



**Paracrine regulation of ovarian function by
Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF)
&
Colony-Stimulating Factor-1 (CSF-1)**

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“Sit down before fact like a little child, and be prepared to give up every preconceived notion, follow humbly wherever to whatever abysses Nature leads you to or you shall learn nothing.”

Thomas Huxley
(1825-1895)

Abstract

The ovaries have two primary functions, the production of oocytes suitably mature for fertilisation, and the secretion of associated substances such as hormones and growth factors that facilitate development of the female reproductive tract and promote fertility. The regulation of ovarian activity has long been considered to be primarily dependent upon hormonal stimuli originating in the pituitary gland in the brain. However, in the past decade, a wide range of additional regulators of ovarian function have been discovered which act locally to modulate hormone action within the ovary.

Study of the cells and mediators shown to be important in ovulation has revealed a surprising similarity between ovulation and the classic inflammatory processes which occur elsewhere in the body in response to pathogenic stimuli or during allergic reactions (Espey, 1980). Most significantly, macrophages and neutrophils have been shown to be the predominant populations of leukocytes present in the ovary and are thought to provide mediators contributing to the regulation of follicular growth, development and rupture (Brännström and Norman, 1993). The recruitment, differentiation, survival and activation of leukocytes within the ovary are likely to be orchestrated by cytokines synthesised by resident ovarian cells. The means by which leukocytes interact with other cells in the ovary to coordinate ovarian events are not well understood, but they are known to secrete a range of cytokines and other biological mediators that can influence the behaviour and secretory function of resident ovarian cells.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) and colony-stimulating factor-1 (CSF-1) are potent regulators of the activation and secretory profiles of macrophages and neutrophils. The GM-CSF receptor (GM-CSFR) is a complex comprised of two subunits, α and β , the latter of which has been found to be expressed only by leukocytes. CSF-1 initiates biological activity through binding its receptor, c-fms. GM-CSF is of particular interest as a potential regulator of ovarian leukocytes since its synthesis in the ovary fluctuates throughout the cycle, peaking around the time of ovulation (Brännström *et al*, 1994). In addition, mice of the naturally occurring CSF-1 deficient *csfm^{op}/csfm^{op}* strain have a major fertility defect (Pollard *et al*, 1991).

The purpose of this study was to investigate the role of colony-stimulating factors in the murine ovary utilising the mice genetically deficient in GM-CSF (GM-/-) (Stanley *et al*,

1994) and CSF-1 (*csfm^{op}/csfm^{op}*). In addition, the expression of components of the GM-CSF and CSF-1 signalling systems in the normal mouse ovary has been investigated.

Expression of mRNA for the GM-CSF and CSF-1 signalling systems in the whole ovary

Whole ovaries were collected from immature, preovulatory, ovulatory and luteinised mice after priming with gonadotrophic hormones. Total cellular RNA was extracted, reverse transcribed into cDNA and subjected to quantitative polymerase chain reaction (PCR) amplification employing *Taq* DNA polymerase. Analysis of the GM-CSF PCR product showed that the initial amount of GM-CSF mRNA was variable throughout the cycle, being highest in the luteinised ovary, when expression increased approximately 7 fold over that in the ovulatory ovary. GM-CSFR α and GM-CSFR β mRNA were detected at all stages of the cycle and levels appeared to increase moderately during the periovulatory period. CSF-1 and c-fms mRNA were detected at all stages of the cycle.

Tissue compartment mRNA expression of the GM-CSF and CSF-1 signalling systems

Whole follicles, theca shells, granulosa cells, corpora lutea and oocyte/cumulus cell complexes were dissected from ovaries, and total cellular RNA was extracted, reverse transcribed and subjected to PCR amplification. Expression of GM-CSF mRNA was restricted to corpora lutea and theca shells of ovulating ovaries. GM-CSFR α and GM-CSFR β mRNA were detected in all tissue compartments except granulosa cells (GM-CSFR β), indicating the capacity of cells in each of these tissue compartments to bind the receptor ligand. For the oocyte/cumulus cell complex, expression of GM-CSF mRNA was detected following ovulation, when the cells had left the confines of the follicle. CSF-1 and c-fms mRNA were detected in all tissue compartments examined.

Oestrous cycle length and ovulation rate

The oestrous cycle of adult mice was tracked by daily vaginal smearing and found to be extended by approximately 1.5 days in GM-/- mice and 3.9 days in *csfm^{op}/csfm^{op}* mice. Comparable numbers of oocytes were ovulated *in vivo* by GM+/+ and GM-/- mice regardless of genotype or hormone priming. However, *csfm^{op}/csfm^{op}* mice ovulated less frequently and ovulated less oocytes. In addition, when development and viability of GM-/- oocytes was assessed, blastocyst development was found to be delayed and the blastocyst comprised fewer inner cell mass cells on day 4 of pregnancy.

Steroidogenesis

Differences in ovarian steroidogenesis were observed in cytokine deficient mice. In *in vitro* perfusion experiments of GM-/- ovaries, ovarian progesterone production per oocyte ovulated appeared reduced. Following natural ovulation, plasma progesterone was unaltered by GM-CSF deficiency but, on day 4 of pregnancy, plasma progesterone was reduced in the GM-/- mouse. In addition, GM-CSF appeared to stimulate androstenedione synthesis *in vitro*. In the *csfm^{op}/csfm^{op}* mouse, plasma progesterone production was also reduced following natural ovulation.

Distribution of ovarian leukocytes

Ovaries were collected from immature and primed mice during the periovulatory period and pregnancy. Frozen sections were fixed in 96% ethanol and leukocytes were detected using lineage-specific rat anti-mouse monoclonal antibodies, followed by biotinylated anti-rat Ig and avidin-horseradish peroxidase, and the chromogenic agent DAB detection system. Leukocytes including macrophages, neutrophils and activated macrophage/dendritic cells were localised predominantly to the thecal layer of follicles, corpora lutea and within stromal tissue of the ovaries. While the number of leukocytes was unaltered by GM-CSF deficiency during the periovulatory period, activated macrophage/dendritic cells were significantly decreased in the stromal region of ovulating ovaries in GM-/- mice. Following pregnancy, the number of macrophages and neutrophils was significantly decreased in the stromal region of postpartum GM-/- ovaries. The macrophage population in the *csfm^{op}/csfm^{op}* ovary was significantly reduced in abundance.

In summary, this study focussed on the synthesis and role of GM-CSF and CSF-1 in ovarian events using mice deficient in these cytokines. mRNAs encoding the ligand and receptor components of the GM-CSF and CSF-1 signalling system were found to be expressed in the normal mouse ovary throughout the oestrous cycle. The findings that the oestrous cycle was extended and the number of activated macrophage and/or mature dendritic cells was markedly reduced in GM-/- mice, along with temporal GM-CSF mRNA fluctuations and variation between tissue compartments suggests that GM-CSF may play a pivotal role in regulating the activation of macrophage/dendritic cells in the ovary at the time of ovulation and during corpus luteum development. In addition, the findings suggest that GM-CSF may play a role in regulating the number of macrophages and neutrophils in the ovary during the

regression of the corpus luteum. The findings that the oestrous cycle was extended, ovulation was reduced and the number of macrophages was markedly diminished in *csfm^{op}/csfm^{op}* mice suggests that CSF-1 may play a pivotal role in regulating the processes of folliculogenesis and ovulation. The data show that leukocytes are a major cellular component of ovarian tissue throughout the oestrous cycle in the mouse and that CSF-1 appears to regulate their abundance in the ovary. Impaired recruitment, viability and activation of myeloid leukocytes in the ovary may directly or indirectly contribute to the disruption of the regulation of ovarian function during the oestrous cycle and early pregnancy.

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university and that, 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.

Melinda Jane Jasper

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Publications arising from these and related studies

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2. **Jasper MJ**, Robertson SA, van der Hoek KH, Norman RJ. Characterisation of ovarian function in granulocyte-macrophage colony-stimulating factor (GM-CSF) deficient mice. *Endocrinology* (submitted).
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Abbreviations

ATP	adenosine triphosphate
ANOVA	analysis of variance
α	alpha, anti-
bp	base pair
Bq	becquerel
β	beta
BSA	bovine serum albumin
cm	centimetre(s)
CSF	colony-stimulating factor
CSF-1	colony-stimulating factor-1
CL	corpus luteum
CTP	cytosine triphosphate
D	dalton(s)
°C	degrees Celsius
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DAB	diaminobenzidine
ELISA	enzyme linked immunosorbent assay
eCG	equine chorionic gonadotrophin
EtBr	ethidium bromide
EDTA	ethylenediamine tetra-acetate
FSH	follicle stimulating hormone
g	gram(s)
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
GTP	guanosine triphosphate
HEPES	<i>N</i> -2-hydroxyethylpiperazine- <i>N'</i> -2-ethane sulfonic acid
hCG	human chorionic gonadotrophin
HRP	horseradish peroxidase
hr	hour(s)
Ig	immunoglobulin

iNOS	inducible nitric oxide synthase
IGF	insulin-like growth factor
IL	interleukin
IO	immature ovary
ICM	inner cell mass
IU	international unit
ip	intraperitoneal(ly)
k	kilo
kb	kilobase pairs
LCA	leukocyte common antigen
l	litre(s)
LH	luteinising hormone
LHRH	luteinising hormone releasing hormone
LIF	leukemic inhibitory factor
LO	luteinised ovary
m	mole(s)/meter(s)
μ	micro (10^{-6})
m	milli (10^{-3})
min	minute(s)
MHC	major histocompatibility complex
M	moles per litre
M_r	molecular weight
mAb	monoclonal antibody
m	murine/mouse
n	nano (10^{-9})
NO	nitric oxide
NMS	normal mouse serum
O+C	oocyte/cumulus cell complex
OD	optical density
OO	ovulatory ovary
%	percent
PO	preovulatory ovary
PG	prostaglandin

PBS	phosphate buffered saline
PCR	polymerase chain reaction
RIA	radioimmunoassay
RT	reverse transcription
RNA	ribonucleic acid
sec	second(s)
SD	standard deviation
SEM	standard error
SCF	stem cell factor
sc	subcutaneous
tRNA	transfer RNA
Tris	tris(hydroxymethyl)-aminomethane
TE	trophectoderm
TNF	tumour necrosis factor
UV	ultraviolet
UTP	uracil triphosphate

Chapter 1

Review of the literature



Aristotle's (384-322BC) "seed and soil" view of reproductive biology, whereby the female played a passive role in providing the soil for the male seed to grow, persisted for almost 2000 years. The presence and role of the ovary, often referred to as the "female testicle" created much confusion and speculation as illustrated by the scientific literature of the time. It was however noted that removal of the ovaries or destruction by disease "arrests the characteristic luxuriances of form which males admire in women, whereby they assume the drier texture, the harder outline, and angular harshness of men" (John Tilt, 1850 reviewed by Short, 1977).

The culmination of centuries of observations has now provided an insight into the role of the ovary in generating the female gamete or oocyte and in synthesising the steroid hormones necessary for the development of female characteristics and the establishment and maintenance of pregnancy.

A further paradigm shift has occurred in recent years as evidence accumulates to demonstrate a role for the immune system in ovarian function. Immunosuppression or immunodeficiency as a result of neonatal thymectomy or congenital athymia (as in the nude mouse) can alter ovarian function, as characterised by delayed puberty, lymphocyte infiltration, reduced numbers of oocytes, increased follicular atresia, decreased ovarian steroidogenesis and premature ovarian failure (Nishizuka and Sakakura, 1969). Restoration of many of these characteristics can be achieved by the grafting of spleen, thymus or lymphoid tissue.

Conversely, the reproductive system appears to influence many aspects of immune function. In the female both humoral and cell mediated immune responses are more active than in the male. The mechanism underlying sexual dimorphism in the immune response is thought to be related to oestrogen synthesis after puberty in females. Steroid hormones have also been implicated to explain the sex-related difference in the incidence of autoimmune disease, which is higher in females.

Leukocytes have been identified in ovarian tissue where they appear to participate in intracellular communication networks as both a source and a target for locally acting mediators of cell function. Cytokines and other cell-signalling molecules often associated with inflammatory events appear to have roles in the processes of folliculogenesis, ovulation, luteinisation and luteolysis. However, the identification and precise functions of regulators of

leukocyte recruitment and behaviour, and characterisation of the exact roles of these cells in tissue remodelling events in the ovary, is still a major experimental challenge.

The aim of the following discussion is to review literature pertaining to the regulation of ovarian function in relation to the classical gonadotrophic hormones and local regulation via the leukocyte-cytokine network.

1.1 Physiology of the ovary

1.1.1 Structure and function of the ovary

The female reproductive system endeavours to sustain orderly development and maturation of an oocyte, its expulsion from the ovary surface and the preparation of a suitable environment for implantation, pending fertilisation. The endocrine activities of the female subserve this function and hormone levels fluctuate extensively throughout the oestrous cycle, notably within the peripheral blood. A finite number of primordial follicles, each containing an immature oocyte, are located within the ovary of the female even before the time of birth (Figure 1.1). Rigorous selection systems act on the oocytes at all stages of development and the number of oocytes eventually ovulated reflects the optimal number of young the female of a species can bear and successfully rear after birth. Continuous and extensive remodelling of the ovarian tissues is a major characteristic of the cyclicity of the female reproductive system. Within the ovary at any given time there is concurrently occurring development and demise of different compartments, with cellular differentiation, growth and development as well as atresia and apoptosis.

Blood supply to the ovary is via the ovarian artery, however supply to the various structures within the ovary including the follicles and corpora lutea varies considerably depending on the stage of development of these structures. The corpus luteum has one of richest blood supplies per gram of any tissue in the body, including the renal cortex. Venules surrounding the arteries drain the ovary, sometimes into the utero-ovarian vein. As a consequence of this close association, countercurrent transfer of substances results in local feedback between the ovary and uterus.

The ovary is innervated by parasympathetic and sympathetic nervous systems; the relative importance of these synapses is unclear.

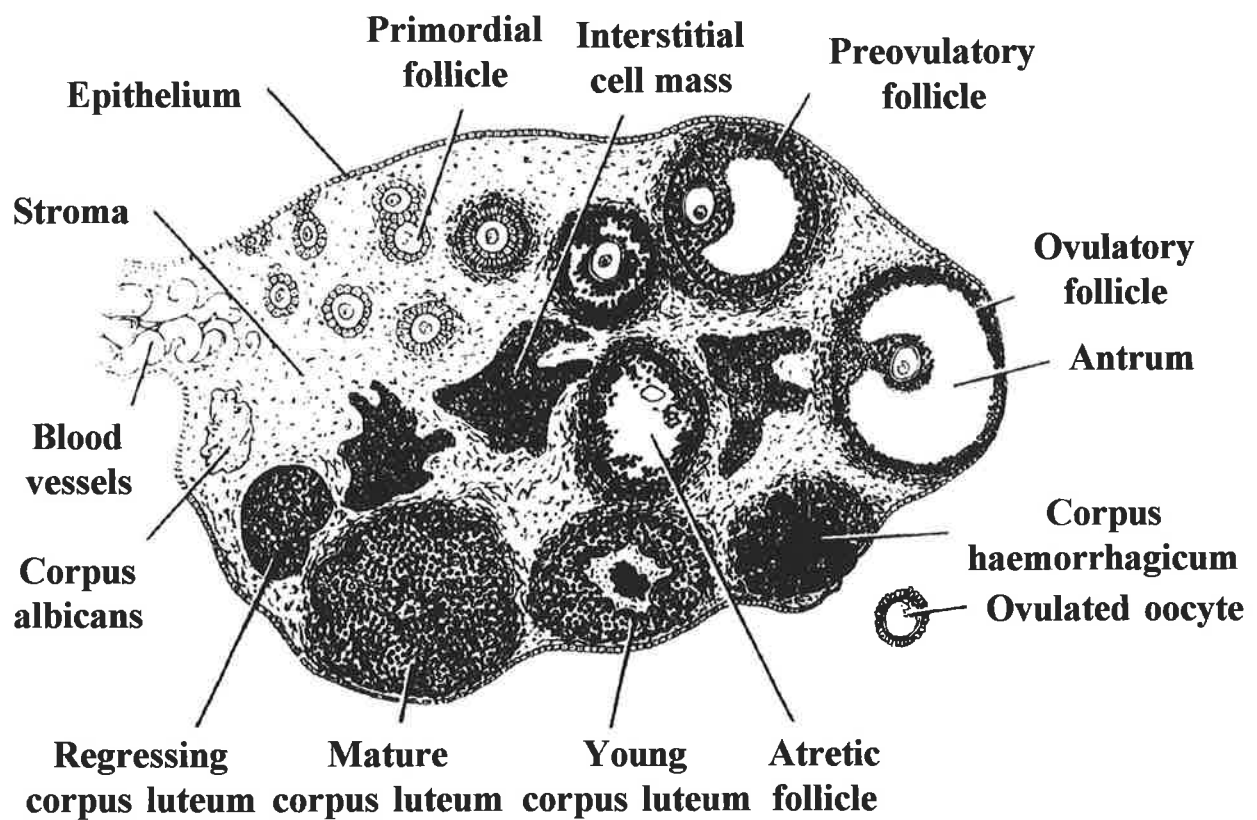


Figure 1.1 The ovary (adapted from Ganong, 1987).

The classical model for the control of ovarian function pivots on the interaction between the glycoprotein hormones, follicle stimulating hormone (FSH) and luteinising hormone (LH) and the locally produced steroid hormones oestradiol and progesterone. Pulsatile release of gonadotrophic releasing hormone (GnRH) by GnRH neurons of the hypothalamus into the portal hypophyseal vessels stimulates the secretion of both FSH and LH by gonadotrophic cells of the anterior pituitary.

1.1.2 The mouse as a model for ovarian regulation

A classic study of the murine oestrous cycle was performed by Allen (1922). The mouse is a polyoestrous mammal and exhibits signs of oestrous every 4 - 5 days. The cycle consists of changes in secretory, anatomical and behavioural characteristics reminiscent of the human menstrual cycle. Allen divided the cycle into 5 stages; the anabolic stages of proestrous and oestrous, followed by the catabolic stages, metoestrous-1 and -2, and dioestrous, the stage of quiescence or slow growth. Examination of vaginal epithelial cells, responsive to circulating steroids, allows convenient, non-invasive tracking of the cycle in the adult mouse.

Ovarian follicular formation and development is almost exclusively postnatal in the mouse (Peters, 1969). By days 12 - 14 of life antrum formation can be detected in some ovarian follicles. Sexual maturity is normally reached between 4 and 6 weeks of age, however gonadotropin priming for ovulation in the immature mouse is successful and elicits maximum response at 3 weeks of age and declines thereafter (Gates and Runner, 1957). In addition, cycles can be synchronised in the adult mouse by the administration of luteinising hormone releasing hormone (LHRH) agonists (Vickery and McRae, 1980).

The oestrous cycle is synchronised with a diurnal rhythm and ovulation occurs just after midnight during the middle of oestrous. Mating coincides with ovulation and fertilisation of the oocyte occurs in the ampulla of the oviduct. Mating is necessary to promote the functional development of the corpus luteum. Temporary development of the corpus luteum may be promoted by the induction of pseudopregnancy via mating with a vasectomised male. Newly formed corpora lutea following ovulation may persist for 2 - 4 subsequent cycles but do not become fully functional.

The mouse is a powerful experimental system for mammalian research due to its technical and practical feasibility (Paigen, 1995). Mice are small, easily contained animals and over the past years the mouse has been widely accepted as a good model for reproductive

studies because of the availability of genetically defined inbred strains and reagents for the identification of leukocytes and cytokines. With the advances of gene technology, they are also suitable candidates for the creation of transgenic mutants that cannot synthesise specific cytokines and hence provide novel insights into the roles of these cytokines in the reproductive tract.

1.1.3 Folliculogenesis

1.1.3.1 Morphological changes associated with folliculogenesis

Folliculogenesis is the growth and development of the follicle and the oocyte and is essential for the production of a fertilisable oocyte. The follicle provides optimal conditions and protection for the oocyte within the confines of a controlled aqueous environment and also acts to prepare the reproductive tract for a pending pregnancy through the synthesis of steroids. Located beneath the surface of the ovary, primordial follicles consist of an oocyte arrested in the dictyotene stage of meiosis, surrounded by a single layer of flattened, quiescent granulosa cells encapsulated in a basement membrane. Primordial follicles are recruited from the pool to develop to preovulatory stage. As the follicle grows it may sink into the centre of the ovary, only to reappear at the surface as ovulation approaches.

The onset of folliculogenesis is signalled by the cuboidal appearance, proliferation and plasma membrane FSH receptor acquisition of granulosa cells (Presl *et al*, 1974). In addition, the oocyte begins to grow in size and the zona pellucida begins to form from glycoproteinaceous material between the oocyte and granulosa cells in the primary follicle.

By the end of development of the preantral follicle, the oocyte is fully grown and covered by a thick zona pellucida, and the granulosa layer is 4 - 8 cells in thickness. Below the basement membrane the prospective theca is formed by the migration of stromal fibroblasts which form concentric layers around the follicle. It is at this stage that the follicle acquires its own network of capillaries, whereas the granulosa cell layer and oocyte remain avascular.

The distinguishing feature of an antral follicle is the presence of an antrum in which follicular fluid collects. As a result of antrum formation the follicle acquires an inherent polarity as the oocyte lies to one side. Gap junctions increase in size and number in the granulosa cell layer enabling molecules of up to 1.8 kD to pass from cell to cell (Pitts and Finbow, 1986), and also develop between the oocyte and corona radiata. A subpopulation of theca interna cells begin to transform into theca interstitial cells. The basement membrane

surrounding the follicle allows molecules of up to 100 kD to pass through into the follicular fluid (Shalgi *et al*, 1973).

Development of the antral follicle to a preovulatory (Graafian) follicle is characterised by growth of the granulosa and theca cells and follicular fluid. The resulting follicle may be up to a tremendous 550 μm in diameter. The preovulatory follicle is located immediately beneath the epithelium and tunica albuginea, in the ovarian cortex (Figure 1.2). A low cuboidal surface epithelium covers the ovary surface and these cells are attached to each other by zona occludens and microvilli. A basal membrane, prolific in laminin and collagen (type IV), forms a mesh like structure and separates the surface epithelium from the tunica albuginea. The tunica albuginea is composed of densely packed collagen (type I and III) fibres and scattered fibroblasts. The theca externa is composed mainly of collagen fibres, which are less densely packed than in the tunica albuginea. Also contained in this outermost follicular region are 4 - 7 layers of elongated fibroblasts and smooth muscle cells. Contained within the theca externa is a vascular network with connections to the theca interna, which is a highly vascularised, 1 - 3 cell layer thick structure embedded in a network of collagen fibres, continuous with the theca externa. Collagen fibres surrounding the follicle provide the tensile strength of the follicular wall. Five to ten layers of granulosa cells comprise the avascular membrana granulosa, lining the basement membrane. The granulosa cell population is heterogeneous with respect to polarity and position in the follicle. Immediately surrounding the oocyte are the corona radiata, and then the cumulus cells which are continuous with the membrana granulosa (reviewed by Erickson, 1995).

1.1.3.2 Endocrine changes associated with folliculogenesis

Granulosa cells are the only FSH responsive cell in the ovary and the growth of a primary follicle to preantral stage of development is dependent on FSH stimulation of granulosa cell proliferation. FSH also stimulates differentiation of granulosa cells which is characterised by the induction of P450_{arom} activity and LH receptor expression. In response to LH, theca interna cells of the preantral follicle synthesise primarily progesterone, due to the expression of P450_{scc}, and also androgens.

The development of the antral follicle to preovulatory stage involves FSH and LH-dependent cell proliferation (Bullough, 1942). Antrum formation is associated with P450_{17 α} expression by the theca, inducing the conversion of progesterone to androgens and a switch to primarily androgen production. The theca cells of the preovulatory follicle are a source of

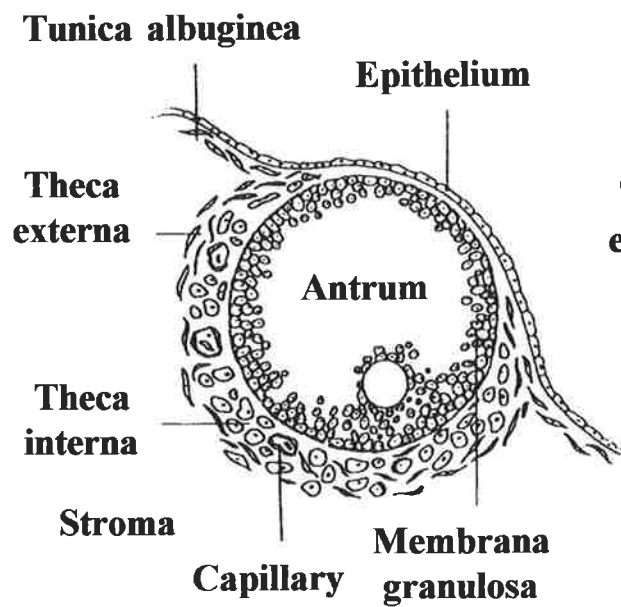
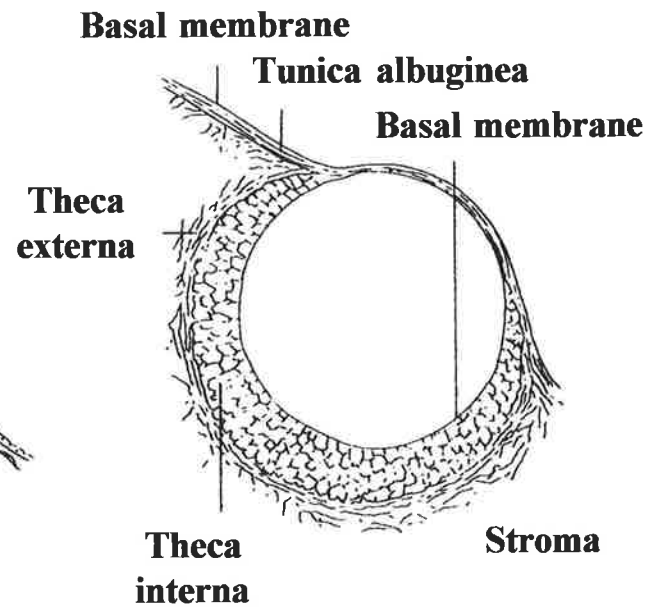
A**B**

Figure 1.2 Structure of the preovulatory follicle **A)** showing the cellular composition and **B)** collagen fibre networks (adapted from Beers *et al*, 1975).

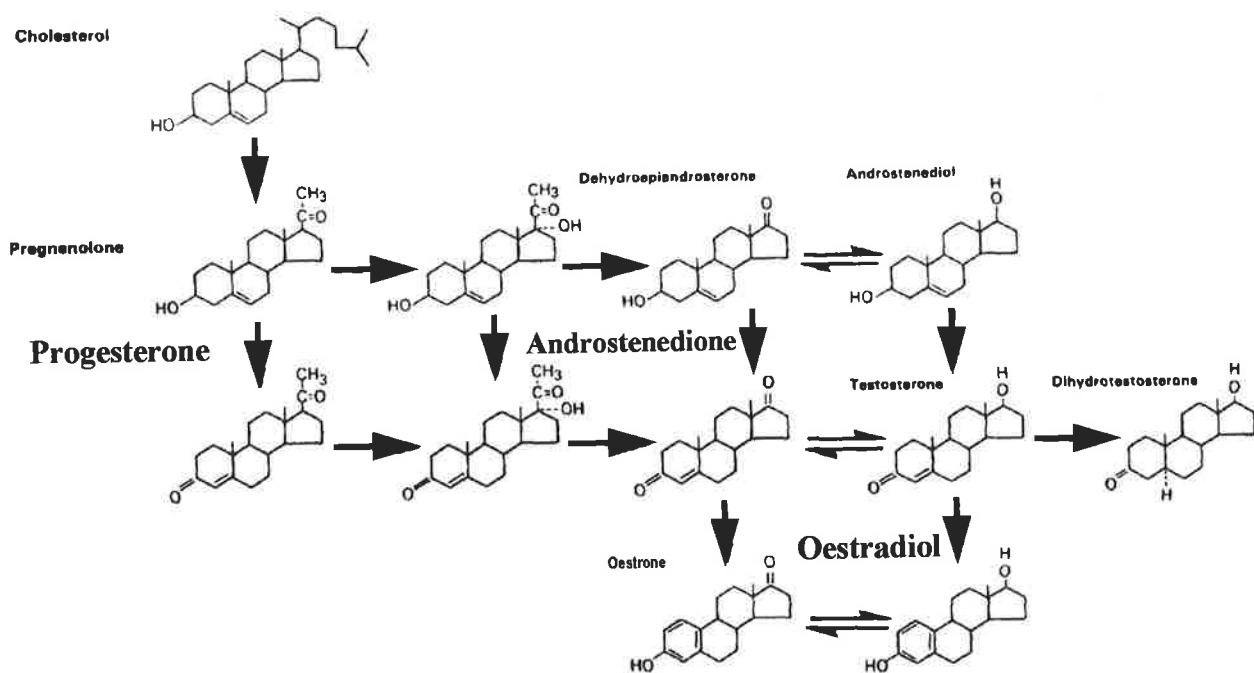


Figure 1.3 Steroid synthesis in the ovary (adapted from Yen and Jaffe, 1991).

androstenedione, which is the precursor for oestrogen synthesis in the granulosa cell under the 'two-cell, two-gonadotrophin' model (Figure 1.3). Androgens synthesised by theca cells diffuse across the basal lamina and are aromatised to oestrogens via FSH-regulated P450_{arom} activity.

The preovulatory period of the oestrous cycle is characterised by growth of ovarian follicles and this is reflected by the increasing synthesis of oestradiol by the ovary. The preovulatory follicle is a source of more than 90% of secreted oestrogen. Circulating oestradiol levels rise from dioestrous and reach peak concentration by the afternoon of proestrous, when circulating LH levels also begin to rise (reviewed by Richards, 1994 and Erickson, 1995).

The antrum of the follicle contains follicular fluid with high levels of oestradiol and progesterone, as well as a variety of other factors which promote oocyte maturation.

1.1.3.3 Follicle atresia associated with folliculogenesis

A recruited primordial follicle is destined to either develop into a preovulatory follicle or undergo atresia. This degenerative process involves apoptosis and removal of the oocyte and granulosa cells but also the reversal of theca cells to stroma or interstitial gland cells. Follicle atresia may occur at any stage of development, as only a few follicles achieve the success of ovulation, with the majority becoming atretic.

The developmental stage of the follicle appears to determine the primary changes associated with atresia. In the case of pre-antral follicles, the oocyte initiates meiotic maturation and then degenerates along with the granulosa cells, while the theca interna cells hypertrophy and become interstitial cells. In preovulatory follicles, the granulosa cells become pyknotic, necrotic and may float freely in the antrum. Following this initial necrosis of the granulosa layer, the cumulus and oocyte degenerate and the follicle collapses. Finally, the theca interna cells hypertrophy and become interstitial cells (reviewed by Richards, 1994 and Erickson, 1995).

1.1.4 Ovulation

1.1.4.1 Morphological changes associated with ovulation

The process of ovulation is initiated by the preovulatory LH surge, and is a dynamic process which, through the weakening of the basal membrane and extra-cellular matrix

(ECM) around the follicle wall, allows follicular rupture and the expulsion of an oocyte from the ovarian follicle.

The resumption of meiosis is triggered by the LH surge and is characterised by the completion of the first meiotic division, the extrusion of the first polar body and initiation of the second meiotic division with arrest at the metaphase II stage. The final stages of oocyte maturation and the completion of meiosis occur when the oocyte is fertilised.

Luteinisation is signalled by the cessation of mitotic activity of granulosa cells and the accumulation of lipid droplets. The basement membrane separating the granulosa and theca begins to breakdown. While cells of the theca interna remain relatively unchanged, quiescent fibroblasts of the tunica albuginea and theca externa enter an active proliferative state, elongate and dissociate from one another. Accompanying these changes in the theca is an increase in vascular permeability of surrounding capillaries. The surface epithelium becomes vacuolated and necrotic above the follicle. This is the stigma and will be the point of follicle rupture. During the final stages the follicle wall is no more than 20% of its preovulatory thickness (Espey, 1967). Cells of the theca interna and granulosa cell layer dissociate and retract to the base of the stigma and above the stigma the surface epithelial cells slough off the ovary. Finally, complete disassociation of collagenous tissue remaining in the follicle wall leads to rupture approximately 12 - 15 hours following the LH surge in the mouse (reviewed by Espey, 1994).

1.1.4.2 Endocrine changes associated with ovulation

During proestrous, rising circulating oestradiol is thought to trigger the ovulation-inducing LH surges. The FSH surge is concomitant with LH levels. Progesterone secretion from granulosa cells of the preovulatory follicle also peaks towards the end of proestrous and then declines to basal levels on the morning of metoestrous-1 (reviewed by Bentley, 1976).

1.1.4.3 Local regulators associated with ovulation

The preovulatory LH surge is thought to initiate biochemical changes which act in a finely tuned cascade to cause increased blood flow, increased vascular permeability and enzymatic degradation and weakening of the collagen fibres, basal membrane and extra cellular matrix of the follicle wall culminating in follicular rupture. There is an extensive list of mediators associated with these well-characterised events and these include nitric oxide (NO), histamine, serotonin, prostaglandins (PG), leukotrienes, bradykinin, metalloproteinases,

collagenases and plasminogen activator (reviewed by Espey, 1980, Tsafiriri and Dekel, 1994, Norman *et al*, 1996).

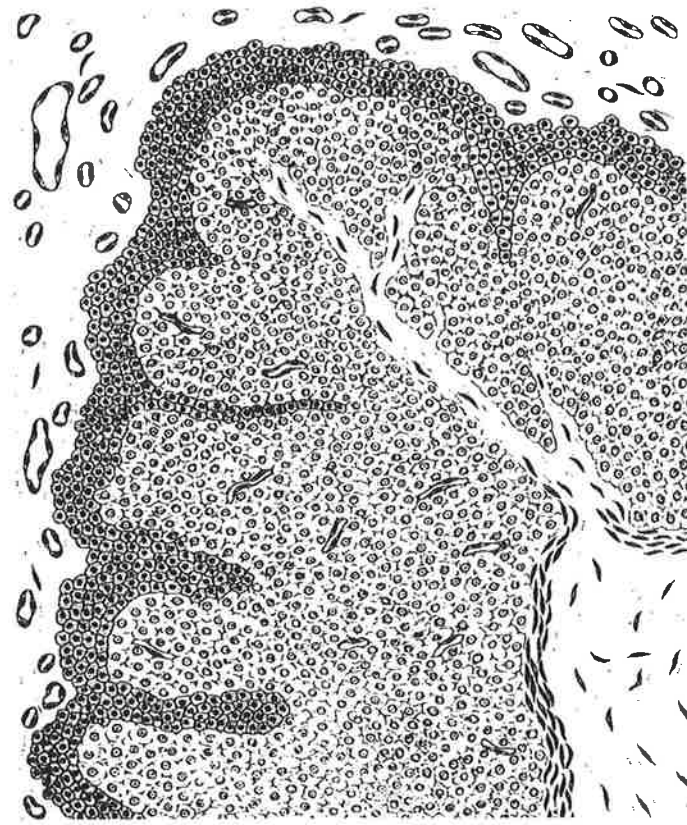
1.1.5 Luteinisation and luteolysis

1.1.5.1 Morphological changes associated with luteinisation and luteolysis

Following the LH surge the basement membrane begins to break down. There is sprouting of theca capillaries, and projections of the theca penetrate and interdigitate with the previously avascular granulosa cell layer. Follicular rupture results in the formation of a substantial cavity in the ovarian surface. Rapid tissue reorganisation results in the formation of the corpus luteum from the follicle remnants, while proliferation of surface epithelium covers the rupture site. The change associated with the transformation of theca interna and granulosa cells into luteal cells is termed luteinisation and actually begins prior to ovulation. Within several hours the process is completed and a dense vascular network is established within the corpus luteum (Figure 1.4), with the blood vessels lying adjacent to strands of theca cells, interspersed between large lutein cells. During this time mitotic activity continues in theca cells as they undergo hypertrophy and hyperplasia, and this is reflected in an increase in ovarian weight at this time.

The corpus luteum is formed from cellular components of the follicle and consists primarily of two morphologically and steroidogenically distinct cell types. Large luteal cells of the corpus luteum are derived from granulosa cells and are spherical or polyhedral in shape. They are the fundamental endocrine cells of the corpus luteum and are abundant in yellow carotenoid pigment. The small luteal cells, derived from the theca, are usually spindle-like in shape. Gap junctions and cytoplasmic projections can be shown to exist between luteal cells which are centred around a fibrin core. As with the follicle, the corpus luteum is surrounded by a theca externa. In sheep, the relative proportions of cells in the corpus luteum by volume are approximately 25 - 35% granulosa-lutein, 22 - 29% connective tissue, 12 - 18% theca-lutein, 11% vascular elements and 7 - 11% fibroblast (Nett *et al*, 1976, Rodgers *et al*, 1984).

Luteolysis refers to the regression of the corpus luteum and is the cellular disorganisation and self-destruction of the corpus luteum. This process occurs when maintenance of progesterone synthesis is no longer required; either at the end of an oestrous cycle, pseudopregnancy or pregnancy. Initially the large lutein cells begin to shrink. Luteal regression is associated with a swelling of endothelial cells and a decrease in vascular elements occupying the corpus luteum. More striking is the accumulation of large lipid



Theca lutein cells

Granulosa lutein cells

Connective tissue

Figure 1.4 Cellular components of the corpus luteum (adapted from Junqueira and Carneiro, 1986).

droplets in the cells. Increasing disorganisation of intracellular organelles is followed by development of large areas of necrosis. Eventually, all that remains of the disintegrated corpus luteum is a nodule of dense connective tissue called the corpus albicans (reviewed by Niswender *et al*, 1994).

1.1.5.2 Endocrine changes associated with luteinisation and luteolysis

The corpus luteum formed following ovulation without mating is only short lived (2 - 3 days) and is considered non-functional. The corpus luteum of pseudopregnancy, following a sterile mating, is functional for 10 - 12 days (Deanesly, 1930). Functional corpora lutea are considered to be those of pseudopregnancy or pregnancy, and in the initial stages of development they have similar hormone secretion profiles (Atkinson and Hooker, 1945). LH induces the formation of the corpus luteum and is also required to maintain its functional activity. Steroid synthesis appears to be regulated as in the case of the preovulatory follicle; LH stimulates both progesterone and oestradiol synthesis in granulosa-lutein cells and over 80% of the progesterone secreted by the corpus luteum (Niswender *et al*, 1985), whereas FSH appears only to promote oestradiol synthesis. LH alone is insufficient to sustain the corpus luteum, and in the absence of other anti-luteolytic factors it undergoes luteolysis. Luteolysis is characterised by a sharp decrease in steroidogenic activity associated with a decrease in steroidogenic cell size and number.

1.1.5.3 Local regulators associated with luteinisation and luteolysis

Temporary rescue of the corpus luteum in the case of pseudopregnancy is a function of mating or cervical stimulation. Activation of the nervous system promotes the release of the luteotrophic hormone prolactin by the anterior pituitary and results in the development of a functional corpus luteum. However, a signal from the conceptus is required to block luteolytic mechanisms and a subsequent luteotrophic stimulus is secreted from the day 10 conceptus to extend the functional life of the corpus luteum to approximately the length of gestation in mice (Critser *et al*, 1982). Maternal recognition of pregnancy is the process which enables the conceptus to rescue and maintain the corpus luteum and therefore ensures the continued production of progesterone required for maintenance of pregnancy.

Luteal regression can be induced by the administration of $\text{PGF}_{2\alpha}$, which causes the demise of large luteal cells (Fitz *et al*, 1984). The antisteroidogenic effects of $\text{PGF}_{2\alpha}$ appear to be mediated by activation of protein kinase C, and cytotoxic effects appear to be mediated

through elevated intracellular calcium concentrations (Wiltbank *et al*, 1991) (reviewed by Braden, 1994).

1.1.6 Local regulation of ovarian function

It is clear that FSH and LH play essential roles in the physiology of the ovary. To explain the variance in follicular development and the extent and rapidity of ovarian tissue remodelling during the cycle in relation to concomitant gonadotrophin secretion, it is necessary to invoke the existence of local regulatory elements.

The ovary has been known for some decades to host large populations of trafficking leukocytes, but it is only recently that the potential for their participation in normal physiological events has been appreciated. An extensive and rapidly growing list of cytokines and growth factors implicated in the regulation of leukocytes or identified as products of activated leukocytes appear to participate in the regulation of ovarian processes. The presence of these factors in the ovary is either the result of sequestration from the peripheral circulation or production by resident and/or infiltrating cells. The sheer number of these molecules illustrates the potential complexity of the regulation of ovarian function at the local level (Table 1.1).

Table 1.1 Potential local regulators of ovarian processes (adapted from Findlay, 1991).

Activin	Histamine	Nitric oxide
α N-inhibin peptide	Inhibin	Oxytocin
Angiotensin II	IGF	Plasminogen activator
Bradykinin	IGF binding protein	Platelet-derived growth factor
Catecholamines	Interferon α , γ	Prolactin
Collagenases	IL-1, 1ra, 2, 6, 8	Prostaglandin
CSFs	Leptin	Substance P
β -Endorphin	Leukotrienes	Transforming growth factor
Epidermal growth factor	Metalloproteinase	Tumour necrosis factor
Fibroblast growth factor	Müllerian inhibiting substance	Vasoactive intestinal peptide
Follistatin	Neuropeptide Y	

CSF = colony-stimulating factors, IGF = insulin-like growth factor, IL = interleukin

In vitro culture experiments have been extensively employed to yield data which indicate possible modes of action and effects of cytokines in the ovary. A plethora of studies now demonstrate the presence of multiple cell signalling systems, complete with proteins, receptors and receptor antagonists. The application of immunohistochemistry and *in situ* hybridisation techniques are providing invaluable information on the location and cellular origin of cytokine and cytokine receptor proteins and mRNA transcripts in the ovary. Local regulators of ovarian function are likely to act in precise spatial and temporal patterns to orchestrate follicle selection and dominance, resumption of meiosis, ovulation and the development and demise of the corpus luteum.

Due to the magnitude of the list of intraovarian regulators, the following discussion will be restricted to the synthesis and potential roles in the local regulation of ovarian function of factors which are the subject of experiments described in this thesis, including granulocyte-macrophage colony-stimulating factor (GM-CSF), colony-stimulating factor-1 (CSF-1), interleukin-1 β (IL-1 β) and tumour necrosis factor α (TNF α).

1.2 Leukocytes as local regulators

1.2.1 Leukocytes in tissue remodelling

Cells of the bone marrow-derived, leukocyte lineages (Figure 1.5) can perform a wide spectrum of actions, with pivotal roles in unplanned events such as infection and wound healing, but also in tissue remodelling associated with the development, growth and homeostasis of peripheral tissues.

Leukocytes participate in the important processes of cell growth, migration and death which occur in the differentiation and formation of tissues during embryonic development. In formation of the retina for example, significant numbers of neuronal cells undergo cell death and phagocytes can be observed throughout the inner layer of the retina where they have roles in the phagocytosis of dead or dying neurons (Hume *et al*, 1983).

After birth, leukocytes persist throughout the juvenile and adult body albeit in varying numbers and forms. In mucosal tissues such as the lung and uterus resident cells modulate 'appropriate' immune reactions to accommodate threatening and non-threatening antigens on a daily basis. With every breath airborne antigens make contact with respiratory epithelium from nasal cavity to alveoli, however tight restriction of the immune response limits

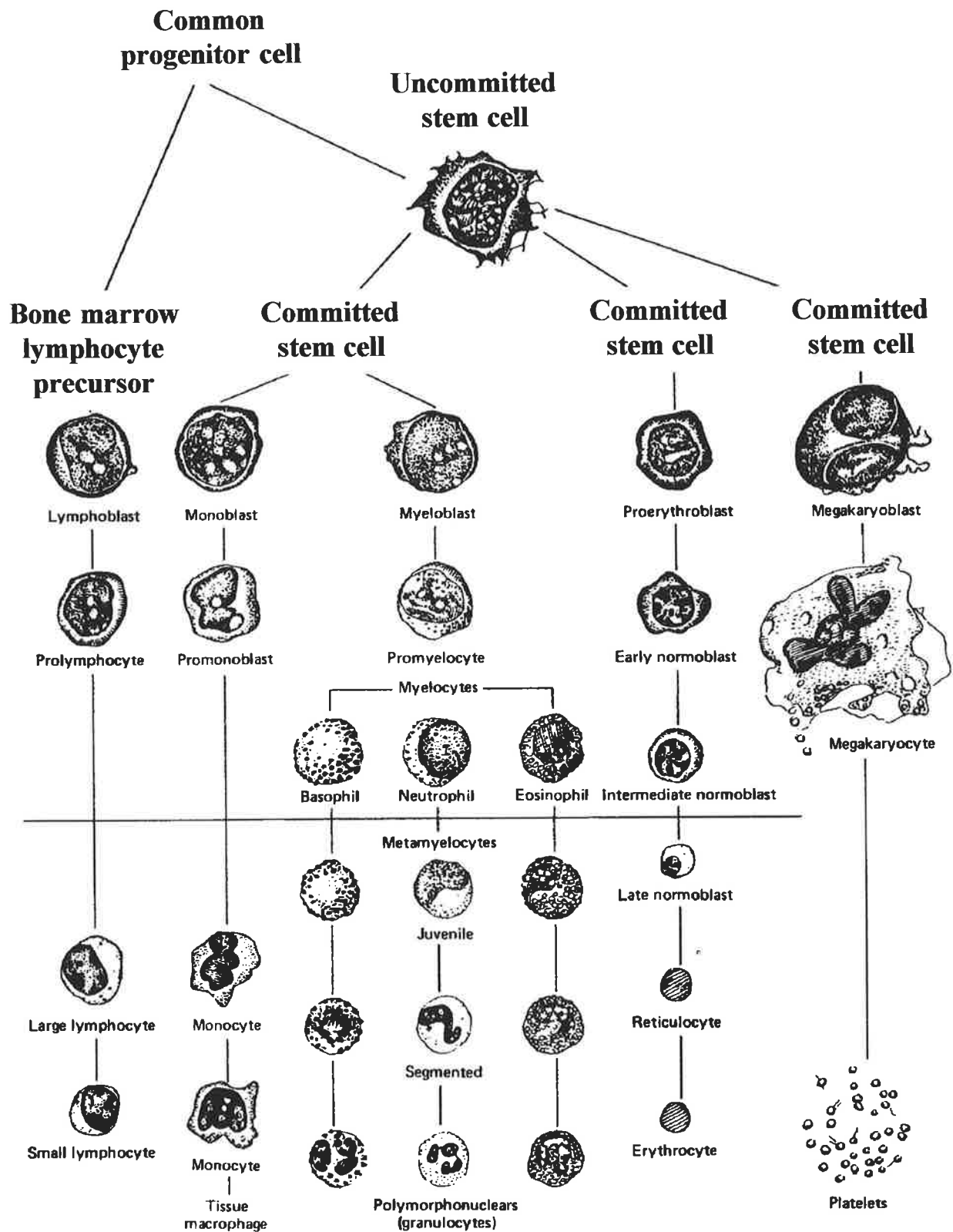


Figure 1.5 Differentiation pathway of lymphohaemopoietic cells in bone marrow and peripheral blood (below the horizontal line) (adapted from Ganong, 1987).

inflammation and the initiation of immune responses (Thepen *et al*, 1994). Bone remodelling is the result of an equilibrium of the processes of bone resorption and bone formation modulated by osteoclasts and osteoblasts, specialised tissue macrophages. Disorders of remodelling are presumed to be influenced by inflammatory cytokines and mediators (Mundy, 1993). Thus, leukocytes are central to the ability of the host to maintain homeostasis. Excessive or inappropriate leukocyte recruitment and/or activation characterises many disease states including asthma, allergies and autoimmunity. During pregnancy, the maternal immune system allows the growth and development of the fetus, considered an allograft (Robertson *et al*, 1994c).

Alterations in the kinetics of leukocyte turnover and in their activation state in any tissue can occur in response to endogenous or exogenous stimuli.

1.2.2 Leukocyte recruitment

The 3 major changes that characterise an inflammatory reaction at sites of antigen challenge or physical tissue damage are an increase in blood supply, increase in vascular permeability and infiltration of leukocytes, particularly neutrophils and macrophages. The kinetics of leukocyte recruitment are different for different lineages, with neutrophils arriving within minutes of stimulation and macrophages and lymphocytes following some hours later. This process provides effective clearance of invading pathogens and of damaged or diseased tissue. While neutrophils reach maximal levels during the first 2 days, these cells are short-lived in the periphery and die within 2 - 3 days. Macrophage populations (including 'resident' tissue macrophages and newly recruited blood-borne monocytes) are the principal phagocytic cell within the wound, reach maximal levels after approximately 3 days and can persist at an inflammatory site.

Wound healing processes replace cells and ECM lost during inflammation and physical damage. Studies examining wound repair in the absence of gross infection demonstrate repair in neutropenic animals is consistent with that in normal animals (Simpson and Ross, 1971). However, the macrophage is essential for wound debridement (removal of foreign material or contaminated tissue) and plays a role in regulation of fibroblast proliferation (Leibovich and Ross, 1975).

Orchestrated migration of leukocytes from the circulation into tissue and accumulation at the focal point occurs by a number of processes. A '3 step model' has been established to describe the emigration of neutrophils from the bloodstream (Butcher, 1991), and appears to

extend to all subclasses of leukocytes, including lymphocytes. This physiological process involves the coordinated expression of endothelial cell adhesion molecules to slow and arrest leukocyte movement and chemoattractant expression to direct cell extravasation and migration (reviewed by Springer, 1994).

Recruitment of leukocytes is initiated when resident cells at a site of inflammation or tissue injury release biological mediators such as prostaglandins, leukotrienes, cytokines, chemokines or histamine which have chemotactic and chemokinetic properties. The diffusion of any of these molecules (but particularly IL-1, interferon- γ (IFN- γ), IL-4, TNF and GM-CSF) towards the local capillaries results in induced or upregulated expression of vascular endothelial cell adhesion molecules including E-selectin, P-selectin, vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1). In the first step, leukocyte 'tethering' to the vessel wall occurs and 'rolling' is mediated by transient selectin-sialylated carbohydrate interactions. This brings leukocytes into proximity with chemoattractants that are associated with or released from endothelial cells. Finally, leukocytes develop firm attachments to endothelial cells through activation of integrin adhesiveness and binding to Ig superfamily proteins on the endothelial cell.

Acting in sequence and not in parallel, the unique combinations or 'area codes' of selectin, chemoattractant and integrin molecules together with cell lineage-specific ligand expression provide a means to selectively regulate migration of leukocytes into different peripheral tissues. Following directional cues from chemoattractants, leukocytes transverse the endothelial cell junction and enter the tissue. Leukocytes then actively migrate through a concentration gradient of soluble ligand or ligand affixed to the surface of other cells, or to ECM. The ensuring location of the cell in the tissue is thought to be the result of chemotaxis and haptotaxis (migration to a region of highest adhesiveness) (Springer, 1994).

Cell-cell and cell-ECM interactions play important roles in the regulation of migration direction and activation of the leukocyte. In part this may be due to the actions of solid-phase cytokines such as GM-CSF and IL-3 which bind ECM, and CSF-1, TNF α , IL-8 and macrophage inflammatory protein-1 β (MIP-1 β) which can associate with cell membranes (Tanaka *et al*, 1993a, 1993b, 1993c, Rot, 1992). Mononuclear phagocyte interactions with ECM have been shown to simulate phagocytosis (Pommier *et al*, 1983), cytotoxicity (Kaplan, 1983), cytokine production (Pacifici *et al*, 1992, Dayer *et al*, 1986) and differentiation (Kaplan and Gaudernack, 1982). The effects of granulocytes binding ECM include regulation of

cytokine production (Pakianathan, 1995), hydrogen peroxide production (Nathan, 1987) and respiratory burst (Nathan *et al*, 1989).

Naive and memory lymphocytes employ different recirculation pathways (Mackay, 1992). At least for peripheral tissue and lymph nodes, memory lymphocytes emigrate preferentially through tissue endothelium, whereas naive lymphocytes enter the lymph node through high endothelial cell venules. The 3 step model has been implicated to regulate localised accumulation of lymphocytes at an inflammatory site. Thus adhesion molecule expression by endothelial cells and local chemoattractants also appear to be primary determinants of memory lymphocyte accumulation at inflammatory sites (reviewed by Springer, 1994).

1.2.3 Leukocyte populations

1.2.3.1 Mononuclear phagocytes

The mononuclear phagocyte system, described as a single lineage, comprises monocytes, macrophages and specialised tissue macrophages including Kupffer (liver), osteoclasts (bone), Langerhans (epidermis), microglia (central nervous system), pleural (alveolar space) and peritoneal cells (serosal cavity). Derived from bone marrow promonoblastic cells, the macrophage undergoes initial differentiation to a blood monocyte and finally, following tissue infiltration, it either becomes a resident or immature macrophage. Resident macrophages are normally distributed in all connective and mucosal tissues, and may occupy tissues for several months as relatively quiescent cells.

However tissue macrophages are profoundly sensitive cells, responding to a vast array of exogenous signals, through at least 60 receptors. Depending on the surrounding tissue microenvironment, macrophages are capable of secreting more than 100 different molecules including growth and cell function regulatory factors (including cytokines), enzymes and enzyme inhibitors, and cytostatic and cytotoxic molecules (Rappolee and Werb, 1989, Gordon *et al*, 1995). Other physiological activities can include phagocytosis, antigen processing and presentation. The activation phenotype achieved will determine whether a given macrophage population becomes involved in defence against foreign antigen and tumour cells, haemostasis, inflammation, wound healing or tissue remodelling. Distinct behavioural and secretory profiles are characteristic of various functional states and tissue phenotypes (Table 1.2).

Table 1.2 Characteristic activities of macrophages (adapted from Male *et al*, 1987).

Characteristic	Activation state		
	Immature	Primed	Activated
Ia (MHC class II) expression	±	++	+++
IL-1	+ or -	+ or -	+ or -
TNF	-	-	++
Secretory neutral proteases	+	++	+++
Reactive oxygen intermediates	-	+	+
Eicosanoids	+	++	+

- = no, ± = minimal, + = low, ++ = medium, +++ = high expression

1.2.3.2 Dendritic cells

Dendritic cells are bone marrow derived cells (Barclay and Mayrhofer, 1981) displaying characteristics of both lymphoid and macrophage cells that are suspected to be derived from myeloid precursors (Peters *et al*, 1996). Very few mature cells are present in the bone marrow or peripheral circulation. Although not all dendritic cells display these characteristics to the same degree, dendritic cells are generally potent antigen presenting cells involved in induction of immune responses through regulating T cell activation and expansion (Steinman, 1991). Dendritic cells express CD45 (LCA), class I and II MHC antigens, integrins and adhesion molecules, but rarely express CD11b (Mac-1), F4/80 and lack most T cell markers (Steinman and Nussenzweig, 1980, Agger *et al*, 1990).

Dendritic cells can process 4 times their volume of extracellular fluid in 1 hr, thus making them very efficient antigen capturing cells. Following capture of foreign antigens, dendritic cells migrate to regional lymph nodes, during which time they undergo maturation events that reduce their antigen processing capacity and enhance their antigen presenting capacity. Migration is thought to be regulated by TNF α and can be blocked by the administration of anti-TNF α , while the activation and maturation processes are thought to be mediated by local factors encountered within the tissue such as GM-CSF, IL-1 β and collagen type I (Caux *et al*, 1995) and possibly neuropeptides (Stingl and Bergstresser, 1995). Upon arrival in lymph nodes, dendritic cells interact with naive or memory T lymphocytes to initiate immune responses.

1.2.3.3 Polymorphonuclear granulocytes

Polymorphonuclear granulocytes are generally the first cells to migrate into tissue and are therefore considered the first line of defence against invading pathogens. They play an important role in inflammation and acute immunity through phagocytosis, killing of bacteria and release of various biochemical mediators (Table 1.3). The lineage consists of neutrophils, eosinophils, basophils and the related mast cells.

Neutrophils are short lived and incapable of proliferation or self-renewal. Nevertheless, they exist at various stages of maturation, from dormant to primed to fully activated. These cells primarily function as phagocytes but can release a spectrum of cytokines including GM-CSF, IL-1 β , IL-1ra, IL-8 and TNF α (Cassatella, 1995).

Eosinophils primarily degranulate intracellular granules, although they appear to also be capable of phagocytosis. These cells are traditionally viewed as parasitic killer-effector cells and modulators of hypersensitivity reactions. They also release factors to dampen inflammatory responses, factors which reduce neutrophil migration and inactivate mast cell products (reviewed by McEwen, 1992).

Basophils are found in very small numbers in the circulation, and mast cells not at all, as these cells undergo maturation in vascularised peripheral tissues. The major mediators are stored in cytoplasmic granules and include histamine, serotonin, proteoglycans, neutral proteases, acid hydrolases (Stevens and Austen, 1989) and TNF α . On activation however these cells can generate PG, leukotrienes, platelet activating factor (PAF), chemokines and GM-CSF, IL-3, IL-4, IL-5, IL-6 and IL-13 (Burd *et al*, 1989, Burd *et al*, 1995).

1.2.3.4 Lymphocytes

Lymphocytes play a key role in both cellular and humoral antigen specific immune responses (Roitt *et al*, 1996). Unlike mononuclear phagocytes and granulocytes, lymphocytes recirculate through the body, from the arterial blood supply and entering the target organ, where they drain into the lymphatic system and re-enter the circulation via the thoracic duct.

T lymphocytes, which mediate cellular immunity and regulate antibody production by B lymphocytes, originate as pre-T cells in the bone marrow, populate the thymus and give rise to mature, discrete T cell subsets, including helper/inducer (T4) and suppressor (T8) T cells. Although T and B cells are morphologically indistinguishable, B cells have the unique ability to produce antibodies in response to foreign antigens, mediating humoral immunity. In contrast to T cell development, postnatal maturation of B cells and differentiation into plasma

and memory B cells occur within the bone marrow. Like other leukocytes, lymphocytes can generate various cell function mediators, and their functional activities are responsive to environmental signals (Table 1.3).

Table 1.3 Secretory and granule stored products of leukocytes (Fujishima and Aikawa, 1994, Galli, 1993, Roitt *et al*, 1996).

Monocytes	Neutrophils	Eosinophils	Mast cells	Lymphocytes
IL-1	IL-1, 1ra, 6, 8	Histaminase	TNF α	GM-CSF
TNF	GM-CSF		Chemokines	IL-2, 4, 5, 6
bFGF	TNF α		TGF β	TNF α
TGF	TGF β			TNF β
CSFs	IFN α		Granules	IFN γ
PGE	MIP-1 α		Histamine	
Nitrites	Oxygen metabolites		Proteoglycans	
Nitrates	Leukotriene B4		Eicosanoids	
PA	PAF		Neutral proteases	
Elastase			IL-1, 3, 4, 6	
Collagenases	Granules		GM-CSF	
Stromelysin	Metalloproteinases		TNF α	
	Myeloperoxidase			
	Acid proteases			
	Neutral proteases			

1.3 Cytokines as local regulators

A local regulator has the ability to control the behavioural and secretory profile of cells within close proximity. The overall biological response of the target cell is often dependent on the cocktail of factors within the adjacent milieu or pre-exposure to specific factors. Local regulators usually have little or no endocrine activity but work as paracrine or juxtacrine agents instead. They are usually highly potent molecules, eliciting a biological response following binding of ligand to perhaps only a few receptors on the target cell membrane (reviewed by Callard and Gearing, 1994, Abbas *et al*, 1991).

Cytokines are a major class of local regulators originally identified as factors produced by or acting on lymphohaemopoietic cells more than thirty years ago. They are a group of soluble, low molecular weight (15 - 60 kD) polypeptides essentially involved in the regulation of immunity, inflammation, wound healing, haemopoiesis and tissue remodelling. The broad class term cytokine includes interleukins (products of leukocytes that regulate lymphoid or myelomonocytic cell growth and differentiation), monokines (products of mononuclear cells), lymphokines (products of lymphocytes), interferons, colony-stimulating factors (which generate progeny cells of the various haemopoietic lineages) and other growth factors.

The cytokine family is characteristically termed pleiotrophic and redundant. Cytokines are produced by many different cell types and have the ability to recruit and influence the behaviour and secretions of both haemopoietic and non-haemopoietic cells, making the original names no longer seem applicable. A single member has the ability to influence a large and diverse population of cells and elicit a variety of biological responses from numerous cell lineages. Although these molecules bind to specific, cell surface receptors, they share a variety of biological and physiochemical properties. In the absence of a cytokine, the compensatory actions of cytokines with similar bioactivities may suffice. It is because of this overlap of functions that the cytokine system is termed redundant.

Their production is subject to complex control. Regulation of cytokine synthesis and effect can occur at the level of gene expression, processing of precursor forms, sequestration by ECM, soluble binding proteins, receptor antagonists and receptor modulation. Evidence suggests some are constitutively expressed, presynthesised and stored in cytoplasmic vesicles, membrane bound or complexed with cell surface binding proteins or ECM. Generally acting in a paracrine or autocrine fashion, they are rarely released singly but instead as part of a cytokine network, exerting potent biological responses in the low nano - picomolar range. Their short half life makes them ideal local regulators, responsible for eliciting potent biological responses from cells within close proximity. Due to the multifocal production of cytokines it is clear that circulating levels do not usually reflect concentration at the site of action.

1.3.1 GM-CSF

1.3.1.1 GM-CSF biochemistry

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a member of the colony stimulating factor family of cytokines which were discovered, characterised and named

according to the types of colonies stimulated from bone marrow stem cells *in vitro* (Bradley and Metcalf, 1966).

Murine GM-CSF is a 124 amino acid glycoprotein with a molecular weight of 18-25 kD depending on the level of post-transcriptional glycosylation (Table 1.4). Crystallographic studies indicate human GM-CSF has a 4- α -helical configuration, yet the carbohydrate moiety is not involved in receptor binding and therefore not critical for biological action (Diederichs *et al*, 1991, Shanafelt *et al*, 1991). Since murine GM-CSF only shares 52% amino acid homology and 70% cDNA homology with human GM-CSF (Miyatake *et al*, 1985), there is no cross-species bioactivity.

The murine genome contains one copy of the GM-CSF gene located on chromosome 11, consisting of 3 introns and 4 exons (Miyatake *et al*, 1985). The transcribed mRNA transcript is 0.78 kb, although alternate transcripts of 5.2, 3.9, 2.4, 2.1 and 1 kb have been described in murine placenta, 4 of which are larger than the conventional T cell mRNA transcript (Crainie *et al*, 1990), but the cellular origin and function of these transcripts are unknown.

GM-CSF is synthesised and then secreted into the extracellular space as a diffusible protein, but may be stored within mast cell cytoplasmic granules. It is known to be sequestered and immobilised by binding the heparan sulphate component of ECM (Roberts *et al*, 1988).

Table 1.4 Molecular and genomic properties of GM-CSF (adapted from Nicola, 1989).

Other names	MGI-1GM, CSF- α , pluripoietin- α
Subunits	1
Leader amino acids	25
Core protein amino acids	124
Mature protein M _r	14.4 kD
Glycosylated M _r	18 - 25 kD
Human protein homology	52%
Chromosomal location	11
Gene size	2.5 kb
Number of exons	4
mRNA sizes	0.78 kb
Alternative splicing	yes

1.3.1.2 Cellular sources of GM-CSF and regulation of expression

Lymphocytes stimulated with phytohaemagglutinin were the first cells discovered to synthesise GM-CSF (Cline and Golde, 1974). GM-CSF is produced in bone marrow but also by a variety of cell lineages at peripheral sites. Bioactive GM-CSF has now been identified in many organs of the body including brain, heart, lymph node, salivary gland, lung, thymus, kidney, spleen, pancreas, small intestine, skeletal muscle, liver, testis and skin (Sheridan and Metcalf, 1972, Nicola *et al*, 1979), however the cellular sources in some of these organs remain to be clarified.

GM-CSF is constitutively transcribed in monocytes, endothelial cells and fibroblasts, although mRNA does not accumulate (Thorens *et al*, 1987, Koeffler *et al*, 1988). In response to immune or inflammatory stimulants, most notably IL-1 β , TNF α and lipopolysaccharide (LPS), T and B lymphocytes, macrophages, mast cells, mesothelial cells, osteoblasts and epithelial cells can be induced to synthesise GM-CSF (Table 1.5).

Table 1.5 Some cellular sources and regulators of expression of GM-CSF (adapted from Gasson, 1991).

Cell	Inducer
Macrophage	LPS, FCS, phagocytosis, adherence
T-lymphocyte	antigen, lectin, IL-1
B-lymphocyte	LPS, TPA
Mast cell	IgE, calcium ionophore
Fibroblast	IL-1, TNF, TPA
Mesothelial cell	EGF + TNF
Endothelial cell	IL-1, TNF, TPA, modified LDL
Osteoblast	PTH, LPS

LPS = lipopolysaccharide, FCS = fetal calf serum, EGF = epidermal growth factor, LDL = low density lipoprotein, PTH = parathyroid hormone, TPA = tetradecanoyl phorbol acetate

Regulation of GM-CSF expression occurs through transcriptional and post-transcriptional control mechanisms which include an increase in mRNA synthesis and/or

increases in mRNA and protein stability. The activation of monocytes, endothelial cells and fibroblasts by a variety of stimuli leads to a transient increase in mRNA as a result of increased transcription and increased stabilisation of the mRNA, possibly by the 3' AT-rich region (Seelentag *et al*, 1987, Kaushansky, 1989). The variations in carbohydrate chain length lead to considerable heterogeneity in the CSF molecules. Glycosylation comprising up to 30% of the complete protein (Metcalf, 1987) may provide stability and resistance to attack by proteolytic enzymes prolific at inflammatory sites (Stanley and Metcalf, 1971, Tsuneoka *et al*, 1981).

The murine uterus is a potent source of bioactive GM-CSF particularly on day 1 of pregnancy. The major source of bioactive GM-CSF was localised to the glandular and luminal epithelial cells of the uterus in cell separation studies (Robertson *et al*, 1992) and has since been confirmed by *in situ* localisation of mRNA (Robertson *et al*, 1996b). At the maternal-fetal interface GM-CSF mRNA expression can be localised to cells in the maternal decidua and placenta (Crainie *et al*, 1990, Kanzaki *et al*, 1991). The significance of the alternative GM-CSF transcripts identified in the murine placenta is unclear. Uterine GM-CSF is induced by ovarian oestradiol, and diminished by progesterone (Robertson *et al*, 1996b). The synthesis of GM-CSF in the uterus can also be stimulated more than 20 fold by seminal factors at mating (Robertson *et al*, 1996a).

1.3.1.3 GM-CSF receptor

Walker and Burgess (1985) have isolated two distinct classes of low and high affinity receptors on murine haemopoietic cells. The high-affinity GM-CSF receptor complex is now known to be comprised of a quaternary complex of 2 ligand-specific α subunits, 2 signal transducing β subunits, and 2 ligand molecules (Bagley *et al*, 1997) (Figure 1.6). The α subunit has low affinity for the GM-CSF protein, whereas the β subunit does not bind the GM-CSF protein, but confers higher affinity to the α subunit in the quaternary complex. GM-CSF receptors are present on all progenitor and mature cells of the myeloid lineage. The α subunit is also present on some non-haemopoietic cells, including placenta, oligodendrocytes, endothelial cells and malignant cells (Gearing *et al*, 1989, Baldwin *et al*, 1989, Baldwin *et al*, 1991), while the β -subunit is only expressed on haemopoietic cells.

The α subunit is a 55 kD amino acid which shares less than 35% homology with the human α subunit (Park *et al*, 1992). Alternative splicing of human GMR α mRNA yields a

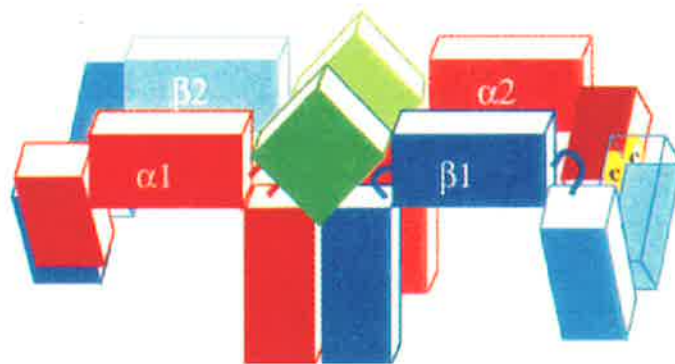


Figure 1.6 Schematic representation of the GM-CSF receptor complex. Ligand (green): α (red): β (blue) stoichiometry of 2:2:2, where the α subunit of receptor 1 forms a bond with the β subunit of receptor 2 and the α subunit of receptor 2 forms a bond with the β subunit of receptor 1 (adapted from Bagley *et al*, 1997). c = cysteine

protein (GM-CSFR α 2) capable of binding GM-CSF with low affinity, but with a cytoplasmic domain rich in serine residue, a feature critical for signal transduction for other receptors (Crosier *et al*, 1991). Another isoform of the α subunit lacks the transmembrane domain and is a soluble GM-CSFR α (sGMR α) receptor for human GM-CSF (Raines *et al*, 1991). The expression of this soluble receptor appears to be regulated independently of the membrane bound form (Heaney *et al*, 1995).

