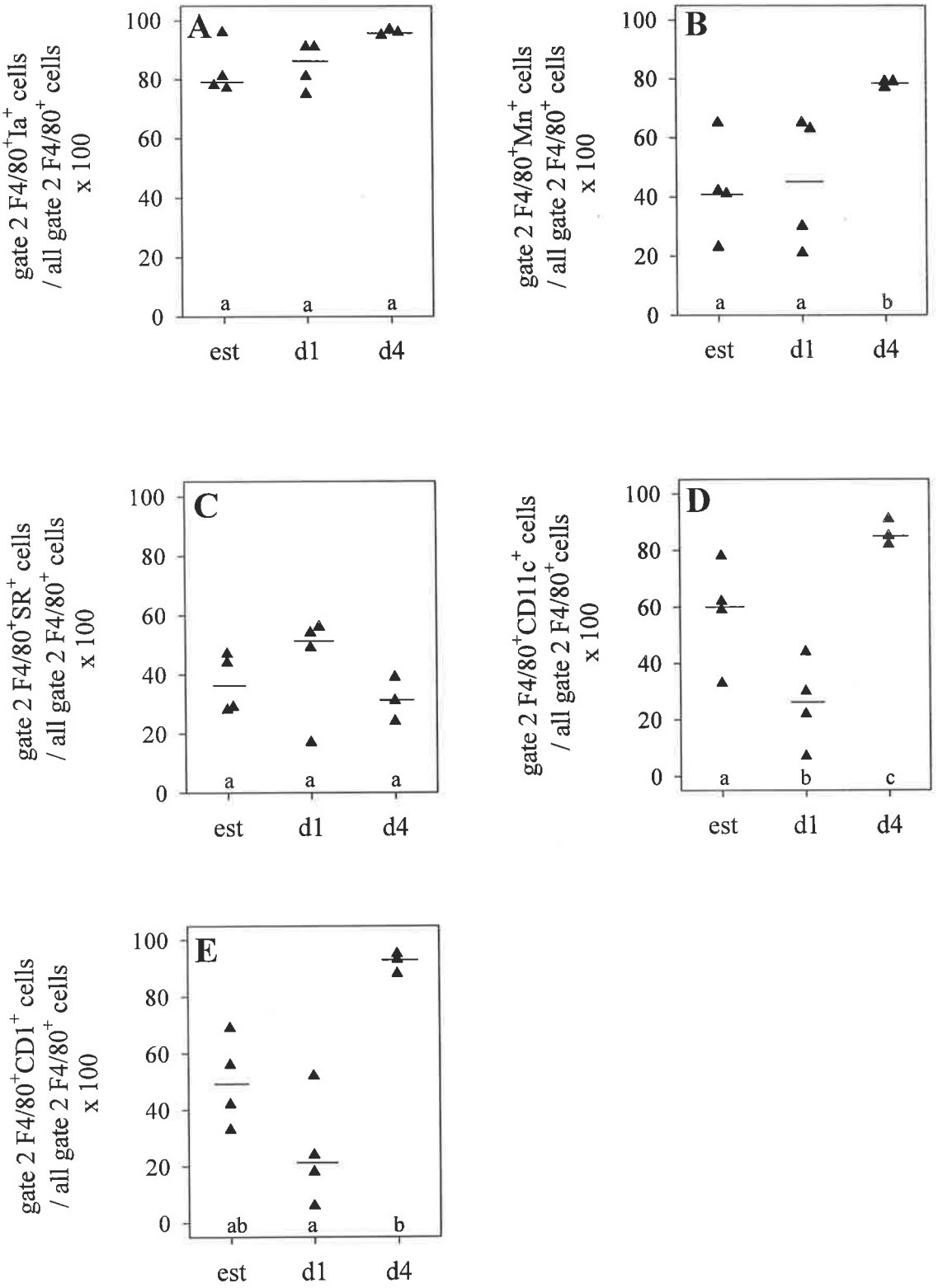


Figure 5.5 The effect of day of pregnancy on the proportion of gate 2 F4/80$^+$ cells expressing phenotypic markers. Cells obtained by enzymatic digestion of individual uteri from estrous (est), day 1 pregnant (d1) and day 4 pregnant (d4) mice were analysed by dual colour flow cytometry following labeling with F4/80-FITC and RPE using mAbs specific for the phenotypic markers Ia (A), macrosialin (Mn; B), class A scavenger receptor (SR; C), CD11c/CD18 (CD11c; D) and CD1 (E). Symbols represent the proportion of gate 2 F4/80$^+$ cells expressing each of the phenotypic markers in individual uteri. Median values are scored. Data were compared by Kruskal-Wallis one-way ANOVA and Mann-Whitney Rank Sum Test. Groups with different lower case letters on the x axis are significantly different for the given mAb ($p < 0.05$).

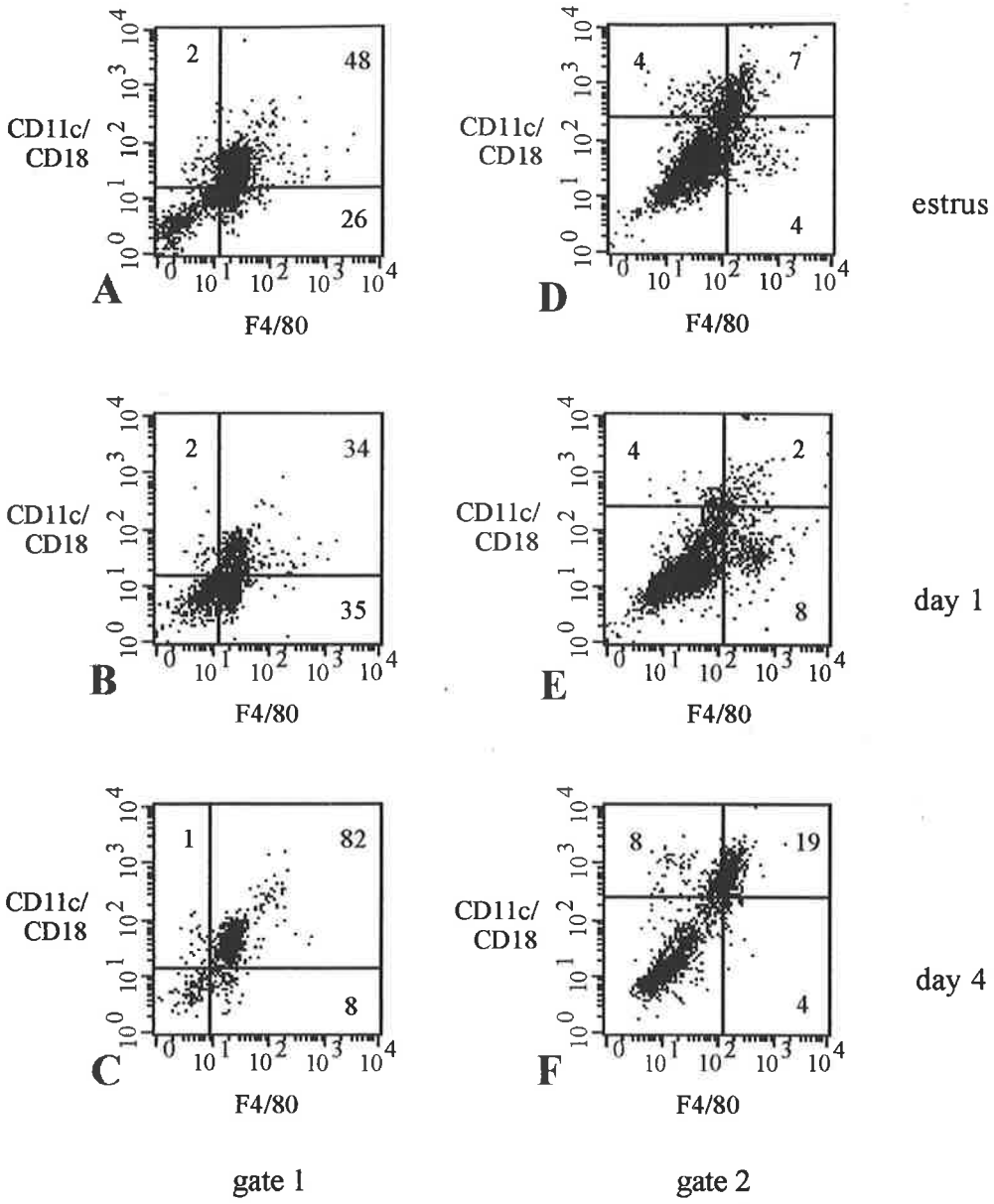


Figure 5.6 Dual colour flow cytometric analysis of gate 1 and gate 2 F4/80$^+$ and CD11c/CD18$^+$ uterine cells at estrus and days 1 and 4 of pregnancy. Cells obtained by enzymatic digestion of individual uteri from estrous (A, D), day 1 pregnant (B, E) and day 4 pregnant mice (C, F) were indirectly labeled with F4/80-FITC and CD11c/CD18-RPE and analysed by dual colour flow cytometry. Fluorescence intensity (in arbitrary units) attributed to FITC (x axis) and RPE (y axis) on cells in gate 1 is shown in A-C, and on cells in gate 2 in D-F, with the numbers of cells comprising each phenotype given as a proportion of gated cells in the lower right, upper right and upper left quadrants respectively. Each of these quadrants contained <1% of gated cells in irrelevant isotype control preparations. Similar results were obtained by analysis of a further 2-3 individual uteri at each of the stages of pregnancy (see Figures 5.4 and 5.5).

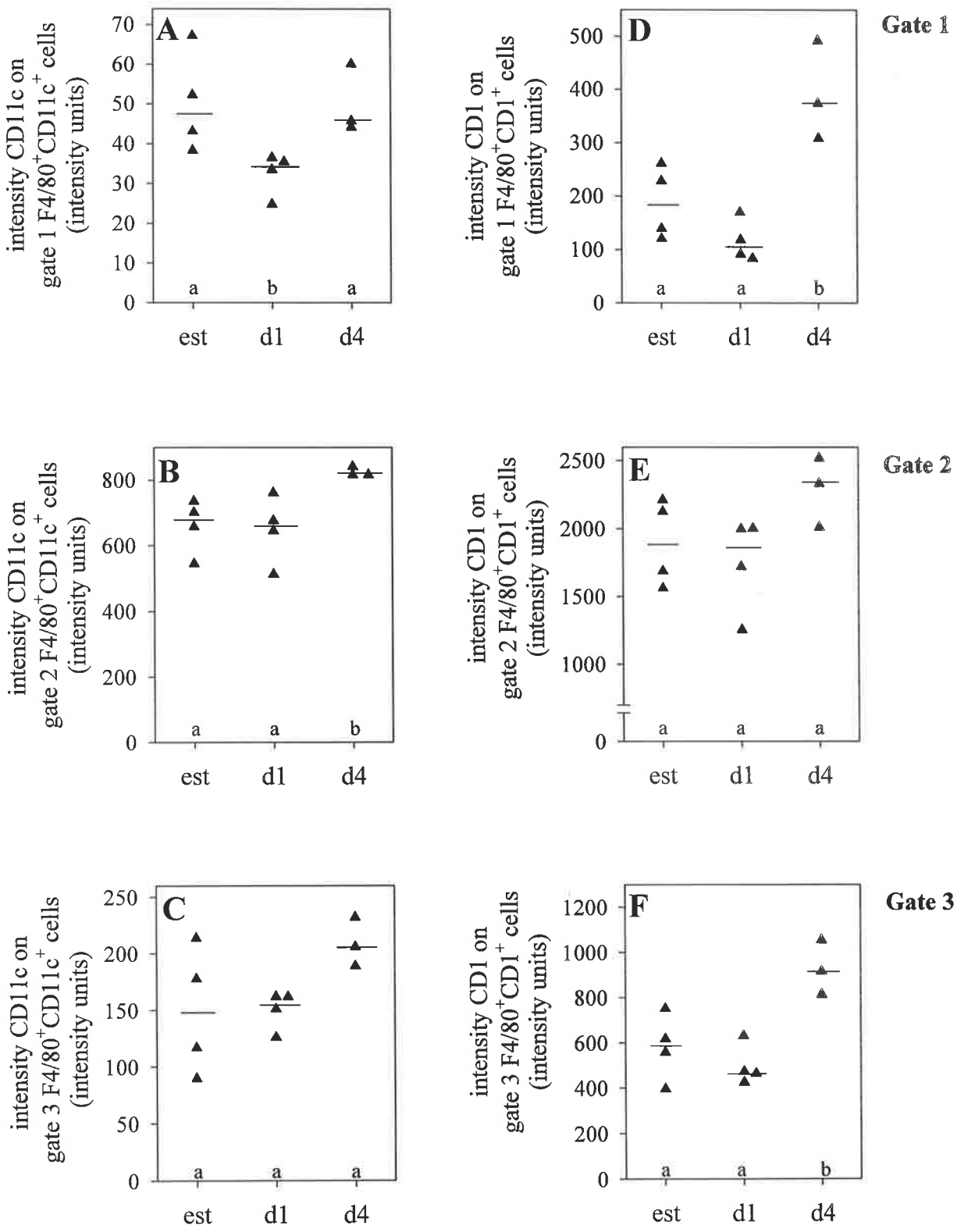


Figure 5.7 The effect of day of pregnancy on the intensity of CD11c/CD18 and CD1 expression by F4/80$^+$ uterine cells. Cells obtained by enzymatic digestion of individual uteri from estrous (est), day 1 pregnant (d1) and day 4 pregnant (d4) mice were analysed by dual colour flow cytometry following indirect labeling with F4/80-FITC and RPE using mAbs specific for CD11c/CD18 (A-C) and CD1 (D-F). For individual uteri, symbols represent the intensity of CD11c/CD18 and CD1 expression on F4/80$^+$ cells in gate 1 (A, D), on F4/80$^+$ cells in gate 2 (B, E) and on F4/80$^+$ cells in gate 3 (C, F). Median values are scored. Data were compared by Kruskal-Wallis one-way ANOVA and Mann-Whitney Rank Sum Test. Groups with different lower case letters on the x axis are significantly different for the given mAb in that gate ($p < 0.05$).

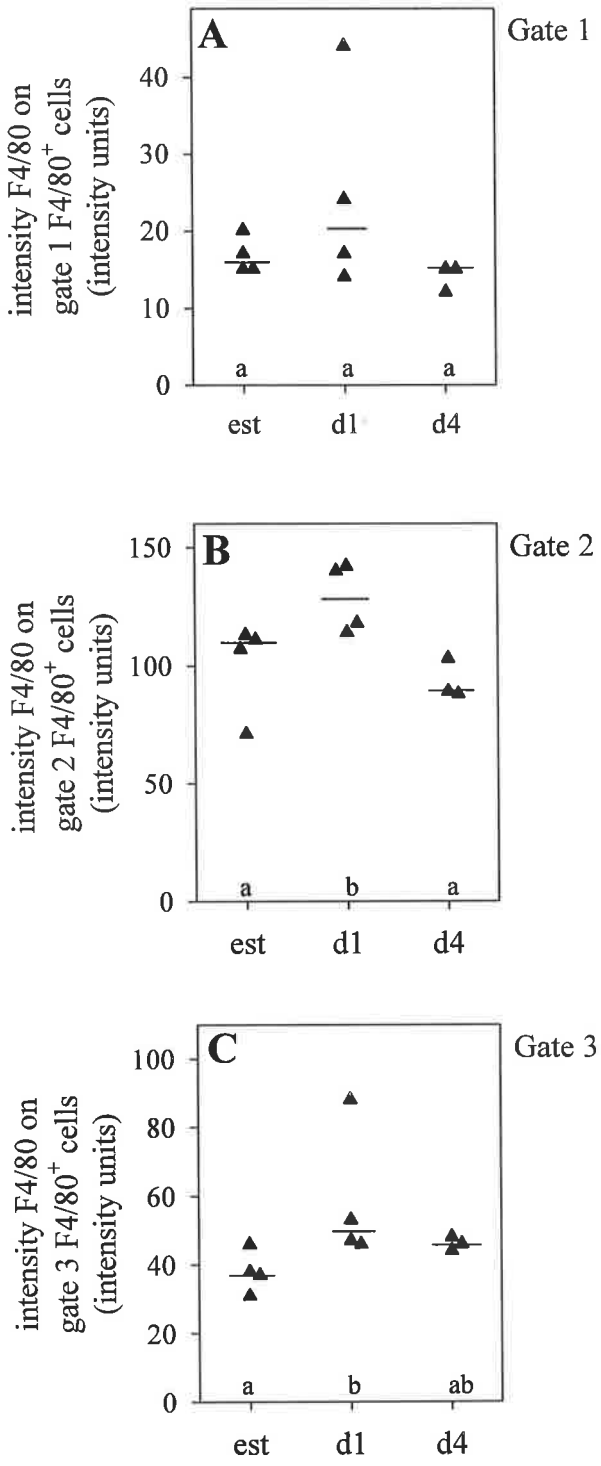


Figure 5.8 The effect of day of pregnancy on intensity of F4/80 expression by uterine cells. Duplicate samples of cells obtained by enzymatic digestion of individual uteri from estrous (est), day 1 pregnant (d1) and day 4 pregnant (d4) mice were analysed by single colour flow cytometry following indirect labeling with F4/80-FITC. For individual uteri, symbols represent the mean intensity of F4/80 expression on $F4/80^+$ cells in gate 1 (A), gate 2 (B) and gate 3 (C). Median values are scored. Data were compared by Kruskal-Wallis one-way ANOVA and Mann-Whitney Rank Sum Test. Groups with different lower case letters on the x axis are significantly different for the given mAb in that gate ($p < 0.05$).

Chapter 6

Antigen presenting cells in the uteri of GM-CSF deficient mice

6.1 Introduction

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a 23-kDa glycoprotein produced by leukocytes (Mϕs, granulocytes and T cells) (Ruef and Coleman 1990, Baldwin 1992) and mesenchymal cells, including the epithelium of the lung (Christensen *et al.* 1995, Nakamura *et al.* 1996) and keratinocytes of the skin (Chodakewitz *et al.* 1988, Kupper *et al.* 1988). The actions of GM-CSF were originally thought to be limited to regulation of the proliferation and differentiation of haematopoietic progenitor cells, but it is now recognised that this cytokine also drives the survival and differentiation of mature Mϕs, dendritic cells and neutrophils during inflammatory responses (Ruef and Coleman 1990, Baldwin 1992). In particular, GM-CSF is thought to upregulate the expression of several cell membrane molecules which have roles in antigen-specific immunity, including Ia (Stumbles *et al.* 1998), macrosialin (Li *et al.* 1998) and the B7 family of costimulatory molecules (Larsen *et al.* 1994, Stumbles *et al.* 1998).

A role for GM-CSF in recruiting and activating APCs within the uterus in a manner analogous to that which occurs in the lung and skin would appear biologically feasible since uterine epithelial cells are a potent source of GM-CSF. During the estrous cycle, expression is positively regulated by estrogen and thus peak levels of bioactive GM-CSF are detected at estrus (Robertson *et al.* 1996b). At the time of mating, GM-CSF expression is further upregulated by 20-fold (Robertson and Seamark 1990), when the uterine epithelial cells are exposed to $TGF\beta_1$ and/or other agents within seminal plasma (Robertson *et al.* 1996a). At this time, expression of the GM-CSF receptor also peaks, and its component subunits can be localised on the cell surface of neutrophils, Mϕs and putative dendritic cells (Robertson *et al.* 2000). A direct role for GM-CSF in regulating the recruitment and phenotype of uterine APCs is further supported by experiments showing that the post-mating recruitment and activation of leukocytes in the uterus can be mimicked by *in utero* administration of GM-CSF to ovariectomised mice (Robertson *et al.* 2000).

The use of cytokine-knockout mice has emerged as a useful means by which to attribute particular cytokines to distinct roles in murine physiology. Mice deficient in GM-CSF were first reported in 1994, and initially their only physiological abnormality appeared to be dysregulation

of surfactant metabolism in the lungs (Dranoff and Mulligan 1994, Stanley *et al.* 1994). Subsequent and more detailed analyses of these mice have since shown subtle differences in APC activation phenotype and secretory profile which become particularly evident after immune or inflammatory challenge (Basu *et al.* 1997, Wada *et al.* 1997, Noguchi *et al.* 1998). The aim of the experiments presented within this chapter was to use immunohistochemistry and flow cytometry to investigate the effect of GM-CSF deficiency on the numbers and phenotype of APCs within the cycling and pregnant murine uterus.

6.2 Effect of GM-CSF deficiency on APCs in the endometrium, mesometrial triangle and myometrium

To determine the number of APCs in the endometrium, mesometrial triangle and myometrium of GM-CSF-replete and -deficient mice, sections of uteri collected from estrous and pregnant mice of each genotype were analysed immunohistochemically. Uteri were collected from estrous, day 1 pregnant and day 4 pregnant mice. Fresh frozen 7μm sections were fixed in ethanol and APCs were detected using a panel of mAbs with an indirect immunoperoxidase technique according the methods outlined in sections 3.2 and 2.12.

The morphologies of APCs expressing each of the target antigens were assessed by light microscopy. The density of APCs in endometrium was quantified by video image analysis (see section 2.12), and the numbers of APCs in mesometrial triangle and myometrium were determined by semi-quantitative analysis (see Table 6.1). When differences in the numbers of scavenger receptor$^+$ and Ia$^+$ cells in mesometrial triangle and myometrium were observed by semi-quantitative analysis, these were then quantified by video image analysis.

6.2.1 APCs expressing F4/80 antigen

APCs expressing F4/80 antigen were detected in the uteri of GM-CSF-replete and -deficient mice by indirect immunohistochemical analysis using the F4/80 mAb (Austyn and Gordon 1981) (see Table 2.1 and section 3.2.2 for mAb specificity and reactive cell lineages).

In GM-CSF-replete mice, F4/80$^+$ cells were evident as abundant, large cells with short dendritic processes, and were distributed throughout the endometrium (Figure 6.2A), as well as in the mesometrial triangle and in the myometrium. On day 1 of pregnancy, the density of endometrial F4/80$^+$ cells increased significantly compared to that seen at estrus (median % positivity increased by 1.4-fold, $p = 0.02$, Figure 6.1A), and the cells were apparent in the superficial and deep endometrium (Figure 6.3A). Morphologically, the cells were large and often

had a 'foamy' intra-cytoplasmic appearance. The number of F4/80$^+$ APCs in the mesometrial triangle (Table 6.1) and myometrium (Table 6.2) also increased at day 1. In both of these locations the F4/80$^+$ cells were large and elongated in shape (not shown).

By day 4 of pregnancy, the densities of F4/80$^+$ cells in the endometrium had decreased significantly compared to day 1 (>60% decrease in median % positivity, $p = 0.01$ compared to day 1) to become slightly but non-significantly lower than the densities observed at estrus ($p = 0.1$ compared to estrus, Figure 6.1A). Small, intensely labeled F4/80$^+$ cells were observed in clusters within the deep endometrium of 2/5 uteri of GM-CSF-replete mice (Figure 6.4A). These were similar in size and appearance to the clusters previously noted in the deep endometrium of uteri of day 4 pregnant Balb/c F1 mice (see section 3.2). The number of F4/80$^+$ cells in the mesometrial triangle (Table 6.1) and the myometrium (Table 6.2) decreased from day 1 to day 4.

In GM-CSF-deficient mice, the density of endometrial F4/80$^+$ cells at estrus was similar to that observed in GM-CSF-replete mice ($p = 0.6$, Figure 6.1A). Also, the morphology of the F4/80$^+$ cells at estrus was not different in the GM-CSF-deficient mice compared to those in wild-type mice (Figure 6.2B). The densities of F4/80$^+$ cells in the uterine tissues of GM-CSF-deficient mice exhibited similar changes at days 1 and 4 of pregnancy as were observed in the GM-CSF-replete mice (Figure 6.3B and Figure 6.4B). Therefore, there were no significant differences between the densities of F4/80$^+$ cells in the endometria of mice of the two genotypes at these times ($p = 0.5$ at day 1, $p = 0.6$ at day 4, Figure 6.1A). Also, clusters of F4/80$^+$ cells were observed in the deep endometrium of 4/5 GM-CSF-deficient mice at day 4 that were similar to those seen in GM-CSF-replete mice.

The numbers of F4/80$^+$ cells in the mesometrial triangle (Table 6.1) and the myometrium (Table 6.2) of uteri from estrous, day 1 and day 4 pregnant GM-CSF-deficient mice were similar to those observed in day 1 pregnant GM-CSF-replete mice.

6.2.2 APCs expressing macrosialin

APCs expressing macrosialin were detected in the uteri of GM-CSF-replete and -deficient mice by indirect immunohistochemical analysis using the FA11 mAb (Rabinowitz and Gordon 1991, Li *et al.* 1998) (see Table 2.1 and section 3.2.3 for mAb specificity and reactive cell lineages).

a. expression in stromal APCs

In the endometrium of uteri from estrous GM-CSF-replete mice, macrosialin$^+$ APCs were evident as abundant, large cells which were very intensely labeled both on the cell surface and in

the cytoplasm. In one of the sections, a large cluster of macrosialin$^+$ cells was observed in the deep endometrium. Abundant macrosialin$^+$ cells were also evident in the mesometrial triangle and the myometrium in uteri of estrous GM-CSF-replete mice at estrus (not shown).

Table 6.1 The effect of GM-CSF deficiency on the number of APCs in the mesometrial triangle. Fresh frozen sections of uteri from estrous, day 1 and day 4 pregnant GM-CSF-replete (+/+) and -deficient (-/-) mice were analysed for number of immunohistochemically-labeled F4/80$^+$, macrosialin$^+$, scavenger receptor$^+$, sialoadhesin$^+$, Ia$^+$ and B7-2$^+$ APCs and endogenous peroxidase$^+$ eosinophils within the mesometrial triangle. The number of reactive cells in each compartment was estimated by counting in uterine fields at 20x magnification and scores are given. - = zero cells/field, +/- = 1-5 cells/field, + = 6-10 cells/field, ++ = 11-20 cells/field, +++ = > 21 cells/field.

	GM-CSF status and day of pregnancy					
	GM-CSF +/+			*GM-CSF -/-*		
	estrus	*day 1*	*day 4*	*estrus*	*day 1*	*day 4*
F4/80$^+$	++	+++	++	++	+++	++
macrosialin$^+$	++	++	++	++	++	++
scavenger receptor$^+$	+	+++	+/-	+	+++	+/-
sialoadhesin$^+$	++	++	+	++	++	+
Ia$^+$	+	+	+/-	+/	+	-
B7-2$^+$	+/-	+/-	+/-	+/-	+/-	+/-
eosinophils	+/-	+/-	+++	+/-	+/-	+++
n/group	*5*	*5*	*5*	*5*	*5*	*5*

In GM-CSF-replete mice the median density of macrosialin$^+$ APCs in the endometrium increased slightly at day 1 of pregnancy, but this was not significant (1.4-fold increase in median % positivity, $p = 0.25$, Figure 6.1B). The number of macrosialin$^+$ cells in the mesometrial triangle at day 1 remained similar to that seen at estrus (Table 6.1), whereas the number in the myometrium increased (Table 6.2). In all locations the macrosialin$^+$ cells at day 1 were elongated and very intensely labeled (not shown).

By day 4 of pregnancy, the density of macrosialin$^+$ cells in the endometrium was significantly lower than that at day 1 (>50% decrease in median % positivity, $p = 0.05$) and lower than that at estrus ($p = 0.03$, Figure 6.1B). The few macrosialin$^+$ cells which were located in the superficial endometrium at day 4 had a 'foamy' cytoplasmic appearance, and were very closely aligned with luminal epithelial cells. Clusters of macrosialin$^+$ cells were observed in the deep endometrium of 4/5 uteri from GM-CSF-replete mice (not shown). The number of macrosialin$^+$ cells in the mesometrial triangle remained consistent from day 1 to day 4 (Table 6.1). The

number of macrosialin$^+$ cells in the myometrium was decreased at day 4 compared to day 1 (Table 6.2).

