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Ovarian Macrophages and the Regulation of Ovarian Function

A thesis submitted for the degree of

Doctor of Philosophy

By

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ABSTRACT

The presence of macrophages within ovarian tissue has been acknowledged for many years. There is substantial evidence showing typical macrophage products such as interleukin-1-beta (IL-1β) and tumour necrosis factor alpha (TNFα), can significantly influence ovarian functions. To date the specific role these cells play in ovarian function has only been postulated. It was the aim of the work presented in this thesis to determine firstly, if these cells are critical to normal ovarian function, secondly to develop a method for isolating these cells from other ovarian cells types and thirdly, to examine the cytokine profile of these cells with the intention of determining which important inflammatory cytokines these cells are producing.

Ovarian macrophages were demonstrated to be critical for normal ovarian function by depleting this population using intrabursal injection of liposome-encapsulated clodronate (CLi), liposome-encapsulated saline (SLi) or saline alone (S) in gonadotrophin-primed adult mice, either 84 hrs (day -3) or 36 hrs (day -1) prior to ovulation. Injection of CLi on day -1 did not affect ovulation rates, while administration on day -3 caused a significant reduction in ovulation rate from 9.13 ± 0.9 down to 5.25 ± 0.6 ($p<0.05$). Examination of macrophage distribution within the theca and stroma of preovulatory ovaries with monoclonal antibodies to the murine macrophage antigens macrosialin (FA/11), MHC class II (anti-Ia) and F4/80 revealed following CLi treatment on day -1 a reduction in macrosialin positive macrophages in the theca at ovulation while CLi treatment on day -3 reduced the numbers of Ia positive and macrosialin positive macrophages present in the theca. When the subsequent estrous cycle was monitored by vaginal smearing the metestrus-2 /diestrus stage was found to be extended in CLi-treated animals, from 3.4 ± 0.4 days to 7.5 ± 1.3 days ($p<0.05$). These results suggest that thecal macrophages may be involved in the regulation of follicular growth or rupture, as

well as being important for the normal progression of the estrous cycle. A method for isolating ovarian macrophages was then developed. Initial experiments optimised the tissue digest conditions and demonstrated that ovarian dissociation at room temperature in collagenase/DNase solution made up in alpha-minimum essential media (αMEM) with added calcium chloride was best for recovery of maximum live macrophages. Ovarian single cell suspensions were then incubated with the specific rat monoclonal antibodies anti-Ia (MHC-II) and F4/80, and then incubated on anti-rat coated antibody panning plates. Cells bound to the plate were shown to be viable, 98% pure and did not produce detectable levels of progesterone. The numbers of cells isolated per mg tissue increased significantly across the gonadotrophin stimulated reproductive cycle with maximum numbers recovered 24 and 48 hrs post ovulation. The isolated cells were more phagocytic after ovulation (75-80%) in comparison to before ovulation (55-60%, $p<0.04$) while conditioned media from cells both before and after ovulation did not significantly influence PHA-stimulated proliferation of adherence purified splenocytes. This isolation method was then used to examine the cytokine profile of ovarian macrophages across the gonadotrophin-stimulated reproductive cycle. Macrophages were isolated from ovaries of groups of 8-10 immature mice at differing stages of the pregnant mare serum gonadotrophin (PMSG)/human chorionic gonadotrophin (hCG)-stimulated cycle using the anti-Ia or F4/80 antibodies. Messenger RNA was isolated from some groups for reverse transcription (RT) and quantitative real-time PCR (QRT-PCR) analysis while others were cultured for 24 hrs and conditioned media collected for analysis of protein content. Messenger RNA expression for the cytokines IL-1β, interleukin-1 receptor antagonist (IL-1ra) and TNFα was transiently stimulated following the administration hCG (increases of 5-7 fold, 4 fold and 5 fold respectively, $p<0.05$) while no significant changes in the levels of mRNA for inducible nitric oxide synthase (iNOS), interleukin-10 (IL-10) or transforming growth factor-beta 1 (TGF-β1) were detected. Protein levels in the conditioned

media did not mirror the changing messenger RNA levels. Ovarian Ia positive cells were found to produce limited amounts of IL-1β following hCG administration while although nitric oxide (NO), IL-10 and TNFα were detected with small fluctuations occurring post hCG administration these changes were not found to be significant. The selection of transcripts and proteins these cells produce show that they are not classical inflammatory cells and thus may not be the primary source of inflammatory molecules with known roles in ovulation.

The work in this thesis has shown that ovarian macrophages are essential for normal ovarian function and that these cells appear to be of an anti-inflammatory phenotype. The major roles of these cells may therefore be in minimising tissue damage occurring following the ovulatory event and tissue reorganisation in subsequent corpus luteum formation and regression.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any University or other Tertiary Institution and, to the best of my knowledge and belief contains no material previously published or written by another person, except where due reference has been made.

I give consent for this thesis, when deposited in the University of Adelaide Library, to be available for loan and photocopying.

K.H. Van der Hoek 6/12/2004

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ABBREVIATIONS

CL	corpus luteum
CLi	clodronate liposome
cNOS	constituitive nitric oxide synthase
CT	threshold cycle
DAB	diaminobenzidene
DE	diestrus
DNAse I	deoxyribonuclease I
EDTA	ethylenediamine tetraacetic acid
eNOS	endothelial nitric oxide synthase
FSH	follicle stimulating hormone
HBSS	hanks buffered saline solution
hCG	human chorionic gonadotrophin
HI-FCS	heat-inactivated foetal calf serum
HPRT	hypoxanthine guanine phosphoribosyl transferase
H-RPMI	hepes buffer RPMI
IL-10	interleukin 10
IL-1β	interleukin-1-beta
IL-1ra	interleukin-1- receptor antangonist
iNOS	inducible nitric oxide synthase
LH	luteinising hormone
LHRH	luteinising hormone releaseing hormone
ME	metestrus
αMEM	minimum essential medium alpha
MHC	major histocompatability complex
mRNA	messenger ribonucleic acid
NMS	normal mouse serum
NO	nitric oxide
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	proestrus
PHA	phytohaemagglutnin
PMSG	preganant mare serum gonadotrophin
RPE	R-phycoerythrin
SLi	saline liposomes
TGFβ	transforming growth factor beta
TNFα	tumor necrosis factor alpha
VIA	video image analysis

Chapter One

1 LITERATURE REVIEW

1.1 INTRODUCTION

The adult ovary is an active endocrine organ that contains and nurtures the female germ cells or oocytes. It is responsible for the regulated release of mature oocytes from the follicles of the ovary into the reproductive tract for fertilisation and the secretion of steroid hormones that will ensure the oviduct and uterus are prepared to support, embryo development and implantation, should fertilisation occur. If fertilisation or implantation is unsuccessful, then the ovary must initiate a new cycle of growth and maturation of new follicles and their oocytes. This results in ovarian cycles of follicular growth and atresia, steroid secretion, and tissue remodelling. The mechanisms by which these important processes are controlled in different mammalian species are complex. Interactions between steroid hormones released from the ovary and gonadotrophins from the pituitary are clearly the main regulatory mechanisms, but it is evident that complex interactions between the reproductive, immune [1, 2] and metabolic systems exist [3, 4]. Furthermore, following gonadotrophin stimulation the local signalling systems that operate within the normal ovary to promote follicular growth, ovulation and corpus luteum growth and regression, are not clearly defined.

Macrophages are cells of the immune system with roles in immunity and tissue homeostasis. They are derived from blood borne monocytes that migrate into the peripheral tissues and differentiate in response to the local microenvironmental signals to assume a functional phenotype. Their roles may include the phagocytosis and degradation of foreign organisms or apoptotic tissues, regulation of local immune and inflammatory responses, and tissue remodelling and repair. The presence of macrophages within the ovary has been recognized for many years, although their function remains undefined. .

This review describes basic ovarian and macrophage functions and then explores current evidence implicating these cells in the regulation of ovarian function.

1.2 BASIC OVARIAN FUNCTION

The ovary is composed primarily of growing and atretic follicles, developing and regressing corpora lutea CL and stromal/interstitial tissue. All components are present simultaneously in the adult ovary until menopause, with the proportion of each dependent on the stage of the reproductive cycle and age.

1.2.1 FOLLICLE GROWTH

The follicles are the components of the ovary that protect and provide for the developing oocytes. Follicles arise from resting primordial follicles consisting of an oocyte arrested in prophase of the first meiosis surrounded by a single layer of flattened epithelial cells and a basement membrane. They are located in the cortical region of the adult ovary and if the appropriate stimulus is provided will grow in size and cell composition and number, to large preovulatory or graafian follicles (Figure 1-1). In the rodent, growth to the preovulatory stage takes 19 days [5, 6]; consequently growing follicles destined for ovulation in subsequent cycles are present during a single estrous cycle. In the mouse follicular development can be divided into 8 stages based on the number of granulosa cells and the stage of oocyte growth [7]. Similar patterns and classification systems have been established in the development of rat and human follicles [8-10]. For the purposes of this introduction follicle growth will be described as preantral, antral and preovulatory growth (reviewed by Johnson [11] and Greenwald [12]).

Primordial follicles become primary follicles following the transformation of the flattened epithelial cells into layers of proliferating cuboidal granulosa cells and, following synthesis of RNA, an increase in oocyte volume. The exact mechanism/s that initiate the growth of quiescent primordial follicles are ill defined although several growth factors have

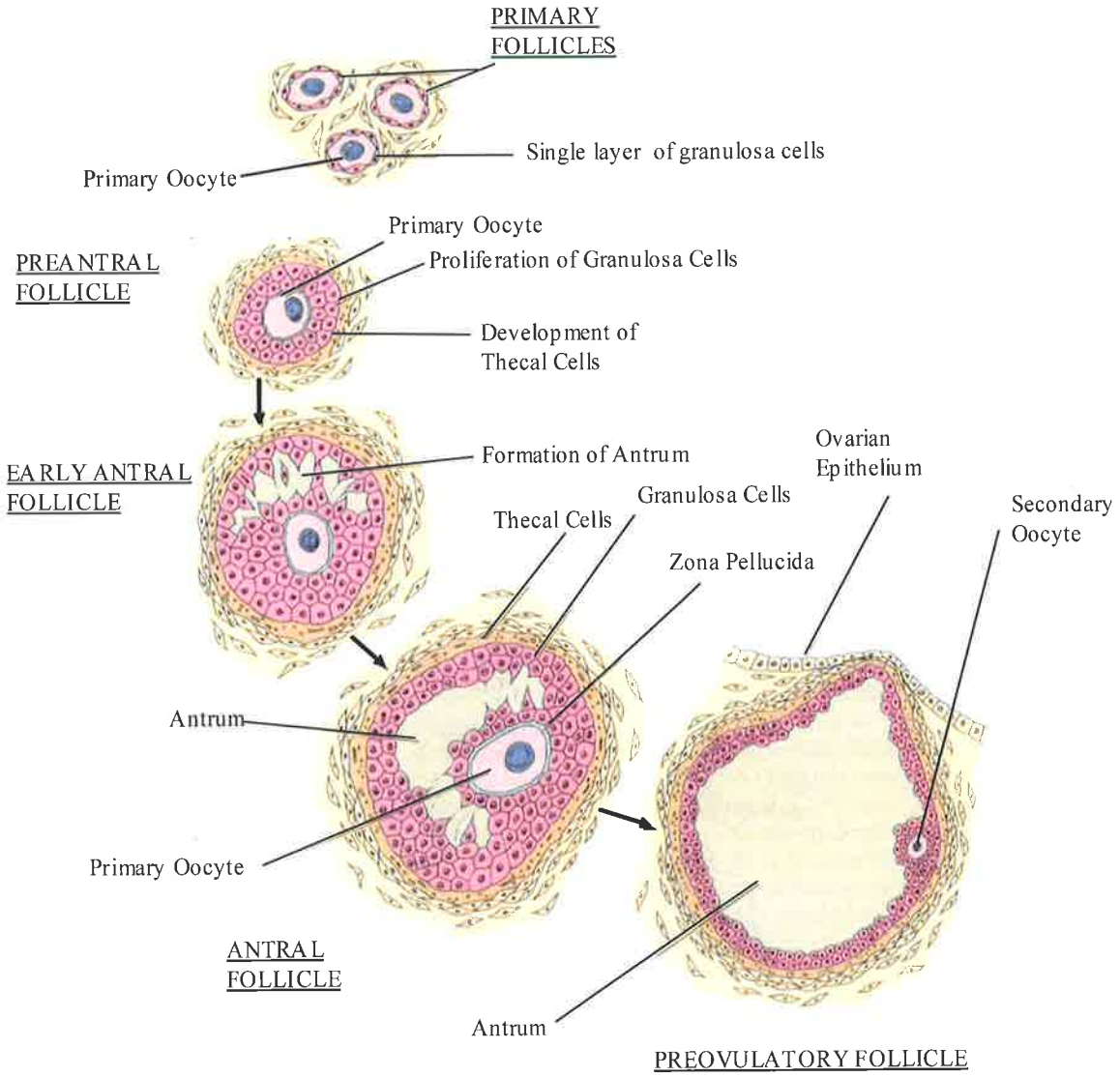


Figure 1-1 The Stages of Follicular Development.