The β subunit of the high affinity receptor has been identified as the 115 kD AIC2B protein in the mouse (Kitamura *et al*, 1991). The subunit is common for the IL-3 and IL-5 receptors, and is closely related to the mouse AIC2A protein which associates specifically with the IL-3 receptor (Park *et al*, 1989, Hara and Miyajima, 1992). The sharing of a common β subunit explains the ability of IL-3 to compete for GM-CSF binding and to minimise GM-CSF responses (Lopez *et al*, 1992). Recent evidence suggests that the quaternary receptor complex may comprise 2 different α subunit specific receptors for GM-CSF with IL-3 or IL-5 in conformation with two β subunits (A. Lopez, personal communication).

GM-CSF receptor activation induces rapid tyrosine phosphorylation of the β subunits and multiple intracellular substrates (Duronio, *et al*, 1992). The signal transducers and activators of transcription (STAT) family members, STAT1 and STAT3, and the product of the *c-fps/fes* proto-oncogene become phosphorylated upon physical association between the β subunit and kinase JAK2 (Brizzi *et al*, 1996, Watanabe *et al*, 1997).

In addition to proliferation, GM-CSF induces various activities depending on the target cell and these activities may be mediated by different cytoplasmic domains. Whether GM-CSF can stimulate proliferation or other cell activities through the α subunit alone is unclear. However, it may mediate glucose uptake in some cell lineages (Ding *et al*, 1994, Spielholz *et al*, 1995) although apparently not in myeloid leukocytes (C. Scott and L. Robb, personal communication).

1.3.1.4 Effects induced by GM-CSF on target cells

The biological activity of GM-CSF is to regulate the survival and stimulation of proliferation of monocytes and macrophages, granulocytes and dendritic cells. In addition, GM-CSF can enhance the function of mature cells (Table 1.5). Although *in vitro* experiments have implicated GM-CSF in the accumulation or peripheralisation of mature cells in non-

haemopoietic organs (Metcalf *et al*, 1987), if they do exist *in vivo*, these effects appear to be largely redundant in the GM-CSF knockout mouse.

Table 1.5 Biological activities of GM-CSF (adapted from Gasson, 1991).

Monocyte/Macrophages	
Adherence	Oxidative metabolism
Chemotaxis	Cytotoxicity
Surface antigen expression	Tumour cell killing
Cytokine expression	Parasite killing
Prostaglandin expression	
Dendritic cells	
Maturation and viability	Antigen presentation
Neutrophils	
Protein synthesis	Degranulation
Adherence	IgA-mediated phagocytosis
Recruitment	Cytotoxicity
Migration inhibition	Cells surface receptor expression
Cytokine secretion	Oxidative metabolism
Arachidonic acid	Superoxide generation
Leukotriene	
Eosinophils	
Cytotoxicity	
Leukotriene synthesis	
Basophils	
Histamine release	
Langerhans cells	
Maturation and viability	Antigen presentation
Endothelial cells	
Migration	
Proliferation	
Adhesion molecule expression	

Uterine GM-CSF expression is evident at important events such as oestrous and during early pregnancy in the female reproductive system. Uterine epithelial synthesised GM-CSF appears to regulate the recruitment and activation of haemopoietic cells in the uterus, since intra-uterine administration of GM-CSF leads to a dramatic inflammatory response (Robertson *et al*, 1994b). Trophoblast cells of the embryo and placenta express GM-CSF receptors and appear to also exhibit biological responses to this cytokine.

1.3.1.5 GM-CSF deficient mice

The GM-CSF knockout (GM^{-/-}) mice have been generated by two independent groups both using gene targeting techniques in embryonic stem cells (Dranoff *et al*, 1994, Stanley *et al*, 1994). Due to the deletion of the first exon of the GM-CSF gene, the mice are devoid of GM-CSF protein. These mice appear to develop normally with no disruption of major haemopoietic populations in blood or marrow, as indicated by normal numbers of total blood leukocytes, monocytes, neutrophils, eosinophils and lymphocytes at 6 - 7 weeks of age. No difference in bone marrow total progenitor cell frequency or peritoneal macrophage numbers have been noted, but there is a moderate increase in splenic progenitor cell number in GM^{-/-} mice (Stanley *et al*, 1994).

However, GM^{-/-} mice develop abnormal lung pathologies absent in heterozygote (GM^{+/-}) or wild-type mice (GM^{+/+}), characterised by extensive infiltration of lymphocytes, (predominantly B cells) and accumulation of surfactant and debris including lamellar bodies, and granular, eosinophilic material. Although no appreciable reduction in alveolar macrophages have been noted, these cells have tended to show morphological abnormalities including an enlarged foamy appearance, indicating they are laden with lipid and surfactant (Dranoff *et al*, 1994, Stanley *et al*, 1994). Stanley *et al* (1994) have also reported the development of lung infections in some mice involving bacterial or fungal organisms. Alveolar proteinosis can be corrected in the GM^{-/-} mouse by the selective expression of GM-CSF directed to the epithelial cells in the lung, under the control of the human surfactant protein-C promoter. Associated with this correction is a restoration of macrophage morphology and an increase in macrophage numbers (Huffman *et al*, 1996). These observations indicate that whilst GM-CSF is not essential for haemopoiesis or maintenance of circulating haemopoietic cells in blood, marrow or spleen, local production of GM-CSF appears to be essential for the maintenance of alveolar macrophage function and lung homeostasis.

Additional studies also support the conclusion that peripheral tissue macrophage behaviour and activation phenotype may be altered in GM-CSF deficient mice. GM-CSF deficient mice are less susceptible to the effects of LPS as observed by diminished hypothermia and loss of body weight; moreover circulating IFN- γ , IL-1 α and TNF α levels do not peak as observed in wild type mice (Basu *et al*, 1997). Although the strength of the TNF α peak is comparable to wild type mice it persists in the circulation for an extended period of time in the absence of GM-CSF. The activation of peritoneal macrophages from GM-/- mice *in vitro* have resulted in secretion of less IL-1 α and NO than macrophages from wild type mice, although TNF α secretion is comparable.

Overtly, the GM-/- mice are 'fertile'. The ratio of offspring from 8 litters of GM+/- crosses is represented in approximately Mendelian ratios, indicating no selective loss of GM-/- pups (Dranoff *et al*, 1994, Stanley *et al*, 1994). However, the fertility and fecundity of GM-/- mice are impaired when larger sample numbers are examined (Seymour *et al*, 1997, Robertson *et al*, in press). Robertson and co-workers have found the mean litter sizes of GM-/- breeding pairs to be 25% smaller at weaning than GM+/- pairs, due to death late in gestation. In addition, the weight of viable fetuses and the fetal : placental weight ratio is diminished. Changes in the cellular composition and function of the placenta may be at least partially responsible for the compromised reproductive outcome in GM-/- mice.

1.3.2 CSF-1

1.3.2.1 CSF-1 biochemistry

CSF-1 was the first of the colony stimulating factors to be identified and has been shown to selectively stimulate the formation of macrophage colonies from haemopoietic progenitor cells *in vitro*. It therefore also referred to as macrophage colony-stimulating factor (M-CSF) (Stanley *et al*, 1976, Stanley and Heard, 1977).

The murine CSF-1 gene is located on chromosome 3 (Gisselbrecht *et al*, 1989), and subsequent alternative splicing yields mRNA products of approximately 4.5 kb and less abundant species of 3.8, 2.3 and 1.4 kb (Ladner *et al*, 1988). Similarly in the human, mRNA transcripts of 4, 3.5 and 1.6 kb have been identified (Ladner *et al*, 1987, Wong *et al*, 1987, Nakamura *et al*, 1989). Unlike the single subunit protein of GM-CSF, bioactive CSF-1 is composed of two identical subunits linked by disulfide bonds (Table 1.6). The subunit, being derived from the 4 kb mRNA transcript, exists as a 43 kD monomer. Apart from the

biologically active 70 - 90 kD homodimer form (Das and Stanley, 1982), at least 2 other forms of CSF-1 are also known to exist. One form is a 150 - 200 kD secreted proteoglycan heterodimer, thought to be derived from the 43 kD monomer (Suzu *et al*, 1992), and the other form, a small disulfide linked homodimer of 36 - 52 kD transcribed from the alternative 1.8 - 2 kD mRNA transcript, is thought to exist as a membrane-anchored protein. Membrane-anchored CSF-1 appears to regulate adjacent CSF-1 responsive cells and adhesion between stromal and c-fms-bearing cells (Uemura *et al*, 1993). Proteolytic cleavage of the extracellular side of the anchor may also yield a soluble CSF-1 protein (Cosman *et al*, 1988). The 82% amino acid sequence conservation between human and mouse species explains partial cross reactivity as human CSF-1 elicits biological effects on murine cells, yet the reverse is not true (reviewed by Morstyn and Burgess, 1988, Nicola, 1989).

Table 1.6 Molecular and genomic properties of CSF-1 (adapted from Nicola, 1989).

Other names	MGI-1M, M-CSF
Subunits	2
Leader amino acids	31
Core protein amino acids	189, 158
Mature protein M _r	21, 18 kD
Glycosylated M _r	70 - 90, 45 - 50 kD
Human protein homology	82%
Chromosomal location	3
mRNA sizes	4.5, 3.8, 2.3, 1.4
Alternative splicing	yes

1.3.2.2 Cellular sources of CSF-1 and regulation of expression

CSF-1 is primarily synthesised by fibroblasts, bone marrow stromal cells, monocytes, activated macrophages, lymphocytes, epithelial cells and endothelial cells, and various tumour cells (Nicola, 1989). Synthesis of CSF-1 has been shown to be induced by a variety of factors including antigen, IL-1, TNF, IL-6, GM-CSF, LPS, IFN- γ and phorbol esters. CSF-1 expression is inhibited by PG, oestradiol, glucocorticoids, TGF β and the cAMP inducers, forskolin and pertussis toxin. Synergism with IL-1, IL-2, IL-3, IL-6, GM-CSF and stem cell factor (SCF) have been observed in various cell systems *in vitro*. The regulation of CSF-1

mRNA expression appears to occur at the transcription level, and expression of the mRNA transcripts in the mouse is tissue-specific (Rajavashisth *et al*, 1987). In addition, regulation of CSF-1 expression is achieved through similar transcriptional and post-transcription control mechanisms as is GM-CSF, including mRNA and protein stabilisation.

During pregnancy in the mouse, uterine concentrations of CSF-1 increase 1000 fold by term (Bartocci *et al*, 1986), and CSF-1 bioactivity is detected in placenta and amniotic fluid. Associated elevation of CSF-1 serum levels, and the abundance of circulating monocytes and neutrophils at the expense of lymphocyte numbers, more than likely reflect this uterine activity (Daiter and Pollard, 1992). CSF-1 mRNA expression has since been localised to the glandular and luminal uterine epithelium (Arceci *et al*, 1989). Expression peaks on day 14 of pregnancy in the mouse, yet is undetectable in the non-gravid uterus (Pollard *et al*, 1987). Uterine epithelial cell expression of CSF-1 is under steroidal regulation, dependent on both oestradiol and progesterone synthesis, synergised by decidual stimulus (Pollard *et al*, 1987). Identification of CSF-1 mRNA by Northern blotting illustrates the predominance of the 2.3 kb (91%) transcript as opposed to the conventional 4.6 kb transcript (9%) (Arceci *et al*, 1989, Pollard *et al*, 1987), which is preferentially expressed by macrophages, fibroblast cells (including L cells) and placenta (Crainie *et al*, 1990). The expression of the shorter transcript may lead to an accumulation of CSF-1 since it exhibits a longer half-life than the 4.6 kb transcript.

1.3.2.3 CSF-1 receptor

CSF-1 initiates biological activity through binding to the 165 kD c-fms proto-oncogene product, which is expressed by virtually all cells of the mononuclear phagocyte lineage (Stanley and Heard, 1977). Receptor binding is followed by autophosphorylation of the tyrosine kinase receptor, internalisation of the receptor protein complex, degradation of CSF-1 and subsequent recycling of the receptor back to the membrane surface. The expression of the receptor is down-regulated by IL-3, GM-CSF, tetradecanoyl phorbol acetate (TPA), LPS and IFN. The receptor belongs to the protein tyrosine kinase family of plasma membrane receptors including EGF, FGF β , PDGF and *c-kit* (Sherr *et al*, 1988, Rothwell and Rohrschneider, 1987, Hampe *et al*, 1989) although unlike the other family members, it is unable to phosphorylate phospholipase C- γ .

The intra-cellular signalling transduction pathway for c-fms is controversial. It is becoming increasingly evident, due to the diverse activities CSF-1 can evoke, that activation

of one particular pathway is unlikely. Activation of calcium/phospholipase-dependent protein kinase C via the agonist phorbol ester only partially elicits CSF-1 activity (Hamilton *et al*, 1991). Activation of phosphatidylinositol kinase (Sherr, 1990) and the G protein family, including G α 1, 2 and 3, and the *ras* family of oncogenes, are alternative pathways (Hume and Denkins, 1987, Sherr, 1990).

1.3.2.4 Effects induced by CSF-1 on target cells

CSF-1 primarily acts on cells of the mononuclear phagocyte lineage in 4 distinct ways, causing differentiation of precursor cells, mitogenicity, changes in cellular function/activation and survival. Specific actions on macrophages have been described including induction of chemotactic activity, adhesiveness *in vitro* (Boocock *et al*, 1989) and the production of numerous cytokines including IL-1, IL-2, IL-3, IL-6, G-CSF, IFN, TNF and SCF. Effects on plasminogen activator, various enzymes and other mediators including prostaglandins, thromboxane and cyclo-oxygenase activity have also been attributed to CSF-1 (reviewed by Stanley, 1992).

The expression of CSF-1 by uterine epithelial and placental cells, and its receptor *c-fms* by the embryo and placental cells, suggest these proteins may contribute to regulation of the environment for pregnancy (Daiter and Pollard, 1992). *c-fms* mRNA is expressed by ovulated, unfertilised oocytes and increases during *in vitro* maturation. Following fertilisation however, mRNA levels drop to undetectable levels by the early 2 cell stage, but later are re-expressed by 2 - 4 cell embryos at low levels throughout preimplantation development (Arceci *et al*, 1992). As the placenta forms, *c-fms* mRNA transcripts can be localised to maternal decidua and the giant trophoblastic and spongiotrophoblastic cells, and to a lesser extent, the labyrinthine layer of the placenta (Arceci *et al*, 1989, Athanassakis-Vassiliadis *et al*, 1993). *In vitro* experiments have CSF-1 implicated in modulating placental cell proliferation, as well as MHC antigen expression by trophoblasts. Effects on the cytotoxic activity of lymphocytes may subsequently lead to reduced fetal loss (Brosh *et al*, 1991, Vassiliadis and Athanassakis, 1994).

1.3.2.5 CSF-1 deficient mice

An excellent tool for the evaluation of the *in vivo* role of CSF-1 in development has been afforded by the identification of the osteopetrotic mutant mouse strain. The osteopetrotic (*csfm^{op}*) mutation is a naturally occurring, recessive mutation on chromosome 3 (Yoshida *et*

al, 1990, Shultz and Sidman, 1987). Mapping of the *csfm* gene to chromosome 3 and sequencing of *csfm* cDNA shows that a single base pair insertion in the coding region of the CSF-1 gene, generating a stop codon 21 base pairs downstream, is responsible for the osteopetrotic phenotype.

While CSF-1 mRNA can be detected in normal levels in *csfm^{op}/csfm^{op}* fibroblasts, the 2.3 kb spliced form is absent and these mice are devoid of bioactive CSF-1 protein (Wiktor-Jedrzejczak *et al*, 1990).

These mice are characterised by bone defects and severe osteopetrosis due to an osteoclast deficiency. They are toothless, diminished in size and exhibit tissue-specific and age-dependent macrophage deficiencies (Wiktor-Jedrzejczak *et al*, 1982, Begg *et al*, 1993). These mice appear to be relatively healthy when maintained under pathogen-free conditions. In early adulthood, *csfm^{op}/csfm^{op}* mice have little or no macrophages in the peritoneal cavity, spleen and lymph nodes (Witmer-Pack *et al*, 1993). Other populations in the thymus, red pulp of the spleen, lymph node medulla, intestinal lamina propria, liver, lung and brain are of reasonable numbers. Tissue macrophages are also smaller in size, and while showing varying degrees of phagocytosis, have poorly developed intracytoplasmic organelles and microvillous projections, indicating they are ultrastructurally immature (Usuda *et al*, 1994). The degree of reduction in macrophage numbers is variable among tissues and corrects to some extent with age (Begg and Bertocello, 1993). The identification of dendritic cells within tissues of the *csfm^{op}/csfm^{op}* mouse supports the contention that CSF-1 is not required for dendritic cell viability, however CSF-1 deficiency does lead to less than usually prominent interdigitation of dendritic (Takahashi *et al*, 1993, Usuda *et al*, 1994) and Langerhans cells (Takahashi *et al*, 1992).

The cytokine profile of the *csfm^{op}/csfm^{op}* mouse is not well studied, but TNF α expression is unaltered in the uterus, placenta and embryo in the absence of CSF-1 (Hunt *et al*, 1993), while the diminished production of IL-1 and IL-4 at 6 weeks returns to normal values by 22 weeks, IL-3 remains partially decreased (Philippart *et al*, 1993).

Various studies have examined the ability of exogenous recombinant CSF-1 treatment to compensate for CSF-1 deficiency. Systemic administration of exogenous CSF-1 reverses osteopetrosis in the *csfm^{op}/csfm^{op}* mouse (Felix *et al*, 1990, Wiktor-Jedrzejczak *et al*, 1990). Administration of recombinant GM-CSF to *csfm^{op}/csfm^{op}* mice increases the abundance and morphological maturation of macrophage populations in some organs (liver, spleen, thymus, marrow, pleural and peritoneal cavity) but has no effect on bone resorption, osteoclast

populations or tooth eruptions (Wiktor-Jedrzejczak *et al*, 1994, Naito *et al*, 1997) suggesting exogenous GM-CSF effects are limited to specific organs. Macrophage numbers in ovary and uterus are unchanged (Naito *et al*, 1997), indicating that local exogenous CSF-1, perhaps bound to membranes, is required in these tissues.

Interestingly, mice deficient in both CSF-1 and GM-CSF (GM-*-/-*/*cfms*^{op}/*cfms*^{op}) exhibit all features corresponding to mice deficient in either factor alone. Some traits, such as the lung pathology, are more prevalent, extensive and often fatal (Lieschke *et al*, 1994).

Homozygous *csfm*^{op} crosses are consistently infertile, although very occasional litters are reported in some pathogen-free breeding facilities (Pollard, 1997). *csfm*^{op}/*csfm*^{op} females mated to heterozygous males *+/-cfms*^{op} have shown reduced fertility (39%), while *+/-cfms*^{op} females mated with *csfm*^{op}/*csfm*^{op} males are almost unaffected by the deficiency, displaying a fertility rate of 85% relative to the *+/-cfms*^{op} female x *+/-cfms*^{op} male matings (Pollard *et al*, 1991). The nature of this fertility defect is unclear.

1.3.3 IL-1

Originally named lymphocyte activating factor (LAF), IL-1 plays a central role in inflammation and immunity. The IL-1 system comprises two polypeptide mediators, termed IL-1 α and IL-1 β , two extracellular binding proteins, referred to as receptor type I and type II (RtI and RtII), and a receptor antagonist (ra). Binding of IL-1 to autoantibodies (Bendtsen *et al*, 1989, Svenson *et al*, 1989) and α_2 -macroglobulins (Borth and Luger, 1989) has been reported, but the interaction and hence significance of these proteins in the IL-1 system is not clear.

IL-1 α and IL-1 β represent the products of two distinct genes, and are initially synthesised as 30 - 32 kD propeptides and subsequently cleaved proteolytically to generate the mature 17 kD protein of 159 and 153 amino acids respectively. A number of cell types have been shown to produce IL-1, including monocytes and macrophages, lymphocytes, neutrophils, endothelial and epithelial cells (reviewed by Dinarello, 1992). IL-1ra has a molecular weight of 18 - 22 kD, depending on the glycosylation of the molecule. It appears to be produced by the same cell lineages which synthesise IL-1 α and β , and to be species-specific in binding the IL-1 receptors (Eisenberg *et al*, 1990).

The IL-1RtI and IL-1RtII receptors are single transmembrane glycoproteins of the immunoglobulin (Ig) superfamily, of 80 kD and 68 kD molecular weights respectively. A naturally occurring soluble form of IL-1RtII has been identified (Symons *et al*, 1991),

however little is known about its binding properties. However, the two membrane-bound receptors bind IL-1 α and β , and IL-1ra with variable affinities (McMahan *et al*, 1991). IL-1RtI binds IL-1 α with highest affinity, and IL-1RtII binds IL-1 β with highest affinity. IL-1ra binds both receptors with comparable affinity to IL-1 α . As IL-1RtII appears not to elicit a biological response, most probably due to a truncated cytoplasmic domain, Colotta *et al* (1994) proposed that this protein may act as a decoy receptor (deceptor), mopping up extracellular IL-1.

The biological actions of IL-1 include T and B cell activation, induction of cytokines, including GM-CSF and CSF-1 (Fibbe *et al*, 1988, Kaushansky *et al*, 1988b) and adhesion molecule expression, stimulation of fibroblasts, mediation of the acute phase reaction and induction of fever (reviewed by Dinarello, 1992, Colotta *et al*, 1994).

IL-1RtI and IL-1 β converting enzyme deficient mice have been reported as fertile and of normal vigour (Labow *et al*, 1997, Glaccum *et al*, 1997, Li *et al*, 1995). Further studies however on IL-1RtI knockout mice suggest that the mean litter size may be reduced (Abbondanzo *et al*, 1996), and blockade of maternal IL-1RtI *in vivo* by the administration of IL-1ra prevents implantation in the mouse (Simón *et al*, 1994a).

1.3.4 TNF

Tumour necrosis factor α was originally identified in serum from tumour-bearing mice injected with LPS, endotoxin or gram-negative bacteria, and named because it induced tumour necrosis in these mice (Carswell *et al*, 1975).

Initially synthesised as a 25 kD nonglycosylated transmembrane protein, the secreted 156 amino acid form of murine TNF α is a 17 kD fragment proteolytically cleaved from the 235 amino acid plasma membrane form, which circulates as a 51 kD homotrimer. Murine TNF α cDNA shares 79% amino acid homology with human TNF α (Beutler and Cerami, 1986).

Cellular sources of TNF α include LPS-stimulated mononuclear phagocytes, antigen stimulated T cells, activated NK cells and activated mast cells.

TNF α appears to bind to 2 distinct cell surface receptors, a 55 - 60 kD receptor and a high affinity, 75 - 80 kD receptor (Tartaglia and Goeddal, 1992), which may mediate discrete TNF α activities (Tartaglia *et al*, 1991). Soluble receptors have been identified (Lantz *et al*, 1994) and autoantibodies to TNF has been reported (Bendtzen *et al*, 1989, Svenson *et al*,

1989, Fomsgaard *et al*, 1989), but the interactions between and hence significance of these proteins are unclear.

TNF α mediates leukocyte accumulation at local sites of inflammation by causing sequential adhesion to vascular endothelial cells and acting as a chemotaxin (Ming *et al*, 1987). TNF α stimulation results in the functional activation of neutrophils, eosinophils and mononuclear phagocytes and initiates a cascade of secretion from various cell targets of cytokines including IL-1, IL-6, TNF α and GM-CSF (Kaushansky *et al*, 1988a). TNF α may coactivate lymphocytes and stimulate antibody production by B cells. As well as mediating local inflammatory events, TNF α is an endogenous pyrogen and induces fever. TNF α -induced endothelial cell necrosis is characteristic of tumour regression (Keyes and Wiltbank, 1988). While sharing a variety of biological actions attributed to IL-1 (Le *et al*, 1988), TNF α also acts synergistically with other cytokines (Fransen *et al*, 1986).

Knockout models of TNF ligand and receptors have focussed on the host response to infection, immunological disorders and tissue injury. However, the impact on fertility is unreported. TNF $^{-/-}$ mice are reported to develop normally and phagocytic activity of macrophages appears relatively normal. In contrast, LPS-induced total colony-stimulating activity is reduced (Marino *et al*, 1997).

1.4 Leukocyte-cytokine axis in the ovary

The ovary is not an immunologically privileged site and leukocytes are present in the ovary. Few studies have examined the presence or role of leukocytes in the immature ovary, however they are present throughout all stages of the cycle in the adult. Leukocytes have been implicated in the paracrine regulation of ovarian processes, primarily because they produce a vast array of biochemical mediators known to be associated with ovarian events including the regulation of steroidogenesis, vascular permeability, ECM remodelling, angiogenesis, apoptosis and immune function. Communication between leukocytes and resident (non-haemopoietic) ovarian cells through the cytokine networks has been suggested to be crucial for successful reproduction. Leukocytes are prevalent in the medulla, surrounding the theca of follicles, and in corpora lutea of the mouse, rat, human and also various other domestic species including cow, pig and sheep.

1.4.1 Leukocyte changes associated with folliculogenesis and ovulation

In 1980, Espey likened ovulation to an inflammatory reaction on the basis of the commonalities between the cellular and biochemical mediators in each event. Macrophages, granulocytes and T lymphocytes have been observed in the ovary of the adult rat, human and pig and are particularly prevalent leading up to ovulation. Studies with follicular fluid have indicated the presence of potent chemotactic molecules which presumably have a direct role in recruiting leukocytes into the follicle (Herriot *et al*, 1986, Murdoch and McCormick, 1989).

1.4.1.1 Mononuclear phagocytes

The macrophage is the major leukocyte subset present in the ovary and implicated in the cascade of events constituting ovulation due to its ability to produce numerous biochemical mediators and cytokines associated with ovulation and possibly steroidogenesis. Early histochemical studies have identified non-specific esterase activity associated with scattered cells throughout the ovary and associated with the theca surrounding developing follicles in the rat (Bulmer, 1964) and guinea pig (Paavola, 1979). The existence of macrophages in the ovary was later confirmed by immunohistochemical labelling of the cells with the macrophage-specific antibody F4/80 (Hume *et al*, 1984), and more recently in the human (Brännström *et al*, 1994c). Macrophages have been identified in the immature rat and pig ovary (Brännström *et al*, 1993a, Standaert *et al*, 1991) and are present in the theca of preantral follicles in the human ovary (Katabuchi *et al*, 1989).

As ovulation approaches, macrophages infiltrate the periovulatory rat ovary and increase 5 fold in density in the theca (Brännström *et al*, 1993a). In the pig ovary, macrophage-like cells increase in density at the time of ovulation (Standaert *et al*, 1991). Macrophages localised to the theca are present in the apical region as well as regions adjacent to the stroma, and those localised to the stroma can be observed close to capillary networks (Katabuchi *et al*, 1989). In addition, many of the cells with a macrophage-like morphology express MHC class II antigens indicating a state of activation, or possibly the presence of dendritic cells. While macrophages have been identified in follicular fluid of women undergoing IVF treatment (Castilla *et al*, 1990, Loukides *et al*, 1990), evidence suggests this is the result of follicular aspiration and associated blood contamination (Beckman *et al*, 1991), since no macrophages nor any other leukocyte are seen in the granulosa layer of healthy follicles in tissue sections.

Co-incubation studies demonstrate the ability of macrophage-derived products to stimulate granulosa cell progesterone production in mice (Kirsch *et al*, 1981), humans (Halme *et al*, 1985) and pigs (Maier and Chew, 1990). During the oestrous cycle, macrophage activity in the ovary may be regulated by changing steroid levels (reviewed by Vernon-Roberts, 1969), since oestrogens and to a lesser degree, progesterone have been demonstrated to stimulate macrophage activities including phagocytosis (Nicol and Bilbey, 1960).

1.4.1.2 Polymorphonuclear granulocytes

Mast cells infiltrate the ovary during the latter part of the follicular phase and degranulate in response to the LH surge (Nakamura *et al*, 1987). Histamine is released into the interstitial tissue, along with various other mediators including serotonin, prostaglandins, leukotrienes, bradykinin and platelet activating factor.

In the peripheral circulation, ovulation is marked by a 5-fold increase in neutrophil alkaline phosphatase activity in the human (Diamaut and Polishuk, 1979) and this increase is also paralleled in salivary peroxidase activity (Cockle and Harkness, 1978) and correlate with plasma and urinary oestrogen levels (Polishuk *et al*, 1968, Polishuk and Diamaut, 1973).

Conventional histological techniques demonstrate a 10-fold increase in the neutrophilic marker, myeloperoxidase in the immature primed rat ovary during the periovulatory period (Abisogun *et al*, 1987). Using immunohistochemical methods, neutrophils are located in the medulla and the theca layer of preovulatory follicles and increase 3 and 8 fold respectively at ovulation (Brännström *et al*, 1993a). This periovulatory increase in theca neutrophils is also observed in the rabbit and sheep using histological staining. In the porcine ovary neutrophils are present in the theca of preovulatory follicles (Standaert *et al*, 1991).

Depletion of circulating neutrophils by administration of a lineage-specific monoclonal antibody causes a significant decrease in ovulation in the immature-primed rat ovary (Brännström *et al*, 1995a).

1.4.1.3 Lymphocytes

T cells have been identified in the stroma of the immature rat (Brännström *et al*, 1993a) and described in the medulla and theca of preovulatory and ovulating follicles, but in low numbers. Lymphocytes comprise < 10% of total blood leukocytes present in the preovulatory porcine ovary (Standaert *et al*, 1991), and subsequent studies employing the use

of lineage-specific monoclonal antibodies against cell surface markers have enabled determination of the specific lymphocyte subsets that are present in the ovary. More specifically, T lymphocytes identified in the medulla and follicle wall of the rat (Brännström *et al*, 1993a) and human (Brännström *et al*, 1994c) are predominantly suppressor/cytotoxic T cells (80%) as identified by the expression of CD8. Comparison with the blood CD4:CD8 ratio (2:1) indicates a selective immigration of CD8+ T cells into ovarian tissue. Earlier reports of CD4+ and CD8+ T cells in human preovulatory follicular fluid (2:1) (Hill *et al*, 1987) are likely to reflect contamination with cells from adjacent tissue and blood. T cells have also been identified in preovulatory and ovulating follicles as well as atretic follicles of the rabbit (Bagavandoss *et al*, 1990). The role of lymphocytes in follicular development and ovulation is unclear, as they comprise a small proportion of blood leukocytes present in the ovary, yet adherence to follicular endothelium in the rat near the time of ovulation has been demonstrated (Parr, 1974). In addition, lymphocytes express steroid receptors, and so ovarian lymphocytes may respond directly to changes in local steroid hormone parameters (Cohen *et al*, 1983).

Immunosuppression or immunodeficiency as a result of neonatal thymectomy or congenital athymia as in the nude mouse, can alter ovarian function, as characterised by delayed puberty, lymphocyte infiltration, reduced oocytes, increased follicular atresia, decreased ovarian steroidogenesis, interstitial cell hyperplasia and premature ovarian failure in animals (Nishizuka and Sakakura, 1969). Restoration of a variety of these characteristics can be achieved by the grafting of spleen, thymus or lymphoid tissue.

1.4.2 Cytokine change associated with folliculogenesis and ovulation

1.4.2.1 GM-CSF

At the onset of these studies, there was little evidence to suggest a role for specific macrophage or granulocyte regulators in the ovary. From a study employing perfusion of the rat ovary, evidence suggests that bioactive GM-CSF is secreted from the ovary and that secretion is maximal at the time of ovulation (Brännström *et al*, 1994b). Immunoreactive GM-CSF has been identified in human plasma (7.1 ± 0.5 pg/ml) throughout the course of the menstrual cycle, with GM-CSF values in 40% of the subjects fluctuating concomitantly with oestradiol concentrations (Jasper *et al*, 1996). Immunoreactive GM-CSF has been identified in preovulatory follicular fluid collected from naturally cycling women (4.9 ± 0.4 pg/ml) and women undergoing IVF treatment (8.9 ± 0.7 pg/ml) (Jasper *et al*, 1996).

Ovarian stimulation for IVF procedures has increased the concentration of GM-CSF in follicular fluid and plasma collected from women (Jasper *et al*, 1996), suggesting that synthesis by ovarian cells may be partially regulated by gonadotrophin or steroid hormones, as is the case for uterine epithelial cell GM-CSF synthesis (Robertson *et al*, 1996b), which is oestrogen dependent. However, GM-CSF secretion by perfused preovulatory rat ovaries is not influenced by LH alone (Brännström *et al*, 1994b). Alternatively, an effect of gonadotrophins may be mediated via intermediary cytokines or through enhanced recruitment of trafficking cells, such as macrophages, which can produce GM-CSF when activated by cytokines, including IL-1, IL-6 and TNF. IL-1 β has been found to stimulate the increased secretion of GM-CSF in the rat perfusion system, as well as TNF α administered with LH or alone (Brännström *et al*, 1994b), although no conclusive data has been generated on the regulation of GM-CSF secretion by cytokines from human granulosa-lutein cells in culture (Jasper *et al*, 1996).

1.4.2.2 CSF-1

There is limited research describing mRNA expression and protein localisation in the ovary. Increased levels of immunoactive CSF-1 in human follicular fluid compared to serum suggests an ovarian source (Witt and Pollard, 1997). CSF-1 is present in conditioned media of oocytes, cumulus and granulosa cells (Arceci *et al*, 1992). Localisation of CSF-1 mRNA suggests cumulus-granulosa cells are likely sources of ovarian CSF-1 (Pollard *et al*, 1987), whereas the 2 cell preimplantation embryo does not express mRNA for CSF-1 in the human (Sharkey *et al*, 1995). Transcripts for the respective receptor, c-fms are expressed in the oocyte from stage III of development and persist through fertilisation (Arceci *et al*, 1992, Haimovici and Anderson, 1993) indicating paracrine regulation of oocyte development may occur.

Administration of exogenous CSF-1 can increase ovulation rate in a dose-dependent manner in immature primed rats when treated between 96 - 49 hr before hCG, and this is temporally associated with the increase in the abundance of macrophages surrounding mature follicles (Nishimura *et al*, 1995). Administration of an anti-CSF-1 antibody results in a reduction in ovulation rate which can be reversed by administration of CSF-1 (Nishimura *et al*, 1995).

1.4.2.3 IL-1

IL-1 was initially detected in porcine and human follicular fluid (Barak *et al*, 1992a, Wang and Norman, 1992, Zolti *et al*, 1992). In addition it is secreted by the ovulating rat ovary *in vitro* (Brännström *et al*, 1994b). Loukides and co workers (1990) have identified IL-1 β mRNA in macrophages derived from human follicular fluid, demonstrating one likely cellular source for IL-1 β in the ovary. During follicular development in the mouse, IL-1 α , IL-1 β and IL-1RtI protein are confined to the theca-interstitial layer of growing follicles (Simón *et al*, 1994b). At ovulation there is a 4 - 5 fold increase in IL-1 β mRNA localised to the theca-interstitial cells of the rat ovary. Follicles ranging from preantral up to early antral stages express mRNA for IL-1RtI and IL-1ra in the granulosa layer, but the signal intensity subsequently fades during the periovulatory period in the rat (Hurwitz *et al*, 1991b, Wang *et al*, 1997). Interestingly, IL-1RtI protein can be detected in the cytoplasm and cell membrane of the murine oocyte regardless of the developmental stage of the follicle (Simón *et al*, 1994b).

In developing follicles, IL-1 appears to stimulates growth of the follicle (Fukuoka *et al*, 1989b) while eliciting an anti-gonadotrophic action on both undifferentiated murine and human granulosa cells *in vitro* by inhibiting LH receptor expression and FSH/hCG stimulated steroidogenesis (Gottschall *et al*, 1988, Kasson and Gorospe, 1989). *In vitro* cultures of preovulatory follicles and/or ovarian dispersates reveal the stimulatory effects of IL-1 β on PGE, PGF_{2 α} , progesterone, prostacyclin and plasminogen activator activity (Kokia *et al*, 1992, Watanabe *et al*, 1993, Nakamura *et al* 1990, Brännström *et al* 1993c, Bonello *et al* 1995). No obvious effects of IL-1 β on androstenedione or oestradiol production have been described (Brännström *et al* 1993c). In contrast, IL-1 β inhibits androgen synthesis in relatively homologous cultures of thecal cells (Hurwitz *et al*, 1991a).

Alterations in IL-1 activity by exogenous administration of cytokine or antagonist can influence ovulation success. The addition of recombinant IL-1 β to the perfused ovary induces a low number of ovulations, but IL-1 β in combination with LH increases ovulation rate 3 fold in the rat (Brännström *et al*, 1993b). IL-1ra inhibits ovulation in the primed rat model *in vitro* (Peterson *et al*, 1993) and *in vivo* (Simón *et al*, 1994c). This contrasts with findings that central administration of IL-1 at proestrous in the cycling rat results in the inhibition of ovulation (Rivest and Rivier, 1993).

In addition to local ovarian effects, IL-1 inhibition of LHRH release consequently alters pituitary LH in the rodent (Rivier and Vale, 1989), as well as FSH secretion in the

primate (Ferin, 1993), and appears to be due to both a reduction in LH frequency and in pulse amplitude (Bonavera *et al*, 1993). The resulting action of IL-1 may be dependent on the oestradiol status of the system, since IL-1 has been shown to stimulate LH release in the presence of oestradiol (Bernton *et al*, 1987, Beach *et al*, 1989, Kalra *et al*, 1990) (reviewed by Rivest and Rivier, 1993).

1.4.2.4 TNF

Plasma TNF α concentrations fluctuate during the human menstrual cycle concomitant with major cyclic events in the ovary, and exhibit a preovulatory and mid-luteal phase rise (Brännström *et al*, submitted). Bioactive TNF is secreted by the ovulating ovary (Brännström *et al*, 1994b), and TNF α protein and mRNA (Sancho-Tello *et al*, 1992) have also been detected in the ovary. Immunoreactive TNF α has been identified in follicular fluid of the human (Wang and Norman, 1992). Immunohistochemical studies localise TNF α to macrophage-like cells in the interstitium of the mouse ovary (Chen *et al*, 1993) and to be associated with the antral layer of granulosa cells in preovulatory rat and bovine follicles (Roby and Terranova, 1989). In the preovulatory mouse, follicle theca and/or granulosa cells express TNF α mRNA (Chen *et al*, 1993). Preovulatory oocytes have also been shown to express TNF α mRNA, protein, and in a more recent study, oocytes collected from preovulatory follicles contained immunoactive and bioactive TNF α and expressed TNF α mRNA (Marcinkiewicz *et al*, 1994).

Like IL-1 β , TNF α has the ability to stimulate progesterone (Roby and Terranova, 1988, Zolti *et al*, 1990) and PGF $_{2\alpha}$ and PGE production by perfused ovulating ovaries (Brännström *et al*, 1995b), preovulatory rat follicles *in vitro* (Brännström *et al*, 1993c) and human granulosa cells (Wang *et al*, 1992).

The addition of recombinant TNF α to the perfused ovulating ovary system has been shown to increase ovulation rate alone and 4 fold in combination with LH (Brännström *et al*, 1995b) (reviewed by Hunt, 1993).

1.4.3 Leukocyte changes associated with luteinisation and luteolysis

After ovulation, accumulation of leukocytes in the stroma but particularly in the theca-interna and antrum of the ruptured follicle, termed the corpus haemorrhagicum, provides many opportunities for direct interactions between leukocytes and granulosa-luteal and theca-luteal cells. The potential for involvement in ECM remodelling, neovascularisation of the corpus

luteum and steroidogenesis, is immense. While formation of the corpus luteum is associated with considerable tissue remodelling, so too is the demise of the corpus luteum and the accompanying removal of cell debris.

1.4.3.1 Mononuclear phagocytes

Macrophages have been identified in the corpus luteum of many species and have been demonstrated to undergo temporal changes in distribution. In the corpus luteum of the rat and human, the density of macrophages has been found to increase from early- to mid-luteal phase (Lei *et al*, 1991, Brännström *et al*, 1993a). In the rabbit however, the density of macrophages is relatively constant throughout the early- and mid-luteal phase (Bagavandoss *et al*, 1990). In the human, macrophages predominate in the theca-lutein area of the corpus luteum (Wang *et al*, 1992, Brännström *et al*, 1994a).

During the formation of the corpus luteum, macrophages have been observed to interact physically with ovarian cells (Bulmer, 1964). Their functions have not been delineated, but they are likely to secrete molecules which promote angiogenesis and also steroidogenesis, since in co-culture experiments macrophages promote the secretion of progesterone from luteal cells (Kirsch *et al*, 1983, Halme *et al*, 1985). Interestingly, peritoneal macrophages are less able to stimulate progesterone production than corpora lutea-derived macrophages (Kirsch *et al*, 1983), indicating a tissue of origin effect on the phenotype of the macrophage. Due to the potent capacity of these cells to participate in phagocytosis, they are prime candidates for the removal of cellular debris during luteolysis and this is suggested by observations of heterophagy of cells in the ovary (Bulmer, 1964).

1.4.3.2 Polymorphonuclear granulocytes

While most studies have focussed on macrophage and lymphoid populations in the regulation of luteinisation and luteolysis, neutrophils are also present in the corpus luteum. In the human a large number of neutrophils can be seen adjacent to the rupture point. Neutrophils are present at early stages of corpus luteum development and are most prolific during the mid-phase of pseudopregnancy and pregnancy. They then tend to decline in numbers and become even further diminished during the early post partum period (Brännström *et al*, 1994a). This pattern of temporal change is not seen in all species, and in the human, numbers appear relatively constant in early, late and regressing corpora lutea (Brännström *et al*, 1994c). In the porcine ovary absolute numbers of neutrophils are highest in

the regressing corpus luteum (Standaert *et al*, 1991). In the rat, the cells are distributed evenly in the corpus luteum, but in the well defined theca-lutein and granulosa-lutein areas of the human corpus luteum, neutrophils are more prevalent in the theca-lutein, where the density of cells is higher than in the surrounding stroma (Brännström *et al*, 1994c).

A soluble neutrophil-derived product exerts a luteolytic effect when cultured with luteal cells of the rat, as indicated by inhibition of LH-sensitive cAMP accumulation and progesterone (Pepperell *et al*, 1992). This moiety may well be a peroxide anion, since neutrophil peroxide has been shown to be luteolytic for luteal cells *in vitro* (Behrman and Preston, 1989), and hydrogen peroxide evokes luteolytic actions in human granulosa luteal cells (Endo *et al*, 1993) and is generated *in vivo* during PGF_{2α}-induced luteolysis during pseudopregnancy.

1.4.3.3 Lymphocytes

T cells were first described in the corpus luteum of the cow (Lobel and Levy, 1968) and rabbit (Bagavandoss *et al*, 1988). Subsequent studies have shown that lymphocytes infiltrate the young corpus luteum (Standaert *et al*, 1991) and persist throughout its life span, both in pseudopregnancy and pregnancy (Bagavandoss *et al*, 1988). Lymphocytes comprise approximately 30% of total blood leukocytes in the porcine ovary in the developing and mature corpus luteum (Standaert *et al*, 1991). Further investigations indicate the activation of some of these leukocytes by the presence of class MHC II antigens (Bagavandoss *et al*, 1990) and the expression of IL-2 receptors (Wang *et al*, 1992). No B cells, but predominantly natural killer cells were identified in the human corpus luteum (Wang *et al*, 1992).

Lymphocytes and lymphocyte-derived products have the ability to stimulate progesterone production by granulosa-lutein cells *in vitro* in the rat (Gorospe and Kasson, 1988, Hughes *et al*, 1991), pig (Hughes *et al*, 1990), and human (Emi *et al*, 1991), suggesting a role in luteinisation and the secretion of luteotropins. Bagavandoss *et al* (1990) suggests that these cells, apparently present before the identification of macrophages, may in fact play a role in attracting macrophages into the corpus luteum and activating them via the production of cytokines.

Lymphocytes are also evident in the regressing corpus luteum (Bagavandoss *et al*, 1988). Although Standaert and co-workers (1991) have reported T cells to comprise a relatively constant (< 8%) proportion of the total leukocyte population, this is an apparent decline from numbers present in the developing and mature corpus luteum. More recently,

these cells have been proposed to be capable of mediating immunoregulated destruction of the corpus luteum (Bukovský *et al*, 1995b), perhaps through apoptotic cytolysis.

1.4.4 Cytokine changes associated with luteinisation and luteolysis

1.4.4.1 GM-CSF

Granulosa-lutein cells from hyperstimulated individuals have been found to secrete immunoactive GM-CSF *in vitro* at 47.6 ± 27 pg/ 10^5 cells, and mRNA for GM-CSF has been detected by RT-PCR in these cell preparations (Jasper *et al*, 1996). However, it cannot be excluded that detection of mRNA and immunoactive GM-CSF in granulosa-lutein cell culture preparations and supernatant respectively is not a result of up to 20% blood cell contamination during follicular aspiration (Beckman *et al*, 1991).

Corpus luteum from early- and mid-luteal phase ovaries have been found to secrete GM-CSF at 0.54 ± 0.22 pg/ μ g DNA and 0.98 ± 0.42 pg/ μ g DNA *in vitro* respectively (Jasper *et al*, 1996). Production by corpus luteum tissue is suggested to be stimulated by IL-1 β and TNF α , in accordance with other cell systems where these cytokines are known to promote GM-CSF production. RT-PCR has demonstrated the presence of GM-CSF mRNA transcripts and protein in early-, mid- and late-luteal phase human corpora lutea tissue indicating local production of the cytokine within the corpus luteum (Jasper *et al*, 1996). The detection of both GM-CSFR α and β chain mRNA transcripts in granulosa-lutein cell preparations and early-, mid- and late-luteal phase corpora lutea suggests the presence of GM-CSF-responsive cells within the ovary, although the cellular source of GM-CSF and target cells have not been identified (Jasper *et al*, 1996).

1.4.4.2 CSF-1

Bartocci and co-workers (1986) measured CSF-1 protein in the homogenised murine ovary by radioimmunoassay, demonstrating relatively constant levels during pregnancy. In the uterus, CSF-1 expression is dependent on progesterone (Pollard *et al*, 1987). However, immunoactive CSF-1 is not significantly elevated in the ovary of the day 10 pregnant mouse compared to the cycling ovary (Bartocci *et al*, 1986), suggesting uterine and not ovary-derived CSF-1 is the primary source of circulating CSF-1 during pregnancy.

1.4.4.3 IL-1

Cannon and Dinarello (1985) consistently observed increased plasma IL-1 bioactivity in women after ovulation, which could be neutralised with antisera to human IL-1. The cellular source of systemic IL-1 is unclear, however peripheral monocytes have been observed to secrete greater levels of bioactive IL-1 and express more mRNA transcripts when isolated in the luteal phase of the human menstrual cycle compared to the follicular phase (Polan *et al*, 1990, Polan *et al*, 1994). Consistent with fluctuating steroid levels during the menstrual cycle, gonadal steroids have been observed to modulate monocyte IL-1 activity, both at the mRNA and protein level (Polan *et al*, 1989, Flynn, 1986, Polan *et al*, 1988).

An alternative or additional tissue source of circulating IL-1 β is the ovary. Within the corpus luteum, IL-1 β can be immunohistochemically localised to macrophages but also granulosa-lutein cells (Simón *et al*, 1994b). This is accompanied by expression of IL-1RtI by macrophages and granulosa-lutein cells in the corpus luteum. The role of IL-1 in the process of luteinisation and luteolysis is controversial as some researchers report a stimulatory effect of IL-1 on granulosa-lutein cell progesterone production (Fukuoka *et al*, 1992, Sjögren *et al*, 1991), while others report no (Wang *et al*, 1991) or an inhibitory effect (Barak *et al*, 1992b). Of particular interest is the fact that IL-1 β increases the release of the potent luteolytic agent PGF_{2 α} from granulosa-lutein cells (Watanabe *et al*, 1993). The action of IL-1 on cells in culture is likely to be highly dependent on the purity of cell preparation, particularly the ratio of leukocyte:macrophages present and perhaps their differentiation phenotype.

1.4.4.4 TNF

The oncolytic effector activity of TNF α implicates this molecule as a major candidate in luteolytic mechanisms, which are reminiscent of the degenerative, self destructive processes similarly associated with tumour regression.

Plasma TNF α concentrations fluctuate and exhibit a preovulatory and mid-luteal phase rise during the human menstrual cycle concomitant with major cyclic events in the ovary (Brännström *et al*, submitted).

The increase in plasma TNF α during the mid-luteal phase might be attributable partly to corpus luteum-derived TNF α . Previous investigators have described secretion of immunoactive and bioactive TNF α from corpus luteum of the rabbit *in vitro* (Bagavandoss *et al*, 1988, 1990) and detection of mRNA for ovine TNF α (Ji *et al*, 1991). During the luteal

phase of the menstrual cycle the corpus luteum is a highly vascularised organ, consisting of large numbers of neutrophils and macrophages, which are potential sources of $\text{TNF}\alpha$. Decreasing luteal concentrations of progesterone precede the rise in bioactivity suggesting $\text{TNF}\alpha$ may not initiate luteal regression (Ji *et al*, 1991).

$\text{TNF}\alpha$ acts to inhibit progesterone production by murine granulosa-luteal cells (Adashi *et al*, 1990). Of particular interest is the fact that $\text{TNF}\alpha$ induces release of $\text{PGF}_{2\alpha}$ from granulosa-lutein cells (Zolti *et al*, 1990) and administration of $\text{TNF}\alpha$ induces functional luteolysis (Wuttke *et al*, 1992). $\text{TNF}\alpha$ may be a mediator in both the processes of luteinisation and luteolysis as a consequence of its pleiotrophic actions in many cell lineages.

1.5 Summary and hypothesis

Rapid and extensive tissue remodelling characterises the ovarian cycle, beginning when a single primordial follicle undergoes the growth and differentiation required to achieve preovulatory status. As the follicle wall is weakened and ruptures during ovulation, the oocyte is expelled and the cellular remnants form the corpus luteum. Rescue of the corpus luteum is a function of the conceptus and in its absence the structure becomes obsolete and regresses. The literature reviewed in this chapter highlights the extensive and rapid cycles of tissue reorganisation occurring within the ovary during the oestrous cycle and pregnancy which are necessary to achieve the:

- production of oocytes suitably mature for fertilisation
- extrusion of the oocyte from the ovary
- secretion of hormones to facilitate development of the female reproductive tract and promote implantation and placental growth.

Events in the ovary are driven at the systemic or endocrine level. However the classical model for the control of ovarian function which pivots on the interaction between the glycoprotein hormones, FSH and LH and the locally produced steroid hormones oestradiol and progesterone, appears inadequate to fully explain diverse and concomitant tissue remodelling events.

A complex leukocyte-cytokine network is now implicated in the paracrine and autocrine mediation of tissue remodelling during each of the stages of ovarian function. Leukocytes are an abundant and dynamic population of cells in the ovary. These macrophages, granulocytes and lymphocytes have the capacity to secrete a diverse array of potent biochemical mediators, many of which are implicated in various ovarian events. The recruitment, differentiation and activation of leukocytes within the ovary is likely to be orchestrated by cytokines synthesised by resident ovarian cells. Their selective immigration and subsequent activation in a given compartment is likely to be orchestrated by precise spatial and temporal patterns of adhesion molecules and regulatory cytokines. Cytokines are ideally suited to provide communication links between the various types of ovarian cells, in view of their pleiotrophic effects, their production by a range of cell types and their ability to exert potent biological responses from cells within close proximity.

Together leukocytes and cytokines have been implicated in ovarian tissue remodelling events including:

- growth factor support for the oocyte
- regulation of steroidogenesis
- extra-cellular matrix remodelling
- angiogenesis
- apoptosis
- phagocytosis
- immunoregulation

The colony stimulating factor family of cytokines may have important roles in the ovary during oestrous and pregnancy in view of their capacity to regulate macrophage and granulocyte survival, proliferation, differentiation and activation.

1.5.1 Hypothesis

The macrophage regulators GM-CSF and CSF-1 contribute to regulation of the tissue remodelling events and immunological changes within the ovary through actions on resident and infiltrating macrophages and granulocytes.

1.5.2 Aims

At the onset of this study, the synthesis and roles of colony-stimulating factors in ovarian function were undefined. The use of knockout models provided an avenue to examine the impact of specific cytokine deficiency on local cell populations in the ovary, including the functional competence of these cells during the tissue remodelling events that characterise the oestrous cycle. Therefore, the aims of this thesis are:

1. To examine the expression of components of the GM-CSF and CSF-1 signalling systems in the ovary.
2. To investigate the role of GM-CSF in the ovary by assessment of functional parameters in the GM-CSF gene knockout mouse.
3. To investigate the role of GM-CSF in the regulation of leukocyte populations in the ovary by assessment of cellular parameters.
4. To examine the developmental competence of oocytes derived from a GM-CSF deficient environment.
5. To investigate the role of CSF-1 in the ovary by assessment of both functional and cellular parameters in the naturally occurring *csf1^{op}* mutant mouse.

Chapter 2

Materials and methods

2.1 Chemicals and solutions

All chemicals were of analytical grade and unless otherwise stated were purchased from Ajax Chemicals, BDH Laboratory Supplies or Sigma Chemical Company. Buffers and solutions were made using milli Q water (milli Q H₂O) (Millipore Corporation); mains water purified by reverse osmosis followed by deionisation, resulting in resistivity > 18 MΩ.cm. The recipes, sterilisation and storage for all solutions and buffers are described in Appendix 1 in alphabetical order. Unless otherwise stated, phosphate buffered saline (PBS) was prepared using Dulbecco's recipe, Ca²⁺, Mg²⁺ free, pH 7.4 as described in Appendix 1. The company and product number or model number for each reagent or piece of equipment are listed in parenthesis after the product in the text. The company address including state and country are listed in alphabetical order in Appendix 3.

2.2 Mice

2.2.1 General

Mice (*Mus musculus*) were housed and bred in a viral free, minimum security holding facility at The University of Adelaide Medical School Animal House, or at The Queen Elizabeth Hospital Conventional Animal House. Dry food (Ridley Agriproducts, formula 8215) and 0.015% hydrochloric acid, ultraviolet (UV)-treated mains water were supplied *ad libitum* and a 12 hr light and 12 hr dark cycle maintained within the enclosure. Mice homozygote for the *csfm^{op}* gene were maintained on a liquid diet of 50% w/v powdered dry food in vanilla flavoured Ensure® nutritional powder (Abbott Australasia, 0750 100-358-100). All female mice were virgins prior to the onset of any experiment. All experiments were approved by The University of Adelaide Animal Ethics Committee and The Queen Elizabeth Hospital Animal Ethics Committee and were performed under the ethics approval numbers M/83/93, M/83/93A and M/83/93B and N/1/94 respectively.

2.2.2 Genetics of the GM-/- mouse

Mice deficient in GM-CSF (GM-/-) were generated using gene targeting techniques in 129/Ola-derived E14 embryonic stem cells, and were propagated from founder mice by

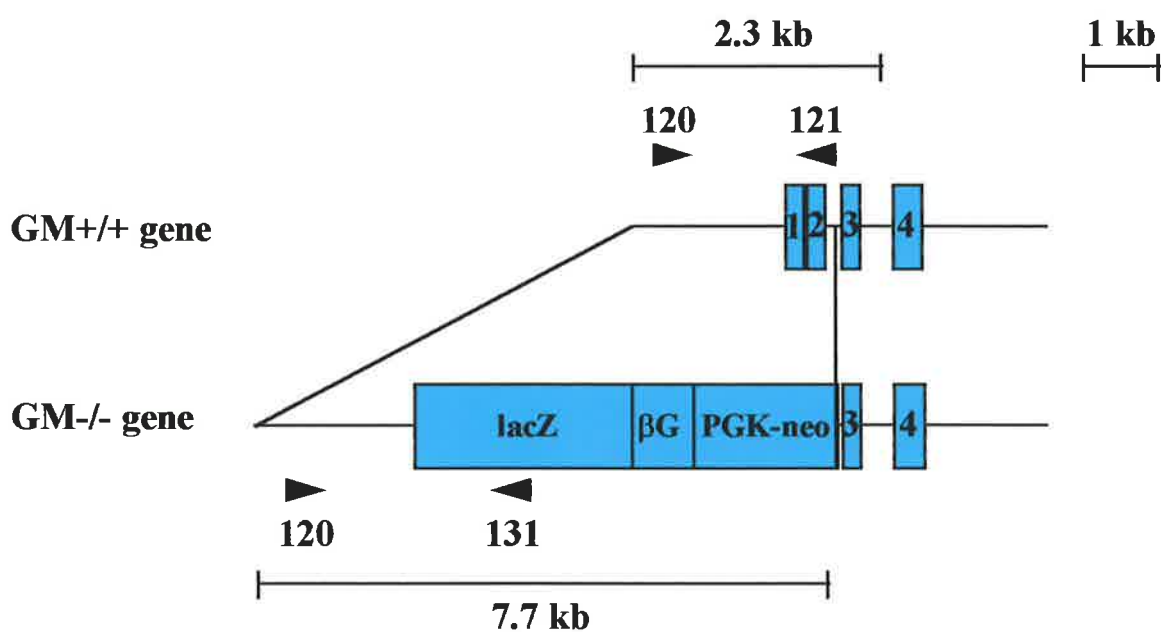
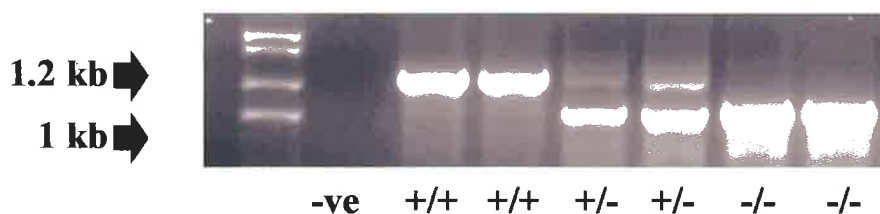
GM+/+**GM-/-****C****D**

Figure 2.1 Genetics of the GM-/- mouse. The genotype of A) GM+/+ and B) GM-/- mice cannot be determined on the basis of phenotype. C) GM-CSF gene of the GM+/+ and GM-/- mouse, illustrating the targeting vector which was constructed to delete exons 1 and 2. The following regions are identified: coding regions of exons (blue) and introns and flanking regions (thin line). PCR primer hybridisation sites are marked by black arrows. D) Confirmation of genotype by analysis of PCR generated products synthesised from GM+/+, GM+/- and GM-/- mouse DNA using primers illustrated in C). lac Z = *Escherichia coli* lacZ, βG = human β-globin, PGK-neo = PGK-neo selectable marker.

mating with C57BL/6J mice (Stanley *et al*, 1994). The GM-CSF targeting vector consisted of from 5' to 3', 900 bp of the GM-CSF gene promoter (from enzyme restriction site *Rsa* I - *Sca* I), the *Escherichia coli lacZ* gene, a 700 bp fragment of the human β -globin gene 3' untranslated and a poly(A) addition motif (*Eco* RI - *Acc* I), the PGK-neo selectable marker and approximately 10 kb of GM-CSF genomic sequence. The targeting vector was constructed to delete GM-CSF exons I and 2 and intron 1 between the *Sca* I and *Sma* I restriction enzyme sites (Figure 2.1). GM-/- breeding pairs were kindly provided by Ashley Dunn, Ludwig Institute for Cancer Research. Since viral-free 129/Ola mice could not be accessed in Australia, GM-CSF replete mice (GM+/+) were derived from F2 offspring of GM-/- females crossed with wild type 129/Sv males obtained from the Animal Resources Centre.

2.2.2.1 Confirmation of the GM-/- genotype by PCR

The genotype of the GM+/+ and GM-/- mice cannot be distinguished on the basis of their phenotypes. DNA was isolated from blood or tissue according to Chapter 2, 2.7.2 and by the addition of three primers in each polymerase chain reaction (PCR), GM+/+, GM+/- and GM-/- mice were simultaneously screened. A single 5' antisense oligonucleotide primer, primer 120 corresponding to the GM-CSF promoter was used in conjunction with two 3' antisense oligonucleotide primers. Primer 121, defined the wild type allele, and primer 131, corresponded to part of the *Escherichia coli lacZ* cassette disrupting the first intron of the GM-CSF allele. PCR (Chapter 2, 2.7.8) was used to simultaneously amplify segments of the GM-CSF gene common to the GM+/+ or GM-/- knockout genome. The genotypes were distinguishable on the basis of the size of the PCR generated product. GM+/+ mice exhibited a single PCR product of 1.2 kb, GM+/- mice exhibited 2 products of 1 kb and 1.2 kb and GM-/- mice exhibited a single PCR product of 1 kb (Figure 2.1). GM-/- spleen conditioned media contained no bioactive or immunoactive GM-CSF as assayed by Stanley and co-workers (1994) and GM-/- uterine conditioned media contained no bioactive GM-CSF as assayed by Robertson and co-workers (in press).

2.2.3 Genetics of the *csfm^{op}/csfm^{op}* mouse

Colony stimulating factor-1 deficient, osteopetrotic mutant (*csfm^{op}/csfm^{op}*) mice on a C57BL/6J-*Pit1^{dw}* background were kindly provided by Ivan Bertoncello, Peter MacCallum Cancer Institute. The mutation results from a spontaneous single base insertion in the CSF-1

CSF^{+/±}



A

csfm^{op}/csfm^{op}



B

C

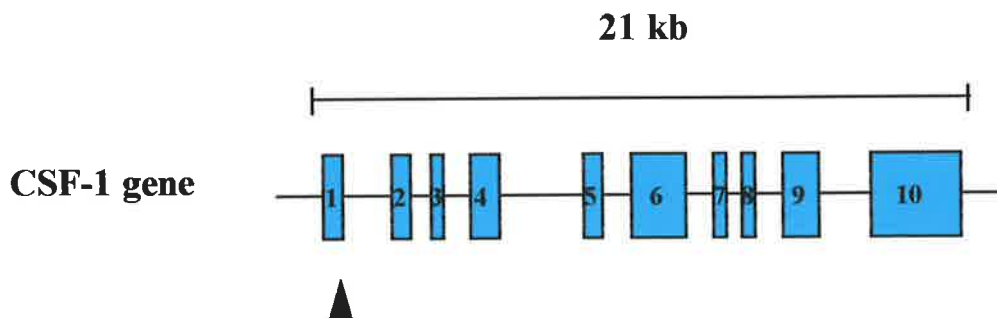


Figure 2.2 Genetics of the *csfm^{op}/csfm^{op}* mouse. A) CSF-1^{+/±} and B) *csfm^{op}/csfm^{op}* mice were distinguished on the basis of phenotype alone, *csfm^{op}/csfm^{op}* mice being smaller in size and lacking incisor teeth. C) Proposed region of the disrupted CSF-1 gene of the *csfm^{op}/csfm^{op}* mouse, arrow indicating location of single base insertion in the human CSF-1 gene. The following regions are identified: coding regions of exons (blue) and introns and flanking regions (thin line).

coding region which generates a stop codon 21 bp downstream of the promoter (Figure 2.2). The mutation appears to be completely recessive and normal CSF-1 levels circulate in heterozygotes (Wiktor-Jedrzejczak *et al*, 1990). Mice homozygous for the *csfm^{op}* allele were bred by mating heterozygous females (CSF-1+/*csfm^{op}*) and homozygous studs (*csfm^{op}/csfm^{op}*). C57BL/6J mice obtained from The University of Adelaide Central Animal House, as well as CSF-1+/+ and CSF-1+/*csfm^{op}* littermates (referred to as CSF-1+/±) were used as controls.

2.2.3.1 Confirmation of the *csfm^{op}/csfm^{op}* genotype

For the purposes of this study *csfm^{op}/csfm^{op}* mice were distinguished on the basis of their smaller size and absence of incisor teeth (Figure 2.2), thus it was not necessary to genotype the mice by PCR. Wiktor-Jedrzejczak *et al* (1990) and Yoshida *et al* (1990) both failed to detect bioactive CSF-1 in lung, bone or skin fibroblasts of *csfm^{op}/csfm^{op}* mice.

2.2.4 Primed ovulation in the immature mouse

In experiments requiring timed follicular development and ovulation, immature mice were primed with sc administered 5 IU equine chorionic gonadotrophin (eCG), Folligon® (Intervet-Boxmeer-Holland), a preparation with predominantly FSH activity, to induce synchronous follicle development. Five IU human chorionic gonadotropin (hCG), Chorulon® (Intervet-Boxmeer-Holland) was administered ip 48 hr later to initiate ovulation approximately 12 hr from the time of administration (Runner and Gates, 1954) (Figure 2.3A). Both eCG and hCG were administered in 100 or 200 µl 0.9% NaCl (Baxter Healthcare, AHF7123) using a 29 gauge needle and insulin syringe (Becton Dickinson, 326719).

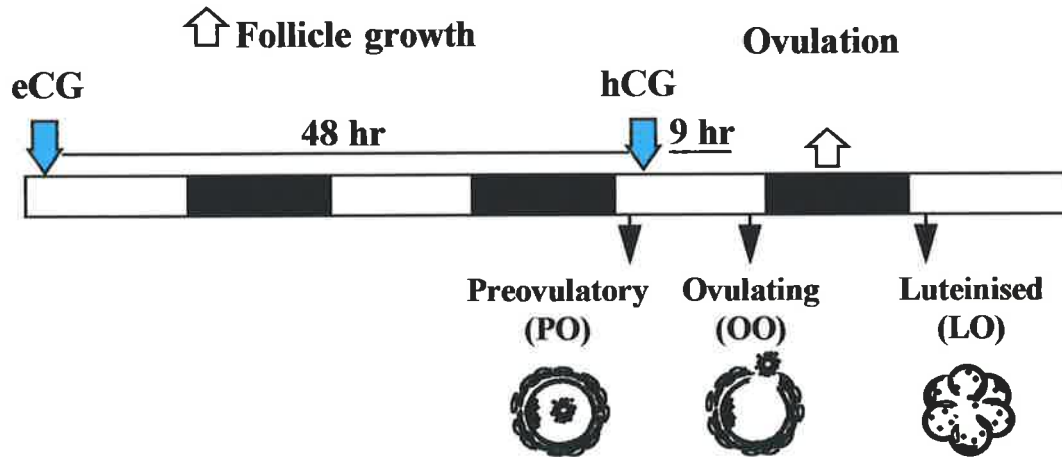
2.2.5 Synchronised ovulation in the adult mouse

In experiments requiring adult mice, ovulation was synchronised using a method modified from Vickery and McRae (1980) and Hamilton and Armstrong (1991). 20 µg LHRH agonist (des-Gly¹⁰, [D-Ala⁶]-LH-RH ethylamide) (Sigma Chemical Company, L4513) was injected ip in 200 µl 0.9% NaCl at 1200 hr on day -4, and 5 IU eCG in 0.9% NaCl administered ip 48 hr later. Ovulation occurred on the morning of day 0 (Figure 2.3B).

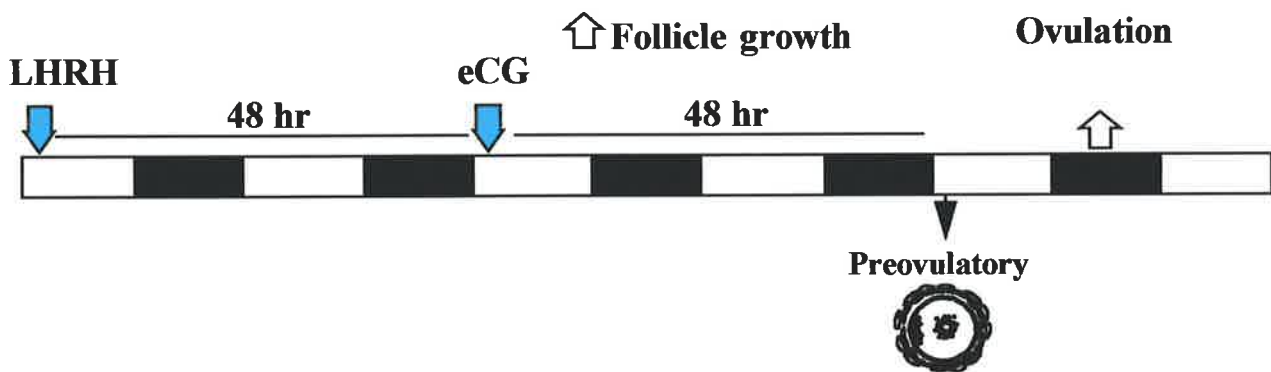
2.2.6 Oestrous cycle tracking by vaginal smear

Oestrous cycles were tracked by daily vaginal smear adapted from Allen (1922) and Bronson and co-workers (1966). The mice were housed 4 per cage (6750 cm³) in close

A Immature mouse



B Adult mouse



C Adult mouse

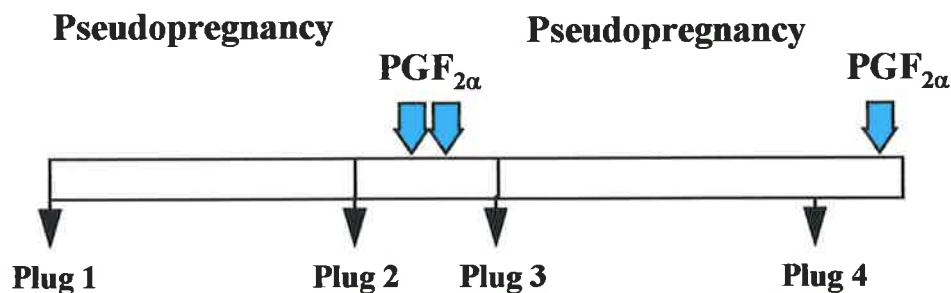


Figure 2.3 Priming protocols for the immature and adult mouse. A) In experiments requiring timed follicular development and ovulation, immature mice were primed with 5 IU equine chorionic gonadotrophin (eCG) administered sc to induce synchronous follicle development. 5 IU human chorionic gonadotrophin (hCG) was administered ip 48 hr later to initiate ovulation, ovulation occurred approximately 12 hr post hCG. B) In experiments requiring adult mice, ovulation was synchronised using 20 μ g LHRH agonist administered ip at 1200 hr on day -4 and 5 IU eCG administered ip 48 hr later. Ovulation occurred on day 0. C) Pseudopregnancy was induced by mating females to vasectomised studs and the presence of a vaginal plug was deemed day 1 of pseudopregnancy. Luteolysis was induced after the completion of one pseudopregnancy by the ip administration of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (3 μ g on day 4 and day 5).

proximity to a stud and acclimatised to the caging for at least 5 days prior to the beginning of the experiment. Oestrous cycles were tracked by daily vaginal smear between 8:00 - 11:00 hr. The vagina was flushed with approximately 15 µl PBS using a pipette and the sample was viewed as an unstained wet preparation on a microscope slide (Sail Brand, 7105) with a coverslip (Mediglass, 18 x 18 mm) under an inverted light microscope (Nikon, TMS or Olympus, CK2). The stage of oestrous was determined according to Bronson and co-workers (1966) and Snell (1941) (Table 2.1).