In estrous GM-CSF-deficient mice, macrosialin$^+$ APCs were of a similar morphological appearance (not shown) and similarly abundant in the endometrium ($p = 0.9$, Figure 6.1B), mesometrial triangle (Table 6.1) and the myometrium (Table 6.2) as in estrous wild-type mice. Also, the density of macrosialin$^+$ cells in the uteri of day 1 pregnant GM-CSF-deficient mice increased from estrous levels in a similar manner to that seen in GM-CSF-replete mice (comparing the two genotypes at day 1, $p = 0.1$, Figure 6.1B). The number of macrosialin$^+$ cells in the mesometrial triangle (Table 6.1) and the myometrium (Table 6.2) of uteri was similar regardless of genotype at day 1 of pregnancy.

By the fourth day of pregnancy, the density and numbers of macrosialin+ cells in the endometrium ($p = 0.5$, Figure 61A), the mesometrial triangle (Table 6.1) and the myometrium (Table 6.2) were similar in uteri of GM-CSF-deficient mice and GM-CSF-replete mice. The foamy macrosialin$^+$ cells which had been observed in the superficial endometrium of wild-type mice were also seen in the endometria of GM-CSF-deficient mice. Also, macrosialin$^+$ cells in the deep endometrium in uteri of 5/5 GM-CSF-deficient mice were arranged in clusters as was evident for other APC markers (not shown).

b. expression in epithelial cells

In previous experiments (see section 3.3) the luminal epithelial cells in uteri of Balb/c F1 mice were found to express macrosialin, and the intensity of expression was positively correlated with circulating estrogens and insemination. In order to determine whether the expression of macrosialin by uterine epithelial cells was influenced by GM-CSF genotype, the expression of macrosialin in uterine epithelial cells was examined in sections of uteri from GM-CSF-replete and GM-CSF-deficient mice.

Uterine epithelial cells in the uteri of estrous GM-CSF-replete mice exhibited high intensity expression of macrosialin, and this intensity was increased at day 1 of pregnancy. By day 4 of pregnancy, the expression of macrosialin by epithelial cells was barely detectable. The epithelial cells in uteri from GM-CSF-deficient mice showed a similar dynamic and intensity of expression of macrosialin from estrus to day 1 to day 4 of pregnancy (not shown).

6.2.3 APCs expressing class A scavenger receptor

APCs expressing class A scavenger receptor (scavenger receptor) were detected in the uteri of GM-CSF-replete and -deficient mice by indirect immunohistochemical analysis using the mAb 2F8 (Fraser *et al.* 1993) (see Table 2.1 and section 3.2.4 for mAb specificity and reactive cell lineages).

Table 6.2 The effect of GM-CSF deficiency on the number of APCs in the myometrium. Fresh frozen sections of uteri from estrous, day 1 and day 4 pregnant GM-CSF-replete (+/+) and -deficient (-/-) mice were analysed for number of immunohistochemically-labeled F4/80$^+$, macrosialin$^+$, scavenger receptor$^+$, sialoadhesin$^+$, Ia$^+$ and B7-2$^+$ APCs and endogenous peroxidase$^+$ eosinophils within the myometrium. The number of reactive cells in each compartment was estimated by counting in uterine fields at 20x magnification and scores are given. - = zero cells/field, +/- = 1-5 cells/field, + = 6-10 cells/field, ++ = 11-20 cells/field, +++ = > 21 cells/field.

	GM-CSF status and day of pregnancy					
	GM-CSF +/+			*GM-CSF -/-*		
	estrus	*day 1*	*day 4*	*estrus*	*day 1*	*day 4*
F4/80$^+$	++	+++	++	++	+++	++
macrosialin$^+$	++	+++	++	++	+++	++
scavenger receptor$^+$	+/++	++	+	+	++	+
sialoadhesin$^+$	++	+++	+	++	+++	+
Ia$^+$	+/-	+/-	+	+/-	+/-	+/-
B7-2$^+$	+/-	+/-	+/-	+/-	+/-	+/-
eosinophils	+	+/-	++	+/-	+/-	++
n/group	*5*	*5*	*5*	*5*	*5*	*5*

In the uteri of estrous GM-CSF-replete mice, scavenger receptor$^+$ APCs were relatively abundant, large cells with a round shape and short, fine dendritic processes. The cells were located throughout the endometrium (Figure 6.2C), in the mesometrial triangle and in the myometrium. After insemination, the density of scavenger receptor$^+$ APCs in the endometrium increased significantly (median % positivity increased by 1.7-fold, $p = 0.01$ compared to estrus, Figure 6.1C and Figure 6.3C). The number of scavenger receptor$^+$ cells in the mesometrial triangle also increased significantly at day 1 ($p = 0.02$ compared to estrus, Figure 6.5A), whereas in the myometrium the number remained similar to that seen at estrus ($p = 0.18$, Figure 6.5C).

By day 4 of pregnancy, the median density of scavenger receptor$^+$ cells in the endometrium decreased by over 80% compared to that observed at day 1 ($p = 0.01$) and this was also significantly lower than the density at estrus (p = 0.04, Figure 6.1C and Figure 6.4C). Clusters of scavenger receptor$^+$ cells were observed in the deep endometrium of 1/5 day 4 pregnant mice (not shown). Also, the number of scavenger receptor$^+$ cells in the mesometrial

triangle at day 4 was significantly reduced compared to the number at day 1 ($p = 0.005$, Figure 6.5A) and the number of myometrial scavenger receptor$^+$ cells also decreased significantly from day 1 to day 4 ($p = 0.008$, Figure 6.5C).

There were significantly fewer scavenger receptor$^+$ APCs in the endometrium of estrous GM-CSF-deficient mice than in the endometrium of estrous GM-CSF-replete mice (median % positivity was 4-fold lower in the knockout mice, $p = 0.005$, Figure 6.1C and Figure 6.2D). In contrast, the number of scavenger receptor$^+$ APCs in the mesometrial triangle ($p = 0.8$, Figure 6.5A) and in the myometrium ($p = 0.39$, Figure 6.5C) was similar in GM-CSF-deficient mice compared to wild-type mice.

The density of scavenger receptor$^+$ APCs in the endometrium of GM-CSF-deficient mice increased only slightly from estrus to day 1 of pregnancy, and this was not significant ($p = 0.17$). Hence, there were fewer scavenger receptor$^+$ cells in the endometria of day 1 pregnant GM-CSF-deficient mice than in GM-CSF-replete mice (median % positivity was diminished by >50%, $p = 0.007$, Figure 6.1C and Figure 6.3D). The number of scavenger receptor$^+$ cells in the mesometrial triangle ($p = 0.6$, Figure 6.5A) and myometrium ($p = 0.6$, Figure 6.5C) of day 1 pregnant GM-CSF-deficient and -replete mice was not influenced by genotype.

By the fourth day of pregnancy, the density of scavenger receptor$^+$ cells in the endometrium ($p = 0.2$, Figure 6.1C and Figure 6.4D), in the mesometrial triangle ($p = 1.0$, Figure 6.5A) and in the myometrium ($p = 0.19$, Figure 6.5C) was similar in the mice of both genotypes. The deep endometrium of 3/5 GM-CSF-deficient mice contained clusters of scavenger receptor$^+$ cells (not shown).

A summary of the effects of GM-CSF deficiency on numbers of scavenger receptor$^+$ APCs in the endometrium, mesometrial triangle and myometrium is provided in Table 6.3.

6.2.4 APCs expressing sialoadhesin

APCs expressing sialoadhesin were detected in the uteri of GM-CSF-replete and -deficient mice by indirect immunohistochemical analysis using the 3D6 mAb (van den Berg *et al.* 1992) (see Table 2.1 and section 3.2.5 for mAb specificity and reactive cell lineages).

APCs expressing sialoadhesin were evident in the endometrium, the mesometrial triangle and the myometrium in uteri of estrous GM-CSF-replete mice as abundant, large, rounded cells with fine dendritic processes (not shown). The density of sialoadhesin$^+$ cells in the endometrium increased significantly after mating (median % positivity increased by 1.8-fold, p = 0.009, Figure 6.1D), whereas the numbers in the mesometrial triangle (Table 6.1) and the myometrium (Table 6.2) remained steady from estrus to day 1. By day 4 of pregnancy, density of sialoadhesin$^+$ cells

in the endometrium was relatively low (median % positivity decreased by >60% compared to day 1, $p = 0.03$, Figure 6.1D). However, small clumps of sialoadhesin+ cells were observed in the deep endometrium in 3/5 day 4 pregnant uteri (not shown). The numbers of sialoadhesin$^+$ cells in the mesometrial triangle (Table 6.1) and the myometrium (Table 6.2) were lower at day 4 than at day 1.

The density of sialoadhesin$^+$ cells in the endometrium of estrous GM-CSF-deficient mice was similar to that in GM-CSF-replete mice ($p = 0.6$, Figure 6.1D). The morphology of the sialoadhesin$^+$ cells was also similar, although those in the endometria of GM-CSF-deficient mice tended to be less intensely labeled than those in wild-type mice (not shown). The numbers of sialoadhesin$^+$ cells in the mesometrial triangle (Table 6.1) and myometrium (Table 6.2) were similar in mice of both genotypes at estrus.

Table 6.3 Summary of the effects of GM-CSF deficiency on the number of scavenger receptor$^+$ and Ia$^+$ APCs in uteri. The numbers of scavenger receptor$^+$ and Ia$^+$ APCs in the endometrium (endo), mesometrial triangle (meso) and myometrium (myo) in fresh frozen sections of immunohistochemically labeled uteri of GM-CSF-replete and GM-CSF-deficient mice are summarised as: - = no cells, +/- = sparse cells, + = some cells, ++ = many cells, +++ = abundant cells. The difference in number of cells in GM-CSF-deficient mice compared to GM-CSF-replete mice are shown as : ↓ = less, - = no change. For original data see Figure 6.1 and Figure 6.5.

			GM-CSF-deficient	GM-CSF-replete	**difference**
scavenger receptor	estrus	*endo*	++	+	↓
		meso	+	+	-
		myo	+/++	+	↓
	day 1	*endo*	+++	+/++	↓
		meso	+++	+++	-
		myo	++	++	-
	day 4	*endo*	+	+	-
		meso	+/-	+/-	-
		myo	+	+	-
Class II MHC (Ia)	estrus	*endo*	++	+	↓
		meso	+	+	-
		myo	+/-	+/-	-
	day 1	*endo*	+++	+++	-
		meso	+	+	-
		myo	+/-	+/-	-
	day 4	*endo*	++	+	↓
		meso	+/-	-	↓
		myo	+	+/-	↓

The densities of sialoadhesin$^+$ cells present on day 1 and day 4 of pregnancy were similar in the endometrium ($p = 0.5$ at day 1, $p = 0.17$ at day 4, Figure 6.1D), in the mesometrial triangle (Table 6.1) and in the myometrium (Table 6.2) in GM-CSF-deficient mice as in GM-CSF-replete mice. Moreover, the clusters of sialoadhesin$^+$ cells observed in the deep endometrium of wild-type mice were also observed in a comparable location in 3/5 GM-CSF-deficient mice (not shown).

6.2.5 APCs expressing MHC class II (Ia)

APCs expressing Ia were detected in the uteri of GM-CSF-replete and -deficient mice by indirect immunohistochemical analysis using the TIB 120 mAb (Bhattacharya *et al.* 1981) (see Table 2.1 and section 3.2.6 for mAb specificity and reactive cell lineages).

In the uteri of estrous GM-CSF-replete mice, Ia$^+$ cells were evident in the endometrium as rounded cells located primarily in the superficial endometrium (Figure 6.2E). Overall, their density was relatively low compared to the densities of endometrial APCs detected with other mAbs at estrus (Figure 6.1E). Also evident in the uteri of estrous GM-CSF-replete mice were relatively sparsely distributed Ia$^+$ cells in the mesometrial triangle and in the myometrium (not shown).

At day 1 of pregnancy, the density of endometrial Ia$^+$ cells increased significantly (median % positivity increased by 2-fold, p = 0.01, Figure 6.1E), and the cells were often found accumulated in large groups directly underneath luminal epithelial cells (Figure 6.3E). The number of Ia$^+$ cells in the mesometrial triangle ($p = 0.47$, Figure 6.5B) and in the myometrium ($p = 0.13$, Figure 6.5D) was not significantly affected by mating.

There was a no significant change in the density of Ia$^+$ cells in the endometrium from day 1 to day 4 in GM-CSF-replete mice (median % positivity decreased by 1.3-fold, p = 0.2, Figure 6.1E). However, the distribution and morphology of the Ia$^+$ cells did change markedly between the two times : at day 4 the Ia$^+$ cells were small and round, and found in the deep endometrium either individually or in small clusters (observed in 4/5 wild-type uteri at day 4, Figure 6.4E). The number of Ia$^+$ cells in the mesometrial triangle fell slightly from day 1 to day 4 ($p = 0.17$, Figure 6.5B), whereas in the myometrium there tended to be more Ia$^+$ cells at day 4 than at day 1, although this was not significant ($p = 0.13$, Figure 6.5D).

There were significantly fewer Ia$^+$ cells in the endometrium of estrous GM-CSF-deficient mice than in estrous GM-CSF-replete mice (median % positivity was reduced by >50%, p = 0.02, Figure 6.1E). Although the morphology of the Ia$^+$ cells was similar in the two genotypes, in uteri of GM-CSF-deficient mice the cells tended to be located in a deeper layer of the endometrium

than those in the wild-type mice (Figure 6.2F). The numbers of Ia^+ cells in the mesometrial triangle ($p = 0.13$, Figure 6.5B) and in the myometrium ($p = 0.61$, Figure 6.5D) at estrus were similar in both genotypes.

On day 1 of pregnancy, the density of Ia^+ cells in the endometrium was comparable in the GM-CSF-deficient mice as in the GM-CSF-replete mice (p= 0.9, Figure 6.1E). The location and the morphology of the endometrial Ia^+ cells was also similar between the two genotypes (Figure 6.3F). The number of Ia^+ cells in the mesometrial triangle ($p = 0.75$, Figure 6.5B) and in the myometrium ($p = 0.13$, Figure 6.5D) was similar in both genotypes.

By day 4 of pregnancy, the density of Ia^+ cells in the endometria of GM-CSF-deficient mice had decreased significantly ($p = 0.001$ compared to day 1), and tended to be lower than the density of Ia^+ cells in the endometria of uteri of day 4 pregnant GM-CSF-replete mice (median % positivity was >50% lower, p = 0.08, Figure 6.1E). The cells were small, pale in staining intensity and round in morphology, and were found in clumps in the deep endometrium of 5/5 GM-CSF-deficient mice (Figure 6.4F). There were significantly fewer Ia^+ cells in the mesometrial triangle of day 4 pregnant GM-CSF-deficient mice than in GM-CSF-replete mice ($p = 0.05$, Figure 6.5B), and a similar trend was evident in the myometrium although this was not significant ($p = 0.13$, Figure 6.5D).

A summary of the effects of GM-CSF deficiency on numbers of Ia^+ APCs in the endometrium, mesometrial triangle and myometrium is provided in Table 6.3.

6.2.6 APCs expressing B7-2

APCs expressing B7-2 were detected in the uteri of GM-CSF-replete and -deficient mice by indirect immunohistochemical analysis using the mAb GL-1 (Freeman *et al.* 1993) (see Table 2.1 and section 3.2.7 for mAb specificity and reactive cell lineages).

$B7\text{-}2^+$ APCs were occasionally evident at estrus in the endometrium of GM-CSF-replete mice as sparsely distributed, large cells which were labeled only faintly by the immunoperoxidase technique. Very few cells were evident in the mesometrial triangle and in the myometrium of estrous uteri (not shown).

After mating, the density of $B7\text{-}2^+$ cells in the endometrium increased significantly (median % positivity increased by over 4-fold, p = 0.01, Figure 6.1F), with the majority of the cells located in the superficial endometrium. By day 4 of pregnancy, the density of $B7\text{-}2^+$ cells in the endometrium had decreased again to a number which was similar to that observed in estrous uteri ($p = 0.01$ compared to day 1, $p = 0.3$ compared to estrus, Figure 6.1F). Also at day 4, clusters of relatively intensely-labeled $B7\text{-}2^+$ cells were seen in the deep endometrium of 2/5

uteri. The number of B7-2$^+$ cells in the mesometrial triangle (Table 6.1) and in the myometrium (Table 6.2) remained low at days 1 and 4 of pregnancy. As had been previously observed in the uteri of Balb/c F1 mice (see Chapter 3), the intensity of B7-2 labeling was considerably lower than that seen using the other mAbs.

The density and appearance of B7-2$^+$ cells in the endometrium ($p = 0.5$, Figure 6.1F), the mesometrial triangle (Table 6.1) and the myometrium (Table 6.2) of uteri from GM-CSF-deficient mice was similar to that in uteri of GM-CSF replete mice at estrus, at day 1 and at day 4 of pregnancy. At day 4, clusters of B7-2$^+$ cells were seen in the deep endometrium of 5/5 uteri from GM-CSF-deficient mice (not shown).

6.2.7 Eosinophils with endogenous peroxidase activity

Eosinophils in sections of uteri of GM-CSF-replete and -deficient mice were detected by virtue of their endogenous peroxidase activity.

In the uteri of estrous GM-CSF-replete mice, eosinophils were distributed throughout the endometrium, particularly in the superficial tissue. Eosinophils were sparse in the mesometrial triangle and the myometrium at estrus (not shown). After mating, the density of eosinophils in the endometrium tended to increase ($p = 0.17$, Figure 6.1G) but remained similar in the mesometrial triangle (Table 6.1) and the myometrium (Table 6.2). On day 4 of pregnancy, the density of eosinophils in the endometrium decreased, although this was not significant compared to day 1 ($p = 0.12$, Figure 6.1G). The number of eosinophils in the mesometrial triangle (Table 6.1) and the myometrium (Table 6.2) increased markedly from day 1 to day 4. There was no clustering of eosinophils observed in the endometrium at any stage.

The numbers and distribution of eosinophils in the endometrium, the mesometrial triangle and the myometrium were not influenced by GM-CSF status (Figure 6.1, Table 6.1 and Table 6.2).

6.3 Effect of GM-CSF deficiency on expression of activation markers by uterine APCs at day 1 of pregnancy

6.3.1 Populations of F4/80$^+$ and Ia$^+$ APCs in the uteri of GM-CSF-deficient mice

The numbers and phenotypes of populations of APCs present in lymphoid and other peripheral organs is thought to arise due to local production of immunoregulatory molecules including cytokines and chemokines (Steinman 1988, Rutherford *et al.* 1993). Precursor and interstitial populations of Mϕs and dendritic cells are thought to be particularly susceptible to

fluctuations in local concentrations of colony stimulating factors such as CSF-1 and GM-CSF (Stanley *et al.* 1997, Santiago Schwarz 1999).

The previous experiments (see Chapters 3 and 4) identified three populations of APCs in the cells released by enzymatic digestion of uteri from estrous and pregnant mice. These were : (1) those that expressed F4/80 and not Ia ($F4/80^+Ia^-$), (2) those that expressed both F4/80 and Ia ($F4/80^+Ia^+$) and (3) those that expressed Ia and not F4/80 ($F4/80^-Ia^+$). The cell populations were designated 'undifferentiated Mϕs', 'differentiated Mϕs' and 'dendritic cells' respectively.

In order to determine whether the relative numbers of Mϕs and dendritic cells were altered in the uteri of GM-CSF deficient mice, aliquots of $5x10^5$ cells release by enzymatic digestion of individual uteri from day 1 pregnant GM-CSF-replete (n = 4) and from day 1 pregnant GM-CSF-deficient (n = 3) mice were assessed by flow cytometry after dual labeling of F4/80 and Ia using protocols 2 and 3 (see Table 4.2). Using the FACS machine, 10 000 events from each aliquot were acquired in a gate designed to exclude dead cells and cellular debris. The FACS machine was set up to detect FITC and RPE emissions as described previously (see Chapter 3) and the thresholds for background fluorescence in both channels 1 and 2 were defined by labeling cells with irrelevant mAbs and fluorochrome-conjugated reagents.

Each of the F4/80-FITC versus Ia-RPE profiles of cells from individual uteri showing $F4/80^+Ia^-$, $F4/80^+$, Ia^+ and $F4/80^-Ia^+$ cells was examined on the basis of several parameters. Firstly, the profiles were used to determine the effect of GM-CSF deficiency on the proportion of total cells comprised by each the three APC phenotypes. Secondly, the profiles were examined in order to determine whether GM-CSF deficiency had any effect on the proportion of $F4/80^+$ cells expressing Ia and the proportion of Ia^+ cells expressing F4/80. Finally, the profiles were examined to determine the effect of GM-CSF deficiency on the intensity of F4/80 expression in $F4/80^+Ia^-$ and $F4/80^+Ia^+$ cells, and effect on the intensity of Ia expression in $F4/80^+Ia^+$ and $F4/80^-Ia^+$ cells.

GM-CSF deficiency had no significant effect on the proportion of total cells comprised by each of the three APC phenotypes ($1.0 > p > 0.2$, Figure 6.6). However, there was a small but non-significant decrease in the proportion of $F4/80^+$ cells which expressed Ia in GM-CSF-deficient mice compared to those in GM-CSF-replete mice ($p = 0.11$, Table 6.4). The proportion of Ia^+ cells which expressed F4/80 was not affected by GM-CSF deficiency ($p = 0.2$, data not shown).

There was no effect of GM-CSF deficiency on the intensity of F4/80 expression on $F4/80^+Ia^-$ or $F4/80^-Ia^+$ cells (data not shown, $p > 0.5$). On $F4/80^+Ia^+$ and $F4/80^-Ia^+$ cells, intensity of Ia expression tended to be higher on cells derived from GM-CSF-deficient mice, although this was not significant ($p = 0.08$, Table 6.5 and Table 6.6).

Table 6.4 The effect of GM-CSF deficiency on the proportion of F4/80$^+$ uterine cells expressing activation markers. Cells derived by enzymatic digestion of uteri from day 1 pregnant GM-CSF-replete (n = 4) and GM-CSF-deficient (n = 3) mice were immunophenotyped by 2-colour fluorometric analysis. The % of F4/80$^+$ cells expressing activation markers Ia, scavenger receptor and B7-2 are given as median (range). Data were analysed by Mann Whitney Rank Sum Test, and p values are given.

	genotype		
	GM-CSF-replete	GM-CSF-*deficient*	*p* value
activation marker:			
Ia	37 (28-42)	26 (22-32)	0.11
scavenger receptor	30 (16-40)	17 (16-29)	0.28
B7-2	25 (13-26)	12 (11-19)	0.11

6.3.2 Effect of GM-CSF deficiency on expression of scavenger receptor by uterine APCs

The previous immunohistochemistry experiments established that on day 1 of pregnancy, uteri of GM-CSF-deficient mice contained significantly fewer APCs expressing scavenger receptor than did uteri of GM-CSF replete mice (see section 6.2.3). Therefore, an experiment was designed to determine whether this effect was preferentially evident in scavenger receptor$^+$ cells expressing F4/80 (ie Mϕs) or scavenger receptor$^+$ cells which did not express F4/80 (ie putative dendritic cells). Aliquots of $5x10^5$ cells released by enzymatic digestion of individual uteri from day 1 pregnant GM-CSF- replete (n = 4) and from day 1 pregnant GM-CSF-deficient (n = 3) mice were dual labeled to detect scavenger receptor and F4/80 using protocols 4 and 1 respectively (see Table 4.2). Data acquisition and analysis was performed as previously (see section 6.3.1). The F4/80-FITC versus scavenger receptor-RPE profiles of uterine cells were analysed to determine the effect of GM-CSF deficiency on scavenger receptor expression by F4/80$^+$ and F4/80$^-$ cells.

a. effect on F4/80$^+$ APCs expressing scavenger receptor

GM-CSF deficiency had no effect on the number of F4/80$^+$, scavenger receptor$^+$ cells as a proportion of total cells (p = 0.48, Figure 6.7A). Similarly, there was no effect of GM-CSF deficiency on the proportion of F4/80$^+$ cells which expressed scavenger receptor (p = 0.28, Table 6.4), or on the intensity of scavenger receptor expression by those cells (p = 0.48, Table 6.5).

b. effect on F4/80⁻ APCs expressing scavenger receptor

GM-CSF deficiency was associated with significant reduction in the number of F4/80⁻, scavenger receptor⁺ cells expressed as a proportion of total cells ($p = 0.03$, Figure 6.7C). The intensity of scavenger receptor expression on F4/80⁻, scavenger receptor⁺ cells tended to be lower in GM-CSF-deficient mice than in GM-CSF-replete mice, although this was not significant ($p = 0.08$, Table 6.6).

Table 6.5 The effect of GM-CSF deficiency on the intensity of activation marker expression by F4/80⁺ uterine cells. Cells derived by enzymatic digestion of uteri from day 1 pregnant GM-CSF-replete and GM-CSF-deficient mice were immunophenotyped by 2-colour fluorometric analysis. The intensity of expression of activation markers Ia, scavenger receptor and B7-2 on F4/80⁺, activation marker⁺ cells are given as median (range) in intensity units. Data were analysed by Mann Whitney Rank Sum Test, and p values are given.

	genotype		
	GM-CSF-replete	*GM-CSF-deficient*	*p* value
activation marker:			
Ia	631 (407-1025)	1258 (862-1334)	0.08
scavenger receptor	551 (422-958)	778 (562-836)	0.48
B7-2	404 (259-552)	588 (397-588)	0.16

6.3.3 Effect of GM-CSF deficiency on expression of B7-2 by uterine APCs

The expression of B7-2 by murine Mϕs and dendritic cells is thought to be positively regulated by GM-CSF (Larsen *et al.* 1994, Stumbles *et al.* 1998). However, the current immunohistochemical studies found no detectable depletion of B7-2+ cells in the uteri of GM-CSF-deficient mice. The sensitivity of detection of B7-2⁺ cells by immunohistochemistry appeared low relative to the detection of other APC markers which were expressed at much higher levels. By comparison with immunohistochemistry, flow cytometry is a more sensitive method by which to quantify cell surface markers. Therefore, a FACS experiment was designed to investigate the effect of GM-CSF deficiency on the expression of B7-2 by F4/80⁺ and F4/80⁻ uterine cells at day 1 of pregnancy. The experimental design was similar to that described in section 6.3.2, except in this case the uterine cells were dual labeled to detect B7-2 and F4/80. The F4/80-FITC versus B7-2-RPE profiles of uterine cells were analysed to determine the effect of GM-CSF deficiency on B7-2 expression by F4/80⁺ and F4/80⁻ cells.

a. effect on F4/80$^+$ cells expressing B7-2

GM-CSF deficiency had no significant effect on the number of F4/80$^+$, B7-2$^+$ cells as a proportion of total cells ($p = 0.29$, Figure 6.7B). There was a slight but non-significant negative effect of GM-CSF deficiency on the proportion of F4/80$^+$ cells which expressed B7-2 ($p = 0.11$, Table 6.4). The intensity of B7-2 expression was similar on F4/80$^+$ cells from GM-CSF-deficient mice compared to F4/80$^+$ cells on wild-type mice ($p = 0.15$, Table 6.5).

b. effect on F4/80$^-$ cells expressing B7-2

GM-CSF deficiency was associated with a small but non-significant reduction in the number of F4/80$^-$, B7-2$^+$ cells as a proportion of total cells ($p = 0.08$, Figure 6.7D). There was no effect of GM-CSF deficiency on the intensity of B7-2 expression on F4/80$^-$, B7-2$^+$ cells ($p = 0.2$, Table 6.6).