Resting primordial follicles develop following unknown signals into primary follicles. These follicles then grow through the proliferation of granulosa cells and formation of the ovarian theca into pre-antral follicles. The development of the antrum and thecal layers together with continued granulosa cell proliferation occurs during antral growth. Maturation of the oocyte occurs in the preovulatory follicle immediately prior to ovulation (Adapted from Sherwood [18]).

been implicated in this process (reviewed by Nilsson [13]), including insulin [14], androgens [15], basic fibroblast growth factor, stem cell factor [16] and growth and differentiation factor-9 [17]. Follicle growth to the preantral stage is characterised by further granulosa cell proliferation, the formation of a zona pellucida surrounding the oocyte, and the development of a primitive thecal layer from ovarian stromal cells surrounding the follicle. In the mouse, gonadotrophins appear important although not critical to maintaining the pool of growing preantral follicles, since the numbers of growing preantral follicles are greatly reduced, but not completely ablated, in the hypogonadal [19, 20] or surgically hypophysectomised [21] mouse. Growth of follicles to the late preantral stage occurs continuously during the lifetime of the female, including in infancy and puberty, although most of these growing follicles undergo atresia via cellular apoptosis, as the hormonal environment will not support further growth (reviewed by Hsueh [22], Tilly [23] and Kaipia [24]).

The antral growth period is completely gonadotrophin dependent, with luteinising hormone receptors expressed on thecal cells and follicle stimulating hormone receptors expressed increasingly on granulosa cells. The antral growth period is characterised by the formation of a fluid filled antrum, the rapid proliferation of the granulosa cell layer with development of LH receptors, and expansion of the thecal layer into two distinct layers, a vascular theca interna and the theca externa. The theca interna is comprised of fibroblasts, steroidogenic cells and, blood vessels and leukocytes, while the theca externa is composed of fibroblasts and smooth muscle cells. Oestradiol production by the granulosa of the growing follicle is also stimulated and plays an important role in the stimulation of granulosa and thecal cell differentiation and the initiation of the LH surge [25]. If gonadotrophin stimulus is not forthcoming or withdrawn then follicles will spontaneously become atretic.

Atresia of antral follicles can be characterised morphologically by the presence of pyknotic granulosa cells, basement membrane breakdown, infiltration of leukocytes and

degeneration of the oocyte. More precise molecular techniques allow the detection of DNA laddering indicative of apoptotic cells, or the expression of death inducing genes, has allowed earlier detection of apoptosis than that achieved with observation of morphological changes (reviewed by Hsueh [22],Tilly [23] and Kaipia [24]). Therefore for a follicle to mature past the preantral and antral stages of development towards ovulation it must be 'rescued' from the follicular atresia pathway by gonadotrophin stimulation.

Preovulatory follicles consist of an oocyte surrounded by layers of cumulus cells, called the corona radiata. This complex extends into the fluid filled antrum of the follicle, via a 'stalk' of granulosa cells that secures the cumulus cell -oocyte complex to one side of the follicular wall (reviewed by Lipner [26] and Brannstrom [27]). At this stage there are many layers of mural granulosa cells lining the basement membrane of the follicle, which is enclosed in several layers of elongated theca interna cells interspersed with blood vessels, and the theca externa. The preovulatory follicle protrudes at one site from the surface of the ovary due to its large size forming a follicular apex. The tunica albuginea (connective tissue), basal lamina and surface epithelium of the ovary must be degraded to expel the oocyte (Figure 1-2) into the reproductive tract. If the LH surge, which stimulates ovulation, is not forthcoming these well-developed follicles will undergo atresia.

1.2.2 OVULATION

Ovulation is the rupture of the preovulatory follicle at the apex and expulsion of the oocyte into the reproductive tract. It occurs only following a surge of the gonadotrophin luteinising hormone (LH) released from the pituitary under the influence of LH releasing hormone and positive feedback of the sex steroids oestrogen and progesterone (reviewed by Brannstrom [27] and Espey [28]). This surge initiates incompletely defined events that lead to rupture of the follicular wall. These events include

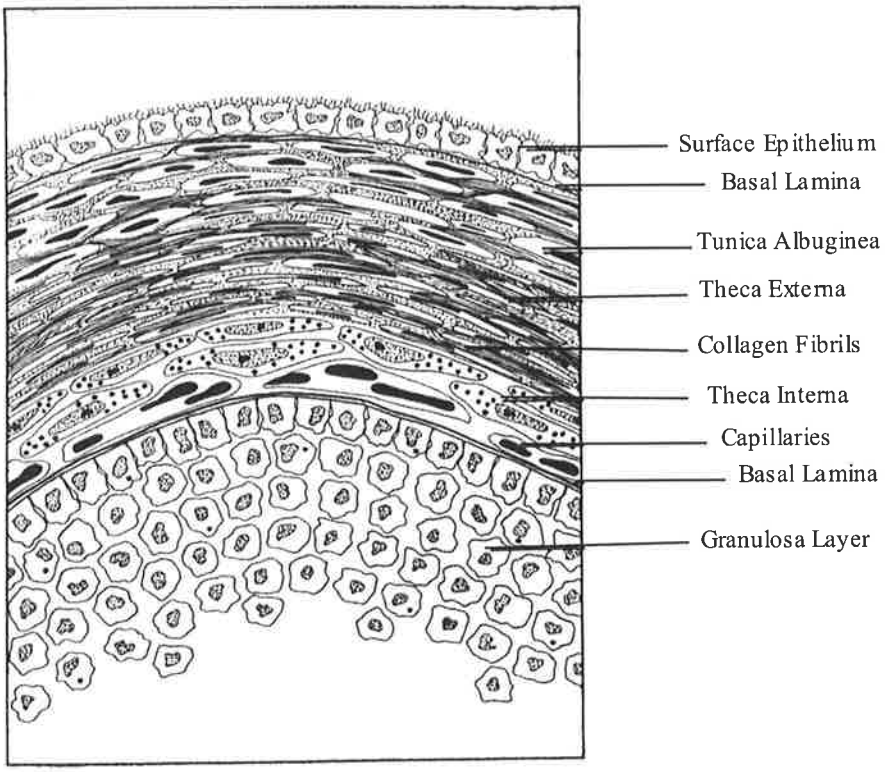


Figure 1-2 The Structure of the Follicular Wall

The many layers of the follicular wall that need to be degraded for ovulation to occur include the basal lamina and tunica albuginea. (Adapted from Espey [28])

vasodilation and an increase in blood supply to the ovary, leukocyte infiltration of the theca of the follicle, increases in intrafollicular pressure, contractions in the theca externa, increases in the activities of proteolytic enzymes, luteinisation of granulosa cells, changes in steroid production, and the initiation of meiosis and extrusion of the first polar body in the oocyte. Several characteristics of this process were recognised as similar to those of a classical inflammatory reaction and a potential role for leukocytes in this process proposed in early 1980's by Espey [29].

1.2.3 THE CORPUS LUTEUM

Following the LH surge and ovulatory event the theca interna and granulosa cells of the follicle transform into a predominantly progesterone secreting corpus luteum (CL) in a process called luteinisation (Figure 1-3; reviewed by Murphy [30] and Niswender [31]). Granulosa and thecal derived luteal cells within the CL can be distinguished as large (LLC's) and small (SLC's) luteal cells respectively. The CL becomes a highly vascularized structure also containing epithelial cells, fibroblasts and connective tissue, pericytes and blood leukocytes. During CL development LLC's grow rapidly in size but not numbers while SLC's, fibroblasts, and endothelial cells proliferate. Both large and small luteal cell types contain βHSD and hence actively produce progesterone. The primary role of the CL is to produce progesterone, which prepares the reproductive tract for embryo development and implantation. If fertilisation occurs, the CL must respond to factors produced by the conceptus with further production of progesterone, preventing the initiation of another follicular growth cycle and thus maintaining pregnancy. If no signal to maintain progesterone secretion is received, the CL deteriorates over several subsequent cycles into a corpus albicans or ovarian scar tissue in a process known as CL regression or luteolysis. This process is stimulated by luteolytic factors predominantly $PGF_{2\alpha}$ derived from the uterus

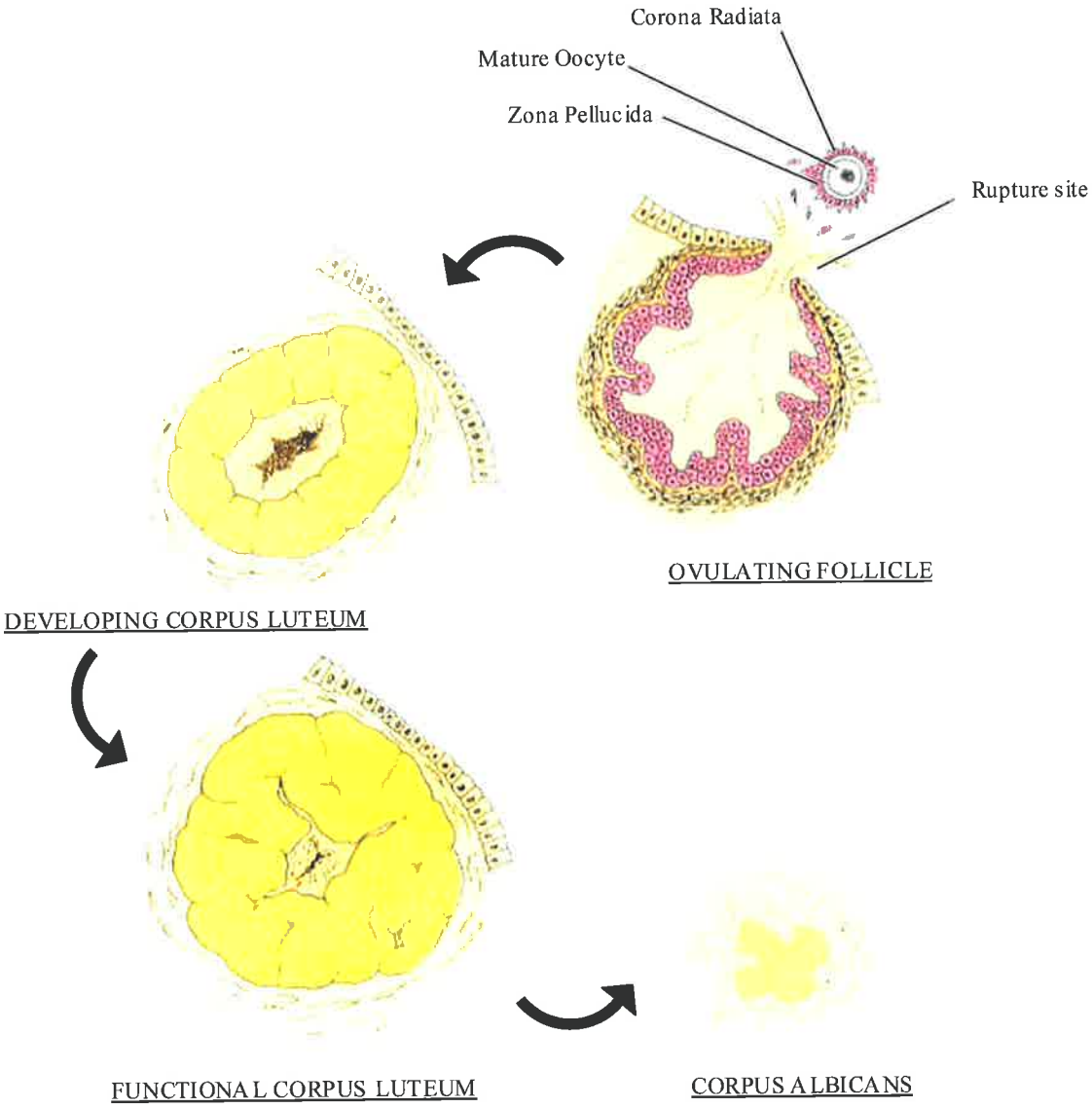


Figure 1-3 Ovulation, and Development and Regression of the Corpus Luteum.