Table 2.1 Murine oestrous cycle. Vaginal smear content and proportions of epithelial cells, cornified epithelial cells and leukocytes. Corresponding activity in the ovary and length of each stage of the oestrous cycle (adapted from Bronson *et al*, 1966, Snell, 1941).

Stage	Smear	Ovary	Length (hr)
Proestrous	E to EC or ECL to EC	Large and distended follicles	24
Oestrous	EC to C+	Ovulation occurs	12
Metoestrous-1	C++ clumped	Early corpora lutea present, many follicles undergoing atresia	24
Metoestrous-2	C++EL++	Growing corpora lutea	24
Dioestrous	EL, +/- mucus	Quiescence, followed by rapid follicle growth towards the end	36

E = epithelial cells, C = epithelial cells cornified, L = leukocytes

2.2.7 Matings

Where pregnant or pseudopregnant mice were required, females were generally housed 2 per cage with 1 stud or vasectomised male (> 14 weeks of age) and checked daily for a vaginal plug. The presence of a plug was regarded as day 1 of pregnancy or pseudopregnancy (Hogan *et al*, 1986).

2.2.8 PGF_{2 α} -induced luteolysis

Luteolysis was induced in pseudopregnant mice by ip administration of 3 μ g of prostaglandin F_{2 α} as the synthetic sodium salt Estrumate® (Jurox) in 0.9% NaCl on day 4 and 5 of pseudopregnancy (Bartke *et al*, 1972) (Figure 2.3C).

2.2.9 Serum and plasma collection

Blood was collected from a nick in the tail. Blood was centrifuged (Eppendorf, 5415C) at 13 000 g for 15 min at 4°C, the blood clot was subsequently removed and the serum centrifuged for a further 3 min. Alternatively, a cardiac puncture was performed on mice following 15 μ l/g body weight 2% Avertin (Aldrich Chemical Company, T4,840-2) anaesthesia. A 26 gauge needle (Becton and Dickinson, 301802) was inserted between the ribs and into the heart. Approximately 1 ml of blood was collected into an EDTA coated tube (Terumo Corporation, T-MQ Capiject) and centrifuged (Eppendorf, 5415C) at 1 400 or 13 000 g for 15 min. Serum and plasma samples were stored at -20°C prior to assaying.

2.2.10 Ovarian Transplants

Instruction on the ovarian transplant technique was given by Jill Shaw (personal communication) and described by Cox and co-workers (1996). Mice were anaesthetised with an ip injection of 15 μ l/g body weight 2% Avertin and swabbed with 0.5% chlorhexidine in 70% alcohol (Orion Laboratories, CHL01458F). A midline incision on the dorsal surface was made level with the ribs and then a single incision through the peritoneal cavity above the ovarian fat pad, avoiding muscle tissue and intestines. The fat pad, ovary and oviduct was pulled through the incision and anchored to the skin with a 25 - 26 gauge needle (Figure 2.4). The incision in the bursa was made through the edge of the fat pad. The bursa was carefully pulled back over the ovary and the connective tissue was located inferior to the ovary. Special care was taken to avoid damage to the fimbria of the oviduct. The connective tissue was torn right through and all ovarian tissue removed. Once the ovarian artery was torn, the blood covering the area was flushed with PBS or 0.9% NaCl to lessen the chance of adhesions. The ovary to be transplanted was cut into quarters or transplanted whole. It was placed in the bursa and orientated curved side to the bursa, as it lies in the donor. The ovary was sutured in place by a single stitch through the ovary and connective tissue at the rear of the bursa using third generation, non-absorbable Novafil® 8-0 or 9-0 suture thread (Davis and Geck, CV310). The bursa was pulled over the ovary and sutured in 1 or 2 places. The ovary was then

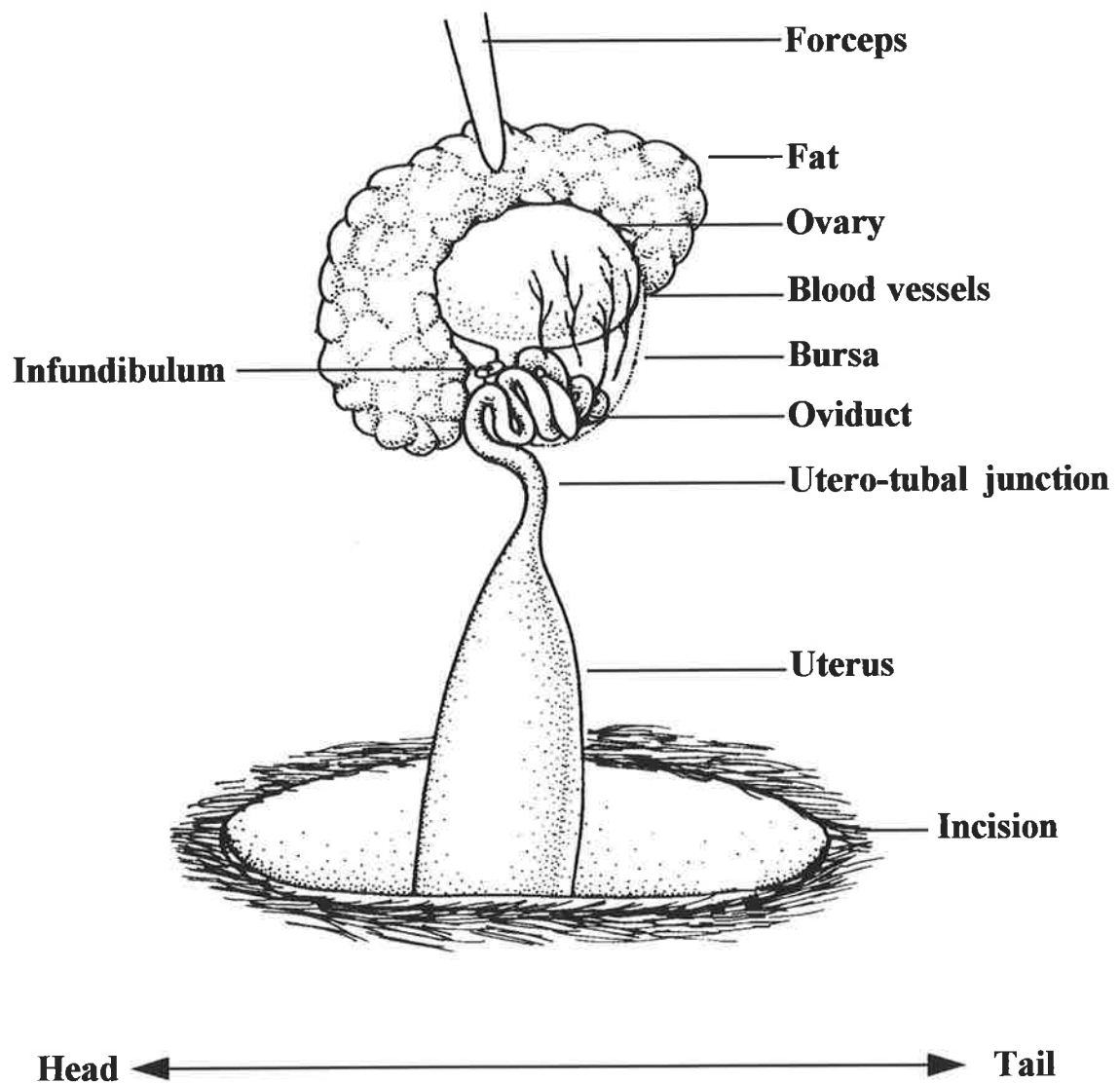


Figure 2.4 Illustration of the ovary and connecting tissue (adapted from Hogan *et al*, 1986). The fat pad, ovary and oviduct are pulled through an incision in the peritoneum and skin during ovary transplantation.

carefully placed back in the peritoneal cavity and the incision sutured closed using 5-0 or 4-0 suture thread (Dynek, V-4024 or Johnson & Johnson, K881). The procedure was then repeated on the opposite side. The skin was closed using 9 mm MikRon® wound clips (Becton Dickinson, 7631). Clips were removed after 2 weeks.

2.2.11 Vasectomy

Adult male mice were vasectomised as described by Hogan *et al* (1986). Each mouse was anaesthetised with an ip injection of 15 µl/g body weight avertin and swabbed with 0.5% chlorhexidine in 70% alcohol. A transverse incision on the ventral surface was made through the skin level with the top of the legs and then a similar size incision was made through the peritoneal cavity. The fat pad, testis, epididymis and vas deferens were pulled through the incision and anchored to the skin with a 25 - 26 gauge needle. Two double knots were tied with suture thread 4 - 5 mm apart. The vas deferens was severed between the 2 knots and the testis carefully placed back inside the body wall. This procedure was repeated on the opposite side. The body wall and skin were sutured and the mice were allowed to recover for at least 2 weeks prior to mating.

2.3 Collection and culture of ovarian tissue

2.3.1 Mixed ovarian cell culture

Mixed ovarian cell cultures were prepared under sterile conditions using a modification of a procedure described by Sherman (1978). Whole ovaries were excised from adult mice, trimmed of mesentery and chopped into pieces using a scalpel blade. Ovary pieces were incubated in 1 ml of 0.5% bovine trypsin, type III (Sigma, T8253) plus 2.5% pancreatin (Sigma) in PBS for 45 min at 4°C, followed by 45 min at 37°C. One ml of media was added and the pieces agitated by passage up and down a plastic pipette. The liberated cells were pelleted at 200 g and washed in M199 media. The cells were placed in M199 plus 3% BSA and were cultured in a humidified 37°C incubator (Forma Scientific, Steri-cult 200) with an atmosphere of 5% CO₂ and 95% air. Conditioned media from ovarian dispersate cultured at a density of 1.6×10^6 cells/ml in 250 µl was collected and stored at -20°C.

2.3.2 Isolation of whole follicles, granulosa cells and theca shells

Each mouse was euthanased and the ovaries were dissected free and trimmed of fat and mesentery in a petri dish of 0.9% NaCl on ice. Whole follicles and theca shells were isolated according to Brännström, personal communication. With the aid of a dissecting microscope, the largest follicles on the surface of the ovary were isolated. These follicles were assumed to have escaped atresia and therefore had the most potential to ovulate. The follicles were trimmed of stromal tissue while care was taken not to rupture the complete structure. For collection of granulosa cells from preovulatory and ovulatory follicles, follicles were ruptured using a 30 gauge needle and granulosa cells were aspirated from the petri dish. The purity of the granulosa cell preparation was not determined but could be thought to contain some theca cells and may be leukocytes as a consequence of the follicle rupturing process. For collection of theca shells, whole follicles were dissected from the ovary, ruptured and scraped free of the granulosa layer. The theca was then rinsed in 0.9% NaCl. The purity of the theca shell preparations was not determined but could be thought to contain some granulosa cells as well as the heterogenous cells which comprise the theca including leukocytes, fibroblasts, smooth muscle cells and endothelial cells.

2.3.3 Follicle culture

Since only one ovary could be isolated from the adult primed mouse for the whole ovary perfusion (Chapter 2, 4), whole follicles were dissected from the discarded ovary according to the method described above. Seven - nine follicles were cultured per well in 200 μ l M199 plus 0.1% bovine serum albumin (BSA) for 48 hr. Conditioned media was collected from 50% of the wells at 24 hr and the remaining wells at 48 hr. All media collected at 24 hr was pooled, as was the media collected at 48 hr, and was stored at -20°C.

2.3.4 Oocyte collection and counting

Oocytes were collected according to the method described by Hogan *et al* (1986). Each mouse was euthanased and the reproductive tract was dissected free and trimmed of fat and mesentery in a petri dish with the aid of a dissecting microscope (Wild, M3Z). For collection of oocyte/cumulus cell complexes (O/C) from preovulatory and ovulatory follicles, follicles were ruptured using a 30 gauge needle (Becton Dickinson, 5106) and O/C were aspirated from the petri dish. For collection of oocytes following ovulation or on day 1 of pregnancy, the swollen ampulla of the oviduct was torn and oocytes were extruded. In the

absence of a swollen ampulla, oocytes were flushed from the oviduct. A blunt 30 gauge needle attached to a 2 ml syringe (Becton Dickinson, 302204) was inserted into the infundibulum and the oviduct was flushed with approximately 50 - 100 μ l 0.9% NaCl or PBS. The number of oocytes in the ampulla region of each oviduct were recorded.

2.4 *In vitro* perfusion of the ovary

2.4.1 Surgical isolation of the ovary

On the morning of synchronised ovulation in the adult (Chapter 2, 2.5), the mice were anaesthetised with an ip injection of 15 μ l/g body weight Avertin (Aldrich Chemical Company) followed by an ip injection of 100 IU heparin sodium (David Bull Laboratories, 3091G). Only the right ovary from each mouse could be used, as the left ovarian artery branches from the renal vessel and not the aorta (Figure 2.5). The right ovary with its vasculature was surgically isolated, with appropriate ligatures to direct the perfusion medium through the ovary (Brännström and Flaherty, 1995). Briefly, the colon was resected through a midline incision in the peritoneum. All vessels branching from the aorta and vena cava were then ligated except the right ovarian artery and vein using 100% polyester thread (Gütermann, M782). The right uterine branch of the ovarian artery and the uterine horn were also ligated at the utero-tubal junction. The aorta was then cannulated in a retrograde direction with a 24 gauge, 1.9 cm Insite® catheter (Becton Dickson, 3873241), and the tip of the catheter was advanced to a position 2 mm from the branching of the right ovarian artery (Figure 2.4). The aortic catheter was sutured in place to encompass the vena cava, the aorta and the inserted catheter. The right ovary was then freed from adherent tissue and the suspensory ligament was cut. The bursa was left intact. All ligated vessels were then severed and the vena cava was pierced to enable perfusate to escape after it had been through the ovary. The preparation was removed and placed in 0.9% NaCl (Baxter Healthcare, AHF7123) at 37°C. Blood was gently flushed from the preparation by perfusion with 0.9% NaCl administered with a 1 ml syringe (Becton Dickinson, 302200). As the blood was displaced from the specimen, the ovary became progressively paler in colour. The preparation was connected to the perfusion apparatus if there was no obvious arterial leakage.

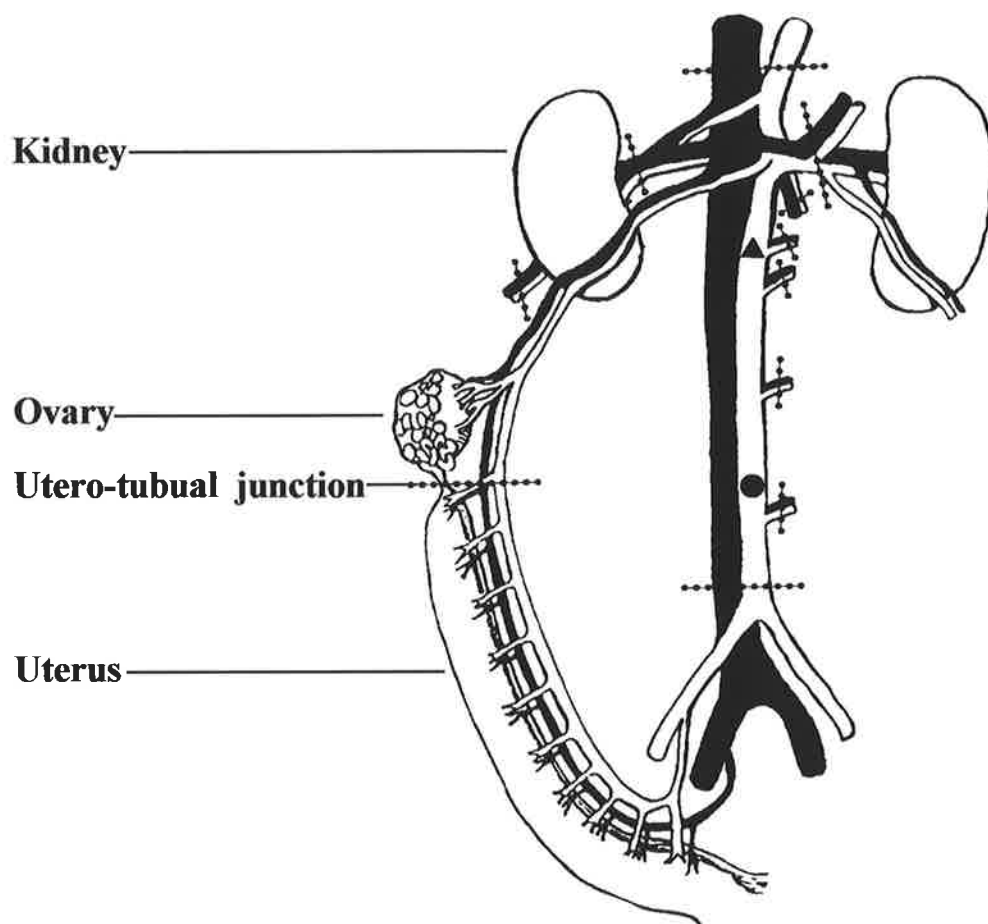


Figure 2.5 Surgical isolation of the ovary for *in vitro* perfusion. Surgical isolation of the right ovary and vasculature with ligations indicated by dotted lines (Brännström and Flaherty, 1995).

2.4.2 Perfusion apparatus

The perfusion apparatus illustrated in Figure 2.5 was a modification of the previously described rat ovary perfusion system (Brännström *et al*, 1987). The medium perfused through the ovary was M199 with Earle's salts (Gibco BRL, Life Technologies Inc, 31100-035), with the addition of 4% BSA (Boehringer Mannheim, 100018), 50 µg/ml gentamycin sulfate (Sigma Chemical Company, G-7632), 26 mM NaHCO₃ (Gibco BRL, Life Technologies Inc, 11810-025) and 0.02 IU/ml insulin Actrapid^R (Novo Nordisk Pharmaceuticals) maintained at 37°C by circulating heated water through jackets of the perfusion apparatus. The initial volume of 25 ml of perfusion medium was continuously recirculated and oxygenated with 95% O₂ and 5% CO₂. During perfusion, the aortic catheter was connected to the outflow part of the bubble trap and the specimen was placed in the perfusion chamber, which was covered by parafilm (American National Can, M). The pressure was maintained at 80 mmHg, producing a flow rate of 0.5 - 0.6 ml/min. The ovaries were perfused for 1 hr to allow metabolic stabilisation of the tissue prior to addition of treatments. Ovaries were perfused alone or with 0.1 µg/ml ovine LH (National Hormone and Pituitary Program, LH-26, Lot# AFP-5551B, specific activity 2.3 U/mg) or 0.1 µg/ml LH plus 4 ng/ml murine GM-CSF (Pharmingen, 19050T, specific activity 10⁷ U/mg) added to the perfusion system as a bolus dose.

2.4.3 Perfusion sample collection

Samples of 1.0 ml of perfusion medium were collected from the perfusion apparatus and an equal volume of perfusion medium replaced at 0, 1, 2, 3, 4, 8 and 22 hr using a 2 ml sampling syringe (Becton Dickinson, 302204) and stored at -20°C for subsequent assays. The times indicate the time of treatment addition which was 1 hr from the initiation of perfusion. Ovulation was assessed by counting the number of oocytes within the bursa and ampulla of the ovary at 22 hr. Total perfusate volume was also determined at 22 hr.

2.4.4 Scoring criteria for perfused ovaries for further analysis

Each perfusion was scored in order to ascertain the 'quality' of each preparation. Scoring took into account the progesterone levels before and during perfusion, flow rate during the perfusion and the actual ovulation event itself (Appendix 2). After scoring all aspects, perfusion samples were either accepted or rejected from the experimental group.

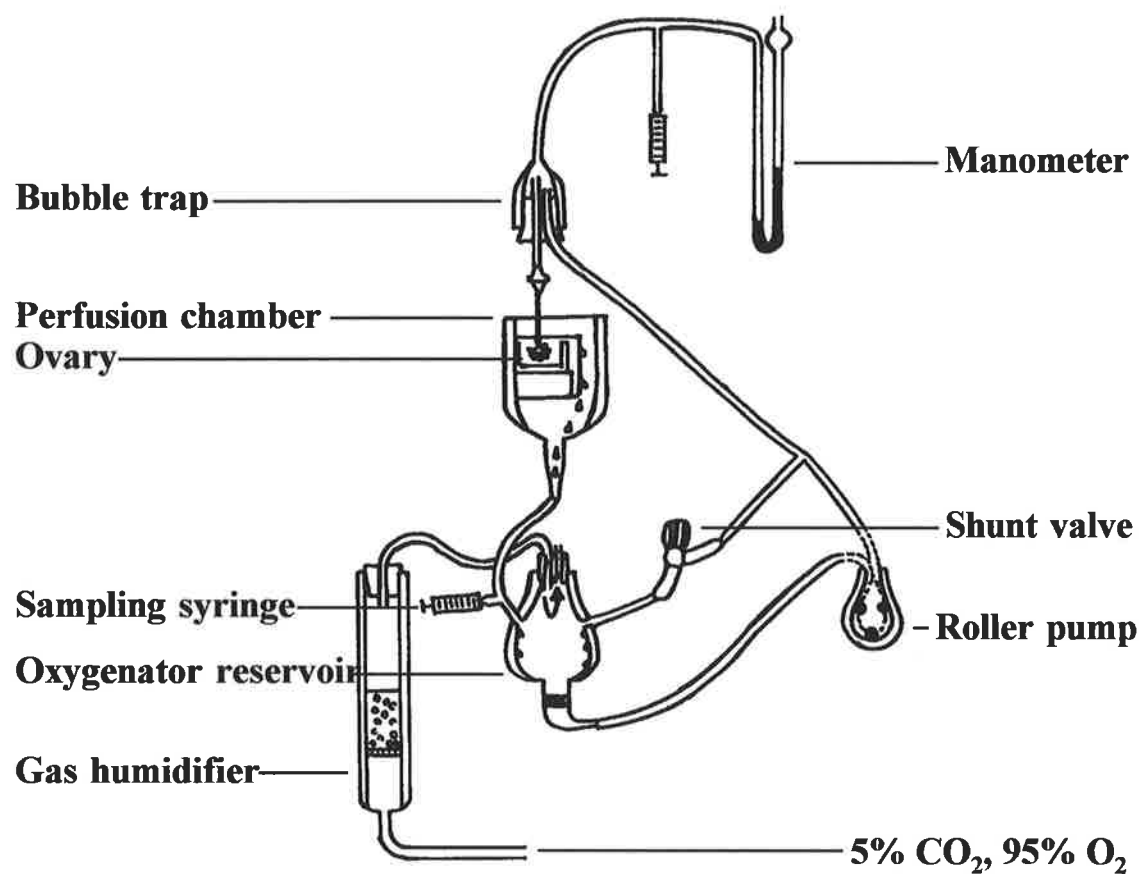


Figure 2.6 Perfusion apparatus. Scale approximately x 5, ovary not drawn to scale.

2.5 Immunohistochemistry

2.5.1 Tissue collection, embedding and sectioning

2.5.1.1 *Fresh frozen*

Fresh tissue collected from mice was embedded in Tissue-Tek OCT compound (Sukura Fintek, 4583) and frozen by immersion in liquid nitrogen-cooled isopentane (Ajax Chemicals, 1521) and stored at -60 to -80°C. 6 µm thick frozen sections were cut on a cryostat (Bright Instrument Company, OTF) and were air-dried overnight onto microscope slides (Sail Brand, 7105) and stained or stored desiccated at -20°C, generally for no longer than 4 weeks.

2.5.1.2 *Paraffin*

Tissue collected for paraffin embedding was fixed in 4% paraformaldehyde (BDH Laboratory Supplies, 29447) for 24 hr at 4°C and subsequently rinsed twice per day for 2 days in PBS prior to storage in 70% ethanol (BDH Laboratory Supplies) at 4°C. Tissues were then dehydrated in serial grades of alcohol: 70%, 80%, 2 changes of 95% and 3 changes of 100% ethanol for 15 min each, and then cleared in 3 changes of Safsolvent (Ajax, 2537) and infiltrated with 2 changes of molten paraffin wax. Tissues were then embedded in paraffin blocks. Paraffin blocks were cut into 5 µm sections, with a microtome and mounted on microscope slides. Sections and blocks were stored at room temperature prior to staining.

2.5.2 Immunoreactive antibodies

Antibodies were purchased from commercial companies or kindly provided as hybridoma supernatants harvested from cell lines cultured to late log phase as listed in Table 2.2.

Table 2.2 Immunoreactive monoclonal and polyclonal antibody characteristics. Distribution and function of antigen adapted from Barclay *et al*, (1993), antibody isotype and source. ATCC hybridoma bank (HB) and tumour immunology bank (TIB) numbers are listed also.

Antibody	Distribution of antigen	Function	Isotype	Source
CSF-1	see Chapter 1, 3.2.2		Rabbit α Ig	ERS
F4/80	Macrophages	Glycoprotein of unknown function	Rat α IgG _{2b}	ATCC HB 198
GM-CSF	see Chapter 1, 3.1.2		Rat α IgG _{2a}	G 1723-01
GM-CSF MP1-22E9			Rat α IgG _{2a}	P 18091D
GM-CSF MP1-31G6			Rat α IgG1-biotin	P 18102D
Ia (MHC class II)	Activated macrophages & dendritic cells	Antigen processing & presentation	Rat α IgG _{2b} (γ _{2b} κ)	ATCC TIB 120
CD45/LCA	All haematopoietic cells except erythrocytes	Leukocyte maturation, T cell receptor signalling	Rat α IgG _{2a}	ATCC TIB 122
CD4/L3T4	CD4+ T cells	MHC class II co-receptor	Rat α IgG _{2b}	ATCC TIB 207
CD5/Lyt 1	All T cells & subsets of mature B cells	Regulation of cell activation?	Rat α Ig G _{2a}	ATCC TIB 104
CD8a/Lyt 2	CD8+ T cells	α chain of MHC class I receptor	Rat α IgG _{2a}	ATCC TIB 105
CD11b/Mac-1	Monocytes, granulocytes & natural killer cells	Adhesion to ICAM-1, fibrinogen & complement component	Rat α IgG _{2b}	ATCC TIB 128
Thy-1.2	All T-cells, immature B cells & some fibroblasts	T cell activation	Rat α IgG _{2b}	ATCC TIB 107

ATCC = American Type Culture Collection, ERS = ER Stanley, G = Genzyme, P = Pharmingen,

2.5.3 Immunohistochemistry protocol

Fresh frozen tissue sections were fixed in 96% ethanol (BDH Laboratory Supplies, 10107) for 10 min at 4°C. Prior to each incubation, slides were rinsed 3 times for 5 min in PBS. The tissue section was encircled with a grease pen (Dako, S 2002) at the onset of the run and then slides were dipped in 1% BSA (Sigma, A-7030) in PBS (PBS-BSA) to reduce non-specific binding. All incubations were performed in a humidified chamber. Sections were incubated with the primary antibody plus 10% normal mouse serum (NMS) (Institute of Medical and Veterinary Science, 2305) for 3 hr at 4°C. This was followed by a 2 hr incubation with a biotinylated secondary antibody diluted 1/300 in PBS-BSA plus 10% NMS

at 4°C and a 40 min incubation in avidin-horseradish peroxidase (HRP) (Dako, P0364) diluted 1/400 in PBS-BSA plus 10% NMS at 4°C. Bound antibody was detected following a 10 min incubation with 3, 3'-diaminobenzidine peroxidase substrate (DAB) (Sigma, D-4293) filtered onto the tissue section using a 10 ml syringe (Becton Dickinson, 302214) and 0.22 µm filter unit (Millipore, SLGSO250S). Slides were rinsed in PBS before being counterstained in haematoxylin (Chapter 2, 2.5.4). DAB was neutralised in a 4% sodium hypochlorite solution (Ajax Chemicals, 484) prior to disposal. All monoclonal antibodies were used as a neat supernatant, unless otherwise stated. Primary antibody binding was detected using biotinylated rabbit α rat IgG (Dako, E0468), or for the antibody Lyt 2, goat α rat IgM μ (Pierce, 31832) was used. Negative controls were incubated as described but with the omission of the primary antibody, while spleen tissue and day 1 pregnant uteri were used as positive controls.

2.5.4 Haematoxylin counterstain

Tissue sections were counterstained with haematoxylin (Sigma Diagnostics, GHS-1-16 or Harris Haematoxylin JJ, Histo Labs). Sections were incubated for approximately 1-2 min in haematoxylin, then rinsed in milli Q H₂O, acid-dipped (0.5% HCl) for 5 sec, then rinsed again in milli Q H₂O. Blue counterstain was enhanced by a 2 min tap water (high Ca²⁺ solution) incubation followed by a rinse in milli Q H₂O. Slides were subsequently dehydrated in 2 changes of 95% or absolute ethanol for 5 min and cleared in 2 changes of Safsolvent (Ajax Chemicals, 2537) or xylene substitute (Shandon Scientific, 99900506) for 5 min. The slides were left to dry overnight and then mounted with coverslips using DPX mountant (BDH Laboratory Supplies, 36029 4H).

2.5.5 Haematoxylin and eosin counterstain

Tissue sections were counterstained with haematoxylin and then stained with eosin for 30 sec prior to dehydration, clearing and mounting as described above.

2.5.6 Quantification of staining by video image analysis

Staining was quantified using a video image analysis system. The system consisted of an Olympus BH-2 microscope bearing a Panasonic wv-CP610/A colour video camera or Olympus AH-3 microscope bearing a Panasonic GP-KR222 colour video camera connected to an IBM-compatible computer. The maximum area of each field analysed was 220 x 220 µm (AH-3 system), as dissimilar tissue compartments were visually erased from the field prior to

computer analysis. The analysis protocol was adapted from Baumgärter *et al* (1992). At the beginning of each session, the microscope, video camera and light intensity were adjusted and calibrated until results accorded with previous measurements using a defined area of a standard slide. The precision of the hardware was determined by recording 10 consecutive fields, revealing an intra-assay coefficient of variation (cv) of 8.9%. Reproducibility of results was determined by analysing an individual field over 4 different sessions, revealing an inter-assay coefficient of variation of 10.3%. Percent positive stain was defined as brown stain (DAB) area/total (haematoxylin + DAB) area expressed as a percent and was determined by analysing multiple fields using Video Pro 32 software (Leading Edge). Percent positive stain was determined by analysing multiple stroma ($n = 4 - 8/\text{ovary}$), theca ($n = 4/\text{follicle}$, diameter $> 350 \mu\text{m}$, since follicles $\sim 380 \mu\text{m}$ at the beginning of oestrous will increase to $\sim 550 \mu\text{m}$ prior to rupture (Bullough, 1942)) and corpora lutea ($n = 4/\text{CL}$) fields. Staining measured using the video image analysis system was compared to staining counted manually.

2.6 Embryo manipulation

2.6.1 Embryo collection

Sterile glassware and aseptic techniques were utilised during embryo handling. Embryos were collected according to the method described by Hogan *et al* (1986). Mice were euthanased and the reproductive tract was dissected free and trimmed of fat and mesentery in a petri dish with the aid of a dissecting microscope (Wild, M3Z). Embryos were extruded through a tear in the swollen ampulla of the oviduct on day 1 of pregnancy or collected on day 4 of pregnancy by flushing the oviduct and uterus (see Figure 2.7 for development in oviduct). A blunt 30 gauge needle (Becton Dickinson, 5106) attached to a 2 ml syringe (Becton Dickinson, 302204) was inserted into the infundibulum and the oviduct and uteri were flushed with approximately 50 μl HEPES buffered human tubal fluid (HTF) media or PBS both supplemented with 5 mg/ml BSA (Sigma, A-7888).

2.6.2 Embryo culture

The embryos were placed in a 50 μl drop of sodium bicarbonate-buffered human tubal fluid (HTF) media plus 5% BSA under paraffin oil in 35 mm culture dishes and were cultured

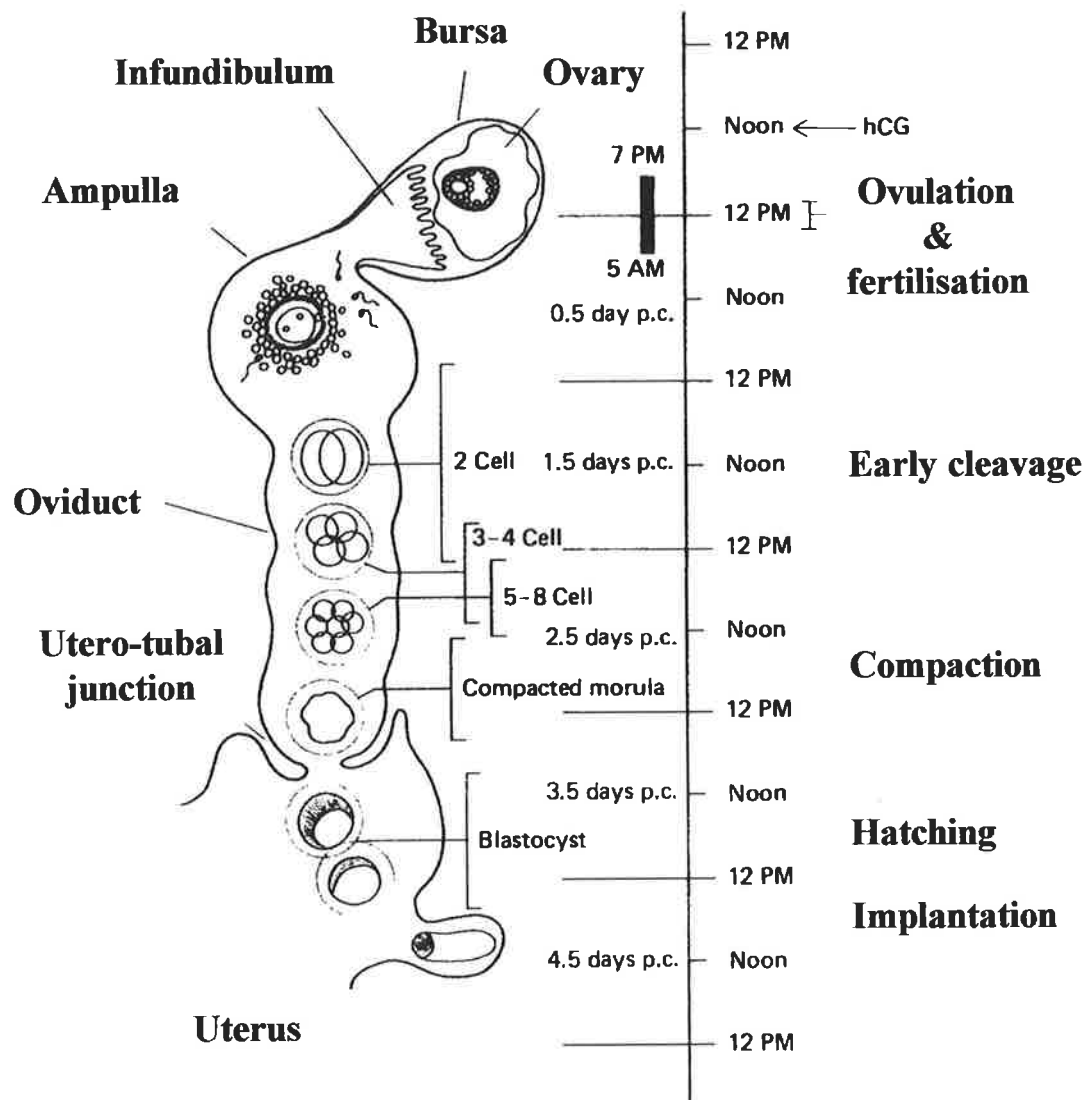


Figure 2.7 Summary of preimplantation development (adapted from Hogan *et al*, 1986).

in a humidified 37°C incubator (Forma Scientific, Steri-cult 200) with an atmosphere of 5% CO₂ and 95% air. Embryos were viewed using an inverted light microscope (Nikon, TMS) and the developmental stage of the embryos were scored at approximately 4 hr intervals.

2.6.3 Differential staining of mouse blastocysts

The embryos were placed in a 200µl drop of PBS-BSA under oil and were grouped according to developmental stage. Blastocysts were differentially stained using a method adapted from Handyside and Hunter (1984). All incubations were performed in 20-50µl microdrops under paraffin oil, except for washing steps. Following the washing steps, embryos were washed once in the subsequent incubation media to avoid dilution during the actual incubation. The zona of each embryo was rapidly removed in acidic Tyrode solution and the embryos were washed twice in PBS-BSA and three times in PBS supplemented with 1 mg/ml polyvinyl-pyrrolidone (PVP), Mr 40000 (Sigma, PVP-40). The inner cell mass (ICM) and trophectoderm (TE) nuclei of embryos were differently labelled *in situ* with the polynucleotide-specific fluorochromes, Hoechst 33258 (Sigma, B2883) and propidium iodide (PI) (Sigma, P4170) respectively. Specific TE labelling was achieved by antibody-mediated complement lysis to render the cells permeable to PI. The embryos were washed once and then incubated in 1/5 dilution of rabbit anti-mouse antiserum (Sigma, M5774) in PBS for 30 min at RT. The embryos were washed three times in PBS-PVP and subsequently incubated in 1/5 dilution of guinea pig complement (Sigma, 51639) plus 10 µg/ml PI in PBS for 30 min at RT. Following three rapid washes in PBS-BSA, the embryos were incubated in 10 µg/ml PI and 10 µg/ml Hoechst 33258 in PBS-BSA for approximately 5 min at RT. On completion of the incubation, embryos were wet mounted in PBS-BSA on microscope slides.

2.6.4 Quantification of blastocyst nuclei

TE and ICM cells were viewed and counted following excitation with the aid of an Olympus BH-2 epi fluorescence microscope fitted with a 400 nm filter (Olympus HBO, 100W/2, USH-102D). TE, PI-labelled nuclei, appeared pink and ICM, bisbenzimidazole-labelled nuclei appeared blue.

2.7 Molecular biology

2.7.1 General procedures for RNase-free conditions

Ribonuclease (RNase)-free conditions were maintained during the preparation of solutions and materials used in the isolation and preparation of total cellular RNA. Precautions included the use of disposable gloves, disposable plastic ware and glassware which had been baked for 3 hr at 170°C prior to use. Solutions were prepared using diethyl pyrocarbonate-treated (DEPC) (Sigma, D-5758) water and sterilised by autoclave for 20 min.

2.7.2 Isolation of genomic DNA

Immediately following dissection, tissue was frozen in liquid nitrogen and stored at -70°C. Total DNA was extracted from frozen tissue using either a QIAamp Blood Kit (Qiagen, 29104) or a method adapted from that described by Chomczynski and Sacchi (1987). Briefly, tissues were disrupted by homogenisation in 700 µl of tissue buffer and 40 µl 10 mg/ml proteinase K (Boehringer Mannheim, 109142) treated at 55°C overnight. RNA activity was destroyed by RNase A (Boehringer Mannheim, 109142) treatment at 37°C for 4 hr. The DNA was phenol/chloroform extracted twice by the sequential addition of 2 M sodium acetate (Sigma, S2889), phenol (Tris saturated) (ICN, 800672), chloroform (Ajax, 152) and isoamyl alcohol (Sigma Chemical Company, I0640) (25:24:1). The solution was vortexed, incubated for 5 min at room temperature and subsequently centrifuged at 7 000 g (Eppendorf, 5415C) for 5 min at 4°C prior to removal of the aqueous DNA phase. The DNA was extracted by the addition of an equal volume of chloroform:isoamyl alcohol (24:1). The solution was mixed well and subsequently centrifuged at 7 000 g for 5 min prior to removal of DNA (aqueous) layer. The DNA was precipitated from the aqueous phase at -20°C overnight, by addition of an equal volume of isopropanol. The DNA precipitate was pelleted at 11 000 g for 15 min at 4°C and then washed twice in ice cold 70% ethanol at 11 000 g for 20 min at 4°C. The pellet was dried *in vacuo* (Savant Instruments, refrigerated vapour trap RVT4104 and Speed Vac plus SC210A) and resuspended in 100 µl TE buffer. In some cases the integrity of the DNA was assessed by gel electrophoresis (Chapter 2, 4.10) and quantified by UV spectrophotometry (Chapter 2, 4.5). DNA was stored in TE buffer at -20°C.

2.7.3 Isolation of total cellular RNA

Immediately following dissection, tissue was frozen in liquid nitrogen and stored at -70°C. Total cellular RNA was extracted from frozen tissue using a method adapted from that described by Chomczynski and Sacchi (1987). Briefly tissues were disrupted by homogenisation in solution D (4 M guanidinium thiocyanate (Fluka Biochemika, 50990), 0.5% sarkosyl (Sigma, L9150) and 0.1 M β -mercaptoethanol (BDH Laboratory Supplies, 44143)). The RNA was phenol/chloroform extracted by the sequential addition of 2 M sodium acetate, phenol (H_2O saturated) and chloroform: isoamyl alcohol (24 : 1) at a ratio of 1 (Sol D) : 0.1 : 1 : 0.2 in a polypropylene tube. The solution was vortexed, incubated on ice for 15 min and subsequently centrifuged at 11 000 g for 15 min at 4°C prior to removal of the aqueous RNA phase, while DNA and protein separated to the interphase and phenol phase respectively. 100 μ g tRNA, RNase-free (Boehringer Mannheim, 109495) was added to some samples to aid in the handling of the RNA. The RNA was precipitated from the aqueous phase by the addition of an equal volume of isopropanol overnight at -20°C. The RNA precipitate was sedimented at 13 000 g for 30 min at 4°C and then washed twice in ice cold 70% ethanol at 11 000 g for 20 min at 4°C. The pellet was dried *in vacuo* and resuspended in 100 μ l milli Q H_2O .

2.7.4 DNase treatment of RNA

Total cellular RNA (90-100 μ g/100 μ l) was Dnase treated as a precautionary measure against contamination by genomic DNA. The reaction solution of 25 μ l 5x DNA buffer, 1 μ l 40 U/ μ l RNase inhibitor (Boehringer Mannheim, 799025) and 5 μ l 10 U/ μ l DNase I (Boehringer Mannheim, 776785) was incubated for 1 hr at 37°C and the RNA was subsequently extracted by the addition of 250 μ l of phenol (H_2O saturated), chloroform and isoamyl alcohol (25 : 24 : 1). The solution was vortexed and centrifuged at 11 000 g for 10 min at RT prior to the removal of the aqueous RNA phase. The RNA was subsequently precipitated from the aqueous phase by the addition of 2.5x volume 100% ethanol and 0.1x volume sodium acetate at -20°C overnight. The RNA precipitate was pelleted at 11 000 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 50 μ l milli Q H_2O .

2.7.5 Quantitation of DNA and RNA

RNA was quantified by measuring the absorption of light at a wavelength of 260 nm (ultraviolet) by spectrophotometer (Beckman, DU-50 or DU-600). One unit of optical density (OD) is equivalent to 50 µg/ml DNA or 40 µg/ml RNA and therefore the concentration of RNA in µg/ml was calculated from the equation $OD_{260} \times 40_{RNA} \times \text{dilution factor}$. The integrity of the RNA was assessed on a 2% agarose-ethidium bromide gel in some cases. An estimate of purity was calculated by also measuring the absorption of light at 280 nm, which corresponds to the wavelength at which proteins absorb light. An OD_{260}/OD_{280} ratio of 1.7 - 2 indicated relatively pure RNA. RNA was subsequently stored in 2.5x volume of 100% ethanol and 0.1x volume 2 M sodium acetate overnight at -20°C. DNA was stored in TE (tris-EDTA) buffer at -20°C.

2.7.6 cDNA generation from RNA by reverse transcription

First strand cDNA synthesis of RNA by reverse transcription (RT) was performed employing a Superscript RNase H- reverse transcription kit (Gibco BRL, 18053-017). RNA (1 µg/10 µl in milli Q H₂O) was initially primed with 1 µl of 500 mg/ml random hexamer (Bresatec, RP-6, 9519) at 70°C for 10 min, then chilled on ice for 5 min. The solution was spun to collect RNA and incubated with 4 µl 5x RT buffer (Gibco BRL, 18053-017), 2 µl DTT (Gibco BRL, 18053-017, 0.1 M) and 2 µl 10 mM 2'-deoxynucleotide 5'-triphosphate (dNTPs) (Pharmacia Biotech, 27-2035-01) at 43°C for 2 min. Following the addition of 1 µl RT Superscript enzyme (Gibco BRL, 18053-017), 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 made up to a final volume of 100 µl by the addition of milli Q H₂O and stored at -20°C.

2.7.7 Oligonucleotide primers and design

Primer pairs specific for cDNAs were designed using published cDNA sequences and Primer Designer version 2 (Scientific and Educational Software). Criteria for the design of primers is listed in Table 2.3.

The presence of alternative CSF-1 mRNA species has been well documented in the literature. CSF-1 PCR primers were designed to amplify both the 2 and 4 kb cDNA clones identified by Ladner *et al* (1988). In effect these primers encompass the hydrophobic membrane-spanning domain of the CSF-1 protein and therefore would amplify both the secreted and cell-surface form of the protein. The region amplified is not required for biological activity, but is proteolytically cleaved to allow secretion (Kawasaki *et al*, 1985) (Figure 2.10A). c-fms PCR primers were designed to encompass an intra-cellular domain which shows homology to tyrosine protein kinases (Rothwell and Rohrschneider, 1987) (Figure 2.10B). The primers also detect the alternative mRNA transcript described in the human placenta to have an extended 3' or transcribed exon 1.

iNOS and TNF α PCR primers were designed to encompass the sequence site of a cysteine residue, believed to be involved in intramolecular disulphide bridge formation of the mature protein (Figure 2.11A and 2.11B).

The oligonucleotide primers and PCR product size are outlined in Table 2.4. The oligonucleotide primers were synthesised from the 3' to the 5' end using phosphoramidite chemistry (Gibco-BRL or Bresatec). The crude primers were then purified by size exclusion chromatography to remove salts and organics. Primers were diluted to a concentration of 100 μ mol/l in milli Q H₂O and stored at -20°C.

β -actin

200 bp
—

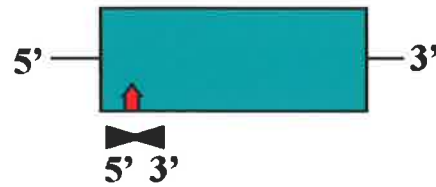
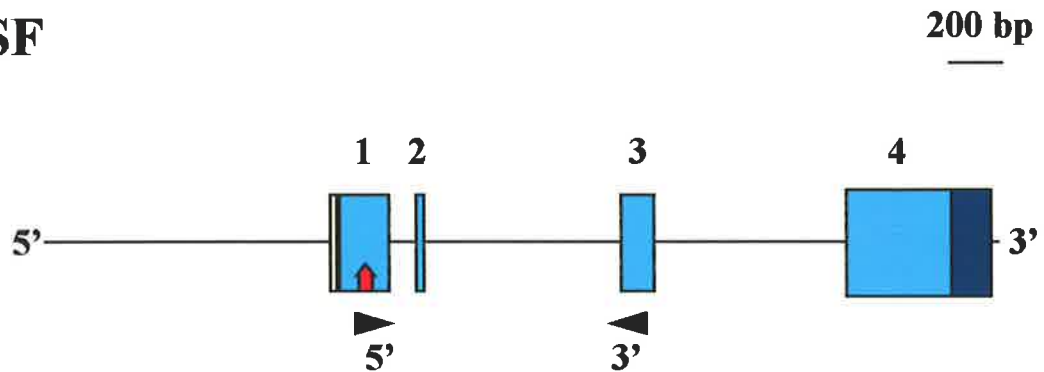
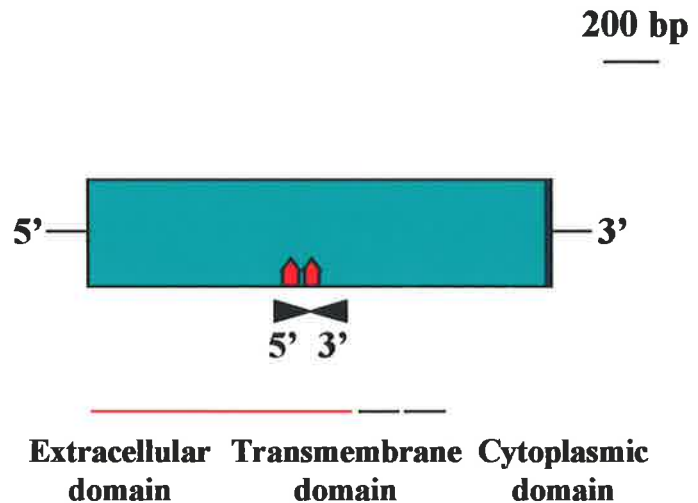


Figure 2.8 Schematic representation of mouse β -actin mRNA. From 5'-flanking region the following sequences are identified: mRNA (green) and flanking region (thin line). PCR primer binding sites are marked by black arrows. The red arrow indicates the proposed site of an intron (Genbank accession number M12481).

GM-CSF



GM-CSFR α



GM-CSFR β

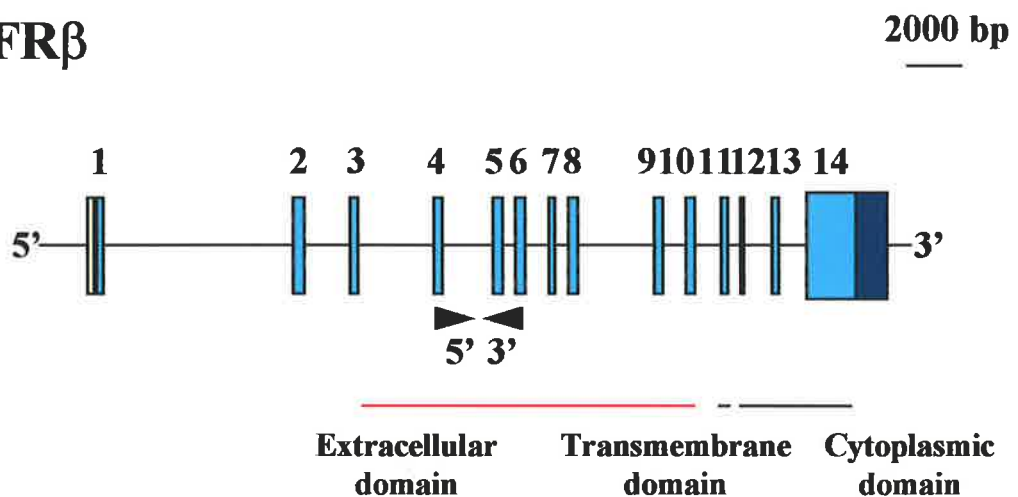
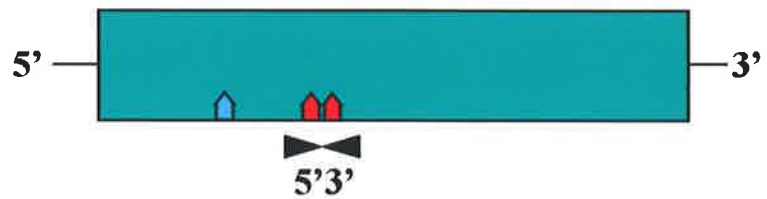


Figure 2.9 Schematic representation of the organisation of the gene components of the mouse GM-CSF signalling system. From 5'-flanking region the following sequences are identified: TATA box (yellow), coding regions of exons (blue) or mRNA (green) and polyadenylation signal (dark blue), introns and flanking region (thin line). cDNA PCR primer binding sites are marked by black arrows. GM-CSF gene, the red arrow indicates the sequence site at which the leader peptide is cleaved from mature protein (Genbank accession number X03020). GM-CSFR α mRNA, the red arrows indicate the sequence site of a cysteine residue and the red line indicates the sequence encoding the extracellular domain of the protein (Genbank accession number M85078 and Park *et al*, 1992). GM-CSFR β gene, the red line indicates the sequence encoding the extracellular domain of the protein (Gorman *et al*, 1992).

CSF-1

200 bp



c-fms

200 bp



Extracellular domain Transmembrane domain

Figure 2.10 Schematic representation of the organisation of the components of the mouse CSF-1 signalling system. From 5'-flanking region the following sequences are identified: mRNA (green), polyadenylation signal (dark blue) and flanking region (thin line). cDNA PCR primer binding sites are marked by black arrows. CSF-1, the blue arrow indicates the sequence site of a single base insertion in the *csfm^{op}/csfm^{op}* mouse CSF-1 gene. The red arrow indicates the sequence site of a cysteine residue believed to be involved in intramolecular disulphide bridge formation of the mature protein (Genbank accession number X05010 and DeLamarter *et al*, 1987). c-fms mRNA (Genbank accession number X06368).

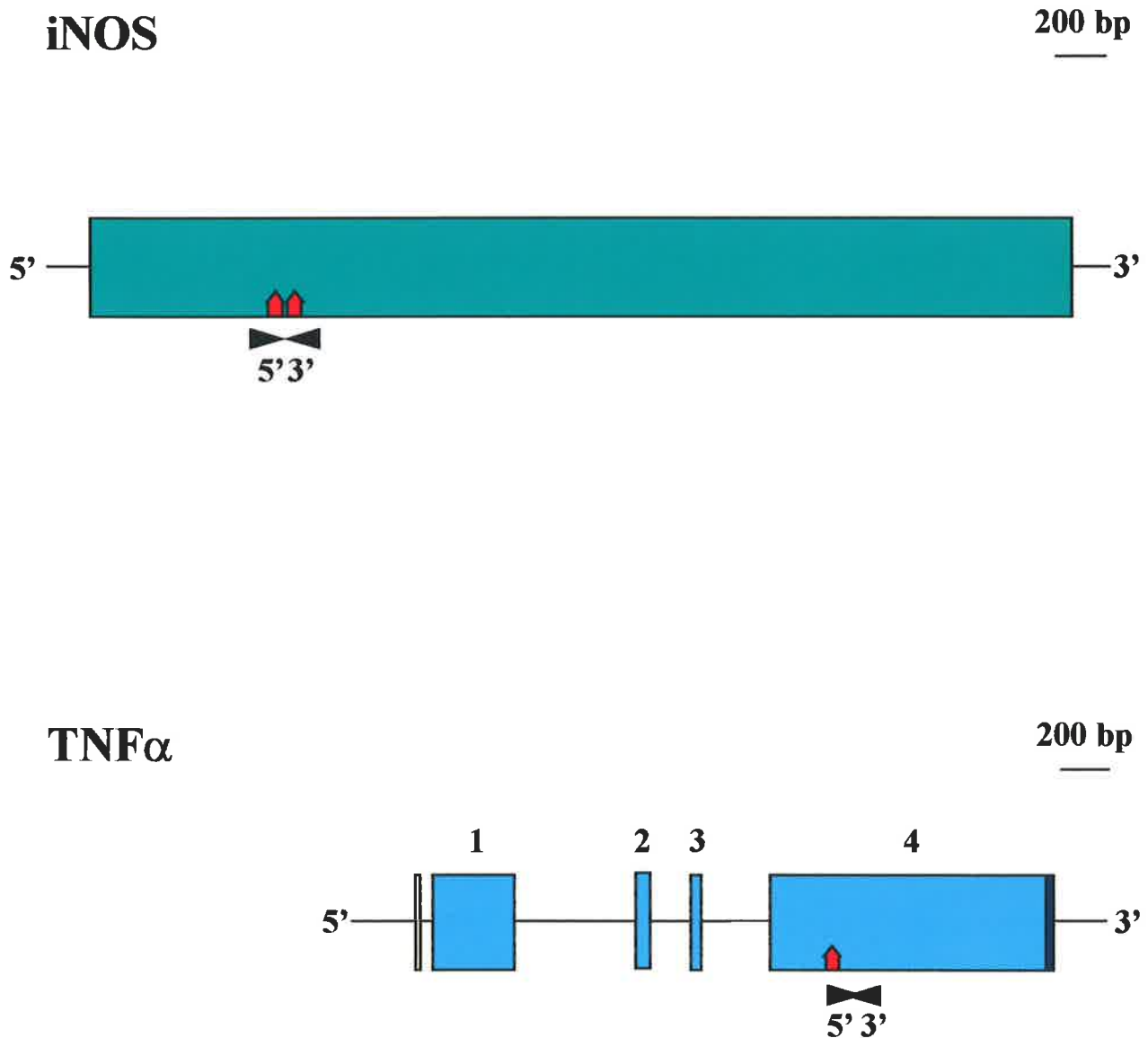


Figure 2.11 Schematic representation of mouse iNOS mRNA and the TNF α gene. From 5'-flanking region the following sequences are identified: TATA box (yellow), coding regions of exons (blue) or mRNA (green) and polyadenylation signal (dark blue), introns and flanking region (thin line). The red arrow indicates the sequence site of a cysteine residue believed to be involved in intramolecular disulphide bridge formation of the mature protein. cDNA PCR primer binding sites are marked by black arrows. iNOS (Genbank accession number M84373 and Lyons *et al*, 1992). TNF α (Genbank accession number Y00467).

Table 2.4 PCR primers. Primer sequence, PCR product size and primer binding sites on the Genbank sequence.

Primers	Sequence	Product size (bp)	Base pair position	Genbank #
120 (wild)	5'-CCAGCCTCAGAGACCCAGGTATCC		200-223	X03020
121 (wild)	5'-GTTAGAGACGACTTCTACCTCTTC	1200	1441-1418	X03020
131 (<i>lacZ</i>)	5'-GATGTGCTGCAAGGCTATTAAGTT	1000		
β-actin	5'-CGTGGGCGCCCTAGGCACCA 5'-ACACGCAGCTCATTG ^C	186	24-44 209-195	M12481
c-fms	5'-GCTGGAAGATCATCGAGA 5'-CCTCCTTCTCATCAGCAT ^C	240	1715-1732 1954-1937	X06368
CSF-1	5'-CCTGTGTCCGAACITTT 5'-TCTACACCACTGGTTC ^C	181	579-594 724-739	XO5010
GM-CSF	5'-CATTGTGGTCTACAGCCTCTC 5'-CGATGATGGTCTGTATGACGG ^C	278	61-81 338-318	XO3221
GM-CSFRα	5'-CCGGTCTCTGTAACITCTT 5'-TGCTCTACTTCGTGACC ^C	234	719-736 951-934	M85078
GM-CSFRβ	5'-ATACACGATTTTCCATCACAACG 5'-GATAAGGATGGGTTGTCTAGAT ^C	325	595-618 919-897	M34397
iNOS	5'-AGGAACCTACCAGCTCA 5'-GATGGCCGACCTGATGT ^C	205	694-710 898-882	M84373
TNFα	5'-CTCCTCTCTGCGGICA 5'-CCAAAGTAGACCTGCC ^C	182	667-682 848-833	X02611

2.7.8 PCR protocol: general

The PCR amplification employed reagents supplied in a *Taq* DNA polymerase kit (Biotech International, TAQ-1). Each reaction volume (25 μ l total) consisted of 67 mM Tris-HCl pH 8.8, 16.6 mM (NH₄)₂SO₄, 0.2 mg/ml gelatin, 0.45% Triton X - 100, 2.5 mM MgCl₂, 0.2 mM dNTPs (Pharmacia Biotech, 27-2035-01), 2 μ M 3' and 5' primer, 2 μ l of cDNA, overlaid with 50 μ l paraffin oil (BDH Laboratory Supplies, 294375J) and 0.55 U *Taq* DNA polymerase in 10 μ l milli Q H₂O added at 94°C. PCR amplification was performed under conditions conducive to denaturation; 5 min at 94°C, followed by multiple 1 min cycles of denaturation at 94°C, annealing at 58 - 61°C, and extension at 72°C, followed by a final extension for 7 min at 72°C using a PCR machine (Hybaid, Omnigene or Omn-E). cDNA

prepared from cell lines was used as a positive control and to optimise reaction conditions for each primer pair listed in Table 2.5. Cell lines included EL-4 (GM-CSF synthesising murine T cell line, Kotlarski, University of Adelaide, Adelaide), FD5/12 (GM-CSF-dependent murine myeloid cell line, A Kelso, WEHI, Melbourne), L (CSF-1 synthesising murine fibroblast cell line, A Lopez, Hanson Centre for Cancer Research, Adelaide), J774 (CSF-1 dependent murine macrophage cell line, ATCC) and MLA 144 (Gibbon T cell line, L Ashman, Hanson Centre for Cancer Research, Adelaide). The negative control included in each reaction consisted of milli Q H₂O substituted for cDNA. PCR reaction products were analysed by gel electrophoresis.

Table 2.5 PCR conditions for each primer pair.

Primers	Annealing °C	Cycle range
120 (wild)	58°C	41 - 50
121 (wild)	58°C	41 - 50
131 (lacZ)	58°C	41 - 50
β-actin	58°C	14 - 28
c-fms	58°C	20 - 34
CSF-1	62°C	22 - 36
GM-CSF	58°C	28 - 42
GM-CSFRα	60°C	24 - 38
GM-CSFRβ	60°C	26 - 40
iNOS	61°C	21 - 35
TNFα	60°C	28 - 42

2.7.9 PCR protocol: quantitative

Semi-quantitative PCR was performed according to Chelly *et al* (1988). After the first strand cDNA synthesis, the PCR reaction mixture including the sample, 15.42 kBq/sample ³²P-labelled dCTP (Bresatec, ADC-3) and specific primer pairs was aliquoted to 8 wells of the PCR tray (Hybaid, HB-TR3-MT). *Taq* DNA polymerase was added to each sample well to initiate the PCR reaction at 2 cycle intervals beginning at the highest cycle number. The reaction products were analysed by gel electrophoresis and then transferred to nylon membrane by rapid alkaline transfer.

2.7.10 Gel electrophoresis and confirmation of PCR products

Reaction products were analysed by gel electrophoresis through a 2% agarose (Promega, V3122), 0.5 mg/ml ethidium bromide (Eastman Kodak, IB40075) gel in TAE buffer, at a voltage of 90 - 100 for 45 - 60 min and visualised over an ultraviolet light box (Fotodyne, 3-4400). PCR product (20 µl PCR product, 4 µl 6x loading dye) size was verified by comparison to molecular weight marker *Hpa II*-digested pUC 19 DNA (Biotech, PUC-H2) for products of approximately 200 - 300 bp, or *Eco R1*-digested SPP-1 DNA (Bresatec, DMW-S1) for products of approximately 1 kb.

PCR products were purified from the agarose gel with the aid of a gel extraction kit (Qiagen, 28704) and sequenced by Rob Skinner (Flinders University) to confirm product specificity. For sequencing each base was fluorescently labelled using dyed deoxy terminator cycling sequencing kit (Applied Bioscience, DDTM) and subsequently denatured and sequenced on an Applied Biosystems 373 DNA sequencer and analysed on a Power Mac.

2.7.11 Rapid alkaline transfer of PCR products

The reaction products were transferred to a 0.45 µm nylon membrane (Schliecher and Schuell, S414596). Initially the membrane was wetted by soaking in milli Q H₂O and then transfer buffer. The gel was equilibrated twice in transfer buffer for 15 min at RT. The transfer assembly included a paper wick (Whatmans, 3030917) over a glass plate and baking dish to facilitate elution of nucleic acid onto the nylon membrane by mass flow of transfer buffer through the gel. A glass plate and 500 g weight overlaid approximately 6 cm of paper towel (Kimberly-Clark, 4419), 4 layers of Whatmans paper and the nylon membrane. The transfer was carried out 6-8 hr or overnight and the membrane was subsequently neutralised by washing with 5x SSC for a few min. The membrane was baked dry at approximately 75°C in 1 hr. The dried nylon membrane was exposed to the phosphorimage screen (Kodak) overnight. A phosphorimage analysis system was then used to quantify ³²P-CTP incorporation into the reaction product.

2.7.12 Quantitation of ³²P-CTP incorporated into PCR products by phosphorimage analysis

A phosphorimage analysis system, consisting of phosphorimager and ImageQuant version 3.3 software (Molecular Dynamics) was used to quantify ³²P-CTP incorporation into the reaction product. The data was plotted against cycle number on a logarithmic graph.

Although with the PCR method amplification is exponential, with theoretical doubling of each strand at each cycle, the efficiency is less than 100%. The initial amount of mRNA was estimated by measuring the extent of ^{32}P -CTP incorporation into the amplified product and by applying the formula $Y = i \times E^n$ to the exponential part of the curve, where i is the initial amount of material, E the efficiency, Y the yield and n the number of cycles. The linear regression was performed using SigmaPlot version 3 (Jandel Corporation). Analysis of the 186 bp β -actin PCR product by gel electrophoresis and phosphorimaging confirmed the initial amount of cDNA to be approximately equivalent in all samples.

2.8 Assays

2.8.1 Progesterone

For analysis of serum and plasma progesterone concentrations, a standard curve ranging from 1.3 - 127 nmol/l was calibrated by inclusion of progesterone in a radioimmunoassay (Amersham Australia, IM.3220). The intra and inter-assay coefficients of variation were calculated as < 5.1% and < 9.4% respectively. The sensitivity, defined as the concentration two standard deviations above the zero standard, was 0.25 nmol/l. The cross-reactivity was stated by the manufacturer to be 100% for progesterone and < 2.79% for all hormones tested, including 17α -hydroxyprogesterone, pregnenolone, pregnenolone-3-sulphate, 11-deoxycorticosterone, corticosterone, testosterone, 5α -pregnane-3,20-dione, 5β -pregnane-3,20-dione, 20α -hydroxypregnen-3-one and <0.01% for cortisol, oestriol, 17β -oestradiol and danzol.

Perfusion media progesterone concentrations were analysed by ACS chemiluminescent immunoassay (Ciba Corning Diagnostics, 672300). A standard curve ranging from 1.9 - 127.2 nmol/l was calibrated by the inclusion of progesterone. The intra and inter-assay coefficients of variation were calculated as $\leq 10\%$ and $\leq 12\%$ respectively (Connolly *et al*, 1993). The sensitivity was 0.35 nmol/l. The cross-reactivity was stated by the manufacturer to be 100% for progesterone and < 1% for all hormones tested, including pregnenolone, corticosterone, 11-deoxycorticosterone and 17α -hydroxyprogesterone, and not detectable for cortisol, testosterone, aldosterone, androstendiol, 11-deoxycortisol, danazol, prednisolone, oestrone, oestriol, 17β -oestradiol, clomiphene and bromocryptine.

2.8.2 Oestradiol

For analysis of serum and plasma oestradiol concentrations, a standard curve ranging from 37 - 7340 pmol/l was calibrated by the inclusion of 17β -oestradiol in a radioimmunoassay (Sorin Biomedica Diagnostics, 200/007-818). The intra and inter-assay coefficients of variation were calculated as $\leq 8\%$ and $\leq 9.7\%$ respectively. The sensitivity, defined as the 95% confidence limit of the zero standard, was 18 pmol/l. The cross-reactivity was stated by the manufacturer to be 100% for oestradiol and $< 0.01\%$ for all hormones tested, including oestrone, oestriol, estradiol-3-glucuronide and oestradiol-17-glucuronide ethinylestradiol and $< 0.001\%$ for progesterone, testosterone and androstenediol.

Perfusion media oestradiol concentrations were analysed by ACS chemiluminescent immunoassay (Ciba Corning Diagnostics, 672297). A standard curve ranging from 108 - 5000 pmol/l was calibrated by inclusion of 17β oestradiol. The intra and inter-assay coefficients of variation were calculated as $\leq 7\%$ and $\leq 10\%$ respectively (Natoli *et al*, 1993). The sensitivity, defined as two standard deviations above the zero standard, was 36.7 pmol/l. The cross-reactivity was stated by the manufacturer to be 100% for oestradiol and $< 1\%$ for all hormones tested, including oestrone, oestriol, 17β -oestradiol- 3β -D-glucuronide and ethinyl oestradiol, clomiphene, danazol and bromocryptine.

2.8.3 Androstenedione

Perfusion media androstendione concentrations were analysed by radioimmunoassay. A standard curve ranging from 0.34 - 34.5 nmol/l was calibrated by inclusion of androstenedione (Diagnostic Systems Laboratories, DSL-4200). The non-specific binding was $< 7\%$ and the intra and inter-assay coefficients of variation were calculated as $\leq 5.9\%$ and $\leq 7\%$ respectively. The sensitivity, defined as two standard deviations above the zero standard, was 0.069 nmol/l. The cross-reactivity was stated by the manufacturer to be 100% for androstenedione and $< 0.33\%$ for androsterone, 17-hydroxyprogesterone and cortisone, $\leq 0.1\%$ for the hormones isoandrosterone, deoxycortisone, etiocholanolone, 5α -dihydrotestosterone, dehydroepiandrosterone, progesterone, corticosterone, cortisol, oestrone and pregnenolone.