Table 6.6 The effect of GM-CSF deficiency on the intensity of activation marker expression by F4/80$^-$ uterine cells. Cells derived by enzymatic digestion of uteri from day 1 pregnant GM-CSF-replete and GM-CSF-deficient mice were immunophenotyped by 2-colour fluorometric analysis. The intensity of expression of activation markers Ia, scavenger receptor and B7-2 on F4/80$^-$, activation marker$^+$ cells are given as median (range) in intensity units. Data were analysed by Mann Whitney Rank Sum Test, and p values are given.

	genotype		
	GM-CSF-replete	*GM-CSF-deficient*	*p* value
activation marker:			
Ia	409 (328-906)	1001 (756-1035)	0.08
scavenger receptor	276 (227-380)	426 (365-559)	0.08
B7-2	279 (228-542)	431 (394-616)	0.20

6.4 Conclusions and discussion

The experiments presented in this chapter investigated the effect of GM-CSF deficiency on the number and phenotype of APCs in the murine uterus. In estrous, day 1 pregnant and day 4 pregnant GM-CSF-deficient and GM-CSF-replete mice, quantitative immunohistochemistry was employed to analyse the number of uterine APCs expressing (1) the Mϕ marker F4/80 antigen, (2) APC phenotypic markers scavenger receptor, macrosialin and sialoadhesin and (3) molecules of antigen presentation (Ia) and T cell co-stimulation (B7-2). By dual-colour FACS analysis, the

expression of scavenger receptor, Ia and B7-2 was then analysed on $F4/80^+$ and $F4/80^-$ uterine cells obtained from day 1 pregnant mice of each genotype.

The data show that the populations of APCs contained within the cycling and pregnant uterus are very similar despite GM-CSF-deficiency, with only subtle differences evident in the number and distribution of APCs expressing some phenotypic markers. An effect of GM-CSF deficiency on the numbers and activation phenotypes of $F4/80^+$ but particularly $F4/80^-$ APCs expressing Ia, scavenger receptor and B7-2 was detected. Thus it appears that GM-CSF has a role in regulating the recruitment and activation of differentiated APCs, particularly dendritic cells, within the uterus.

During the course of these studies, the data derived by FACs analysis of uterine cells was found to be highly variable, and in combination with the relatively small numbers of mice used in these experiments, it was difficult to detect significant differences between the mice of the two genotypes. Future experiments are designed at replicating these data with more mice.

The processes driving the derivation of interstitial Mϕ and dendritic cell populations are precisely regulated by local microenvironmental factors, most notably the receptor-specific actions of a diverse group of cytokines and chemokines (Steinman 1988, Rutherford *et al.* 1993). The factors which determine the nature of the APCs in mucosal tissues include the development and proliferation of progenitor cells in the bone marrow, the recruitment of precursor cells from the blood stream into mucosal organs, the differentiation of precursor cells into mature cell phenotypes and the activation and finally the trafficking of antigen-loaded APCs from inflamed organs to the lymph nodes and spleen. GM-CSF is just one of the many candidate molecules involved in these processes, and is relatively unique in that it can play a role in each one of the steps outlined above (Ruef and Coleman 1990, Baldwin 1992). *In vivo* and *in vitro* experiments show that GM-CSF can (1) drive the proliferation and maturation of myeloid progenitors and precursor cells (Socolovsky *et al.* 1998), (2) specifically recruit Mϕs and dendritic cells into peripheral organs (Kaplan *et al.* 1992, LeVine *et al.* 1999) and (3) activate precursors to differentiate into mature APCs (Heufler *et al.* 1988, Steinman 1988).

Despite these many proposed activities, the current and previous studies have shown that the effects of 'knockout' of the GM-CSF gene are surprisingly minor, since in the steady state normal numbers of myeloid cell precursors (Dranoff and Mulligan 1994, Stanley *et al.* 1994) and differentiated cells in peripheral organs (Vremec *et al.* 1997, Zhan *et al.* 1998, Zhan *et al.* 1999) are observed. Other molecules, including IL-3 and IL-5 (Dranoff and Mulligan 1994, Stanley *et al.* 1994, Burdach *et al.* 1998), must therefore fulfill roles that are similar or even indistinguishable from those of GM-CSF (Socolovsky *et al.* 1998). Whether such a comprehensive degree of compensation would be observed under conditions of more short-term

or localised depletion of GM-CSF is not known. In the lung, a direct role for local rather that systemic GM-CSF in regulating the phenotype of alveolar Mϕs is likely, given that the phenotypic abnormalities observed in GM-CSF-deficient mice can be corrected by aerosol delivery or promoter-induced local synthesis of GM-CSF (LeVine *et al.* 1999).

The key cytokines and chemokines involved in the recruitment and phenotypic regulation of Mϕs and dendritic cells within the murine reproductive tract remain only partially elucidated. It is recognised that during the estrous cycle, steroid hormone-driven synthesis of CSF-1 by uterine epithelial cells (Wood *et al.* 1992) is crucial for the maintenance of normal numbers and phenotypes of F4/80$^+$ Mϕs, since the cycling uteri of congenitally CSF-1-deficient *csfm^{op}/csfm^{op}* mice contain few phenotypically normal Mϕs despite having normal numbers of circulating monocytes (Pollard *et al.* 1991, Pollard *et al.* 1998). A comparable role for uterine epithelial cell-derived GM-CSF (Robertson *et al.* 1992, Robertson *et al.* 1996b) is refuted by the current and other studies (Robertson *et al.* 1998, Robertson *et al.* 1999) showing that uteri of GM-CSF-deficient mice contain similar numbers of F4/80$^+$ Mϕs within the endometrium, mesometrial triangle and myometrium as are observed in GM-CSF-replete mice.

Cytokines are also implicated in regulating the recruitment and differentiation of APCs in the uterus after insemination. Synthesis of GM-CSF by uterine epithelial cells has been shown to increase markedly upon exposure to TGFβ_1 and other components of seminal plasma (Robertson and Seamark 1990, Robertson *et al.* 1996, Tremellen *et al.* 1998), and the leukocytes present in the uterus at the time of insemination express both the α- and β-subunits of the GM-CSF receptor (Robertson *et al.* 2000). Despite this, as shown here and previously (Robertson *et al.* 1998, Robertson *et al.* 1999), the number of F4/80$^+$ cells present in the uterus of GM-CSF-deficient mice at day 1 is similar to the number found in wild-type mice. Thus it appears that GM-CSF is not absolutely essential for recruitment of F4/80$^+$ Mϕs to the inseminated uterus. Since uterine epithelial CSF-1 also appears not to be critical at day 1 of pregnancy for Mϕ recruitment (Robertson *et al.* 1998), chemotactic factors including RANTES, MIP-1α, MIP-1β, MCP-1 (Wood *et al.* 1997, Pollard *et al.* 1998, Robertson *et al.* 1998) or other inflammatory cytokines (McMaster *et al.* 1992, Sanford *et al.* 1992) must fulfill this role (see Figure 4.13).

Despite the presence of normal numbers of endometrial F4/80$^+$ Mϕs, APC expression of activation markers was diminished in GM-CSF-deficient mice. The number of Ia$^+$ cells was depleted at estrus, and scavenger receptor$^+$ cells were depleted at both estrus and day 1 of pregnancy. Such a result is consistent with *in vitro* experiments showing that the expression of both of these molecules can be upregulated on APCs by GM-CSF (Guidez *et al.* 1998, Stumbles *et al.* 1998). Expression of scavenger receptor is believed to be reflective of the capacity of

APCs to participate in tissue remodeling and scavenging of polyanionic ligands (de Villiers *et al.* 1994, Hughes *et al.* 1995), and Ia is one of several antigen presentation molecules expressed by APCs (Unanue 1984). Thus the data could be interpreted to suggest that GM-CSF plays a role within the uterus in driving the recruitment or differentiation of APCs with scavenger and antigen presenting roles. The extent to which this altered phenotype might translate into a functional deficit in scavenging or antigen-presenting function remains to be explored.

By immunohistochemistry it was not possible to ascertain which sub-populations of uterine APCs were affected by GM-CSF deficiency. However, flow cytometric analysis of cells obtained from uteri at day 1 of pregnancy showed that while scavenger receptor$^+$ cells expressing F4/80 were slightly diminished in the GM-CSF-deficient mice, the major deficit was in scavenger receptor$^+$ cells which did not express F4/80. Cells expressing B7-2 but not F4/80 were also diminished in the cytokine-deficient mice.

That the cells most affected by GM-CSF deficiency did not express F4/80 suggests that they are more likely to be dendritic cells than Mϕs. A role for GM-CSF in regulating the function of such uterine dendritic cells seems probable given the large body of literature describing the *in vitro* and *in vivo* regulation by GM-CSF of dendritic cell generation and differentiation into potent immunostimulatory cells (reviewed in Banchereau and Steinman 1998). Whether populations of dendritic cells are altered in phenotype or number in other peripheral and mucosal organs in GM-CSF-deficient mice has not been analysed. However, GM-CSF-deficiency does have a small effect on the numbers of dendritic cells found in lymphoid organs (Vremec *et al.* 1997) and furthermore these APCs tend to activate T cells in an abnormal manner (Wada *et al.* 1997, Noguchi *et al.* 1998).

There may also be a role for GM-CSF in ensuring normal trafficking of undifferentiated and mature APCs between uterine compartments. While the number of APCs expressing scavenger receptor was decreased in the endometrium of day 1 pregnant GM-CSF-deficient mice, in the same mice the number of scavenger receptor$^+$ APCs in the mesometrial triangle and myometrium were apparently normal, suggesting that perhaps their movement into the endometrium at the time of insemination may have been compromised in the absence of GM-CSF. Similarly, the numbers of APCs expressing scavenger receptor and Ia at estrus were differently affected in the endometrium, the mesometrial triangle and the myometrium. In contrast, Ia$^+$ APCs were depleted in all three uterine compartments at day 4, perhaps an indication that all of these cell populations were dependent on GM-CSF for recruitment, differentiation or viability.

Interestingly the formation of APC clusters within the deep endometrial tissue at day 4 of pregnancy did not appear to be compromised in the GM-CSF deficient mice. The significance of these aggregations of cells is not known.

In contrast to APCs expressing scavenger receptor, Ia and B7-2, there was no effect of GM-CSF deficiency on the number of uterine APCs expressing macrosialin or sialoadhesin at either estrus, day 1 or day 4. The regulation of sialoadhesin expression is still largely uncharacterised, but this molecule is known to be upregulated on bone marrow-derived Mϕs after exposure to supernatant from Con A-activated splenocytes (Damoiseaux *et al.* 1989) and an unidentified serum factor (Crocker *et al.* 1988) [the inducing factor is not CSF-1, IL-2 or IFNγ]. Macrosialin mRNA levels have been shown to be markedly upregulated during Mϕ differentiation of bone marrow progenitor cells in response to GM-CSF but other cytokines (most likely CSF-1) also appear to perform this role *in vivo* (Li *et al.* 1998). The current study also showed no effect of GM-CSF-deficiency on macrosialin expression by uterine epithelial cells: whether the expression of macrosialin by epithelial cells is cytokine-responsive to any extent is not known.

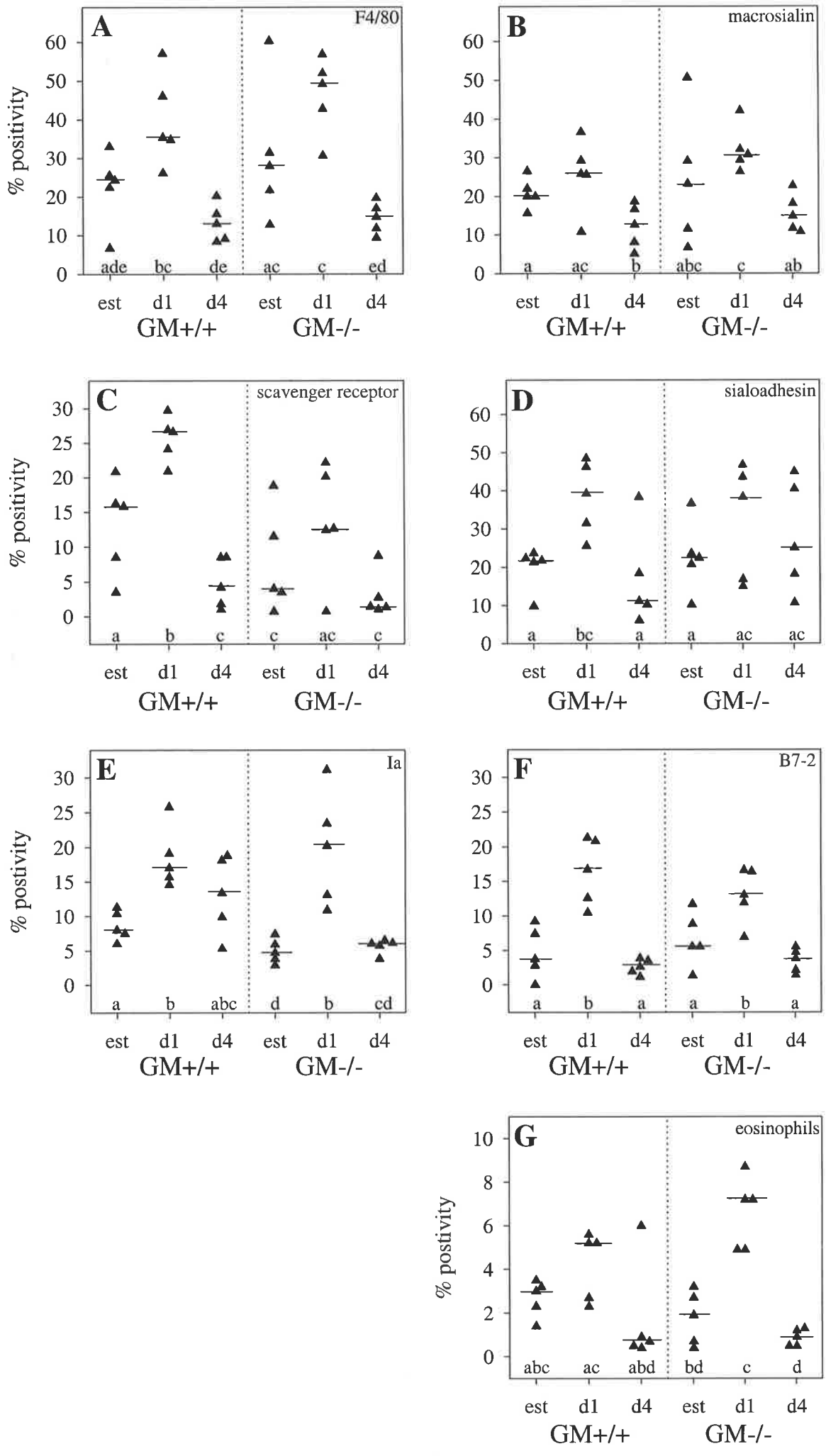
There are several potential consequences of dysregulated APC phenotypes within the inseminated uterus. Since the normally sterile uterine milieu is exposed to pathogens and paternal cells introduced with the ejaculate at mating, the generation of appropriate immune responses within the uterus at this time would be vital to ensure uterine homeostasis. Interestingly, an elevated rate of bacterial infections has been noted in the uteri of GM-CSF-deficient mice (Robertson *et al.* 1999), suggesting that perhaps GM-CSF is necessary to activate uterine APCs for effective anti-pathogen immunity in a manner similar to that which is believed to occur in the lung (LeVine *et al.* 1999) and peritoneal cavity (Zhan *et al.* 1998, Zhan *et al.* 1999).

There is evidence that immune responses initiated in the uterus at the time of insemination are involved in the generation of tolerogenic immune responses to paternal transplantation antigens which might facilitate implantation and placental development (Tafuri *et al.* 1995, Robertson *et al.* 1997). It is reasonable to speculate that in the uterus of day 1 pregnant GM-CSF-deficient mice, the abnormal phenotype of Mϕs and dendritic cells might interfere with the normal generation of such a response, and that this may contribute to the reproductive anomalies observed in pregnant GM-CSF-deficient mice (Robertson *et al.* 1999). Alternatively, since haemopoiesis in GM-CSF-deficient mice can be compromised in demanding situations (Zhan *et al.* 1998), it is possible that in the pregnant state the cytokine knockout mice may have difficulty maintaining the required numbers of Mϕs for scavenger and tissue remodeling roles

(Kyaw *et al.* 1998) or immunosuppression (Hunt *et al.* 1984) within the uterus. A study of uterine Mϕs in pregnant GM-CSF-deficient mice would be needed to test this hypothesis. Furthermore, since GM-CSF has been postulated to have a role in regulating the growth and function of GM-CSF receptor α-subunit$^+$ trophoblast cells (Jokhi *et al.* 1994a), a better determination of the role of GM-CSF in the various facets of murine reproduction could be achieved by a detailed analysis of the uterine APC phenotypes and reproductive outcome in mice deficient in the signal-transducing β-subunit of the GM-CSF receptor, the expression of which appears to be limited to haematopoietic cells (Scott *et al.* 1998).

In summary, the studies presented in this chapter show that APCs within the uteri of GM-CSF-deficient mice exhibit phenotypic aberrations comparable in nature to those described in other tissues. A role for GM-CSF in regulating the recruitment and activation of differentiated Mϕs, dendritic cells and their precursor cells within the uterus is consistent with a number of studies showing similar roles of this pleiotrophic cytokine in other mucosal and epithelial organs. Perturbations in the activities of uterine Mϕs appears to be most evident during the inflammatory response to insemination, and might have consequences for the generation of appropriate immune responses within the uterus at this time.

Figure 6.1 The effect of GM-CSF deficiency on the density of APCs in the endometrium. Fresh frozen sections of uteri collected from GM-CSF-replete (GM+/+) and GM-CSF-deficient (GM-/-) mice at estrus (est), day 1 of pregnancy (d1) and day 4 of pregnancy (d4) were labeled with an indirect immunoperoxidase technique using mAbs specific for F4/80 antigen (A), macrosialin (B), class A scavenger receptor (C), sialoadhesin (D), Ia (E) and B7-2 (F). Eosinophils were detected by virtue of their endogenous peroxidase activity (G). The density of peroxidase-labeled cells in the endometrium was quantified by video image analysis. Symbols represent the mean density (% positivity) of peroxidase-labeled cells in individual uteri, and median values for each group are scored. Data were compared by Kruskal-Wallis one-way ANOVA and Mann-Whitney Rank Sum Test. Groups with different lower case letters on the x axis are significantly different for the given mAb ($p < 0.05$).


A
F4/80
% positivity
ade bc de ac c ed
B
macrosialin
a ac b abc c ab
C
scavenger receptor
a b c c ac c
D
sialoadhesin
a bc a a ac ac
E
Ia
% postivity
a b abc d b cd
F
B7-2
a b a a b a
G
eosinophils
abc ac abd bd c d
est d1 d4 est d1 d4
GM+/+ GM-/-

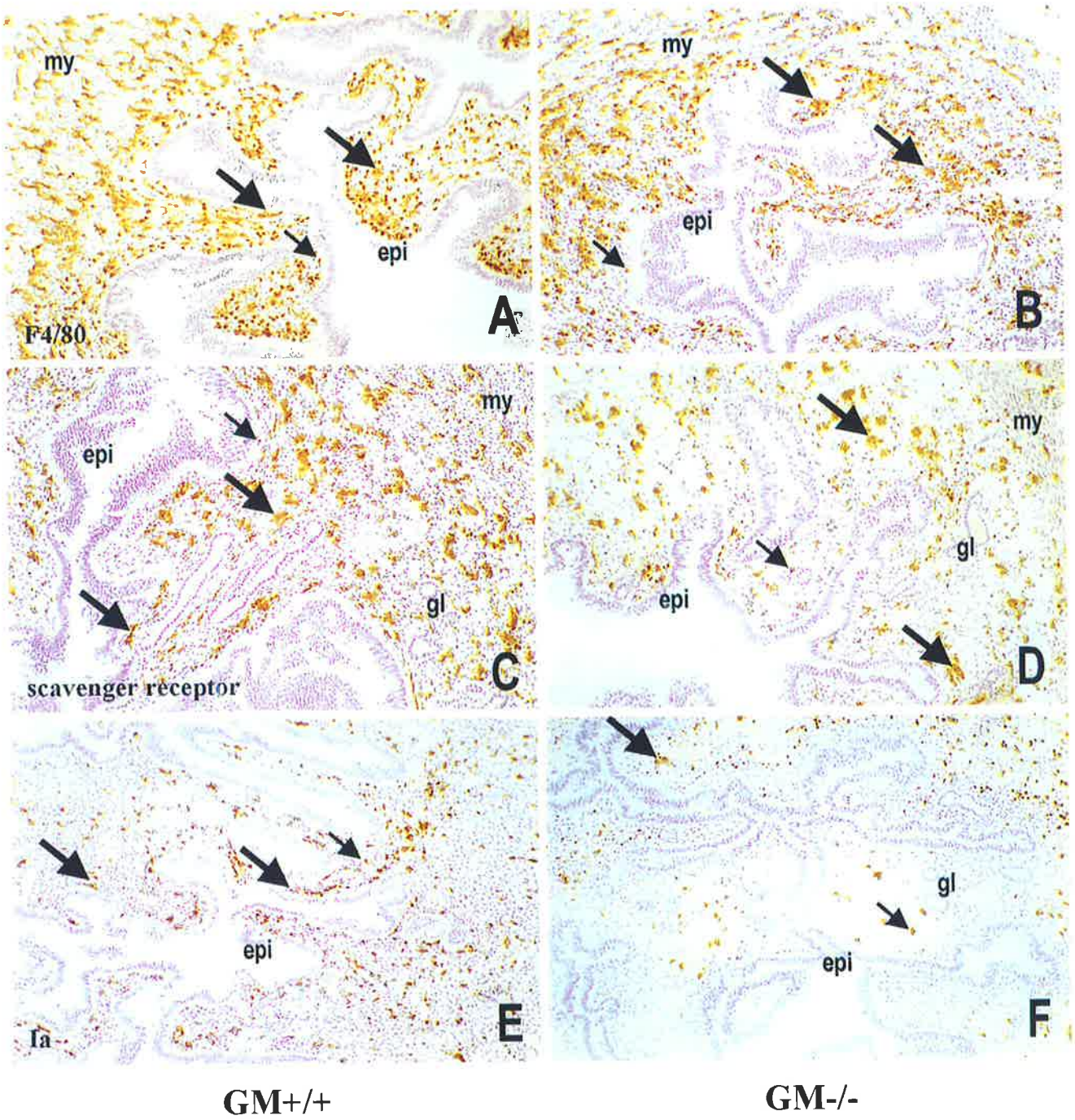


Figure 6.2 APCs in the uteri of GM-CSF-replete and GM-CSF-deficient mice at estrus. Fresh frozen sections of uteri collected from estrous GM-CSF-replete (GM+/+; A, C, E) and GM-CSF-deficient mice (GM-/-; B, D, F) mice were labeled with an indirect immunoperoxidase technique using mAbs specific for F4/80 antigen (A, B), class A scavenger receptor (C, D) and Ia (E, F). Immunoperoxidase-labeled antigen presenting cells are evident in the endometrium and myometrium (my) as large, dendriform cells (large arrows). Endogenous peroxidase$^+$ eosinophils are evident as small, dark brown cells predominantly in the endometrium (small arrows). epi = luminal epithelium, gl = glandular epithelium. x25 magnification.

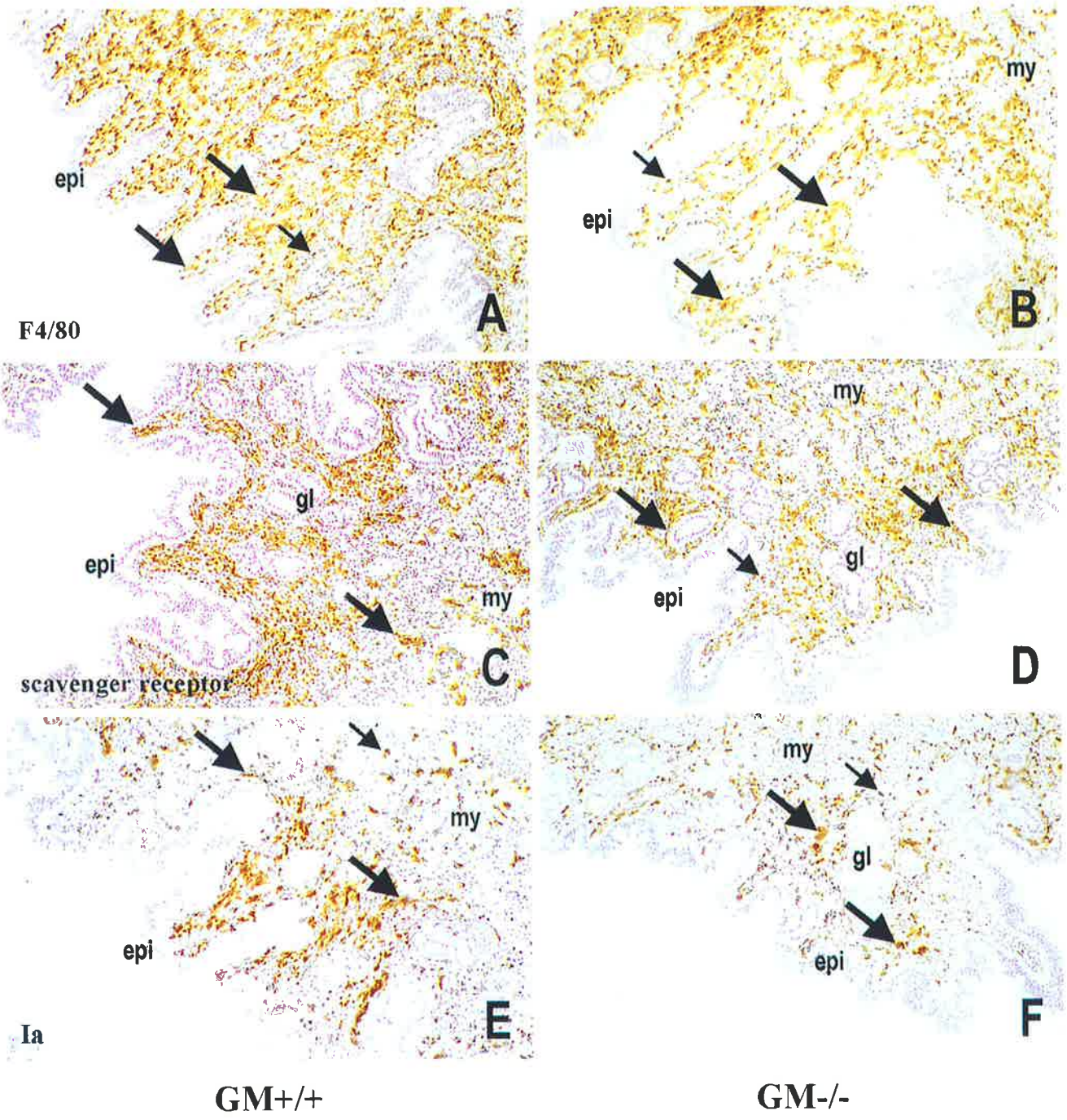


Figure 6.3 APCs in the uteri of GM-CSF-replete and GM-CSF-deficient mice at day 1 of pregnancy. Fresh frozen sections of uteri collected from day 1 pregnant GM-CSF-replete (GM+/+; A, C, E) and GM-CSF-deficient mice (GM-/-; B, D, F) mice were labeled with an indirect immunoperoxidase technique using mAbs specific for F4/80 antigen (A, B), class A scavenger receptor (C, D) and Ia (E, F). Immunoperoxidase-labeled antigen presenting cells are evident in the endometrium and myometrium (my) as large, dendriform cells (large arrows). Endogenous peroxidase$^+$ eosinophils are evident as small, dark brown cells predominantly in the endometrium (small arrows). epi = luminal epithelium, gl = glandular epithelium. x25 magnification.