The oocyte is expelled from the preovulatory follicles which then transforms into the progesterone producing CL. If no pregnancy occurs then the CL rapidly regresses into a corpus albicans and subsequently to ovarian scar tissue. (Adapted from Sherwood [18])

and is characterised by the loss of vascularization and progesterone synthesis, followed by loss of the cells comprising the CL through apoptosis (reviewed by Murphy [30] and Niswender [31]). In the human this luteal phase last 12-15 days with most other mammalian species having a luteal phase of 15-19 days [11]. In the rodent and rabbit the luteal phase of the cycle varies in length depending on whether or not they have mated. In the rodent if mating does not occur then the CL begins to deteriorate within 2 days of ovulation, never establishing its full progesterone secreting potential. Therefore in a cycling non-pregnant animal several CL's at differing stages of regression from the preceding cycles may be present during any one reproductive cycle. If a non-fertile mating occurs the stimulation of the cervix initiates the release of prolactin from the pituitary and the CL goes on to produce progesterone for a full luteal phase of 11-12 days[6]. This is known as pseudopregnancy. In the rabbit the act of mating stimulates the release of LH from the pituitary and hence ovulation 10-12 hours post mating. However if a female is housed with an infertile male she will exhibit a 14-day ovarian cycle, similar to that of the pig, consisting of a 2-day follicular phase followed by a 12-day luteal phase [11].

1.3 THE CYCLIC PATTERN OF GONADOTROPHIN AND STEROID SECRETION

The female sex steroid hormones, produced by the granulosa and theca of the ovary, 'feedback' systemically to the pituitary, controlling the release of the gonadotrophins required to stimulate follicular growth and ovulation (reviewed by Couzinet [32]). This results in fluctuating systemic hormone levels that coordinate follicle growth and development, tissue restructuring, and behavioural changes to optimise the chances of conception (Figure 1-4). In the rodent this is called the estrous cycle based on the characteristic female behaviours associated with approaching ovulation. In the human, behavioural estrus does not occur and the reproductive cycle is recognized only by the event of menstruation. It is therefore termed

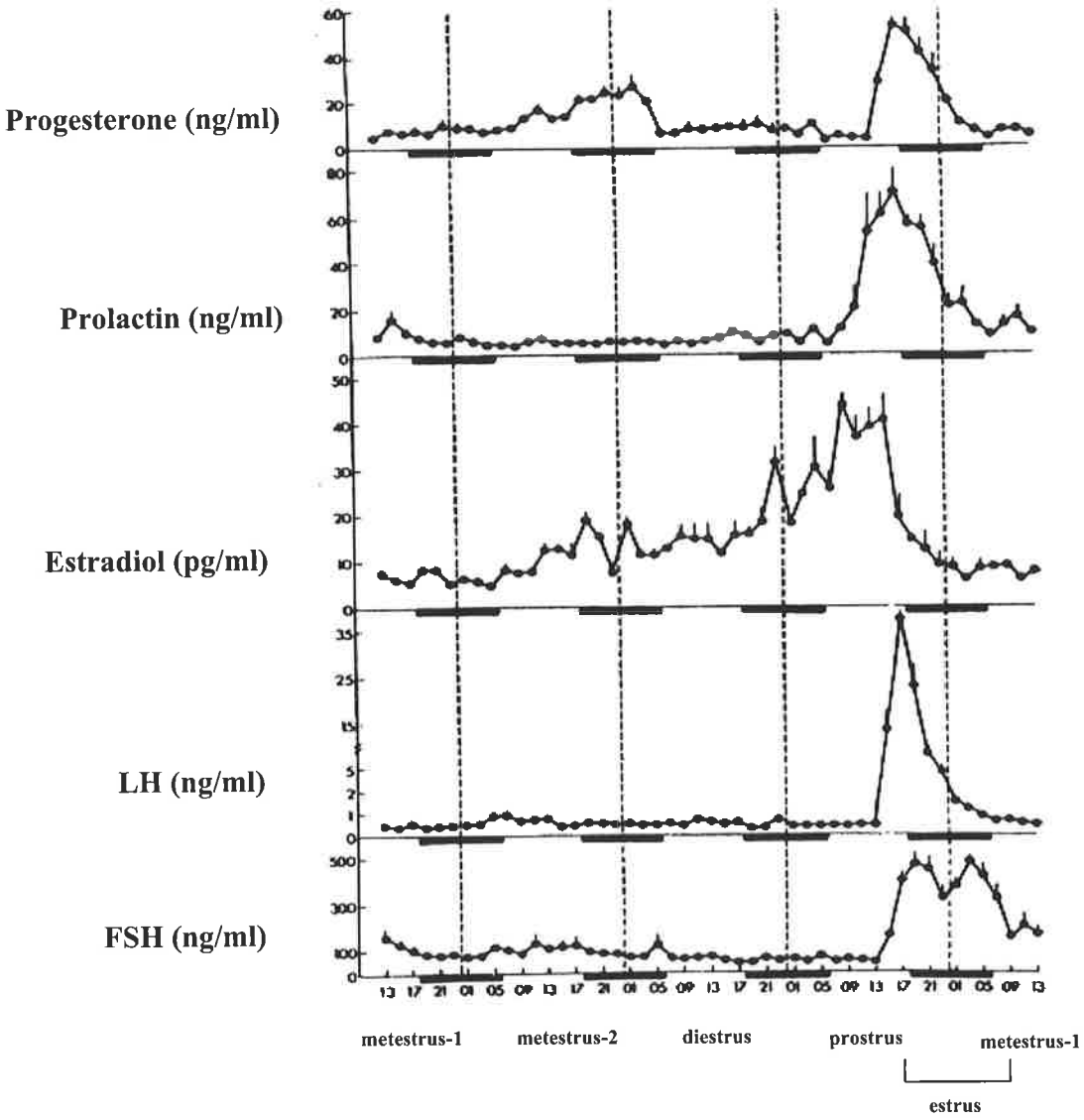


Figure 1-4 Fluctuating Hormone Levels During The Estrous Cycle Of The Rodent.

The systemic levels of the ovarian steroids (progesterone and oestradiol) and pituitary hormones (LH/FSH/prolactin) that reguate follicle growth, ovulation and CL development and demise. (Adapted from Freeman [6]) Levels in the mouse are comparable to those seen in the rat [34]

the menstrual cycle. Both cycles are regulated via complex hormonal interactions between the hypothalamus, pituitary and ovary. The estrous cycle of the rodent is continuous throughout the year and ovulation occurs spontaneously every 4-5 days (reviewed by Bronson [33]). This cycle can be divided into stages by examining smears of the vaginal epithelium that is sensitive to changes in systemic steroid levels (Table 1-1).

1.3.1 LUTEINISING HORMONE AND FOLLICLE STIMULATING HORMONE (FSH)

LH and FSH are glycoproteins consisting of similar alpha subunits but differing beta subunits. They are secreted from the anterior pituitary following gonadotrophin releasing hormone secretion by the hypothalamus. (reviewed by Freeman [6]) They act on the ovary via specific receptors expressed on the thecal (LH) and granulosa cells (FSH, and LH-during late follicle development) of the follicle and stimulate proliferation, differentiation and steroidogenesis in these cell types.

On the morning of metestrus-1, following ovulation, serum levels of LH and FSH are low, remaining so until proestrus. On the afternoon of proestrus there is a simultaneous increase in systemic levels of both FSH and LH that leads to follicular rupture. In the rat it has been reported that during the estrous cycle of periods low levels of LH are characterised by a pulsatile pattern of LH secretion that on the morning of proestrus increases in amplitude and decreases in frequency to a single surge in some animals [35, 36]. More recently it has been reported that LH pulse frequency does not change in the lead up to the LH surge [37]. On the morning of metestrus-1 there is a small secondary rise in FSH levels.

1.3.2 PROGESTERONE

Progesterone is produced from pregnenolone by a $\triangle^5$–3β-hydroxysteroid dehydrogenase-$\triangle^{5\text{-}4}$-isomerase (3βHSD) complex, predominantly by cells in the corpus

ESTROUS CYCLE STAGE	APPEARANCE OF VAGINAL SMEAR	UTERINE MORPHOLOGY	OVARIAN MORPHOLOGY
PROESTRUS	Mainly rounded epithelial cells with some cornified epithelial cells, few leukocytes may be present	Becoming distended	Follicles growing rapidly
ESTRUS	Cornified epithelial cells with some rounded epithelial cells. Few leukocytes present.	Maximum distension reached	Ovulation occurs
METESTRUS - 1	Sheets or clumps of many cornified epithelial cells. No epithelial cells and few leukocytes	The uterus becomes less distended and leukocyte invasion begins	Oocytes can be found in the ampulla region of the oviduct and early corpora lutea form at the site of the ruptured follicle
METESTRUS - 2	Many leukocytes and cornified epithelium, may be some epithelial cells present	Walls collapsed with degenerating epithelium and many leukocytes present	Oocytes found in the oviduct and growing corpora lutea in the ovary.
DIESTRUS	Rounded epithelial cells and leukocytes	Walls collapsed with healthy epithelium and many leukocytes present	Quiescence but follicles begin to grow towards the end of this stage

Table 1-1 Morphological Features Of The Rodent Estrous Cycle.

The relationship between the morphologies of the vaginal epithelium and, the ovarian and uterine tissues during the estrous cycle of the mouse. (adapted from Bronson[33])

luteum but also to a lesser extent by granulosa and thecal cells during follicle development, as a substrate for the production of androgens and oestradiol [11, 38]. A surge in progesterone secretion occurs at proestrus almost simultaneously with the increase in oestradiol and LH and then falls again on the morning of metestrus-1[34]. A second increase derived from the activity of the developing CL occurs in metestrus-2 and dies away again in diestrus. Progesterone alone cannot reduce systemic LH levels, but it can enhance the inhibitory effect of low levels of oestrogen on pituitary LH secretion and the magnitude of the oestrogen induced LH surge [6, 32].

1.3.3 Oestradiol and Androgens

Androgens are produced from progesterone or pregnenolone by a cytochrome P450 oxidase enzyme complex that carries out two reactions: 17α–hydroxylation and cleavage of the C 17,20-bond in the thecal cells [11, 38]. It is an important substrate in the synthesis of oestradiol but is also thought to have direct effects on the ovary itself. Oestradiol is produced during follicular growth by a P450 oxidase (or 'aromatase') enzyme complex that performs several reactions resulting in the formation of the aromatic oestrogens (reviewed by Simpson et al [39]). In the rodent this occurs in the granulosa cells of the follicle, using androgens provided by the thecal cells as the substrate. In some other species, such as the ovine and porcine, the thecal cells are also capable of producing oestrogens. Oestradiol is the major feedback mechanism from the ovary to the pituitary. It acts at low levels found in metestrus through to diestrus, to reduce the amplitude or amount of LH and FSH released. In proestrus, when due to developing follicles oestradiol levels become elevated, a positive feedback mechanism is initiated and a surge in LH levels occurs resulting in the maturation of preovulatory follicles and the initiation of the ovulatory cascade [6, 32, 38].