2.8.4 Nitrate/nitrite

Nitrate and nitrite was detected in perfusion media by conversion of nitrate to nitrite and analysis of total nitrite in a colorimetric assay. A standard curve ranging from 5 - 35 $\mu\text{mol/l}$ was calibrated by inclusion of nitrate (Caymen Chemical Company, 780001). The intra-assay coefficient of variation was calculated $\leq 4\%$. Chemicals stated by the manufacturer not to interfere in assay colour development included alkyl amines, most sugars, lipids or amino acids not containing thiol groups. Chemicals known to interfere with assay colour development through blocking the conversion of nitrate to nitrite include 100 μM azide, ascorbic acid, dithiothreitol, and β -mercaptoethanol and phosphate concentrations $> 50 \text{ mM}$. Colour development was assessed by measurement of absorbance at 540 nm by ELISA plate reader (Dynatech Laboratories, Immunoassay System DIAS).

2.8.5 GM-CSF

Immunoactive murine GM-CSF was detected by enzyme-linked immunosorbent assay (ELISA). The assay principle employs the coating of a microwell plate with a monoclonal antibody to capture sample GM-CSF, and the addition of a polyclonal antibody to sandwich the captured GM-CSF. Bound, immunoactive GM-CSF was quantitated by the enzymatic reaction resulting in a colour change. Optical absorbance measured was proportional to the concentration of GM-CSF.

96-well ELISA plates (Disposable Products, 23148) were coated with 4 $\mu\text{g/ml}$ rat IgG_{2a} monoclonal α mouse GM-CSF antibody (PharMingen, 18091D) in 50 μl coating buffer (0.1M NaHCO₃, pH 8.2) overnight at 4°C. Non-specific binding was blocked by an incubation of 200 μl of 3% BSA PBS (pH 7.4) for 2 hr at room temperature. Remaining incubations were performed in 3% BSA PBS (pH 7.4) assay diluent. 100 μl standards and samples were incubated overnight at 4°C. 100 μl Biotinylated rat IgG₁ polyclonal α mouse GM-CSF antibody at a concentration of 1 $\mu\text{g/ml}$ (PharMingen, 18102D) sandwiched the captured GM-CSF following a 1 hr incubation at 37°C. Subsequently, 100 μl avidin and biotinylated horseradish peroxidase diluted 1:300 (Dako, K355) was incubated for 40 min at room temperature. Unbound enzyme was removed by aspiration and washing. Then, 100 μl tetramethylbenzidine dihydrochloride (TMB) (Sigma Chemical Company, T-3405) peroxidase substrate was added to the wells, colour development was halted with the addition of 2 M sulfuric acid and assessed by measurement of absorbance at 540 nm. A standard curve

ranging from 7.5 - 2000 pg/ml was calibrated by inclusion of recombinant murine GM-CSF (PharMingen, 19050T). The intra and inter-assay coefficients of variation were calculated as 11.1%, 7.7%, 3.1%, 0.4%, 1.6% and 2.0% for the standards 15, 31, 63, 125, 250 and 500 pg/ml respectively. The cross-reactivity was stated by the manufacturer to be 100% for murine GM-CSF and 0% above background for all recombinant cytokines tested, including human GM-CSF, murine IL-2, IL-3, IL-4, IL-5, IL-6, TNF α and IFN γ , with only very weak cross-reactivity for IL-10.

2.9 Data Analyses

Data are presented as mean $\pm$ SE and where necessary percent and proportion data were arcsin transformed for statistical analysis. Graphpad InStat version 2.04a (Graphpad Software) was used to analyse all data except perfusion data which were ranked prior to assessment by general linear model repeated measures and Tukey HSD post hoc Test using SPSS (SPSS). Statistical significance was assessed at $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$. The details of data analysis and P values are presented within each chapter.

2.10 Photography and image capture

Digital images of immunohistochemistry were captured in tagged image file format (TIFF) using the video image analysis system (Chapter 2, 3.7). Digital images of 35 mm slides were captured in TIFF format using a scanner (Polaroid, Sprintscan 35) connected to a computer (Macintosh, Apple Quadra 605) running Adobe Photoshop version 2.5.1. A direct screen instant (Polaroid, DS-34) or land (Polaroid, MP-4) camera with black and white film (Polaroid, 665 or 666) was used to photograph electrophoresis gels over a UV transilluminator (Chapter 2, 7.10). Photographs were subsequently scanned at approximately 600 dpi (Hewlett Packard, ScanJet 11c) and stored as TIFF files.

Chapter 3

GM-CSF and CSF-1 signalling systems in the mouse ovary

3.1 Introduction

The likening of ovulation to an inflammatory response in the early 1980's laid the foundations for a branch of biological science referred to as reproductive immunology. The union of two previously distinct systems provided a conceptual framework to begin to investigate the previously noted abundance of macrophages in the ovary. It was hypothesised that these leukocytes, through the release of their multitude of biochemical mediators, could be involved in the process of degradation necessary for the expulsion of an oocyte from the ovary surface at ovulation. The haemopoietic cells present in the ovary were recognised to be an important constituent of the cellular remodelling processes (Chapter 1, 1.2) and the multitude of cytokines found to be in the ovary were proposed to mediate the communication between cells of haemopoietic and non-haemopoietic lineages (Chapter 1, 1.3), acting on infiltrating and resident cells in an autocrine/paracrine manner. As the potential roles of these networks were better understood, it was eventually hypothesised that haemopoietic cells and cytokines could contribute to the tissue remodelling processes of follicular development, luteinisation and luteolysis as well as ovulation (Chapter 1, 1.4).

The fundamental hypothesis of the studies presented within this chapter is that colony-stimulating factors, specifically GM-CSF and CSF-1, are synthesised within the mouse ovary and regulate the function of CSF receptor-bearing cells.

In order to examine the function of GM-CSF and CSF-1 in the ovary, it was initially considered important to determine the cellular sources and target cells of GM-CSF and CSF-1. The studies described in this chapter aim to elucidate these points through the employment of various techniques to identify protein and mRNA expression. The detection of protein was considered the primary aim and immunohistochemistry and enzyme linked immunosorbent assay (ELISA) were undertaken to assess localisation and secretion of the protein respectively. However, the attempts to establish immunohistochemistry proved increasingly futile and the GM-CSF specific ELISA was insufficiently sensitive. RT-PCR was subsequently performed on whole ovaries and ovarian tissue compartments to assess the presence, localisation and level of expression of both ligand and receptors. The expression of mRNA was viewed as an indicator of the potential for production of protein by cells constituting the ovary. Detection of receptor was interpreted to suggest the presence of ligand-responsive cells in the ovary.

3.2 Immunohistochemical localisation of GM-CSF and CSF-1 protein

The purpose of this initial study was to employ immunohistochemistry to localise GM-CSF and CSF-1 protein in the mouse ovary at various stages of follicle development and rupture, in order to investigate the identity of the cells that express GM-CSF and CSF-1. To examine protein expression in the ovary it was first necessary to establish and validate the immunohistochemistry protocol for detection of immunoactive GM-CSF and CSF-1.

Three α mGM-CSF antibodies, derived from the clones MPI-22E9, MPI-31G6 and the monoclonal rat α GM-CSF antibody (Table 2.2), and the rabbit α mCSF-1 antibody were trialled for immunohistochemistry in various protocols using combinations of 0 - 0.01% trypsin treatment and fixatives including 96% ethanol, 2% and 4% paraformaldehyde, 10% formalin, 4% formaldehyde, 100% acetone and Bouin's on fresh frozen tissue (Chapter 2, 2.5). On day 1 of pregnancy the epithelial cells of the uterus secrete very large quantities of GM-CSF (Robertson *et al*, 1992), and so uterus and also spleen tissue were used as positive control tissues for GM-CSF and CSF-1 immunohistochemistry. Ovulatory ovaries (OO) and luteinised ovaries (LO) were collected from immature primed mice (Chapter 2, 2.2.4) and included in the trial. The protocol for immunohistochemical localisation of protein described in Chapter 2, 2.5.3 was followed with the exception of the initial incubation of primary antibody which was extended from 3 hr to an overnight incubation. The appropriate secondary antibodies were used where necessary. The combinations of antibodies and fixatives employed for the detection of GM-CSF are summarised in Table 3.1.

Table 3.1 Fixatives and antibodies trialled for immunohistochemical localisation of GM-CSF.

Fixative	Antibody	Dilution	Pretreatment
96% ethanol	α mGM-CSF (Genzyme)	1:90	$\pm$ 0.01% trypsin
	α mGM-CSF (MPI-22E9)	1:5 - 1:40	
	α mGM-CSF (MPI-31G6)	1:5 - 1:40	
2% paraformaldehyde	α mGM-CSF (Genzyme)	1:90	1:5 - 1:40
	α mGM-CSF (MPI-22E9)	1:5 - 1:40	
4% paraformaldehyde	α mGM-CSF (Genzyme)	1:90	1:5 - 1:40
	α mGM-CSF (MPI-22E9)	1:5 - 1:40	
	α mGM-CSF (MPI-31G6)	1:5 - 1:40	
4% formaldehyde	α mGM-CSF (Genzyme)	1:90	1:5 - 1:40
	α mGM-CSF (MPI-22E9)	1:5 - 1:40	
10% formalin	α mGM-CSF (Genzyme)	1:90	0.01% trypsin
Bouin's	α mGM-CSF (Genzyme)	1:50	0.01% trypsin

Disappointingly, no signal was detected for GM-CSF in day 1 pregnant uterus, spleen or ovary tissue regardless of antibody and fixatives (Table 3.1). The detection of CSF-1 binding was hampered by non-specific binding of rabbit immunoglobulins to all tissues investigated, regardless of fixative. This experiment was abandoned when it was concluded that the available reagents were not appropriate for use with immunohistochemical techniques.

3.3 ELISA detection of GM-CSF

When immunohistochemistry was found to be unsuitable for the localisation and/or quantitation of cytokine proteins in the ovary, the capacity of an alternative assay to measure immunoactive GM-CSF protein concentrations in ovarian cell conditioned media and ovary perfusion media was investigated. In comparison to immunohistochemistry, this method would permit more accurate quantitation of the protein. The sensitivity and lack of need for radioactive labelled GM-CSF made the ELISA the assay of choice. It was first necessary to develop a GM-CSF standard curve from recombinant GM-CSF.

Preliminary experiments using the protocol supplied by the manufacturer of the antibodies and avidin-conjugated horseradish peroxidase (Pierce, 29994) and ABTS detection system (Pierce, 34026) identified the need for a more sensitive detection system than that initially used. In addition, the establishment of a standard curve was hindered by the initial use of seemingly inactive recombinant GM-CSF and therefore took many months to achieve. However, replacement of the recombinant material yielded a standard curve of 7.5 - 2000 pg/ml (Figure 3.1). The capture antibody, MPI-22E9, was used to coat the ELISA plate at a concentration of 4 µg/ml and the biotinylated detecting antibody, MPI-31G6, was titrated to cover a range of concentrations from 1 - 4 µg/ml. The optimised protocol is described in Chapter 2, 2.8.5.

Conditioned media from mixed ovarian cell cultures, and whole follicles and media from *in vitro* perfused ovaries were then assayed for GM-CSF content to determine whether the assay would be sensitive enough to detect cumulative secretion of protein during culture and perfusion periods of 24 - 96 hr. Conditioned media was collected from dispersate ovarian cells cultured at a density of 1.6×10^6 cell/ml (Chapter 2, 2.3.1) and from 7 - 9 follicles cultured per well in 200 µl M199 plus 0.1% BSA for 48 hr (Chapter 2, 2.3.3). Ovarian perfusate was collected at 4, 8 and 22 hr (Chapter 2, 2.4).

Immunoactive GM-CSF was detected in media collected at various times from between 4 and 22 hr of perfusion and was found to increase from 6 pg/ml to 448 pg/ml during that time (Table 3.2). In another perfusion sample collected at 22 hr, sample GM-CSF was detected at a concentration of 455 pg/ml. Immunoactive GM-CSF was detected at a concentration of 168 pg/ml in conditioned media collected from ovarian cells at 96 hr, but could not be detected at previous time points. Immunoactive GM-CSF was not detected in conditioned media of whole follicles.

Table 3.2 Immunoactive GM-CSF (pg/ml) in ovarian conditioned media and perfusion media as assessed by in-house ELISA.

Hours	4	8	22 - 24	48	72	96
Ovarian perfusate	6	120	448			
Ovarian dispersate			ND	ND	ND	168
Whole follicle			ND	ND		

ND = not detected

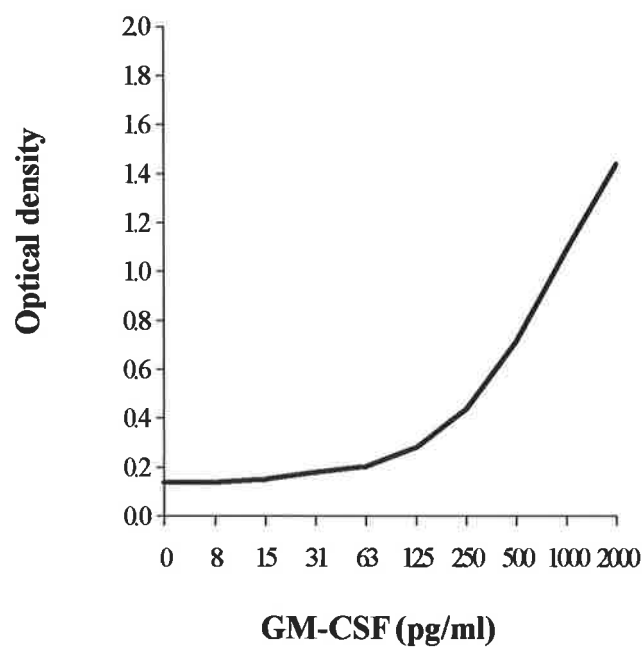


Figure 3.1 Typical standard curve for the GM-CSF ELISA. The principle of the assay is to sandwich recombinant or native GM-CSF between a solid phase capture rat α GM-CSF mAb (4 μ g/ml) and a second biotinylated rat α GM-CSF mAb (1 μ g/ml). The assay employs an avidin-horseradish peroxidase, TMB detection system and colour development was assessed by measurement of absorbance at 540 nm.

The GM-CSF ELISA was considered sensitive enough to determine concentrations in the 22 hr ovarian perfusion sample, however it appeared to be insufficiently sensitive to determine the concentration of GM-CSF in ovarian cell and whole follicle conditioned media. Due to the lack of sensitivity of this assay, methods for the detection of mRNA were investigated.

3.4 Development and optimisation of an RT-PCR assay for the components of the GM-CSF signalling system

The purpose of this study was to investigate a technique thought to be sufficiently sensitive to detect low quantities of cytokine and cytokine receptor mRNA. Since cytokines are generally expressed in relatively low abundance, and often by only small proportions of the cells in a tissue, Northern blotting was considered to lack the sensitivity required. RT-PCR was considered to provide the most sensitive means of detecting mRNA in small amounts of tissue (Dallman *et al*, 1991). RT-PCR was employed to detect the presence of mRNA transcripts for GM-CSF and its receptor subunits GM-CSFR α and GM-CSFR β at various stages of follicle development and rupture in the immature primed mouse ovary. To examine mRNA expression in the ovary it was first necessary to fulfil an initial requirement which was to develop the RT-PCR assay to specifically detect mRNA transcripts for GM-CSF and its receptor subunits.

To ensure the specificity of the assay, primer pairs were tested for their capacity to amplify product from cDNAs prepared from various cell lines and tissues known to express or not to express GM-CSF and GM-CSF receptor mRNAs. Total cellular RNA was extracted from $\sim 10^6$ EL-4 cells and $\sim 10^6$ FD5/12 cells, $\sim 10^6$ L cells, murine liver and random cycling ovary as well as liver from a GM-/- mouse by guanidine thiocyanate extraction (Chapter 2, 2.7.3). Following RNase-free DNase treatment (Chapter 2, 2.7.4), cDNA was synthesised by random-primed reverse transcription (Chapter 2, 2.7.6). cDNA prepared from cell lines and murine tissues were used as positive and negative controls to determine primer specificity and to optimise PCR conditions (Chapter 2, 2.7.8). The integrity of each cDNA preparation was analysed using β -actin primers.

PCR conditions which were found to give a strong signal-to-noise ratio (ie a single product in the absence of products in the negative controls) for each primer pair were for β -actin, 58°C/28 cycles; GM-CSF, 60°C/36; GM-CSF R α , 60°C/34 and GM-CSF R β 60°C/34 (Figure 3.2A). The β -actin primers amplified a single product from each of the cell lines and murine tissue. Each cDNA generated a single product of the expected size, with no evidence of product generated from contaminating genomic DNA. The GM-CSF primers amplified a single product from EL-4 cell, murine liver and random cycling ovary cDNA but not from GM-/- liver. The GM-CSFR α and GM-CSFR β primers amplified single products from FD5/12 cell, murine liver and random cycling ovary cDNA but not L cell cDNA. The identities of the GM-CSF, GM-CSFR α , GM-CSFR β and β -actin products (278, 234, 325, 186 bp respectively) was confirmed by sequence analysis (Chapter 2, 2.7.10).

To investigate whether mRNA for GM-CSF and receptor subunits are expressed by the murine ovary at different stages of follicle growth and rupture, cDNAs were prepared from immature primed whole ovaries at stages reflecting follicle growth (PO), ovulation (OO) and corpus luteum formation (LO) following priming with eCG and hCG (Chapter 2, 2.2.4), and subjected to RT-PCR analysis. Serum progesterone and oestradiol concentrations were assessed (Chapter 2, 2.8.1, 2.8.2) as an indicator of follicle development and of the success of priming. Serum was collected from anaesthetised mice by cardiac puncture (Chapter 2, 2.2.9) and prior to assaying, serum was pooled for each time point due to the small total blood volume of immature mice. Oocytes were collected from the ampulla of the luteinised mouse oviduct and counted to confirm ovulation (Chapter 2, 2.3.4).

The β -actin primers amplified a single product from each of the positive control cell lines, EL-4 and FD5/12 and all ovarian cDNAs. Each cDNA generated a single product of the expected size, with no evidence of product generated from contaminating genomic DNA. Expression of mRNA for GM-CSF was detected in all ovarian preparations (Figure 3.2B). The GM-CSFR α and GM-CSFR β primers amplified single products from FD5/12 cell cDNA, and expression of mRNAs for the GM-CSF receptor subunits GM-CSFR α and GM-CSFR β were detected in all ovarian preparations. Steroid hormone profiles and ovulation rate were within the expected range and confirmed the priming to be effective in stimulating follicle growth and ovulation (Table 3.3).

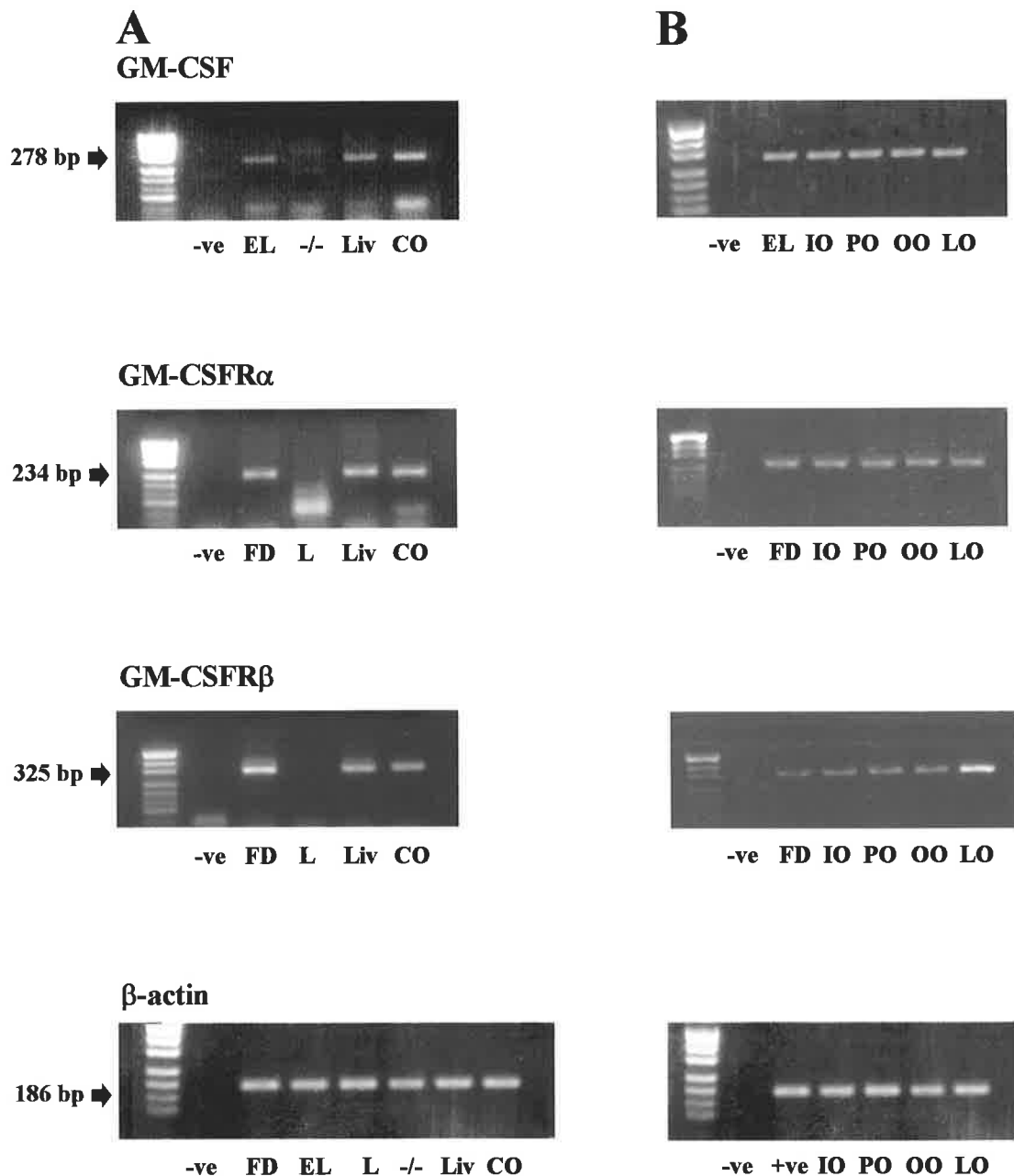


Figure 3.2 GM-CSF ligand and receptor PCR primer specificity. Total cellular RNA was extracted from FD5/12 (FD), L (L), EL-4 (EL) cell lines, murine liver (Liv) and random cycling ovary (CO), GM-/- ovary (-/-) and also from immature (IO) and immature primed preovulatory (PO), ovulatory (OO) and luteinised (LO) ovary. First strand cDNA was reverse transcribed from RNA using random primers. PCR products were amplified from cDNA using *Taq* DNA polymerase and specific primer pairs and analysed by gel electrophoresis in parallel with the molecular weight marker. A) GM-CSF, GM-CSFR α , GM-CSFR β and β -actin PCR products amplified from positive cell lines, murine liver and random cycling ovary. B) GM-CSF, GM-CSFR α , GM-CSFR β and β -actin PCR products amplified from positive cell lines and IO, PO, OO and LO.

Table 3.3 Serum steroid concentrations and ovulation rate of immature primed mice. Both serum and whole ovaries collected from immature primed mice following avertin anaesthesia were pooled for each stage of development prior to assaying.

	Progesterone (nmol/l)	Oestradiol (pmol/l)	Ovulation rate (oocytes/ovary)	Total mice (n)
IO	3.5	40	-	6
PO	5.8	450	-	2
OO	9.3	750	-	2
LO	31.2	100	38, 42, 28, 34	2

IO = immature ovary, PO = preovulatory ovary, OO = ovulatory ovary, LO = luteinised ovary

These data showed RT-PCR to be an appropriate method for quantitative assessment of GM-CSF and receptor mRNA expression by the murine ovary and demonstrate that the murine ovary expresses mRNAs for GM-CSF and both the α and β -chain of the GM-CSF receptor, throughout the oestrous cycle.

3.5 Quantification of mRNA expression for components of the GM-CSF signalling system

In order to determine whether the extent of mRNA expression of GM-CSF, GM-CSFR α and GM-CSFR β was altered by hormone priming during follicle development and rupture, quantitative RT-PCR was employed to measure the relative amounts of mRNA expressed in immature, preovulatory, ovulatory and luteinised whole ovaries.

Whole ovary cDNA collected at various stages of follicle development and rupture (Chapter 3, 3.4) was subjected to quantitative PCR amplification (Chapter 2, 2.7.9). *Taq* DNA polymerase was added to the sample wells containing cDNA, ^{32}P -CTP and specific primer pairs at two cycle intervals. PCR products were analysed by gel electrophoresis, transferred to a nylon membrane (Chapter 2, 2.7.11) and quantified by phosphorimage analysis. Linear regressions were performed on the data to determine the initial amount of specific mRNA (Chapter 2, 2.7.12). For each primer pair, PCR reactions were replicated and

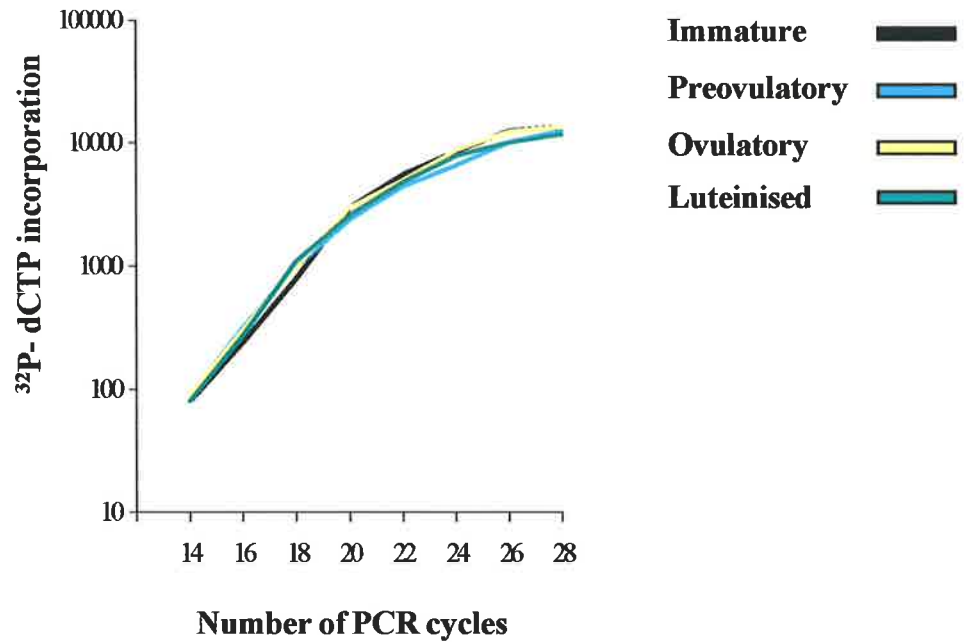
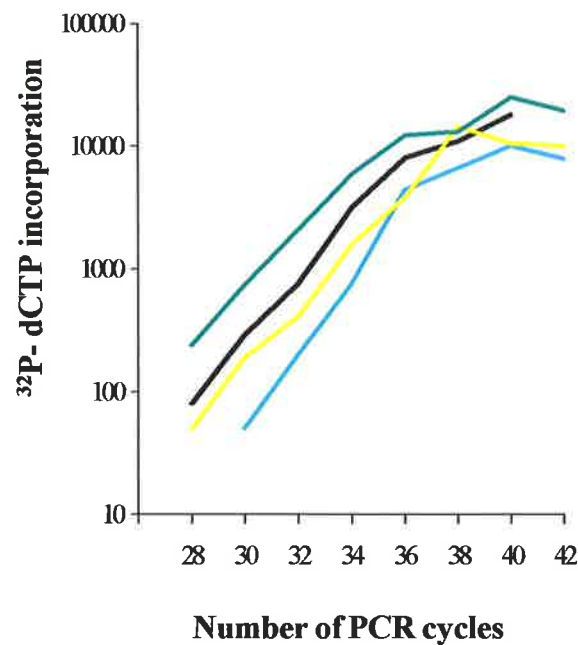
A**B**

Figure 3.3 ^{32}P -dCTP incorporation into the β -actin and GM-CSF PCR products. Total cellular RNA was extracted from whole ovaries, and cDNA was synthesised by reverse transcription primed by random oligonucleotides. *Taq* DNA polymerase was added to the sample wells containing cDNA, ^{32}P -CTP and primer pairs at 2 cycle intervals. PCR products were analysed by gel electrophoresis, transferred to a nylon membrane and quantified by phosphorimage analysis. A) ^{32}P -dCTP incorporation into the β -actin PCR product. B) ^{32}P -dCTP incorporation into the GM-CSF PCR product. Data graphed are representative of one experiment.

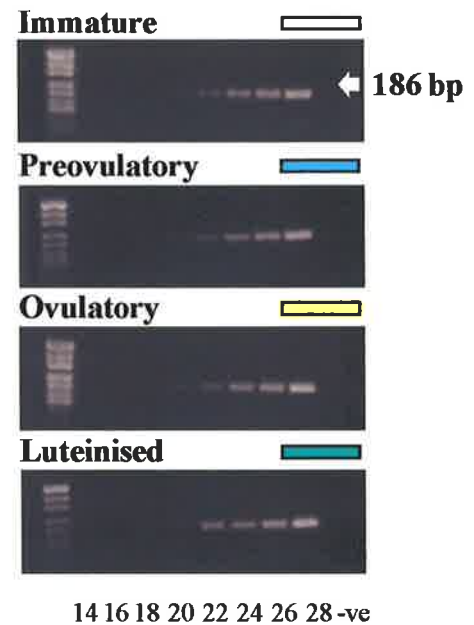
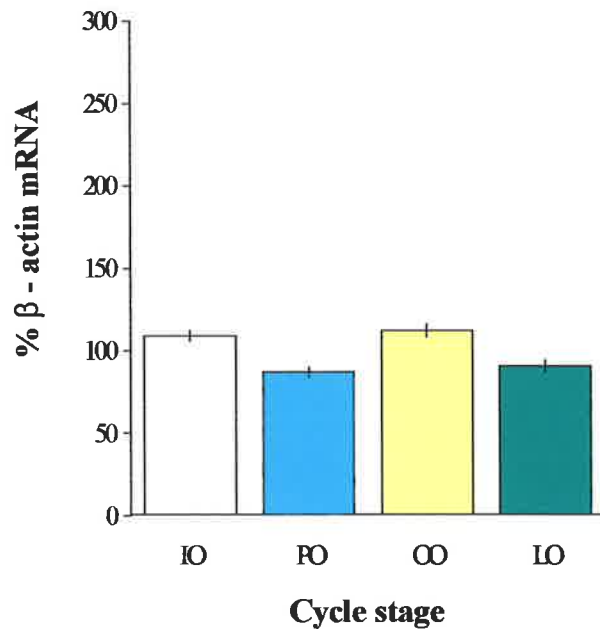
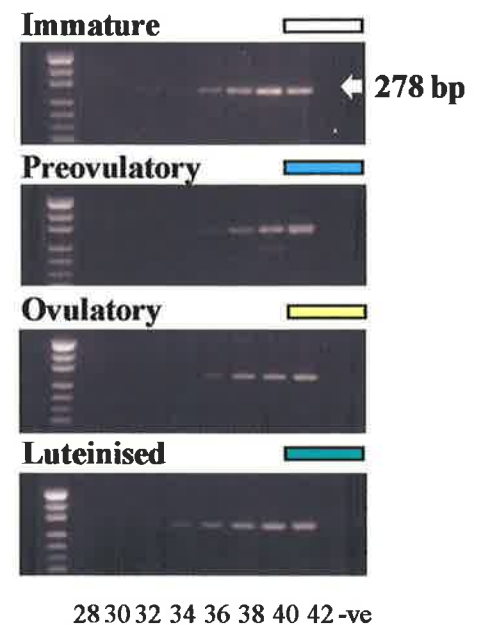
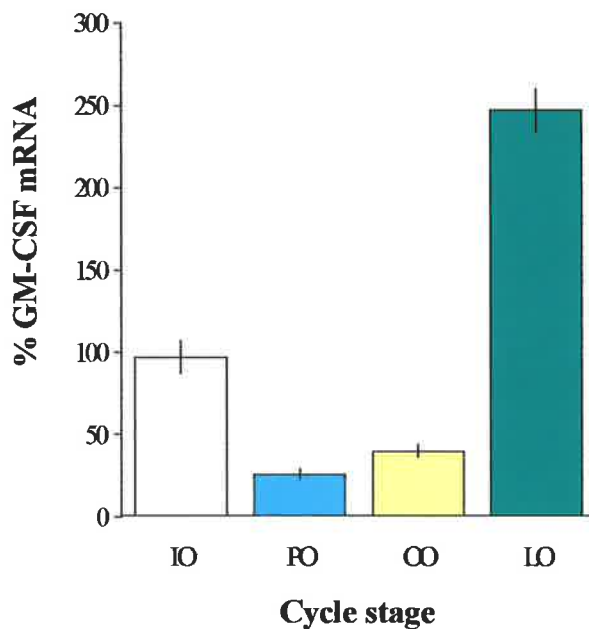
A**B**

Figure 3.4 β -actin and GM-CSF mRNA expression in the ovary during the peri-ovulatory period. Total cellular RNA was extracted from whole ovaries, and cDNA was synthesised by reverse transcription primed by random oligonucleotides. *Taq* DNA polymerase was added to the sample wells containing cDNA, ^{32}P -CTP and primer pairs at 2 cycle intervals. PCR products were analysed by gel electrophoresis in parallel with the molecular weight marker, then transferred to a nylon membrane and quantified by phosphorimage analysis. The relative amount of specific mRNA in each sample was determined by linear regression. Data graphed as mean $\pm$ SE. A) β -actin mRNA levels in the whole ovary ($n = 2$ PCR experiments). B) GM-CSF mRNA levels in the whole ovary, expressed as a % of β -actin mRNA levels ($n = 4$ PCR experiments). IO = immature, PO = preovulatory, OO = ovulatory, LO = luteinised ovary

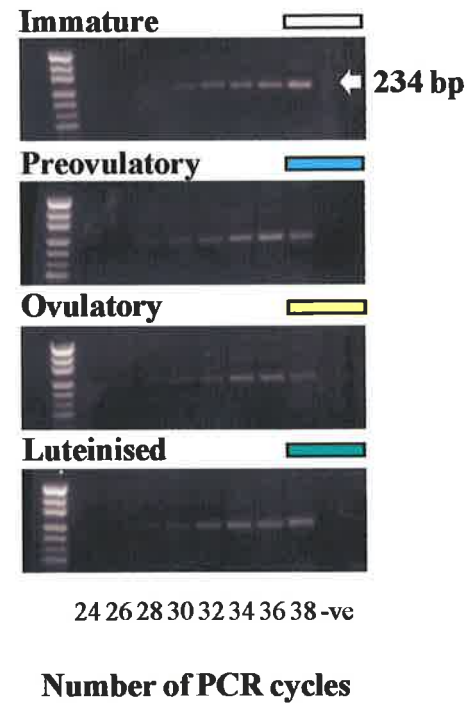
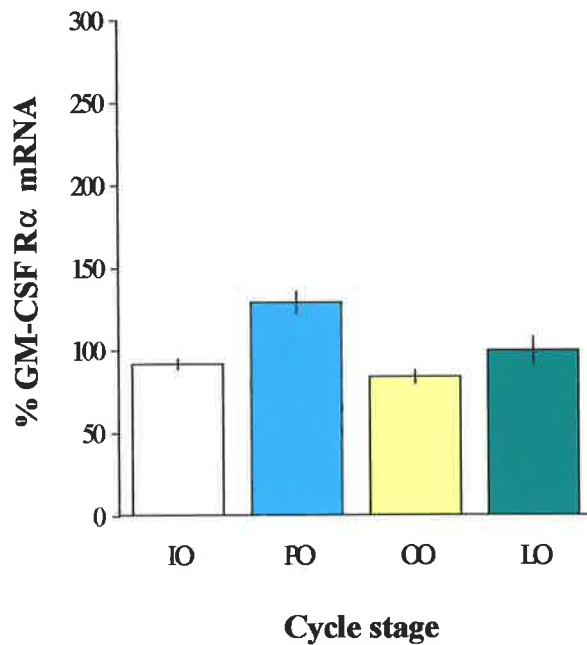
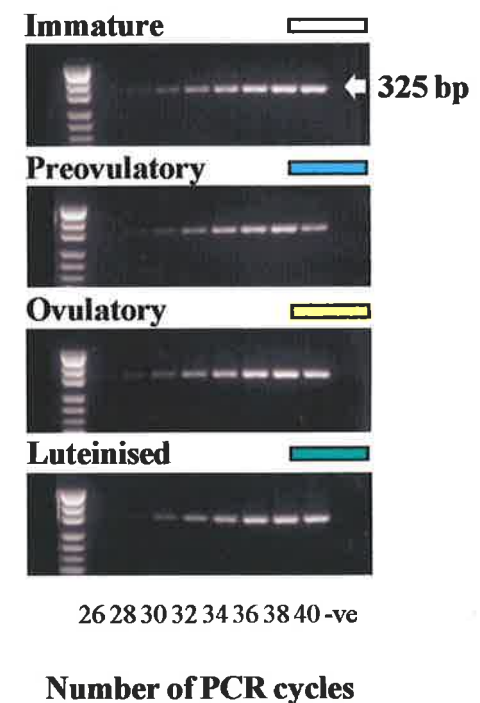
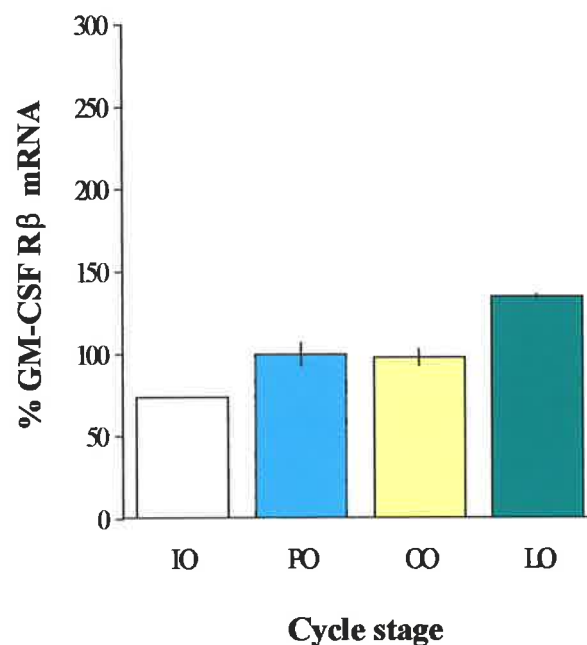
A**B**

Figure 3.5 GM-CSF receptor subunit mRNA expression in the ovary during the peri-ovulatory period. Total cellular RNA was extracted from whole ovaries, and cDNA was synthesised by reverse transcription primed by random oligonucleotides. *Taq* DNA polymerase was added to the sample wells containing cDNA, ^{32}P -CTP and primer pairs at 2 cycle intervals. PCR products were analysed by gel electrophoresis in parallel with the molecular weight marker, then transferred to a nylon membrane and quantified by phosphorimage analysis. The relative amount of specific mRNA in each sample was determined by linear regression and expressed as a % of β -actin mRNA levels. Data graphed as mean $\pm$ SE. A) GM-CSFR α mRNA levels in the whole ovary (n = 3 PCR experiments). B) GM-CSFR β mRNA levels in the whole ovary (n = 2 PCR experiments).

accepted for analysis if results demonstrated a comparable trend. Data was expressed as a percent of the mean of all four samples and normalised for β -actin mRNA expression.

Analysis of the 186 bp β -actin PCR product by gel electrophoresis and phosphorimaging (Figure 3.3A, 3.4A) confirmed the initial amount of cDNA to be approximately equivalent in all samples. The GM-CSF PCR was replicated 4 times, and on each occasion the data showed a comparable trend. The inter-assay coefficient of variation calculated from these experiments was 21, 25, 21 and 10% for the samples of immature, preovulatory, ovulatory and luteinised ovary respectively. The intra-assay coefficient of variation calculated by Robertson *et al* (1996a) was 20%. Analysis of the 278 bp GM-CSF PCR product by gel electrophoresis and phosphorimaging determined GM-CSF mRNA was differentially expressed in the ovary, with levels reaching approximately 7 fold higher in the luteinised ovary compared to the ovulating ovary (Figure 3.3B, 3.4B). Analysis of the 234 bp GM-CSFR α PCR product by gel electrophoresis and phosphorimaging determined GM-CSFR α mRNA levels may increase moderately (1.3 fold) in the preovulatory over immature ovary sample, but this increase was not observed in the ovulatory or luteinised ovary samples (Figure 3.5A). Analysis of the 325 bp GM-CSFR β PCR product by gel electrophoresis and phosphorimaging determined GM-CSFR β mRNA was differentially expressed at different time points with moderately increasing levels in the preovulatory and ovulatory samples, reaching the highest level of expression in the luteinised ovary (1.8 fold immature expression) (Figure 3.5B).

In summary, the finding of substantially elevated GM-CSF mRNA expression in the luteinised ovary suggests that GM-CSF may be involved in the formation of the corpus luteum from follicle remnants or atresia of non-ovulated follicles. Expression of receptor mRNA indicated the presence of potential target cells within the ovary, and suggested that these may be moderately increased during the periovulatory and luteinised periods.

3.6 Tissue compartment mRNA expression of components of the GM-CSF signalling system

The purpose of this experiment was to employ qualitative RT-PCR to determine whether expression of mRNA transcripts for GM-CSF, GM-CSFR α and GM-CSFR β were

restricted to specific tissue compartments in the ovary which may account for the changes in the level of expression in whole ovaries at different stages of follicle development and rupture. To determine whether expression of mRNA was restricted to specific tissue compartments in the ovary, cDNA was prepared from corpora lutea, whole follicles, theca shells, granulosa cells and oocyte/cumulus cell complexes.

Tissue compartments of the ovary, including granulosa cells and theca shells, as well as whole follicles and corpora lutea were collected for RT-PCR analysis. Preovulatory (PO), ovulatory (OO) and luteinised ovaries (LO) were collected from immature primed C57BL/6J mice and pooled. Whole follicles were dissected from the PO (n = 6 mice) and OO ovaries (n = 6 mice). Thecal shells, granulosa cells and oocyte/cumulus cell complexes were collected from half of these mice (n = 3 per group) (Chapter 2, 2.3.2). Corpora lutea were dissected from LO ovaries (n = 6 mice) and oocyte/cumulus cell complexes were flushed from the ampullae of the oviducts of these mice. Total cellular RNA was extracted, DNase treated, reverse transcribed and subjected to PCR analysis using specific primers. PCR conditions were GM-CSF, 60°C/40 - 50 cycles; GM-CSF R α , 60°C/40 - 50 cycles; GM-CSF R β 60°C/40 - 50, cycles and β -actin, 58°C/28 cycles. The PCR assay was repeated at least 3 times on each cDNA sample.

Expression of mRNA (Figure 3.6) for GM-CSF was restricted to corpora lutea, theca shells (OO) and ovulated oocyte/cumulus cell complexes (LO). Expression of GM-CSF was not observed in oocyte/cumulus cell complexes collected prior to ovulation, before the cell complex had left the confines of the follicle. GM-CSFR α mRNA was detected in all preparations, however expression in theca and granulosa preparations collected from PO was transient and may reflect extremely low expression. GM-CSFR β mRNA was detected in all but granulosa cell preparations. The expression of the positive control, β -actin was observed in all preparations. The amplified products were all of the expected size.

A summary of expression for mRNA of components of the GM-CSF cell signalling system is given in Table 3.4. The site-restricted expression of mRNA for GM-CSF is consistent with the views that this cytokine may play a role in the development of the corpus luteum from follicle remnants.

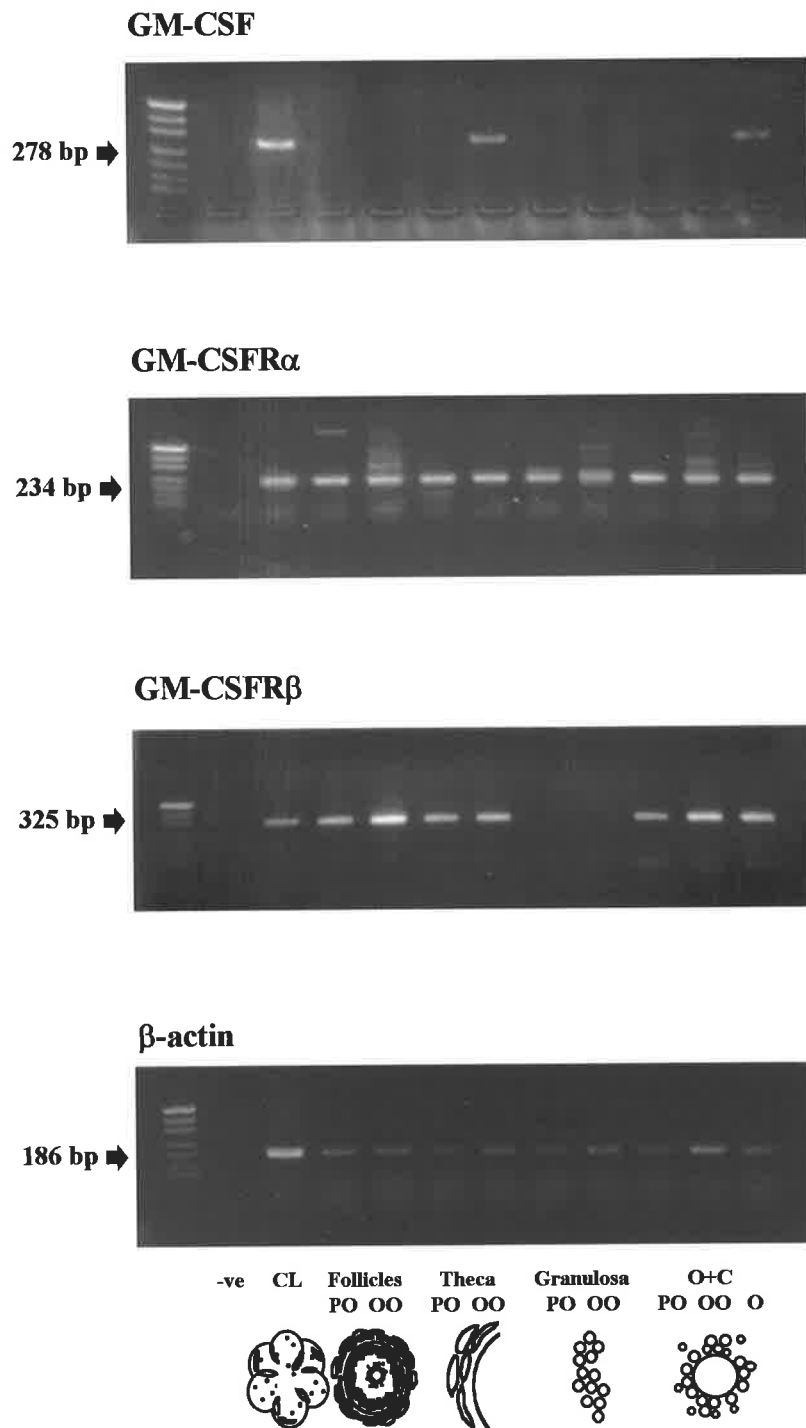


Figure 3.6 GM-CSF ligand and receptor expression of mRNA within ovarian tissue compartments as determined by RT-PCR. Total cellular RNA was extracted from tissue compartments and cDNA was synthesised by reverse transcription primed by random oligonucleotides. *Taq* DNA polymerase was added to the sample wells containing cDNA and specific primer pairs. PCR products were analysed by gel electrophoresis in parallel with the molecular weight marker. PO = preovulatory, OO = ovulatory ovary, O = ovulated.

Table 3.4 Expression of mRNA within ovarian tissue compartments as determined by RT-PCR. Total RNA was extracted, DNase treated and cDNA was synthesised by random-primed reverse transcription. cDNA was subjected to PCR amplification using specific primer pairs.

	CL	Whole follicle		Theca shells		Granulosa cells		Oocyte/cumulus		
		(PO)	(OO)	(PO)	(OO)	(PO)	(OO)	(PO)	(OO)	(LO)
GM-CSF	+	-	-	-	+	-	-	-	-	+
GM-CSFRα	+	+	+	+/-	+	+/-	+	+	+	+
GM-CSFRβ	+	+	+	+	+	-	-	+	+	+
β-actin	+	+	+	+	+	+	+	+	+	+

PO = preovulatory ovary, OO = ovulating ovary, LO = luteinised ovary

+ = expression detected, - = no expression detected

3.7 Development and optimisation of an RT-PCR assay for the components of the CSF-1 signalling system

The purpose of this experiment was to employ RT-PCR to detect the presence of mRNA transcripts for CSF-1 and the CSF-1 receptor, c-fms at various stages of follicle development and rupture in the immature primed mouse ovary. To examine mRNA expression in the ovary it was first necessary to fulfil an initial requirement, which was to establish and validate the RT-PCR technique for detecting mRNA transcripts for CSF-1 and its receptor.

To ensure the specificity of the assay, primer pairs were tested for their capacity to amplify product from cDNAs prepared from various cell lines and tissues known to express or not to express CSF-1 and c-fms mRNAs. Total cellular RNA was extracted from $\sim 10^6$ L cells and $\sim 10^6$ J774 cells, murine liver and random cycling ovary and *cfms^{op}/cfms^{op}* ovary by guanidine thiocyanate extraction. Following RNase-free DNase treatment, cDNA was synthesised by random-primed reverse transcription. cDNA prepared from cell lines and murine tissues were used as positive and negative controls to determine primer specificity and optimise PCR conditions. The integrity of each cDNA preparation was analysed using β -actin primers.

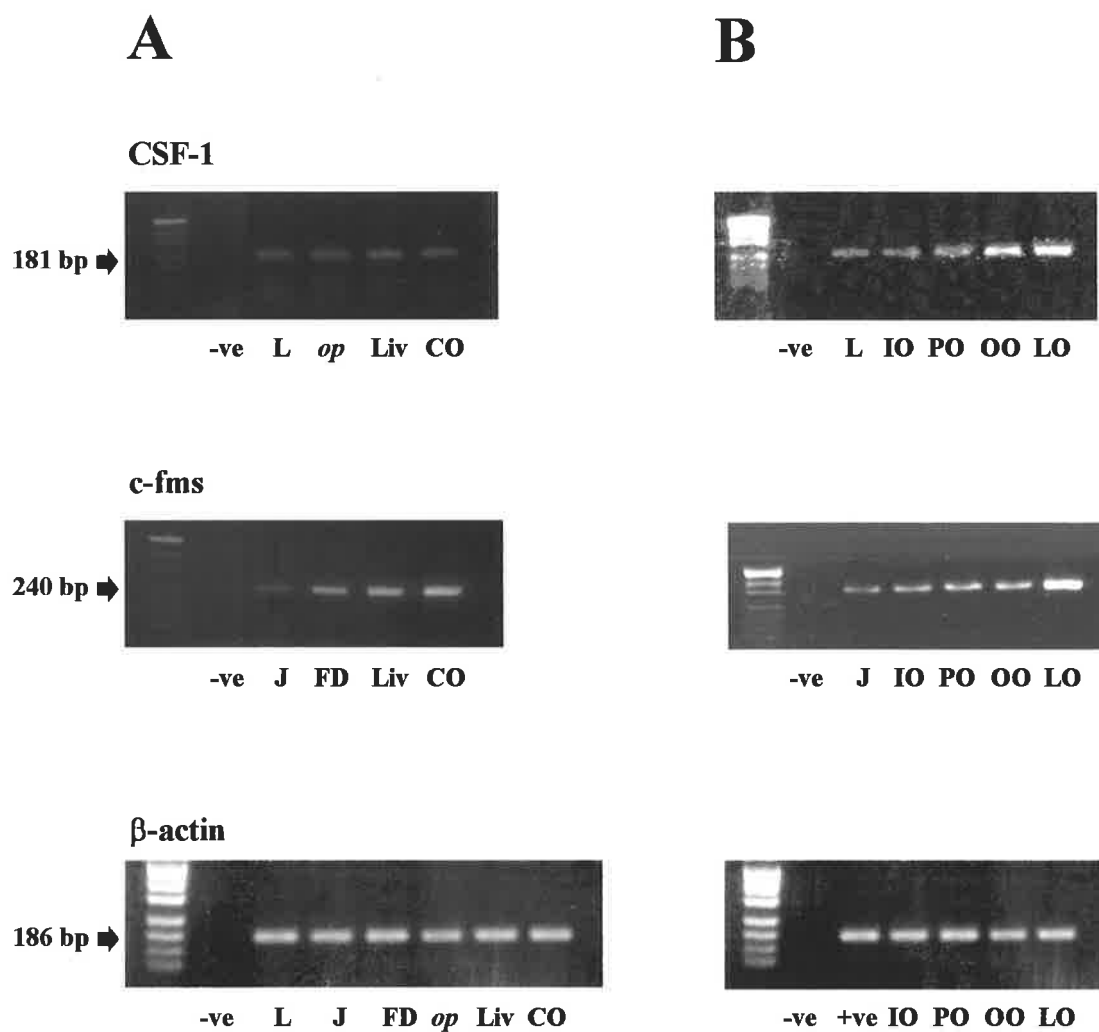


Figure 3.7 CSF-1 ligand and receptor PCR primer specificity. Total cellular RNA was extracted from murine L (L), J774 (J), FD5/12 (FD) cells, murine liver (Liv), random cycling ovary (CO), *csfm^{op}/csfm^{op}* ovary (*op*), and also from immature (IO) and immature primed preovulatory (PO), ovulatory (OO) and luteinised (LO) ovary. First strand cDNA was reverse transcribed from RNA using random oligonucleotides. PCR products were amplified from cDNA using *Taq* DNA polymerase and specific primer pairs and analysed by gel electrophoresis in parallel with the molecular weight marker. PCR products A) CSF-1, c-fms and β -actin amplified from positive cell lines, murine liver and random cycling ovary. B) CSF-1, c-fms and β -actin amplified from positive cell lines and IO, PO, OO and LO.

PCR conditions which were found to give a strong signal to noise ratio (ie a single product in the absence of bands in the negative controls) for each primer pair were β -actin, 58°C/28 cycles; CSF-1 60°C/34 cycles and c-fms 58°C/34 cycles. The β -actin primers amplified a single product from each of the control cell line and murine tissue cDNAs. Each cDNA generated a single product of the expected size, with no evidence of product generated from contaminating genomic DNA. The CSF-1 primers amplified a single product from L cell, murine liver and random cycling ovary but also *cfms^{op}/cfms^{op}* ovary cDNA due to the design of the primers (Figure 3.7A). The c-fms primers amplified a single product from J774 cell, murine liver and random cycling ovary but also FD5/12 cell cDNA which was unexpected (Figure 3.7A). The identities of the CSF-1, c-fms and β -actin products (181, 240 and 186 bp respectively) were confirmed by sequence analysis.

To investigate whether mRNA for CSF-1 and its receptor, c-fms are expressed by the murine ovary at various stages of follicle development and rupture, cDNAs were prepared from immature primed whole ovaries (Chapter 3, 3.4) and subjected to RT-PCR analysis. The β -actin primers amplified a single product from each of the positive control cell lines, L, J774 and all ovarian cDNAs. Each cDNA generated a single product of the expected size, with no evidence of product generated from contaminating genomic DNA. The CSF-1 primers amplified a single product from L cell cDNA and all ovarian preparations (Figure 3.7B). The c-fms primers amplified a single product from J774 cell cDNA and all ovarian preparations (Figure 3.7B).

These data demonstrate that the murine ovary expresses mRNAs for CSF-1 and the CSF-1 receptor throughout the oestrous cycle.

3.8 Quantification of mRNA expression for components of the CSF-1 signalling system

The purpose of this experiment was to employ quantitative RT-PCR to measure the relative amounts of mRNA transcripts for the macrophage mediator CSF-1 and its receptor c-fms at various stages of follicle development and rupture in the immature primed whole ovary.

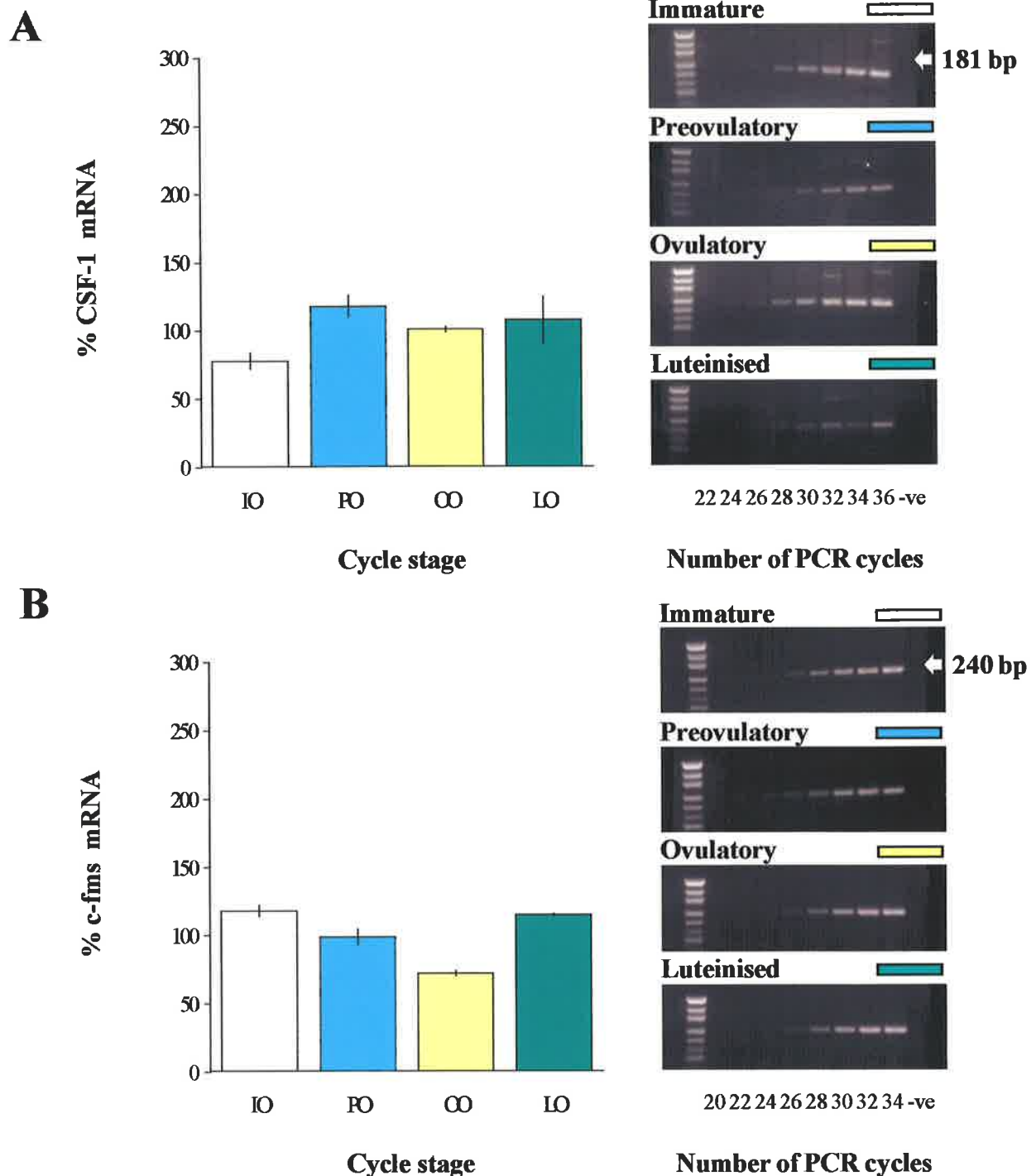


Figure 3.8 CSF-1 and receptor mRNA expression in the ovary during the peri-ovulatory period. Total cellular RNA was extracted from whole ovaries, and cDNA was synthesised by reverse transcription primed by random oligonucleotides. *Taq* DNA polymerase was added to the sample wells containing cDNA, ^{32}P -CTP and primer pairs at 2 cycle intervals. PCR products were analysed by gel electrophoresis in parallel with the molecular weight marker, transferred to a nylon membrane and phosphorimaged. The relative amount of specific mRNA in each sample was determined by linear regression and expressed as a % of β -actin mRNA levels. Data graphed as mean $\pm$ SE. A) CSF-1 mRNA levels in the whole ovary (n = 2 PCR experiments). B) c-fms mRNA levels in the whole ovary (n = 2 PCR experiments).

The quantitative PCR amplification for CSF-1 and c-fms was performed on the samples described in Chapter 3, 3.2. *Taq* DNA polymerase was added to the sample wells containing cDNA, ³²P-CTP and primer pairs at two cycle intervals. PCR products were analysed by gel electrophoresis, transferred to a nylon membrane and quantified by phosphorimage analysis. Linear regressions were performed on the data to determine the initial amount of each mRNA. For each primer pair, PCR reactions were replicated and accepted for analysis if results demonstrated a comparable trend. Data was expressed as a percent of the mean of all four samples and normalised for β -actin mRNA expression.

Analysis of the 186 bp β -actin PCR product by gel electrophoresis and phosphorimaging (Figure 3.4A) confirmed the initial amount of cDNA to be approximately equivalent in all samples. Analysis of the 181 bp CSF-1 PCR product by gel electrophoresis and phosphorimaging showed that CSF-1 mRNA levels were moderately elevated (0.5 fold over immature) following the induction of follicle development, and then remained relatively constant during follicle development and rupture (Figure 3.8A). Expression of receptor mRNA was considered an indicator of the presence of potential target cells within the ovary. Analysis of the 240 bp c-fms PCR product by gel electrophoresis and phosphorimaging showed that expression of CSF-1 receptor mRNA in whole ovary declined to 60% of the immature value, and was lowest at the time of ovulation and subsequently returned to immature levels after rupture (Figure 3.8B).

In summary, CSF-1 mRNA expression was relatively constant during follicle growth and rupture. Expression of CSF-1 receptor mRNA identified the presence of potential target cells within the ovary, and suggested that these cells may be reduced during the ovulatory process.

3.9 Tissue compartment mRNA expression of components of the CSF-1 signalling system

The purpose of this experiment was to employ RT-PCR to determine whether expression of mRNA transcripts for CSF-1 and c-fms were restricted to specific tissue compartments in the ovary. cDNA was prepared from corpora lutea, whole follicles, theca

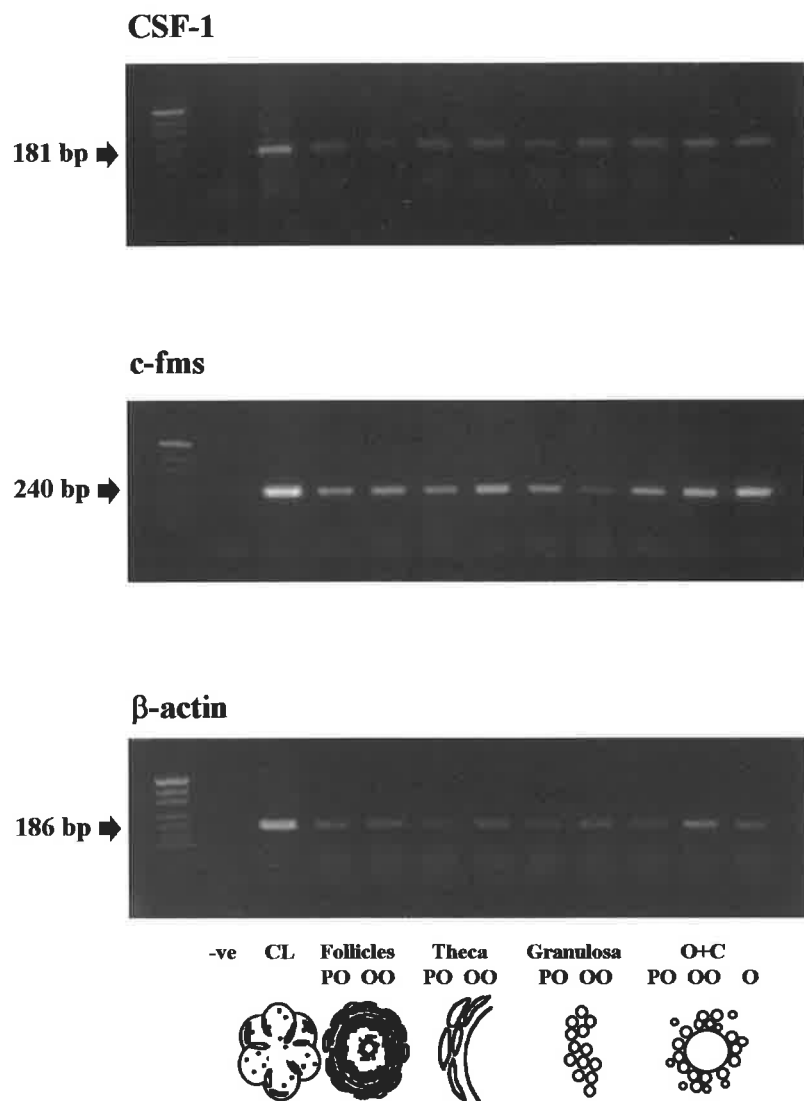


Figure 3.9 CSF-1 ligand and receptor mRNA expression within ovarian tissue compartments as determined by RT-PCR. Total cellular RNA was extracted from tissue compartments and cDNA was synthesised by reverse transcription primed by random oligonucleotides. *Taq* DNA polymerase was added to the sample wells containing cDNA and specific primer pairs. PCR products were analysed by gel electrophoresis in parallel with the molecular weight marker.

shells, granulosa cells and oocyte/cumulus cell complexes and subjected to PCR primed by specific primers.

cDNA prepared from specific ovarian tissue compartments collected in experiment 3.4 was subjected to PCR amplification in order to assess expression of mRNAs for components of the CSF-1 cell signalling system. PCR conditions for amplification of CSF-1 were 60°C/40 - 50 cycles; c-fms, 58°C/50 cycles and β -actin, 58°C/28 cycles. PCR assays were repeated a minimum of 3 times on each cDNA sample.

CSF-1 and c-fms mRNAs were detected in all preparations (Figure 3.9), as were mRNA for the positive control, β -actin. cDNA generated products were all of the expected size.

A summary of the location of CSF-1 and c-fms mRNA expression in ovarian tissue compartments is given in Table 3.5.

Table 3.5 Expression of mRNA within ovarian tissue compartments as determined by RT-PCR. Total RNA was extracted, DNase treated and cDNA was synthesised by random-primed reverse transcription. cDNA was subjected to PCR amplification using specific primer pairs.

	CL	Whole follicle		Theca shells		Granulosa cells		Oocyte/cumulus		
		(PO)	(OO)	(PO)	(OO)	(PO)	(OO)	(PO)	(OO)	(LO)
CSF-1	+	+	+	+	+	+	+	+	+	+
c-fms	+	+	+	+	+	+	+	+	+	+
β-actin	+	+	+	+	+	+	+	+	+	+

PO = preovulatory ovary, OO = ovulatory ovary, LO = luteinised ovary

+ = expression detected, - = no expression detected

3.10 Discussion and conclusion

The experiments described in this chapter have identified expression of GM-CSF and CSF-1 mRNA within the mouse ovary, where they may contribute to the tissue remodelling processes of follicular development, ovulation, luteinisation and luteolysis through their actions on local receptor-bearing cells.

In an attempt to determine the location and to give information on the potential identity of cell types secreting ligand and expressing the receptor proteins within the ovary, immunohistochemistry was trialed but the technique was not successfully established. Described in the literature are two methods for the immunolocalisation of GM-CSF protein in murine tissues; Robertson *et al* (1994a) demonstrated GM-CSF localisation following 0.1% trypsin treatment on 10% formalin-fixed, paraffin embedded skin using a rat α mGM-CSF mAb (Genzyme), and Sander *et al* (1993) illustrated intracellular localisation of GM-CSF in 4% paraformaldehyde-fixed T cell clones using rat α mGM-CSF mAbs MPI-22E9 and MPI-31G6. The failure to detect immunoactive GM-CSF may have been due to inadequate or inappropriate fixation procedures and/or the use of fresh frozen, as opposed to paraffin-embedded tissue. Generally detection of antigens is considered easier in fresh frozen as opposed to paraffin embedded tissue, so it was surprising that protein was not detected following similar protocols to Robertson *et al* (1994a). However, it is possible that the need to wash tissue sections following an incubation may have successively reduced the retention of soluble GM-CSF in the fresh frozen tissues. Eventually, due to exhaustion of the options for fixation of fresh frozen tissue and time restraints, this method of detection was abandoned.

The measurement of immunoactive protein by ELISA was considered the next best option and development of the assay was pursued. Initially following the methods described by the manufacturer and later by Judith Layton (Ludwig Institute for Cancer Research; personal communication) and Sander and co-workers (1993), a standard curve of 7.5 - 2000 pg/ml was established. Sander constructed a standard curve using the same antibodies from 19 - 5000 pg/ml *E. coli*-derived recombinant GM-CSF and reported the sensitivity of their assay to be 100 pg/ml. However, following the establishment of a standard curve, the assay was considered insufficiently sensitive for the experimental requirements. Primarily this was because the small size of the murine ovary limited the density of cell cultures that could be established, and consequently the concentration of GM-CSF that could be achieved *in vitro*. Furthermore, the necessity of using *in vitro* techniques to investigate ovarian synthesis could have further confounded these studies. Both methods for the detection of immunoactive protein were abandoned and the expression of mRNA encoding ligand and receptor was examined.