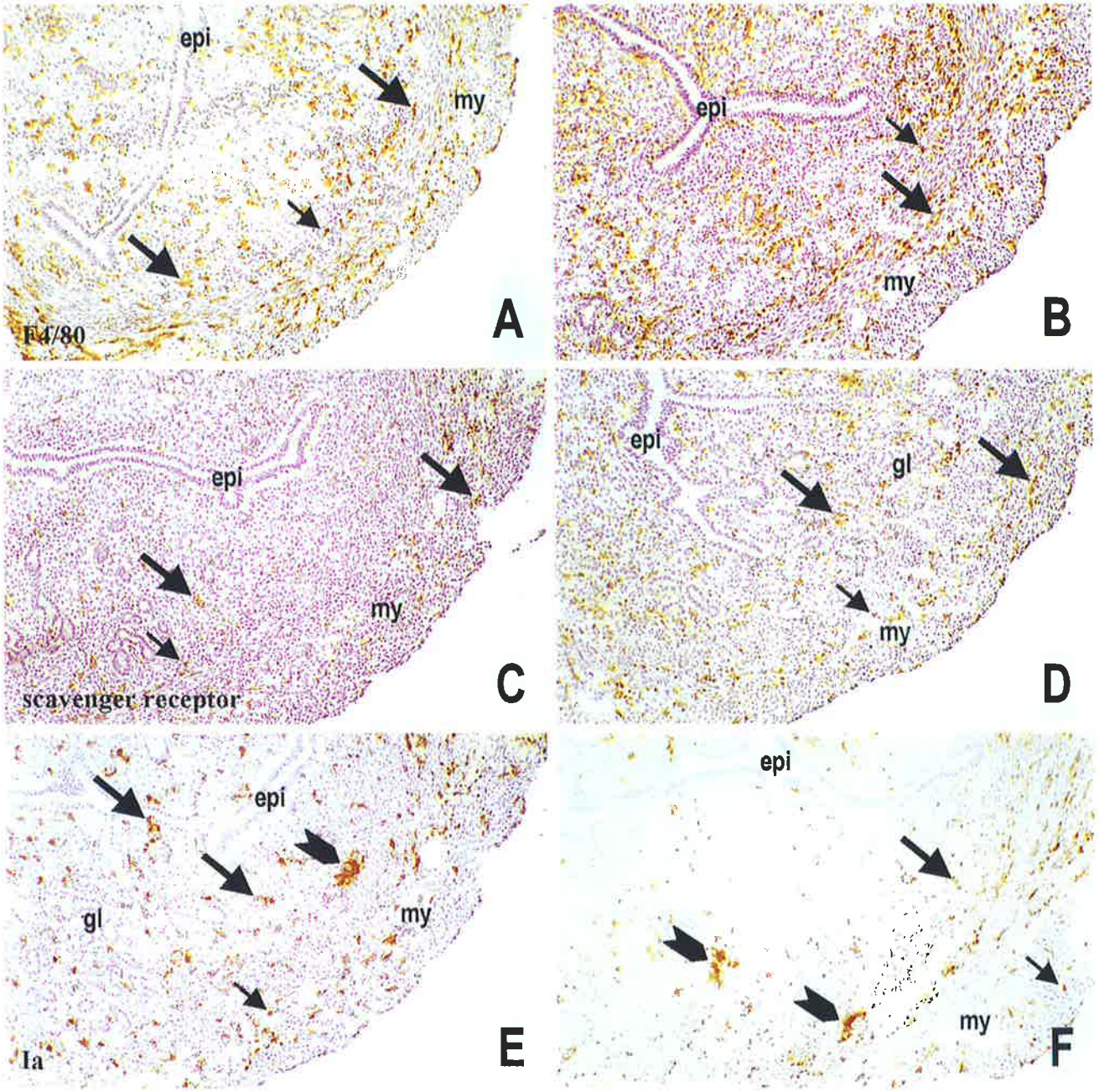


Figure 6.4 APCs in the uteri of GM-CSF-replete and GM-CSF-deficient mice at day 4 of pregnancy. Fresh frozen sections of uteri collected from day 4 pregnant GM-CSF-replete (GM+/+; A, C, E) and GM-CSF-deficient mice (GM-/-; B, D, F) mice were labeled with an indirect immunoperoxidase technique using mAbs specific for F4/80 antigen (A, B), class A scavenger receptor (C, D) and Ia (E, F). Immunoperoxidase-labeled antigen presenting cells are evident in the endometrium and myometrium (my) as rounded and dendriform cells of intermediate size (large arrows). Aggregations of Ia^+ antigen presenting cells are present in the deep endometrium in E and F (block arrow). Endogenous $peroxidase^+$ eosinophils are evident as small, dark brown cells in the deep endometrium and myometrium (small arrows). epi = luminal epithelium, gl = glandular epithelium. x25 magnification.

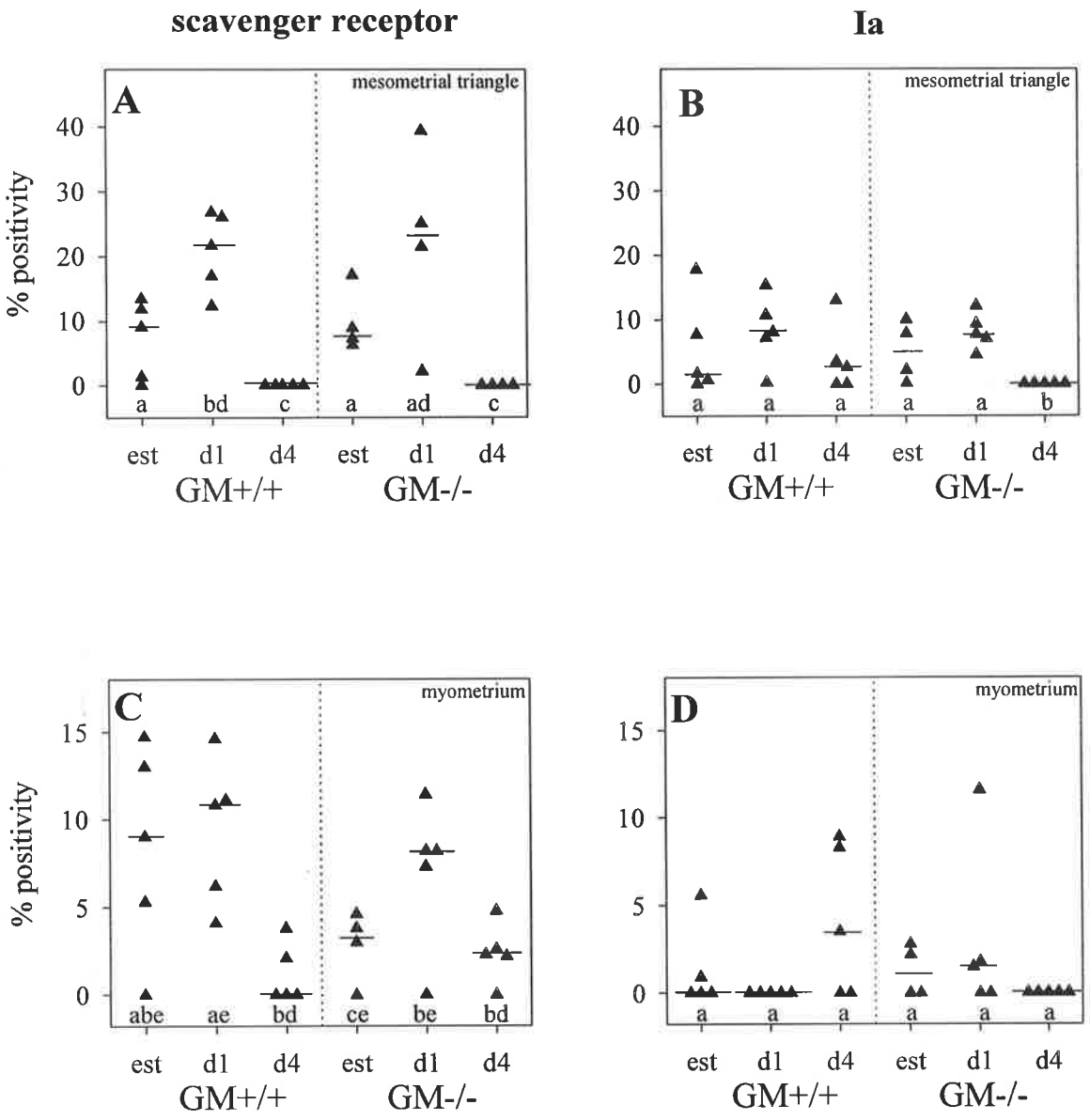


Figure 6.5 The effect of GM-CSF deficiency on the density of class A scavenger receptor$^+$ and Ia$^+$ APCs in the mesometrial triangle and myometrium. Fresh frozen sections of uteri collected from GM-CSF-replete (GM+/+) and GM-CSF-deficient (GM-/-) mice at estrus (est), day 1 of pregnancy (d1) and day 4 of pregnancy (d4) were labeled with an indirect immunoperoxidase technique using mAbs specific for class A scavenger receptor (A, C) and Ia (B, D). The density of peroxidase-labeled cells in the mesometrial triangle (A, B) and myometrium (C, D) was quantified by video image analysis. Symbols represent the mean density (% positivity) of immunoreactive cells in individual uteri, and median values for each group are scored. Data were compared by Kruskal-Wallis one-way ANOVA and Mann-Whitney Rank Sum Test. Groups with different lower case letters on the x axis are significantly different for the given mAb ($p < 0.05$).

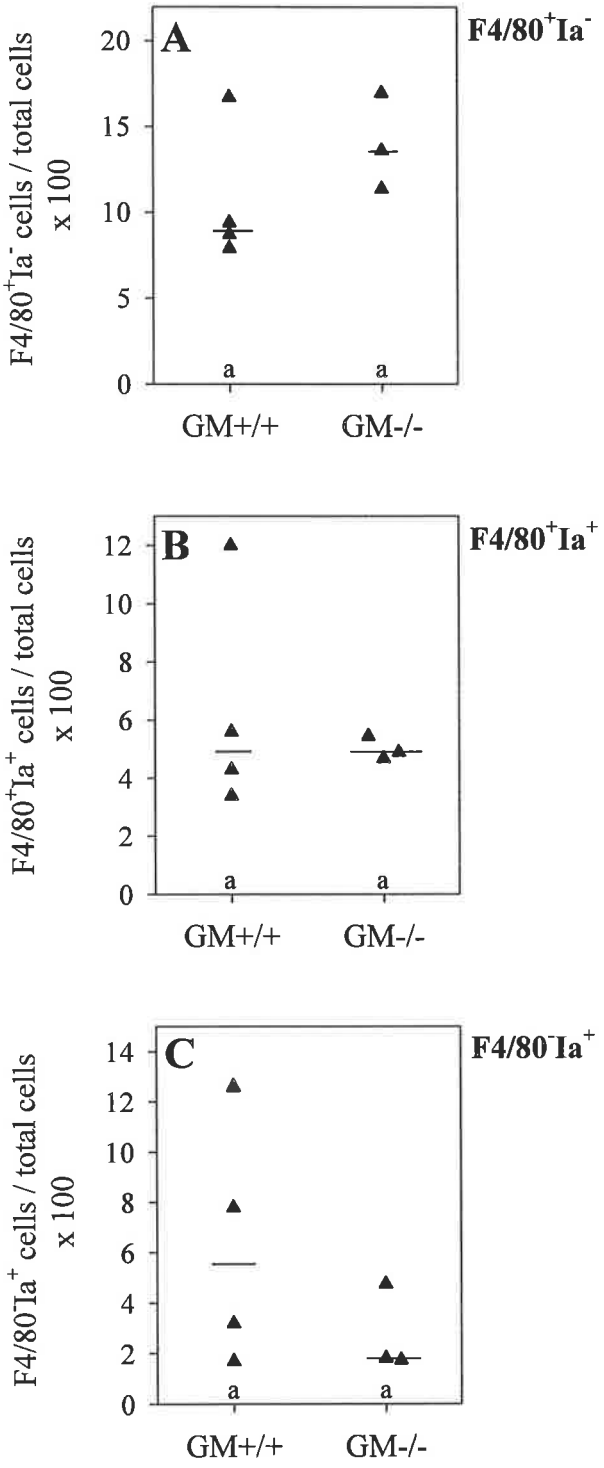


Figure 6.6 The effect of GM-CSF deficiency on the number of F4/80$^+$ and Ia$^+$ uterine APCs at day 1 of pregnancy. Cells derived by enzymatic digestion of uteri from day 1 pregnant GM-CSF-replete (GM+/+) and GM-CSF-deficient (GM-/-) mice were immunophenotyped by 2-colour flow cytometric analysis. Symbols represent the number of F4/80$^+$Ia$^-$ (A), F4/80$^+$Ia$^+$ (B) and F4/80$^-$ Ia$^+$ (C) antigen presenting cells as a % of total cells in individual uteri. Data were compared by Mann-Whitney Rank Sum Test, and groups with different lower case letters on the x axis are significantly different for the given mAb ($p < 0.05$).

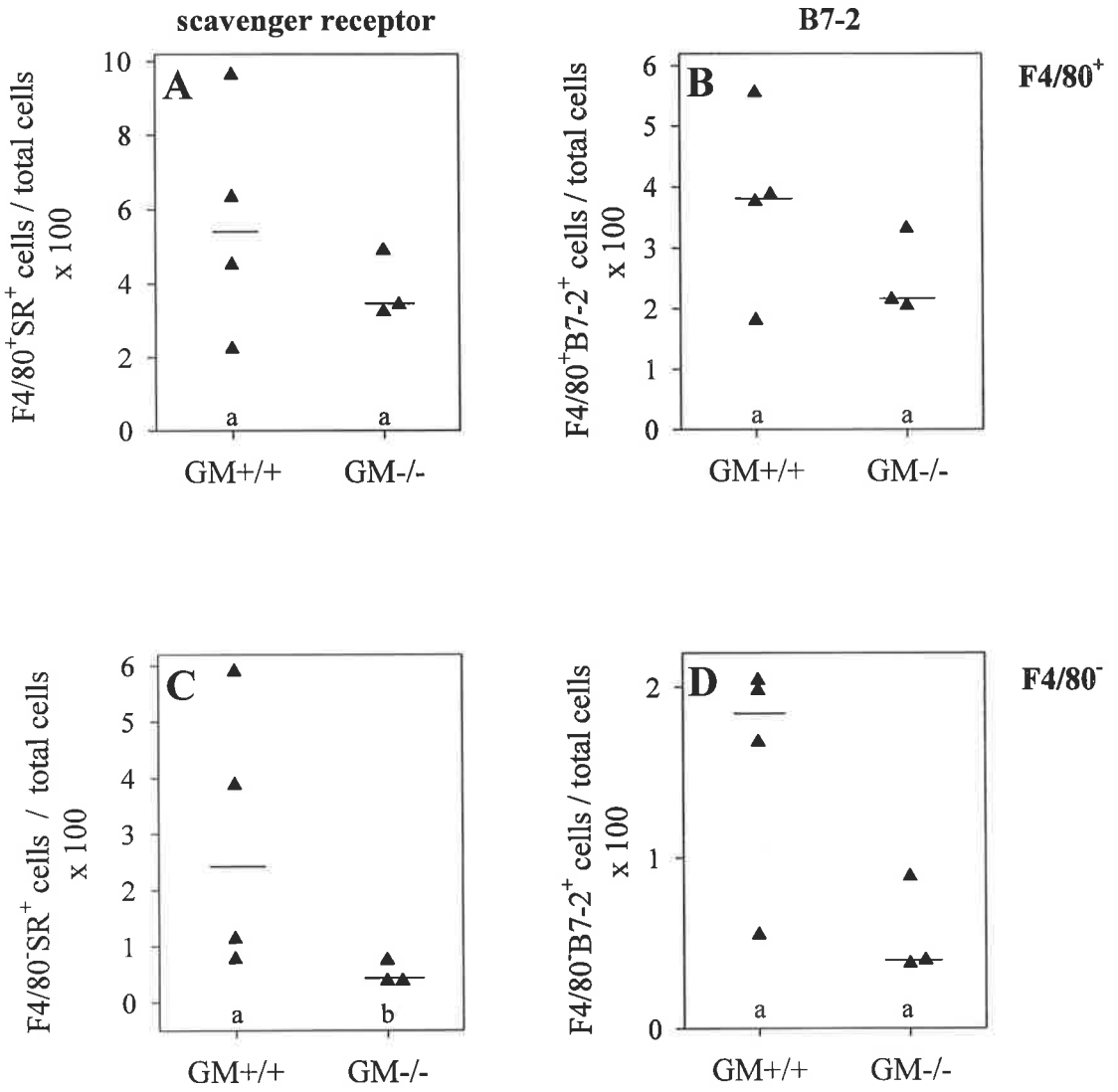


Figure 6.7 The effect of GM-CSF deficiency on the proportion of F4/80$^+$ and F4/80$^-$ uterine APCs expressing class A scavenger receptor and B7-2 at day 1 of pregnancy. Cells derived by enzymatic digestion of uteri from day 1 pregnant GM-CSF-replete (GM+/+) and GM-CSF-deficient (GM-/-) mice were immunophenotyped by 2-colour flow cytometric analysis. Symbols represent the proportion of F4/80$^+$ (A, B) and F4/80$^-$ (C, D) uterine antigen presenting cells expressing class A scavenger receptor (A, C) and B7-2 (B, D) in individual uteri. Data were compared by Mann-Whitney Rank Sum Test, and groups with different lower case letters on the x axis are significantly different ($p < 0.05$).

Chapter 7

The immunoaccessory phenotype of uterine macrophages

7.1 Introduction

The experiments already presented in this thesis have established that diverse populations of APCs exist in the cycling and early pregnant murine uterus, including undifferentiated and differentiated Mϕs and putative dendritic cells. Detailed analyses of interstitial and lymphoid organ dendritic cells have suggested that these cells are specialised for the generation of antigen-specific immunity (Banchereau and Steinman 1998). A similar role for dendritic cells within the uterus would appear likely given their cell membrane phenotype (described in Chapters 3-6). In contrast, the possible roles of Mϕs within the uterine milieu are much more diverse since analogous cells in other mucosal and epithelial organs show evidence of participation in many aspects of immune regulation. These may include phagocytosis, secretion of toxic and cytostatic molecules for innate immune protection, antigen processing and presentation for antigen-specific immunity, secretion of mediators with tissue remodeling activities and secretion of cytokines and other immune regulators for regulation and recruitment of other leukocytes (Nathan 1987, Gordon 1995, Gordon 1998).

Considerable information about the functional activities of Mϕs and dendritic cells can be acquired by *in vitro* studies. *Ex vivo* dendritic cells have been found to exhibit a range of behaviours indicative of their differentiation state at the time of collection. Thus, relatively 'immature' dendritic cells exhibit rapid fluid phase endocytosis (Sallusto *et al.* 1995, Stumbles *et al.* 1998) and highly efficient antigen processing (Streilein and Grammer 1989) consistent with their roles as cells specialised for the uptake and processing of antigens. Dendritic cells collected from inflamed tissues or lymphoid organs, or those analysed after *in vitro* culture in cytokines such as GM-CSF instead show a predilection to behaviours such as stimulation of lymphocyte proliferation (Stumbles *et al.* 1998, Holt *et al.* 1999) and activation of naïve T cells (Inaba *et al.* 1990, Maldonado Lopez *et al.* 1999).

In contrast to the potent immunostimulatory capacity of mature dendritic cells, Mϕs collected from rodent ocular, lung and gastrointestinal tissues function poorly as APCs and in most cases are inhibitory toward T cell activation (Holt 1979, Pavli *et al.* 1993, Steptoe *et al.* 1995) due to their constitutive production of inhibitory molecules including nitric oxide (Strickland *et al.* 1996). This action is thought be physiologically important in maintaining

homeostasis within the local tissue environment, an effect achieved by down-regulating the induction of immune responses to innocuous antigens in the steady state (Stumbles *et al.* 1999). It has been documented that Mϕs collected from the uteri of mice in the mid-late stages of gestation can inhibit the proliferation of lymphocytes *in vitro* (Hunt *et al.* 1984), but whether Mϕs present in the uteri of cycling mice or those in the early stages of pregnancy have a similar phenotype has not been explored.

The aim of the studies presented in this chapter was to characterise the functional phenotype of Mϕs purified from the uteri of mice during the estrous cycle and the early stages of pregnancy. The experiments begin with an investigation of the immunoaccessory phenotype of uterine Mϕs obtained from estrous mice. Subsequent experiments were designed to examine the effect of ovarian steroid hormones and days 1 and 4 of pregnancy on this phenotype. In addition, the molecular nature of an immunosuppressive activity associated with uterine Mϕs was investigated.

7.2 *In vitro* assessment of uterine Mϕ immunoaccessory phenotype

The previous experiments identified three populations of APCs in the cells released by enzymatic digestion of cycling and pregnant uteri (see Chapters 3 and 4). Cells designated 'Mϕs' were those of high intra-cellular density which expressed F4/80 (and a range of other membrane molecules known to be expressed on other populations of mucosal Mϕs) and exhibited adherent and phagocytic activity *in vitro*. Uterine dendritic cells were distinguished from uterine Mϕs on the basis of their lack of F4/80 expression, their Ia^+ cell membrane phenotype and their less complex internal structure. Therefore, in the following experiments, $F4/80^+$ cells purified from single cell suspensions of enzyme-digested uteri were designated 'uterine Mϕs'. In some experiments, uterine Mϕs were also purified from single cell suspensions on the basis of their expression of Fc receptor [FcR, the expression of which is common to most Mϕ populations (Gordon *et al.* 1995)].

For assessment of Mϕ immunoaccessory phenotype *in vitro*, a spleen cell mitogenesis assay was employed, in which uterine or peritoneal Mϕs were utilised as stimulator cells and mitogen-activated spleen cells were utilised as responder cells. Prior to their use in these assays, the spleen cells were purified by depletion of $F4/80^+$, Ia^+, adherent APCs (preparations referred to as 'purified spleen cells', see section 2.4.3c). In each mitogenesis assay, Mϕs were cultured in RPMI-FCS containing 10 μg/ml phytohaemaglutinin (PHA) with $5x10^3$-$5x10^5$ purified spleen cells for 72-96 h (including a 24 h pulse with ^{3}H-thymidine). The proliferation of responder cells

was assessed by measurement of the incorporation of ^{3}H-thymidine by liquid scintillation β-counting (see section 2.9).

In order to obtain uterine Mϕs for *in vitro* experiments, uteri were digested in collagenase and DNase (as described in section 2.4.2) and Mϕs were selected from the resulting single-cell suspensions on the basis of their expression of FcR by rosetting, or on the basis of their F4/80 expression by miniMACs purification (see section 2.6.3). Mϕ preparations were confirmed as >80% and >95% pure respectively based on their phagocytic activity *in vitro* and their F4/80 expression assessed by immunofluorescence (see section 4.2 and Table 4.1). Peritoneal Mϕs were collected from resident or elicited peritoneal exudates by adherence, or by rosetting or miniMACS purification on the basis of FcR- or F4/80-expression respectively.

7.2.1 Immunoaccessory phenotype of uterine Mϕs at estrus

In order to examine the immunoaccessory phenotype of uterine Mϕs at the estrous phase of the cycle, mice were sacrificed at estrus after diagnosis of cycle stage by vaginal smear cytology (see section 2.1). In two separate experiments, FcR^{+} and $F4/80^{+}$ Mϕs were collected from single cell suspensions released from those uteri and assessed for their effect on spleen cell mitogenesis. The immunoaccessory phenotype of adherent peritoneal Mϕs collected from the same mice was also assessed.

In the absence of exogenous Mϕs, purified spleen cells exhibited baseline proliferation associated with ^{3}H-thymidine incorporation in the range of 10^{3}-10^{4} disintegrations per minute (DPM), depending on the initial number of cells and the total time in culture. Elicited peritoneal Mϕs purified by adherence and added at concentrations of 1:1000 to 1:100 (concentrations henceforth expressed as ratio of peritoneal or uterine Mϕs : spleen cells) stimulated their proliferation in a dose dependent manner. At a concentration of 1:100, proliferation induced by elicited peritoneal Mϕs was 5-fold greater than baseline (Figure 7.1A). Similar results were obtained in > 5 further experiments (data not shown).

Resident peritoneal Mϕs also stimulated spleen cell proliferation, although at high concentrations the net stimulatory effect of resident peritoneal Mϕs began to diminish in a dose-dependent manner until at ratios approximating 1:2, proliferation was inhibited to 50% of baseline (Figure 7.1B). Similar results were obtained in 2 similar experiments (another example provided in Figure 7.6A).

In contrast to the effect of peritoneal Mϕs, at all concentrations examined except 1:40, uterine Mϕs collected from the uteri of estrous mice inhibited spleen cell proliferation. However,

this effect was not dose-dependent in these experiments since across all concentrations (1:1000-1:2), proliferation remained quite steady at 75-80% of baseline. Inhibition was observed regardless of whether the uterine Mϕs were collected on the basis of F4/80- or FcR-expression (Figure 7.1A and B).

In order to confirm that the rosetting and immunomagnetic selection procedures were not responsible for the induction of an inhibitory immunoaccessory phenotype in uterine Mϕs, in two additional experiments peritoneal Mϕs were prepared using each of these two selection methods. Peritoneal Mϕs selected using either of the miniMACs or the rosetting procedures did not exhibit an immunoinhibitory phenotype: indeed they were up to 6-fold more stimulatory towards spleen cell proliferation (Figure 7.1C and D). The proliferation of uterine or peritoneal Mϕs alone contributed less than 2% of baseline in all experiments (not shown).

For all subsequent experiments, uterine Mϕs were collected from single cell suspensions of enzyme-digested uteri by miniMACs purification on the basis of their F4/80 expression.

7.2.2 Effect of ovarian steroid hormones on the immunoaccessory phenotype of uterine Mϕs

During the murine estrous cycle, the steroid hormones estrogen and progesterone are released into the bloodstream in a cyclical manner in accordance with the continual development and demise of follicles within the ovary. The hormones have a number of actions on non-reproductive and reproductive target tissues. In the uterus, luminal and glandular epithelial cells respond to estrogen and progesterone by synthesising various cytokines, many of which are known to influence the phenotype of Mϕs in other mucosal tissues and *in vitro*, most notably CSF-1 (Wood *et al.* 1992) and GM-CSF (Robertson *et al.* 1996b). Other cytokines produced by uterine epithelial cells in response to ovarian steroid hormones include IFNγ (Platt and Hunt 1998), TNFα, IL-1 and IL-6 (De *et al.* 1992, Robertson *et al.* 1992, Sanford *et al.* 1992). As a result, the cytokine milieu within the uterus is continuously fluctuating, with GM-CSF, IL-6 and IFNγ appearing to be the most abundant cytokines present in the uterus at estrus, whereas at diestrus CSF-1 dominates.

The previous studies have established that the number and phenotype of Mϕs present within the uterine stroma is also continually fluxing in parallel with the changes in the steroid hormone environment. Such changes could be due to a direct action of the steroid hormones on the Mϕs themselves (Hunt *et al.*, 1998), but would likely be primarily a result of the prevailing cytokine milieu within the uterus (Balkwill 1997, Hunt and Robertson, 1996).

To determine the effect of removal of ovary-derived steroid hormones on uterine Mϕ immunoaccessory phenotype, F4/80$^+$ uterine Mϕs were collected by immunomagnetic selection from mice which had been ovariectomised three weeks earlier (see section 2.1). For comparison, Mϕs were also collected from the uteri of estrous mice in the same experiment.

Compared to the numbers of F4/80$^+$ cells released from the uteri of cycling or pregnant mice (see Table 5.2), the yield of F4/80$^+$ Mϕs from the uteri of ovariectomised mice was 10-30-fold lower since F4/80$^+$ cells are severely diminished following ovariectomy (see Chapters 3 and 4). Indeed, >20 ovariectomised mice were routinely sacrificed in order to obtain enough uterine Mϕs for immunoaccessory assays, in contrast to estrous mice, where 5-8 uteri were sufficient. Consequently, fewer stimulator cells were employed when the effect of ovariectomy on Mϕ immunoaccessory phenotype was investigated.

As previously, at concentrations of 1:1000-1:20, uterine Mϕs from estrous mice inhibited spleen cell proliferation to 52% of baseline. In contrast, uterine Mϕs collected from ovariectomised mice stimulated spleen cell proliferation in a roughly dose-dependent manner. At the highest concentration (1:20), spleen cell proliferation was stimulated 1.6-fold above baseline (Figure 7.2A).

To determine the effect of cyclic variation in ovarian steroid hormones and to confirm the effect of ovariectomy on uterine Mϕ immunoaccessory phenotype, a second experiment examined the immunoaccessory phenotype of uterine Mϕs collected from estrous, diestrous and ovariectomised mice. As expected, at a concentration of 1:10, uterine Mϕs from estrous mice were inhibitory, and uterine Mϕs from ovariectomised mice strongly stimulated proliferation by over 20-fold. At the same concentration, uterine Mϕs from diestrous mice stimulated spleen cell proliferation 6.5-fold above baseline (Figure 7.2B). At the same ratios of stimulator : responder cells, the immunoaccessory phenotype of peritoneal Mϕs was not sensitive to stage of estrous cycle or ovariectomy, although differences did become apparent at higher concentrations of cells (Figure 7.2B).