1.3.4 Stimulation of Ovulation in the Mouse via Exogenous Gonadotrophins

In immature mice follicle growth can be stimulated from around 16-17 days of age up to 28-30 days [40] using pregnant mare serum gonadotrophin (PMSG), also known as equine chorionic gonadotrophin (eCG). This stimulates the growth of antral follicles to the preovulatory stage while human chorionic gonadotrophin (hCG) or LH stimulates ovulation of mature oocytes. These hormones stimulate follicle growth through the prevention of follicular atresia in both rats [41] and mice [41]. Consequently, in animals stimulated with PMSG/eCG and hCG few atretic follicles are present. The number of oocytes ovulated using this protocol can also be influenced by mouse strain and body weight [40].

In adult animals, the time of ovulation may be manipulated using a LH releasing hormone antagonist, which blocks release of LH from the pituitary and generates ovarian quiescence, followed by PMSG/eCG and hCG to stimulate a new wave of follicle growth and ovulation. These types of gonadotrophin priming protocols are conventional methods of ovarian stimulation used in rodents when experimental design requires the synchronisation of estrous cycles. Although the ovarian events following gonadotrophin stimulation closely reflect those that occur in naturally cycling animals, it has been found that stimulation can result in the development of embryos with a higher incidence of polyploidy [42]. Steroid levels in stimulated animals have also been shown to differ from those seen in normally maturing animals [43].

1.4 Leukocyte Distribution in The Ovary

Blood leukocyte types are all derived from a common stem cell precursor found in the bone marrow (reviewed by Abbas [44], Figure 1-5). The main function of these mature cells is to coordinate the surveillance and defence of the body against foreign organisms, as well as

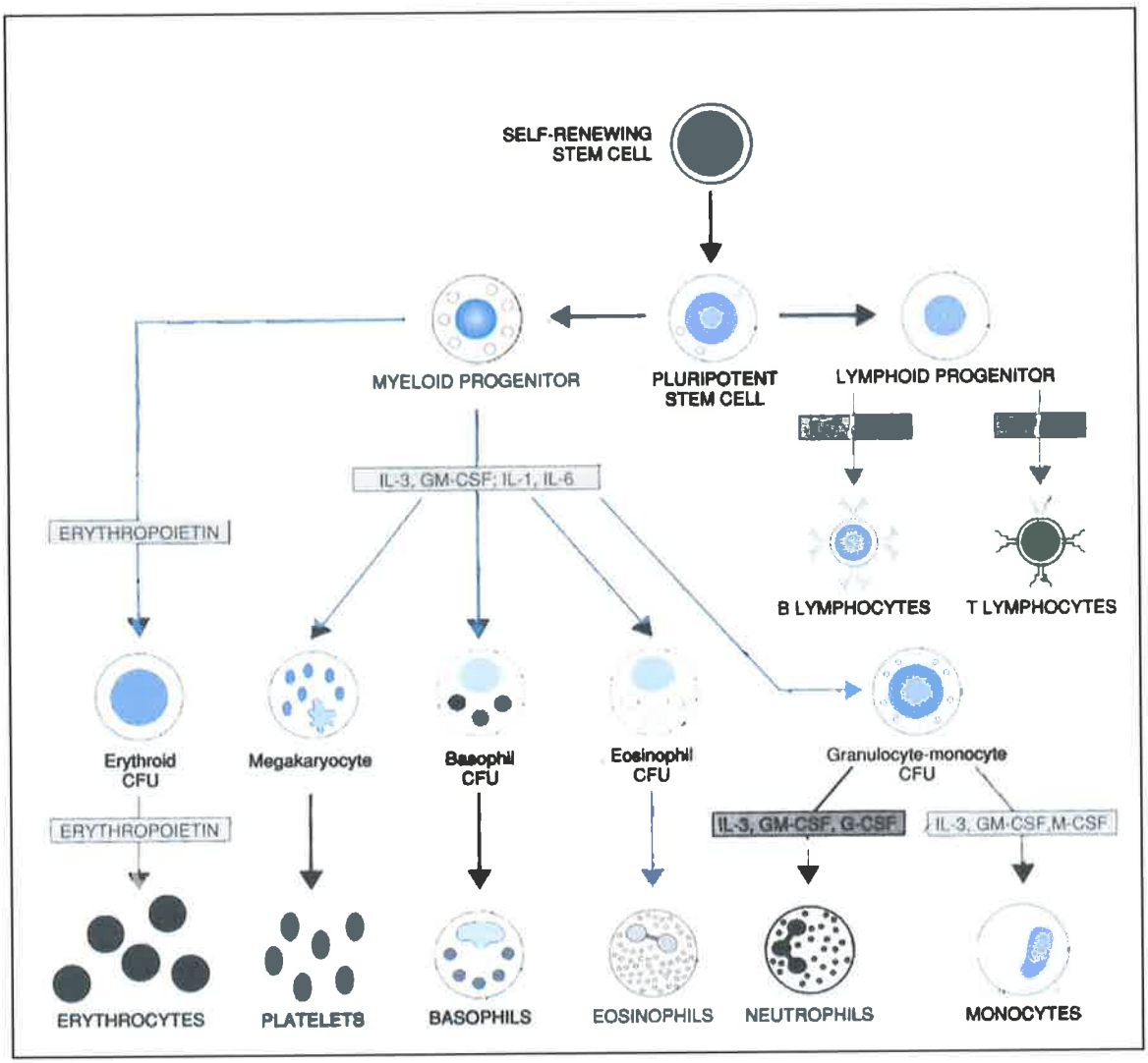


Figure 1-5 The Development of the Blood Cell Lineages

Concise representation of the development of all blood cell types from the pluripotent stem cells in the bone marrow. Each lineage develops under the regulation of different cytokines which act at different stages of development to produce the different cell types. Macrophages develop from monocytes that migrate into the tissues. (From Abbas [44])

mediating tissue inflammation and repair following injury or infection. These cells can therefore readily migrate from the blood stream into the majority of tissues and organs of the body. Since the events that occur in the mature ovary involve substantial tissue growth, atresia and reorganization it follows that cells playing significant roles in these processes elsewhere in the body would be active in the ovary. Early evidence that leukocytes play a role in the regulation of the reproductive system demonstrated a possible link between these two systems without clearly defining which components of each system were involved. Splenectomy in rats was found to delay ovulation [45] and could be reversed by the injection of splenocytes. Neonatal thymectomy of mice prevents normal follicular development and ovulation in later life [46], while treatment of rats with thymocyte antiserum results in reduced frequency of ovulation, concluded to be due to persistent CLs [47]. More specifically, the supplementation of the recirculating media of a perfused preovulatory rat ovary with blood leukocytes increases the number of oocytes released following the administration of LH [48], suggesting that these cells may have a role in the complex ovulatory cascade.

1.4.1 LYMPHOCYTES

The identification of B and T cell types in the ovary has been attempted in several species. B cells have been demonstrated at very low levels in the human ovary while T cells are found in small numbers in the stroma, the theca of developing and atretic follicles [49, 50] and the CL [49-51]. The highest numbers of T cells are found in the regressing CL. Small numbers of T cells can also be found in the chicken ovary [52] and in the medulla and stroma of the rat ovary [53]. Examination of the specific T cell type (expressing CD4 (cluster of differentiation) 4 - "helper" or CD8 -"effector" molecules) in the human ovary has revealed both no difference between the numbers of these T cell types present [54] and more CD8 + cells than CD4 + cells [49]. The latter finding is supported by results from the same research

group showing significantly more CD8+ than CD4+ cells in the rat ovary [53] as well as results published by Suzuki [50], although in this study no statistically comparison of the types of ovarian T cells was made. The role of T cells in the ovary has not been investigated although reproductive function is significantly disrupted following surgical removal of the thymus [46] or in animals with congenital athymia [55]. Elsewhere lymphocytes have been found to play important roles in wound healing (reviewed by Schaffer [56]) with which events in the CL are comparable. Hence it appears these cells must play a role in the regulation of ovarian function, through either aiding in the control of the immune response following ovulation, or alternatively through assisting in the regulation of apoptosis in cells of atretic follicles or the degenerating CLs.

1.4.2 NEUTROPHILS

In the rat granulocyte numbers have been shown to vary, according to the stage of the estrous cycle, in the medulla of the ovary and the thecal region of the follicle [53]. A large increase in the numbers of neutrophils in the theca of the follicle was also shown to occur immediately prior to ovulation. Neutrophils have also been demonstrated in the thecal layer and CL of the human [49, 57] with numbers in the theca increasing as ovulation approaches. Similar results were also obtained in the preovulatory follicles of rabbit ovaries using histochemical analysis [58], and the preovulatory follicle and regressing CL of the pig ovary [59]. In addition, experimental depletion of neutrophils in the rat ovary with either an administered antibody [60] or by treatment with inhibitor of nitric oxide (NO) production [61] results in reduced ovulation rates.

1.4.3 MACROPHAGES

Studies carried out 40 years ago examining the distribution of the enzymes alkaline phosphatase, esterase and beta-glucuronidase led to the conclusions that these cells are present in the ovary [62]. Experiments carried out during the same period observing the uptake of dianil blue (trypan blue) by cells in the ovary further suggested that these cells were macrophages, however at this time no role for these cells in ovarian function was proposed. A variation between species was noted with some of the enzymes studied more intense in some species than others. With the advent of immunohistochemistry and the ability to produce specific antibodies to identify any desired cells type, the role of leukocytes in the ovary, including macrophages, was more closely examined.

Macrophages have been found to be the predominant leukocyte type in the rat ovary, with numbers found to vary in the medulla of the ovary and the thecal region of the follicle [53], in relation to the stage of the estrous cycle. A large increase in thecal macrophage numbers immediately prior to ovulation has been described. In the mouse ovary Cohen et al [63] found no change in stromal macrophage distribution across the cycle but an increase in thecal macrophage numbers as the follicle increases in size. Petrovska et al [64] also demonstrated macrophages in the thecal layer of healthy antral and preovulatory mouse follicles and in the granulosa cell layer of atretic follicles. Macrophages have also been demonstrated in the thecal layer of the follicles in the ovary of the chicken [65] and the human [49, 54, 57] with numbers similarly increasing as ovulation approaches. In addition, macrophages are prevalent in the CLs of most species. They have been reported to be more common in the layer of thecal derived luteal cells than the granulosa derived luteal cell layer of the developing CL in the rat and human [49, 57, 66, 67]. In the mouse ovary, macrophage density in these locations has found to be highest in proestrus and metestrus [64] with numbers in the stroma and CL of luteinised mouse ovaries increasing as luteinisation

progresses [68]. Similarly, in the human the numbers of macrophages in early CL's increases significantly with progressing CL regression [49, 54]. Significant increases in macrophage numbers in the newly formed and regressing CL of the pig have also been described [59, 69].