3.10.1 GM-CSF signalling system expression in the mouse ovary

The experiments presented in this chapter show the pattern of GM-CSF mRNA expression to be consistent with a major role for GM-CSF in the development of the corpus luteum from follicle remnants. The association of GM-CSF mRNA with this event was demonstrated in two experiments. Firstly, it was found that expression increased 7 fold after ovulation in the whole ovary (3.5). Second, it was found that expression of mRNA was restricted to the theca just prior to ovulation, and then to the corpus luteum (3.6). The expression of both α and β subunit mRNA coincides with the trafficking of myeloid leukocytes into the theca prior to ovulation and into the corpus luteum.

The expression of GM-CSF mRNA was restricted to the theca, corpus luteum and oocyte/cumulus cells complex following ovulation. Interestingly, the induction of GM-CSF mRNA expression in the theca by hCG administration is reminiscent of the gonadotrophin dependency of other cytokines, most notably IL-1 β mRNA (Hurwitz *et al*, 1991b). The finding of strong expression in the theca concurs with a previous finding showing that maximal secretion of bioactive GM-CSF by whole perfused ovaries is at ovulation (Brännström *et al*, 1994). The lack of detection of GM-CSF mRNA in cDNA of whole follicles may reflect the smaller ratio of positive to negative cells in this preparation as compared to the theca shells alone. At a time, when the immature primed luteinised ovary consists primarily of ruptured follicles and atretic follicles, the ovary expresses more GM-CSF mRNA than at any other stage investigated. In addition, these findings are consistent with those of Tamura and colleagues (in press) reporting both mRNA and protein for GM-CSF in the theca and corpus luteum of the rat and human (Zhao *et al*, 1995) (for summary of results see Figure 3.6). Oocyte/cumulus cell complexes extracted from the preovulatory and ovulatory follicles did not express GM-CSF mRNA. This is consistent with the findings of Zhao *et al* (1995), but in contrast to the localisation of protein and mRNA by Tamura and co-workers (in press), who may have detected receptor-bound GM-CSF on the oocyte surface. Interestingly, mRNA for GM-CSF was detected in ovulated oocyte/cumulus cell complexes by RT-PCR. It could be proposed that these conflicting data may be the result of differences in assay sensitivity, incorporation of leukocytes into the oocyte/cumulus complex in the oviduct following ovulation, or species differences.

Table 3.6 Summary of expression of protein and mRNA for components of the GM-CSF signalling system within the ovary. Localisation determined by immunohistochemistry or *in situ* hybridisation in the mouse (Tamura *et al*, in press) and human (Zhao *et al*, 1995).

	Corpus luteum	Theca	Granulosa cells	Oocyte
GM-CSF	mP, mRNA hP, mRNA	mP, mRNA hP, mRNA		mP, mRNA
GM-CSFR α	mmRNA hP, mRNA	mmRNA hP, mRNA	hP	
GM-CSFR β	mmRNA hP, mRNA	mmRNA hP, mRNA	hP	

h = human, m = mouse, P = protein

GM-CSF may target cells of the theca, granulosa, oocyte/cumulus cell complex and corpus luteum as indicated by the expression of components of the receptor. GM-CSF synthesised in the theca may target granulosa cells, as a consequence of diffusion across the basement membrane, as well as cells of the theca expressing both subunits of the receptor. GM-CSF has not been detected on granulosa cells. This model of diffusion across the basement membrane may explain the detection of GM-CSF in follicular fluid (Jasper *et al*, 1996). However GM-CSF is known to bind heparan sulphate, a constituent of the basement membrane, so that this immobilisation in the membrane may actually sequester GM-CSF and perhaps even prevent its passage into the follicular fluid. The possibility that detection of GM-CSF in follicular fluid and granulosa-lutein cell culture supernatant and mRNA preparations (Jasper *et al*, 1996), may occur as a result of up to 20% blood cell contamination during follicle aspiration (Beckman *et al*, 1991), and subsequent activation of these cells during sample processing, also remains to be elucidated. Granulosa cells were found to express the α but not the β subunit of the GM-CSF receptor. The detection of the α subunit is consistent with the findings of Zhao and co-worker (1995), but in contrast to the findings of Tamura and co-workers (in press). The lack of detection of the β subunit mRNA is consistent with the findings of Tamura and co-workers (in press), but in contrast to the results of immunohistochemistry and RT-PCR studies conducted in the human (Zhao *et al*, 1995, Jasper *et al*, 1996). The biological significance of α chain expression alone by granulosa cells is

unclear, since haemopoietic cells require both for effective signal transduction, but the expression may play a role in glucose transport (Ding *et al*, 1994, Spielholz *et al*, 1995) and thus prolong cell survival. There is only one report in the literature which examines the effect of GM-CSF on ovarian cells, and no effect of GM-CSF on granulosa cell LH-stimulated progesterone secretion was found (Tamura *et al*, 1997). Oocyte/cumulus cell complexes were found to express the α and β subunit of the GM-CSF receptor. The detection of mRNA for the α subunit is consistent with the findings of Robertson and co-workers (1994b), whereas the detection of the β subunit is in contrast to the results of studies conducted in the mouse (Robertson *et al*, 1994b), rat (Tamura *et al*, in press) and human (Zhao *et al*, 1995). The discrepancies between detection of GM-CSF receptor mRNA in this and earlier studies suggests the presence of contaminating cells, possibly leukocytes, in the oocyte/cumulus complex at the time of sample collection or expression by cumulus cells alone, which remains to be elucidated.

The pattern of mRNA expression of the α and β receptor subunits in the whole ovary was not identical. Expression of GM-CSFR β mRNA expression was highest in the luteinised ovary, and this is significant given that availability of β chain is thought to be the limiting factor in GM-CSF signalling transduction in haemopoietic cells. The increase in leukocytes, particularly macrophages and neutrophils, which occurs in the corpora lutea and ovarian stroma during pregnancy, may account to a great extent for the increase in β receptor subunits.

By using RT-PCR for detection of ligand and receptor subunits mRNA, this study has provided new observations on the GM-CSF cell signalling system in the mouse and confirmed the separate observations of previous investigators in the human and rat (Zhao *et al*, 1995, Jasper *et al*, 1996, Tamura *et al*, in press).

3.10.2 CSF-1 signalling expression in the mouse ovary

The experiments presented in this chapter show CSF-1 mRNA expression in the ovary at all stages of oocyte and follicle development and rupture (Chapter 3, 3.8), and in all ovarian tissue compartments examined (Chapter 3, 3.9).

There is limited research describing mRNA expression and protein localisation in the ovary, unlike those in the uterus. However, increased levels of immunoactive CSF-1 in human follicular fluid compared to serum suggest an ovarian source (Witt and Pollard, 1997).

By using RT-PCR for detection of ligand and receptor mRNA, this study has provided new observations on components of the CSF-1 signalling system in the mouse ovary and suggests a broad range of actions.

The expression of CSF-1 ligand mRNA in the granulosa layer, oocyte/cumulus cell complex and in the theca and corpus luteum coincides with the trafficking of myeloid cells into the theca prior to ovulation and corpus luteum development. Granulosa cell data presented in this chapter are supported by the findings indicating mRNA expression of ligand in the granulosa cell layer (Arceci *et al*, 1992, Witt and Pollard, 1997).

CSF-1 may target cells of the theca, granulosa, oocyte/cumulus cell complex and corpus luteum as indicated by the expression of c-fms mRNA. The detection of expression of c-fms in the granulosa cell layer and oocyte is consistent with the findings of Arceci *et al* (1992), Haimovici and Anderson (1993), Tekpetey *et al*, (1996), and suggests that paracrine regulation of oocyte development may occur. Interestingly, expression of c-fms mRNA was lowest at the time of ovulation. An increase of leukocytes, particularly macrophages at ovulation in the ovary and corpus luteum, may have been expected to be associated with an increase in c-fms mRNA expression, however this was not observed.

The presence of mRNA can be an indicator of protein expression and biological activity, however mRNA expression does not always occur concomitantly with protein expression. In many instances cytokine mRNAs are transcribed but not translated unless stabilisation of the mRNA occurs as a consequence of cell activation. In addition, GM-CSF is known to exist in presynthesised cytoplasmic stores and to bind to ECM.

The results presented in this chapter are based solely on RT-PCR detection. PCR provided the most sensitive means of detecting mRNA expression and was particularly useful due to the limited amounts of ovary tissue available. However, it did not provide conclusive information on the cells or tissue compartments contributing to this expression. The cellular source and target cells remained undetermined by this technique due to the heterogeneous population of cells which comprise the theca and corpus luteum. *In situ* hybridisation and/or immunohistochemical studies localise gene/protein expression at the single-cell level. Being a technically demanding approach, *in situ* hybridisation was considered for analysing mRNA but was not pursued to the stage of validation. However, as discussed in previous sections, there is adequate evidence both in humans and rodents to suggest that proteins for both ligand and receptors exist in the ovary.

Quantification of mRNA according to the method described by Chelly *et al*, 1988 assumes amplification is exponential. The advantage of this method is that it is not necessary to construct an internal standard for each primer pair as is the case for other quantitative methods, thereby making this method relatively simple. Another assumption made was that β -actin mRNA expression was a marker of total mRNA to normalise data. This study made no attempt to discriminate between alternative mRNA species that have been described for CSF-1, GM-CSF as well as the GM-CSFR α (Chapter 2, 2.7.7). The frequency of alternative CSF-1 mRNA transcripts in the ovary remains undetermined although Witt and Pollard (1997) reported the presence of numerous species in human follicular fluid cell preparations.

Further studies including *in situ* localisation are required to determine the sites of synthesis and action of GM-CSF and CSF-1 in the mouse ovary. Northern blotting would provide information as to the size of spliced mRNA variants expressed in the ovary. Preferential expression of mRNA transcripts may also provide a mechanism to regulate biological activity. Definitive investigations of the cell types expressing GM-CSF and CSF-1 and the hormonal regulation of these genes is required in order to further understand the events controlling the regulation of expression of the CSFs in the ovary. Differential expression of GM-CSF mRNA in the ovary may reflect regulation by gonadotrophin, LH, FSH or ovarian steroid regulation, or may reflect changes in expression in the cellular composition of the major structures dominating the ovary at the various time points. The anti-progesterone RU 486, the oestrogen antagonist ZK 119010 and LHRH agonist could be administered alone and in combinations to examine gonadotrophin and or steroid regulation of GM-CSF and CSF-1 secretion in the ovary. Alternatively, an effect of gonadotropins could be mediated via cytokines or through enhanced recruitment of trafficking cells such as macrophages, which can produce GM-CSF and CSF-1 when activated.

These data show that expression of the GM-CSF signalling system is tissue compartment specific in the normal mouse ovary. While the compartments consist of more than one cell type in some cases, it would appear GM-CSF influences corpus luteum development, possibly through its actions on macrophages and granulocytes surrounding the ovulatory follicle, and the development of the oocytes/cumulus cell complex following ovulation. The expression of the CSF-1 signalling system appears to be more widely spread and could be implicated in follicle development and rupture.

Chapter 4

Characterisation of the GM-CSF deficient ovary

4.1 Introduction

The rhythmicity of the oestrous cycle is the direct result of endocrine hormone driven tissue remodelling events occurring in the ovary, which in turn are orchestrated by cyclic activity in the hypothalamic-pituitary axis. Peptide growth factors including lymphohaemopoietic cytokines are increasingly implicated in mediating the effects of endocrine hormones at the cellular level. The profile of cytokines derived from ovarian cells includes the IL-1 β , TNF α and more recently GM-CSF (Chapter 1, 1.4.2). Both protein and mRNA encoding components of the GM-CSF signalling system have been detected within or demonstrated to be secreted by the human and rodent ovary (Chapter 1, 1.4.2 and Chapter 3). Cytokines, together with the abundant population of leukocytes which infiltrate ovarian tissues, are thought to mediate processes including the regulation of steroidogenesis, local vascular permeability, ECM remodelling, angiogenesis and apoptosis.

It has been proposed that GM-CSF contributes to regulation of the tissue remodelling events and immunological changes within the ovary that maintain the rhythmicity of oestrous and ovulation.

This study was undertaken to determine whether GM-CSF deficiency due to disruption of the GM-CSF gene altered ovarian parameters including oestrous cycle length, ovulation rate, steroidogenesis, and the duration of pseudopregnancy. Oestrous cycle length was examined as a measure of hypothalamic-pituitary-ovarian axis activity. *In vivo* ovulation rates were assessed in immature mice after priming with exogenous gonadotrophins and in adult naturally cycling mice. *In vitro* ovulation was assessed using a perfusion model, which enables the examination of ovarian derived steroids and NO released during the periovulatory period. In addition, development and function of the corpus luteum was assessed during pregnancy by measurement of ovarian weight and of the duration of pseudopregnancy.

4.2 Oestrous cycle length

In order to determine whether GM-/- adult female mice display regular signs of oestrous and to determine the length of the oestrous cycle, the cellular characteristics of daily vaginal smears were examined in GM+/+ (12.8 $\pm$ 0.3 weeks of age) and GM-/- (11.8 $\pm$ 0.6

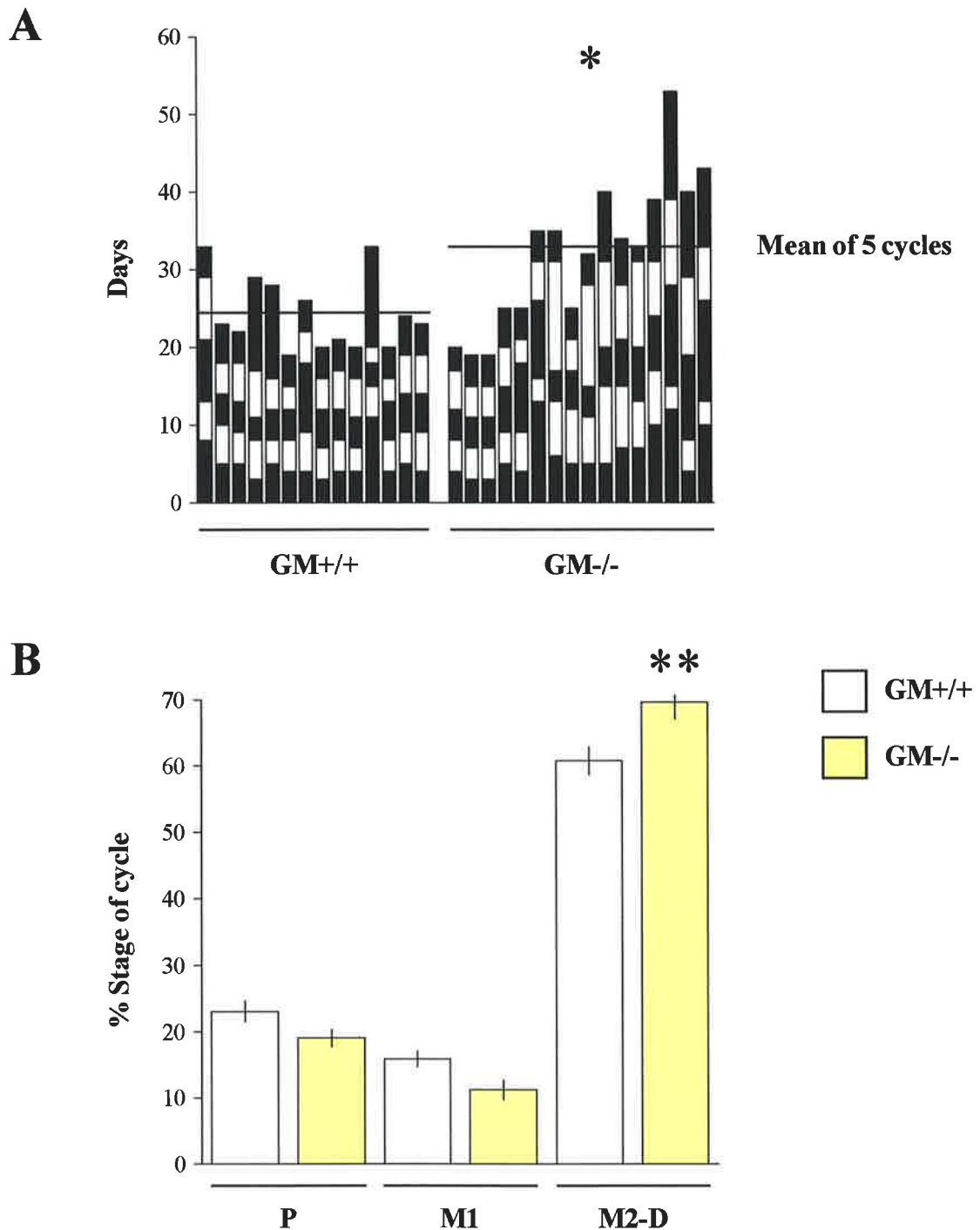


Figure 4.1 Oestrous cycle characteristics of naturally cycling adult GM+/+ and GM-/ mice. The cellular characteristics of daily vaginal smears recorded over 5 cycles were analysed to determine the stage of oestrous. A) Oestrous cycle length. Each oestrous cycle is represented by a segment on the bar for each mouse. The horizontal lines across the graph indicate the mean duration of 5 cycles (days). * significantly different from GM+/+ as assessed by Mann-Whitney Test, two tailed $P = 0.0222$. B) Days spent at each stage of the oestrous cycle. ** significantly different from GM+/+ as assessed by one-way ANOVA and Tukey-Kramer Multiple Comparisons Test, $P < 0.01$. Data presented as mean $\pm$ SE, $n = 14$ GM+/+, 16 GM-/ mice. P = prooestrous, M1 = metoestrous-1, M2 = metoestrous-2 and D = dioestrous.

weeks of age) mice housed in close proximity to a stud male (Chapter 2, 2.2.6). Oestrous cycle length was averaged over 5 consecutively completed cycles.

Vaginal smears taken from GM-/- mice reflected changes in the structure of the vaginal epithelium and followed a regular and predictable course during the cycle. GM+/- mice displayed signs of oestrous every 4.9 ± 0.3 days ($n = 14$). In contrast, GM-/- mice exhibited an extended oestrous cycle of 6.5 ± 0.5 days ($n = 16$), an extension of approximately 1.5 days (two-tailed $P = 0.0222$, Mann-Whitney Test) (Figure 4.1A). The GM-/- females exhibited the cellular characteristics of a metoestrous-2 and/or dioestrous smear for a significantly longer period of time than the GM+/+ females (Figure 4.1B). The extended oestrous cycle in GM-/- mice could result from delayed remodelling of the corpus luteum or follicle recruitment and growth for the subsequent cycle.

4.3 Ovulation rate

4.3.1 *In vivo* ovulation rate

To investigate ovulation rates, oocytes were collected and counted from immature primed and naturally cycling adult mice. Ovulation rate was assessed in immature, primed 129/Sv (4 ± 0 weeks of age), GM+/+ (3.3 ± 0.1 weeks of age) and GM-/- (3.4 ± 0.1 weeks of age) mice, according to the regime described in Chapter 2, 2.2.4. Mice were sacrificed on the morning of ovulation between 9:00 - 12:00 hr and oocytes in the ampulla region of each oviduct were counted (Chapter 2, 2.3.4). Ovulation rate was assessed in naturally cycling adult GM+/+ (18.7 ± 0.7 weeks of age) and GM-/- (18.3 ± 1.2 weeks of age) mice following oestrous cycle tracking. At the first metoestrous-1, following a minimum of 5 completed oestrous cycles, mice from experiment 4.2 were euthanased and oocytes were dissected from the ampulla or flushed from the oviduct.

Comparable numbers of oocytes were ovulated from GM+/+ and GM-/- mice regardless of genotype or hormone priming. The numbers of oocytes ovulated from immature primed GM+/+ and 129/Sv mice were similar (Table 4.1) ($P = 0.9437$, one-way ANOVA). Comparable numbers of oocytes were also ovulated from naturally cycling adult GM+/+ and GM-/- mice (Table 4.1) ($P = 0.9791$, unpaired t-Test). Oocytes collected from the oviduct of naturally cycling adult mice were retrieved equally from the ampulla or flushed from the

remaining oviduct (two-tailed $P = 0.4136$, Fisher's Exact Test), indicating that rate of transport through the oviduct was similar in GM+/+ and GM-/- mice. These results would suggest that follicle recruitment and ovulation is unaltered in the case of GM-CSF deficiency.

Table 4.1 *In vivo* ovulation rate of immature primed and naturally cycling adult 129/Sv, GM+/+ and GM-/- mice. Immature mice were primed to ovulate by administration of 5 IU eCG and 5 IU hCG. Oocytes were retrieved from adult mice following the completion of a minimum of 5 oestrous cycles. Data are mean $\pm$ SE.

	# Ovulated/total (%)	Oocytes/mouse	Ovulation range
Immature			
129/Sv	8/8 (100%)	31.0 $\pm$ 4.9	16 - 59
GM+/+	6/6 (100%)	31.8 $\pm$ 7.7	15 - 56
GM-/-	7/7 (100%)	28.9 $\pm$ 6.4	9 - 52
Adult			
GM+/+	11/12 (92%)	10.4 $\pm$ 0.8	7 - 15
GM-/-	12/12 (100%)	10.3 $\pm$ 0.8	5 - 17

4.3.2 *In vitro* ovulation rate of perfused ovaries

Ovulation rates of GM+/+ and GM-/- mice were assessed following the perfusion of the whole ovary *in vitro*. Adult female, 12 - 14 week old GM+/+ and GM-/- mice were synchronised to ovulate by ip administration of an LHRH agonist followed 48 hr later by sc administration of 5 IU eCG (Chapter 2, 2.2.5). On the morning prior to ovulation, the right ovary with its vasculature was surgically isolated and perfused (Chapter 2, 2.4). Following 1 hr of perfusion, 0.1 μ g/ml ovine LH alone or in combination with 4 ng/ml rmGM-CSF was added to the perfusion media at time 0 hr. Ovulation rate was assessed at 22 hr. Eleven GM+/+ mice were set aside and allowed to ovulate *in vivo* under the same hormone priming regime as those perfused. These mice were sacrificed 16 - 20 hr following ip administration of 5 IU hCG and the number of oocytes in the ampulla region of the oviduct were counted. For statistical analysis, data were ranked prior to assessment by univariate ANOVA and Tukey HSD post hoc Test.

The ovulation rate of GM+/+ ovaries perfused with LH for 22 hr was compared with rates in GM-/- ovaries. Of the ovaries perfused with LH, 70% GM+/+ ovulated 1 or more oocytes compared to 93% of GM-/- ovaries (Table 4.2). There appeared to be no difference in the number of oocytes ovulated per GM-/- ovary compared to the GM+/+ ovary when non-ovulators were excluded from the analysis ($P = 0.302$, univariate ANOVA). Ovulation of GM+/+ ovaries was however reduced 6 fold in the *in vitro* perfusion system compared with an *in vivo* ovulation rate of 17.2 ± 2.7 per ovary using the same hormone priming regime, indicating that although the priming regime was successful a reduction in ovulation rate is associated with the *in vitro* model. The addition of rmGM-CSF to GM-/- ovaries perfused with LH did not alter the ovulation rate of GM-/- ovaries. Of the GM-/- ovaries perfused with LH plus rmGM-CSF, 88% ovulated 1 or more oocytes (Table 4.2). Although tenuous, these results suggest *in vitro* ovulation may be less inhibited in the case of GM-CSF deficiency.

Table 4.2 *In vitro* perfused ovulation rate of ovaries collected from adult GM+/+ and GM-/- mice. Mice were primed with 20 μ g LHRH and 5 IU eCG. The right ovary was surgically isolated from the mouse and perfused alone or with LH (0.1 μ g/ml) and rmGM-CSF (4 ng/ml). Oocytes were counted following 22 hr perfusion. Ovulation rate was assessed *in vivo* in mice given the same hormone priming regime. Nitrate/nitrite accumulation in the perfusion media of GM+/+ and GM-/- ovaries perfused for 22 hr (n = 8, 10, 14, 8 respectively). Data are mean $\pm$ SE.

Genotype Treatment	<i>in vivo</i>	<i>in vitro</i>			
	GM+/+	GM+/+ Control	GM+/+ LH	GM-/- LH	GM-/- LH+GM- CSF
# Perfused	-		15	22	9
# Meet criteria	-	10	10 (67%)	14 (63%)	8 (89%)
# Ovulated/total(%)	11/11 (100%)	5/9 (56%)	7/10 (70%)	13/14 (93%)	7/8 (88%)
# Oocytes/ovary	17.2 ± 2.7	1.6 ± 0.2	2.9 ± 0.7	6 ± 0.9	4.7 ± 1.5
Ovulation range	5 - 34	1 - 2	1 - 6	1 - 11	1 - 10
Nitrate/nitrite (μ mol/l)	-	1.5 ± 0.1	1.4 ± 0.1	$2.2 \pm 0.2^*$	$2.7 \pm 0.3^*$

* significantly different from GM+/+ (control) and GM+/+ (LH) as assessed by univariate ANOVA, $P < 0.05$.

To investigate the impact of GM-CSF deficiency on NO production by the ovary at ovulation, nitrate/nitrite concentrations were measured in the perfusion media at 22 hr after the addition of the treatments (Chapter 2, 2.8.4). The accumulation of NO was analysed by measuring nitrate/nitrite in the perfusion media at 22 hr. Nitrate/nitrite secretion by GM-/- ovaries perfused with LH or LH + rmGM-CSF was significantly greater than control and LH perfused GM+/+ ovaries (Table 4.2). The addition of LH did not alter nitrate/nitrite secretion by the GM+/+ ovary compared to the GM+/+ control ovary perfused with media alone ($P = 0.999$, univariate ANOVA) and the addition of rmGM-CSF to the GM-/- ovary did not increase nitrate/nitrite secretion by the GM-/- ovaries ($P = 0.568$, univariate ANOVA). These data suggest that the NO, an inflammatory mediator of ovulation, may be more prolific in the absence of GM-CSF.

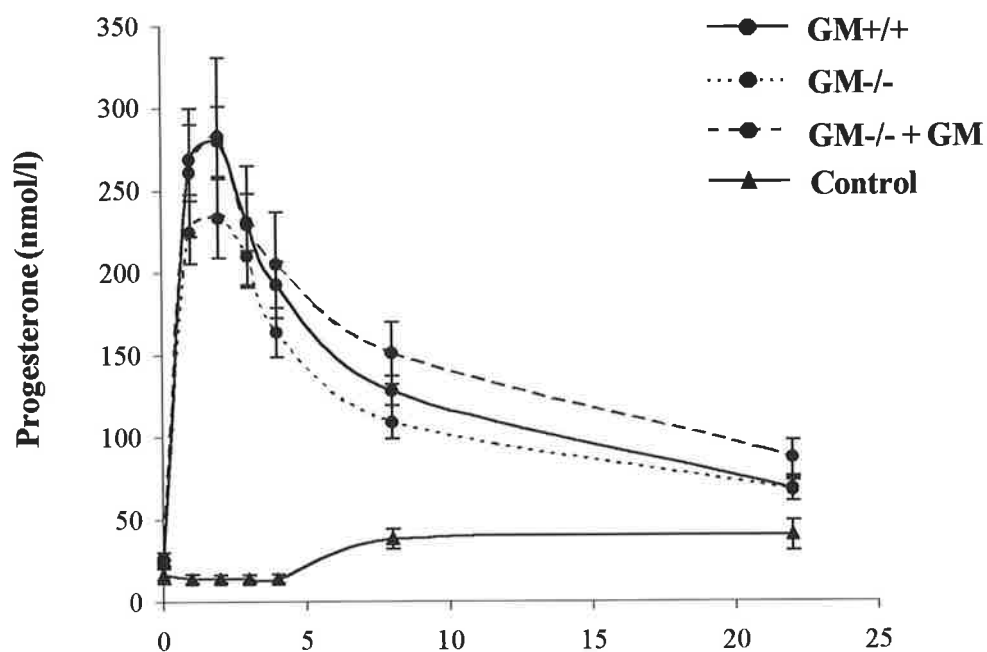
4.4 Steroid synthesis

4.4.1 *In vivo* steroid production following ovulation

To investigate the impact of GM-CSF deficiency on steroid production by the ovary, plasma was collected from adult mice following natural ovulation (Chapter 2, 2.2.9) and assayed for progesterone (Chapter 2, 2.8.1) and oestradiol (Chapter 2, 2.8.2). Comparable plasma progesterone levels following natural ovulation were recorded from mice regardless of genotype (GM+/+, 9.1 ± 1.3 and GM-/-, 10.2 ± 1.2 nmol/l) (two-tailed $P = 0.5379$, t-Test). Only 50% ($n = 6$) of GM+/+ and 42% ($n = 5$) of GM-/- mice had detectable levels of plasma oestradiol following natural ovulation, but where measurable (GM+/+, 50 ± 2.6 and GM-/-, 46 ± 2.4 pmol/l), there appeared to be no difference (two-tailed $P = 0.2967$, t-Test).

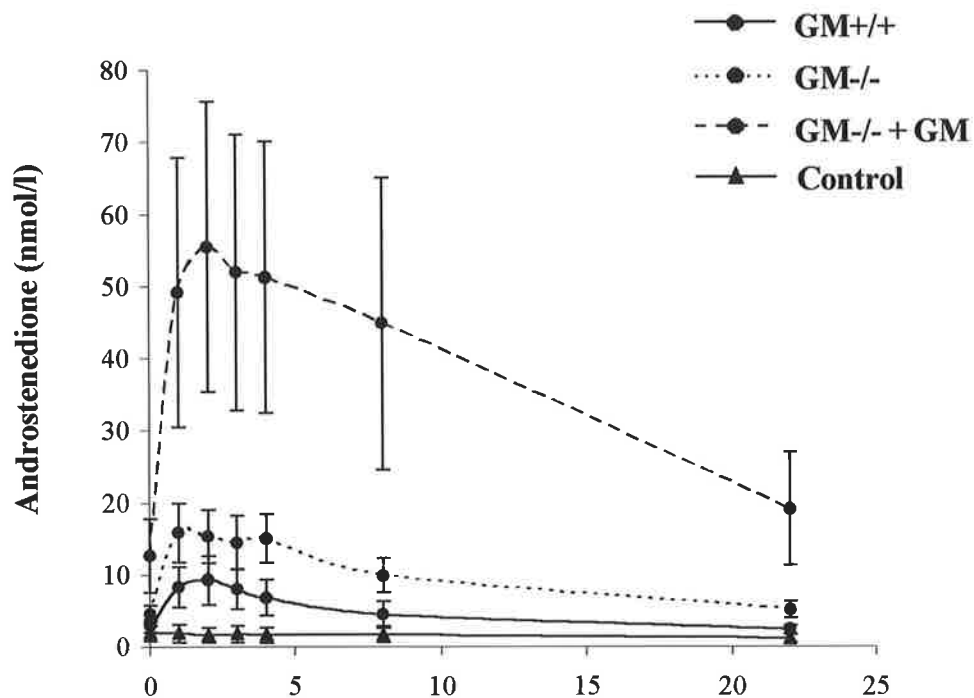
4.4.2 *In vitro* perfusion steroid production during the periovulatory period

Cumulative progesterone, androstenedione and oestradiol concentrations were determined in GM+/+ and GM-/- ovary perfusion media during a 22 hr perfusion period. Following 1 hr of perfusion, samples of the recirculating perfusion medium were collected at 0, 1, 2, 3, 4, 8 and 22 hr, stored at -20°C and subsequently assayed for progesterone, androstenedione (Chapter 2, 2.8.3) and oestradiol. For statistical analysis, data were ranked prior to assessment by repeated measures general linear model and Tukey HSD post hoc Test.



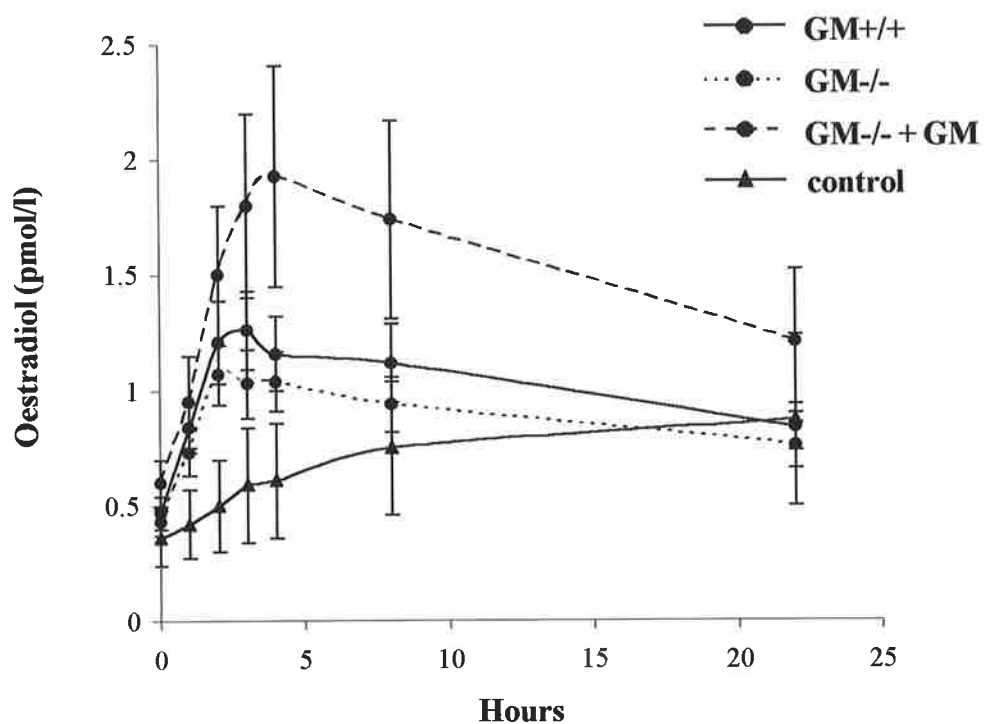
	0 hr			1 hr			2 hr			3 hr			4 hr			8 hr			22 hr		
	+/+	-/-	-/- GM	+/+	-/-	-/- GM	+/+	-/-	-/- GM	+/+	-/-	-/- GM	+/+	-/-	-/- GM	+/+	-/-	-/- GM	+/+	-/-	-/- GM
Control				*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
+/+																					
-/-																					
-/- GM																					

Figure 4.2 Progesterone concentrations in the perfusion media of GM+/+ and GM-/- ovaries perfused for 22 hr. The right ovary was surgically isolated from the mouse and perfused alone (control) or with LH (0.1 µg/ml) and rmGM-CSF (4 ng/ml). Data are presented as mean ± SE, control n = 9 GM+/+, LH perfused n = 10 GM+/+, 14 GM-/-, 8 GM-/- + rmGM-CSF. Significance assessed by general linear model, $P < 0.05$ indicated in the table by *.



	0 hr			1 hr			2 hr			3 hr			4 hr			8 hr			22 hr		
	+/+	-/-	-/- GM	+/+	-/-	-/- GM	+/+	-/-	-/- GM	+/+	-/-	-/- GM	+/+	-/-	-/- GM	+/+	-/-	-/- GM	+/+	-/-	-/- GM
Control			*	*	*	*	*	*	*	*	*	*	*	*	*		*	*		*	*
+/+			*			*			*			*			*			*			*
-/-																					
-/- GM	*			*			*			*			*			*			*		

Figure 4.3 Androstenedione concentrations in the perfusion media of GM+/+ and GM-/- ovaries perfused for 22 hr. The right ovary was surgically isolated from the mouse and perfused alone (control) or with LH (0.1 µg/ml) and rmGM-CSF (4 ng/ml). Data are presented as mean ± SE, control n = 8 GM+/+, LH perfused n = 10 GM+/+, 14 GM-/-, 7 GM-/- + rmGM-CSF. Significance assessed by general linear model, $P < 0.05$ indicated in the table by *.



	0 hr			1 hr			2 hr			3 hr			4 hr			8 hr			22 hr		
	+/+	-/-	-/- GM	+/+	-/-	-/- GM	+/+	-/-	-/- GM	+/+	-/-	-/- GM	+/+	-/-	-/- GM	+/+	-/-	-/- GM	+/+	-/-	-/- GM
Control							*	*		*			*								
+/+																					
-/-																					
-/- GM																					

Figure 4.4 Oestradiol concentrations in the perfusion media of GM+/+ and GM-/- ovaries perfused for 22 hr. The right ovary was surgically isolated from the mouse and perfused alone (control) or with LH (0.1 µg/ml) and rmGM-CSF (4 ng/ml). Data are presented as mean ± SE, control n = 7 GM+/+, LH perfused n = 10 GM+/+, 13 GM-/-, 6 GM-/- + rmGM-CSF. Significance assessed by general linear model, $P < 0.05$ indicated in the table by *.

The addition of LH resulted in an approximate 20 fold increase in progesterone secretion from the GM+/+ ovaries, with maximum concentrations in the perfusion media 2 - 3 hr after LH addition, and levels subsequently declining due to rapid adsorption of steroid into the glass walls of the perfusion system (Brännström *et al*, 1987) (Figure 4.2). This profile of secretion was observed for all perfused ovaries except the control group perfused with media alone. Progesterone concentrations in perfusion media of GM+/+, GM-/- and GM-/- + rmGM-CSF perfused with LH were comparable but significantly greater than those of the control group at all times except at 0 hr.

The addition of LH resulted in an approximate 4.5 fold increase in androstenedione secretion from the GM+/+ ovary, with maximum concentrations in the perfusion media 2 hr after LH addition (Figure 4.3). The addition of LH to ovaries of both genotypes stimulated androstenedione secretion above that of the control at the majority of sampling times. The addition of rmGM-CSF to the GM-/- perfused ovary resulted in production of mean androstenedione 3.7 fold greater than that of the GM-/- ovaries perfused with LH alone, although this increase was not statistically significant ($P = 0.130$, general linear model). The addition of rmGM-CSF stimulated LH-induced androstenedione secretion by GM-/- ovaries above that secreted by GM+/+ ovaries at ovulation *in vitro*. Androstenedione concentrations in perfusion media of GM+/+ and GM-/- perfused with LH were comparable.

The addition of LH resulted in an approximate 2.3 fold increase in oestradiol secretion from the GM+/+ ovary, with maximum concentrations in the perfusion media 3 hr after LH addition (Figure 4.4). Peak secretion of oestradiol by GM-/- ovaries perfused with LH + rmGM-CSF occurred 1 hr later at 4 hr. The addition of rmGM-CSF to the GM-/- perfused ovary resulted in production of oestradiol approximately 2 fold greater than that of the GM-/- ovaries, although not statistically significant ($P = 0.387$, general linear model).

GM-CSF deficiency does not appear to inhibit androstenedione production but exogenous GM-CSF may promote production of this steroid. Progesterone and oestradiol secretion at ovulation appears unaltered by GM-CSF deficiency.

4.4.3 *In vivo* steroid production on day 4 of pregnancy

To investigate the impact of GM-CSF deficiency on steroid production by the ovary, plasma was collected from adult mice on day 4 of pregnancy. However, on day 4 of pregnancy, plasma progesterone was significantly reduced in the GM-/- (116.5 ± 6 nmol/l)

compared to the GM+/+ mouse (141.6 ± 10.3 nmol/l) (two-tailed $P = 0.05$, t-Test). Oestradiol was not detected in the plasma of mice on day 4 of pregnancy.

4.5 Ovarian weight on day 4 of pregnancy

To investigate the impact of GM-CSF deficiency on cell proliferation within the corpus luteum, ovarian weight and the number of corpora lutea present in the ovary were assessed on day 4 of pregnancy. On day 4 of pregnancy, GM+/+ ($n = 11$) and GM-/- ($n = 11$) mice mated with studs of the same strain were euthanased and the ovaries were dissected free from the mesentery and fixed in 10% formalin in PBS. The wet weight of ovaries was determined and then the ovaries were embedded in paraffin (Chapter 2, 2.5.1.2). Serial sections were cut at 50 μ m intervals and counterstained with haematoxylin and eosin (Chapter 2, 2.5.5). The total number of corpora lutea were counted in both ovaries.

On day 4 of pregnancy, ovarian weight was significantly reduced in the GM-/- mouse compared to the GM+/+ mouse. However, the number of corpora lutea in the GM-/- ovary counted on day 4 of pregnancy was similar to those in the GM+/+ mouse. (Table 4.3).

Table 4.3 Ovarian wet weight and number of corpora lutea of GM+/+ and GM-/- day 4 pregnant ovaries. Data are mean $\pm$ SE, $n = 11$ per group.

	GM+/+	GM-/-
Ovarian weight (mg)	14.9 ± 0.9	$11.9 \pm 0.7^*$
# Corpora lutea/mouse	20.4 ± 3.1	21.5 ± 4.3

* significantly different from GM+/+ as assessed by t-Test, two-tailed $P = 0.0169$

Based on these results, it would appear that GM-CSF may promote cell proliferation prior to or during corpus luteum development, or it may prevent apoptosis of cells that comprise the corpus luteum.

4.6 Duration of pseudopregnancy

In order to assess the origin of the extended oestrous cycle in the GM-/- mouse, this experiment examined corpus luteum function as a measure of the duration of pseudopregnancy and the length of PGF_{2α}-induced luteolysis. One GM+/+ (n = 9) and 1 GM-/- (n = 10) adult female mouse were housed per cage with a vasectomised adult male aged (10.2 ± 0.1 weeks) until the completion of three consecutive pseudopregnancies (Chapter 2, 2.2.7). The presence of a vaginal plug was regarded as day 1 of pseudopregnancy. The duration of pseudopregnancy was calculated as the number of days lapsed until a second vaginal plug was detected. During the second pseudopregnancy, luteolysis was induced by the ip administration of 3 µg of PGF_{2α} between 09:00 and 12:00 hr on days 4 and 5 following the citing of the second vaginal plug (Chapter 2, 2.2.8). The days lapsed until detection of the third, and finally the fourth vaginal plug were determined.

The duration of pseudopregnancy in GM+/+ and GM-/- mice was comparable (Figure 4.5). PGF_{2α} administration induced luteal regression and premature termination of pseudopregnancy ($P < 0.05$, Kruskal Wallis ANOVA) as seen by the onset of oestrous generally within 2 days of administration in the GM-/- and GM+/+ mouse. The duration of the pseudopregnancy post-PGF_{2α} treatment was comparable to that prior to PGF_{2α} treatment, illustrating no delayed effect of PGF_{2α} administration. The number of days prior to the citing of the first vaginal plug in the GM+/+ mouse on day 2.3 ± 0.4 and the GM-/- mouse on day 1.6 ± 0.2 was comparable (two-tailed $P = 0.2345$, Mann-Whitney Test), suggesting normal induction of oestrous and follicle recruitment following exposure to a male.

4.7 Ovary transplant: an ovarian knockout model

Ovarian transplantation is a surgical technique enabling essentially the production of an ovary-specific knockout. The aim of establishing the technique of ovarian transplantation was to assess the effect of local gene deficiency in a replete peripheral environment.

In order to determine the impact of surgery and the effect of transplanting ovaries on the rhythmicity of oestrous, in a preliminary experiment, GM-/- ovaries were transplanted into GM+/+ mice (Chapter 2, 2.2.10). Ovaries from immature GM-/- mice (3 - 4 weeks of age)

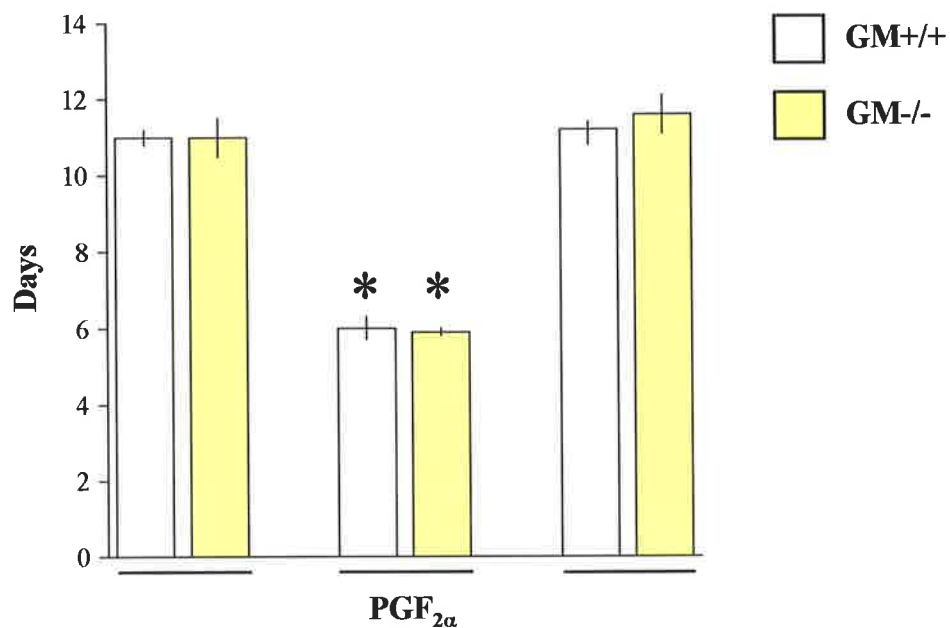


Figure 4.5 The duration of pseudopregnancy in adult GM+/+ and GM-/- mice. Mice were mated to vasectomised studs and the presence of a vaginal plug was deemed day 1 of pseudopregnancy. Pseudopregnancy length was determined, then luteal regression was induced by PGF_{2α}-treatment (3 µg on day 4 and day 5) and the duration of the subsequent pseudopregnancy was measured. * significantly different from pre- and post-treatment pseudopregnancy as assessed by Kruskal-Wallis Test and Dunn's Multiple Comparisons Test, $P < 0.05$. Data presented as mean ± SE, n = 9 GM+/+, 10 GM-/- mice.

were transplanted into the bursa of early adult GM+/+ mice (8 weeks of age). A sham operation was performed on early adult GM+/+ mice, which included the removal of all endogenous ovarian tissue and the transplantation of ovaries from immature GM+/+ mice (3 - 4 weeks of age). Following a rest period of 4 weeks, the mice were tracked by daily vaginal smear to determine the success of the surgical transplant.

Vaginal smears taken from 88% of sham-operated GM+/+ mice and 61% of GM+/+ mice transplanted with GM-/- ovary tissue reflected changes in the structure of the vaginal epithelium and followed a regular and predictable course during the cycle (Table 4.4).

Table 4.4 Ovarian transplantation trial. Ovaries from immature GM+/+ (sham) or GM-/- mice were transplanted into the bursa of early adult GM+/+ mice. Mice were then tracked by daily vaginal smear.

Genotype of ovary tissue	GM+/+	GM-/-
# Operated	17	24
# Survived (%)	16/17 (94%)	18/24 (75%)
# Cycle (%)	14/16 (88%)	11/18 (61%)

At metoestrous-1, the reproductive tract of GM+/+ mice transplanted with ovarian tissue was dissected. On examination of the bursa, ovarian tissue was found in most cases and as expected at metoestrous-1, the uterine horns of these mice were distended. In addition, oocytes were occasionally collected in the ampulla of the oviduct. In some cases, ovarian tissue was not identified in either bursa or was incorporated onto the bursa, which was noted as a thickening of the tissue. Although smearing of the vaginal epithelial cell gives an indication of steroid activity, it does not confirm complete success of ovary grafting and the vascularisation of the transplant.

In conclusion, the surgical technique for transplanting ovaries into the bursa of a recipient was mastered and considered to be a practical method for future investigations. However, further analysis is required to assess the histology of the transplant and genotype the transplant tissue by PCR to confirm the success of the procedure. Unfortunately, due to time restrictions, no further experiments were conducted. This technique would appear to provide an avenue to assess ovarian parameters, including oestrous cycle length, ovulation

rate, ovarian leukocyte populations, and also pregnancy outcome of gene deficient ovaries in a 'normal' endocrine environment and a means to decipher the role of ovarian and peripheral effects of cytokines.

4.8 Discussion and conclusions

The primary purpose for undertaking this study was to determine whether the deficiency of GM-CSF would perturb the functional parameters of the ovary.

Our results illustrated an extended oestrous cycle, decreased steroidogenesis and decreased ovarian weight in the GM-/- mouse, despite GM-CSF not appearing to be required for ovulation. Disruption of the rhythmicity of oestrous may result from any one or a combination of levels including hypothalamus, pituitary and/or ovary. At the local level, inadequate or delayed tissue remodelling may be the source of disruption of the oestrous cycle, which was seen to be extended by 1.5 days. To what specific stage of oestrous this relates is unclear. However, evidence suggests that GM-CSF expression is important during the formation of the corpus luteum and may regulate its functional capacity perhaps in terms of steroidogenesis.

GM-CSF was initially implicated in the ovulatory process, as peak secretion from the rat ovary occurred during the periovulatory period (Brännström *et al*, 1994b). The lack effect of GM-CSF on the ovulatory process was unexpected and contrasts with the beneficial effect of the pro-ovulatory cytokines, IL-1 β and TNF α (Brännström *et al*, 1993b, 1995b). *In vivo* ovulation was unperturbed in both the immature primed and naturally cycling models, suggesting GM-CSF is not essential for the process of ovulation, perhaps as a consequence of the redundancy of the cytokine network (Chapter 1, 1.3). This data is consistent with comparable numbers of corpora lutea counted on day 4 of pregnancy and implantation sites in GM+/+ and GM-/- mice on day 8 of pregnancy (Robertson *et al*, in press). In addition, no effect of exogenous rmGM-CSF on *in vivo* ovulation rate in the rat has been observed (Nishimura *et al*, 1995). Likewise, the ovulation rates of GM+/+, GM-/- and GM-/- + rmGM-CSF ovaries perfused *in vitro* with LH were comparable. While the precise effect of GM-CSF on the ovulatory process remains unclear, *in vivo* and *in vitro* ovulation data suggests ovulation is unaltered by GM-CSF deficiency.

In a previous study, LH induced ovulation in the wild type mouse ovary to a similar degree as seen in this study, whereas ovaries perfused with media alone did not ovulate (Brännström and Flaherty, 1995). That ovulation was achieved in the absence of LH in the current study suggests that ovaries in this experiment may have been exposed to endogenous LH prior to their removal at surgery. The reduction in ovulation rate *in vitro* compared to *in vivo* observed in this study is not unique but associated with the perfusion model and is observed in both the rabbit and rat (Janson *et al*, 1982, Brännström *et al*, 1987).

One role IL-1 β and TNF α have been demonstrated to play in the regulation of ovarian function is in the modulation of steroidogenesis (Nakamura *et al*, 1990, Roby and Terranova, 1990). There was a tendency for reduced progesterone production by the GM-/- ovary in the *in vitro* perfusion system when results were expressed per ovulated oocyte. In addition, on day 4 of pregnancy, progesterone was significantly reduced in the GM-/- mouse. This data suggests that while progesterone production during an oestrous cycle may occur in the absence of GM-CSF, the increased demand in the case of pregnancy cannot be met. The precise cell targeted by GM-CSF in the theca or corpus luteum is unclear, but GM-CSF may act directly on the luteal cells or through ovarian leukocytes may influence progesterone secretion. GM-CSF had no effect on LH-stimulated progesterone synthesis by luteinised granulosa cells in the rat (Tamura *et al*, 1997), suggesting effects may be mediated via leukocytes. In the case of androstenedione synthesis by the *in vitro* perfused ovary, this study demonstrated that rmGM-CSF stimulates synthesis above that of the GM+/+ ovary throughout the perfusion period. However, the mechanism for GM-CSF-stimulated androstenedione synthesis by the GM-/- ovary is unknown. Perhaps the pro-androstenedione action of GM-CSF may be enzyme P450_{17 α} -specific, as this increase was not concomitant with progesterone synthesis, and oestradiol synthesis was moderately increased perhaps as a consequence of increased precursor.

GM-CSF does not appear to impact on the time frame of corpus luteum tissue remodelling during pseudopregnancy, but the reduction in ovarian weight on day 4 of pregnancy suggests GM-CSF may influence proliferation of cells which comprise the corpus luteum. GM-CSF may promote granulosa and/or theca proliferation prior to ovulation, and may also promote endothelial cell migration and proliferation (Bussolino *et al*, 1989, 1991) during the development of the corpus luteum. Vascularisation is an important process during the development of the corpus luteum; expression of the receptor subunits correlates with this proposed role (Zhao *et al*, 1995, Jasper *et al*, 1996), and incomplete vascular penetration of

the corpus luteum may explain reduced circulating steroid levels during pregnancy. In addition, GM-CSF may protect myeloid cells against apoptosis in the corpus luteum as in other cell systems (Brach *et al*, 1992, Kinoshita *et al*, 1995), thereby ensuring their survival and maintenance in the corpus luteum.

Disruption of the oestrous cycle and ovarian biochemical perturbations may be a consequence of inadequately primed macrophages at the local level. Interestingly, LPS-stimulated peritoneal macrophages from GM-/- mice secrete less IL-1 α and NO (Basu *et al*, 1997), suggesting inadequate responses to immune stimulation in the absence of GM-CSF. In contrast, nitrate/nitrite secretion was elevated in ovaries from GM-CSF deficient mice at ovulation in the *in vitro* perfused model, and was unaltered by the addition of rmGM-CSF to the perfusion. As indicated previously, NO production is not increased by LH alone in the GM+/+ ovary or in the rat (Bonello *et al*, 1996).

In summary, our results indicate that GM-CSF is not essential for ovulation but that it may be involved in the formation of the corpus luteum from follicle remnants via the promotion of cell proliferation and steroidogenesis.

Chapter 5

Leukocyte populations in the GM-CSF deficient ovary

5.1 Introduction

Immunohistochemical studies have revealed precise spatial and temporal distribution patterns for leukocytes in the ovaries of various species including the human, domestic species and rodents, which fluctuate across the oestrous cycle. During the processes of folliculogenesis, follicle atresia, ovulation and corpora lutea development and demise, leukocytes of the macrophage and granulocyte lineage display close association with follicles and corpora lutea (Chapter 1, 1.4). However, little is known about the molecular mechanisms which coordinate the precise spatial and temporal dynamics of leukocyte recruitment and activation in ovarian tissue.

GM-CSF may influence infiltration of myeloid leukocytes into tissues through its actions as a specific chemoattractant and through its ability to promote integrin expression in endothelial cells. In addition, GM-CSF may influence functional activities of these cells which span a spectrum of cellular and biochemical profiles including phagocytosis, cytotoxicity, antigen presentation, extracellular matrix remodelling, angiogenesis, nerve regeneration and fibroblast proliferation.

It is proposed that GM-CSF contributes to regulation of the tissue remodelling events and immunological changes within the ovary through the recruitment and activation of myeloid cells.

The studies described in the previous chapter suggest that GM-CSF deficiency leads to perturbation in steroidogenesis and tissue remodelling associated with the development of the corpus luteum. In order to assess the effect of GM-CSF deficiency on leukocyte infiltration and activation within the ovary, this study examined the spatial and temporal patterns of GM-CSF responsive leukocytes by immunohistochemical staining of lineage-specific leukocyte markers in the ovary during the periovulatory period and pregnancy.

5.2 General ovarian morphology

Ovaries were collected from GM+/+ and GM-/- mice (5 - 6 mice per group). Immature ovaries (IO) were collected from unprimed mice, preovulatory ovaries (PO) were collected from mice 48 hr following the administration of 5 IU eCG and ovulatory ovaries

(OO) were collected 9 hr following the administration of 5 IU eCG/hCG. Luteinised ovaries were collected from immature primed mice following ovulation on day 1 of pregnancy (Day 1), day 3 of pregnancy (Day 3) and 24 - 48 hr postpartum (PP). Ovaries collected were embedded in OCT compound, frozen in liquid nitrogen-cooled isopentane and stored at -20°C (Chapter 2, 2.5.1.1). Fresh frozen tissue sections (6 µm) were fixed in 96% ethanol (Chapter 2, 2.5.3), counterstained in haematoxylin, cleared and mounted (Chapter 2, 2.5.4).

Periovulatory ovaries collected from GM+/+ and GM-/- mice comprised follicles from primordial to antral stages. Ovaries collected from day 1, day 3 pregnant and postpartum mice also comprised follicles, but predominantly corpora lutea.

5.3 Leukocytes in the periovulatory ovary

To assess the effect of GM-CSF deficiency on the distribution and activation of leukocytes in the ovary, immunohistochemistry using an indirect immunoperoxidase technique was performed on sections cut from periovulatory GM+/+ and GM-/- ovaries. Fresh frozen tissue sections (6 µm) were fixed in 96% ethanol and leukocytes were detected using mAbs (Chapter 2, 2.5.2, 2.5.3). Slides were counterstained in haematoxylin, cleared and mounted. Staining was quantified using a video image analysis system and Video Pro software (Chapter 2, 2.5.6). All slides were stained in the same run to reduce assay variation and were analysed on the one system. Mean stroma and theca data were arcsin transformed and statistically analysed using ANOVA followed by the Tukey-Kramer Multiple Comparisons Test.

All CD45 positive cells including monocyte/macrophages, granulocytes and lymphocytes were localised predominantly within stromal tissue and surrounding blood vessels but were also found within the theca cell layer of follicles, adjacent to the basement membrane, in GM+/+ and GM-/- ovaries. Seldom were leukocytes found within the granulosa cell layer of follicles, and in such cases these follicles were suspected to be atretic (Gaytán *et al*, 1998) (Figure 5.3D). No significant differences were observed for CD45+ staining between GM+/+ and GM-/- mice in the stroma or theca during the periovulatory period (Figure 5.1). Evaluation of sections labelled with lineage-specific mAbs showed that the leukocytes present in the ovary were predominantly of macrophage, neutrophil and/or dendritic lineages. Macrophages were localised by binding of the macrophage-specific mAb

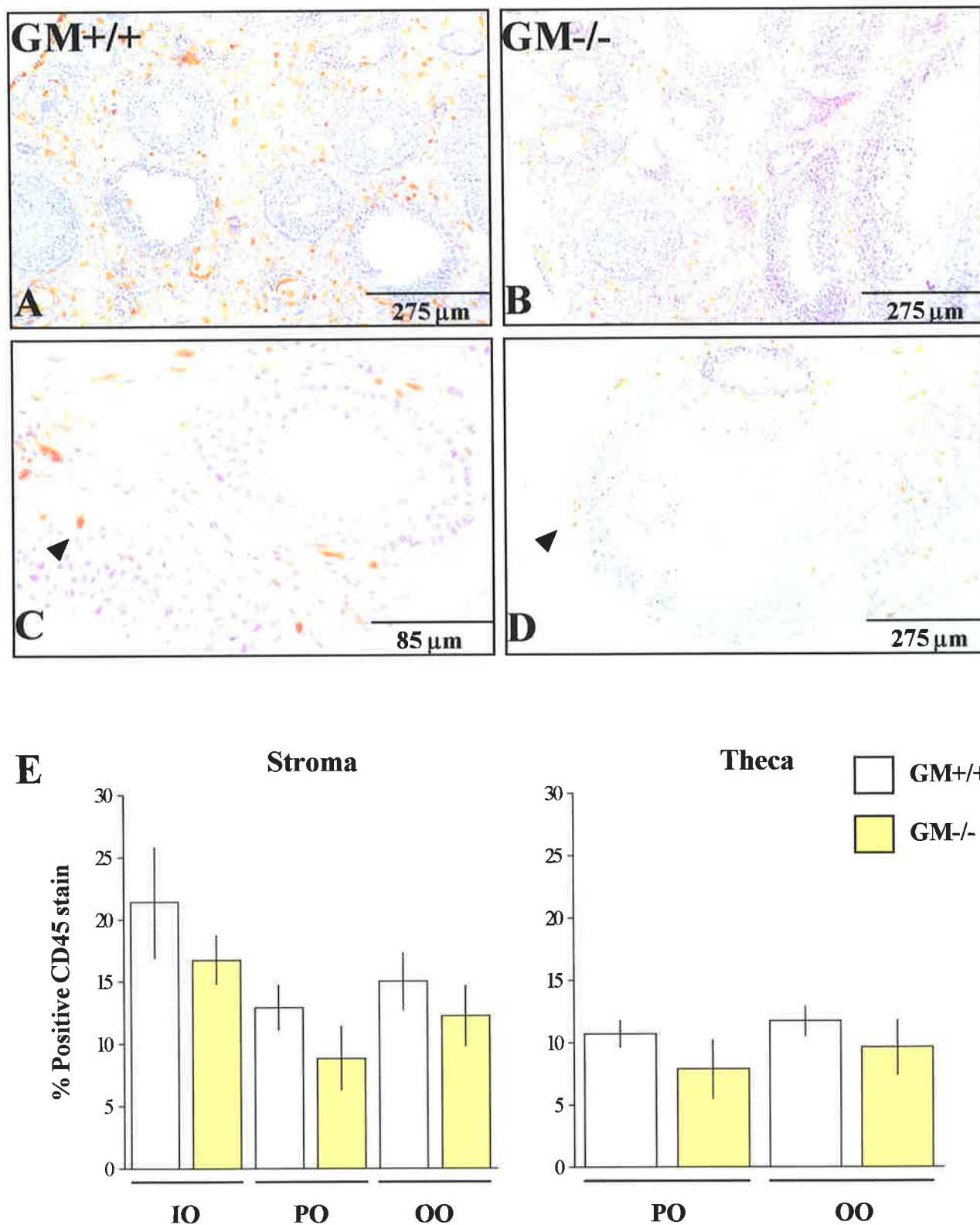


Figure 5.1 Immunohistochemical localisation of leukocytes in the periovulatory $GM^{+/+}$ and $GM^{-/-}$ ovary. Ovaries were collected from immature (IO), preovulatory (PO) and ovulatory (OO) primed mice. Leukocytes are stained with a mAb against the leukocyte marker CD45. CD45 positive staining in the stroma of A) $GM^{+/+}$ and B) $GM^{-/-}$ and theca of C) $GM^{+/+}$ and D) $GM^{-/-}$ ovulatory ovaries. E) Percent positive stain as assessed by video image analysis. Data are mean $\pm$ SE, n = 5 per group.

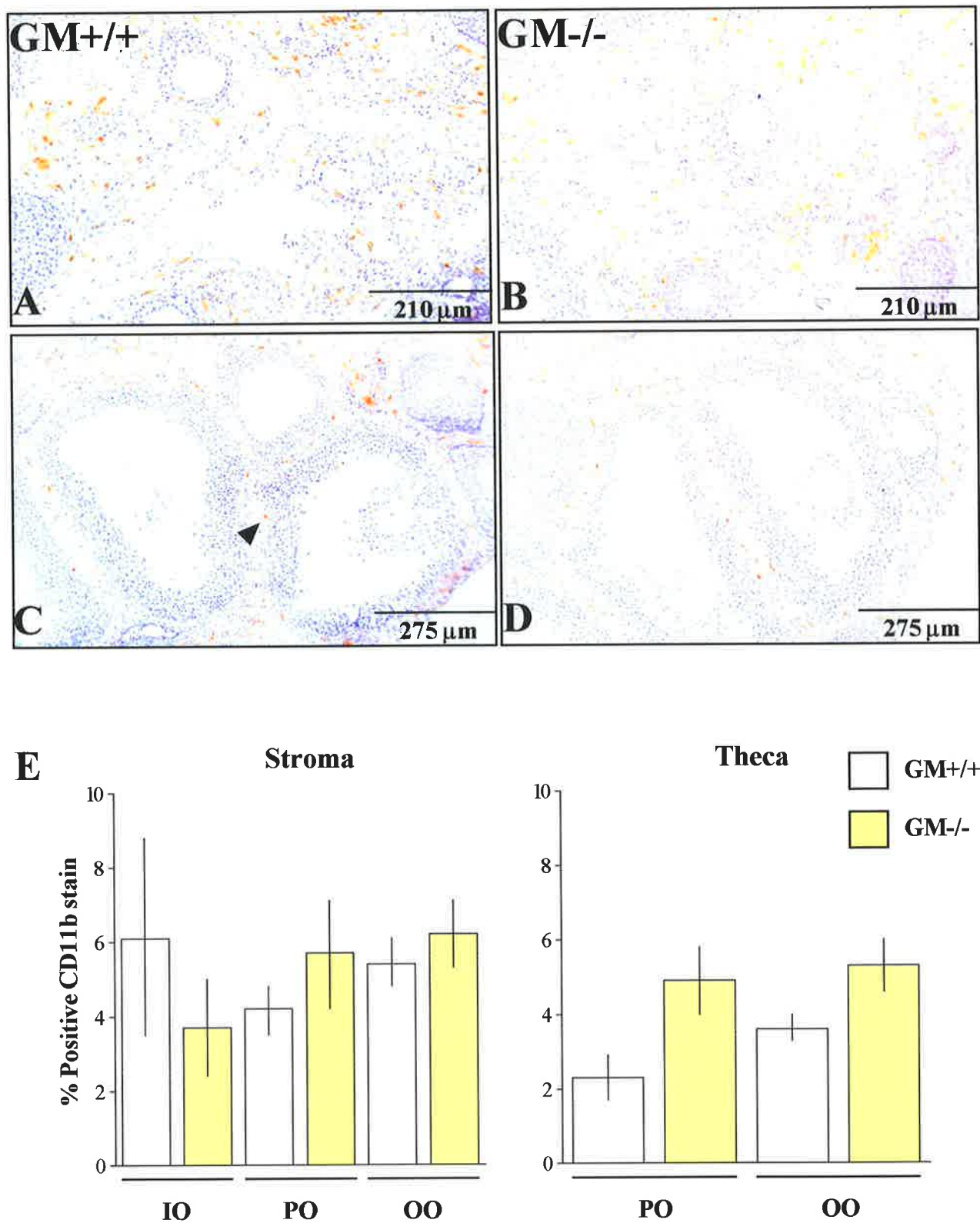


Figure 5.2 Immunohistochemical localisation of macrophages and neutrophils in the periovarian GM^{+/+} and GM^{-/-} ovary. Ovaries were collected from immature (IO), preovulatory (PO) and ovulatory (OO) primed mice. Macrophages and neutrophils are stained with a lineage-specific mAb against CD11b. CD11b positive staining in the stroma of A) GM^{+/+} and B) GM^{-/-} and theca of C) GM^{+/+} and D) GM^{-/-} ovulatory ovaries. E) Percent positive stain as assessed by video image analysis. Data are mean $\pm$ SE, n = 5 per group.

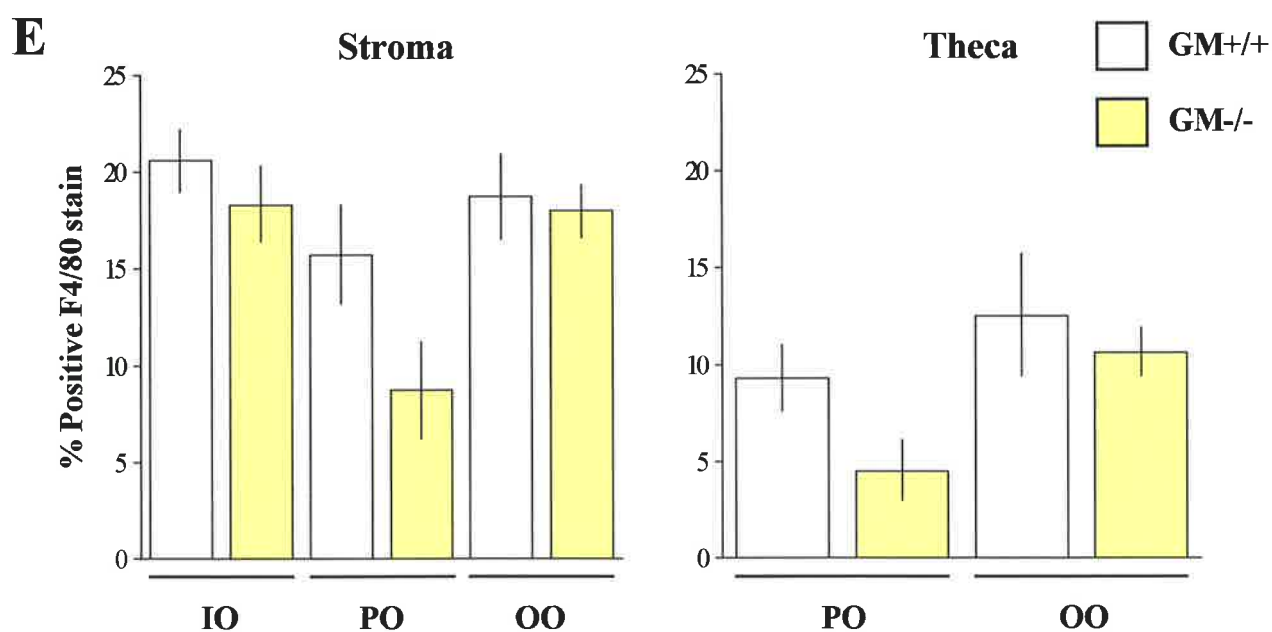
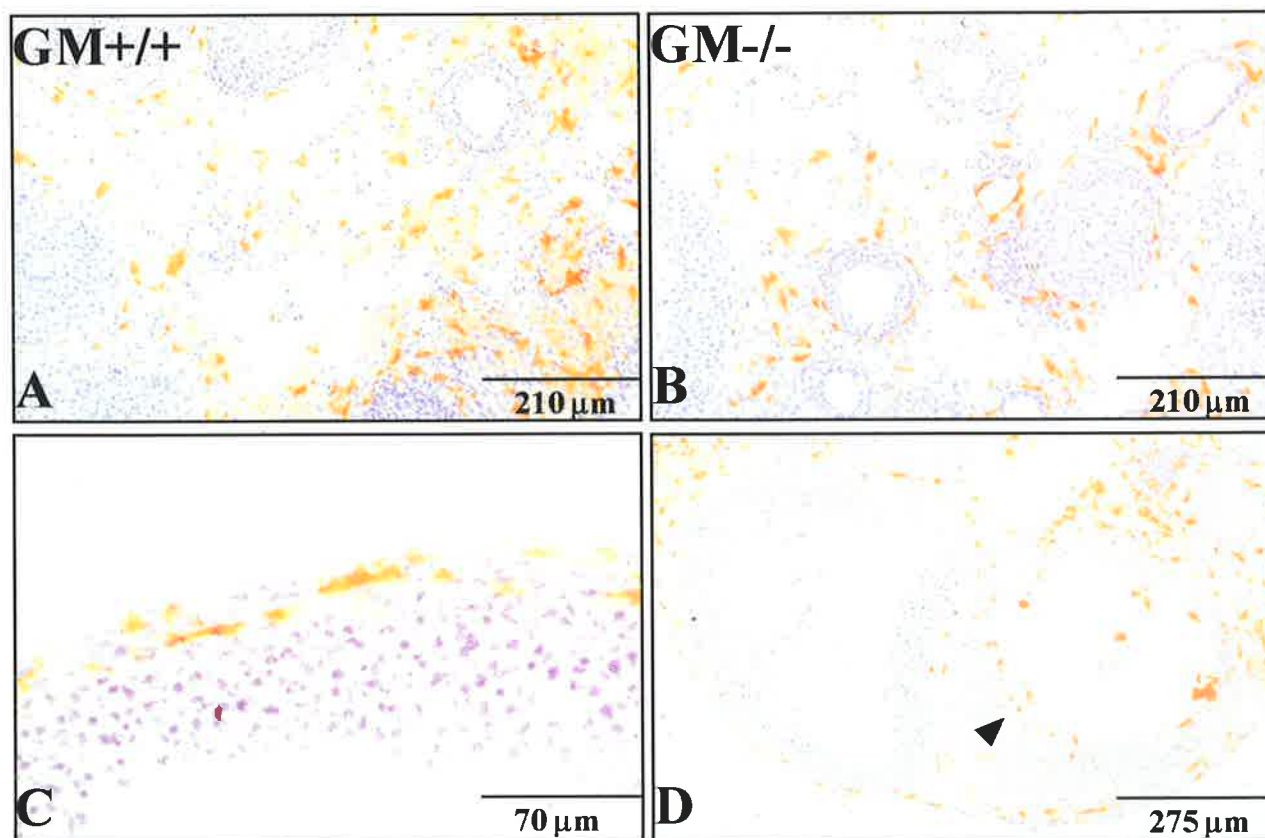


Figure 5.3 Immunohistochemical localisation of macrophages in the periovulatory GM+/+ and GM-/- ovary. Ovaries were collected from immature (IO), preovulatory (PO) and ovulatory (OO) primed mice. Macrophages are stained with a lineage-specific mAb against F4/80. F4/80 positive staining in the stroma of A) GM+/+ and B) GM-/- and C) follicle theca of GM+/+ ovulatory ovaries and D) granulosa layer of an atretic GM-/- follicle at ovulation. E) Percent positive stain as assessed by video image analysis. Data are mean $\pm$ SE, n = 5 per group.