7.2.3 Effect of mating and day 4 of pregnancy on the immunoaccessory phenotype of uterine Mϕs

The previous experiments established that dynamic alterations in the recruitment and activation of Mϕs and dendritic cells accompany insemination and early pregnancy. The patterns of expression of phenotypic markers on the uterine Mϕ cell surface suggest that the phenotype of these cells is also sensitive to microenvironmental factors fluctuating over early pregnancy. To examine potential differences between uterine Mϕ immunoaccessory phenotype at estrus and on

days 1 and 4 of pregnancy, F4/80$^+$ uterine Mϕs were collected by miniMACs purification from the uterine cells of estrous, day 1 pregnant and day 4 pregnant mice and examined for their effect on spleen cell proliferation in two separate experiments. The collection of uteri from pregnant mice is described in section 2.1.

In the first experiment, uterine Mϕs collected from estrous mice and added at concentrations of 1:2500-1:20 had a dose-dependent inhibitory effect on spleen cell proliferation. At the highest concentration (1:20), they inhibited proliferation to less than 35% of baseline (Figure 7.3A). In the same assay, peritoneal Mϕs stimulated and then inhibited spleen cell proliferation in a dose-dependent manner at concentrations greater than 1:160 (Figure 7.6A).

Although uterine Mϕs collected from day 1 pregnant mice were as inhibitory as uterine Mϕs from estrous mice at high concentrations, their inhibitory effect titrated out much faster so that at a concentration of 1:40 they only inhibited proliferation to 80% of baseline. Uterine Mϕs collected from day 4 pregnant mice were very potent inhibitors of spleen cell proliferation, and even at very low concentrations (<1:1000) they exerted considerably greater inhibition than cells from estrous and day 1 pregnant mice, inhibiting baseline proliferation by 50% at 1:2500 (Figure 7.3A).

In the second experiment investigating the effect of day of pregnancy on uterine Mϕ immunoaccessory phenotype, uterine Mϕs from estrous and day 4 pregnant mice inhibited the proliferation of these purified spleen cells to 80% and 69% of baseline respectively at a concentration of 1:20. In contrast, uterine Mϕs from day 1 pregnant uteri stimulated proliferation almost 2-fold (Figure 7.3B). The effect of day of pregnancy on the immunoaccessory phenotype of peritoneal Mϕs was not examined in these experiments.

7.3 Molecular nature of the immunosuppressive activity of uterine Mϕs

The experiments described above clearly demonstrate that uterine Mϕs can inhibit mitogen-induced proliferation of spleen cells and that suppressive activity fluctuates depending on the stage of the estrous cycle or pregnancy. This suppressive activity may be due to a number of possible mechanisms, acting individually or in combination. It could be the result of a failure of uterine cells to produce sufficient soluble immunostimulatory molecules, or alternatively be attributed to (1) active suppression of the immunostimulatory activities of residual spleen APCs, or (2) inhibition of the proliferation of splenic lymphocytes. A suppressive effect of uterine Mϕs could result from uterine Mϕ secretion of soluble inhibitory molecules such as prostaglandin (Boraschi *et al.* 1984, Stevens *et al.* 1989) or nitric oxide (Ding *et al.* 1988, Stuehr and Nathan

1989), or inhibitory cytokines such as IL-10 (Wilkes *et al.* 1995), TGFβ (Letterio and Roberts 1998) or an IL-1 inhibitor (Arend 1993). Alternatively, direct inhibition of lymphocyte proliferation could be mediated by Mϕ delivery of a cytotoxic signal such as Fas ligand (Badley *et al.* 1996), or by starvation through Mϕ catabolism of tryptophan or other essential nutrients (Munn *et al.* 1999).

In order to determine the nature of the immunoinhibitory effect of uterine Mϕs on spleen cell proliferation, a two-tiered approach was applied. Firstly, co-culture and supernatant transfer experiments were employed to investigate whether the immunoinhibitory phenotype of uterine Mϕs could be attributed to the accumulation of a soluble inhibitory molecule within the culture supernatant. Secondly, the identity of an inhibitory molecule synthesised by uterine Mϕs was examined by the addition of specific antagonists of candidate inhibitory molecules to assays of immunoaccessory function. As previously, uterine and peritoneal Mϕs were obtained from uterine digests and peritoneal washouts by miniMACs purification and adherence respectively and were assessed for their immunoaccessory function by analysis of their effect on spleen cell mitogenesis.

7.3.1 Ability of uterine Mϕs to inhibit peritoneal Mϕ-induced spleen cell mitogenesis

The previous experiments showed that culture with peritoneal Mϕs caused stimulation of the proliferation of purified spleen cells. To determine whether uterine Mϕs can inhibit peritoneal Mϕ-induced spleen cell mitogenesis, peritoneal Mϕs were cultured with 10^5 purified spleen cells / well and various concentrations of uterine Mϕs. When no uterine Mϕs were added, 10^5 elicited peritoneal Mϕs stimulated the proliferation of spleen cells to levels 6-fold higher than baseline. In the presence of uterine Mϕs, a dose dependent inhibition of the stimulatory effect of peritoneal Mϕs was observed as the concentration of uterine Mϕs was increased. At a ratio of 1:10 uterine Mϕs:peritoneal Mϕs, proliferation was reduced to only 2.2-fold above baseline and significant inhibition was observed at ratios as low as 1:100 uterine Mϕs:peritoneal Mϕs (Figure 7.4A). The addition of similar numbers of peritoneal Mϕs stimulated purified spleen cells to proliferate 10-fold above baseline (not shown).

7.3.2 Ability of uterine Mϕs to inhibit spleen cell mitogenesis across a permeable membrane

To determine whether the immunoinhibitory effect of uterine Mϕs on spleen cell proliferation could be mediated across a permeable membrane whereby there was no physical contact between stimulator and responder cells, $5x10^5$ purified spleen cells were cultured in 1

millilitre wells with 10^5 uterine Mϕs, which were present within the same well or contained within a permeable 12 mm well insert (pore size 1.4 μm). As previously, at a concentration of 1:5, uterine Mϕs inhibited the proliferation of purified spleen cells when the two cell populations were contained within the same well (proliferation was inhibited to 20% of baseline). In contrast, when the uterine Mϕs were separated from the spleen cells, proliferation was inhibited to only 86% of baseline. The same concentration of peritoneal Mϕs stimulated spleen cell proliferation by 2.7-fold and 1.9-fold above baseline when contained within the same well or in a permeable insert respectively (Figure 7.4B).

7.3.3 Ability of supernatant from uterine Mϕ cultures to inhibit spleen cell mitogenesis

The failure of membrane-separated uterine Mϕs to inhibit spleen cell proliferation to the same extent as cultures in which the stimulator and responder cells were in physical contact may reflect several features of the Mϕ-spleen cell interaction. It may be that inhibition requires direct physical contact between the purified spleen cells and the uterine Mϕs. Alternatively, it may indicate that the uterine Mϕs and spleen cells needed to be in close proximity in order to deliver biological signals at adequate concentrations to be effective. To determine whether uterine Mϕs cultured in physical contact with purified spleen cells release a soluble inhibitory factor, supernatants collected from mitogenesis assays containing co-cultured purified spleen cells and uterine Mϕs were added to fresh cultures of purified spleen cells in RPMI-FCS with PHA.

Purified spleen cells in RPMI-FCS with PHA and no additional Mϕs or supernatant proliferated at expected levels, and this was stimulated 2-fold by addition of supernatant from rapidly proliferating spleen cell cultures. Supernatant which was collected from uterine Mϕ / spleen cell mitogenesis assays, diluted 1:2 and then added to purified spleen cells in RPMI-FCS with PHA inhibited baseline proliferation to 53%. The inhibitory effect was dose-dependent, with decreasing inhibition observed as the supernatant was titrated, with an effect on proliferation observed to a dilution of 1:8 (Figure 7.4C).

7.3.4 Synthesis of prostaglandin and nitric oxide by uterine Mϕs

The experiments described in sections 7.3.1-7.3.3 suggested that uterine Mϕs release a soluble inhibitory molecule. Two candidate molecules which might have mediate this inhibition are prostaglandin E_2 (PGE_2) and nitric oxide (NO).

PGE_2 is synthesised by Mϕ populations in many tissues, where it has diverse endocrine, paracrine and autocrine actions. PGE_2 is a metabolite of arachadonic acid and its synthesis

depends on the actions of the enzyme cyclooxygenase. Indomethacin is a specific inhibitor of cyclooxygenase (Stevens *et al.* 1989).

NO is a small molecule produced in Mϕs by an inducible form of the enzyme nitric oxide synthase (iNOS) (Collier and Vallance 1989, Nathan 1992) which in response to IFNγ and other pro-inflammatory cytokines (Ding *et al.* 1988, Drapier *et al.* 1988) rapidly synthesises large amounts of NO by metabolism of arginine. The released NO has cytostatic effects on a number of different cell lineages, including bacteria, tumour cells and proliferating lymphocytes (Nathan 1992) and then decomposes quickly to form nitrite and nitrate (Collier and Vallance 1989). Stereo-specific blockade of NO synthesis by Mϕ iNOS can be accomplished with an arginine analogue such as N^G-monomethyl-L-arginine (L-NMA) (Collier and Vallance 1989).

a. effect of indomethacin on uterine Mϕ immunoaccessory phenotype

In order to determine whether PGE_2 might contribute to the inhibitory activity released by uterine Mϕs, the effect of indomethacin on the immunoaccessory phenotype of uterine Mϕs was examined. When purified spleen cells were cultured with peritoneal or uterine Mϕs (from estrous mice) at concentrations of 1:330-1:25, peritoneal Mϕs stimulated spleen cell proliferation in a dose dependent manner (at 1:25, the peritoneal Mϕs stimulated spleen cell proliferation by over 2-fold above baseline). Inclusion of 5 μg/ml indomethacin to the culture media increased the stimulatory capacity of peritoneal Mϕs at all concentrations such that they stimulated spleen cell proliferation nearly 5-fold above baseline at a concentration of 1:25 (Figure 7.5A).

As expected, in this experiment uterine Mϕs inhibited the proliferation of purified spleen cells at all concentrations analysed: at 1:25, proliferation was inhibited to 53% of baseline. Addition of indomethacin at 5μg/ml had no effect on this inhibition at any of the Mϕ concentrations examined (Figure 7.5B). Similar results were obtained in 2 further experiments (not shown).

b. effect of L-NMA and IFNγ on uterine Mϕ immunoaccessory phenotype and synthesis of NO

In order to determine whether NO contributed to the inhibitory activity released by uterine Mϕs, the effect of L-NMA on spleen cell mitogenesis and accumulation of nitrate and nitrite in mitogenesis assay supernatants was examined. To further examine the potential for NO synthesis by uterine Mϕs, the effect of *in vitro* culture in IFNγ on Mϕ immunoaccessory function

and nitrate/nitrite accumulation was also assessed since this cytokine has been reported to specifically activate the iNOS gene in Mϕs (Ding *et al.* 1988).

Resident peritoneal Mϕs at concentrations of approximately 1:2500 – 1:160 stimulated spleen cell proliferation and synthesised small quantities of NO (< 10 μM nitrite was detected in each well). As previously observed with resident peritoneal Mϕs (see section 7.2.1), as the concentration of these cells increased from 1:160-1:2, their effect on spleen cell proliferation became inhibitory in a dose-dependent manner, and this was accompanied by a dose-dependent accumulation of nitrate/nitrite in the culture supernatant. Peritoneal Mϕs at the highest concentration (1:2) inhibited proliferation to 23% of baseline and synthesised over 6-fold more nitrate/nitrite than did purified spleen cells alone (Figure 7.6A). In a separate experiment in which peritoneal Mϕs were cultured with purified spleen cells at a concentration of 1:5, the baseline and IFNγ-stimulated accumulation of nitrate/nitrite was nearly completely inhibited by L-NMA but not by the D-enantiomer of this enzyme (D-NMA) (Figure 7.6B).

Uterine Mϕs collected from estrous mice and added to mitogenesis assays at concentrations of 1:2500-1:20 inhibited spleen cell proliferation in a dose-dependent manner such that at the highest dose proliferation was inhibited to 35% of baseline. However, the accumulation of nitrate/nitrite in the media of these wells did not increase above baseline levels at any of the uterine Mϕ concentrations examined (Figure 7.6C). In accordance with this, an additional experiment showed that L-NMA had no effect on the immunoaccessory phenotype of uterine Mϕs (Figure 7.6D). Furthermore, additional experiments showed that culture of uterine Mϕs in IFNγ had no effect on their immunoaccessory phenotype (Figure 7.6E) nor on the accumulation of NO breakdown products in the culture media (Figure 7.6F).

Furthermore, there was no accumulation of nitrate/nitrite above baseline levels in supernatants of spleen cells cultured with uterine Mϕs from day 1 pregnant or day 4 pregnant mice (proliferation curves shown in Figure 7.3A, nitrite curves shown in 7.6C).

7.4 Modulation of the immunoaccessory phenotype of uterine Mϕs by ovarian steroid hormones and cytokines

The experiments described in section 7.2 illustrated that the immunoaccessory phenotype of uterine Mϕs was very sensitive to the stage of early pregnancy and the fluxes in ovarian steroid hormone levels encountered during the estrous cycle or after ovariectomy. An effect of ovarian steroid hormones was not seen on peritoneal Mϕs, suggesting that modulation of Mϕ phenotype is specific to uterine cells and hence is likely to occur at a local rather than a systemic level. The

induction of an inhibitory phenotype in uterine Mϕs might result from a direct effect of estrogen and progesterone on the Mϕs, and/or be due to the steroid hormone-induced fluctuations in concentrations of cytokines encountered in the uterine milieu during the estrous cycle and in early pregnancy. In particular, the dramatic changes in concentrations of CSF-1 and GM-CSF in the cycling and mated uterus could specifically induce changes in the phenotype of uterine Mϕs in a manner similar to that which has been observed for other populations of mucosal Mϕs (Ruef and Coleman 1990, Balkwill 1997). The following experiments were designed in order to determine whether administration of ovarian steroid hormones to ovariectomised mice could induce the acquisition of an inhibitory phenotype in uterine Mϕs, and whether the inhibitory phenotype of uterine Mϕs collected from estrous mice could be altered by *in vitro* culture in CSF-1 or GM-CSF.

7.4.1 Effect of estrogen and progesterone treatment of ovariectomised mice on uterine Mϕ immunoaccessory phenotype

To investigate whether estrogen and progesterone are active in inducing an immunoinhibitory phenotype in uterine Mϕs, an experiment was designed to examine the effects of administering exogenous 17β-estradiol and progesterone on the ability of uterine Mϕs to inhibit spleen cell mitogenesis. A hormone replacement protocol was utilised that mimics the pre- and peri-implantation period of pregnancy. This protocol has been shown previously to allow implantation and decidualisation to occur (Finn and Martin 1969, Finn and Martin 1972) and to induce uterine epithelial cell CSF-1 synthesis at physiological levels (Pollard *et al.* 1987). In two separate experiments, uterine Mϕs were collected from ovariectomised and steroid hormone-treated mice (see section 2.2.5) and assessed for their immunoaccessory function in a spleen cell mitogenesis assay.

The biological efficacy of the steroid hormone treatments was confirmed by analysis of uterine weights. Uteri from mice treated with progesterone were significantly heavier than those treated with vehicle ($p = 0.02$). Treatment of ovariectomised mice with 17β-estrodiol +/- progesterone had a further significant effect on uterine weight ($p < 0.001$ compared to vehicle-treated or progesterone-treated mice, Figure 7.7A).

As was observed in previous experiments (see section 7.2.3), at concentrations of 1:900-1:80, uterine Mϕs from ovariectomised, vehicle-treated mice stimulated spleen cell proliferation up to 2-fold above baseline. Similar effects on baseline spleen cell proliferation were observed at the same concentrations of uterine Mϕs obtained from steroid hormone-treated mice, regardless of treatment with 17β-estrodiol alone, progesterone alone or the two in combination (Figure 7.7

B-D). The stimulatory effect of uterine Mϕs obtained from estrogen + progesterone-treated mice did not increase further beyond concentrations of 1:100, whereas peritoneal Mϕs obtained from the same mice were stimulatory in a dose-dependent manner up to a concentration of 1:10 (Figure 7.7E). A similar investigation of the effects of higher numbers of uterine Mϕs from vehicle-, progesterone- or estrogen-treated mice was not possible due to insufficient yields.

7.4.2 Effect of culture in CSF-1 and GM-CSF on uterine Mϕ immunoaccessory phenotype

To investigate whether the immunoinhibitory phenotype of uterine Mϕs might be susceptible to modulation by CSF-1 or GM-CSF, in two separate experiments peritoneal and uterine Mϕs collected from estrous mice were cultured in CSF-1 or GM-CSF *in vitro* prior to assessment of their immunoaccessory phenotype in a spleen cell mitogenesis assay.

While CSF-1 had only a small effect on the phenotype of peritoneal Mϕs, culture of peritoneal Mϕs in GM-CSF caused a 5-fold increase in their capacity to stimulate the proliferation of purified spleen cells. In contrast, neither CSF-1 nor GM-CSF induced a comparable shift in the phenotype of uterine Mϕs, which were still inhibitory to spleen cell proliferation relative to the baseline proliferation regardless of preculture with cytokine (CSF-1 and GM-CSF had a small stimulatory effect on baseline proliferation of spleen cells, Figure 7.8B). Similar effects of CSF-1 and GM-CSF on peritoneal and uterine Mϕs were obtained in a third experiment of similar design (not shown).

7.5 Conclusions and discussion

The experiments presented in previous chapters describe the cell membrane expression of phenotypic markers by uterine APCs, which allowed some predictions to be made concerning the participation of uterine Mϕs in behaviours such as antigen scavenging, antigen processing and antigen presentation. The studies presented in this chapter focus on *in vitro* analysis of the immunoaccessory phenotype of F4/80$^+$ Mϕs collected from the uteri of mice.

Uterine Mϕs collected from estrous mice were found to exert a potent inhibitory effect on the mitogen-induced proliferation of purified spleen cells *in vitro*. Such a phenotype is not unique to Mϕs obtained from the uterus. Holt and colleagues have firmly established that resident, unstimulated rodent and human alveolar Mϕs suppress mitogen-induced and allo-antigen-induced lymphocyte proliferation (reviewed in Stumbles *et al.* 1999). Mϕs purified from the gastrointestinal tract have a similar effect (Pavli *et al.* 1993) and, as was observed in the

current study, at high concentrations peritoneal Mϕs, blood monocytes and splenic Mϕs can also inhibit lymphocyte proliferation (Holt 1979, Stein and Strejan 1993).

That the inhibitory effect of uterine Mϕs on spleen cells is mediated by the synthesis of a soluble inhibitory molecule was suggested by co-culture and supernatant transfer experiments. The inhibitory phenotype of alveolar (Holt *et al.* 1993, Bilyk and Holt 1995, Strickland *et al.* 1996) and other Mϕs (Ding *et al.* 1988, Stuehr and Nathan 1989) has been attributed to the production of NO, and immunoreactive iNOS co-localises with Mϕs in sections of cycling and pregnant murine uterus (Huang *et al.* 1995, Hunt *et al.* 1997b). Despite this, in the current study a specific inhibitor of the iNOS enzyme had no effect on the phenotype of *ex vivo* uterine Mϕs. In addition, no abrogation of Mϕ-induced inhibition was mediated by the cyclooxygenase inhibitor indomethacin, which prevents PGE_2 production by Mϕs (Stevens *et al.* 1989). Thus the identity of the inhibitory molecule synthesised by uterine Mϕs remains elusive, but several possibilities warrant further examination. Firstly, the inhibitory effect could have been due to the synthesis of an interleukin (IL)-1 inhibitor, such as an IL-1 receptor antagonist (Arend 1993) or IL-1 decoy receptors (Colotta *et al.* 1994) by the uterine Mϕs. Such molecules act by decreasing the availability of IL-1α and/or IL-1β molecules required for inducing lymphocyte proliferation. Other cytokines potentially produced by uterine Mϕs which would be expected to inhibit lymphocyte proliferation include TGFβ or IL-10 (Wilkes *et al.* 1995, Letterio and Roberts 1998), the activities of which could be investigated by addition of specific antibodies to the immunoaccessory assays.

Another mechanism by which uterine Mϕs could prevent the proliferation of lymphocytes is by tryptophan catabolism. Both murine and human Mϕs produce the enzyme indoleamine 2,3 dioxygenase (IDO), which degrades the amino acid tryptophan (Mellor and Munn 1999). Since T cells appear to possess a cell cycle regulatory checkpoint that is sensitive to the level of free tryptophan, the synthesis of IDO can prevent T cell proliferation within the confines of the local microenvironment. To investigate whether inhibition of spleen cell proliferation observed in these studies might have been mediated by Mϕ depletion of tryptophan, the 1-methyl analogue of tryptophan (a potent inhibitor of IDO) could be added to the immunoaccessory assays (Munn *et al.* 1999).

The immunoinhibitory phenotype of uterine Mϕs was found to be highly sensitive to ovariectomy and stage of estrous cycle, suggesting that the induction of an immunosuppressive phenotype in uterine Mϕs might be mediated by ovarian steroid hormones and/or the cytokines whose synthesis they regulate (Hunt and Robertson 1996). That ovarian steroid hormones might be responsible is suggested by *in vitro* studies showing that both progesterone and estradiol can

induce an inhibitory phenotype in Mϕs (Chao *et al.* 1995, Hunt and Robertson 1996, Deshpande *et al.* 1997). However, there are two lines of evidence in the current study which would suggest that ovarian steroid hormones alone are insufficient for this role. Firstly, it would be expected that all tissue Mϕs would be similarly susceptible to steroid regulation, and the immunoaccessory phenotype of Mϕs recovered from the peritoneal cavity was found to be relatively insensitive to ovariectomy and stage of estrous cycle. Secondly, the inhibitory phenotype was not evident in uterine Mϕs collected from ovariectomised mice which had been treated with physiological doses of ovarian steroid hormones. Thus, a factor(s) specific to the uterine microenvironment and not induced by this regime of steroid hormones replacement is implicated.

CSF-1 has been described by several different laboratories as being responsible for the induction of an immunoinhibitory phenotype in Mϕs. Munn and others (Mellor and Munn 1999, Munn *et al.* 1999) suggest that expression of IDO in human Mϕs only occurs when the cells are differentiated from monocytes under the influence of CSF-1 but not other cytokines. Interestingly, a role for tryptophan catabolism by trophoblast cells and uterine Mϕs in preventing T-cell mediated immune rejection of the conceptus has been postulated (Munn *et al.* 1998). Other authors (Sakurai *et al.* 1996) suggest that the production of an as yet unidentified inhibitory molecule by murine spleen Mϕs is also dependent on CSF-1 (the inhibitory molecule produced by the Mϕs in this case appeared not to be PGE_2, NO, IL-10, an IL-1 inhibitor, or TGFβ).

A role for CSF-1 in inducing an inhibitory phenotype in uterine Mϕs appears possible since the known pattern of synthesis of this cytokine in uterine epithelial cells parallels the potency of the inhibitory phenotype observed in uterine Mϕs of estrous, diestrous, ovariectomised and also pregnant mice in the current study. Interestingly, CSF-1 has also been shown to induce production of IL-1 inhibitors by cells of the Mϕ lineage (Matsushime *et al.* 1991), and high concentrations of an IL-inhibitor accumulate in the urine of pregnant women (Brown *et al.* 1986). However, a role for other steroid hormone-induced polypeptides either alone or in combination with CSF-1 in regulating uterine Mϕ suppressive activity cannot be discounted. Other possibilities include TGFβ and IL-10, both of which are recognised to be produced by uterine cells (Robertson *et al.* 1994) and which have potent immunosuppressive effects on murine Mϕs (Huaux *et al.* 1998, Letterio and Roberts 1998).

That the immunoinhibitory effect of uterine Mϕs was somewhat diminished, even abrogated at day 1 of pregnancy suggests that the Mϕs contained within the uterus at this time are phenotypically different from those present during the estrous cycle. This observation could have been a consequence of activation of Mϕs resident within the uterine tissue, a possibility

which seems likely given the upregulated synthesis of GM-CSF and other inflammatory cytokines in the inseminated uterus (Robertson and Seamark 1990, McMaster *et al.* 1992, Sanford *et al.* 1992, Robertson *et al.* 1996a, Tremellen *et al.* 1998). However, that uterine Mϕs were found to be relatively phenotypically resistant to activation by *in vitro* culture in GM-CSF suggests that other mechanisms for phenotypic regulation might exist *in vivo*. One possible explanation lies with the observations in the current studies (see Chapters 3 and 5) and previously published works (De and Wood 1991, McMaster *et al.* 1992, Robertson *et al.* 1996a) that insemination is associated with a recruitment of new Mϕs into the uterine endometrium. Data derived from analysis of alveolar Mϕs shows that freshly recruited cells initially exhibit an immunostimulatory phenotype, which is progressively lost once the cells begin to differentiate according to the profile of microenvironmental signals within the tissue (Bilyk and Holt 1995). If a similar situation occurs in the uterus, this could be reflected as an enhanced immunostimulatory phenotype as was observed in the current study.

Correspondingly, the potent inhibitory phenotype of Mϕs purified from the uteri of day 4 pregnant mice could be a result of the cessation of local pro-inflammatory cytokine (McMaster *et al.* 1992, Sanford *et al.* 1992, Robertson *et al.* 1996a) and chemokine synthesis (Robertson *et al.* 1998) and/or an exodus of immunostimulatory Mϕs through draining lymphatic vessels. Although the current study did not make any attempt to separate resident and freshly recruited uterine Mϕs prior to assessment of their immunoaccessory phenotype, this could be accomplished in future experiments with the use of cell selection methods which exploit the differential expression patterns of molecules such as CD11c on resident and freshly recruited cells (see Chapter 5).

Some degree of caution needs to be exercised in the interpretation of data derived from these immunoaccessory assays since the phenotype of Mϕs has been shown to be remarkably sensitive to many features of the *in vitro* culture environment, including exposure to fetal calf serum and adherence to fibronectin (Thorens *et al.* 1987). However, it would seem unlikely that the observed phenotype in uterine Mϕs is purely a result of *in vitro* factors since peritoneal Mϕs treated in the same manner were entirely different in their phenotype. Furthermore, the assays were sufficiently sensitive to detect differences in uterine Mϕ phenotype when the cells were collected after ovariectomy or following mating and clear differences between treatment groups were evident.

Constitutive production of an inhibitory molecule by Mϕs within the cycling uterus would have a number of implications. Firstly, the molecule could prevent activation of local dendritic cells in a similar manner to that postulated to occur in the airways (Holt *et al.* 1993, Stumbles *et*

al. 1999) and gastrointestinal tract (Pavli *et al.* 1993). In the uterus, this could have the effect of preventing the generation of immune responses to innocuous or commensal antigens. In the case of secondary immune responses, an inhibitory molecule secreted by uterine Mϕs could limit the activation and proliferation of resident lymphocytes. These two potential immunoinhibitory effects mediated by uterine Mϕs could be crucial to prevent the initiation and mediation of immune responses which might otherwise have a devastating effect on uterine homeostasis. In the event of the perception of 'danger' in the uterus, these local regulatory mechanisms might be bypassed through pro-inflammatory cytokine-induced recruitment and activation of uterine Mϕs, dendritic cells and lymphocytes (Stumbles *et al.* 1999).

In summary, the studies presented in this chapter have identified that $F4/80^+$ Mϕs collected from the uteri of estrous mice exhibit a potent immunoinhibitory phenotype in a spleen cell mitogenesis assay due to their production of a soluble molecule which was shown not to be NO nor PGE_2. That the induction of such a phenotype is regulated in an ovarian steroid hormone-dependent manner was suggested by analysis of the phenotype of uterine Mϕs harvested from diestrous and ovariectomised mice. However, the phenotype could not be induced by administration of steroid hormones to ovariectomised mice. After insemination, uterine Mϕs were found to exhibit a markedly reduced inhibitory phenotype, perhaps due to their phenotypic conversion by locally produced pro-inflammatory cytokines and/or as a result of a dilution effect by newly recruited, undifferentiated cells. By the fourth day of pregnancy, uterine Mϕs exhibited an inhibitory phenotype which was similar in nature to that observed at estrus.