1.5 General Characteristics and Functions of Macrophages

Macrophages develop from self-renewing stem cells found in the bone marrow from which all haematopoietic cells develop (Figure 1-5). When these totipotent cells are stimulated by the cytokines interleukin (IL) -1, IL-3 and IL-6 they develop into pluripotent myeloid cells. Further exposure to IL-1 and IL-3 commits these cells to becoming granulocyte-macrophage colony forming units that will proliferate following exposure to granulocyte-macrophage colony-stimulating factor (GM-CSF) [70]. The presence of macrophage colony-stimulating factor (M-CSF) induces both proliferation and differentiation of these cells into monocytic precursors or monoblasts that further divide into promonocytes and then monocytes in the bone marrow. These monocytes then migrate into the blood stream where the final maturation step for these cells occurs when they migrate via endothelial cell adhesion molecule expression and chemotactic cytokines into tissues. Once within the tissue the environment of that particular tissue can determine the specific characteristics of the mature tissue macrophage, leading to widespread heterogeneous macrophage populations [71]. Classically macrophages are considered cells of the immune system with a central role in the regulation of immunity (Figure 1-6). Their most basic and critical role being that of a scavenging phagocytic cell recognizing foreign molecules and apoptotic cells and disposing of them by phagocytosis. Macrophages can also process ingested molecules intracellularly into peptide fragments and present them, in association with a molecule called the major histocompatibility complex or MHC, to the T cell repertoire with whom they interact via

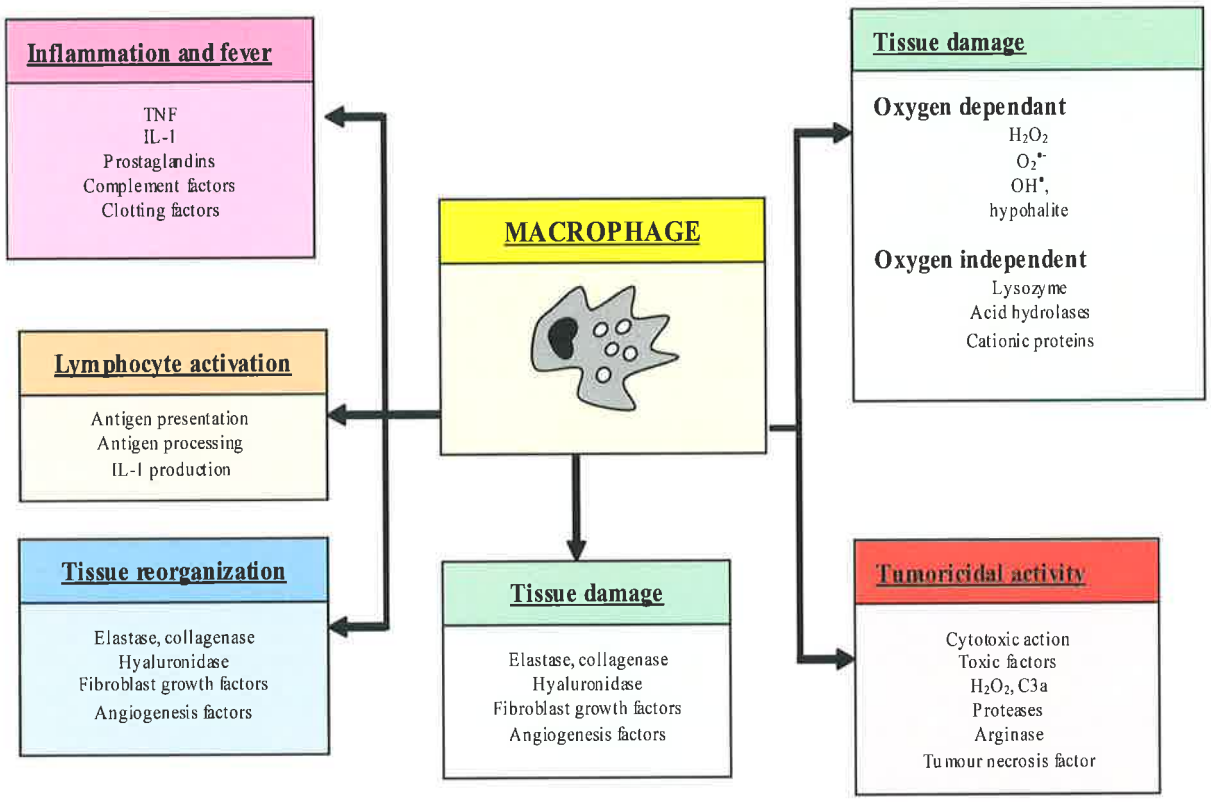


Figure 1-6 The Central Role Of Macrophages In Immunity

Macrophages and their products play a central role in the induction and regulation of immune responses as well as tissue reorganisation and repair. They also have effector functions in defence of the body against microbial infection and tumour development. (Adapted from Roitt [75])

cytokine messages to initiate an appropriate immune response. In addition to these roles in the initiation of immune responses they also participate as effector cells migrating into established wound or inflammatory sites and producing a large number of different factors (Table 1-2) with numerous effects, including tumoricidal and microbicidal activities, stimulation of tissue growth and repair, and the infiltration of other leukocytes.

1.5.1 Monocyte Migration

All leukocytes, including monocytes, infiltrate inflammatory tissues via the processes of tethering and rolling, activation, adhesion and transendothelial migration. Each of these steps requires the expression of appropriate molecules by both the endothelium and the leukocyte (reviewed by Ebnet [72], Berton [73] and Weber [74]). In addition, chemotactic cytokines or chemokines expressed at the site of inflammation are thought to play a crucial role in the regulation of endothelial cell and leukocyte activation, as well as manipulation of the expression of the molecules required for secure leukocyte binding and transendothelial migration (reviewed by Gale [80], Greaves [81] and Middleton [82]).

Leukocyte tethering and rolling is mediated by the expression of selectins and their ligands. L- Selectin is expressed constitutively on the leukocyte surface, while E- and P-selectin expression on the endothelium is induced [72]. Combined, these selectins initiate tethering and rolling of leukocytes along endothelium expressing appropriate ligands. The ligands that bind the selectins are numerous and fall into different classes of molecules (reviewed by Varki [83]) with the different selectins binding with differing affinities to some of the same ligands.

Once rolling has been initiated cells become activated by selectin binding and the presence of chemokines presented on the surface of the endothelium [72, 82]. Specific chemokine combinations are thought to attract specific leukocytes, monocytes respond to

Growth Factors	Cytokines	Extracellular matrix
Basic fibroblast growth factor (bFGF) Epidermal Growth Factor (EGF) Transforming Growth Factor – alpha/beta (TGF-α/β) Insulin-like growth factor 1 (IGF-1) Platelet derived growth factor (PDGF) Vascular Permeability Factor (VPF) Vascular Endothelial growth factor (VEGF)	Interleukin-1 (IL-1) Interleukin-8 Interleukin-12 Interleukin-6 (IL-6) Interferon –alpha/gamma (IFN-α/γ) Tumour Necrosis Factor –alpha (TNFα) Macrophage inflammatory protein (MIP) Granulocyte Colony Stimulating Factor (G-CSF) Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) Macrophage Colony Stimulating Factor (M-CSF)	Fibronectin Proteoglycans
Reactive intermediates	**Bioactive Lipids**	**Enzymes**
Superoxide Hydrogen peroxide Hydroxyl radical Nitrites/nitrates	Prostaglandins E2 and F2 Leukotreines Prostacyclin	Plasminogen Activator and inhibitors of Elastase Collagenase and inhibitors

Table 1-2 Factors Known to be Produced by Macrophages in Different Environments

(Compiled from references [76-79])

monocyte chemotactic peptide –1 (MCP-1), MIP-1α, -1β, RANTES, I –309, Fractalkine [80, 81]. Activation leads to stronger adhesion instigated by integrins expressed on the monocyte and adhesion molecules expressed on the endothelium. The beta 2 (LFA-1, Mac-1) and beta 1 (VLA4) integrins appear most important for monocyte adhesion and bind ICAM-1, -2, -3 and VCAM-1 and fibronectin, respectively [73, 84]. Under the influence of chemokines transendothelial migration is thought to occur by diapedesis at inter-endothelial cell junctions through binding interactions between LFA-1 on the leukocytes and junctional adhesion molecule (JAM-1)[74]. Intergrins expressed by macrophages also bind extracellular matrix allowing migration into the tissue and further activation specific to that tissue environment [73]. The recruiting environment can, through the expression of integrins and chemokines, dictate the characteristics recruited macrophages develop, generating regional populations that differ widely in their functional characteristics.

1.5.2 MACROPHAGES IN INFLAMMATION

Inflammation occurs as a result of tissue injury or infection, and involves the characteristic pathologies of swelling, redness and pain. These symptoms are a result of the release of early mediators of inflammation, such as histamine, and products of the complement and kinin enzyme systems, and are released by cells, including tissue macrophages, located at the damaged site. They induce the infiltration and activation of leukocytes (described in 1.5.1) and production of factors to promote vasodilation (reviewed by Mutsaers [85] and Greenhalgh [86]). Neutrophils are the first immune cells recruited to the site releasing free radicals and proteases, which may cause some host tissue damage, to eliminate foreign organisms and then dying via apoptotic mechanisms. Macrophages are the second cell type recruited and are considered central regulators of inflammation and tissue repair mechanisms. In inflammation these cells can promote vascular dilation, leukocyte

infiltration and T and B cell proliferation and maturation through the production of classical inflammatory mediators, such as prostaglandins, NO, IL-1β, tumour necrosis factor-alpha (TNFα) and platelet activating factor (PAF). This leads to the destruction of the invading bacterial organism or virally infected cells. The resolution of this type of destructive inflammatory response is regulated by the overriding expression of anti-inflammatory cytokines such as IL-10, transforming growth factor beta 1 (TGFβ1) and IL-1 receptor antagonist (IL-1ra), while healing of damaged tissues and clearance of apoptotic cells occurring after the inflammatory phase is also a central role of the recruited macrophages.

1.5.3 Macrophage Activation

The activation of macrophages has been a recognised developmental stage for many years, characterised by changes in functions that increase the ability of these cells to combat infections (Table 1-3)[87]. This type of 'classical' activation is bought about by the presence of inflammatory mediators, bacterial products such as lipopolysaccharides (LPS), interferon gamma (IFNγ) released by T cells during the course of an infection to stimulate the production of pro-inflammatory mediators such as IL-1β, TNFα, IL-12, and reactive oxygen species. This promotes the 'classical' inflammatory reaction with the production of prostaglandins, leukotrienes and numerous cytokines chemotactic and stimulatory to T and B cells. Anti-inflammatory agents such as IL-4 and IL-13 [88, 89] have the ability to reduce the production of pro-inflammatory cytokines by these activated macrophages (reviewed by Doherty [90] and Ma [91]), promoting deactivation of the macrophages and resolution of the inflammatory response. These factors have also been found to promote what is now termed the alternative route of macrophage activation resulting in increased expression of mannose receptor, increased endocytosis [92], lower pro-inflammatory cytokine levels

Microbial activity (↑)	Complement proteins (↑)
Tumoricidal activity (↑)	Acid hydrolases (↑)
Chemotaxis (↑)	Collagenase (↑)
Phagocytosis (↑)	Plasminogen activator (↑)
Pinocytosis (↑)	Cytolytic proteinase (↑)
Glucose transport and metabolism (↑)	Arginase (↑)
Respiratory burst (↑)	Fibronectin (↑)
Antigen presentation (↑)	Interleukin-1 (↑)
Prostaglandins, leukotrienes (↓)	Tumour Necrosis Factor (↑)
Apolipoprotein E and lipoprotein lipase (↓)	Interferon α and β (↑)
Elastase (↓)	Angiogenesis factors (↑)

Table 1-3 Major Macrophage Functions And Their Regulation By The Process Of Activation. Adapted from Johnston [87].

[93], and production of the anti-inflammatory cytokines IL-10 and TGFβ [94]. These cells are highly angiogenic *in vitro* and *in vivo* with the ability to actively inhibit mitogen induced proliferation of PBL or CD4+ T cells *in vitro* [95, 96] while promoting the differentiation of Th2 type cells involved in the stimulation of antibody responses [97]. It follows that these cell types play an important role in the down regulation of inflammatory responses, and subsequent wound healing.

1.5.4 MACROPHAGE INTERACTIONS WITH T CELLS

Following the phagocytosis of a foreign body the macrophage is capable of intracellular degradation of the particle and presentation of peptide fragments from the particle on the surface of the cell in association with the class II MHC (MHC II). These MHC-II –antigen complexes can be recognised by helper T (Th) cells via the T cell receptor (TcR) and the CD4 molecule. This results in the initiation of an immune response through the activation of both the T cell and the antigen presenting macrophage. The type of immune response mounted and the activation state of the macrophage that results has been found to be dependant on the co-stimulatory molecules present on the cells and the cytokines in the environment (reviewed by Reiner [98], Constant [99] and Goerdt [92]). In a cell-mediated inflammatory response, binding of the TcR/CD4 on the helper T cell to the antigen/MHC II molecule complex must be accompanied by interaction of co-stimulatory molecules. This initiates the production of IL-2, IFN-γ, and TNFβ by the T cell, and IL-12 by the macrophage, which in turn stimulate proliferation of the T cells and activation of the presenting macrophage (Figure 1-7).