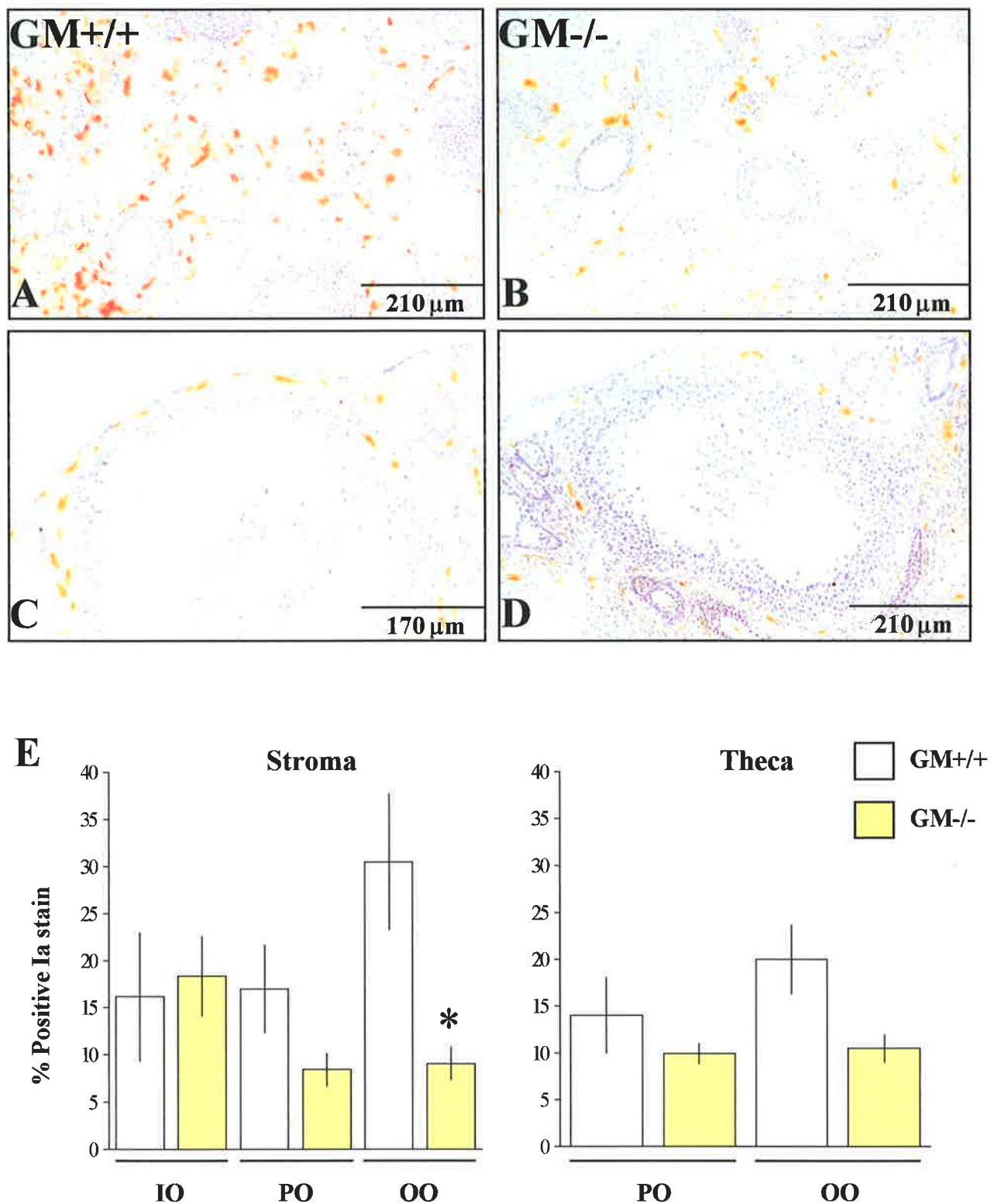


Figure 5.4 Immunohistochemical localisation of activated macrophages and dendritic cells in the periovarian GM^{+/+} and GM^{-/-} ovary. Ovaries were collected from immature (IO), preovulatory (PO) and ovulatory (OO) primed mice. Activated macrophages and dendritic cells are stained with a lineage-specific mAb against MHC class II (Ia). Ia positive staining in the stroma of A) GM^{+/+} and B) GM^{-/-} and follicle theca of C) GM^{+/+} and D) GM^{-/-} ovulatory ovaries. E) Percent positive stain as assessed by video image analysis. Data are mean $\pm$ SE, $n = 5$ per group. * significantly different from GM^{+/+} as assessed by Mann-Whitney Test, two tailed $P < 0.05$.

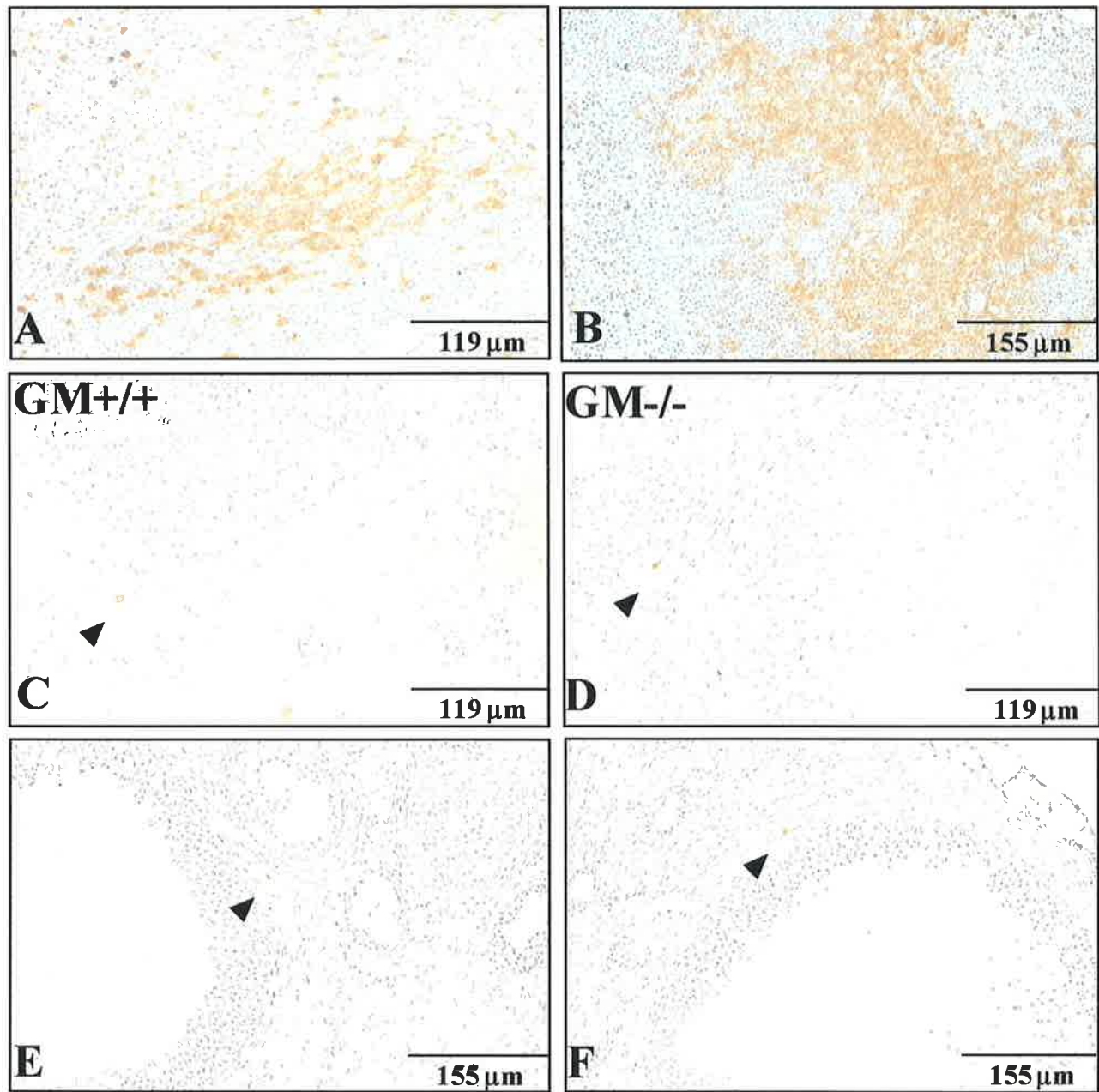


Figure 5.5 Immunohistochemical localisation of lymphocytes in the periovarian GM+/+ and GM-/- ovary. Ovaries were collected from immature primed ovulatory mice. T and B lymphocytes are stained with a lineage-specific mAb against CD5, and CD4 positive T lymphocytes are stained with a lineage-specific mAb against CD4. A) CD5 and B) CD4 positive lymphocytes in the spleen. CD5 positive stained cells in the stroma or theca of C) GM+/+ and D) GM-/- ovulatory ovaries. CD4 positive stained cells in the theca of E) GM+/+ and F) GM-/- ovulatory ovaries.

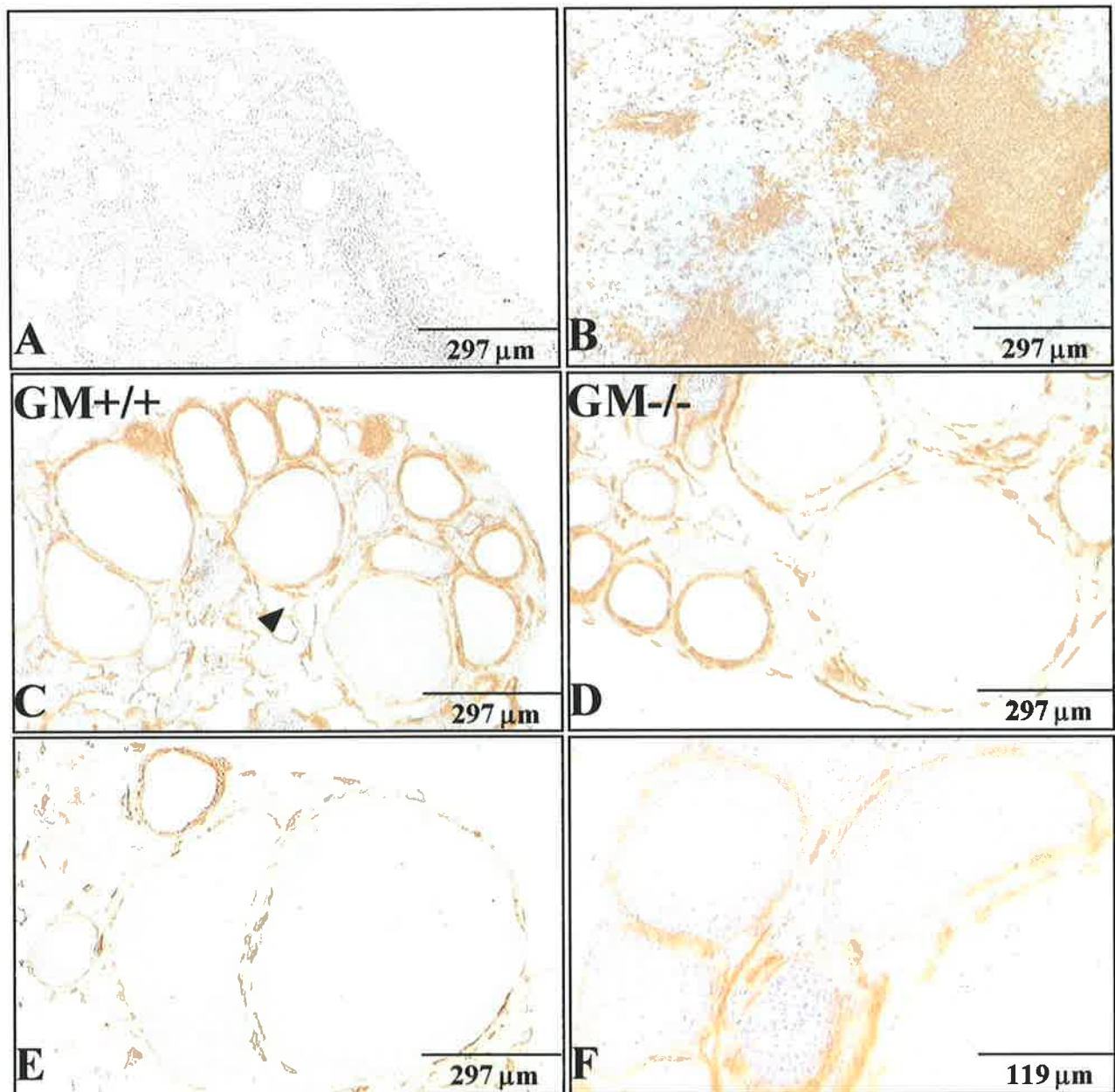


Figure 5.6 Immunohistochemical localisation of Thy 1.2 in the periovulatory GM^{+/+} and GM^{-/-} ovary. Ovaries were collected from immature primed ovulatory mice. A) Negative (ovary) and B) positive (spleen) immunohistochemistry controls. Thy 1.2 positive stained cells in the theca of C) E) GM^{+/+} and D) F) GM^{-/-} ovulatory ovaries.

F4/80, which reacted with comparatively large cells with membranous processes. Cells of similar macrophage-like morphology were also CD11b reactive, while the smaller CD11b reactive cells with granular nuclei were presumed to be neutrophils (Figures 5.2). The area of positive staining in the stroma and theca during the periovulatory period was relatively constant, and no significant differences were observed for CD11b+ or F4/80+ staining (Figures 5.3) between GM+/+ and GM-/- mice. A large population of Ia reactive, MHC class II antigen expressing cells of dendritic morphology were identified in both the stroma and theca, and potentially included both activated macrophages and dendritic cells (Figure 5.4). Percent positive Ia stain was significantly decreased in the stromal region of ovulating ovaries in GM-/- mice compared to the GM+/+, however no significant differences were observed in the theca between GM+/+ and GM-/- mice during the periovulatory period. Eosinophils were identified on a few of the negative control sections due to endogenous DAB reactive peroxidase activity. Generally only 1 cell on average was observed per 1 - 2 sections. CD5+ lymphocytes, including both T and B subsets along with CD4+ T cells were identified, but due to the very sparse distribution of lymphocytes, staining was not quantified using the video image analysis system (Figure 5.5). Anti-Thy 1.2 was found to react with a large number of cells within the stroma and theca of periovulatory ovaries (Figure 5.6). These cells were not round like T and NK cells but were elongated and had a fibroblast-like morphology. The degree of reactivity varied in relation to the developmental stage of follicles; the most strongly labelled cells surrounded preantral follicles and a progressive decrease in intensity of labelling was evident with more developed follicles. Similarly, staining was not quantified using the video image analysis system.

5.4 Leukocytes in the pregnant ovary

To assess the influence of GM-CSF deficiency on the distribution and activation of leukocytes in the ovary during pregnancy, immunohistochemistry was performed on sections cut from fresh frozen ovaries collected from day 1, day 3 and postpartum GM+/+ and GM-/- mice. Slides were counterstained in haematoxylin, cleared, mounted and quantified using video image analysis. Mean stroma and corpus luteum data were arcsin transformed and statistically analysed using ANOVA followed by the Tukey-Kramer Multiple Comparisons Test.

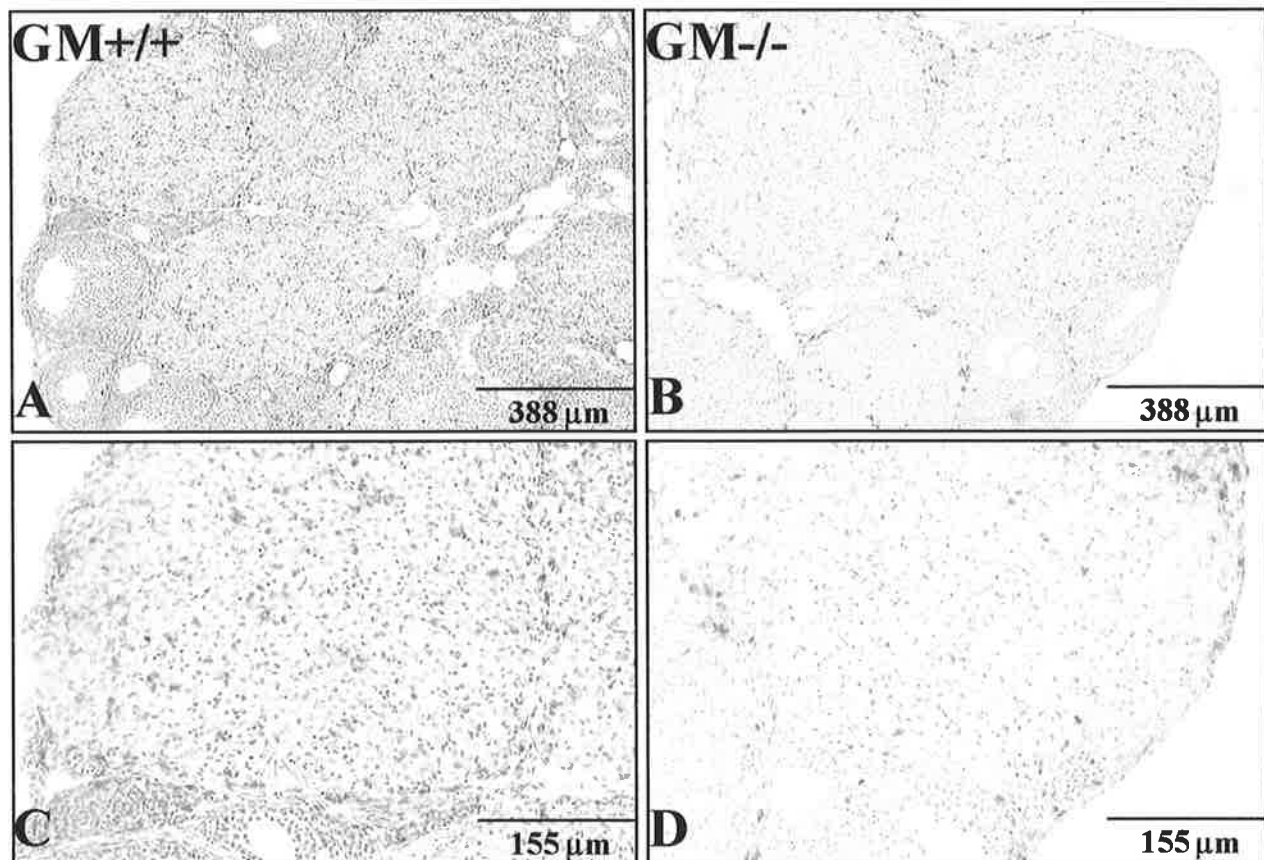


Figure 5.7 Immunohistochemical localisation of leukocytes in the pregnant GM+/+ and GM-/- ovary. Luteinised (Day 1), early luteal (Day 3) and postpartum (PP) ovaries were collected from immature primed pregnant mice and immunohistochemically stained using the lineage-specific mAb against CD45. CD45 positive staining in the stroma and regressing corpus luteum of A) C) GM+/+ and B) D) GM-/- postpartum ovaries.

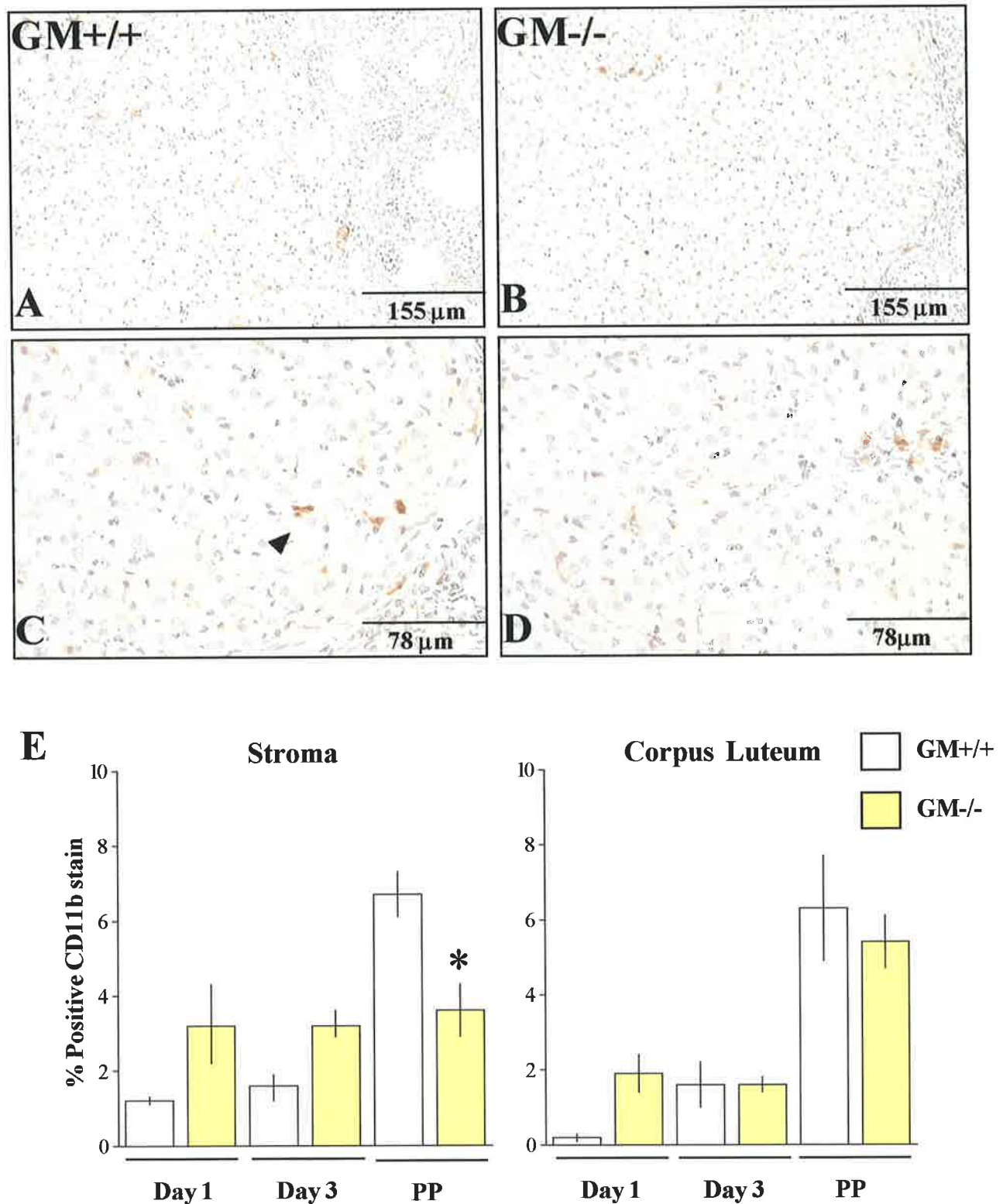


Figure 5.8 Immunohistochemical localisation of macrophages and neutrophils in the pregnant GM+/+ and GM-/- ovary. Luteinised (Day 1), early luteal (Day 3) and postpartum (PP) ovaries were collected from immature primed pregnant mice. Macrophages and neutrophils are stained with a lineage-specific mAb against CD11b. CD11b positive staining in the stroma and regressing corpus luteum of A) C) GM+/+ and B) D) GM-/- postpartum ovaries. E) Percent positive stain as assessed by video image analysis. Data are mean $\pm$ SE, $n = 4 - 6$ per group. * significantly different from GM+/+ as assessed by Mann-Whitney Test, two tailed $P < 0.05$.

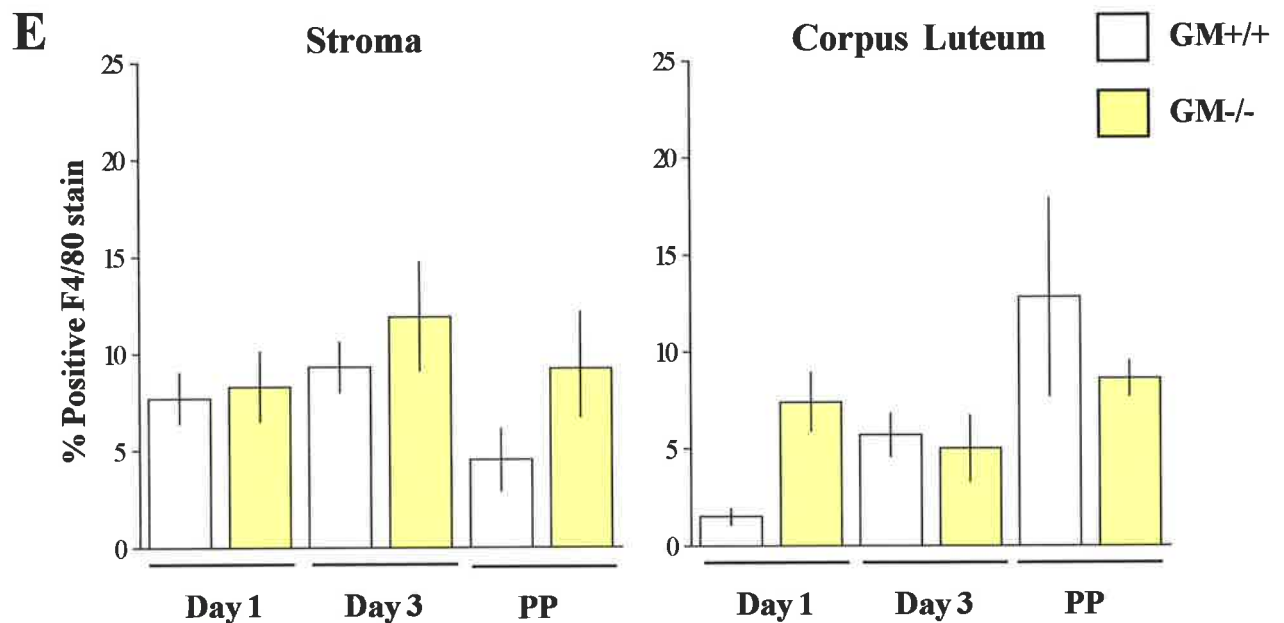
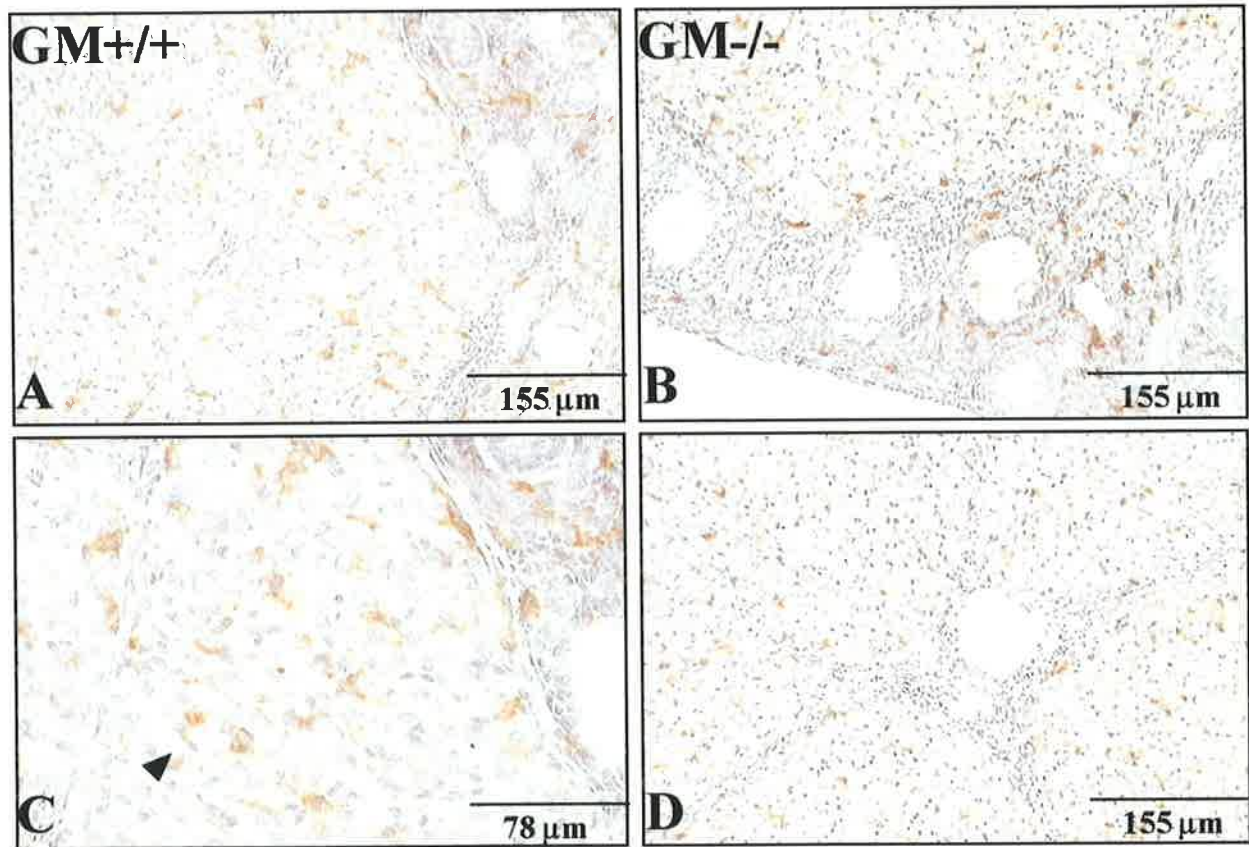


Figure 5.9 Immunohistochemical localisation of macrophages in the pregnant GM+/+ and GM-/- ovary. Luteinised (Day 1), early luteal (Day 3) and postpartum (PP) ovaries were collected from immature primed pregnant mice. Macrophages are stained with a lineage-specific mAb against F4/80. F4/80 positive staining in the stroma and regressing corpus luteum of A) C) GM+/+ and B) D) GM-/- postpartum ovaries. E) Percent positive stain as assessed by video image analysis. Data are mean $\pm$ SE, n = 4 - 6 per group.

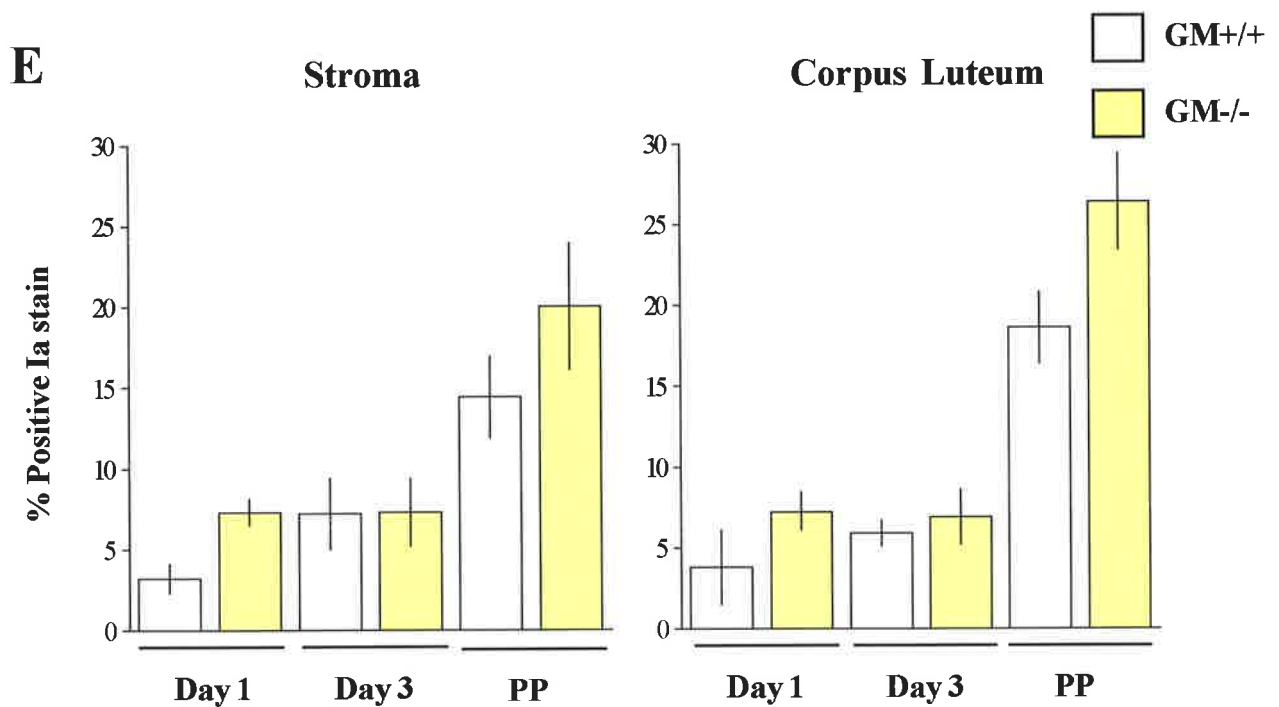
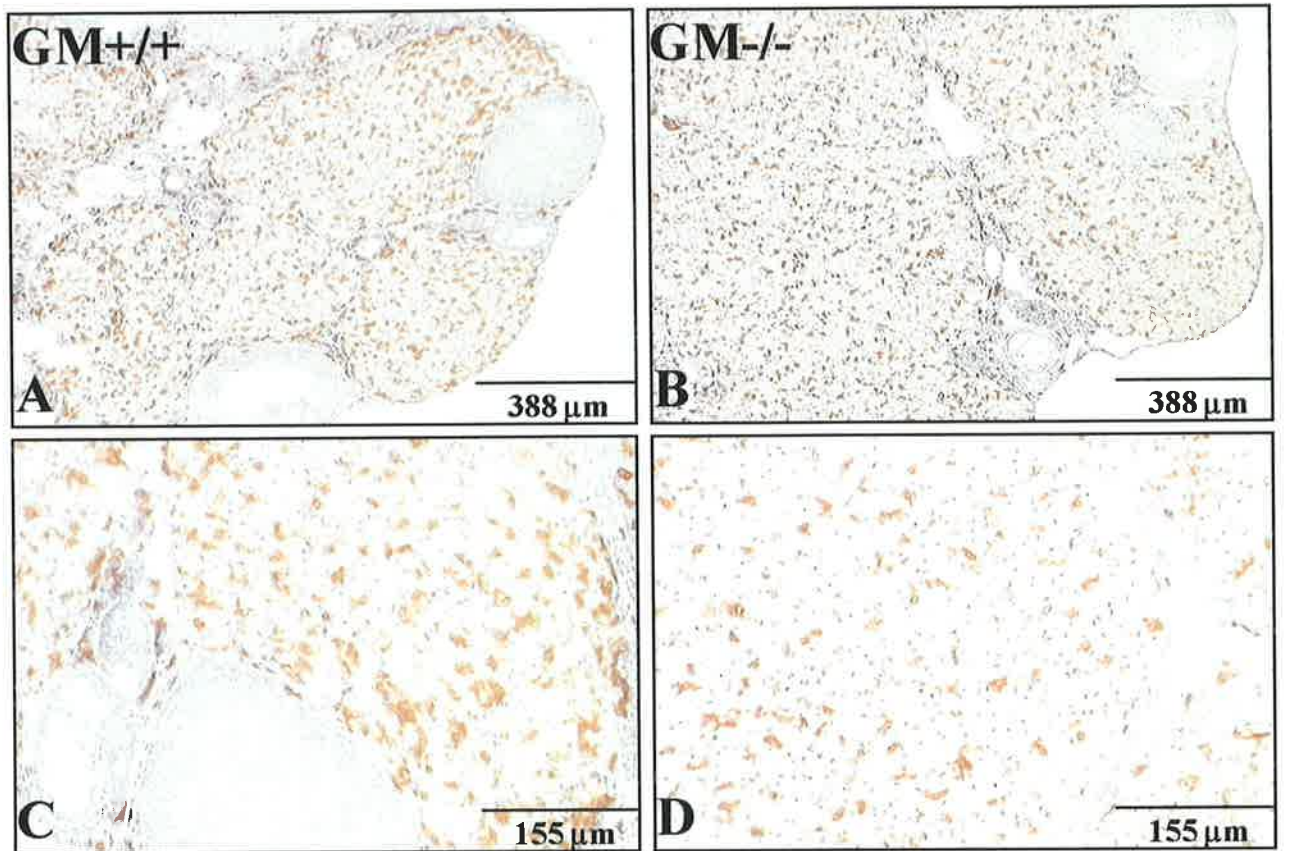


Figure 5.10 Immunohistochemical localisation of activated macrophages and dendritic cells in the pregnant GM+/+ and GM-/- ovary. Luteinised (Day 1), early luteal (Day 3) and postpartum (PP) ovaries were collected from immature primed pregnant mice. Activated macrophages and dendritic cells are stained with a lineage-specific mAb against MHC class II (Ia). Ia positive staining in the stroma and regressing corpus luteum of A) C) GM+/+ and B) D) GM-/- postpartum ovaries. E) Percent positive stain as assessed by video image analysis. Data are mean $\pm$ SE, n = 4 - 6 per group.

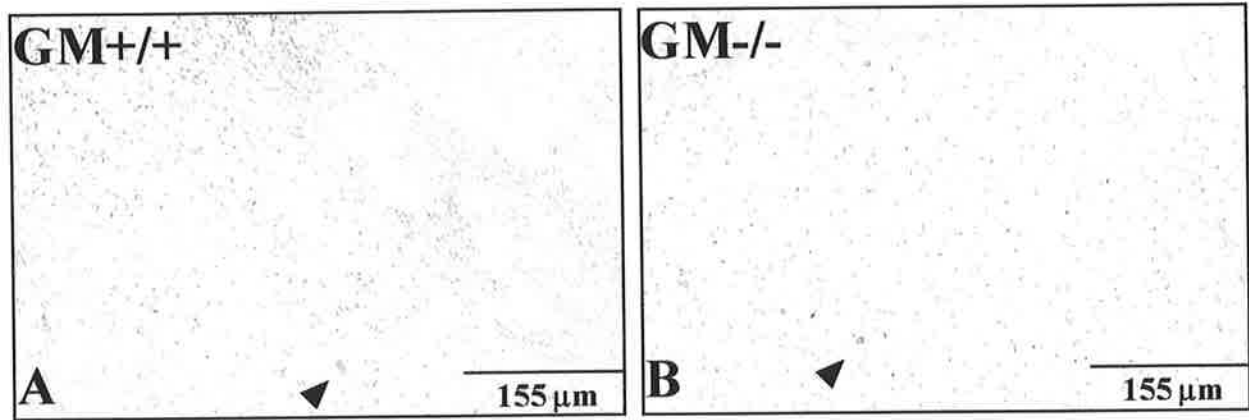


Figure 5.11 Immunohistochemical localisation of lymphocytes in the pregnant GM+/+ and GM-/- ovary. Ovaries were collected from immature primed postpartum mice. T and B lymphocytes are stained with a lineage-specific mAb against CD5 in ovary of A) GM+/+ and B) GM-/- mice postpartum.

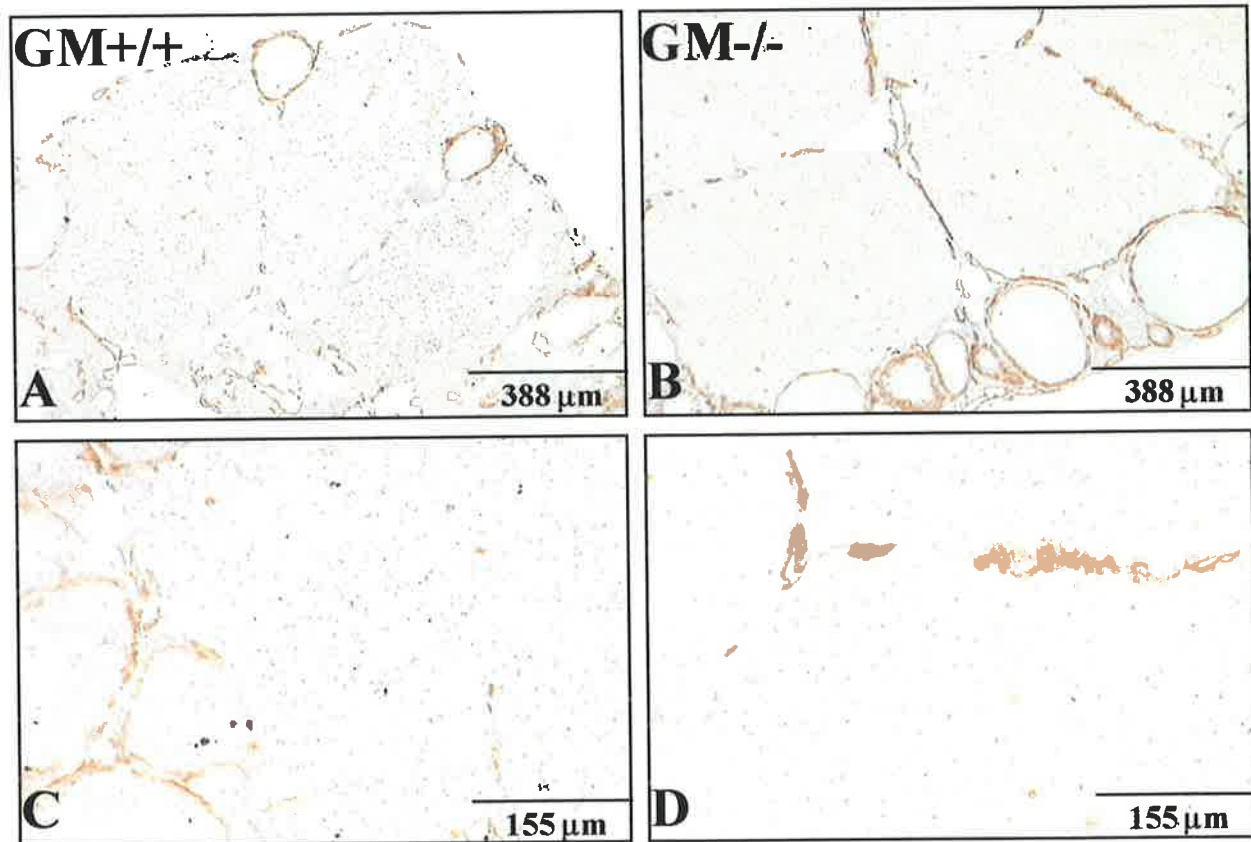


Figure 5.12 Immunohistochemical localisation of Thy 1.2 in the pregnant GM+/+ and GM-/- ovary. Ovaries were collected from immature primed mice postpartum. Thy 1.2 positive stained cells in the stroma and regressing corpus luteum of A) C) GM+/+ and B) D) GM-/- postpartum ovaries.

Leukocytes (CD45+) were localised predominantly within stromal tissue and to corpora lutea of the GM+/+ and GM-/- ovaries (Figure 5.7). The area of positive CD11b and Ia staining in the stroma and corpora lutea during pregnancy increased dramatically in ovaries collected from postpartum mice. Percent positive CD11b+ stain (Figure 5.8) was significantly decreased in the stromal region of postpartum GM-/- ovaries compared to GM+/+ ovaries, however no significant differences were observed in the corpus luteum. No significant differences were observed for F4/80+ (Figures 5.9) staining between GM+/+ and GM-/- mice in either the stroma or corpora lutea. Similar to the periovulatory ovary, a large population of Ia reactive cells of dendritic morphology were identified in the stroma and corpora lutea (Figure 5.10). In contrast, no significant differences were observed in Ia positive staining between GM+/+ and GM-/- mice during pregnancy. Eosinophils were identified on a few of the negative control sections, although generally only 1 cell was observed per 1 - 2 sections. Both T and B subsets of lymphocytes were very sparsely distributed as seen by limited CD5+ and CD8+ staining (Figure 5.11). Anti-Thy 1.2 was found to react with a large number of cells surrounding corpora lutea and theca of immature follicles (Figure 5.12).

5.5 Discussion and conclusions

GM-CSF at sites of inflammation acts as a paracrine regulator of myeloid and dendritic cell recruitment, survival and activation, and is implicated in the regulation of tissue breakdown and cellular reorganisation as exemplified in the uterus during the oestrus cycle and early pregnancy (Chapter 1, 1.3.1.4). Tissue compartment-specific mRNA expression of GM-CSF coincides with the trafficking of leukocytes in the theca of ovulatory follicles and corpora lutea during the oestrous cycle (Chapter 3, 3.6). The primary purpose of this study was to determine whether deficiency in GM-CSF would perturb the ovarian cellular events associated with the oestrous cycle and early pregnancy in the mouse.

These data show that, as in other species, leukocytes are a major cellular component of ovarian tissue throughout the oestrous cycle and pregnancy in the mouse, and that GM-CSF deficiency does not appear to alter their abundance during the periovulatory period. However, the findings that the numbers of activated macrophage and dendritic cells were markedly reduced in the GM-CSF deficient ovary at ovulation suggests that GM-CSF may play a

pivotal role in regulating the behaviour of these cells in the ovary during the formation of the corpus luteum from follicle remnants. In addition, GM-CSF may regulate the number of macrophages and neutrophils in the ovary during the regression of the corpus luteum postpartum.

Disruption of the rhythmicity of oestrous (Chapter 4, 4.2) at the local level may result from inadequate or delayed tissue remodelling as a consequence of inadequate numbers or priming of leukocytes. Since the phenotype of a macrophage is influenced by the large and diverse array of soluble and membrane or ECM-associated mediators in the local environment, the specific activity of the cell may be compromised in the absence of a single factor. GM-CSF-derived macrophages express more Ia antigen, secrete more PGE₂ and are more toxic after exposure to inflammatory conditions, as compared to CSF-1-derived macrophages (Rutherford *et al*, 1993). Conversely, LPS-stimulated peritoneal macrophages from GM-/- mice secrete less IL-1 α and nitric oxide (Basu *et al*, 1997), suggesting inadequate responses to immune stimulation in the absence of GM-CSF. In addition, dendritic cells also express Ia and under the influence of GM-CSF become more competent antigen presenting cells and less competent antigen processing cells. The role of dendritic cells in the ovary is unclear but these cells may promote lymphocyte activation in the tissue and immunoregulated killing of superfluous cells perhaps during corpus luteum formation and/or demise. Furthermore, these cells may behave as they do in other tissues, where after antigen uptake they emigrate to local lymph nodes and initiate antigen-specific T lymphocyte responses. The detection and trafficking of dendritic cells in the ovary may be aided by immunolocalisation of B7-2, the ligand of CD 28, and a co-stimulatory molecule expressed on antigen presenting cells.

The role of the GM-CSF-derived Ia positive cells in the ovary remains to be elucidated but Ia cell numbers are also reduced in the uterus of the GM-/- mouse (Robertson *et al*, 1998). During oestrous, expression of the GM-CSF cell signalling system in the theca and localisation of GM-CSF responsive cells coincides with rapid tissue remodelling of the theca, including basement membrane degradation, luteinisation of granulosa cells and angiogenesis occurring during the formation of the corpus luteum from follicle remnants. In addition, reduced Ia expression coincides with reduced progesterone production per oocyte ovulated. By day 4 of pregnancy, progesterone synthesis is compromised and pregnancy is then associated with reduced ovarian weight. Thus, these data suggest GM-CSF activation of macrophages may influence steroid synthesis.

While no neutrophil-specific mAb was available for immunohistochemistry in the mouse, small rounded CD11b reactive cells were presumed to be neutrophils. Neutrophils have been reported to comprise a large proportion of leukocytes present in the immature primed rat ovary and increase 3 fold in medulla and 8 fold in theca at ovulation (Brännström *et al*, 1993a). This ovulatory increase seen as CD11b positive staining was not evident in this study during the periovulatory period. However, during pregnancy there was a dramatic increase in CD11b positive macrophages and neutrophils in both the stroma and corpus luteum, but this increase was absent in the stroma of GM-CSF deficient ovaries postpartum. During luteal regression, these cells may play a role in the structural regression of the corpus luteum, but appear not to impact on the functional regression of the corpus luteum as reflected in the length of pseudopregnancy (Chapter 4, 4.6).

The temporal patterns of leukocyte trafficking were most notable during pregnancy. Macrophages were found to comprise a large proportion of ovarian leucocytes, but unlike the study by Brännström and co-workers (1993a) demonstrating a 5 fold increase in theca macrophages at the time of ovulation, percent positive F4/80 staining was relatively consistent during the periovulatory period. However, the infiltration of the corpus luteum by macrophages was associated with pregnancy, particularly during postpartum regression of the corpus luteum. T lymphocytes were reported to be present in the stroma and theca of follicles and corpus luteum, but in low numbers. Although no previous studies have attempted to detect lymphocytes in the murine ovary, our findings were similar to those in the rat as well as human, cow and pig, which detected the presence of these cells, although this study did not quantify cell numbers. The trafficking of lymphocytes into the ovary and in particular the corpus luteum, possibly as a result of antigen presentation at local lymph nodes, may mediate cytotoxic killing of luteal cells in the regression process. Certainly only small numbers of these cells are required to efficiently carry out this process. Associated with luteolysis is a decrease in steroidogenic activity. The finding of strong Thy 1.2 positive cells in the theca of periovulatory ovaries is supported by the work of Presl and Bukovský (1986), who suggested that these cells in rats are involved in folliculogenesis and that this may occur through cell contact with Ia positive cells.

Discrepancies between the temporal dynamics of the murine F4/80 and CD11b positive population and the equivalent populations in the rat may be due to differences in the mode of data collection. In this study, positive staining was analysed by video image analysis. Therefore the area of positive stain was normalised to cell density, in contrast with

manually counting cells, which does not take into account tissue density. Automated analysis reduces the bias associated with manual counting but unfortunately is not necessarily any more time efficient.

The control of leukocyte extravasation in the ovary is unknown. Although macrophages can proliferate in non-lymphoid tissues, the upregulation of cell adhesion molecules in the rat ovary around the time of ovulation (Bonello and Norman, 1997) suggest that increased staining is at least partly the result of infiltration. This data suggests that recruitment factors other than GM-CSF are the primary chemotaxins in the ovary during the periovulatory period, however GM-CSF may regulate recruitment of macrophages and neutrophils into the ovary during luteolysis. IL-8 is likely to have a role in neutrophil trafficking (Ina *et al*, 1997). In addition, CSF-1 and the chemokines JE (MCP-1), C10, RANTES and MIP1 α may be candidates, as they have been implicated in leukocyte recruitment in the uterus (Robertson *et al*, 1998, Pollard *et al*, 1998). Leukocytes present in the immature mouse ovary may act to potentiate the recruiting activities of cytokines, possibly by the release of factors active on endothelial cells. Identification of the chemotactic factor observed in follicular fluid (Herriot *et al*, 1986) may provide information as to the mechanism through which leukocytes are drawn to the theca and align with the basement membrane.

While this study provided information as to the cellular content of the ovary, the functional capacity/phenotype of myeloid cells present within the ovary in the absence of GM-CSF is unclear. Future experiments would include purification of these cells and careful assessment of cytokine profiles and phagocytic activity, including the determination of CSF-1 and GM-CSF-derived macrophage functions in the ovary. *In situ* localisation of cytokines by immunohistochemistry on serial GM-/- and *csfm^{op}/csfm^{op}* ovary sections also stained for F4/80 and CD11b would provide conclusive data on the cellular source of these factors and activation of macrophages by CSFs.

In summary, our results indicate that GM-CSF is not a primary regulator of ovarian leukocyte trafficking during the periovulatory period and early pregnancy. This suggests that the redundancy of the cytokine network permits normal trafficking of ovarian leukocytes in the absence of GM-CSF, perhaps through the compensatory actions of other cytokines with similar bioactivities. However, GM-CSF appears to regulate macrophage and/or dendritic cell function at the time of ovulation as reflected by increased Ia positive staining in the wild type mouse ovary. In addition, it appears to regulate recruitment of macrophages and neutrophils during luteal regression.

Chapter 6

Development and viability of GM-CSF deficient embryos

6.1 Introduction

Granulocyte-macrophage colony-stimulating factor deficiency is associated with reduced fetal size in late gestation and increased postnatal mortality rate (Robertson *et al*, submitted). GM-CSF mRNA expression and protein secretion has been described throughout the female reproductive tract, in the murine ovary (Chapter 3), human fallopian tube (Zhao and Chegini, 1994) and murine uterus (Robertson *et al*, 1992), in addition, placental tissue of the rodent expresses GM-CSF (Crainie *et al*, 1990) and both the oocyte/embryo and placenta express components of the GM-CSFR. It is therefore unclear specifically where within the tract the expression of GM-CSF is critical to ensure long-term offspring viability. As ovulation rate appears to be unaltered by GM-CSF deficiency (Chapter 4), prenatal embryonic loss and reduced postnatal survival in the GM-/- mouse may be a result of the ovulation of immature oocytes or dysregulation in early embryonic development. The manipulation of the environment during early pregnancy appears to impact on the development and viability of the embryo (Minami and Iritani, 1993). The addition of insulin (Harvey and Kaye, 1990), IGFs (Gardner and Kaye, 1991) and LIF (Robertson *et al*, 1991) have been shown to stimulate embryo development. In contrast, blastocysts do not implant in LIF deficient female mice (Stewart *et al*, 1992). *In vitro*, GM-CSF acts to increase developmental rate, enhances implantation rate of blastocysts (Robertson *et al*, 1991, Pampfer *et al*, 1991, de Moraes and Hansen, 1997), and regulates proliferation, differentiation and secretory activity of cytotrophoblast cells (Imakawa *et al*, 1993). In addition, GM-CSF prevents spontaneous abortion in the mouse (Chaouat *et al*, 1990, Clark *et al*, 1994).

In order to assess the effect of ovarian GM-CSF deficiency on the developmental potential of oocytes, this study analysed *in vitro* development of embryos to the first cell division. *In vivo*, embryo development and allocation of cells to trophectoderm and inner cell mass in the blastocyst was determined on day 4 of pregnancy to examine embryo differentiation and viability.

6.2 *In vitro* development of GM-CSF deficient embryos

To investigate early development of embryos, embryos were collected and scored according to developmental stage and timing of the first cell division during *in vitro* culture.

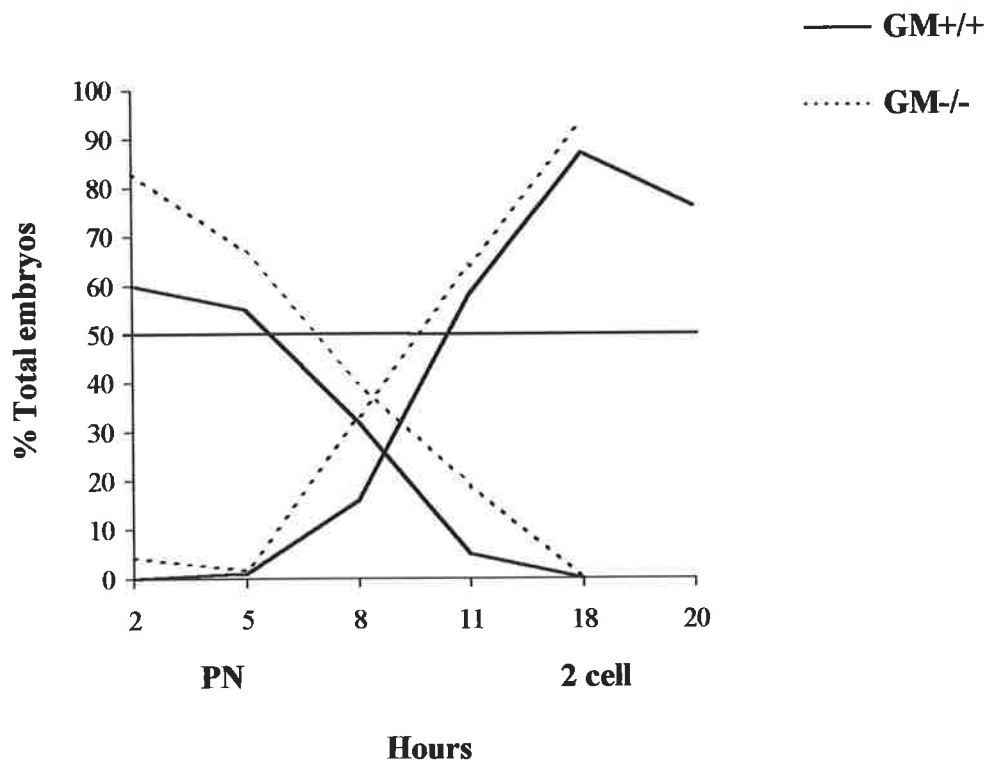


Figure 6.1 Proportion of 1 cell, pronuclei and 2 cell embryos of total GM+/+ and GM-/- embryos *in vitro*. Embryos were collected from adult naturally mated mice at 15:30 hr on day 1, cultured and scored according to developmental stage at 2, 5, 8, 11, 18 and 20 hr post collection time. Data graphed as percent 1 cell, pronuclei embryos on the left and percent 2 cell embryos of total embryos (n = 99 GM+/+, 58 GM-/- embryos) on the right as a function of time. The bar across the graph represents 50 %. PN = 1 cell embryo, formation of pronuclei, 2 cell = 2 cell embryo.

Adult virgin female GM+/+ and GM-/- mice were mated with fertile males of the same strain to induce pregnancy (Chapter 2, 2.2.7). Females were euthanased on day 1 of pregnancy at 15:30 hr by cervical dislocation, and embryos were retrieved from the oviduct (Chapter 2, 2.6.1). The embryos were cultured (Chapter 2, 2.5.2), viewed using an inverted light microscope and scored according to developmental stage (Hogan *et al*, 1986) at 2, 5, 8, 11, 18 and 20 hr post collection time.

Timing of the first cell division appeared to be unaltered in the GM-/- embryo as determined by the time taken for 50% of embryos to develop from the pronuclei stage to 2 cell embryos, and the time at which these stages intersect (Figure 6.1).

6.3 *In vivo* development of GM-CSF deficient embryos

In order to assess the affect of GM-CSF deficiency on the developmental potential of oocytes, this study analysed *in vivo* development of embryos and the allocation of cells to trophoctoderm and inner cell mass in the blastocyst on day 4 of pregnancy.

In order to determine the optimal blastocyst collection time on day 4 of pregnancy, a preliminary experiment was performed recovering embryos at two time points: 13:30 hr and 19:30 hr. Adult female mice were mated with fertile males of the same strain to induce pregnancy. Embryos were flushed from the uteri and oviducts on day 4 of pregnancy (Chapter 2, 2.6.1) and scored according to developmental stage. The collection time of 19:30 hr was chosen since the proportion of embryos at blastocyst stage was greater at the latter time point from both GM+/+ and GM-/- mice.

Immature primed and adult female mice (GM+/+ age range 19-27 weeks, GM-/- age range 14-24 weeks) were mated with fertile males of the same strain to induce pregnancy. For the experiment, embryos were collected on day 4 of pregnancy from immature primed and naturally mated adult mice at 13:30 hr (25 ± 0.5 hr post hCG) and 19:30 hr respectively. The embryos were scored according to developmental stage and the inner cell mass (ICM) and trophoctoderm (TE) cell nuclei of blastocysts were differentially stained (Chapter 2, 2.6.3). On completion of the staining, embryos were wet mounted, viewed and scored (Chapter 2, 2.6.4) (Figure 6.2).

Only 80 % of GM+/+ and 50% of GM-/- immature primed mice observed to be plugged yielded embryos. Of the naturally mated adult mice, 71% GM+/+ and 50% GM-/- mice yielded embryos (Table 6.1). This low frequency of embryo retrieval was due partially to a high rate of fertilisation failure.

Table 6.1 Mice yielding embryos and oocytes on day 4 of pregnancy. Embryos were flushed from the oviducts and uteri of day 4 immature primed and adult naturally mated GM+/+ and GM-/- mice. Mice were scored according to the yield. Data are the total number of embryos recovered, with the percent of mice yielding embryos shown in brackets.

	Primed		Natural	
	GM+/+	GM-/-	GM+/+	GM-/-
None	1	3	3	6
Oocytes only	0	0	1	5
Embryos (%)	4 (80%)	3 (50%)	10 (71%)	11 (50%)
Total mice (n)	5	6	14	22

6.3.1 Developmental stage of embryos

The number and developmental stage of embryos immediately prior to implantation was determined to examine the effect of GM-CSF deficiency on the embryo *in vivo*. There was considerable variation in fertilisation rate, number and stage of development of embryos collected from both immature primed and naturally mated adults (Table 6.2). Hatching of GM-/- blastocysts was significantly delayed as compared to GM+/+ blastocysts.

Table 6.2 Developmental stage of recovered embryos and oocytes on day 4 of pregnancy. Embryos were flushed from the oviduct and uteri of day 4 immature primed and adult naturally mated GM+/+ and GM-/- pregnant mice and scored according to developmental stage. Data are the total number of embryos recovered, with the percent of total embryos recovered shown in brackets.

	Primed		Natural	
	GM+/+	GM-/-	GM+/+	GM-/-
Degenerated	14 (10.9%)	13 (11.1%)		
Unfertilised	15 (11.7%)	1 (0.9%)	7 (10%)	23 (26.1%)
16 cell (uncompacted)	0	0	11 (14.3%)	0
Morula	8 (6.3%)	26 (22.2%)	19 (24.7%)	16 (18.2%)
Blastocyst	84 (65.6%)	77 (65.8%)	7 (10.0%)	23 (26.1%)
Blastocyst-hatched	7 (5.5%)	0*	33 (42.9%)	26 (29.5%)**
Total embryos (n)	128	117	77	88

* significantly different from immature primed GM+/+ as assessed by Fisher's Exact Test, two tail $P = 0.0159$.

** significantly different from adult naturally mated GM+/+ as assessed by Fisher's Exact Test, two tail $P = 0.0038$.

6.3.2 Blastocyst cell allocation

Differential staining was employed to investigate whether GM-CSF deficiency alters the allocation of embryonic cells to trophectoderm and inner cell mass. The total number of cells which comprised the blastocyst was significantly reduced by 18% in the GM-/- blastocysts collected from immature primed mice and by 19% in the GM-/- blastocysts collected from naturally mated adult mice (Figure 6.3). The decrease in total cell number was due to a specific decrease of ICM cells in blastocysts collected from both immature primed and adult mice. The apparent reduction in the total cell number of GM+/+ and GM-/- blastocysts collected from immature compared to adult mice was possibly due to the difference in collection time by 6 hr and/or the dynamics of embryo development following superovulation as opposed to naturally mated mice (Allen and McLaren, 1971).

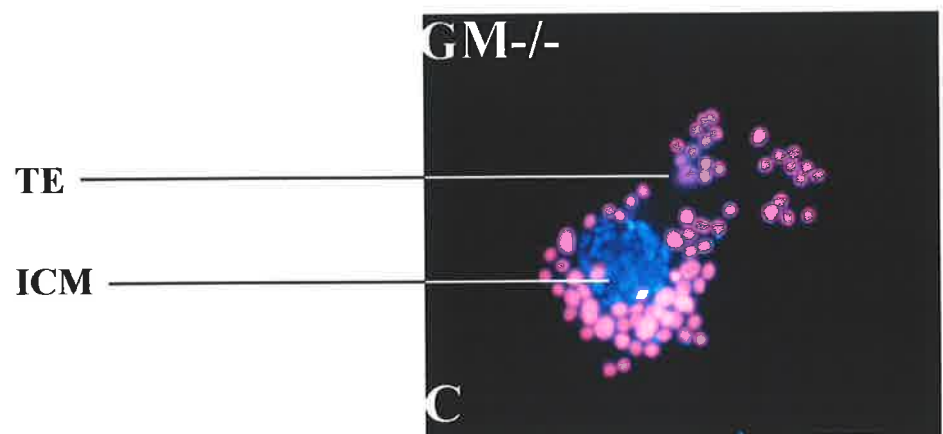
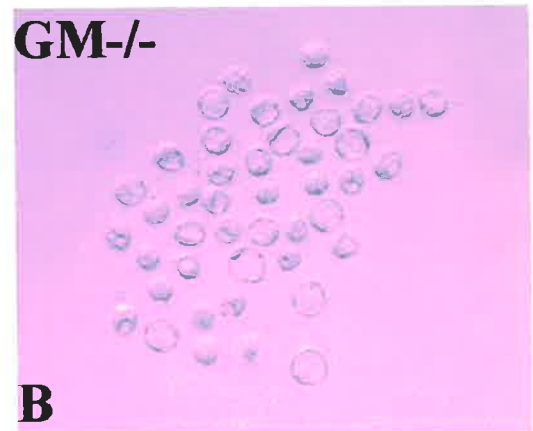
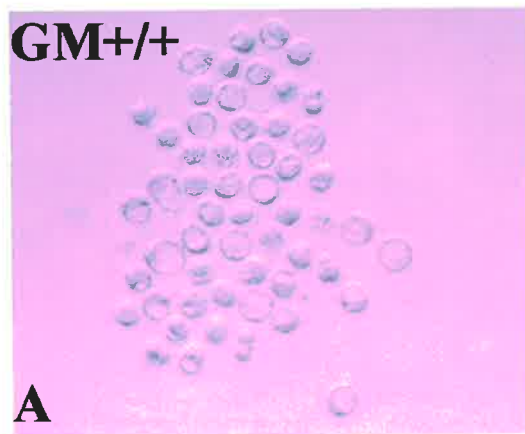


Figure 6.2 Embryos collected from day 4 pregnant **GM+/+** and **GM-/-** mice. Embryos were flushed from the oviduct and uteri and differentially stained. Embryos collected from A) **GM+/+** and B) **GM-/-** adult naturally mated mice range from unfertilised oocytes through to blastocyst stage of development. C) **GM-/-** blastocyst collected from an immature primed mouse and differentially stained to enable quantification of trophectoderm (TE) and inner cell mass (ICM) cell numbers.