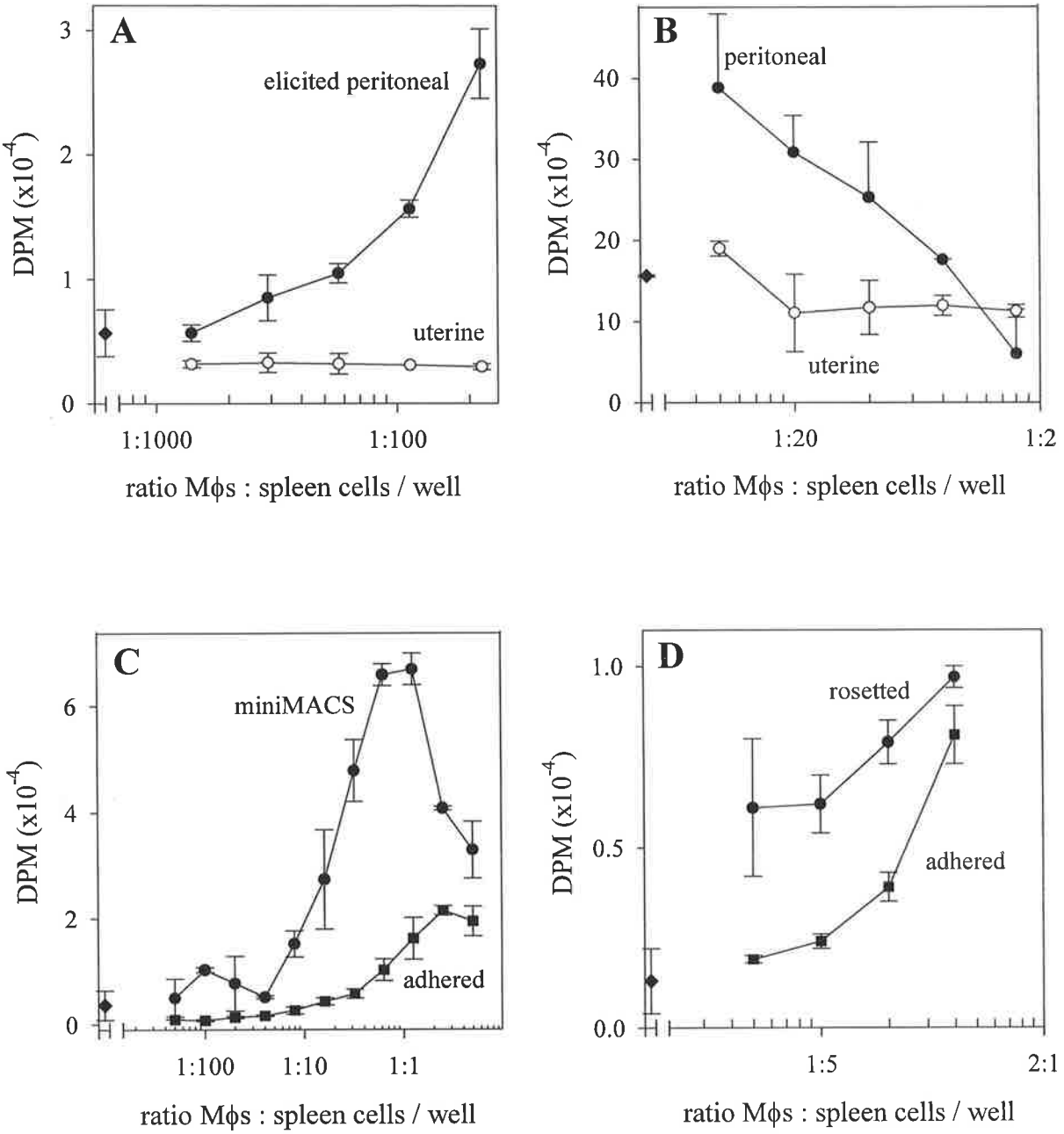


Figure 7.1 The immunoaccessory phenotype of peritoneal and uterine Mϕs. In 4 separate experiments (A-D), peritoneal (filled symbols) or uterine (unfilled circles) Mϕs were cultured with purified spleen cells in 200µl with PHA (10 µg/ml) for 72 h (A-C) or 96 h (D). Cells were harvested after pulsing with ^{3}H-thymidine (1 µCi/ml) during the final 24 h of the culture period. Results are mean proliferation (DPM) +/- SD for duplicate wells. The proliferation of spleen cells alone is shown in each graph (diamond).

A. *Experiment 1.* Elicited peritoneal Mϕs purified by adherence and F4/80^{+} uterine Mϕs purified by miniMACs, cultured with 10^{5} spleen cells.
B. *Experiment 2.* Resident peritoneal Mϕs purified by adherence and FcR+ uterine Mϕs purified by rosetting with SRBC, cultured with $2x10^{5}$ spleen cells.
C. *Experiment 3.* Resident peritoneal Mϕs purified by adherence or on the basis of F4/80 expression by miniMACS, cultured with 10^{5} spleen cells.
D. *Experiment 4.* Resident peritoneal Mϕs purified by adherence or on the basis of FcR expression by rosetting with SRBC, cultured with $5x10^{4}$ spleen cells.

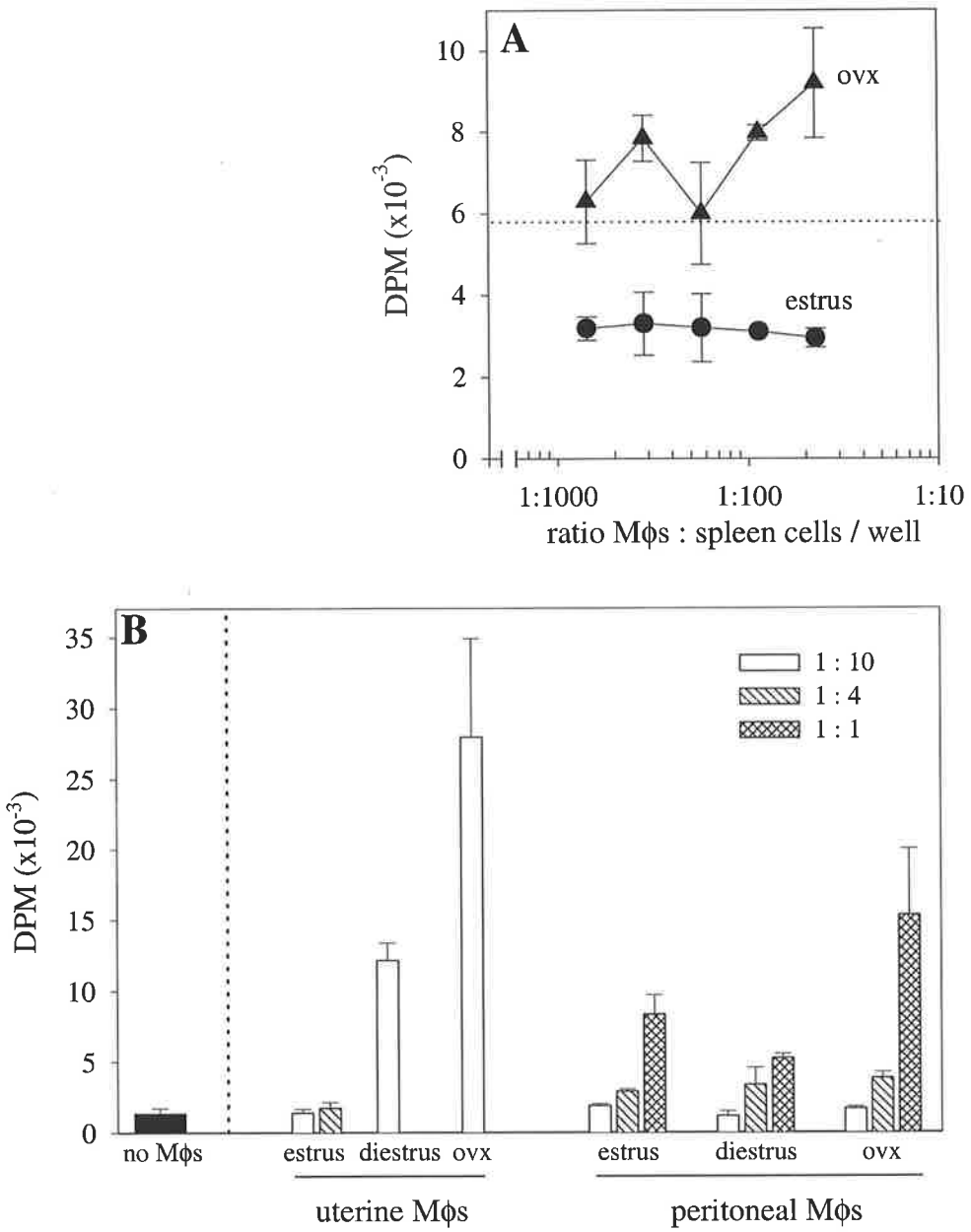


Figure 7.2 The effect of ovariectomy and stage of estrous cycle on the immunoaccessory phenotype of uterine and peritoneal Mϕs. In two separate experiments, the effect of ovariectomy and stage of estrous cycle on the immunoaccessory phenotype of F4/80$^+$ uterine (A & B) and adherent peritoneal (B) Mϕs was examined with a spleen cell mitogenesis assay. Uterine or peritoneal Mϕs were cultured with purified spleen cells in 200µl with PHA (10 µg/ml) for 72 h (A) or 120 h (B). Cells were harvested after pulsing with ^{3}H-thymidine (1 µCi/ml) during the final 24 h of the culture period. Results are mean proliferation (DPM) +/- SD for duplicate wells.

A. *Experiment 1.* F4/80$^+$ uterine Mϕs purified by miniMACs at estrus (circle) or following ovariectomy (ovx, triangle), cultured with 10^5 spleen cells. The proliferation of spleen cells alone is shown (dotted line).

B. *Experiment 2.* Uterine or peritoneal Mϕs, collected by miniMACs or adherence respectively, at estrus, diestrus or following ovariectomy (ovx), cultured with $4x10^4$ spleen cells. The number of Mϕs/well is indicated relative to the number of spleen cells. The proliferation of spleen cells alone is shown (filled bar, 'no Mϕs').

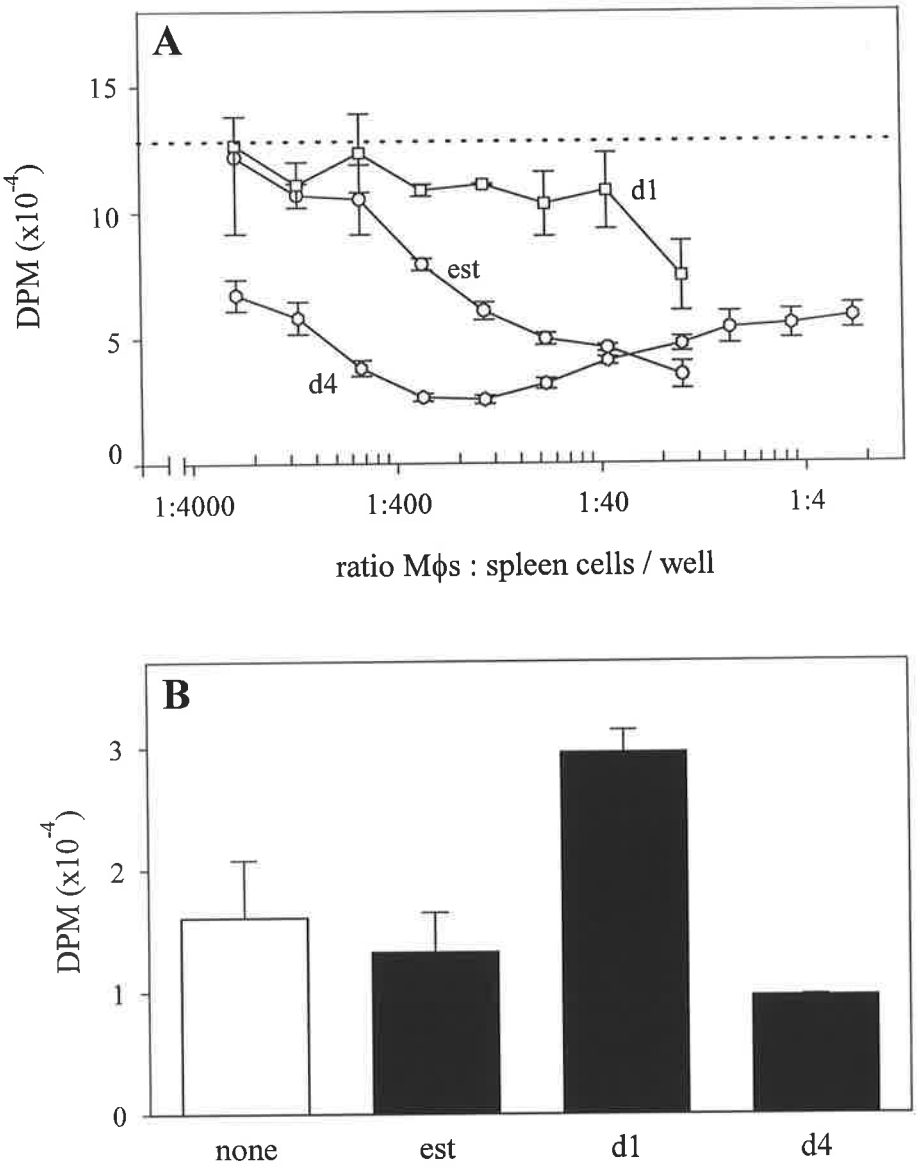


Figure 7.3 The effect of day of early pregnancy on the immunoaccessory phenotype of uterine Mϕs. In two separate experiments, the effect of day of early pregnancy on the immunoaccessory phenotype of uterine Mϕs was examined with a spleen cell mitogenesis assay. F4/80$^+$ uterine Mϕs collected by miniMACs from estrous (est, circle), day 1 pregnant (d1, square) or day 4 pregnant (d4, hexagon) mice were cultured with purified spleen cells in 200µl with PHA (10 µg/ml) for 72 h, and cells were harvested after pulsing with ^{3}H-thymidine (1 µCi/ml) during the final 24 h of the culture period. Results are mean proliferation (DPM) +/- SD for duplicate wells.

A. *Experiment 1.* Uterine Mϕs titrated into wells containing 4x10^5 spleen cells. The proliferation of spleen cells alone is shown (dotted line).

B. *Experiment 2.* Uterine Mϕs (10^4) cultured with 2x10^5 spleen cells (concentration Mϕs:spleen cells = 1:20). The proliferation of spleen cells alone is shown (unfilled bar, 'none').

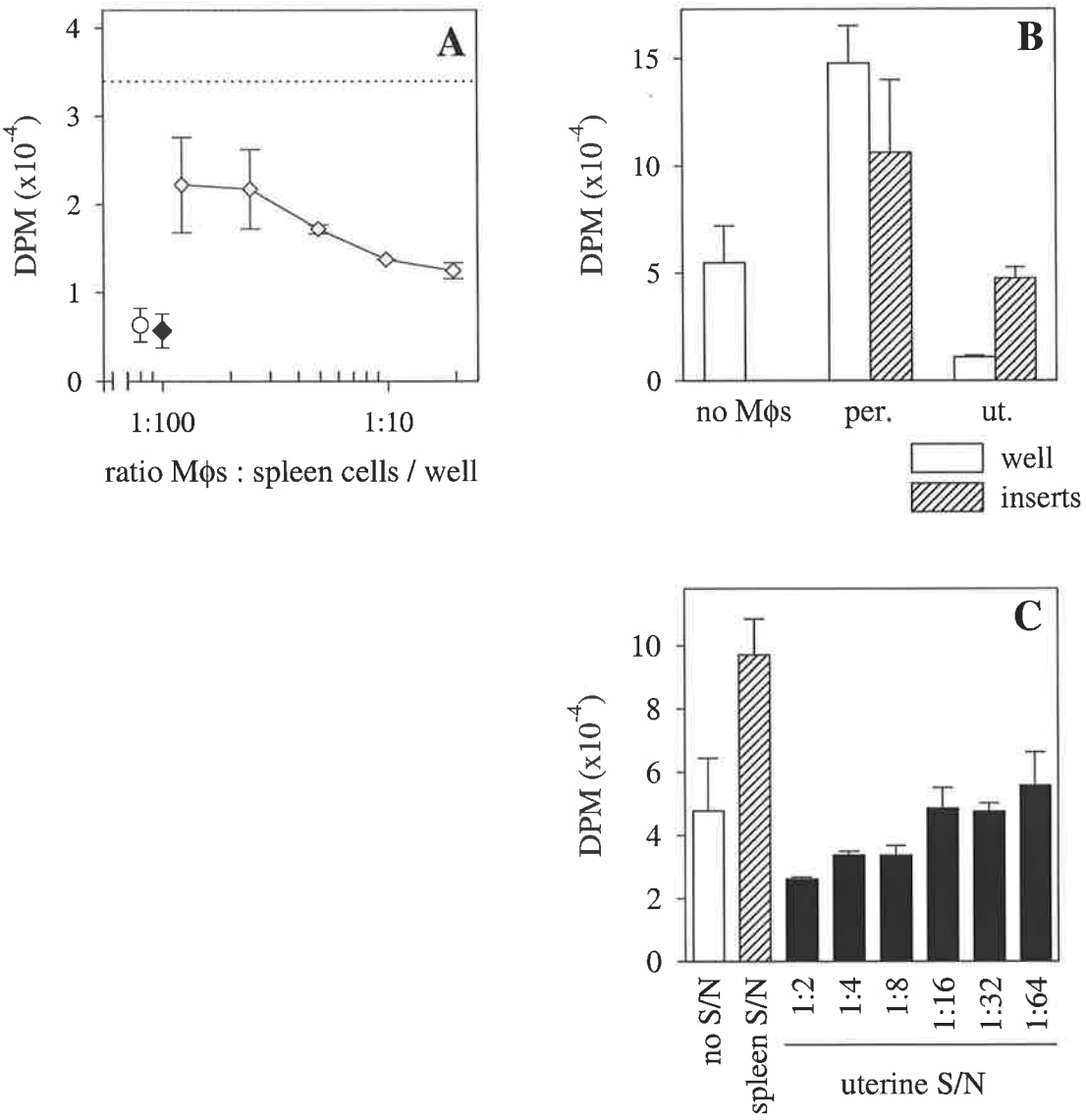


Figure 7.4 Molecular nature of the immunosuppressive activity of uterine Mϕs. In three separate experiments, the molecular nature of the immunosuppressive activity of F4/80$^+$ uterine Mϕs at estrus was examined with a spleen cell mitogenesis assay. Purified spleen cells were cultured in PHA (10 μg/ml) with uterine and/or peritoneal Mϕs (purified by miniMACs and adherence respectively) for 72 h (A) or 96 h (B, C) and cells were harvested after pulsing with ^{3}H-thymidine (1 μCi/ml) during the final 24 h of the culture period. Results are mean proliferation (DPM) +/- SD for duplicate wells.

A. *Experiment 1.* Uterine Mϕs titrated into 200 μl wells with 10^5 peritoneal Mϕs and 10^5 spleen cells (unfilled diamond). The proliferation of spleen cells alone (filled diamond), spleen cells with 10^5 peritoneal Mϕs and no uterine Mϕs (dotted line) and spleen cells with $5x10^3$ uterine Mϕs and no peritoneal Mϕs (unfilled circle) are shown.

B. *Experiment 2.* Uterine (ut) or peritoneal (per) Mϕs (10^5/well) cultured in 1 millilitre wells with $5x10^5$ spleen cells (filled bars) or in culture plate inserts with 0.4 μm pores suspended above $5x10^5$ spleen cells (unfilled bars). The proliferation of spleen cells alone is shown (no Mϕs).

C. *Experiment 3.* Spleen cells (10^5) cultured in 200μl wells in media (no S/N), or in media diluted with supernatant from proliferating cultures of spleen cells (spleen S/N, diluted 1:2), or media from 72 h cultures of spleen cells and $4x10^4$ uterine Mϕs (uterine S/N, diluted 1:2 – 1:64).

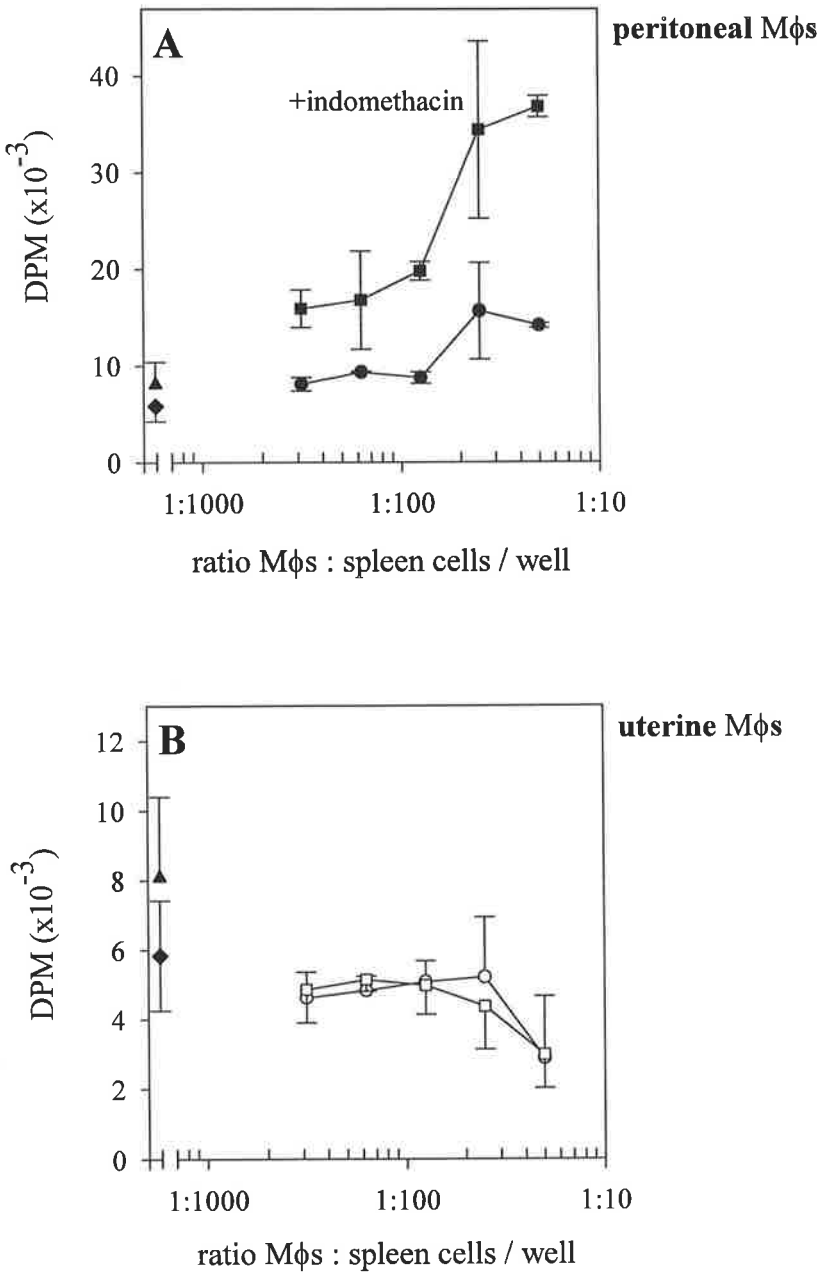


Figure 7.5 The effect of indomethacin on the immunoaccessory phenotype of uterine and peritoneal Mϕs. In a single experiment, peritoneal (A) or uterine (B) Mϕs were cultured with 10^5 purified spleen cells in 200µl with PHA (10 µg/ml) with (squares) or without (circles) indomethacin (5 µg/ml) for 72 h. Cells were harvested after pulsing with ^{3}H-thymidine (1 µCi/ml) during the final 24 h of the culture period. Results are mean proliferation (DPM) +/- SD for duplicate wells. The proliferation of spleen cells alone either with (triangle) or without (diamond) indomethacin is shown in each graph. Results are representative of 3 experiments.

Figure 7.6 Synthesis of nitric oxide (NO) by uterine and peritoneal Mϕs. In 4 separate experiments, the proliferation of spleen cells and the accumulation of NO breakdown products in immunoaccessory assays containing spleen cells and peritoneal (A, B) or uterine (C-F) Mϕs collected from estrous mice were compared. In some assays, 2 mM of an inhibitor of inducible NO synthase (L-NMA) or its D-enatiomer (D-NMA), and/or 1000 U/ml IFNγ were present during part or all of the culture period. All assays were performed in 200μl with PHA (10 μg/ml). Supernatants were collected and cells were harvested after pulsing with ^{3}H-thymidine (1 μCi/ml) during the final 24 h of the culture period. Results are mean proliferation (DPM), or concentration of nitrite/nitrate (μM) in culture supernatant (measured as total nitrite after conversion of nitrate to nitrite) +/- SD for duplicate wells.

A. *Experiment 1.* Resident peritoneal Mϕs cultured with $4x10^5$ spleen cells for 72 h. Proliferation is shown in filled circles, and nitrite/nitrate concentration in culture supernatant in unfilled circles. For cultures of spleen cells alone, proliferation (filled bar) and nitrite/nitrate accumulation (unfilled bar) are shown.

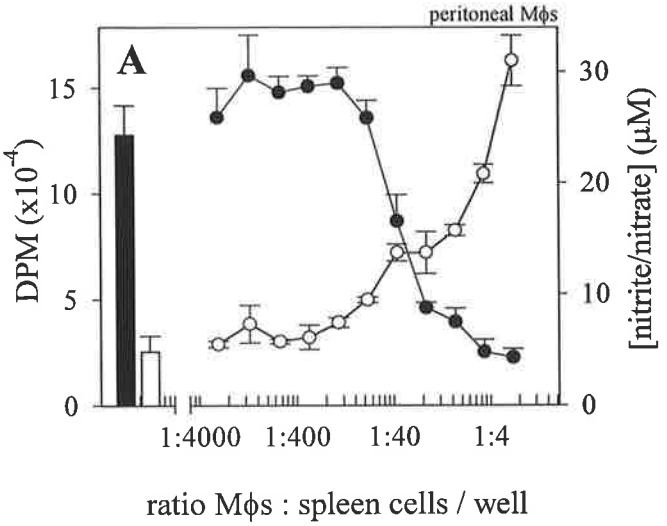
B. *Experiment 2.* Accumulation of nitrite/nitrate in the supernatants of elicited peritoneal Mϕs ($2x10^4$) cultured with 10^5 spleen cells alone, with L-NMA +/- IFNγ or with D-NMA +/- IFNγ.

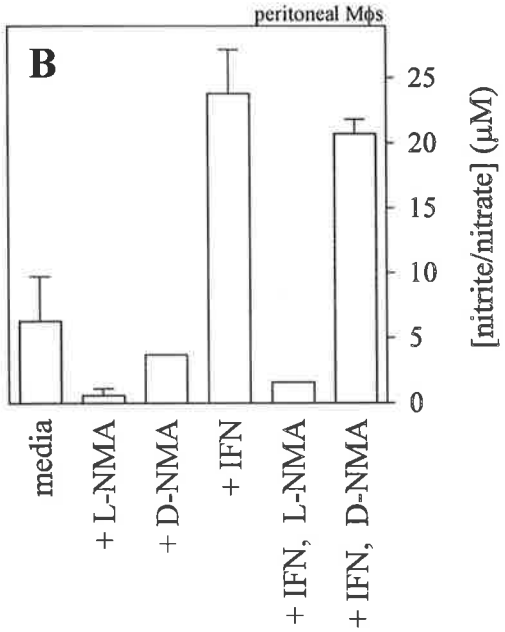
C. *Experiment 1.* Uterine Mϕs collected at estrus cultured with $4x10^5$ spleen cells for 72 h. Proliferation is shown in filled circles, and nitrite/nitrate concentration in culture supernatant in unfilled circles. For cultures of spleen cells alone, proliferation (filled bar) and nitrite/nitrate accumulation (unfilled bar) are shown. Also shown is the nitrite/nitrate accumulation in the supernatants of immunoaccessory assays using uterine Mϕs collected from day 1 pregnant (dashed line) and day 4 pregnant (dotted line) [corresponding proliferation shown in Figure 7.3].