Activation of the macrophage is characterised by the production of pro-inflammatory cytokines, increased expression of MHC II molecules and the ability to produce reactive oxygen intermediates. The proliferating T cells also produce pro-inflammatory cytokines

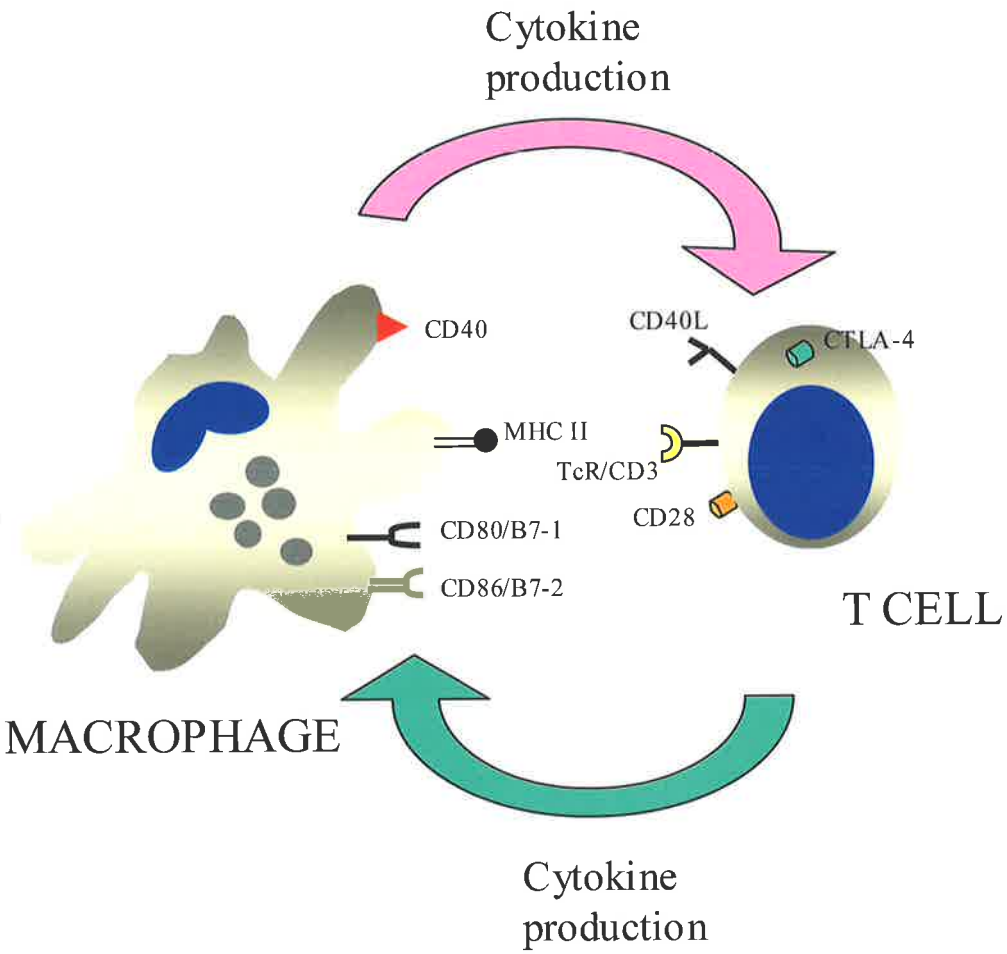


Figure 1-7 The Interactions Between Macrophages And T Cells During An Immune Response. The MHC II, major histocompatibility complex II, on the macrophages presents processed antigens to the T cell receptor (TcR). Co-stimulatory molecules must be present to initiate an immune response. CD40 on the macrophages binds with the CD40 ligand on the T cell and the B7 molecules (CD80/86) of the macrophage bind the CD28 or CTLA-4 which is externalised in certain conditions on the surface of the T cell. Cytokines are produced by both the macrophage and the T cell.

promoting antibody production by B cells, and assist in the initiation of a tissue destructive cytotoxic response. This is termed a Th1 or cell mediated response. If the appropriate co-stimulatory molecules are not present and an apoptotic-inducing ligand is present on the macrophage cell membrane, T cell deletion can occur [92]. In the presence of the cytokine IL-4 , an alternative immune response can also be generated resulting in proliferation of Th2 cells, which produce IL-4 and IL-5 [88]. This stimulates B cell proliferation and alternatively activates macrophages to produce anti-inflammatory cytokines, such as IL-10 and TGFβ, promoting down regulation of the inflammatory response [100].

1.5.5 Macrophages in Wound healing and Tissue Repair

The macrophages that pervade a wound site have the ability to release, proteases such as collagenase or elastase which instigate tissue degradation, and cytokines that stimulate fibroblastic proliferation and blood vessel growth, particularly in hypoxic conditions [101]. They also have the capacity to eliminate cell debris and other non-viable or apoptotic material. The wound healing process itself can be divided into inflammatory, proliferative and maturation phases [86]. The inflammatory, proliferative and maturation phases can co-exist in a single wound site with the earlier phases existing in the central open areas of the wound and later phases existing at the periphery of the wound. Members of the TGF-β family also have critical roles in regulating wound healing [102].

1.5.6 Macrophage Phagocytosis and Phagocytic Receptors

Phagocytosis is a complex process mediated by binding of the molecule to the cell surface which in turn initiates actin polymerisation and internalisation of the attached particle into a phagosome or vacuole in the cell cytoplasm. Two models of internalisation have been

proposed; a 'zipper' model, in which sequential interactions between the surface of the molecule and receptors on the phagocyte cell surface are required as the molecule is engulfed, and a 'triggering' model in which binding of the molecule to receptors on the surface of the phagocyte initiates engulfment independent of further receptor interactions [103, 104]. Phagocytosis may be followed by fusion of the phagosome with enzyme containing lysosomes, and degradation of the particle. Even though most cells have some phagocytic capacity, macrophages are 'professional' phagocytes and can internalise particles much more rapidly and efficiently than normal cells, due mainly to the expression of innate immune receptors (Table 1-4). These receptors recognise various non-specific entities which are expressed on the surface of pathogens or whose production is triggered by the presence of a pathogen. Particle uptake has also been shown to be dependent on particle size and surface charge [105]. Although the phagocytosis of a particle generally leads to the activation of the phagocytosing cell and release of pro-inflammatory cytokines this is not always the case. In the human the uptake of apoptotic cells by blood monocyte derived macrophages via CD14, has been found to result in the inhibition of the release of pro-inflammatory cytokines, such as IL-1β, IL-8 TNFα and GM-CSF, and stimulation of the release of anti-inflammatory factors such as TGFβ, PGE_2 and PAF [106, 107]. The anti-inflammatory response to apoptotic cell uptake is hence thought to be dependent on the receptors used to internalise the altered 'self' cells [108], the cytokines released in response to uptake [106], and/or to the lack of expression of macrophage co-stimulatory molecules [109].

1.5.7 Macrophage Surface Markers and their Regulated Expression

Monoclonal antibodies are the principal tool for the identification and classification of macrophages. Several monoclonal antibodies raised against different proteins found on macrophage cell membranes have been developed and are currently in common use

TARGET ANTIGEN (ANTIBODY NAME)	CELLULAR LOCATION	CELL TYPE	MACROPHAGE EXPRESSION PATTERN	FUNCTION
Macrosialin CD68 (mFA11)	Transmembrane, mostly intracellular, some cell surface	Macrophages Dendritic cells	Increased in inflammation	Endosomal/Lysosomal associated lectin binding protein
Sialoadhesion (SER-4, 3D6, MOMA-1)	Cell surface Transmembrane	Stromal macrophages	Not expressed on monocytes	Non-phagocytic receptor that binds a particular oligosaccharide sequence expressed on other immune cells
Complement receptors, CD11b (Mac-1)	Cell surface	Macrophages Neutrophils NK Cells Dendritic cells	Reduced on activated cells but variable in different populations	Bind particles opsonised with complement proteins activated by microbial infection or antigen-antibody complexes
Fc receptors FcRI	Cell surface	Macrophages Neutrophils NK cells Lymphocytes	Up regulated by IFNγ	Receptors for all immunoglobulin classes enables binding of particles opsonised with immunoglobulin
Scavenger receptors Class A receptors (2F8)	Cell surface	Macrophages	Not expressed on monocytes	Six different classes-binding various lipoproteins on pathogens and some apoptotic cells
Mannose receptor CD206 (murine N/A)	Cell Surface	Mature tissue macrophages and Dendritic cells	Th1 cytokines (↓) Th2 cytokines (↑)	Binds terminal sugar molecules found uniquely on pathogens

Table 1-4 Macrophage Receptors and Antigens: their Location, Expression Pattern and Function. (complied from Gordon [110], Leenen [115] and McKnight [116].

(Table 1-4). An understanding of how prevalent expression is in other cell types and the circumstances in which they are expressed by macrophages is important when interpreting results obtained using this technology. Since several excellent reviews of these and other antigen markers in murine tissues are available [110-112], only the antigens used to isolate and identify cells in this thesis will be discussed here.

1.5.7.1 F4/80

Austyn and Gordon developed a monoclonal antibody to this antigen in 1981 by immunization of rats with thioglycollate elicited mouse peritoneal cells [113]. At the time the specific function of the antigen was unknown although it was found to be a unique 160KDa glycoprotein expressed on the cell surface that bound exclusively to all macrophages and monocytes and was not expressed on neutrophils or dendritic cells which have some cell surface molecules in common with macrophages. It has subsequently been used as a pan macrophage marker in the mouse even though the level of its expression varies (low on blood monocytes and higher on tissue macrophages) with expression additionally decreasing with increasing activation state or time in culture [113]. This antigen has since been cloned [114] and found to have structural similarities to both the epidermal growth factor family (at the amino-terminal end) and the seven transmembrane-spanning family of hormone receptors (at the carboxy Terminal). Five isoforms of the F4/80 molecule varying in the number and combination of EGF domains are possible due to the fact that each domain is encoded as a separate exon (Figure 1-8). A human homologue EGF module-containing mucin-like hormone receptor (EMR 1) sharing 68% homology has also been identified [112]. Based on the structural features of this molecule it has been postulated that this molecule may have a role in cell-cell adhesion following adhesion to the extracellular matrix, as well as some signalling capacity, although the ligand for the mouse molecules is, as yet, undefined [112, 114].

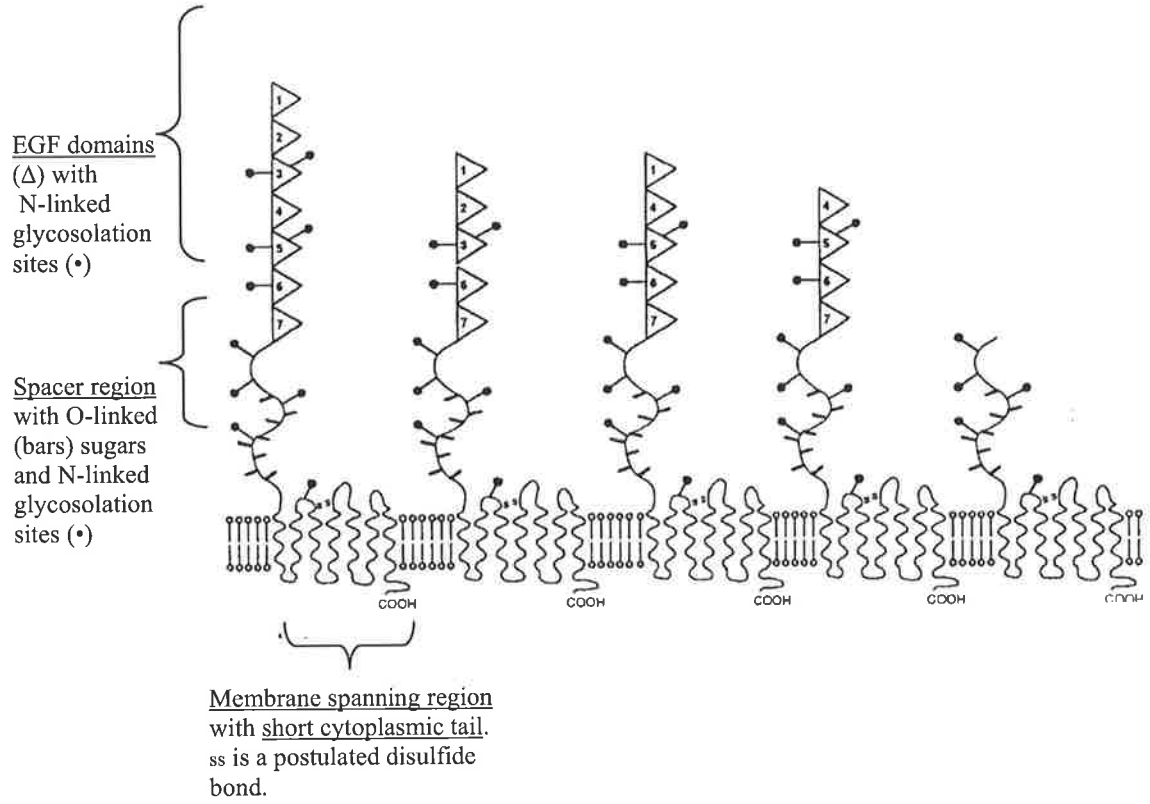


Figure 1-8 Alternative Isoforms of the F4/80 Molecule

The five alternative isoforms identified to date are generated by alternative splicing of the F4/80 messenger RNA transcripts. Each translated protein is made up of combinations of the seven extracellular EGF domains, numbered here for each isofrom, followed by a spacer region and then the seven transmembrane spanning hydrophobic regions and short cytoplasmic tail. (from McKnight and Gordon [112])