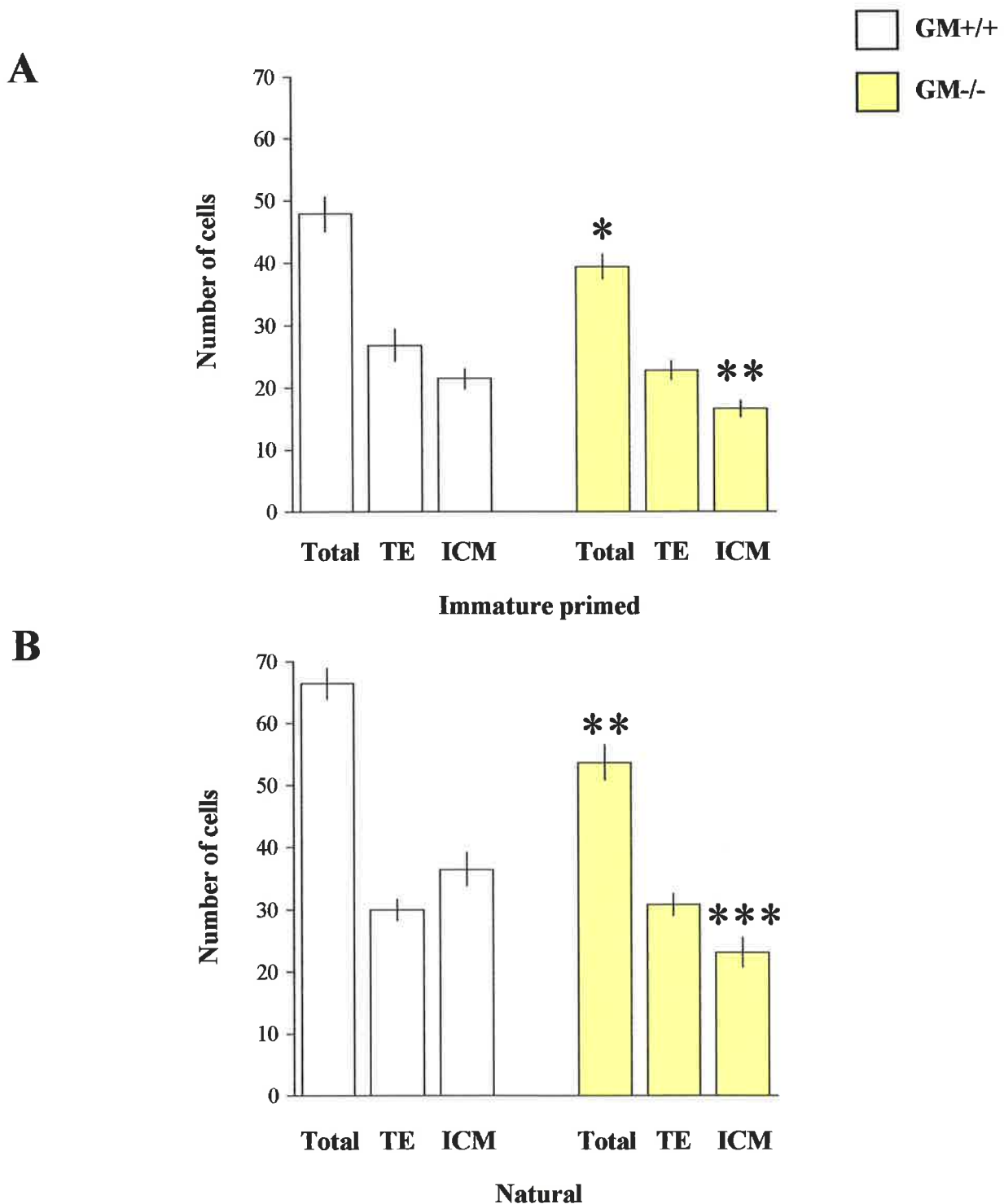


Figure 6.3 GM+/+ and GM-/- blastocyst cell allocation to trophoblast (TE) and inner cell mass (ICM) cells. Embryos were flushed from the oviduct and uteri of immature primed and adult naturally mated mice and differentially stained. A) Total, TE and ICM cell numbers of blastocysts collected from immature primed mice. Data graphed as mean $\pm$ SE for $n = 24$ GM+/+, 55 GM-/- embryos. *, ** significantly different from GM+/+ as assessed by Mann-Whitney Test, two tailed $*P = 0.0167$, $**P = 0.0076$. B) Total, TE and ICM cell numbers of blastocysts collected from adult naturally mated mice. Data graphed as mean $\pm$ SE for $n = 36$ GM+/+, 39 GM-/- embryos. **, *** significantly different from GM+/+ as assessed by Mann-Whitney Test, two-tailed $**P = 0.0013$, $***P = 0.0003$.

6.4 Discussion and conclusions

GM-CSF is one of a number of cytokines secreted by uterine epithelial cells implicated in a network of inter-cellular communications in the female reproductive tract during early pregnancy. This cytokine appears to act through mediating the recruitment and activation of uterine neutrophils and macrophages and has also been found to possess embryotrophic properties. In the absence of GM-CSF, embryo and postnatal survival is significantly impaired.

In the current study, the heterogeneity in developmental stage of the embryos collected on day 4 of pregnancy from both immature primed and adult mice was consistent with a moderate delay in the rate of embryonic development in the absence of GM-CSF. Blastocysts of GM-CSF gene knockout mice were found to comprise fewer ICM cells on day 4 of pregnancy. Together with the finding of reduced plasma progesterone on day 4 of pregnancy (Chapter 4), these data demonstrate measurable cellular and biochemical shifts associated with GM-CSF deficiency from the preimplantation stages of development.

The embryo appears to be a target for an array of growth factors even prior to implantation (Sharkey *et al*, 1995), and IGF-1 promotes 2 cell embryo growth by increasing the number of ICM cells alone (Harvey and Kaye, 1992). The ICM of the blastocyst gives rise to the fetus and amnion, visceral yolk sac and chorioallantois of the placenta, whereas the TE cells are responsible for the initial adhesion and latter burrowing associated with implantation, and will eventually give rise to the parietal yolk sac of the placenta. It has been suggested that alterations in weight ratio of fetus and placenta can be caused by asynchrony between the development of the embryo and reproductive tract in early development, and results in less than optimal postnatal development and survival (Seamark and Robinson, 1995). In the mouse, embryo transfer results in disruption of normal fetal and placental growth (Harlow and Quin, 1975). Placental weight is normal but fetal weight of GM-/- pups is reduced compared to wild type on day 17 of pregnancy (Robertson *et al*, in press). The data reported in this chapter show that this is associated with increased TE:ICM ratio in the blastocyst prior to implantation. Several mechanisms could be responsible for a shift in synchrony of embryo and tract development which may be explained by 3 scenarios.

First, a delay in ovulation of the GM-/- oocyte. Braden (1957) found that 75% of total oocytes ovulated are ovulated in 30 - 60 min. A delay in GM-/- ovulation would possibly result in delays of subsequent events including cleavage, hatching and implantation. A delay

of a few hours may be enough to disrupt embryo development and long-term viability. Although *in vivo* findings suggesting delayed development are in line with this hypothesis, this can be discounted by the findings that GM-/- oocyte transport rate to the ampulla following ovulation (Chapter 4, 4.3.1) and GM-/- embryo development is not delayed when the time of cleavage of 1 cell embryos is examined *in vitro*. In a similar experiment, *in vitro* development of GM-/- embryos is also found to be not altered (R. Gilchrist, unpublished data).

A second explanation for these findings would be that embryotrophic and uterotrophic actions of GM-CSF are lacking in GM-/- mice. *In vitro*, GM-CSF promotes development of embryos, attachment and outgrowths in the human (Sjöblom *et al*, 1998) and mouse (Robertson *et al*, 1991). The mechanism for this action is unclear due to the lack of expression of the β receptor subunit (Robertson *et al*, 1994b). Originally, the α subunit of the receptor was thought not to elicit biological activity, but it has since been proposed to mediate glucose uptake (Ding *et al*, 1994, Spielholz *et al*, 1995). Exposure of the mouse embryo to glucose is required to ensure optimal embryo development to blastocyst and hatching (Martin and Leese, 1995).

The maintenance of TE cell number to aid attachment and implantation at the expense of the ICM may explain why day 8 implantation rates were normal in GM-/- mice. It could be speculated that compromised embryo growth results from diminished ICM cell numbers at implantation. A comparable phenomena of reduced ICM is observed in insulin-deprived blastocysts (Lea *et al*, 1996) and also in blastocysts cultured with TNF α (Pampfer *et al*, 1994).

Alternatively, GM-CSF may influence the cellular composition and secretory profile of the tract, indirectly affecting embryonic development. Leukocytes are an integral and dynamic cell population of the uterus and are potential targets of GM-CSF. Seminal plasma-induced GM-CSF synthesis, or administration of exogenous GM-CSF elicits a massive infiltration of leukocytes during early pregnancy (Robertson *et al*, 1994b). Interestingly, the GM-/- genotype is associated with diminished numbers of activated macrophage/dendritic cells in the uterus (Robertson *et al*, 1998). GM-CSF-responsive leukocytes are also present in the oviduct (Farr *et al*, 1995) and while less is known about the roles of these cells, subtle differences in their numbers or activation status could influence tissue remodelling in the oviduct. The significance of this indirect effect of GM-CSF on embryo development, mediated through the leukocyte - cytokine networks of the oviduct and uterus this early in pregnancy remains unclear, but is perhaps the less likely of options.

A final mechanism to shift the synchrony of embryo and tract may result from the effect of reduced ovarian production of progesterone on the secretion of embryotrophic agents from the oviduct and uterus. The reduced progesterone production noted on day 4 in naturally pregnant adult mice is also supported by findings suggesting reduced progesterone production per oocyte ovulated in the *in vitro* ovary perfusion model (Chapter 4). When performed early in pregnancy (day 3), ovariectomy results in spontaneous abortion, suggesting ovarian-derived progesterone is the necessary signal ensuring maintenance of early pregnancy (McLaren *et al*, 1971). In addition, blocking progesterone production delays embryo movement through the oviduct (Kendle and Lee, 1980). A major role of progesterone in pregnancy is the maintenance of uterine quiescence through decidualisation of the endometrium, and inhibition of detrimental immune responses (Siiteri *et al*, 1977), perhaps mediated through the cytokine network. Most notably, it may promote uterine and oviduct secretions of growth factors and cytokines such as CSF-1, TNF α and LIF which appear to be dependent on progesterone (Pollard *et al*, 1987, Bhatt *et al*, 1991). An excess of this hormone may compromise the system as seen by treatment with gonadotrophin which impairs implantation and retards embryo development (Ertzeid *et al*, 1993).

Even though the timing of embryo cleavage was unaltered by GM-CSF deficiency, these experiments do not preclude an effect of GM-CSF on the oocyte, and further experiments are warranted. Since DNA synthesis of GM-/- preovulatory oocyte cumulus cell complexes appears perturbed *in vitro* (D. Rowe and R. Gilchrist, unpublished data), paracrine regulation of oocyte development by GM-CSF may be important prior to fertilisation.

The experimental technique of transplanting GM-CSF gene knockout ovaries into the bursa of GM-CSF replete mice may provide information on the significance of the uterotrophic versus oocyte and/or embryotrophic actions of GM-CSF during early pregnancy.

In summary, a lack of GM-CSF in early embryonic development disrupts the allocation of blastocyst cells and embryo development. Requirement by the oocyte and/or the early embryo for GM-CSF to regulate the uterine environment, the rate and degree of embryonic development as well as the differentiation and proliferation of placental trophoblast cells may be necessary for successful pregnancy outcome to guard against pre and postnatal loss.

Chapter 7

Characterisation of the CSF-1 deficient *csfm^{op}/csfm^{op}* ovary

7.1 Introduction

The experiments described in chapter 4 provide evidence that while GM-CSF is not essential for ovulation, it may act to promote ovarian steroidogenesis. In addition, data from chapter 5 suggest GM-CSF may act as a regulator of activation of macrophage and dendritic cell populations in the ovary at ovulation. The experiments presented in this chapter were undertaken to investigate the role of CSF-1 in ovarian function using the CSF-1 deficient (*csfm^{op}/csfm^{op}*) mouse model.

Like GM-CSF, CSF-1 is a potent regulator of macrophage survival, proliferation, differentiation and activation (Chapter 1, 1.3.2). The macrophage lineage displays great heterogeneity in terms of phenotype, and it is thought that the tissue of maturation determines the phenotype of the mature cell. While both GM-CSF and CSF-1 are potent activators of macrophage function, they have different regulatory effects on macrophage populations.

The description of the naturally occurring *op/op* mutant to be CSF-1 deficient makes this mouse model (Chapter 1, 1.3.2.5, Chapter 2, 2.2.3) suitable to investigate the role of CSF-1 in ovary function *in vivo*. Interestingly, *csfm^{op}/csfm^{op}* female mice have marked fertility defects whereas GM-CSF deficient mice display less severe fertility defects. Considering that macrophage phenotype is dependent on the tissue of maturation, it was considered essential to examine the ovarian function of the *csfm^{op}/csfm^{op}* mouse to fully understand the contribution of CSF to the regulation of ovarian leukocyte populations and ovarian function.

The fundamental hypothesis of the studies presented within this chapter is that CSF-1 synthesised within the mouse ovary (Chapter 3) acts to regulate the function of CSF-1 receptor-bearing cells and in effect contributes to the regulation of ovarian function.

The primary purpose for undertaking this study was to determine whether infertility associated with the *csfm^{op}/csfm^{op}* genotype was associated with ovarian dysfunction. This investigation was carried out to determine whether CSF-1 deficiency affects cellular and biochemical ovarian parameters including oestrus cycle and pseudopregnancy length, ovulation rate, corpora lutea development, steroidogenesis and the leukocyte population. Oestrous cycle and pseudopregnancy length were examined as a measure of hypothalamic/pituitary/ovarian axis activity. *In vivo* ovulation rates were assessed in immature primed and adult naturally cycling mice. Corpus luteum development was examined by assessment of the expression of mRNAs for the macrophage mediators GM-CSF, TNF α and iNOS and the CSF-1 receptor, c-fms. The expression of mRNA was viewed

as an indicator of the potential for production of protein by cells constituting the corpus luteum. Detection of receptor was interpreted to suggest the presence of ligand-responsive cells in the ovary. *In vivo* production of progesterone was assessed in the immature primed, naturally cycling adult and day 5 pseudopregnant mouse. In order to assess the effect of CSF-1 deficiency on macrophage infiltration and activation within the ovary, we examined spatial immunohistochemical staining of macrophage markers, CD11b, F4/80 and Ia in the ovary.

7.2 Oestrous cycle length

In order to determine whether *csfm^{op}/csfm^{op}* adult female mice display signs of oestrous and to determine the length of an oestrous cycle, the cellular characteristics of daily vaginal smears were examined. CSF-1^{+/-} (n = 12) and *csfm^{op}/csfm^{op}* (n = 15) mice both aged 12 ± 0 weeks were tracked by vaginal smear until the completion of 5 oestrous cycles (Chapter 2, 2.6). During acclimatisation and throughout the tracking period, as in all subsequent experiments, CSF-1^{+/-} were given access to the liquid diet of *csfm^{op}/csfm^{op}* mice.

Cellular characteristics of vaginal smears taken from *csfm^{op}/csfm^{op}* mice reflected changes in the structure of the vaginal epithelium and followed a regular and predictable course during the cycle. CSF-1^{+/-} mice displayed signs of oestrous every 4.2 ± 0.1 days (n = 12). In contrast, *csfm^{op}/csfm^{op}* mice exhibited an extended oestrous cycle of 8.3 ± 1.1 days (n = 11), an extension of approximately 3.9 days (Figure 7.1A). On closer examination of the data, there was considerable variation in the length of oestrous of *csfm^{op}/csfm^{op}* mice. It would appear that oestrous cycle length in this genotype could be divided into 3 classes of cycles: relatively normal, extended and those which did not cycle at all. The *csfm^{op}/csfm^{op}* mice exhibited the cellular characteristics of a metoestrous-2 and/or dioestrous smear for a significantly longer period of time than the CSF-1^{+/-} mice (Figure 7.1B). The first display of oestrous after the onset of tracking occurred in CSF-1^{+/-} mice on day 1.9 ± 0.4, but significantly later in the *csfm^{op}/csfm^{op}* mice on day 4.4 ± 0.9 (two-tailed *P* = 0.0268, Mann-Whitney Test). The ages of each genotype were matched, but *csfm^{op}/csfm^{op}* mice weighed less than control mice at the onset and completion of the study (Table 7.1). Data for 4 *csfm^{op}/csfm^{op}* mice were excluded as follows; 2 because they did not complete 5 cycles, 1 did not exhibit any signs of oestrous in the 60 days of tracking, and 1 was withdrawn from the

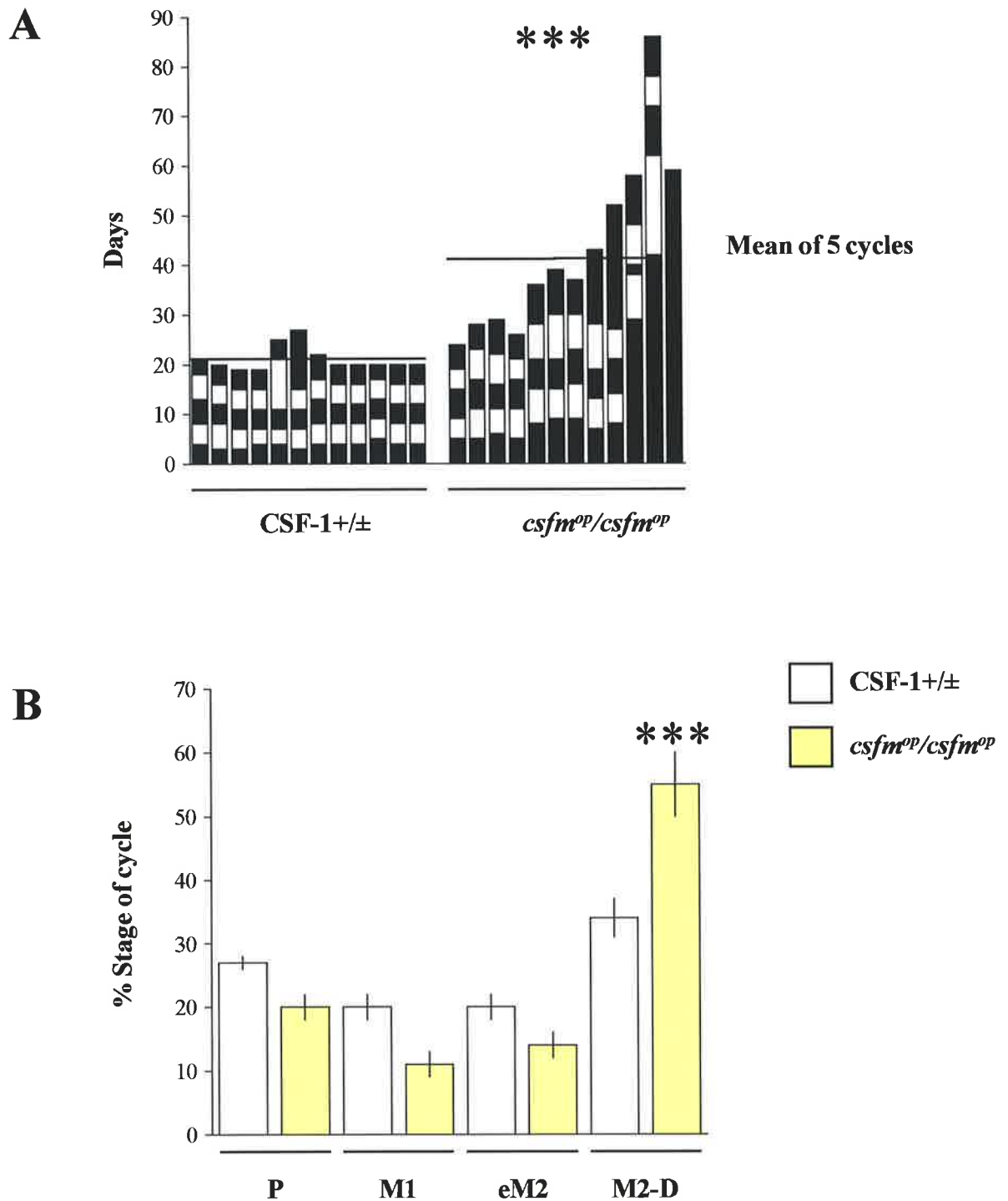


Figure 7.1 Oestrous cycle characteristics of naturally cycling adult CSF-1+/± and *csfm^{op}/csfm^{op}* mice. The cellular characteristics of daily vaginal smears recorded over 5 cycles were analysed to determine the stage of oestrous. A) Oestrous cycle length. Each oestrous cycle is represented by a segment on the bar for each mouse. The horizontal lines across the graph represent the mean of 5 cycles. *** significantly different from CSF-1+/± as assessed by Mann-Whitney Test, two tailed $P < 0.0001$. B) Percent days spent at each stage of the oestrous cycle. *** significantly different from CSF-1+/± as assessed by one-way ANOVA and Tukey-Kramer Multiple Comparisons Test, $P < 0.001$. Data presented as mean $\pm$ SE, $n = 12$ CSF-1+/±, 11 *csfm^{op}/csfm^{op}* mice.

study due to consistently poor health and failure to gain weight during 60 days of tracking in which it did not display any signs of oestrous.

Table 7.1 Weight of naturally cycling adult CSF-1+/± and *csfm^{op}/csfm^{op}* mice at the onset and end of the oestrous cycling tracking study. Data are presented as mean ± SE.

	CSF-1+/±	n	<i>csfm^{op}/csfm^{op}</i>	n
Weight - onset (g)	24 ± 1	12	19 ± 1*	11
Weight - end (g)	30 ± 1	12	22 ± 1*	9

* significantly different from CSF-1+/± as assessed by Mann-Whitney Test, two-tailed $P < 0.0001$.

The extended time to the first oestrous and extension of the oestrous cycle, in particular metoestrous-2 and/or dioestrous, suggests delayed tissue remodelling and follicle growth for the subsequent cycle. Whether this extension of oestrous is a consequence of local tissue remodelling or as a consequence of inadequate gonadotrophin signalling at the hypothalamic-pituitary level is unclear.

7.3 Pseudopregnancy length

In order to further assess the effect of CSF-1 deficiency on the hypothalamic/pituitary/ovarian axis and specifically examine the duration of corpus luteum persistence and demise, this experiment examined the effect of PGF_{2α} administration on pseudopregnancy length in the *csfm^{op}/csfm^{op}* mouse. One CSF-1+/± (16.2 ± 1.2 weeks, n = 10) and 1 *csfm^{op}/csfm^{op}* (15.4 ± 0.6 weeks, n = 8) adult female mouse were housed per cage with a vasectomised adult male (21.4 ± 0.3 weeks) until the completion of three consecutive pseudopregnancies (Chapter 2, 2.2.7). The presence of a vaginal plug was regarded as day 1 of pseudopregnancy. The duration of pseudopregnancy was calculated as the number of days lapsed until a second vaginal plug was detected. During the second pseudopregnancy, luteolysis was induced by the ip administration of 3 µg of PGF_{2α} between 09:00 and 12:00 hr

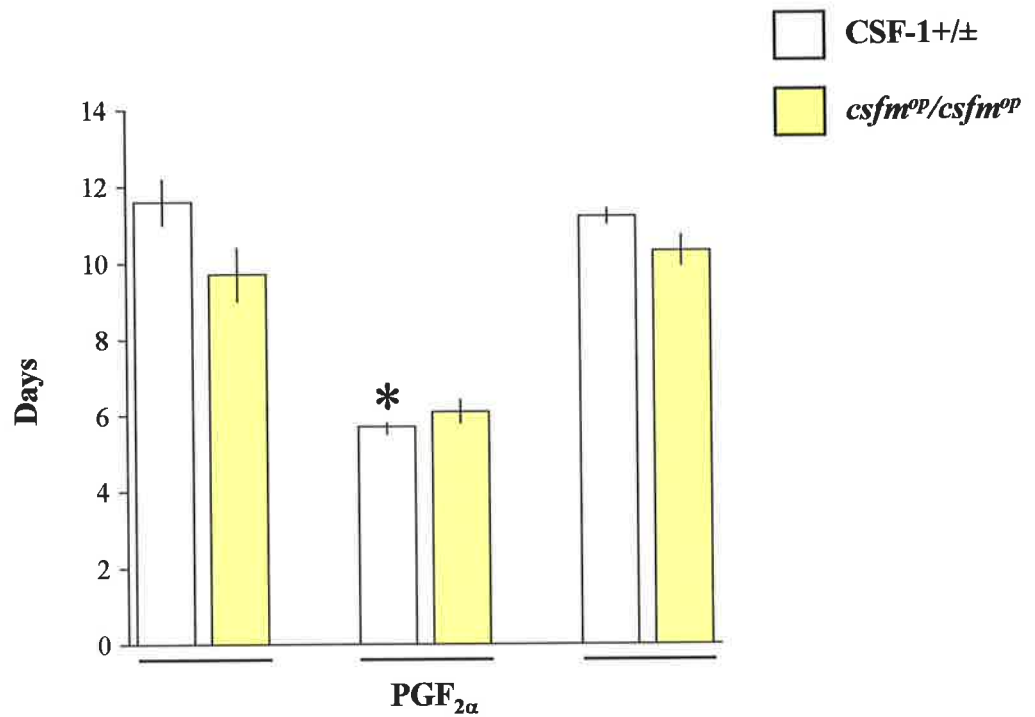


Figure 7.2 The duration of pseudopregnancy in CSF-1^{+/±} and *csfm*^{op}/*csfm*^{op} adult mice. Mice were mated to vasectomised studs and the presence of a vaginal plug was deemed day 1 of pseudopregnancy. Pseudopregnancy length was determined, then luteal regression was induced by PGF_{2α}-treatment (3 µg on day 4 and day 5) and the duration of the subsequent pseudopregnancy was measured. * significantly different from CSF-1^{+/±} mice pre- and post-treatment as assessed by Kruskal-Wallis Test and Dunn's Multiple Comparisons Test, $P < 0.001$. Data presented as mean $\pm$ SE, $n = 10$ CSF-1^{+/±}, 8 *csfm*^{op}/*csfm*^{op} mice.

on days 4 and 5 following the citing of the second vaginal plug (Chapter 2, 2.2.8). The days lapsed until detection of the third, and finally the fourth vaginal plug were determined.

The duration of pseudopregnancy in CSF-1+/± and *csfm^{op}/csfm^{op}* mice was comparable (Figure 7.2). However, PGF_{2α} administration did not induce significant premature termination of pseudopregnancy in the *csfm^{op}/csfm^{op}* mouse, but PGF_{2α} administration did induce luteal regression and premature termination of pseudopregnancy as seen by the onset of oestrous generally within 1 day of administration in the CSF-1+/± mouse. Pseudopregnancy length of CSF-1+/± and *csfm^{op}/csfm^{op}* mice post-treatment was comparable to pre-treatment illustrating no delayed effect of PGF_{2α} administration. The number of days prior to the citing of the first vaginal plug in the CSF-1+/± mouse on day 1.1 ± 0.4 and the *csfm^{op}/csfm^{op}* mouse on day 1.2 ± 0.5 was comparable (Mann-Whitney Test, two-tailed $P > 0.9999$).

In summary, pseudopregnancy of *csfm^{op}/csfm^{op}* mice appeared comparable or moderately reduced in the case of CSF-1 deficiency.

7.4 *In vivo* ovulation rate

To investigate *in vivo* ovulation rate, oocytes were collected and counted from immature primed and naturally cycling adult CSF-1+/± and *csfm^{op}/csfm^{op}* mice. Immature CSF-1+/± mice matched for weight with *csfm^{op}/csfm^{op}* mice were primed to ovulate by administration of 5 IU eCG and 5 IU hCG (Chapter 2, 2.4). Oocytes were retrieved from the ampulla of the oviduct on the morning of ovulation and counted.

While all CSF-1+/± and *csfm^{op}/csfm^{op}* immature primed mice ovulated regardless of genotype, *csfm^{op}/csfm^{op}* mice ovulated significantly less oocytes *in vivo* than CSF-1+/± mice (Figure 7.3). The weight of CSF-1+/± and *csfm^{op}/csfm^{op}* mice was comparable, however this was only achieved through the use of *csfm^{op}/csfm^{op}* mice 3.6 weeks older than CSF-1+/± mice (Table 7.2).

In vivo ovulation rate was also assessed in naturally cycling adult CSF-1+/± and *csfm^{op}/csfm^{op}* mice. On completion of a minimum of 5 oestrous cycles, oocytes were retrieved from the ampulla or flushed from the oviduct at metoestrous-1 (Chapter 7, 7.2). While 100% of CSF-1+/± mice ovulated, only 66% of *csfm^{op}/csfm^{op}* mice ovulated. Naturally cycling adult

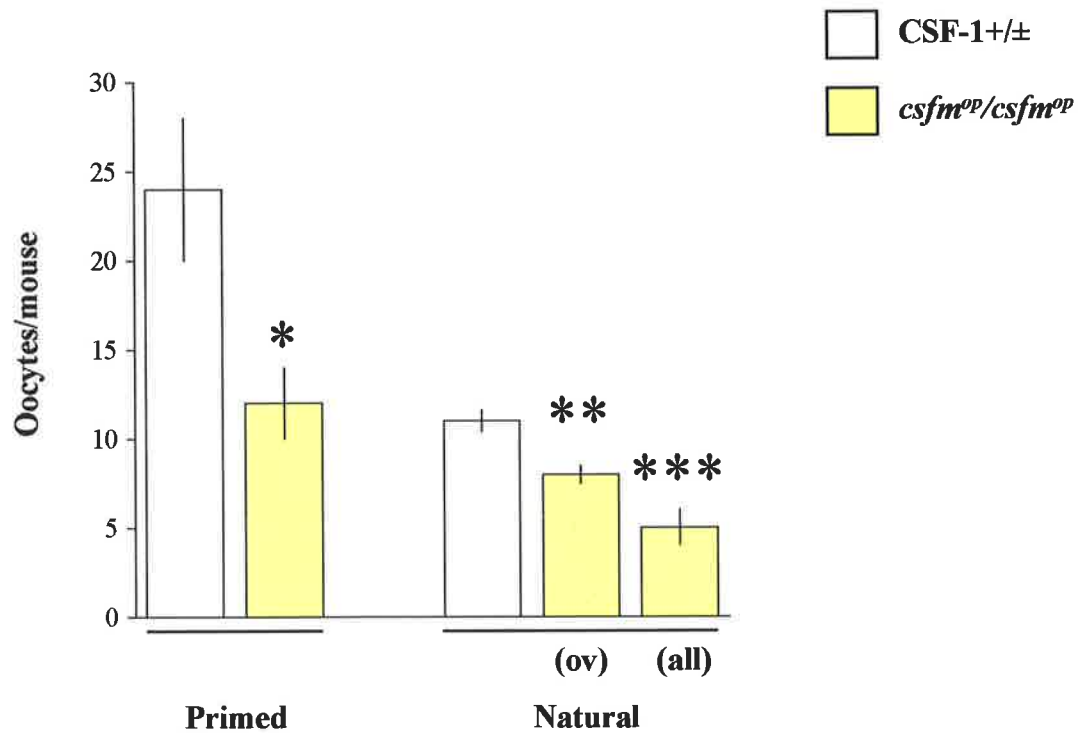


Figure 7.3 *In vivo* ovulation rate of immature primed and naturally cycling adult CSF-1+/± and *csfm^{op}/csfm^{op}* adult mice. Immature mice were primed to ovulate by the administration of 5 IU eCG and 5 IU hCG. Oocytes were retrieved from the ampulla of the oviduct on the morning of ovulation. Oestrous cycles were tracked in adult mice by daily vaginal smear and on completion of a minimum of 5 oestrous cycles, oocytes were retrieved from the ampulla or flushed from the oviduct at metoestrous-1. Data are presented as mean ± SE, immature primed n = 8, naturally cycling n = 11 CSF-1+/±, 6 *csfm^{op}/csfm^{op}* (ov = ovulating), 9 *csfm^{op}/csfm^{op}* (all). * significantly different from immature primed CSF-1+/± mice as assessed by Mann-Whitney Test, two tailed $P = 0.0148$. **, *** significantly different from natural cycling adult CSF-1+/± mice as assessed by Mann-Whitney Test, two tailed $P = 0.0011$, $P < 0.0001$.

csfm^{op}/csfm^{op} mice ovulated significantly less oocytes *in vivo* than CSF-1+/± mice, even when non-ovulators were excluded from the *csfm^{op}/csfm^{op}* group, (ov = ovulating) (Figure 7.3).

Table 7.2 Age and weight of immature primed CSF-1+/± and *csfm^{op}/csfm^{op}* mice.

Data are presented as mean ± SE.

	CSF-1+/±	<i>csfm^{op}/csfm^{op}</i>
Age (weeks)	4.5 ± 0	8.1 ± 0.4
Weight (g)	14.9 ± 0.4	13.8 ± 0.8
n	8	8

These results would suggest that ovulation is compromised in *csfm^{op}/csfm^{op}* mice. Diminished ovulation in immature *csfm^{op}/csfm^{op}* mice suggests they may be less responsive to exogenous gonadotrophins at an equal weight to CSF-1+/± mice. *Csfm^{op}/csfm^{op}* adult mice ovulate less frequently and ovulate less oocytes/oestrous, which may be as a result of reduced endogenous gonadotrophin secretion.

7.5 mRNA expression of macrophage-derived mediators in the developing corpus luteum

In order to assess the effect of CSF-1 deficiency on cytokine expression during the development of the corpus luteum, this experiment examined the expression of the macrophage products GM-CSF, TNFα and iNOS as well as c-fms mRNA in the naturally cycling adult ovary following ovulation (metoestrous-1). Oestrous cycle was tracked by daily vaginal smear in adult CSF-1+/± and *csfm^{op}/csfm^{op}* mice and on completion of a minimum of 5 cycles (Chapter 7, 7.2), ovaries were collected for total RNA extraction. *Csfm^{op}/csfm^{op}* mice were divided into 2 groups on the basis of a relatively normal oestrous cycle and ovulation (ovulating), and a relatively normal - disrupted oestrous cycle and no ovulation (non-ovulating).

Total cellular RNA was extracted from immature (IO), immature eCG/hCG primed preovulatory (PO), ovulatory (OO) and luteinised ovaries (LO) as well as from ~10⁶ L cells,

~10⁶ MLA cells and murine liver by guanidine thiocyanate extraction (Chapter 2, 2.7.3). Following RNase-free DNase treatment (Chapter 2, 2.7.4), cDNA was synthesised by random primed reverse transcription (Chapter 2, 2.7.6). cDNA prepared from cell lines and murine tissues were used as positive and negative controls to determine primer specificity and to optimise PCR conditions for TNF α and iNOS.

PCR conditions which were found to give a strong signal-to-noise ratio (ie a single product in the absence of products in the negative controls) for each primer pair were for TNF α , 60°C/40 and iNOS, 61°C/40 (Figure 7.4). The β -actin primers amplified a single product from each of the cell lines and murine tissues. Each cDNA generated a single product of the expected size, with no evidence of product generated from contaminating genomic DNA. The TNF α primers amplified a single product from L cell, murine liver and all ovary cDNAs. The iNOS primers amplified a single product from L cell, murine liver and all ovary cDNAs but not from MLA cells.

Whole ovary cDNA collected at metoestrous-1 from naturally cycling adult CSF-1+/± and *csfm^{op}/csfm^{op}* mice was subjected to quantitative PCR amplification (Chapter 2, 6.9). *Taq* DNA polymerase was added to the sample wells containing cDNA, ³²P-CTP and specific primer pairs at two cycle intervals. PCR conditions for each primer pair were those optimised in chapter 3 for β -actin, GM-CSF and c-fms or described above for TNF α and iNOS (Chapter 2, 7.8). PCR products were analysed by gel electrophoresis, transferred to a nylon membrane and quantified by phosphorimage analysis. Linear regressions were performed on the data to determine the initial amount of specific mRNA (Chapter 2, 6.12). For each primer pair, PCR reactions were performed in singleton. Data were expressed as a percent of the mean of CSF-1+/± samples and normalised for β -actin mRNA expression.

Analysis of the 186 bp β -actin PCR product by gel electrophoresis and phosphorimaging (Figure 7.5) confirmed the initial amount of cDNA to be approximately equivalent in most samples, but with some variation. Analysis of the 240 bp c-fms PCR product by gel electrophoresis and phosphorimaging determined mRNA expression to be reduced by approximately 50% in the *csfm^{op}/csfm^{op}* ovary in comparison to the CSF-1+/± ovary regardless of ovulation status (Figure 7.6) (Kruskal-Wallis Test, $P = 0.1042$). Analysis of the 278 bp GM-CSF PCR product by gel electrophoresis and phosphorimaging determined mRNA expression to be approximately 2.5 fold higher in the non-ovulating *csfm^{op}/csfm^{op}* ovary compared to the CSF-1+/± and ovulating *csfm^{op}/csfm^{op}* ovaries (Figure 7.7) (Kruskal-Wallis Test, $P = 0.1139$). Analysis of the 205 bp iNOS PCR product by gel electrophoresis

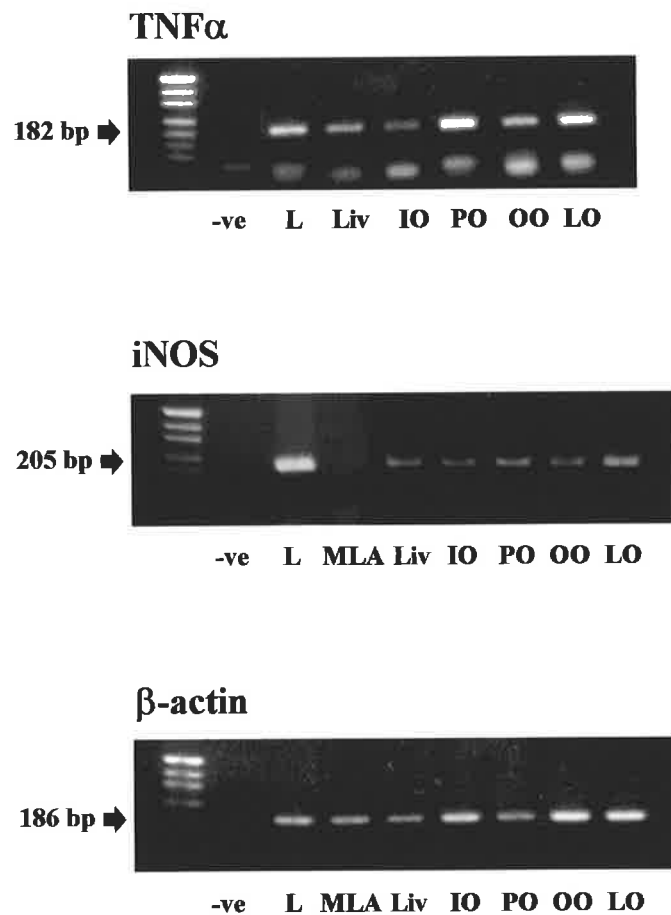


Figure 7.4 TNF α and iNOS PCR primer specificity. Total cellular RNA was extracted from L cells (L), MLA cells (MLA), murine liver (Liv) and also from immature (IO) and immature primed preovulatory (PO), ovulatory (OO) and luteinised (LO) ovary. First strand cDNA was reverse transcribed from RNA using random oligonucleotides. TNF α , iNOS and β -actin PCR products were amplified from positive cell lines, murine liver and ovary cDNAs using *Taq* DNA polymerase and specific primer pairs and analysed by gel electrophoresis in parallel with the molecular weight marker.

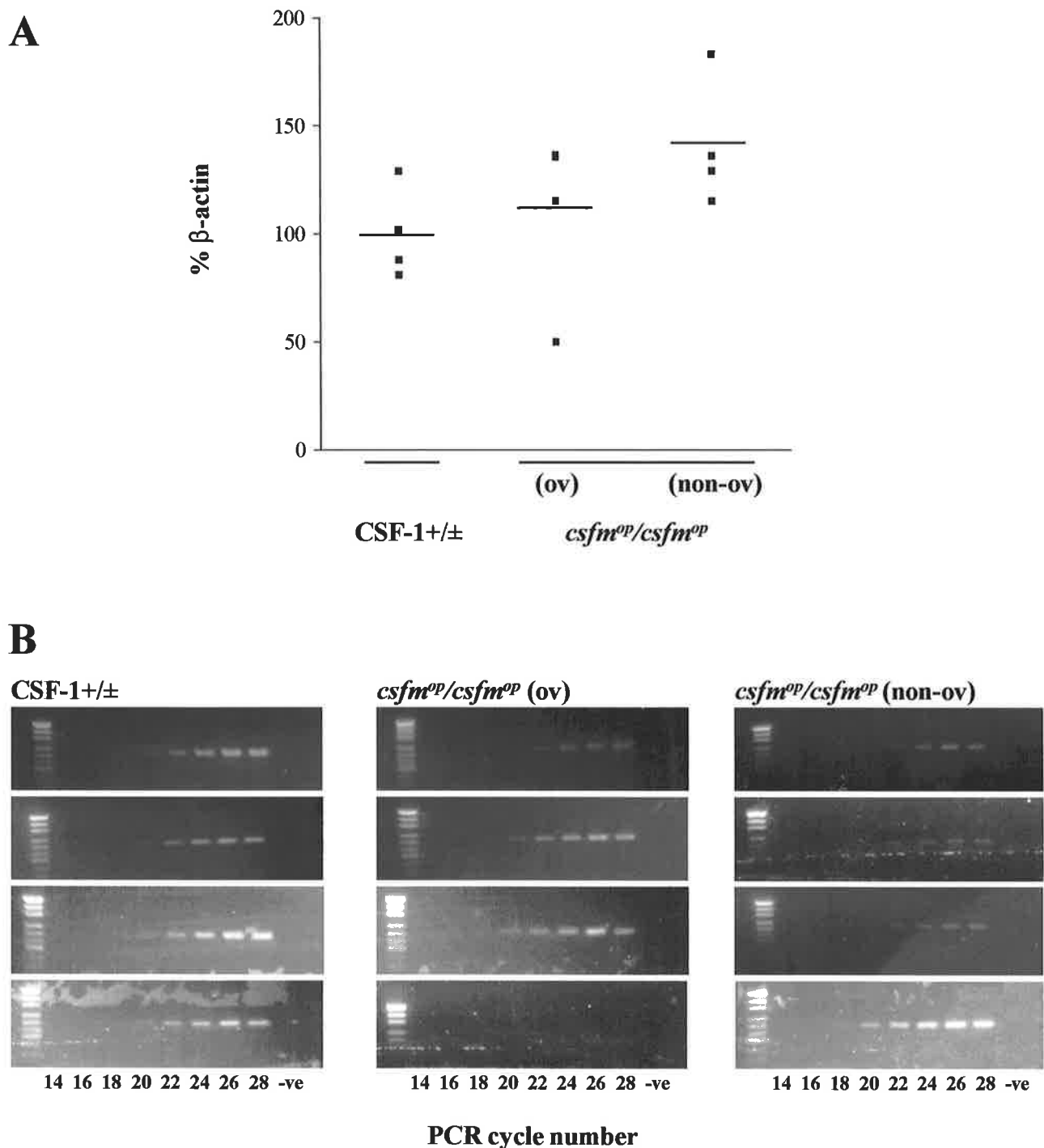


Figure 7.5 β -actin mRNA expression in the ovary of ovulating (ov) CSF-1+/± and *csfm^{op}/csfm^{op}* mice and non-ovulating (non-ov) *csfm^{op}/csfm^{op}* mice. Ovaries were collected at metoestrous-1 following a minimum of 5 completed oestrous cycles. Total cellular RNA was extracted from whole ovaries and cDNA was synthesised by reverse transcription primed by random oligonucleotides. *Taq* DNA polymerase was added to the sample wells containing cDNA, ^{32}P -CTP and primer pairs at 2 cycle intervals. PCR products were analysed by gel electrophoresis along with the molecular weight marker, transferred to a nylon membrane and phosphorimaged. The relative amount of specific mRNA in each sample was determined by linear regression. A) β -actin mRNA levels in the whole ovary. Data graphed as mean and individual data points. B) RT-PCR products.

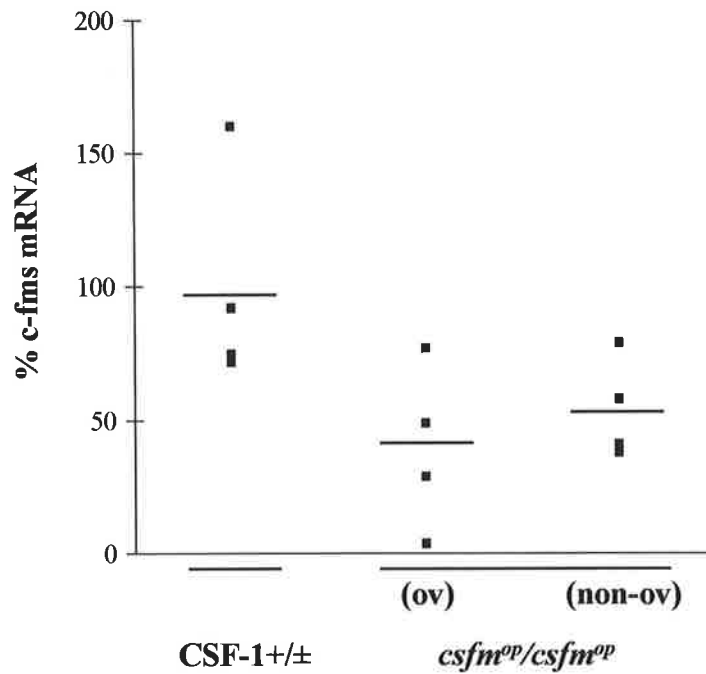
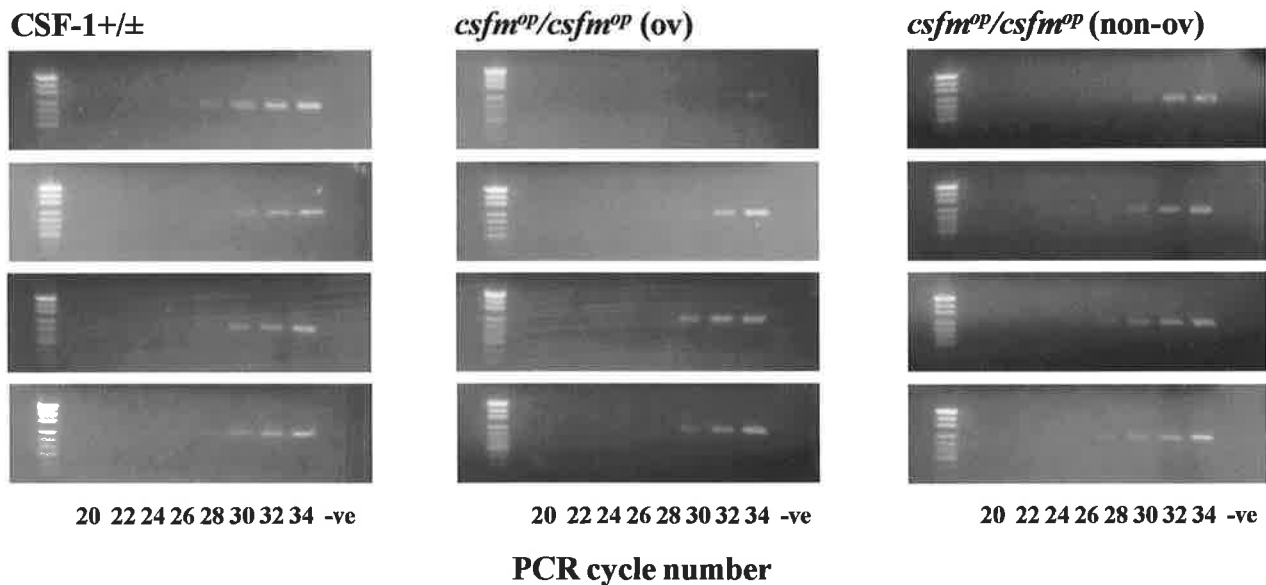
A**B**

Figure 7.6 *c-fms* mRNA expression in the ovary of ovulating (ov) CSF-1+/± and *csfm^{op}/csfm^{op}* mice and non-ovulating (non-ov) *csfm^{op}/csfm^{op}* mice. Ovaries were collected at metoestrous-1 following a minimum of 5 completed oestrous cycles. Total cellular RNA was extracted from whole ovaries and cDNA was synthesised by reverse transcription primed by random oligonucleotides. *Taq* DNA polymerase was added to the sample wells containing cDNA, ³²P-CTP and primer pairs at 2 cycle intervals. PCR products were analysed by gel electrophoresis along with the molecular weight marker, transferred to a nylon membrane and phosphorimaged. The relative amount of specific mRNA in each sample was determined by linear regression. A) *c-fms* mRNA levels in the whole ovary. Data graphed as mean and individual data points, normalised to β -actin mRNA levels. B) RT-PCR products.

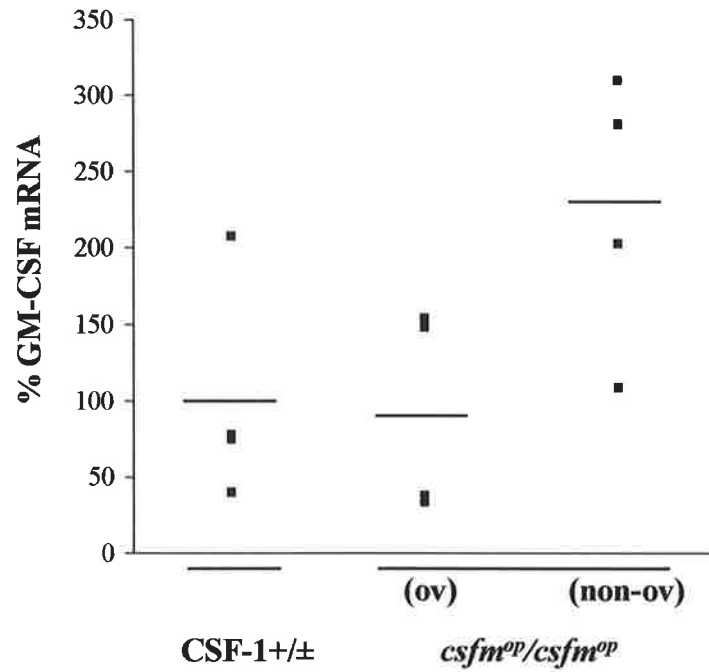
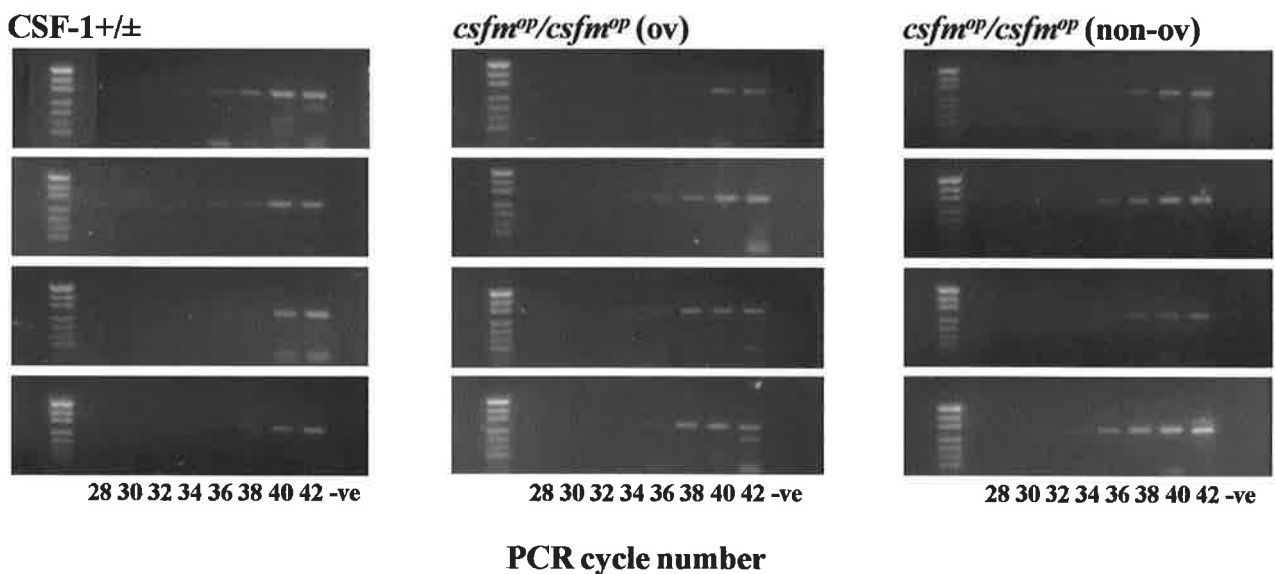
A**B**

Figure 7.7 GM-CSF mRNA expression in the ovary of ovulating (ov) CSF-1+/± and *csfm^{op}/csfm^{op}* mice and non-ovulating (non-ov) *csfm^{op}/csfm^{op}* mice. Ovaries were collected at metoestrous-1 following a minimum of 5 completed oestrous cycles. Total cellular RNA was extracted from whole ovaries and cDNA was synthesised by reverse transcription primed by random oligonucleotides. *Taq* DNA polymerase was added to the sample wells containing cDNA, ³²P-CTP and primer pairs at 2 cycle intervals. PCR products were analysed by gel electrophoresis along with the molecular weight marker, transferred to a nylon membrane and phosphorimaged. The relative amount of specific mRNA in each sample was determined by linear regression. A) GM-CSF mRNA levels in the whole ovary. Data graphed as mean and individual data points, normalised to β -actin mRNA levels. B) RT-PCR products.

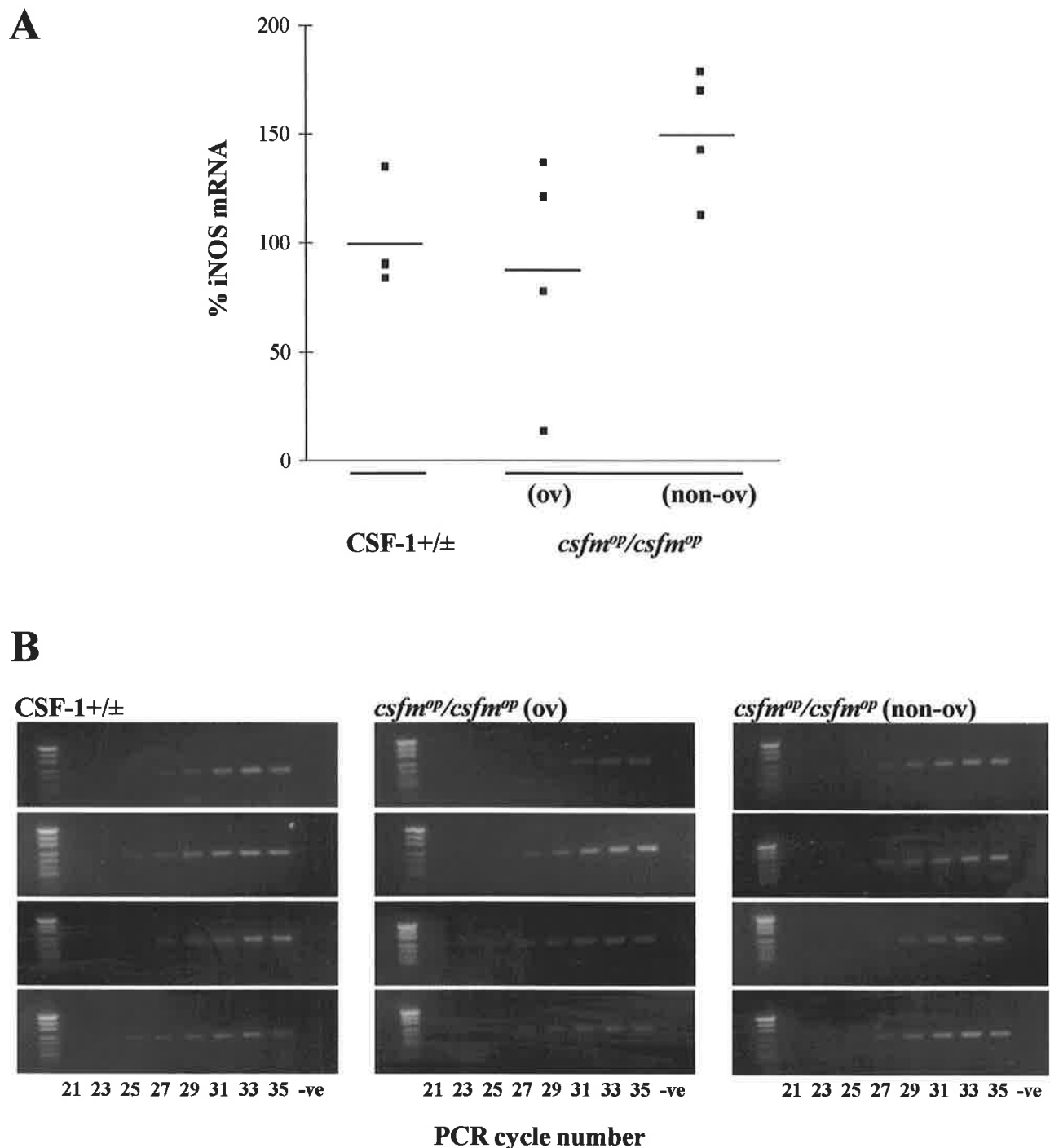


Figure 7.8 iNOS mRNA expression in the ovary of ovulating (ov) CSF-1+/± and *csfm^{op}/csfm^{op}* mice and non-ovulating (non-ov) *csfm^{op}/csfm^{op}* mice. Ovaries were collected at metoestrous-1 following a minimum of 5 completed oestrous cycles. Total cellular RNA was extracted from whole ovaries and cDNA was synthesised by reverse transcription primed by random oligonucleotides. *Taq* DNA polymerase was added to the sample wells containing cDNA, ³²P-CTP and primer pairs at 2 cycle intervals. PCR products were analysed by gel electrophoresis along with the molecular weight marker, transferred to a nylon membrane and phosphorimaged. The relative amount of specific mRNA in each sample was determined by linear regression. A) iNOS mRNA levels in the whole ovary. Data graphed as mean and individual data points, normalised to β -actin mRNA levels. B) RT-PCR products.

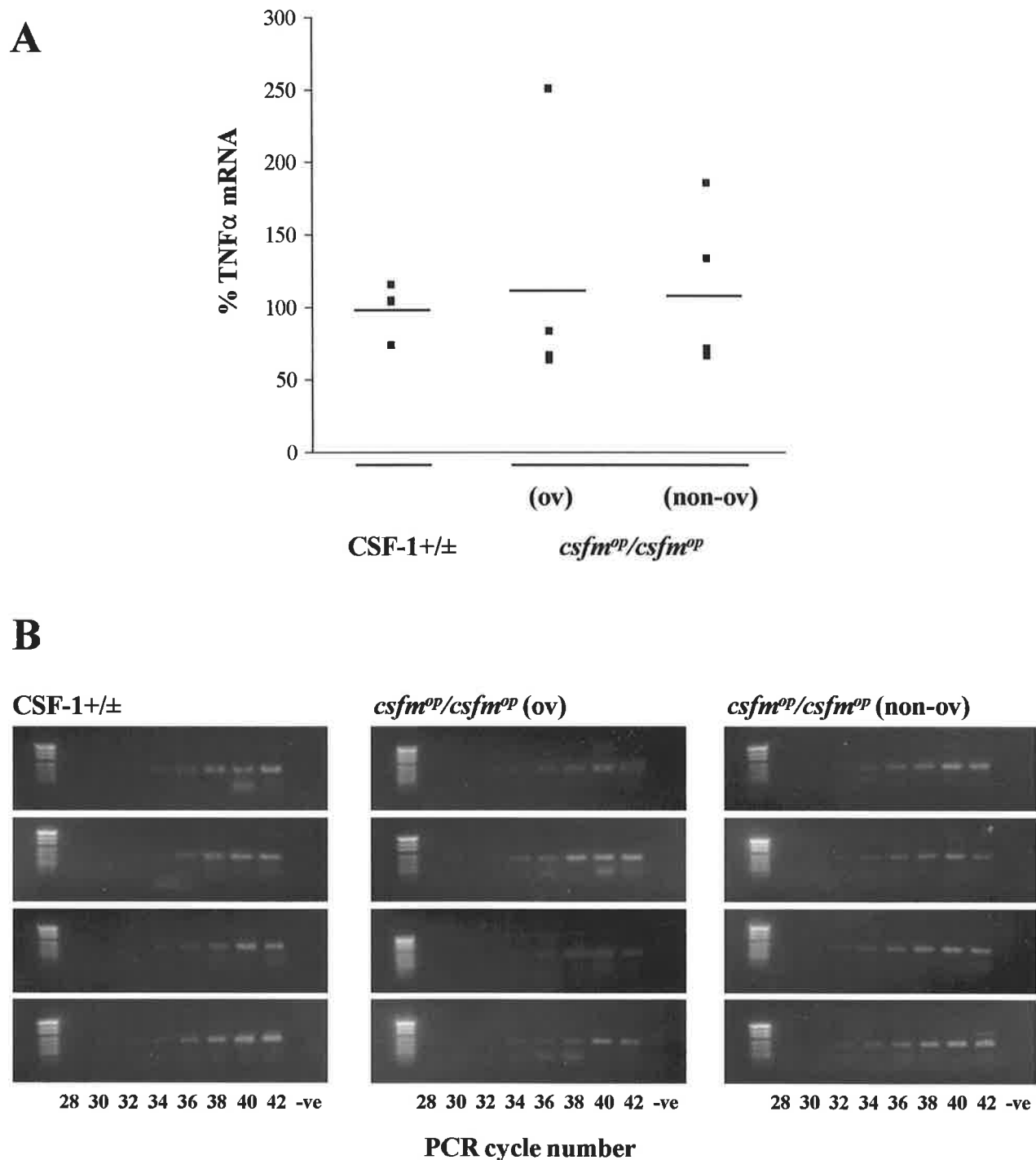


Figure 7.9 TNF α mRNA expression in the ovary of ovulating (ov) CSF-1+/± and *csfm^{op}/csfm^{op}* mice and non-ovulating (non-ov) *csfm^{op}/csfm^{op}* mice. Ovaries were collected at metoestrous-1 following a minimum of 5 completed oestrous cycles. Total cellular RNA was extracted from whole ovaries and cDNA was synthesised by reverse transcription primed by random oligonucleotides. *Taq* DNA polymerase was added to the sample wells containing cDNA, ³²P-CTP and primer pairs at 2 cycle intervals. PCR products were analysed by gel electrophoresis along with the molecular weight marker, transferred to a nylon membrane and phosphorimaged. The relative amount of specific mRNA in each sample was determined by linear regression. A) TNF α mRNA levels in the whole ovary. Data graphed as mean and individual data points, normalised to β -actin mRNA levels. B) RT-PCR products.

and phosphorimaging determined mRNA levels to be increased by approximately 50% in the non-ovulating *csfm^{op}/csfm^{op}* ovary in comparison to the CSF-1^{+/±} and ovulating *csfm^{op}/csfm^{op}* ovaries (Figure 7.8) (Kruskal-Wallis Test, $P = 0.0859$). Analysis of the 182 bp TNF α PCR product by gel electrophoresis and phosphorimaging determined mRNA expression to be comparable in the CSF-1^{+/±} and *csfm^{op}/csfm^{op}* ovaries regardless of ovulation status (Figure 7.9) (Kruskal-Wallis Test, $P = 0.7697$).

In summary, the finding of moderately reduced c-fms mRNA expression in the *csfm^{op}/csfm^{op}* ovary suggests reduced numbers of CSF-1 responsive cells, presumably macrophages in the ovary. Examination of expression of GM-CSF and iNOS mRNA suggests that they may be moderately increased in the non-ovulating ovary, but it is unclear if this increase is associated as a cause or a consequence of ovulation failure. While these findings were not statistically significant and must be considered in the context of the complexity of the experimental technique, the moderate changes in expression are worthy of further investigation.

7.6 *In vivo* steroid production

To investigate the impact of CSF-1 deficiency on steroid production by the ovary, plasma collected from immature primed mice, adult virgin mice following natural ovulation and on day 5 of pseudopregnancy was assayed for progesterone and oestradiol (Chapter 2, 2.8.1, 2.8.2).

Plasma progesterone was significantly reduced in the *csfm^{op}/csfm^{op}* compared to CSF-1^{+/±} mouse (Table 7.3) in the naturally cycling adult following ovulation when non-ovulating animal data are included in the group. However, at all other stages of collection, even when non-ovulating animal data were excluded from the naturally cycling adult (9.5 ± 1.0 , $n = 6$), plasma progesterone concentrations were comparable. These data indicate that progesterone production may be similar or even greater per oocyte ovulated in *csfm^{op}/csfm^{op}* compared to CSF-1^{+/±} mice due to the significant reduction in ovulation rate reported earlier in this chapter (7.4).

Table 7.3 Plasma progesterone of CSF-1+/± and *csfm^{op}/csfm^{op}* mice. Plasma was collected from immature primed and adult naturally cycling mice at metoestrous-1, regardless of ovulation and also from day 5 pseudopregnant mice or following PGF_{2α} (3μg) treatment 18 hr previously. Data presented as mean ± SE, nmol/l.

	CSF-1+/±	n	<i>csfm^{op}/csfm^{op}</i>	n
Immature primed	24.6 ± 3.6	8	27.7 ± 2.2	7
Naturally cycling adult	13.8 ± 1.6	11	8.9 ± 0.8*	8
Day 5 pseudopregnant	148.8 ± 6.9	6	162 ± 25.5	5
Day 5 pseudopregnant (PGF_{2α})	44.5 ± 6.9	5	38.4 ± 14.3	3

* significantly different from CSF-1+/± as assessed by Mann-Whitney Test, *P* = 0.0259.

Plasma oestradiol was not detected in the majority of samples as indicated in Table 7.4. Due to the sensitivity of the assay and the small sample sizes it was not possible to make any conclusions from this data.

Table 7.4 Plasma oestradiol of CSF-1+/± and *csfm^{op}/csfm^{op}* mice. Plasma was collected from immature primed and adult naturally cycling mice at metoestrous-1, regardless of ovulation and also from day 5 pseudopregnant mice or following PGF_{2α} (3μg) treatment 18 hr previously. Data presented as mean ± SE, pmol/l. n = sample number detected/total sample number assayed.

	CSF-1+/±	n	<i>csfm^{op}/csfm^{op}</i>	n
Immature primed	330	1/8	43.3 ± 3.3	3/8
Naturally cycling adult	ND	11	80 ± 20	2/9
Day 5 pseudopregnant	ND	6	ND	5
Day 5 pseudopregnant (PGF_{2α})	ND	5	ND	3

ND = not detected

7.7 Ovarian leukocyte populations

The finding of reduced *c-fms* mRNA expression in the *csfm^{op}/csfm^{op}* ovary in 7.5 suggests that the numbers of macrophages in the ovary may be reduced. To assess the effect of CSF-1 deficiency on the distribution and activation of macrophages in the ovary, immunohistochemistry using mAbs reactive with CD11b, F4/80 and Ia antigens with an indirect immunoperoxidase technique was performed in a preliminary experiment. Ovaries were collected from 2 naturally cycling adult *csfm^{op}/csfm^{op}* mice aged 12 and 16 weeks determined to be in dioestrous and oestrus respectively by vaginal smear on the day of tissue collection. One 12 week *csfm^{op}/csfm^{op}* mouse was administered 20 µg LH-RH agonist (Chapter 2, 2.2.5), followed by 5 IU eCG and 5 IU hCG, and the ovaries were retrieved on the morning following the ovulation of 18 oocytes. Tissue sections were cut from fresh frozen *csfm^{op}/csfm^{op}* and random cycling adult wild type (C57BL/6J) ovaries and stained according to the methods described in chapter 2, 4.

7.7.1 General ovarian morphology

Ovaries collected from naturally cycling *csfm^{op}/csfm^{op}* mice comprised follicles from primordial to antral stages and corpora lutea. The nuclei of granulosa cells of a large proportion of antral follicles appeared pyknotic and displayed signs of migration towards the centre of the follicle, indicative of atresia. Theca cells of these follicles were relatively densely packed and did not appear to be actively steroidogenic in contrast to wild type mice.