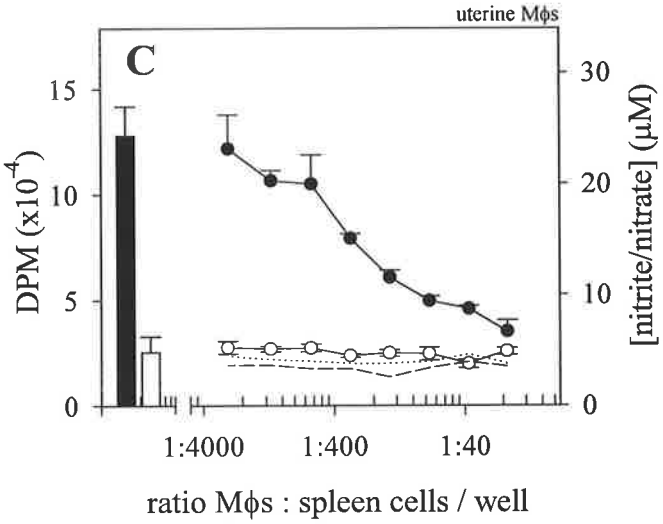
D. *Experiment 3.* Proliferation of $4x10^4$ spleen cells cultured with uterine Mϕs, with (unfilled square) or without (unfilled circle) L-NMA for 72 h. The proliferation of spleen cells alone with (filled square) or without (filled circle) L-NMA is also shown.

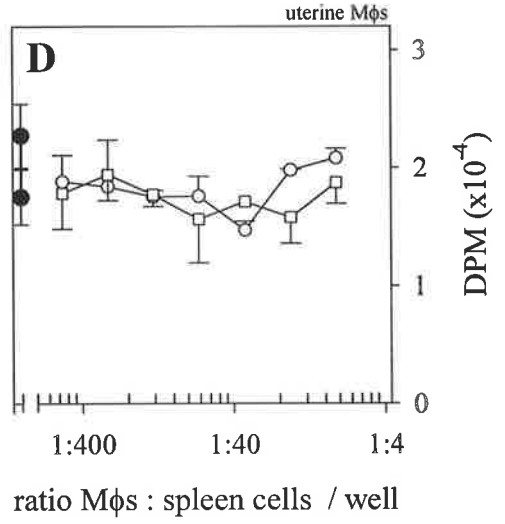
E. *Experiment 3.* Proliferation of $4x10^4$ spleen cells cultured with uterine Mϕs, with (unfilled diamond) or without (unfilled circle) IFNγ for 72 h. The proliferation of spleen cells alone with (filled square) or without (filled circle) IFNγ is also shown.

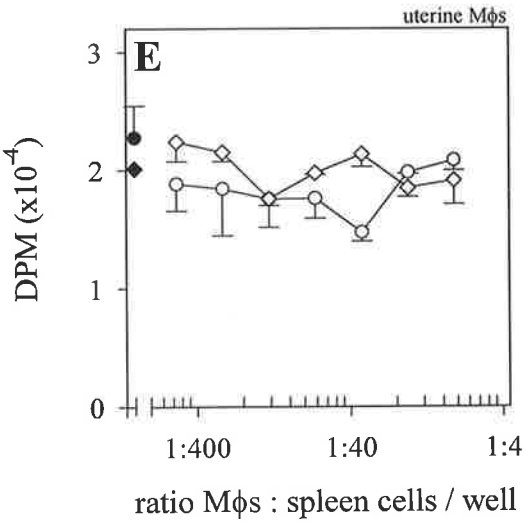
F. *Experiment 4.* Accumulation of nitrite/nitrate in the supernatants of $5x10^5$ spleen cells cultured with 10^4 uterine Mϕs with (+IFN) or without (media) IFNγ for 72h.


peritoneal Mϕs
A
DPM (x10^{-4})
[nitrite/nitrate] (μM)
1:4000
1:400
1:40
1:4
ratio Mϕs : spleen cells / well


peritoneal Mϕs
B
[nitrite/nitrate] (μM)
media
+ L-NMA
+ D-NMA
+ IFN
+ IFN, L-NMA
+ IFN, D-NMA


uterine Mϕs
C
DPM (x10^{-4})
[nitrite/nitrate] (μM)
1:4000
1:400
1:40
ratio Mϕs : spleen cells / well


uterine Mϕs
D
DPM (x10^{-4})
1:400
1:40
1:4
ratio Mϕs : spleen cells / well


uterine Mϕs
E
DPM (x10^{-4})
1:400
1:40
1:4
ratio Mϕs : spleen cells / well

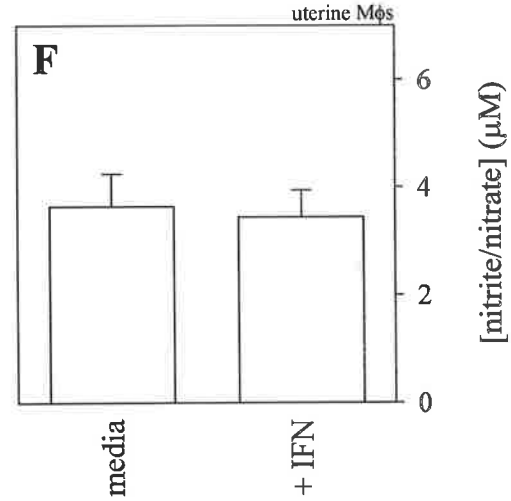
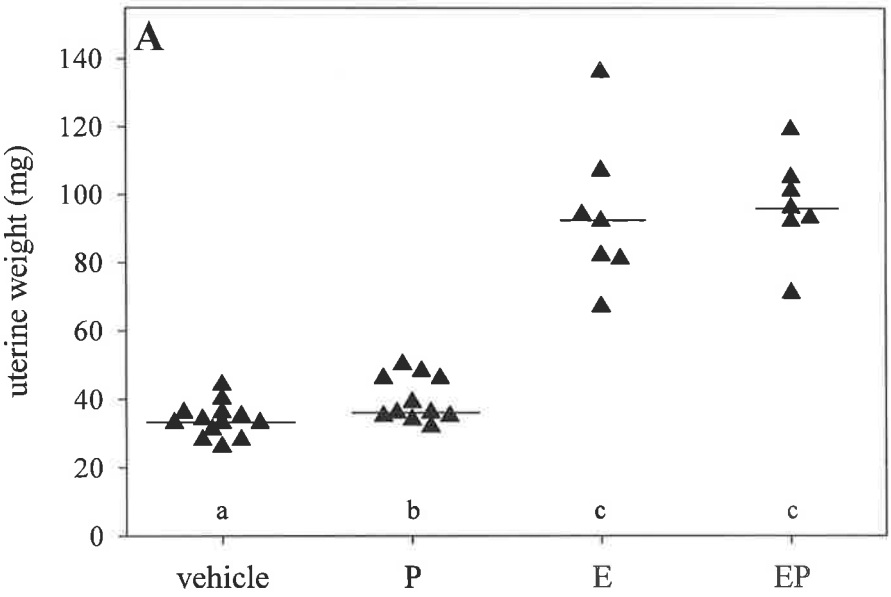

uterine Mϕs
F
[nitrite/nitrate] (μM)
media
+ IFN

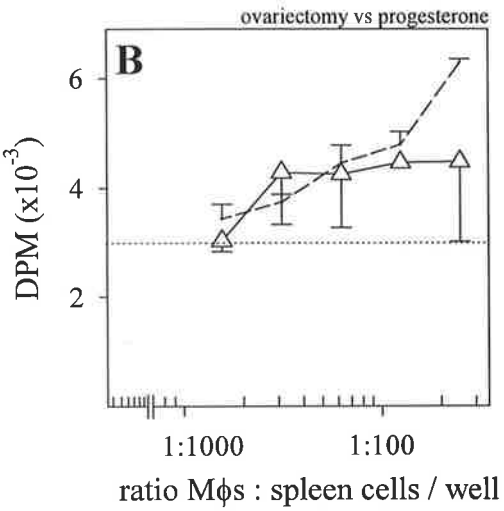
Figure 7.7 The effect of ovarian steroid hormones on the immunoaccessory phenotype of uterine Mϕs. The effect of administration of estrogen +/- progesterone to ovariectomised mice on the immunoaccessory phenotype of uterine Mϕs was examined with a spleen cell mitogenesis assay. $F4/80^+$ Mϕs collected by miniMACs from the uteri of mice in each treatment group were cultured with 10^5 purified spleen cells in 200µl with PHA (10 µg/ml) for 72 h. Cells were harvested after pulsing with ^{3}H-thymidine (1 µCi/ml) during the final 24 h of the culture period. Results are mean proliferation (DPM) +/- SD for duplicate wells. The proliferation of spleen cells alone is shown in each graph (dotted line). Similar results were obtained in a second experiment of similar design.

A. Symbols represent the weight of uteri collected from individual mice which had been ovariectomised and then treated (for treatment regimen see section 2.2.5) with vehicle (n = 13), progesterone (P; n = 11), 17β-estradiol (E; n = 7) or progesterone and 17β-estradiol (EP; n = 7). Data were compared by Kruskal-Wallis one-way ANOVA and Mann-Whitney Rank Sum Test. Groups with different lower case letters on the x axis are significantly different for the given mAb ($p < 0.02$).

B-D. The effect of uterine Mϕs collected from vehicle-treated mice on proliferation of spleen cells is shown as a dotted line in each graph. The effect of Mϕs collected from progesterone-treated (B), 17β-estradiol-treated (C) and progesterone + 17β-estradiol-treated (D) mice on proliferation of spleen cells is shown as a solid line.

E. The effect of uterine (unfilled triangles) or peritoneal (filled triangles; purified by adherence) Mϕs collected from progesterone + 17β-estradiol-treated mice on proliferation of spleen cells.


A
uterine weight (mg)
140
120
100
80
60
40
20
0
a
b
c
c
vehicle
P
E
EP


ovariectomy vs progesterone
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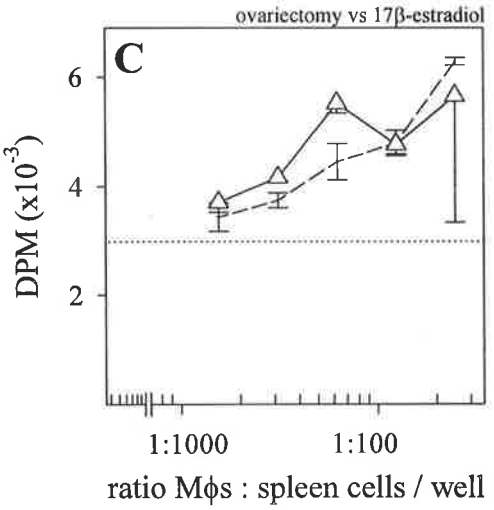
ratio Mϕs : spleen cells / well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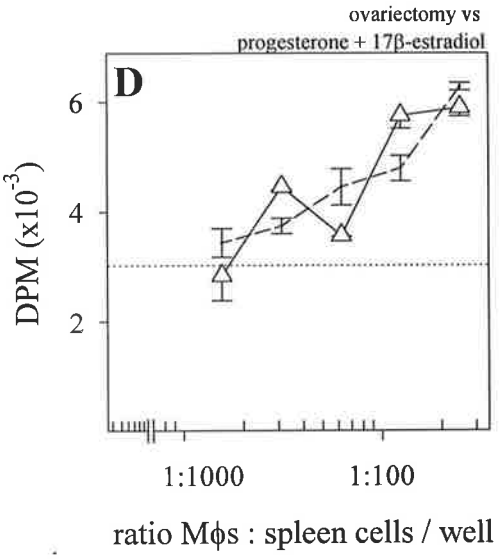
ovariectomy vs
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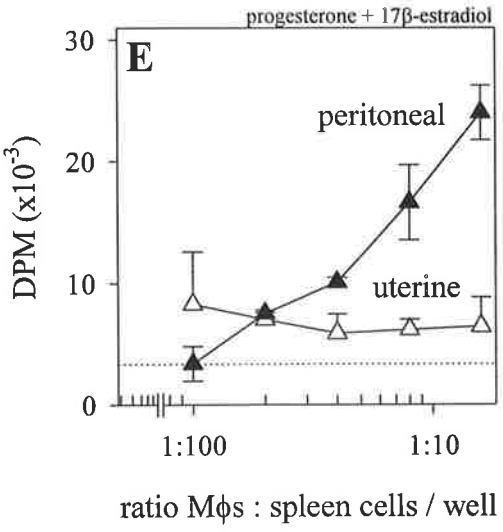
progesterone + 17β-estradiol
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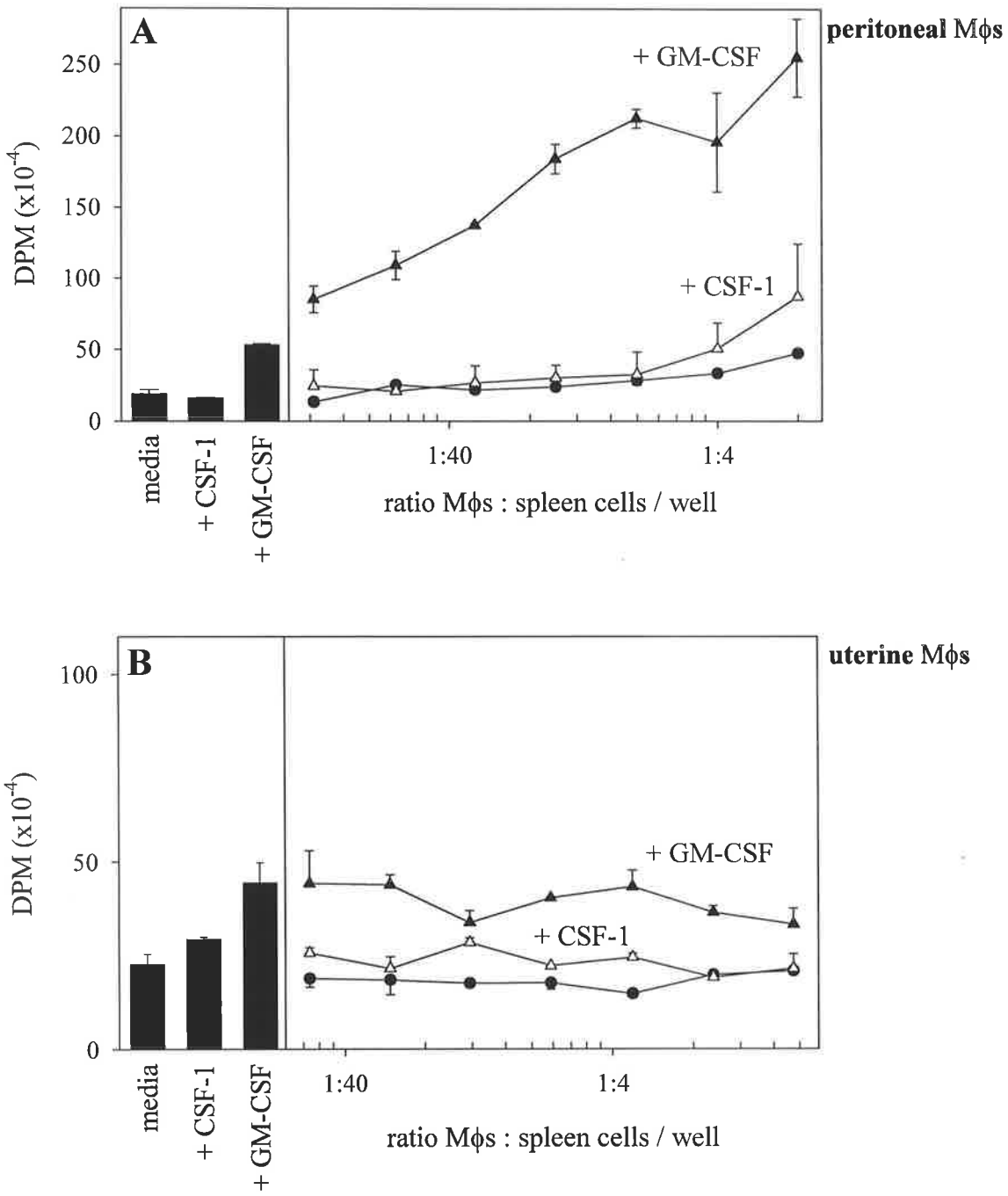


Figure 7.8 The effect of CSF-1 and GM-CSF on the immunoaccessory phenotype of uterine and peritoneal Mϕs. In two separate experiments, the effect of CSF-1 and GM-CSF on the immunoaccessory phenotype of peritoneal and uterine Mϕs was examined with a spleen cell mitogenesis assay. Peritoneal Mϕs (A; purified by adherence) and F4/80$^+$ uterine Mϕs (B; purified by miniMACs) were collected from estrous mice and cultured for 24 h in media (filled circles) or in 100 U/ml CSF-1 (unfilled triangles) or 100 U/ml GM-CSF (filled triangles). $4x10^4$ purified spleen cells were subsequently added, all cells were cultured for a further 48 h in 200µl with PHA (10 µg/ml), and then harvested after pulsing with ^{3}H-thymidine (1 µCi/ml) during the final 24 h of the culture period. Results are mean proliferation (DPM) +/- SD for duplicate wells. The proliferation of spleen cells alone added to wells containing media or 100 U/ml CSF-1 or GM-CSF are shown as filled bars in each graph. Similar results for both peritoneal and uterine Mϕs were obtained in a second experiment of similar design.

Chapter 8

General discussion and conclusions

The cells and intercellular signaling mechanisms involved in the generation and regulation of cell mediated immunity in mucosal and other peripheral organs have been studied extensively in the past few decades. It has now emerged that myeloid antigen presenting cells (APCs), including macrophages (Mϕs) and dendritic cells, are critical participants in most aspects of mucosal immunity. In organs such as the lung and the gastrointestinal tract, the roles of these cells include tissue remodeling, maintenance of homeostasis through the secretion of immunoregulatory molecules and the generation of antigen-specific immunity. The current and previous studies provide compelling evidence to suggest that similar populations of myeloid APCs and the regulatory networks which govern their behaviour also exist in the uterus. The following discussion will consider the significance of the new findings described herein in the context of previous studies, with a focus on the potential roles for myeloid APCs which exist in the regulation of uterine homeostasis and immunity.

8.1 APC populations in the murine uterus

The experiments described in this thesis provide the best evidence to date that the populations of APCs contained within the murine uterus are analogous to populations of APCs found in other mucosal organs. By immunohistochemistry, APCs were identified in the uteri of cycling, ovariectomised and pregnant mice on the basis of their morphology and their expression of an array of cell membrane markers, including F4/80, macrosialin, class A scavenger receptor, sialoadhesin, Ia and B7-2. The development of a method for dual-colour flow cytometric analysis allowed further characterisation of these cells, and provided evidence of the existence of three separate populations. Cells referred to as undifferentiated and differentiated Mϕs were both $F4/80^+$, but exhibited different sizes, cell membrane phenotypes and sensitivity to ovarian steroid hormones. $F4/80^-$ uterine APCs were designated dendritic cells based on their relatively small size and simple intra-cellular structure, their Ia^+ membrane phenotype, and their mixed expression of macrosialin, class A scavenger receptor, sialoadhesin, β2 integrins, 33D1, DEC-205 and CD1. These dendritic cells were considerably less responsive to ovarian steroids, and persisted in a relatively unaltered phenotype after ovariectomy. Within the Mϕ and dendritic cell populations, considerable heterogeneity was evident, and it will be of key interest in future studies to further define these heterogeneous populations using 3- or 4-colour FACS labeling to

define the patterns of expression of multiple membrane markers on these cells. For example, analysis of uterine cells labeled with mAbs to DEC-205, macrosialin and mannose receptor might detect a population of uterine APCs specialised to internalise and process antigens, whereas the concurrent use of mAbs to Ia, 33D1 and B7-2 could label a subpopulation of cells specialised for trafficking to draining lymph nodes and activation of naïve T lymphocytes. Such an approach could also be useful for identifying differentiation pathways linking the different sub-populations. The possible developmental and differentiation pathways which might exist in the cycling uterus have been proposed earlier in this thesis (see section 4.8 and Figure 4.13).

8.2 The role of Mϕs in maintaining uterine homeostasis during the estrous cycle

The current and other studies suggest a role for uterine Mϕs in maintenance of uterine homeostasis during the estrous cycle. Data presented in this thesis shows that during the estrous cycle, Mϕs in the endometrium and myometrium express endocytic and phagocytic receptors, which indicates a capacity to take up and degrade necrotic and apoptotic cells, and extra-cellular debris generated during tissue remodeling (Figure 8.1). The phagocytic ability of uterine Mϕs might also afford a key role for these cells as providers of innate immune defence through uptake and destruction of opportunistic microorganisms. Uterine Mϕs may also be involved in tissue remodeling through their secretion of enzymes and growth factors. It has been postulated that the differentiation of Mϕs equipped for these activities is driven by high local concentrations of CSF-1 (Stanley *et al.* 1997), a cytokine known to be expressed in abundance in the uterus. That uterine Mϕs express class A scavenger receptor, a molecule induced by CSF-1, is in accordance with this.

By analysis of their immunoaccessory function *in vitro*, the studies presented herein show that uterine Mϕs exhibit an inhibitory phenotype comparable to that of Mϕs derived from other mucosal sites such as the lung and anterior chamber of the eye. However, in contrast to other Mϕ populations, the molecule secreted by uterine Mϕs was determined not to be a prostaglandin nor nitric oxide. The aim of future studies in this laboratory will be to identify the nature of this inhibition, and of particular focus will be an investigation of the synthesis of inhibitory cytokines such as IL-10 or TGFβ, or enzymes such as indoleamine 2,3 dioxygenase by these cells. Furthermore, the identification of the ovarian steroid hormone-regulated factor(s), responsible for inducing the inhibitory phenotype in uterine Mϕs remains to be explored.

The synthesis and secretion of inhibitory and/or cytostatic molecules by endometrial Mϕs during the estrous cycle could have a number of consequences for uterine homeostasis (Figure

8.1). The *in vitro* experiments conducted in Chapter 7 suggest that uterine Mϕ synthesis of immunosuppressive molecules could be important in down-regulating the function and differentiation of local populations of leukocytes. In the uterus, as in the lung and the anterior chamber of the eye, the generation of a functional tolerance to 'self' or innocuous antigens encountered in the steady-state would be critical for homeostasis to be maintained. One plausible scenario by which such functional tolerance might be achieved is at the level of the local APC populations; namely, uterine dendritic cells which differentiate in the context of Mϕ-derived immunosuppressive molecules such as IL-10 or TFGβ might be expected to have a limited capacity to initiate Th1-polarised immune responses and instead might display DC-2 or DC-3 phenotypes, preferentially driving Th2- or Th3-deviated immune responses. Furthermore, if the cytostatic molecules secreted by uterine Mϕs are found to have broad specificity they could act to directly inhibit or kill invading pathogenic microorganisms. The cell lineage and species specificity of the uterine Mϕ-derived suppressive factors could be explored *in vitro* with the use of co-culture techniques; one key experiment would be to examine whether uterine Mϕs are able to inhibit the potent immunostimulatory capacity of uterine dendritic cells *in vitro* (S. Robertson, unpublished).

Another means by which functional tolerance might be achieved in the steady state could operate at the level of the cell membrane phenotype of uterine APCs. Immature dendritic cells or Mϕs trafficking to the draining lymph node during the steady state could elicit T cell anergy or deletion if their delivery of costimulatory molecules to T lymphocytes at the time of antigen presentation was inadequate for activation. Indeed, the current studies show that although uterine APCs expressing F4/80 and macrosialin are abundant in the vicinity of the draining lymphatics within the mesometrial triangle at estrus, cells expressing Ia and B7-2 are relatively sparse. Whether the APCs of the steady state uterus exhibit rapid rates of cell turnover in a manner similar to that which occurs in the lung, or very slow turnover as seen in the skin also requires further investigation, as this might provide another level of control over the antigen load delivered to the draining lymph node.

Apart from their synthesis of immunosuppressive molecules, other studies show that uterine Mϕs can secrete an array of cytokines, including TNFα, IFNγ and IL-1, and these may also be implicated in the maintenance of homeostasis within the cycling uterus through regulatory actions on uterine NK cells and other leukocytes, even Mϕs themselves (Hunt 1993, Hunt and Robertson 1996) (Figure 8.1).

8.3 The response of uterine APCs to insemination

In the 8-12 hours following insemination, uterine APCs exhibit a number of changes in their density and activation status of a nature comparable to that which accompanies inflammation in other organs. The current and previous immunohistochemical studies show that as well as Mϕs expressing F4/80, the numbers of uterine APCs expressing macrosialin, class A scavenger receptor, sialoadhesin, Ia and B7-2 are all markedly enhanced in the myometrium and particularly the endometrium after mating. It also appears that uterine Mϕs are functionally different following mating, since the studies in Chapter 7 show that these cells exhibit a diminished immunosuppressive phenotype at day 1 of pregnancy, and it was proposed that this be due to either (1) 'dilution' of highly suppressive resident Mϕs by undifferentiated, newly recruited cohorts of cells or (2) differentiation of resident cells to become more immunostimulatory in nature, perhaps through their cessation of inhibitory molecule secretion and/or preferential secretion of inflammatory cytokines such as IL-1, TNFα and IFNγ. Correspondingly, the current studies provide several lines of evidence that the dynamics of uterine APC arrival into the tissue, activation and exodus through draining lymph vessels is enhanced following insemination. For example, undifferentiated and differentiated uterine Mϕs exhibited diminished expression of CD11c/CD18 at day 1 of pregnancy, and enhanced numbers of APCs expressing F4/80, macrosialin and scavenger receptor were present in the mesometrial triangle following mating. By extrapolation from the literature describing inflammatory responses in other mucosae, it is reasonable to suggest that a considerable proportion of the APCs recruited to, differentiating within and trafficking from the inseminated uterus would be dendritic cells and their precursors, particularly during the first few 1-2 hours following mating (McWilliam *et al.* 1994) (Figure 8.2).

It is likely that the enhanced recruitment, activation and exodus of uterine APCs to draining lymph nodes following mating is driven by the enhanced expression of inflammatory cytokines and chemokines in uterine epithelial and stromal cells at this time. Although uterine APCs expressing all of the examined phenotypic markers were evident in the uteri of GM-CSF-deficient mice, APCs expressing scavenger receptor and Ia were found to be diminished. Thus GM-CSF appears to play a specific role in recruiting and/or activating specific subsets of uterine APCs in a manner analogous to that proposed to operate in other mucosal organs (Figure 8.2). The flow cytometric studies performed in these studies suggest a particular role for GM-CSF in regulating uterine dendritic cell populations, although it is clear that cytokines and chemokines other than GM-CSF can perform similar or indistinguishable roles. However, whether uterine APCs in GM-CSF-deficient mice are functionally different from those in wildtype mice remains

to be explored. Other cytokines synthesised in the uterus which might regulate the recruitment, activation and differentiation of uterine Mϕs and dendritic cells include CSF-1, TNFα, IFNγ, TGFβ and IL-6. Evidence that steroid hormones may directly regulate uterine leukocytes is also emerging (see Figures 8.1 and 8.3).