1.5.7.2 Class II Major Histocompatibility Complex (Ia)

The MHC is a region of polymorphic genes that are expressed on the surface of many cell types. Individuals who express the same MHC antigens will accept tissue grafts from each other while those who differ will rapidly reject grafts. The complex can be divided into class I MHC molecules, which bind endogenously derived peptides and are present on nearly all cells; class II MHC molecules, which bind exogenously derived peptides and are expressed only on B cells and antigen presenting cells; and an S region encoding components of the complement system. In the mouse the class I region can be divided into H-2K, H-2D and H2-L, and the class II region can be divided in I-A (Ia) and I-E regions [44]. In the human the equivalent genes are called human leukocyte antigens (HLA); HLA -A, -B, or –C are class I genes and HLA -DP, -DQ, or –DR are class II genes. In the mouse the glycoprotein products of the I-A and I-E regions are called I region associated antigens and monoclonal antibodies that detect these antigens are readily available. Class II MHC molecules are expressed at high levels on all antigen presenting cells such as dendritic cells, macrophages, and some fibroblasts and epithelial cells.

1.5.8 MACROPHAGES RESPOND TO THE FEMALE SEX STEROID HORMONES

Studies investigating the effect of progesterone on macrophages have been carried out in other fields, while there is ample evidence indicating oestradiol has significant effects on macrophage and monocyte function. Unfortunately, the results for both steroids are divergent, probably due to the varying sources of macrophages used and their resulting activation states. Thus it can be difficult to interpret these results in terms of the function these interactions may play in regulating ovarian function.

1.5.8.1 Progesterone

Progesterone has been shown to inhibit superoxide release by unstimulated peritoneal macrophages isolated during pseudopregnancy in the rat [117]. In contrast, progesterone has also been reported to enhance the release of reactive oxygen intermediates and inhibit nitrite production in 12-phorbol 13-myristate acetate (PMA) and opsonized zymosan (OZ) stimulated mineral-oil-elicited peritoneal cells from male rats [118]. Progesterone treatment of guinea pigs impairs Fc mediated clearance of erythrocytes *in vivo* through a reduction in the numbers of Fc receptors expressed by macrophages in the spleen [119], while *in vitro* Fc mediated phagocytosis by mineral oil elicited peritoneal cells from male rats was up regulated by progesterone treatment [120]. These varying results may be explained by the proposition that activated macrophages when exposed to progesterone are stimulated whereas resting or inactive macrophages are inhibited by the presence of progesterone. In context of the ovary the largest amounts of progesterone are found in the functional CL and perhaps during CL development or pregnancy the macrophages present are 'resting' macrophages and their activity is inhibited by the progesterone present. Following the initiation of luteolysis they may become activated and the progesterone present further stimulates these cells leading to luteolysis.

1.5.8.2 Oestradiol

Peritoneal cells from male rats pre-treated *in vitro* with oestradiol and then stimulated with phorbol myristate acetate or opsonized zymoson exhibit increased release of reactive oxygen intermediates and inhibited nitrite production [118]when compared to untreated cells. Endogenous chemiluminescence, reflecting the production of reactive oxygen intermediates, was also found to be stimulated by pre-exposure to oestradiol [120]. In addition, in these same cells Fc receptor mediated phagocytosis of SRBC was also stimulated by oestradiol.

Treatment of isolated human monocytes with oestradiol has also been found to stimulate the release of NO [121]. High levels of oestradiol are found immediately prior to ovulation and hence these effects of oestradiol on macrophages may play a role in the stimulation of the production of reactive species involved in the ovulatory event. It has also been demonstrated that oestradiol can inhibit human monocyte chemotaxis in response to MCP-1 *in vitro* [122]. This effect was reversed by the addition of the oestrogen receptor antagonists, tamoxifen and clomiphene, while blood monocytes and peritoneal macrophages from oestradiol treated animals are more phagocytically active, but have reduced intracellular bactericidal activities [123].

In addition to the effects of oestradiol presented above this steroid has also been shown to modulate cytokine production by monocytes and macrophages. Adherent peritoneal cells from oestradiol treated Listeria monocytogenes infected animals produce less IL-12, TNFα and IL-10 although it was suggested this may be mediated through the nonadherent cell population (possibly T cells) which in oestradiol treated animals produced less IFN-gamma and more IL-4, IL10 and TGF-beta than untreated cells [123]. Elsewhere oestradiol has been shown to reduce TNFα production by peritoneal macrophages isolated from rats subject to myocardial ischaemia–reperfusion [124], and to significantly increase the level of platelet derived growth factor-A messenger RNA (mRNA) in the monocyte cell line THP-1 following stimulation with 12-O-tetradecanoylphorbol 13 acetate (TPA) [125]. Oestradiol also inhibited LPS stimulated MCP-1/JE mRNA expression by thioglycollate elicited peritoneal macrophages and two murine macrophage cell lines, but had no effect on TNFα expression. The effect could be reversed by oestrogen antagonists and could not be replicated using progesterone [126]. It appears that oestradiol inhibits the production of inflammatory cytokines by stimulated macrophages but enhances their phagocytic activity and production of reactive oxygen.

1.6 Characteristics of Tissue Macrophages

Macrophages located in the tissues develop from blood borne monocytes that migrate out of the blood stream via adhesion molecules and chemotactic factors. They differentiate in response to the local microenvironmenal signals to assume a functional phenotype[127]. Their roles may include the phagocytosis and degradation of foreign organisms or apoptotic tissues, the regulation of local immune and inflammatory responses, and/or tissue remodelling. The studies presented in this section demonstrate that macrophages have a specific tissue phenotype, presumably generated by exposure to factors found in the local environment that stimulate or inhibit selected activities of developing macrophages. This creates specialised macrophage cells capable of performing the specific tasks required for normal function of that particular tissue. The method of isolation and characteristics of macrophages recovered from some complex tissues or organs deemed not to have a primary immune function are examined.

1.6.1 Testis

Macrophages have been demonstrated in the interstitial tissue of the testis closely associated with the Leydig cells of the testicular interstitium. Within the testis these macrophages are thought to regulate steroid synthesis (reviewed by Hales [128]) and to maintain the immunosuppressive environment of the testis (reviewed by Hedger [129]). In the rat the testicular macrophage population has been demonstrated to be heterogeneous by the expression of specific macrophage markers [130] with their numbers closely correlated to the numbers of Leydig cells. Testicular macrophages have been shown to significantly influence normal testicular function. Macrophage conditioned media has been shown to significantly influence Leydig cell steroidogenesis [131, 132] while cytotoxic depletion of the leydig cell

population or inhibition of Leydig cell function via testosterone implants both significantly alter the numbers of testicular macrophages [130, 133]. Macrophages have been isolated from this reproductive organ via adherence [134], collagenase digestion followed by cell elutriation [135], and by immunoaffinity [136]. These cells have been reported to be poorly pro-inflammatory, inhibiting the proliferation of lymphocytes both directly in co-culture experiments through the secretion of prostaglandins[135], and to have a cytokine profile significantly different to that of peritoneal macrophages. In addition when compared to peritoneal cells they were found to respond in a unique manner to regular macrophage stimulants [137]. Interestingly if inflammation is induced in the testis *in vivo* then testicular function is compromised [138] and this is proposed to occur through the production of inflammatory cytokines some of which may be derived from infiltrating monocytes [129].

1.6.2 UTERUS

Uterine macrophage distribution and numbers vary according to the stage estrous cycle. It is evident that this occurs due to the production of chemotactic factors produced by the uterus in response to fluctuating steroid levels. Chemokines such as colony stimulating factor –1 [139], granulocyte-macrophage colony stimulating factor [140] and others [141] have been shown be regulated by steroid levels, as well as by normal mating. The uterus displays an inflammatory type reaction to mating, with a rapid but transient infiltration of macrophages that subsides on day 3 of pregnancy [141, 142]. Uterine macrophages have been shown to express GM-CSF receptor following mating [143] indicating these cells respond to the surge of GM-CSF from the uterine epithelium that occurs mating [144]. Uterine macrophages are consequently thought to play an important role in successful implantation and maintenance pregnancy (reviewed by Hunt and Pollard [145] and Hunt [142]). Macrophages have been isolated from the uteri of cycling mice by collagenase digestion followed by rosetting with sheep red blood

cells [146, 147] or immunomagnetic beads [148]. Uterine macrophages were shown to be inhibitory in T cell mitogenesis assays in which elicited peritoneal cells were stimulatory [147]. In contrast, uterine macrophages isolated from ovariectomised mice were found to be stimulatory in the same assay. More recently it has been shown that macrophage/dendritic cells located in the mouse uterus can be divided into three heterogeneous populations, defined by their expression of cell surface markers [148]. These distinct uterine macrophage populations have further been found to exhibit discrete immunostimulatory phenotypes [148].

1.6.3 Human Placenta and Decidua

Macrophages can be found in the human decidua but are less evident in the decidua of the mouse and rat [145]. In the human, macrophages comprise about 20% of the decidual tissue [149] and are they thought to play an immunosuppressive role in this tissue [150]. Incidentally these cells can stimulate lymphocyte proliferation although it is not comparable to that stimulated by peripheral monocytes [151] and it has been proposed elsewhere that the stimulated cells may be suppressor or regulatory T cells required to promote survival of the conceptus [145]. Evidence suggests decidual macrophages may play a role in the regulation of trophoblast invasion [149] as well as parturition [152]. The macrophages in these studies have been isolated from the decidua via numerous methods including collagenase or dispase digestion followed by, density gradient centrifugation, immunomagnetic beads, adherence and/or flow cytometry [151, 153, 154].

In the placenta, foetal macrophages or Hofbauer cells can be found in close proximity to the trophoblast cells they comprise 25% of the human placental stromal cells [145]. Evidence suggests these cells may have a significant role in the regulation of trophoblast function [155] potentially through the secretion of cytokines. In the rat placenta macrophages have been shown to express only low levels of MHC class II molecules [145] while in the

human comparing macrophages isolated from placenta early and late in gestation shows class II MHC molecules were expressed at lower and higher levels respectively [156]. Macrophages from human placenta have been isolated by a variety of methods including enzyme digestion, adherence, density gradient centrifugation and magnetic depletion [157-159].

1.6.4 BRAIN MACROPHAGES

Several cell types found in the central nervous system display characteristics of macrophages. Whether or not these cells are related, representing different stages of maturation is unclear, although evidence does exist to suggest this (reviewed by Thomas [160]). The microglial cells are the main immune effector cells or macrophages of the brain. These cells appear to be a resting cell type becoming activated by inflammatory signals or injury in the brain. This activation has been graded into two steps (reviewed by Gehrmann [161]) the first being characterised by proliferation, an increase in antigenic markers and the released of common inflammatory mediators followed by the second step or development of phagocytic activity if neuronal and/or terminal degeneration occurs. In addition, these cells have been reported to present antigen to T cells and have cytotoxic activity. All antibodies developed so far against microglial cells are cross- reactive with tissue macrophages while in the human they have been reported to express the CD45RB (LCA) antigen [162]. Microglial cells have been isolated from the brain by mechanical dissociation and adherence, magnetic selection [163] and enzymatic digestion [164].