7.7.2 Leukocytes in the ovary

Macrophages in addition to neutrophils and dendritic cells were localised predominantly within stromal tissue and the theca layer of follicles of *csfm^{op}/csfm^{op}* ovaries, however positive staining for F4/80, CD11b and Ia were reduced compared to the wild type mice. Macrophages were localised by binding of the macrophage-specific monoclonal antibody F4/80 which reacted with comparatively round cells (Figure 7.10) in contrast to the larger cells with membranous processes identified in wild type ovaries. Cells of similar morphology were also CD11b reactive (Figure 7.11). A population of Ia+ cells with dendritic morphology were identified in the *csfm^{op}/csfm^{op}* ovary (Figure 7.12). These Ia+ cells, present in both the stroma and theca, potentially included both activated macrophages and dendritic

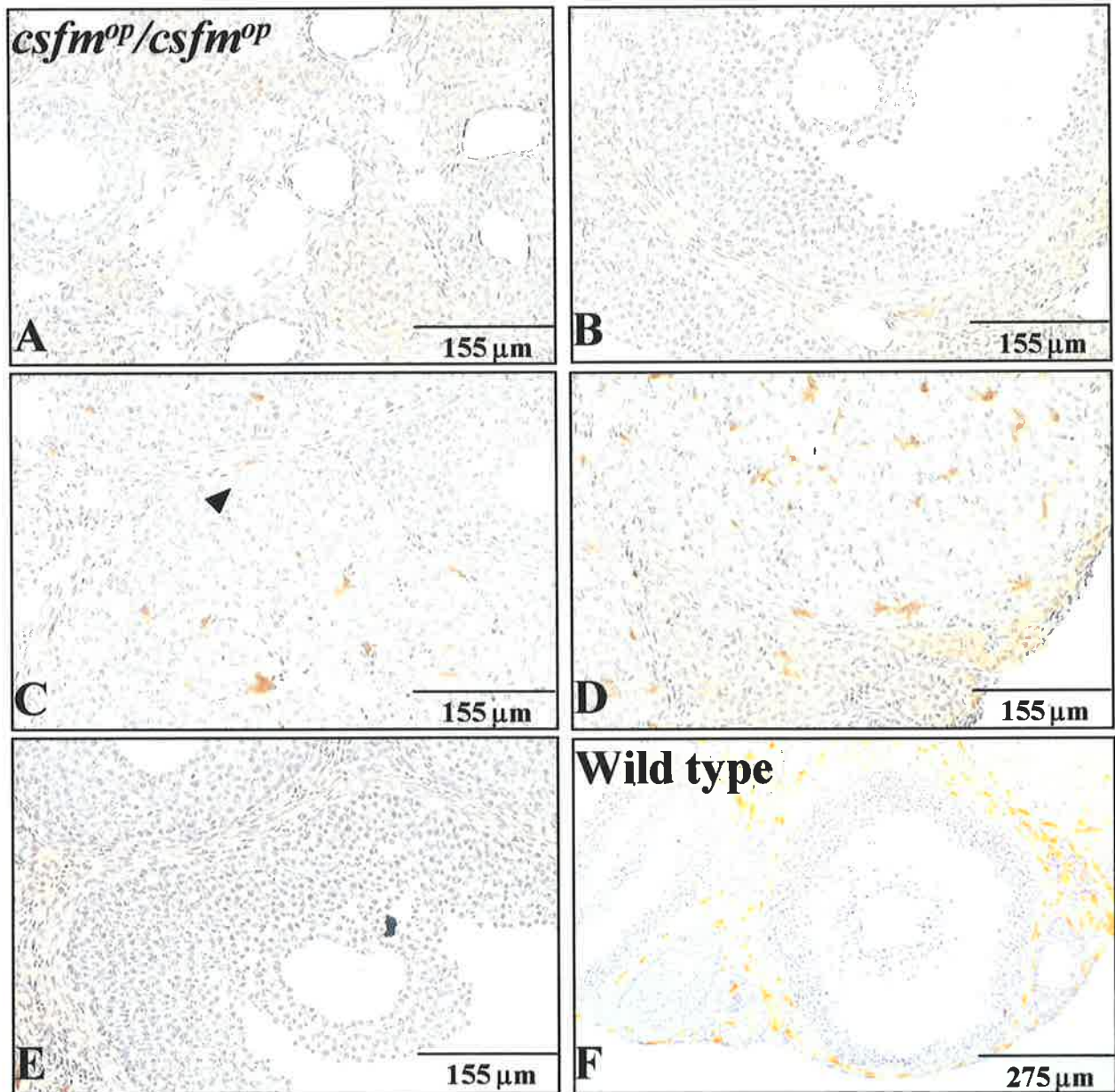


Figure 7.10 Macrophages in the *csfm^{op}/csfm^{op}* ovary. Ovaries were collected from adult mice and sections immunohistochemically stained using the lineage-specific mAb F4/80. No positive staining in the A) stroma and B) theca and minimal positive staining in the C) corpus luteum of a 12 week old dioestrous *csfm^{op}/csfm^{op}* ovary. D) Positive staining in the corpus luteum and stroma the day following ovulation of a 12 week old LHRH primed *csfm^{op}/csfm^{op}* ovary. E) No F4/80 reactive cells in the theca of a 16 week old oestrous *csfm^{op}/csfm^{op}* ovary. F) Macrophages in the stroma and theca of a wild type ovary.

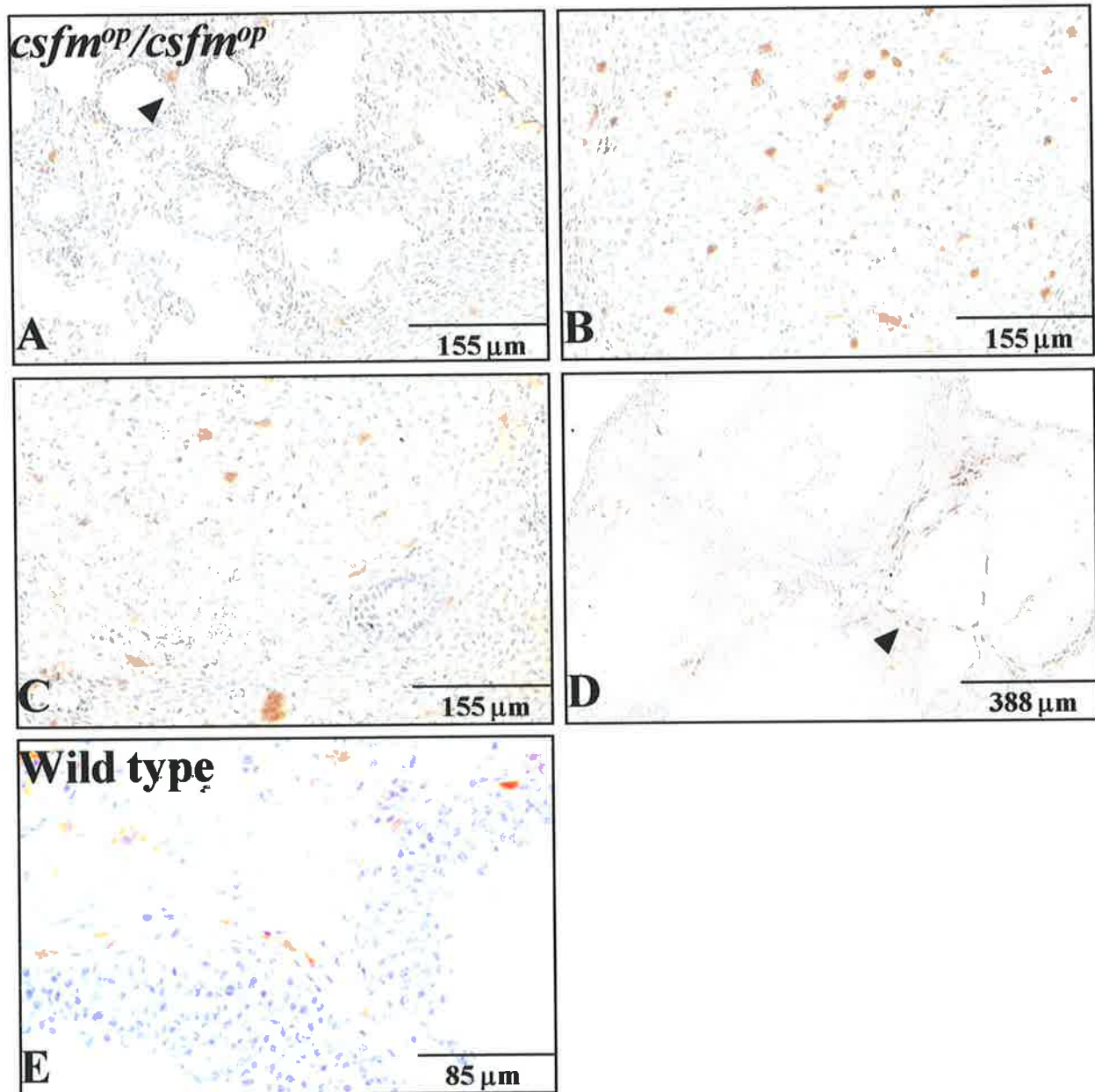


Figure 7.11 Macrophages and neutrophils in the *csfm^{op}/csfm^{op}* ovary. Ovaries were collected from adult mice and sections immunohistochemically stained using the lineage-specific mAb CD11b. Minimal positive staining in the A) stroma and B) corpus luteum of a 12 week old dioestrous *csfm^{op}/csfm^{op}* ovary. C) Positive staining in the corpus luteum and stroma the day following ovulation of a 12 week old LHRH primed *csfm^{op}/csfm^{op}* ovary. CD11b positive cells in the ovary of D) a 16 week old oestrous *csfm^{op}/csfm^{op}* ovary and E) wild type ovary.

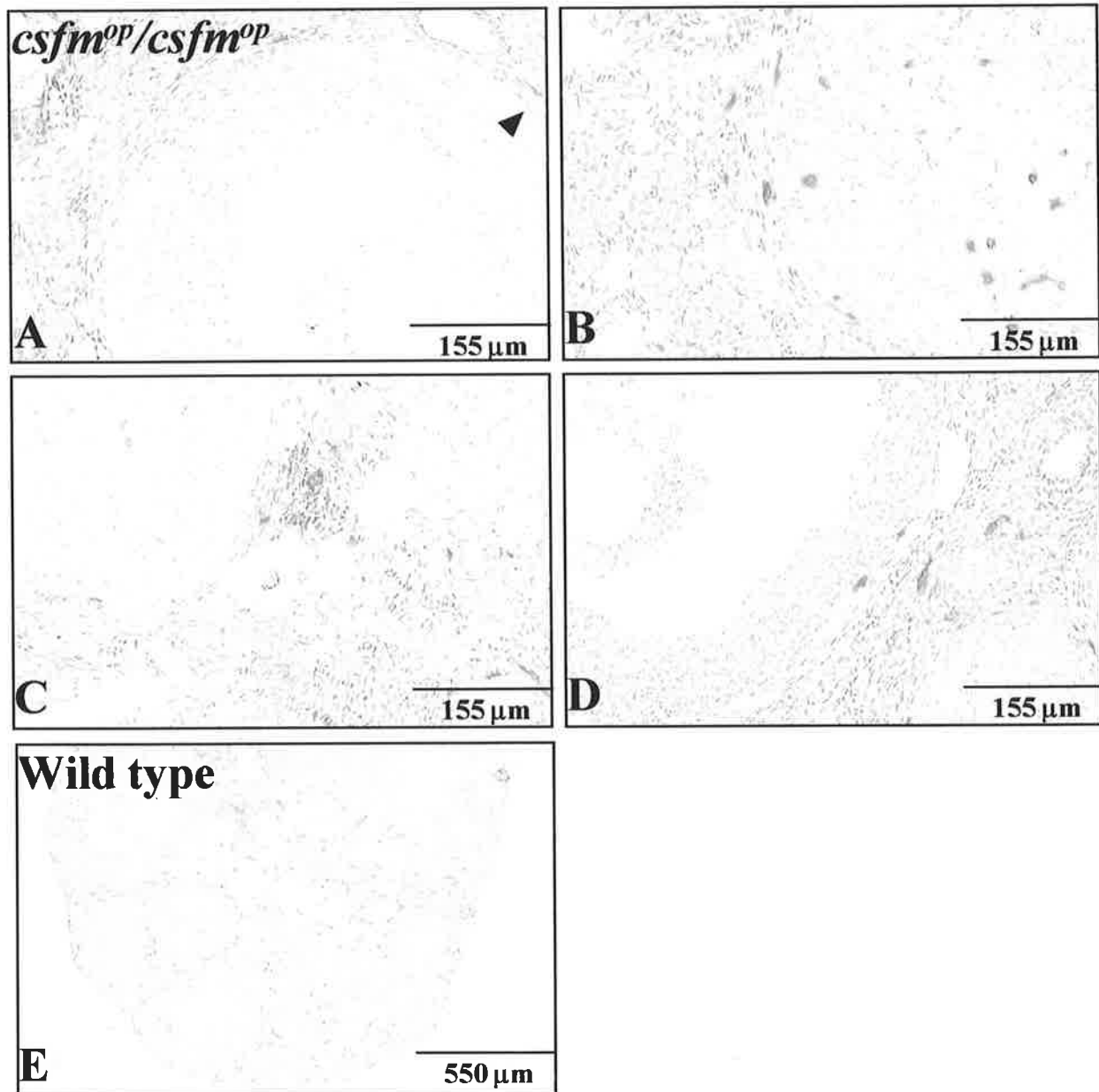


Figure 7.12 Activated macrophages and dendritic cells in the *csfm^{op}/csfm^{op}* ovary. Ovaries were collected from adult mice and sections immunohistochemically stained using the lineage-specific mAb Ia. Minimal positive staining in the A) theca and B) corpus luteum of a 12 week old dioestrous *csfm^{op}/csfm^{op}* ovary. C) Positive staining in the corpus luteum and stroma the day following ovulation of a 12 week old LHRH primed *csfm^{op}/csfm^{op}* ovary. D) Ia reactive cells in the theca of a 16 week old oestrous *csfm^{op}/csfm^{op}* ovary and E) wild type ovary.

cells. Following treatment with LHRH, F4/80, CD11b and Ia positive staining in the stroma was comparable or moderately greater than that in the unprimed *csfm^{op}/csfm^{op}* mice.

Neutrophils have been reported to comprise a large proportion of leukocytes present in the mouse ovary, however they were difficult to distinguish morphologically due to the rounder, less dendriform appearance of macrophages which also stain positive for CD11b.

Ovaries of *csfm^{op}/csfm^{op}* mice contain only a small proportion of myeloid cells in comparison to wild type ovaries. In summary, the finding of reduced macrophages in the *csfm^{op}/csfm^{op}* ovary is the likely explanation for the reduced c-fms mRNA expression in the *csfm^{op}/csfm^{op}* ovary, suggesting these are the predominant cells expressing the CSF-1 receptor. Interestingly, exogenous gonadotrophin agonist stimulated ovulation above that determined for naturally cycling adult *csfm^{op}/csfm^{op}* mice, as reported earlier in this chapter (7.4). This may be as a result of a moderate promotion of chemotactic activity in the ovary. It would be of interest to examine this hypothesis in future experiments.

7.8 Discussion and conclusions

The experiments presented in this chapter provide compelling evidence that infertility associated with the *csfm^{op}/csfm^{op}* genotype is at least in part as a result of ovarian dysfunction, as CSF-1 deficiency perturbed the cellular and biochemical events associated with the oestrous cycle and ovulation. This is illustrated by the extended oestrous cycle and decreased ovulation rate. In addition, the myeloid population is vastly reduced in the *csfm^{op}/csfm^{op}* mouse ovary. These data show that CSF-1-regulated trafficking and survival of macrophages appears to be important in remodelling of the follicle during folliculogenesis and ovulation.

Disruption of the rhythmicity of oestrus by an extension of approximately 4 days in the cycle of some mice or the complete lack of cycling in a few may result from events occurring in the hypothalamus, pituitary and/or ovary. During the course of these studies, Cohen and co-workers (1997) published a study investigating oestrous cycle length of *csfm^{op}/csfm^{op}* mice, reporting an extension of the oestrous cycle to 14 days. While the *csfm^{op}/csfm^{op}* mice appear to stall in metoestrous-2 - dioestrous in both studies, oestrous cycle length differs greatly between the two studies. The length of pseudopregnancies were slightly reduced but comparable to wild type following induced luteolysis, suggesting adequate follicle recruitment to induce oestrous. The GM-/- mouse displays an extended oestrous cycle of 1.5 days

(Chapter 4), suggesting CSF-1 plays a major role in the rhythmicity of oestrous. In contrast to the GM-/- mouse, CSF-1 expression appears important during folliculogenesis and ovulation.

Pollard (personal communication) suggests a feedback defect of the *csfm^{op}/csfm^{op}* hypothalamus. The adult ovary appears responsive to exogenous gonadotrophins, as seen by induced ovulation and a moderate increase in ovarian macrophages following LHRH, eCG and hCG administration. However, the immature primed *csfm^{op}/csfm^{op}* ovary was not overtly sensitive to exogenous gonadotrophins as seen by reduced ovulation rates, but this was in a weight-matched and not age-matched experiment.

In the absence of CSF-1, ovulation is less frequent in the naturally cycling adult and the number of oocytes ovulated is reduced compared to CSF-1+/± mice in both the immature primed and naturally cycling models, suggesting CSF-1 is essential for the process of ovulation to occur optimally. CSF-1 acts as a pro-ovulatory cytokine when administered ip between 96 and 49 hr prior to hCG administration (Nishimura *et al*, 1995). Cohen and co-workers (1997) and Araki and co-workers (1996) reported reduced ovulation for adult *csfm^{op}/csfm^{op}* mice, which increased slightly with the exclusion of non-ovulators from the group (Table 7.5) but still remained significantly less than wild types. Daily rCSF-1 treatment of *csfm^{op}/csfm^{op}* mice restores ovulation rate 100%, and oocytes ovulated to 8.1 ± 0.8 (Araki *et al*, 1996). The precise mechanism of action of CSF-1 on the ovulatory process remains unclear.

Table 7.5 Published ovulation rates of *csfm^{op}/csfm^{op}* mice. Data presented as mean ± SE, as determined by number of oocytes or the number of corpora lutea counted per mouse.

	Ovulators	All	% ovulating
Cohen <i>et al</i> (1997)	$8.6 \pm 0.3^*$	$6.8 \pm 0.4^*$	~ 80%
Araki <i>et al</i> (1996)	$6.0 \pm 0.9^*$	$4.1 \pm 0.9^*$	69%
Chapter 7, 7.4	$8.0 \pm 0.5^*$	$5.0 \pm 1.0^*$	66%

* significantly different from wild type, $P < 0.05$

At least part of the ovulation defect is intrinsic to the ovary, as ovulation can be enhanced in *csfm^{op}/csfm^{op}* follicle culture *in vitro* by the addition of rCSF-1 (J Pollard,

personal communication). CSF-1 may be involved in oocyte maturation through the interaction of CSF-1 produced by granulosa and cumulus cells, and the c-fms receptor present on the oocyte (Arechi *et al*, 1992, Pampfer *et al*, 1991, Haimovici and Anderson, 1993). These data suggest paracrine regulation of oocyte development. Expression of the CSF-1 cell signalling system (Chapter 3, 3.9) in the theca and granulosa cell layers, and also expression of the receptor by the oocyte, coincides with oocyte maturation and rapid tissue remodelling of the theca, including basement membrane degradation and luteinisation of granulosa cells which culminates in ovulation.

One role cytokines have been demonstrated to play in the regulation of ovarian function is in the modulation of steroidogenesis. Progesterone production at ovulation and during pseudopregnancy appears unaltered in the case of CSF-1 deficiency except for a moderate reduction following ovulation in the naturally cycling adult *csfm^{op}/csfm^{op}* mice (including non-ovulators), and although not in this study, on day 5 of pregnancy serum progesterone of *csfm^{op}/csfm^{op}* mice was reduced by 50% of wild type (Cohen *et al*, 1997). Cohen and co-worker (1997) detected serum oestradiol concentrations at all stages of the oestrous cycle, however the surge at proestrous was not observed in *csfm^{op}/csfm^{op}* mice. These data suggest that CSF-1 does not influence steroidogenesis to a large degree.

The reduction in ovulation associated with the *csfm^{op}/csfm^{op}* phenotype could be compared to reductions in ovulation following depletion of ovarian leukocyte populations (Chapter 1, 4.2). In the current study, macrophages were found to be present in the *csfm^{op}/csfm^{op}* mouse ovary, but numbers were severely compromised in comparison to those of the wild type mouse ovary. In addition, F4/80 positive cell density was significantly reduced in *csfm^{op}/csfm^{op}* mouse ovaries associated with the theca cell layer of follicles ranging from stage 3 - 7/8, atretic follicles and corpora lutea at oestrous and day 1 of pregnancy (Cohen *et al*, 1997, Araki *et al*, 1996). Treatment of rCSF-1 increases F4/80 positive cells associated with antral follicles in the wild type (Nishimura *et al*, 1995), but not in the *csfm^{op}/csfm^{op}* ovary to the same degree as the wild type (Araki *et al*, 1996).

CSF-1 appears to be essential for macrophages trafficking and survival in the ovary as seen by vastly reduced populations in the *csfm^{op}/csfm^{op}* mouse ovary devoid of CSF-1. This is in contrast with the relatively normal trafficking of leukocytes into the ovary occurring in the absence of GM-CSF. Macrophages present also displayed a rounded morphology, presumably due to poor intracytoplasmic and microvillous projections (Usuda *et al*, 1994).

The *csfm^{op}/csfm^{op}* ovary is not completely devoid of macrophages, and the phenotype of gross deficiency in tissue macrophages also corrects with age (Begg *et al*, 1993, Begg and Bertoncello, 1993), suggesting that various other chemoattractants, cytokines and/or chemokines must be regulating leukocyte infiltration to a degree. GM-CSF was proposed to provide the necessary support for macrophage growth in the *csfm^{op}/csfm^{op}* mouse, however in GM-/-*csfm^{op}/csfm^{op}* mice deficient in both cytokines, F4/80 monocytes are still present in the peripheral blood, and tissues are occupied by macrophages (Lieschke *et al*, 1994, Nilsson *et al*, 1995).

Although a moderate increase in GM-CSF and iNOS mRNA expression was noted in the non-ovulating *csfm^{op}/csfm^{op}* ovary, it was not seen in the ovulating ovary, implying it was not compensating for the lack of CSF-1 at ovulation. In the uterus and placenta of the *csfm^{op}/csfm^{op}* mouse, TNF α expression did not differ in any major respect from that in the wild type (Hunt *et al*, 1993). However, endotoxin-induced TNF α secretion in *csfm^{op}/csfm^{op}* mice is significantly reduced (Wiktor-Jedrzejczak *et al*, 1992). In the uterus, expression of mRNA for JE (MCP-1), C10, RANTES and MIP1 α suggests the chemokines may regulate leukocyte trafficking during oestrous and early pregnancy (Robertson *et al*, 1998, Pollard, 1998). The follicular fluid is a source of chemoattractant and contains cytokines including IL-1, IL-2, IL-6, TNF and GM-CSF. At the local level, inadequate numbers and/or priming of leukocytes may be the source of disruption of the oestrous cycle and ovulation.

An issue that developed during the course of these studies was whether weight-matched or age-matched wild type mice were a better control for the *csfm^{op}/csfm^{op}* mice. *Csfm^{op}/csfm^{op}* mice are indisputably smaller in size than their littermate controls, and this is an issue, as weight is a confounding factor in the onset of sexual maturity and as gonadotrophin priming decreases in effectiveness with increasing age (reviewed by Hogan *et al*, 1986). As observed in experiment 7.4, ovulation rate was reduced in the *csfm^{op}/csfm^{op}* mouse compared to wild type when matched for weight, but the effect of superovulation in mice matched for age was not determined. Since even moderate undernutrition affects oestrous cycle activity (Hudson *et al*, in preparation), the compromised nutritional intake of *csfm^{op}/csfm^{op}* mice may partially explain their infertility defects. While the liquid diet was fed daily, it did become somewhat dry and therefore increasingly difficult for the *csfm^{op}/csfm^{op}* mice to consume over the course of the day. To aid feed intake, small aluminium frames with a flat top were designed to raise feeding dishes above bedding and limit the chances of the dishes being spilt

and becoming contaminated. Feeding could have been increased to twice daily to better approximate *ad libitum* feed intake, but this was considered too costly and time consuming.

Since *csfm^{op}/csfm^{op}* mice could be subdivided on the basis of oestrous cycle length and ovulation status, factors contributing to this phenomenon were considered. Firstly, compensation by other factors regulating macrophage trafficking, activation and survival may increase cell numbers in some mice more readily than others, and this may be due to the nutritional status of the mouse (Begg and Bertoncello, 1993). Secondly, this effect may be attributed to husbandry, since the colony was established from a small number of founders; it is possible that the selection pressures of breeding may have selected for *csfm^{op}/csfm^{op}* mice with better ovarian function. Hume and Favot (1995) have published a controversial paper suggesting that the *csfm^{op}/csfm^{op}* mouse may not be completely devoid of biologically active CSF-1 due to the existence of splice variants, the potential for expression of these thereby making the system leaky.

Future work would include a more complete characterisation of the leukocyte subsets in the *csfm^{op}/csfm^{op}* ovary. An examination of correlations between ovarian leukocyte numbers and the oestrous cycle length and ovulation rate in individual mice may provide an insight into the factors contributing to the subpopulations of *csfm^{op}/csfm^{op}* mice observed in this study. The perfusion model would allow examination of secretion of biochemical mediators associated with ovulation over the perfusion period and may prove useful in examining the mechanism through which CSF-1 promotes ovulation. The action of CSF-1 on adhesion molecule expression by ovarian epithelial cells is unknown, as is the action of CSF-1 on gonadotrophin production and secretion. These two components of the system may play important roles in the trafficking of leukocytes into the ovary. As *csfm^{op}/csfm^{op}* ovaries are not completely devoid of myeloid cells, other cytokines or chemokines must be acting to promote some trafficking of myeloid cells into the ovary. Certainly prior to the onset of further studies, I believe a refinement of the feeding regime for the *csfm^{op}/csfm^{op}* mice is necessary. The diminished rate of weight gain in these animals is a great concern, as nutrition is clearly a prime determinant of reproductive function.

It is proposed that disruption of the *csfm^{op}/csfm^{op}* oestrous cycle is a result of inadequate macrophage-mediated tissue remodelling. Together, these data suggest that CSF-1 plays a pivotal role in the processes of regulation of folliculogenesis and ovulation. This may be due to direct effects on the oocyte, in addition to the requirement for leukocytes which are crucial for tissue remodelling processes for reproductive function.

Chapter 8

General discussion and conclusions

8.1 Introduction

While it is clear that gonadotrophins and steroids play essential roles in the physiology of the ovary, their action may be mediated locally through the leukocyte-cytokine network. The experiments described in this thesis have identified expression of GM-CSF and CSF-1 within the mouse ovary, where they appear to contribute to the tissue remodelling processes of follicular development, ovulation, luteinisation and luteolysis through their actions on local receptor-bearing cells. The spatial and temporal patterns of expression coincide with the rapid and extensive tissue remodelling required to maintain the rhythmicity of oestrous and functionality of the ovary. The studies described in this thesis indicate that these factors regulate the recruitment, survival and function of leukocyte populations in the ovary. In addition, they may also regulate the function of non-haemopoietic cells. Thus these factors modulate both cellular and biochemical parameters in the ovary during the oestrous cycle and early pregnancy.

8.2 GM-CSF and CSF-1 as mediators of ovarian leukocytes

Ovarian macrophages, granulocytes and possibly dendritic cells appear to be responsive to locally synthesised GM-CSF and CSF-1. CSF-1 plays a pivotal role in regulating the recruitment and survival of macrophages into the ovary as seen by the vastly reduced population of macrophages in the *csfm^{op}/csfm^{op}* mouse ovary which is devoid of CSF-1 protein (Chapter 7). While GM-CSF does not regulate recruitment to any large degree, it does appear to regulate activation (Ia expression) of macrophages and/or dendritic cells at ovulation (Chapter 5). It is proposed that CSF-1-responsive and GM-CSF-responsive macrophages are involved in specific events associated with ovarian function. In addition, the data indicate that these 2 cytokines have independent functions which would not necessarily compensate for each other (Figure 8.1). It is suggested that CSF-orchestrated recruitment and survival of leukocytes is imperative for ovarian function, and that disruption of oestrous, ovulation and biochemical events associated with the cycle are a consequence of inadequate or inappropriate management of these processes (Chapter 4, Chapter 7). These cells are active components of the tissue remodelling process and provide a diverse array of

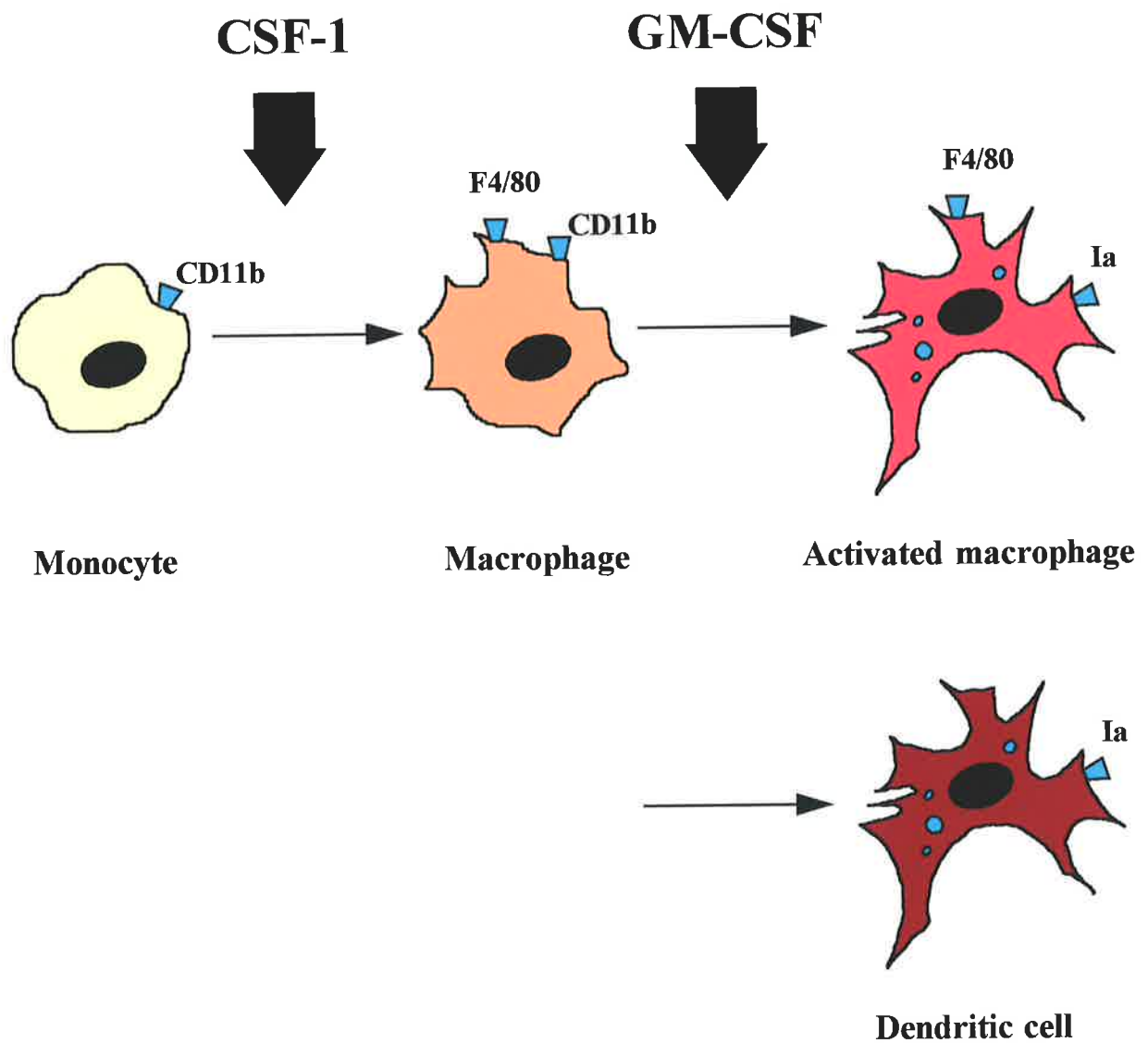


Figure 8.1 The proposed role of CSF-1 and GM-CSF in the determination of macrophage phenotype in the ovary. CSF-1 appears to promote the recruitment and survival of macrophages in the ovary and GM-CSF regulates macrophage activation and/or dendritic cell maturation, reflected by increased Ia expression.

inflammatory mediators, enzymes, enzyme inhibitors, growth and cell function regulatory factors and cytostatic and cytotoxic molecules, they contribute to the tissue remodelling process.

8.3 GM-CSF and CSF-1 as mediators of folliculogenesis and ovulation

The growth and maturation of the follicle culminates in ovulation; the cascade of cellular and biochemical events leading to the degradation of the ovary surface and expulsion of the oocyte. Expression of components of the CSF-1 cell signalling system in the theca and granulosa cell layers and also expression of the receptor by the oocyte coincides with oocyte maturation and the rapid tissue remodelling of the theca, including basement membrane degradation associated with ovulation. In the absence of CSF-1, ovulation is less frequent and the number of oocytes ovulated is reduced (Chapter 7), suggesting CSF-1 is essential for the process of folliculogenesis and ovulation. The contribution of CSF-1 and CSF-1-regulated macrophages to these processes is unclear, but the experimental evidence suggests CSF-1-regulated macrophage recruitment and survival in the ovary is a crucial step in the processes of folliculogenesis and ovulation. In contrast, GM-CSF deficiency does not appear to impact on ovulation (Chapter 4). However, GM-CSF may promote steroidogenesis during the periovulatory period as observed by increased synthesis of androstenedione and moderately reduced ovarian progesterone synthesis per oocyte ovulated in the GM-CSF deficient mouse *in vitro* (Chapter 4).

8.4 GM-CSF and CSF-1 as mediators of luteinisation and luteolysis

The experiments presented in this thesis show the pattern of GM-CSF expression to be consistent with a predominant role for GM-CSF in the development of the corpus luteum from follicle remnants (Figure 8.2). Expression of components of the GM-CSF signalling system in the theca coincides with its rapid remodelling after ovulation, including basement membrane degradation, cell proliferation and angiogenesis. In addition, GM-CSF appears to promote steroidogenesis in the corpus luteum, since reduced ovarian progesterone synthesis

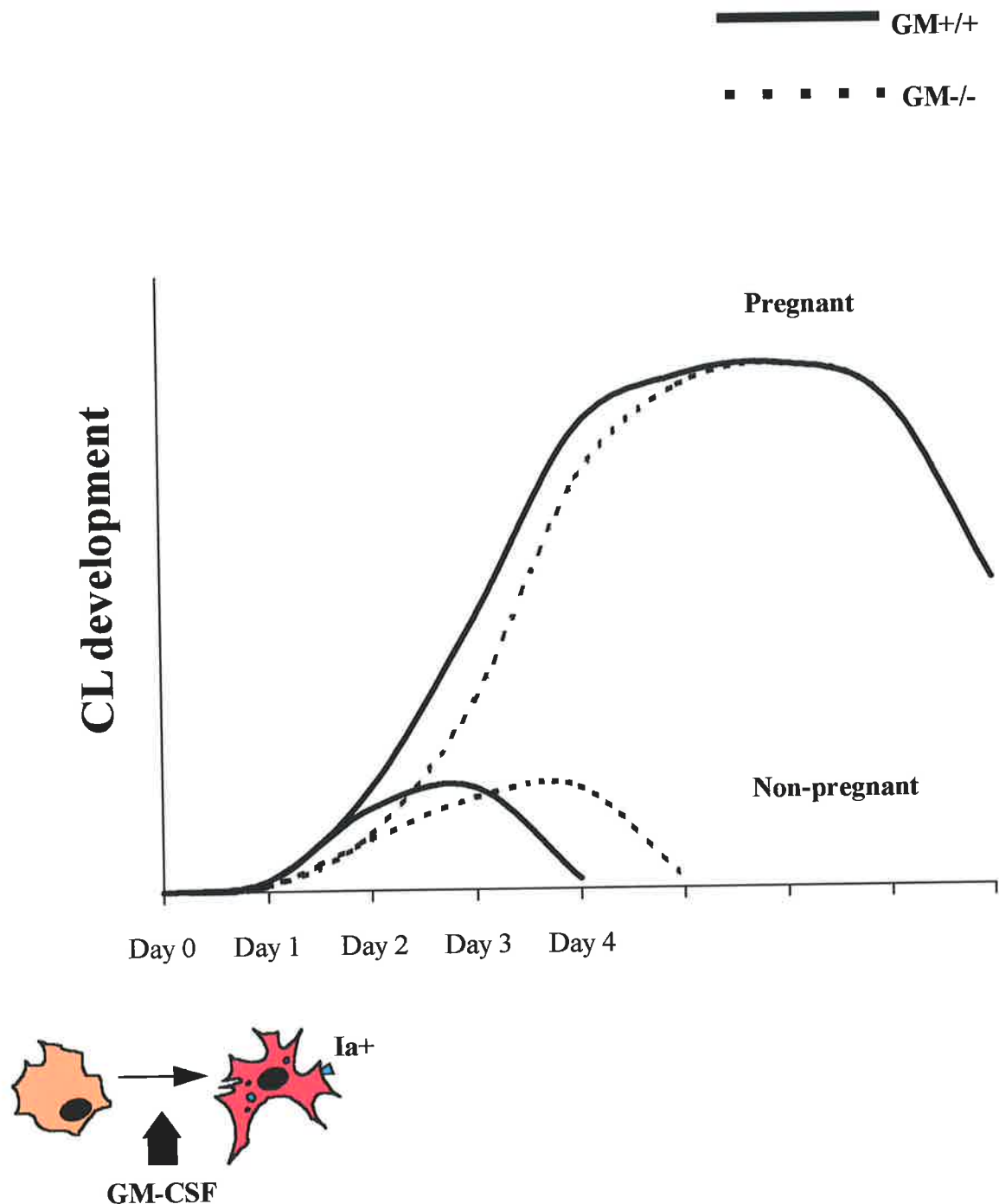


Figure 8.2 A schematic illustration of the proposed role of GM-CSF in the development and demise of the corpus luteum. This model is based on findings that activation of macrophages and/or dendritic cell maturation (Ia expression) at ovulation is required for optimal remodelling of the follicle remnants into the corpus luteum. In the case of a non-pregnant cycle, GM-CSF deficiency results in reduced numbers of activated macrophages and delays the time frame of tissue remodelling. This is reflected in an extension of the oestrous cycle. During pregnancy, development of the corpus luteum is delayed in the GM-CSF deficient mouse. This is reflected in a) diminished ovarian size and b) reduced circulating progesterone. Once the corpus luteum is functional, GM-CSF deficiency does not appear to alter functional regression, as reflected in the normality of pseudopregnancy length.

occurs on day 4 of pregnancy (Chapter 4) in the GM-CSF deficient mouse. During pregnancy, inadequate steroid synthesis may result in inadequate priming and preparation of the uterine environment for optimal pregnancy outcome. During luteal regression, GM-CSF-mediated recruitment and/or survival of macrophages and neutrophils (Chapter 4) may be necessary for the structural, but probably not functional regression of the corpus luteum (Chapter 5).

8.5 GM-CSF and CSF-1 as mediators of steroidogenesis

One role cytokines have been demonstrated to play in the regulation of ovarian function is in the modulation of steroidogenesis. The findings of this thesis (Chapter 4) implicate GM-CSF in the regulation of steroidogenesis, both at ovulation and during development of the corpus luteum. This effect may be worthy of follow-up experiments examining the synthesis of progesterone and androstenedione in the GM-CSF deficient mouse.

8.6 GM-CSF and CSF-1 as components of a leukocyte-cytokine network in the ovary

The findings of this thesis together with current literature can be integrated into a model for the role of GM-CSF and CSF-1 in ovarian function. In this model, both cytokines act to regulate distinct cell populations (Table 8.1, Figure 8.3).

During follicle development, chemotactic factors signal the recruitment of leukocytes, particularly macrophage, into the ovary and survival factors (CSF-1) promote their persistence in the stroma and also their congregation in the theca of the developing follicle. Release of CSFs and other cytokines by haemopoietic and non-haemopoietic cells in the theca and granulosa cell layers acts to regulate oocyte maturation and promote proliferation of the granulosa cells (Araki *et al*, 1996). In the event of follicle atresia, adjacent phagocytic cells are prime candidates to dispose of cellular debris, but may also mediate this activity by the retention of growth factors or release of cytotoxic molecules.

During the periovulatory period, the LH surge is associated with a release of chemotactic factors which act to further promote the recruitment and activation of leukocytes

in the ovary, but particularly in the theca of the preovulatory follicle. The activation of these cells signals the initiation of an inflammatory reaction in the theca, which leads to the eventual weakening and degradation of the ovarian surface and the subsequent expulsion of the oocyte.

The remodelling of the follicle remnants into the highly vascularised endocrine structure of the corpus luteum is then orchestrated. GM-CSF may promote proliferation of endothelial cells and also their migration to ensure complete vascularisation of the corpus luteum. In the lead up to and during pregnancy, leukocyte-derived factors modulate steroidogenesis. Leukocytes may withdraw cell-growth factor support during the demise of the corpus luteum and mediate the clean-up process by phagocytosis of cells and cellular debris. While present, GM-CSF protects macrophages and neutrophils against apoptosis. Perhaps its down-regulation eventually leads to neutrophil and/or macrophage cell death. In addition, GM-CSF may promote antigen presentation by dendritic cells at local lymph nodes and initiate lymphocyte-mediated destruction of the corpus luteum.

Table 8.1 Summary of the proposed functions of GM-CSF and CSF-1 in the ovary during oestrous and early pregnancy.

	GM-CSF	CSF-1
Folliculogenesis		
Oocyte maturation		+++
Leukocyte recruitment/survival		++++
Ovulation		
Leukocyte recruitment/survival		++++
Leukocyte activation	++++	
Steroidogenesis	+	+++
Corpus luteum development		
Leukocyte activation	++++	ND
Steroidogenesis	+++	
Cell proliferation	+	ND
Angiogenesis	+	ND
ECM remodelling	+	ND
Corpus luteum demise		
Leukocyte recruitment/survival	+++	ND

+ = suggested role, ++++ = strongly implicated, ND = not determined

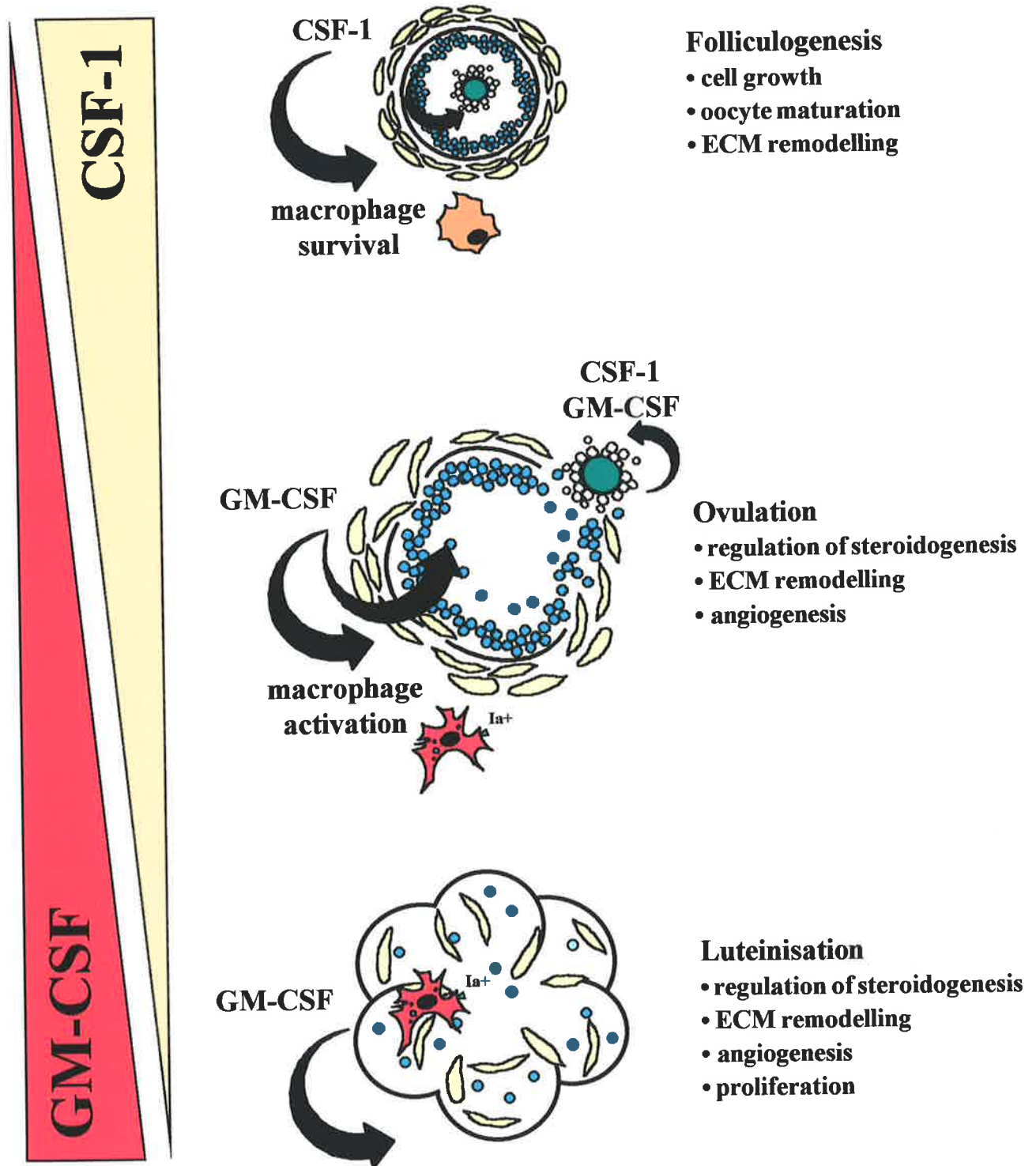


Figure 8.3 A schematic illustration of the proposed roles and importance of GM-CSF and CSF-1 in the leukocyte-cytokine network during folliculogenesis, ovulation and luteinisation.

8.7 Embryotrophic actions of GM-CSF and CSF-1

Paracrine regulation of oocyte maturation and embryonic growth may provide a fine-tuning mechanism to enable regulation of growth and cell differentiation of the embryo prior to and during its invasion into maternal tissues. Temporal and spatial secretion patterns of oviduct and uterine cytokines may target discrete stages of oocyte/embryo growth, and perturbations of this profile both in degree and/or in timing may have long-term repercussions. As early as day 4 of pregnancy, cellular (Chapter 6) and biochemical (Chapter 4) perturbation can be detected in the GM-CSF deficient mouse. These changes suggest that GM-CSF promotes development of the inner cell mass of the embryo, which eventually gives rise to the fetus and also promotes circulating progesterone. The earliest time at which oocyte exposure to GM-CSF is required to maintain optimal pregnancy outcome is undetermined. The developmental potential of *csfm^{op}/csfm^{op}* oocytes remains unclear, but studies by Cohen and co-workers (1997) suggest embryonic viability is reduced in the *csfm^{op}/csfm^{op}* mouse. CSF-1 influences the preimplantation embryo, increasing both its rate of development and, in contrast to GM-CSF, the number of trophectoderm cells in the blastocyst (Bhatnagar *et al*, 1995). The degree to which GM-CSF and CSF-1 impact on oocyte maturation and cumulus cell proliferation will be of great interest to further understand paracrine regulation of folliculogenesis.

8.8 Implications for the human

Experimental evidence derived from human tissues suggests that the findings of this study are relevant to the human situation. The extent to which the knowledge gained from this study will eventually be applied to the clinical setting can only be speculated upon, but in view of the findings, administration of CSF-1 may eventually provide an alternative treatment for anovulation and menstrual disorders. In relation to the optimisation of pregnancy outcomes, certainly the embryotrophic effects of GM-CSF are already being exploited in the clinical research setting (Sjöblom *et al*, 1998).

In addition, cytokines have been implicated in the pathogenesis of ovarian cancer because their production by tumours may contribute to the pathophysiology of the disease

(Malik and Balkwill, 1991). GM-CSF can be shown to enhance colony formation by human ovarian cancer cells (Cimoli *et al*, 1991) and to regulate expression of adhesion molecules, cytotoxicity and production of cytokines by ovarian tumour-associated macrophages (Bernasconi *et al*, 1995). GM-CSF mRNA and CSF-1 mRNA and protein can be found to be expressed in biopsies from cancerous ovarian cells (Kacinski *et al*, 1990, Pisa *et al*, 1992, Ziltner *et al*, 1993, Bauknecht *et al*, 1994, Kommoss *et al*, 1994, Kacinski, 1995, Burke *et al*, 1996, Asschert *et al*, 1997). In addition, elevated serum levels of CSF-1 have been suggest as a marker for gynaecologic cancers and are possibly a prognostic indicator. Cytokines are suitable candidates for involvement in the tissue remodelling associated with cancer and may provide optimal tumour growth-promoting conditions. This may be through their cell growth-promoting characteristics as well as through their ability to influence the local immunological response. Future therapies for ovarian cancer may need to confront the role of cytokines in the pathophysiology of the disease, and perhaps negate their effects by the use of antagonists or inhibitors.

8.9 Future perspectives

This is the first comprehensive study detailing ovarian function of GM-/- mice, and in addition one of the first few studies to examine ovarian function and potential mechanism of infertility of *csfm^{op}/csfm^{op}* mice. The studies described in this thesis along with the studies described by Tamura *et al* (1997), Cohen *et al* (1997), Araki *et al* (1996) and Nishimura *et al* (1995) identify GM-CSF and CSF-1 as components of the leukocyte-cytokine network operating in the ovary during the oestrous cycle and early pregnancy. These studies have, using a variety of techniques, detected expression of mRNA and protein for the ligands and receptors of these CSF cell signalling systems in the ovary, while functional studies in CSF deficient mice have demonstrated disturbances in ovarian function. The degree to which these studies complement or contradict each other has been discussed in detail at the end of each chapter. In addition, these studies demonstrate that while these cytokines are not obligatory, most likely due to the redundancy of the cytokine network, they are required to maintain optimal ovarian function and perhaps indirectly influence processes supported by ovarian steroid hormones, principally the maintenance of pregnancy.

While the studies described in this thesis focussed on the expression of mRNA for components of the GM-CSF and CSF-1 cell signalling systems in the ovary and also the

work for further investigation into the specific and obligatory roles these proteins play in the regulation of ovarian function.

Acidic tyrode solution

NaCl	0.8 g	
KCl	0.02 g	
CaCl ₂ .2H ₂ O	0.024 g	
MgCl ₂ .6H ₂ O	0.01 g	
Glucose	0.1 g	
Polyvinylpyrrolidone	0.4 g	0.4%

Dissolve in milli Q H₂O. Adjust to pH 2.5, make up to 100 ml with milli Q H₂O and filter sterilise. Store in aliquots at -20°C.

Avertin

2,2,2-tribromomethanol	1 g	2%
2 methyl-2-butanol (Ajax, 1306)	1 ml	2%

Make up to 50 ml with 0.9% NaCl. Shake vigorously until completely dissolved or heat to 50°C if necessary, aliquot and store at -20°C. Final concentration 20 µg/µl.

Chloroform : isoamyl alcohol (24 : 1)

Chloroform	96 ml
Isoamyl alcohol	4 ml

Make on the day of use or store in the dark.

DNA Ladder

Ladder	2 µl
Loading buffer 6x	2 µl
Milli Q H ₂ O	8 µl

Make on the day of use.

DNase buffer 5x

Tris-Hcl (2M)	1.25 ml
MgCl ₂ (500 mM)	1 ml
Milli Q H ₂ O	7.75 ml

Autoclave and filter into 1 ml aliquots and store at -20°C.

DNase treatment of RNA

RNA (in Milli Q H ₂ O)	100 µl
DNase buffer 5x	25 µl
RNAse inhibitor	1 µl
DNase I	5 µl

dNTPs (10 mM)

dATP (100 mM)	20 µl	10 mM
dCTP (100 mM)	20 µl	10 mM
dGTP (100 mM)	20 µl	10 mM
dUTP (100 mM)	20 µl	10 mM
Milli Q H ₂ O	120 µl	

Combine, aliquot and store at -20°C.

DAB peroxidase substrate

DAB tablet	1
Urea hydrogen tablet	1

Dissolve tablets in 5 ml Milli Q H₂O, filter through a 0.22 µm filter. Prepare within 30 min of use.

DEPC H₂O

Diethy pyrocarbonate (DEPC) 1 ml

Milli Q H₂O 1 l

Shake, stand overnight at room temperature and then autoclave. Store at room temperature.

Dry food

Caloric content	100g		
MJ/kg	14.3832	Leucine	1.4880
kcal	3437.1084	Arginine	1.1873
Protein	20.8826	Phenylalanine	0.9606
Fat	6.892	Valine	1.0437
Fibre	5.4394	Histidine	0.4948
Calcium	0.7620	Glycine	1.1643
Phosphate	0.5942	Choline	1675.3741
Lysine	1.2381	Salt	0.8216
Methionine	0.3639	Linoleic acid	2.2516
Threonine	0.7733	Ash	5.2176
Isoleucine	0.8782	Moisture	10.0833
Tryptophan	0.2421	Density	0.6755

Dulbelco's PBS (Ca²⁺, Mg²⁺ free, pH 7.4, 10x)

NaCl 80.0 g

Na₂HPO₄ 11.6 g

KH₂PO₄ 2.0 g

KCl 2.0 g

Initially make up to 1l to make 10x stock, dilute 1:10 and adjust pH to 7.4.

Ensure nutritional powder

100g			
Caloric content		Iron	4.37 mg
kcal	450	Manganese	1.2 mg
kJ	1883	Copper	0.52 mg
Caloric distribution		Iodide	34 mcg
Protein	14%	Selenium	20 mcg
Fat	31.5%	Chromium	20 mcg
Carbohydrate	54.5%	Molybdenum	38 mcg
Nutrients		Vitamins	
Protein	15.9 g	A	351 mcg
Fat	15.9 g	D	2.375 mcg
Linoleic acid	8.7 g	E	10.7 mg
Carbohydrate	61.8 g	C	68 mg
Minerals (Ash)	2.5 g	B1	0.72 mg
Moisture	5 g	B2	0.8 mg
Minerals		Niacinamide	10 mg
Potassium	0.67 g	B6	1 mg
Chloride	0.61 g	Pantothenic acid	5 mg
Sodium	0.36 g	K1	18 mcg
Calcium	0.23 g	B12	3.1 mcg
Phosphorus	0.23 g	Choline	136 mg
Magnesium	90 mg	Biotin	150 mcg
Zinc	5.4 mg	Folic acid	200 mcg

Eosin

Eosin (1%)	40 ml
Phloxine (1%)	10 ml
Ethanol (100%)	330 ml
Acetic acid	1.6 ml
Milli Q H ₂ O	20 ml

Combine and store at room temperature.

Ethanol (96%)

Ethanol 100%	240 ml
Milli Q H ₂ O	10 ml

Ethanol (70%)

Ethanol 100%	70 ml
Milli Q H ₂ O	30 ml

Store at -20°C.

Ethidium Bromide

Ethidium bromide	0.5 g	10 mg/ml
Milli Q H ₂ O	50 ml	

Following several hours of stirring. Do not autoclave, but store at room temperature in the dark.

eCG

Equine chorionic gonadotrophin	1000 IU	5 IU/200 µl
NaCl (0.9%)	40 ml	

Dissolve, aliquot and store at -20°C. Use within 2 months.

First strand RT Buffer 5x

Tris-Hcl (pH 8.3)	250 mM
KCl	375 mM
MgCl ₂	15 mM

Supplied.

Guanidine thiocyanate

Guanidine thiocyanate	47.264 g	4 M
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Make up to 100 ml with DEPC H₂O. Autoclave and store at room temperature.

HEPES

HEPES	23.83 g	1 M
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Add to 80 ml Milli Q H₂O, adjust pH with 10 M NaOH to 7.4. Add 1 ml of phenol red, make up to 100 ml and filter sterilise. Store at 4°C.

HCl

HCl	1.25 ml	0.5%
Milli Q H ₂ O	248.75 ml	

Hoechst 33258

Dissolve in milli Q H₂O, aliquot and store at -20°C. Final concentration 1 mg/ml.

hCG

Human chorionic gonadotrophin	1500 IU	5 IU/200 µl.
0.9% NaCl	60 ml	

Dissolve, aliquot and store at -20°C. Use within 2 months.

HTF stock solutions**Stock A**

NaCl	2.965 g
KCl	0.175 g
MgSO ₄	0.025 g
KH ₂ PO ₄	0.025 g
Glucose	0.25 g
Penicillin-G	0.03 g
Na Lactate	1.85 ml

Make up to 50 ml with Milli Q H₂O. Solution keeps for 3 months at 4°C.

Stock B

NaHCO ₃	1.05 g	
phenol red	0.005 g	0.001%

Make up to 50 ml with Milli Q H₂O. Solution keeps for 2 weeks at 4°C.

Stock C

Na Pyruvate	0.051 g
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Make up to 10 ml with Milli Q H₂O. Solution keeps for 2 weeks at 4°C.

Stock D

CaCl ₂ .2H ₂ O	0.262 g
--------------------------------------	---------

Make up to 10 ml with Milli Q H₂O. Solution keeps for 3 months at 4°C.

Stock E

Na HEPES	3.254 g
phenol red	0.005 g

Add to approximately 35 ml Milli Q H₂O, adjust pH to 7.5 using 1M HCl and then make up to a final volume of 50 ml. Solution keeps for 3 months.

HTF-HCO₃ (for culturing in 5% CO₂)

A	1 ml
B	1 ml
C	0.071 ml
D	0.115 ml

Combine with 7.81 ml Milli Q H₂O to make 10 ml. Adjust osmolarity to 280, add 50 mg BSA (Sigma fraction V) for HCO₃-BSA and then filter sterilise. Store at 4°C.

HTF-HEPES (for handling embryos outside incubator)

A	1 ml
B	0.16 ml
C	0.071 ml
D	0.115 ml
E	0.84 ml

Combine with 7.81 ml Milli Q H₂O to make 10 ml. Adjust osmolarity to 280, add 50 mg BSA (Sigma, Fraction V) for Hepes-BSA and then filter sterilise. Store at 4°C.

Loading Buffer 6x

Bromophenol blue	0.05 g	0.25%
Xylene cyanol FF	0.05 g	0.25%
Glycerol	6 ml	30%

Adjust volume to 20 ml with Milli Q H₂O, aliquot and store at -20°C.

LHRH

LHRH agonist	5 mg
Milli Q water	1 ml

Dilute 200 µl of working stock in 4.8 ml 0.9% NaCl. Vortex, aliquot and store at -20°C. Final concentration 20 µg/100 µl.

M199 plus 4% BSA

NaHCO ₃	2 g	26 mM
Gentamycin sulfate	50 mg	50 mg/l
Insulin	21 IU	21 IU/l
BSA	4 g	4%

Dissolve M199 powder, with Earle's salts and L-glutamine, without NaHCO₃ in Milli Q H₂O with other reagents. Adjust osmolarity to 280 - 290 and pH to 7.4 and then make up to 1 l. Filter sterilise and store at 4°C.

Mouse data (adapted from Fox *et al*, 1984, UFAW Handbook, 1987)

Weight		
Weight adult male	20 - 40 g	
Weight adult female	18 - 35 g	
Weight birth	1 - 1.5 g	
Life span	1 - 3 years	
Puberty male	28 - 49 days	
Puberty female	28 - 49 days	
Cleavage of 1 cell embryo	17 - 20 hr post-insemination	
Gestation	19 - 21 days	
Litter size	4 - 12 pups	
Weaning	21 days	
Whole blood volume	5.85 ml/100 g	

Paraformaldehyde in 70 mM phosphate buffer

Solution A

Paraformaldehyde	40 g	4%
Milli Q water	600 ml	

Heat water to 60°C, stir in paraformaldehyde slowly, add a few drops of 1N NaOH until solution clears.

Solution B

Na ₂ HPO ₄	4 g
NaH ₂ PO ₄ ·2H ₂ O	8.45 g

Mix solutions A and B, adjust pH to 7 and make up to 1L. Store at 4°C and use within 1 - 2 weeks.

Phenol (H₂O saturated)

Melt phenol at 70°C and equilibrate with milli Q H₂O using three changes. Aliquot and store at -20°C in the dark.

Phenol (Tris saturated)

Melt phenol at 70°C and tris equilibrate using one change of 0.5 M tris - HCl (pH 8) and then changes of 0.1 M tris - HCl (pH 8) until the phenol pH is > 7.8. Aliquot and store at -20°C in the dark.

PBS plus 1% BSA

Dissolve 0.5 g BSA in 50 ml PBS.

PBS plus 3% BSA

Dissolve 3 g BSA to 100 ml PBS. Store at 4°C.

PBS plus 0.05% Tween-20

Add 0.5 ml Tween-20 to 1 l PBS. Store at 4°C.

Phosphate-citrate buffer

Citric acid	0.51 g	0.1M
NaPO ₄	0.3 g	

Dissolve in Milli Q H₂O, pH to 5 and then make up to 50 ml. Store at 4°C.

PCR buffer 10x

1x reaction buffer is equivalent to:

Tris-HCl (pH 8.8)	67 mM
(NH ₄) ₂ SO ₄	16.6 mM
Triton X-100	0.45%
Gelatin	0.2 mg/ml

PCR cycles

Temperature	Time	Cycle #	Effect
94°C	5 mins	1 cycle	Denaturation
94°C	1 min	40 cycles	Denaturation
58 - 64°C	1 min		Annealing
72°C	1 min		Extension
72°C	7 mins	1 cycle	Final extension

PCR master mix:general

Master mix equivalent to 1 reaction tube:

Milli Q H ₂ O	6.6 µl	
PCR buffer (10x)	2.5 µl	
MgCl ₂ (25 mM)	2.5 µl	2.5 mM
dNTPs (10 mM)	0.5 µl	0.2 mM each
3' primer (100 µM)	0.5 µl	2 µm
5' primer (100 µM)	0.5 µl	2 µm

Combine master mix reagents and aliquot into 13 µl aliquots. Add 2 µl cDNA.

Following the addition of 10 µl *Taq* mix, the final reaction volume is 25 µl.

PCR master mix:quantitative

Milli Q H ₂ O	6.6 µl	
PCR buffer (10x)	2.5 µl	
MgCl ₂ (25 mM)	2.5 µl	2.5 mM
dNTPs (10 mM)	0.5 µl	0.2 mM each
³² P-CTP		15.42 kBq
3' primer (100 µM)	0.5 µl	2 µm
5' primer (100 µM)	0.5 µl	2 µm

Combine master mix reagents and aliquot into 116 μ l aliquots. Add 16 μ l cDNA.

Combine and aliquot down the PCR tray. Following the addition of 10 μ l *Taq* mix, the final reaction volume is 25 μ l.

Powdered dry food and Ensure nutritional powder (50% w/v)

H ₂ O-HCl treated	800 ml
Ensure nutritional powder	225 g
Dry food, stock ration fines	410 g

Total 1 l of diet. Made 2 days prior to feeding and stored in the refrigerator at 4°C.

Primers

Dilute to 100 μ m/l in milli Q H₂O, aliquot and store at -20°C.

PGF_{2 α}

PGF _{2α} (250 μ g/ml)	120 μ l	3 μ g/100 μ l.
NaCl (0.9%)	880 μ l	

Vortex, aliquot and store at 4°C or -20°C. Use within 2 months.

RNA Extraction

Solution D	500 μ l
Sodium acetate	50 μ l
Phenol	500 μ l
Chloroform: isoamly alcohol (24 : 1)	100 μ l

SSC 5x neutralising buffer

Tris-HCl	78.8 g	0.5 M
NaCl	58.44 g	1 M

Make up to 1 l, adjust to pH 7 and autoclave.

Sarkosyl

Sarkosyl	2.5 g	10%
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Dissolve in DEPC milli Q H₂O. Make up to 25 ml, autoclave and store at room temperature.

Sodium acetate 2M, pH 4

Sodium acetate	16.406 g	2 M
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Dissolve in DEPC milli Q H₂O. Make up to 100 ml, autoclave and store at 4°C.

Sodium carbonate coating buffer

NaHCO ₃	8.4g	0.1 M
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Dissolve in milli Q H₂O, adjust pH to 8.2 then make up to 1l. Store at 4°C.

Solution D

Guanidine thiocyanate	2 ml	4 M
Sarkosyl	100 µl	10%
β mercaptoethanol	14.4 µl	0.1 M

Combine and store at 4°C.

Sulphuric acid

Sulphuric acid	98.07 ml	2 M
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Add to milli Q H₂O and make up to 250 ml. Store at room temperature.

TAE Buffer 50x

0.5 M EDTA; pH 8 (or 18.61g EDTA)	100 ml	1 mM
Glacial acetic acid	57.1 ml	20 mM
Tris base (Sigma, T-1503)	242 g	40 mM

Dissolve in milli Q H₂O to a volume of 1l, adjust pH to 7.2 and store at room temperature.

Tissue buffer

Tris (1 M; pH 8)	5 ml
NaCl (4 M)	2 ml
EDTA (0.5 M; pH 8)	20 ml
SDS (10%)	10 ml
Milli Q H ₂ O	63 ml

TE buffer

Tris - HCl (1 M; pH 8)	5 ml
EDTA (0.5 M; pH 8)	1 ml

Dissolved in milli Q H₂O to a volume of 0.5 l. Autoclave and store at room temperature.

Transfer buffer

NaCl	35.06 g	0.6 M
NaOH	16 g	0.4 M

Make up to 1 l on the day.

tRNA

Dissolve in milli Q H₂O and store at -20°C.

Appendix 2 Scoring criteria for perfused ovaries for further analysis

Each perfusion was scored in order to ascertain the 'quality' of each preparation. The method below was used to score points for each perfusion according to each parameter of the perfusion, indicating importance to the normal function of the ovary. Scoring took into account the progesterone levels before and during perfusion, flow rate during the perfusion and the actual ovulation event itself. Initial progesterone level (indicative of the LH surge and *in vivo* ovulation) was assessed. If 60 nmol/l, the perfusion scored -5, between 50 and 60 nmol/l the perfusion scored -4, and if > 450 nmol/l the perfusion scored -1. Peak progesterone not occurring at $t = 1, 2$ or 3 , scored -5 as irregular progesterone levels throughout the perfusion period can indicate improper perfusion of blood vessels in the ovary. To assess flow rate, a mean $\pm$ SD flow rate over the perfusion period was determined which took into account any changes in flow rate during the perfusion. Changes could indicate leakage or blockage of the preparation, however smaller changes could be indicative of changes in pressure not of the state of the preparation. A standard deviation of flow rate > 20 scored the perfusion -1 and > 40 scored the perfusion -2. The preparation was then scored as in Table 1.

Table 1 Perfusion flow rate scoring system. Flow rate defined as the time taken for 1 ml to perfuse the ovary.

Mean flow rate (secs)	Grade	Score
55 - 74	Excellent	0
45 - 54	Good	0
75 - 84	Good	0
85 - 109	Slow	-2
> 110	Very slow	-4
30 - 44	Fast	-4
< 29	Very fast	-5

If no oocytes were ovulated, the perfusion sample scored -1. After scoring all aspects, a total of -5 resulted in rejection of the perfusion sample from the experimental group.

Abbott Australasia Kurnell NSW **Australia**
Ajax Chemicals Auburn NSW **Australia**
Albert Einstein College of Medicine of Yeshiva University Bronx NY **USA**
Aldrich Chemical Company Milwaukee WIS **USA**
American National Can Greenwich CT **USA**
American Type Culture Collection Rockville MD **USA**
Amersham International Amersham **UK**
Animal Resources Centre Canning Vale WA **Australia**
Baxter Healthcare Old Toongabbie NSW **Australia**
BDH Laboratory Supplies Poole **UK**
Beckman Instruments Somerset NJ **USA**
Becton Dickinson & Co Franklin Lakes NJ **USA**
Biotech International Bentley WA **Australia**
Boehringer Mannheim Mannheim **Germany**
Bresatec Thebarton SA **Australia**
Bright Instrument Company Huntingdon **England**
Caymen Chemical Company Ann Arbor MI **USA**
Dako Glostrup **Denmark**
David Bull Laboratories Mulgrave VIC **Australia**
Davis and Geck Cyanamid Medical Device, Anyang **Korea**
Delta West Bentley WA **Australia**
Diagnostic Systems Laboratories Webster TEX **USA**
Dynatech Laboratories Gernsey Channel Islands **UK**
Dynek Hendon SA **Australia**
Eastman Kodak Company New Haven CT **USA**
Eppendorf Hamburg **Germany**
Fluka Biochemica Buchs **Switzerland**
Forma Scientific Marietta OH **USA**
Fotodyne Incorporated New Berlin WI **USA**
Genzyme Corporation Cambridge MA **USA**

Gibco-BRL, Life Technologies Inc Gaithersburg MD USA
Graphpad Software San Diego CA USA
Hewlett Packard Palo Alto CA USA
Histo Labs Riverstone NSW Australia
Hybaid Teddington Middlesex UK
ICN Pharmaceuticals Costa Mesa CA USA
Institute of Medical and Veterinary Science Gillies Plains SA Australia
Intervet International BV-Boxmeer-Holland Lane Cove NSW Australia
Jandel Corporation San Rafael CA USA
Johnson and Johnson Medical Products North Ryde NSW Australia
Jurox Silverwater NSW Australia
Kimberly-Clark Milsons Point NSW Australia
Leading Edge Marion SA Australia
Ludwig Institute for Cancer Research Parkville VIC Australia
Merck Kilsyth VIC Australia
Miles Laboratories Elkhart IN USA
Millipore Corporation Bedford MA USA
Molecular Dynamics Sunnyvale CA USA
National Institute of Diabetes and Digestive and Kidney Diseases Bethesda MD USA
Novo Nordisk Pharmaceuticals Pty Ltd North Rocks NSW Australia
Orion Laboratories Welshpool WA Australia
Peter MacCullum Cancer Institute East Melbourne VIC Australia
Pharmacia Biotech Uppsala Sweden
Pharmingen San Diego CA USA
Pierce Chemical Company Rockford IL USA
Polaroid Australia Pty Ltd North Ryde NSW Australia
Polaroid Corporation Cambridge MA USA
Promega Corporation Madison WI USA
Ridley Agriproducts Epping NSW Australia
Savant Instruments Farmingdale NY USA
Sakura Finetek Torrance CA USA
Schliecher and Schuell Dassel Germany
Shandon Scientific Cheshire UK

Sigma Chemical Company St Louis MO USA

Sorin Biomedica Diagnostics Vercelli Italy

SPSS Chicago ILL USA

Terumo Corporation Piscataway NJ USA

The University of Adelaide Adelaide SA Australia

The Queen Elizabeth Hospital Woodville SA Australia

The Walter and Elisa Hall Institute of Medical Research Parkville VIC Australia

Whatmans International Maidstone UK

Wild Herbrugg Switzerland

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Erratum

Page 26 disulfide should read disulphide

Page 75 reproductive should read ovarian

Page 124 oestrus should read oestrous

Page 125 oestrous should read oestrus

Page 127 citing should read sighting