8.4 The role of uterine APCs in the initiation of immune responses in the inseminated uterus

Insemination is a time at which the uterus is exposed to many and diverse antigens, including sperm and other cells of paternal origin, and presumably commensal but potentially also pathogenic microorganisms. The maintenance of uterine homeostasis and the establishment of an immunological environment conducive to pregnancy would require that appropriate immune responses are generated against these antigens. The nature and magnitude of immune responses initiated against paternal antigens might prove critical not only to ensure rapid and non-disruptive clearance of sperm and other paternal cells, but also for the success of blastocyst implantation. The ability to mount disparate immune responses to different microorganisms would also have consequences; for example, pathogenic bacteria require active defence whereas an immune response of a permissive nature would presumably be appropriate for commensal bacteria. It is now commonly believed that the context in which a foreign antigen is first encountered is critical in determining the nature of an ensuing immune response. Specifically, the cell membrane features of APCs involved in antigen uptake, processing and presentation, and the cytokines to which they are exposed and which they secrete can result in polarised T cells of various phenotypes, or mediate tolerance through T cell anergy or deletion. To date, there has been very little specific examination of the roles of uterine APCs in initiating antigen-specific immune responses in the uterus following mating. However, a number of speculations are justified based on the many similarities which exist between the cell populations and regulatory networks present in the uterus and those observed in other mucosal organs in which immune responses are better characterised. The possible events leading to the generation of antigen-specific immunity in the inseminated uterus are summarised in Figure 8.2.

8.4.1 Mechanisms contributing to heterogeneity in immune responses initiated at insemination

One of the principal determinants of the nature of an immune response elicited against a particular uterine antigen would be the type of APC predominantly involved. Immune responses initiated by mature uterine dendritic cells could be expected to be rapidly generated and of a high

affinity due to the efficient antigen uptake and processing behaviours and potent T lymphocyte-activating capabilities of these cells. While uterine Mϕs could also be expected to exhibit a number of the features of dendritic cells, whether they can generate the same types of immune responses is debatable (Banchereau and Steinman 1998). Instead, their relatively slow and inefficient antigen processing behaviour combined with their poor ability to effectively bind and co-stimulate naïve T cells could lead to the generation of anergised T cells or their deletion (Croft and Dubey 1997). Antigen presentation by non-professional APCs such as uterine epithelial cells could also generate T cell anergy due to a paucity of costimulatory molecule delivery (Croft and Dubey 1997) or subtle differences in the structure of antigen presentation molecules (Porcelli and Modlin 1999). Alternatively, uterine Mϕs, dendritic cells or epithelial cells expressing non-classical MHC molecules such as CD1 might be expected to activate unusual T cell subsets, such as NKT cells, whose activation-induced secretion of IL-4 or other cytokines could have a marked influence on the phenotypes of other leukocytes in the vicinity (Bendelac *et al.* 1997).

Heterogeneity within the phenotypes of specific APC subsets would be a further determinant of the quality of a T cell response to a particular uterine antigen. For example, emerging information shows clearly that alternative phenotypes in dendritic cells are associated with skewing the differentiation of Th0 cells to assume Th1, Th2, Th3 or Tr1 phenotypes. There are many cytokines and other biological mediators induced at mating or present in the ejaculate which could provide the final differentiation signals to dendritic cells such that they might preferentially activate certain T-lymphocyte subsets. Specifically, IFNγ and GM-CSF derived from uterine epithelial cells in combination with LPS introduced with the ejaculate would be expected to induce Th1-polarising dendritic cells (DC-1 cells). Other uterine dendritic cells might be induced to assume DC-2 or DC-3 phenotypes following exposure to prostaglandins and/or seminal vesicle-derived or locally synthesised TGFβ respectively. Uterine Mϕs could prove to be an important source of many of the differentiation factors active on dendritic cells, including IL-12 and IL-10. Thus, the profile of cytokines and other locally synthesised factors prevailing at the time of introduction of a particular uterine antigen would be critical in determining the nature of any ensuing immune response. Although currently the DC-1, -2 and -3 dendritic cell subsets are defined according to the profile of cytokines they secrete, better characterisation of these cells in the future might allow the use of specific mAbs to label their cell membranes in uterine tissue sections or by flow cytometric analysis.

A final mechanism by which uterine APCs might achieve differential responses to antigens would be mediated by their varied abilities to take up and process different antigenic

moieties, presumably due to diversity in expression of cell membrane molecules. For example, the expression of different endocytic and phagocytic receptors by Mϕs versus dendritic cells or epithelial cells might direct certain antigens towards specific cells and even different intra-cellular processing pathways. For example, the presence of sialyated moieties on the membrane of sperm cells might target them to uptake by uterine APCs expressing class A scavenger receptors or macrosialin. Other lectins on bacteria would preferentially bind to APCs expressing DEC-205. Furthermore, whether endometrial APCs acquire antigens directly from the uterine lumen or by phagocytosis of apoptotic antigen-containing epithelial cells might also have an impact on an ensuing immune response, since it has been proposed that antigens acquired in the latter manner may be processed in the class I MHC processing pathway. The presentation of such antigens in the context of class I MHC would activate $CD8^+$ T cells. Also, the expression of classical or non-classical MHC molecules such as CD1 by uterine APCs could limit the cells to presenting protein or glycolipid antigens respectively (Porcelli and Modlin 1999).

8.4.2 The generation of immune tolerance to paternal antigens at insemination

While the generation of appropriate immune responses to cells, tissue debris and micro-organisms present in semen would be critical in order to maintain uterine homeostasis and prevent infection, the consequences of the immune response initiated against paternal antigens following mating are likely to be of paramount importance for pregnancy. A cell mediated or cytotoxic antibody-response mounted against paternal antigens encountered during the clearance of sperm and other paternal cells after mating is known to limit the availability of viable sperm for fertilisation. Furthermore, a destructive immune response initiated against paternal antigens common to sperm and the conceptus could lead to pregnancy loss. A state of paternal antigen-specific tolerance which is believed to be initiated at the time of mating (Robertson *et al.* 1997) and active throughout pregnancy has been attributed to the deletion and functional anergy of paternal antigen-specific T cells (Tafuri *et al.* 1995, Jiang and Vacchio 1998). The generation of such responses would clearly implicate uterine APCs of tolerance-inducing phenotypes present in the inseminated uterus. The synthesis of cytokines such as IL-10 by uterine APCs might lead to TCR downregulation and anergy. Alternatively, antigen-specific T lymphocyte deletion, although less likely, might be accomplished through inadequate APC expression of costimulatory molecules at the time of activation.

8.5 The roles of uterine APCs at implantation

Of necessity for successful pregnancy is suppression of uterine immune responses with the potential to damage the implanting conceptus or maternal structures critical for its survival. Clearly, preventing the generation and/or activity of Th1-deviated conceptus-reactive T cells during pregnancy would seem vital. However, the mechanisms by which this might be achieved are still unclear. For example, whether uterine APCs persisting in the uterus at implantation are active in the uptake, processing and presentation of conceptus antigens at implantation and during the ensuing days of pregnancy has not been determined. The current studies show that distinct, highly differentiated populations of APCs are retained in the uterus on day 4 of pregnancy and that a proportion of these accumulate in unusual aggregates in the deep endometrium. Better characterisation of the cells contained within these aggregations might prove illuminating in understanding the immune response to pregnancy. In particular, it would be interesting to determine whether they contain Mϕs of an immunosuppressive nature, dendritic cells of distinct phenotypes (such as DC-2 or DC-3), or indeed expanding clones of T-helper or other T lymphocytes. One intriguing possibility is that these aggregates represent expanding populations of T lymphocytes induced during the post-mating response that are reactive with antigens shared by the conceptus. If such cells were found to have a Th3 or Tr1 phenotype it might be expected that the phenomenon of 'bystander suppression' could act to diminish responses to other antigens within the implantation site (Figure 8.3).

As well as their involvement in antigen-specific immunity, there are many other potential roles of Mϕs within the pregnant uterus (Figure 8.3). The current and other studies find that uterine Mϕs during pregnancy are highly immunosuppressive (Hunt *et al.* 1984), perhaps reducing the likelihood of any new immune responses, particularly those of a Th1 nature, being induced after implantation. Mϕs in the uterus at the time of implantation express diverse cell membrane endocytic and phagocytic receptors which implicate them in roles of tissue repair and turnover (Hunt and Pollard 1992, Stewart and Mitchell 1992, Kyaw *et al.* 1998). The susceptibility of these Mϕs to further fluctuations in phenotype is unknown, but it could be hypothesised that in the event of infection, the action of bacterial products such as LPS and locally produced cytokines including IFNγ and TNFα might lead uterine Mϕs to assume highly activated phenotypes for the clearance of the invading pathogens (Hunt 1990). In the case of overwhelming infection or fetal demise, it might be speculated that the accompanying cascade of pro-inflammatory cytokines would provide a means by which a pregnancy could be terminated

through activating local Mϕ populations with apoptosis-inducing and cellular remodeling properties.

8.6 Implications for the human

A better understanding of the nature and the roles of APCs in the uterus will have considerable implications for human reproductive medicine. It is already clear that the human uterus contains populations of myeloid APCs which are regulated during the menstrual cycle in a steroid hormone-dependent manner (Prabhala and Wira 1995, Wira and Rossoll 1995, Yeaman *et al.* 1998), and at least in cervical tissues these cells respond to insemination in terms of recruitment and activation in a manner similar to that observed in rodents (D. Sharkey and S. Robertson, unpublished). Furthermore, human uterine tissues are known to synthesise GM-CSF and other cytokines which are implicated in the regulation of APCs and other leukocytes (reviewed in Robertson *et al.* 1994) and human seminal plasma is a potent source of immunomodulatory cytokines such as TGFβ (Nocera and Chu 1993, Nocera and Chu 1995). However, some key differences in the physiology of the human and rodent reproductive systems limit the direct application of the conclusions drawn herein to the human situation. Firstly, in humans and many other mammals, insemination is not intra-uterine but instead is intra-vaginal, so that the APC populations which might be exposed to antigens and cytokines in semen are those present within the vaginal epithelium and the cervix rather than those contained in the endometrium. Also, that the female reproductive tract might be exposed to semen at any stage of the menstrual cycle and not only in the peri-ovulatory period might mean that the nature and extent of APC responsiveness to insemination could vary according to the prevailing steroid hormone milieu.

A better characterisation of the processes governing immune responses in the female reproductive tract would provide some opportunity for manipulation of immune responses by therapeutic application of specific cytokines or their analogues or antagonists (Kelso, 1998). For example, the modification of detrimental immune responses associated with recurrent spontaneous abortion is an exciting prospect. Alternatively, the deviation of existing Th2-polarised immune responses to sexually transmitted diseases such as chlamydia (Westbay *et al.* 1995) and HIV (de Martino *et al.* 1999) might improve clinical outcomes by providing protective cell mediated immunity. Implications might also extend to manipulation of immune responses at other mucosal surfaces during infection or following allogeneic tissue grafts.

8.7 Future perspectives

The experiments presented in this thesis complement and extend many previous studies which suggest that the murine uterus contains heterogeneous and dynamic populations of myeloid APCs. There are many exciting possibilities for the further extension of these studies, and a number of these have been highlighted in Chapters 3-7. Overall, the aim of future studies would be to more precisely define the lineages and phenotypes of uterine APCs, their regulation by local cytokines, growth factors and steroids, and the types of immune responses which they initiate in the uterus.

A better understanding of the phenotypes and lineages of uterine APCs and the populations of T lymphocytes with which they interact could be achieved with the use of multiple-colour confocal microscopy and FACS analysis of uterine and draining lymph node tissues. In combination with RT PCR and cell culture analysis, this would allow better understanding of the profiles of cytokines produced by uterine APCs and lymphocytes during the estrous cycle, and of key interest would be whether these are induced to 'switch' during the post-mating inflammatory response. Specifically, whether uterine dendritic cells synthesise potent T lymphocyte-polarising cytokines such as IL-10 or IL-12 associated with DC-2, DC-2 or DC-3 phenotypes (Stumbles *et al.* 1998), and the phenotyping of cytokine secretion by T lymphocytes within draining lymph nodes (Constant and Bottomly 1997) would be informative of the type of immune responses initiated to uterine antigens.

The use of cytokine-deficient or -depleted mice remains a highly informative strategy for defining roles of specific cytokines in regulation of uterine APCs. An alternative approach would be to undertake a phenotypic analysis of uterine APCs following their culture in specific cocktails of cytokines, or after their isolation from animals following administration of exogenous cytokines or cytokine antagonists. Cytokines which might prove enlightening in this regard include those involved in early dendritic cell development (such as GM-CSF and IL-3), those thought to play a role in regulating peripheral dendritic cell populations in the steady state (examples include Flt3-ligand, GM-CSF and TGFβ) and cytokines believed to initiate differentiation and phenotypic maturation of alternative phenotypes of dendritic cells during inflammatory responses, such as GM-CSF, IL-12, IL-10, TNFα and TGFβ.

The fate of antigens introduced into the uterus might be explored by passive transfer of soluble, particulate or cellular proteins or lipids into the uterine lumen. Subsequent isolation of Mϕs, dendritic cells or epithelial cells from these uteri and their culture with naïve or sensitised T cells would permit assessment of their potency as APCs (Prabhala and Wira 1995). T cell clones generated during such experiments could be phenotyped by analysis of intracellular cytokine

expression with a view to discerning the roles of specific cytokines in driving certain immune responses. A similar approach, for example using fluorescent labeled antigens of various forms, might be exploited to investigate the existence of alternative pathways for the differential uptake and processing of antigens. The administration of labeled Mϕ and dendritic cell precursors to the blood or directly into the uterus would allow tracking of differentiation and emigration pathways in the steady state and following insemination.

These are just some of the approaches which could be persued to better understand the regulation of immunity in the murine uterus. The adoption of experimental strategies from studies of the networks governing myeloid APCs in other mucosal tissues is likely to remain a fruitful approach and the clear similarities between mucosal tissues will almost certainly ensure that principals emerging from studies in other tissues will guide work in the reproductive tract. However, any investigation of immunity in reproductive tissues needs to be considerate of the unique endocrine and paracrine networks which operate in these organs. Ovarian steroid hormones and the locally synthesised cytokines which they regulate, the introduction of semen at the time of mating and subsequently the development of the conceptus and its secreted hormones and cytokines are all unique factors which will dramatically influence local APC populations.

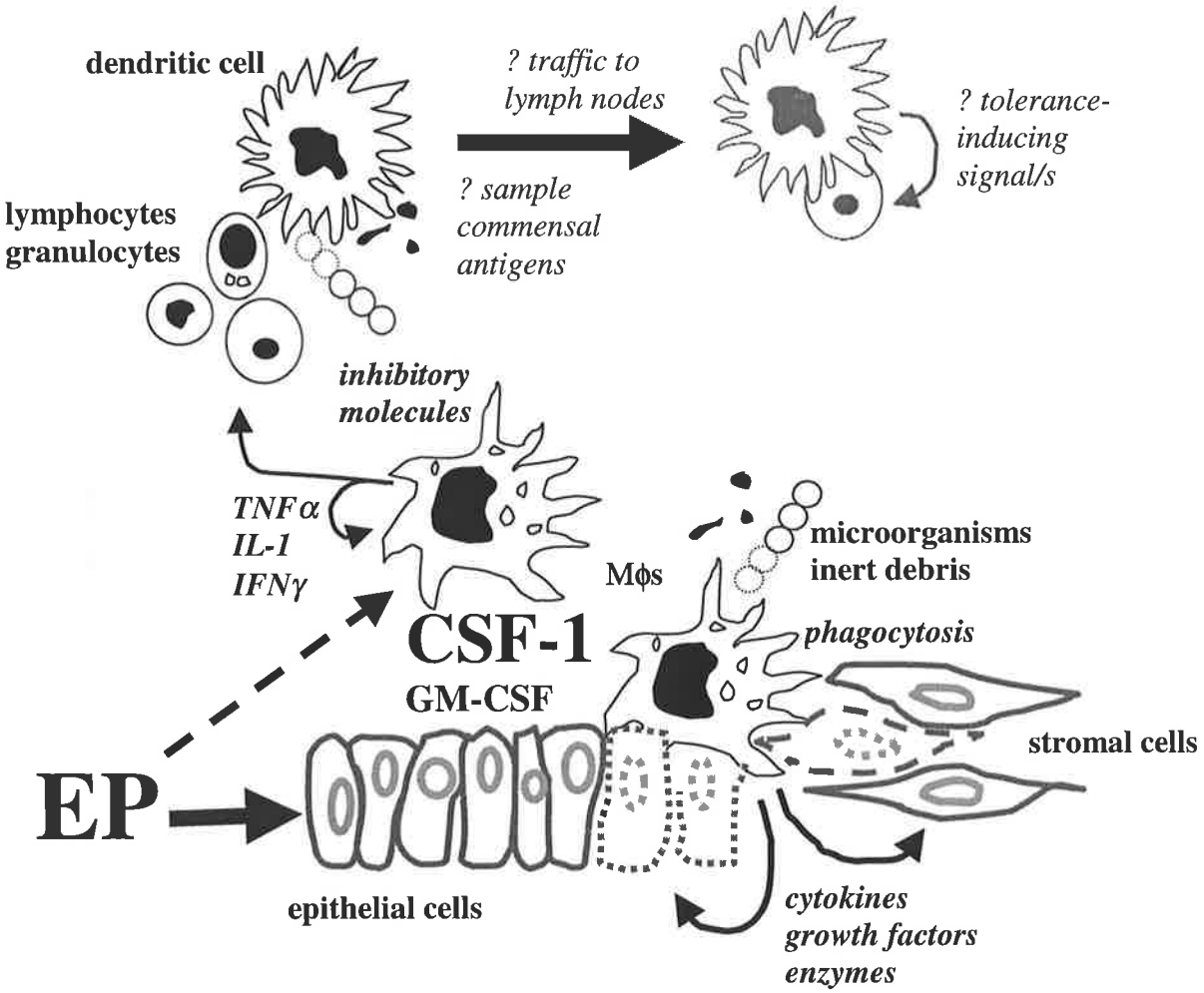
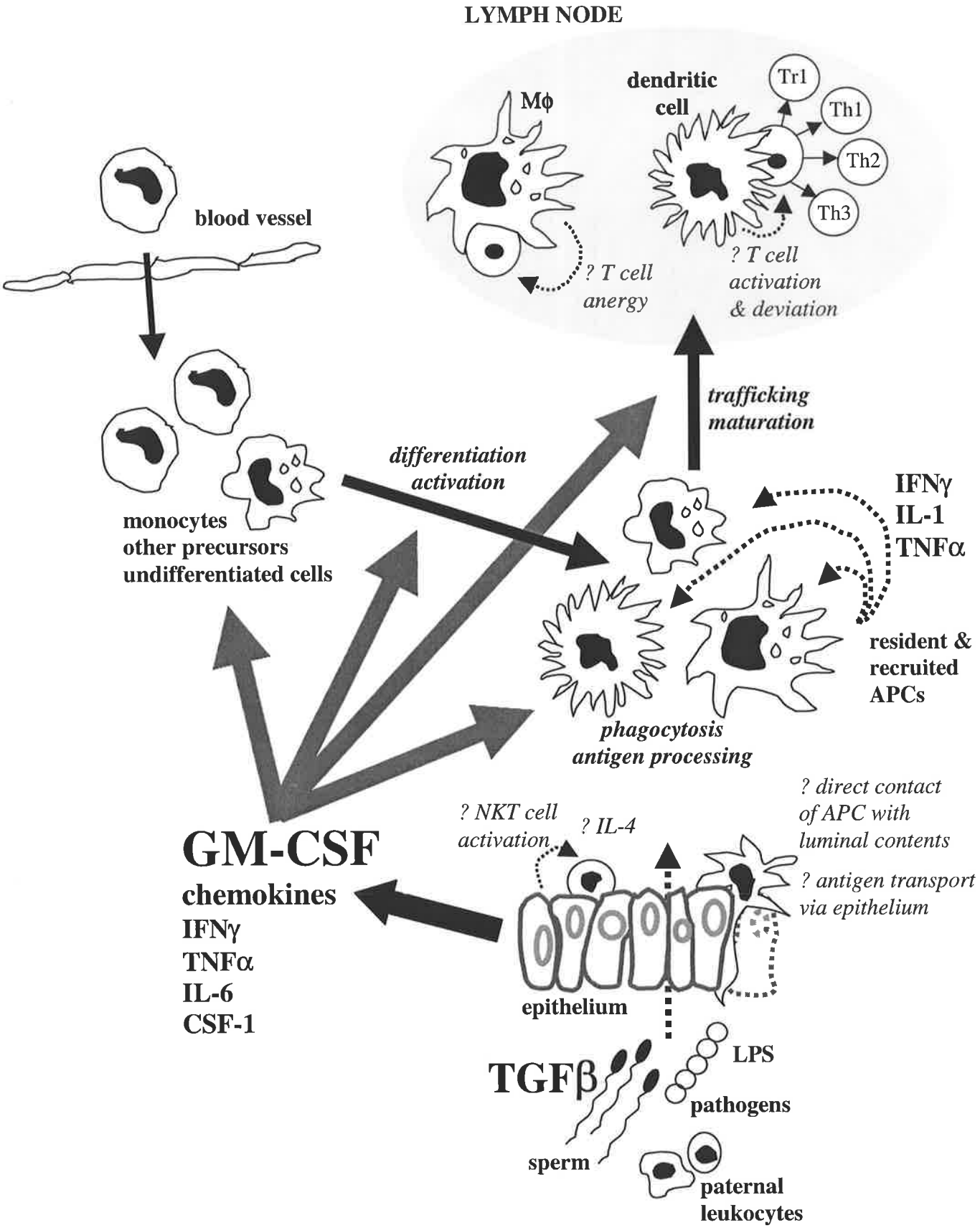


Figure 8.1 A model for regulation and roles of uterine APCs during the estrous cycle. The steroid hormone-regulated synthesis of CSF-1, GM-CSF and other cytokines by uterine luminal and glandular epithelial cells is thought to regulate the recruitment and function of populations of uterine Mϕs and dendritic cells during the estrous cycle. By virtue of their phagocytic activity and secretion of cytokines, growth factors and enzymes, uterine Mϕs are implicated in mediating tissue breakdown and remodeling, as well as providing innate immune protection against opportunistic microorganisms. The synthesis of cytokines and inhibitory molecules by uterine Mϕs during the estrous cycle is likely to regulate other populations of uterine leukocytes, including lymphocytes, granulocytes and dendritic cells. It is postulated that the action of Mϕ-derived inhibitory molecules might induce a phenotype in uterine dendritic cells such that a state of functional tolerance is induced towards 'self' antigens and those associated with commensal microorganisms.

Figure 8.2 A model for regulation and roles of uterine APCs after insemination. It is proposed that seminal factors including $TGF\beta_1$ stimulate enhanced release of GM-CSF, chemokines and other inflammatory cytokines from uterine epithelial cells. Together, these cytokines would have a number of actions on myeloid APCs, including enhanced recruitment of precursor and other undifferentiated cells from the blood stream, differentiation of resident and recruited cells, uptake and processing of paternal and other antigens acquired directly from the uterine lumen or by phagocytosis of epithelial cells, and phenotypic maturation and trafficking of antigen-bearing APCs to the draining lymph nodes. Other cytokines and factors synthesised within the uterine milieu which may impact on these processes are derived from Mϕs, and perhaps also unusual T lymphocytes such as NKT cells which might become activated through their interaction with myeloid or other APCs. In the draining lymph node, activation of antigen-specific T lymphocytes is determined by the lineage of the APC as well as the nature of costimulatory signals delivered at the time of interaction. Possible outcomes include anergy, or the polarisation of Th cells to assume Th1, Th2, Th3 or Tr1 phenotypes.


LYMPH NODE
Mϕ
dendritic cell
Tr1
Th1
Th2
Th3
? T cell anergy
? T cell activation & deviation
blood vessel
trafficking maturation
differentiation activation
monocytes
other precursors
undifferentiated cells
IFNγ
IL-1
TNFα
resident & recruited APCs
phagocytosis antigen processing
? direct contact of APC with luminal contents
? NKT cell activation
? IL-4
? antigen transport via epithelium
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IL-6
CSF-1
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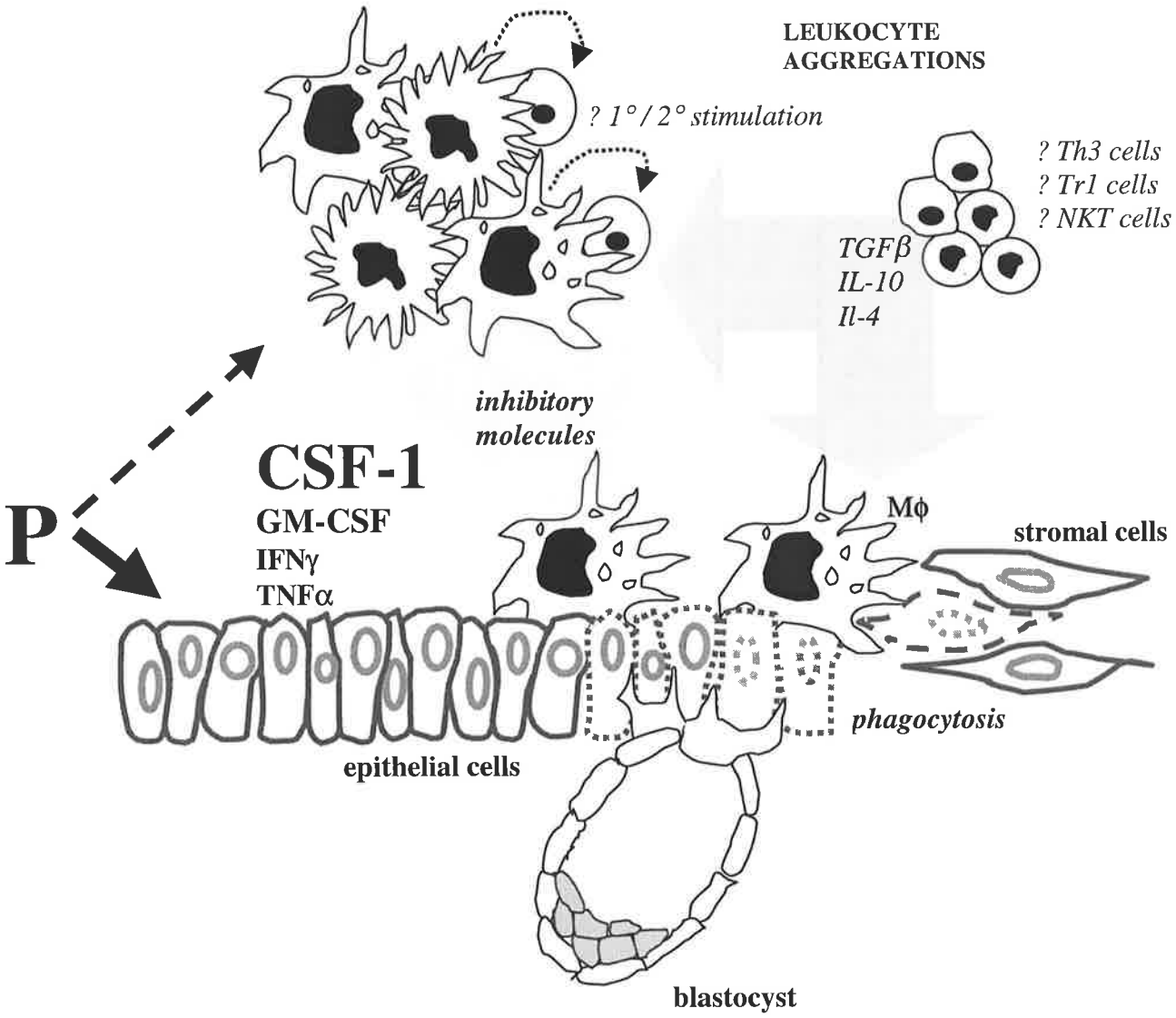

Figure 8.3 A model for regulation and roles of uterine APCs at embryo implantation. It is hypothesised that single or aggregated populations of uterine APCs present in the uterus at day 4 of pregnancy are dependent upon CSF-1 and other cytokines derived from the progesterone-primed uterine epithelium. Uterine Mϕs at this time are likely to have tissue breakdown and remodeling roles, as well as maintaining immunological homeostasis through the synthesis of inhibitory molecules. Mϕs and dendritic cells may also be involved in the uptake and processing of antigens expressed on the conceptus, and presentation of these antigens to naïve or memory T cells in the uterus or draining lymph node. The outcome of immune responses initiated against these antigens could be influenced by the secretion of cytokines by local populations of lymphocytes recruited following their antigen-specific activation during the inflammatory response to insemination. The bystander suppressive effects of such T cells, perhaps of Tr1, Th3 or NKT phenotypes, would be antigen non-specific.

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