1.7 Potential Macrophage Functions in the Ovary

1.7.1 Macrophages and Follicular Growth and Atresia

The mechanisms initiating primary follicle growth are unknown although recent evidence suggests the production of various growth factors by the oocyte and early granulosa cells may lead to the initiation of follicle growth (reviewed by McNatty [165] and Nilsson [13]). Macrophages have not been localised around primordial or primary follicles and are unlikely to have any significant role in this initial stimulation.

Following the initiation of follicle growth, numerous factors potentially derived from macrophages located in the thecal layer of growing follicles, are believed to play a role in either stimulating the proliferation and growth of follicles or regulating follicular apoptosis. Molecules such as hepatocyte growth factor [166], basic fibroblast growth factor (bFGF) [167, 168], epidermal growth factor (EGF) [167-169], TGFβ 11 [170], and NO [171, 172] with roles in stimulating the proliferation and/or growth of follicles or regulating follicular apoptosis have all been shown to be produced by macrophages in other systems [77-79, 96, 173]. In agreement with this the co-culture of peritoneal macrophages with granulosa cells results in stimulated proliferation of the granulosa cells above that of cells cultured alone [174].

The presence of macrophages in follicles at advanced stages of atresia suggests these cells participate in the removal of cell debris created during follicular atresia [175]. Macrophages have the ability to identify molecules in the cell membranes of apoptotic cells through the expression of scavenger receptors discussed earlier. Interestingly, macrophages have also been found to stimulate apoptosis in some cell lines [176, 177], while several cytokines that macrophages produce such as TNFα [178, 179] and IL-6 [180-182] have been found *in vitro* to induce apoptosis in ovarian and other cell types.

1.7.2 MACROPHAGES AND THE STIMULATION OF OVULATION

It was proposed in 1980 [29] that the ovulatory event was analogous to an inflammatory response with vasodilation, infiltration of leukocytes and production of inflammatory cytokines and prostaglandins triggered by the preovulatory LH surge. To date studies examining these events have explored and reinforced this hypothesis showing classical inflammatory mediators can influence many aspects of ovarian function. Macrophages have a pivotal role in the inflammatory process producing or stimulating the production of many of the inflammatory mediators required to regulate inflammation, tissue growth and restructuring processes (Section 1.5). Macrophages have the capacity to release numerous cytokines that have been demonstrated to be important in the ovulatory process, as well as some metallo-proteinases required for degrading the follicle wall and basement membrane. Here several key products of classically activated macrophages with roles in ovulation will be discussed.

1.7.2.1 Interleukin-1Beta (IL-1β)

IL-1β is a potent pro-inflammatory cytokine, released by classically activated macrophages, with divergent effects on different cell types [183]. In the ovary it has been implicated as a local regulator of follicle atresia [24], ovulation [184] and CL function [31]. Within the ovary the expression of the type I receptor for this cytokine has been demonstrated in the granulosa, and to a lesser degree the theca of growing and preovulatory follicles of the rat [185-187] and mouse [188]. The IL-1β protein can be found in human preovulatory follicular fluid [189] and in samples taken from *in vitro* perfused preovulatory rat ovary [190], with concentrations increasing as ovulation approaches. Levels of mRNA measured in the whole rat ovary similarly increase leading up to ovulation and have been localised in both the theca and granulosa layers[185] of the follicle. In the mouse IL-1β protein has been localised

prior to ovulation only in the thecal layer of the mouse preovulatory follicle [188], although following ovulation granulosa cells also express the protein. Culture of preovulatory follicles with IL-1β inhibits the spontaneous apoptosis of isolated preovulatory follicles *in vitro* through the production of NO [171], also known to be important for ovulation to proceed [61], while in the same system the 'rescuing' effects of gonadotrophins on follicles can be significantly reduced by the treatment with IL-1ra [171]. Addition of IL-1β to the media of *in vitro* perfused ovaries increases ovulation rates in the rat [191, 192] and rabbit [193], where it was also reported to promote oocyte maturation. In the mouse, injection of the IL-1ra, a naturally occurring receptor antagonist which has also been localised in the ovary [186, 194], inhibits ovulation *in vivo* [192, 195]. In the mare, intrafollicular injection of IL-1β has also been reported to promote oocyte maturation and ovulation *in vivo* [196]. More recently this cytokine has been shown to stimulate the expression of the vascular endothelial growth factor (VEGF) gene, a factor important for vascularisation occurring during the pre- and post-ovulatory stages, in whole ovarian dispersates and granulosa cell cultures [197].

1.7.2.2 Tumour Necrosis Factor alpha (TNFα)

TNFα is also an inflammatory cytokine produced by activated macrophages usually in combination with IL-1β. It is part of a TNF-like ligand and receptor superfamily, but individually has only two specific receptors, type 1 (p55/p60) and type 2 (p75/p80), which differ in their intracellular domain [198, 199]. It is believed that these receptors and their associating factors (TRAFs) orchestrate the divergent cellular responses of TNFα binding. Specific localisation of these receptors in the ovary has not been carried out although type 1 receptor mRNA has been detected in whole rat ovarian dispersates [200], which have also been shown to bind TNFα protein. Similar binding of the TNFα protein to pig [201] and bovine [202] granulosa cells has also been demonstrated. TNF protein has been measured in

human follicular fluid [203] and samples taken from the *in vitro* perfused preovulatory rat ovary [190], while mRNA for this protein has been localized in the oocyte and macrophage-like cells of preovulatory follicles [204]. Like IL-1β, TNFα can stimulate LH induced ovulation in the perfused rat ovary [205] and the production of ovulatory mediators, such as prostaglandin and progesterone, in cultured preovulatory rat follicles [206]. In addition, it has been proposed that in the sheep ovary, at the apex of the ovulatory follicle, the release of this cytokine from thecal cells stimulates local cellular apoptosis and thus facilitates follicular rupture [207].

1.7.2.3 Nitric Oxide (NO)

Nitric oxide, a prominent product of activated macrophages [208], is a reactive oxygen intermediate produced by the NO synthase enzyme, of which there is a constitutive (cNOS) and an inducible form (iNOS). Two isoforms of the latter have been found one, expressed in the endothelium (eNOS) and the other in the brain (nNOS). Both the inducible and endothelial forms of this enzyme have been reported to be expressed in the ovary in a regulated manner [209, 210]. In the immature and preovulatory ovaries iNOS has been localised to the theca and stroma [211], with an increase in protein levels occurring following LH administration [212]. Several studies have examined the effects of NO inhibitors on ovulation, reporting that this molecule is critical in follicular rupture in the rat [61, 212, 213] and rabbit [214]. Interestingly, it has also been hypothesised that IL-1β has its effects by stimulating the production of this molecule [215, 216]. The NO molecule has been postulated to have a role in regulating leukocyte adhesion and vascular permeability and dilation [217, 218]. Research in the ovary suggests it may also have a role in the prevention of follicular and luteal cell apoptosis [171, 219-221]. It has also been shown to have significant stimulatory effects on prostaglandin production, through activation of the cyclo-oxygenase enzymes in the ovary [222, 223] and other tissues [224, 225].

1.7.3 Macrophages And the Corpus Luteum

Studies of the distribution of macrophages within the ovary have found the greatest numbers in the CL, in particular the regressing CL. During the development and regression of this endocrine organ substantial tissue restructuring, angiogenesis and apoptosis occurs, in a manner similar to that seen in wound healing and resolution, events which macrophages are known to regulate through the secretion of many different cytokines and growth factors.

1.7.3.1 Macrophages in Corpus Luteum Formation

Following ovulation, the granulosa cells of the CL luteinise and enlarge to become LLC's while the SLC's derived from the theca rapidly proliferate. Both cell types become much more steroidogenically active and begin to produce progesterone. This development of the CL has been found to be dependent on angiogenesis, stimulated predominantly by VEGF [226, 227] with an intricate vascular network developing and enabling the CL to function as an endocrine gland. Macrophages have elsewhere been shown to secrete many factors capable of stimulating angiogenesis, including VEGF (reviewed by Sunderkotter [78, 79]), hence they may play a role in the establishment of the vasculature of CL through the secretion of factors such as EGF, bFGF (mentioned in 1.6.1) and VEGF, which can regulate vascular dilation and permeability, and stimulate endothelial growth or angiogenesis *in vivo*. It has been suggested elsewhere that macrophage inhibitory factor (MIF) is present in the early CL to prevent macrophage migration from the ovary and promote these processes, although it was also acknowledged that this factor is regulator of cellular differentiation [228]. Macrophages may also play a role in stimulating progesterone production seen in the forming CL. The addition of peritoneal macrophage conditioned media to granulosa cells results in increased levels of progesterone produced by granulosa cells [229], while several macrophage derived

cytokines such as IL-1β [230], EGF [231] and TNFα [232] have been shown to stimulate the production of progesterone and proliferation of granulosa-luteal cells derived from preovulatory follicles.

1.7.3.2 Macrophages and Luteal Regression

Luteolysis or regression of the CL can be divided into; functional regression involving reduced progesterone production by luteal cells; and structural regression involving decreased blood flow and cellular apoptosis. The means by which these processes occur is not clearly defined although the immune system is thought to be intricately involved [233]. In most mammals regression is stimulated by PGF2α derived from the non-pregnant uterus (reviewed by Niswender [31]) and ultimately leads to apoptosis and regression of the CL. The initiation of luteal regression stimulates the CL itself to produce endogenous PGF2α, although macrophages can themselves produce PGF2α and may therefore contribute to endogenous levels of this factor. Other work has shown that macrophage derived factors such as TNFα and IL-1β can stimulate the production of endogenous PGF2α by the CL [203, 234-236]. The inhibition of luteal cell progesterone production following PGF2α initiation of luteal regression may also be enhanced by the production of local mediators potentially derived from macrophages. The removal of leukocytes from luteal cell cultures has been shown to increase progesterone production in purified luteal cells [237], whilst progesterone production in these same cultures was stimulated by IL-1β only in the presence of leukocytes. The presence of peritoneal macrophages has also been shown to inhibit granulosa progesterone synthesis [238, 239], while potentially macrophage secreted cytokines such as NO [240] and IFN-γ [241] have been shown to inhibit progesterone secretion by luteinized cells.

Luteal regression is also characterized by the apoptotic death of luteal cells and an active Fas-Fas ligand system has been demonstrated in the CL [242-244]. Classically this type of system

is involved in the deletion of auto reactive FasL expressing T cells [245, 246]. It has been implicated as a mechanism of CL regression. Macrophages possess receptors that recognize apoptotic self cells and allow rapid clearance of these dying cells without the release of pro-inflammatory cytokines. In addition to this basic function macrophages also have the capacity to secrete factors that may stimulate apoptosis in the CL through this Fas system, such as TNFα and IFNγ[247]. It has been also demonstrated that IFN-γ mRNA [248, 249] is present in the bovine CL although the source of this cytokine within the tissue has not been clarified - T cells or macrophages further infiltrating at the commencement of regression are both potential sources. This cytokine has been found *in vitro* to stimulate apoptosis [250] and Fas mRNA expression [247] in mouse and bovine luteal cells respectively, when added to cultures in combination with TNFα, yet it is also potent stimulator of macrophage activation. The TNFα protein itself has also been demonstrated in the CL of the mouse [251], human [252] and rat [204] and has been equated with macrophage numbers in the CL [253, 254]. This protein has further been shown to stimulate of MHC antigens in luteal cells [235] expression of which has in the bovine been shown to change at regression [255]. Finally the demonstration that luteal cells can stimulate T cell proliferation provides convincing evidence of a role for the immune system in CL regression [256, 257].

1.8 SUMMARY AND HYPOTHESIS

1.8.1 SUMMARY

Research demonstrating the presence of macrophages in the ovary in association with growing antral follicles, ovulatory or atretic follicles and the CL initially prompted suggestions these cells may have some role in regulating processes occurring in these ovarian compartments. The observation that the ovulatory cascade, initiated by the LH surge, has characteristics similar to those of a classical inflammatory reaction, involving vasodilation and leukocyte infiltration, led to the hypothesis that cells of the immune system could play an important role in the regulation of reproductive events [29]. Macrophages are versatile cells of the immune system with the ability to perform various functions and, depending on the environmental cues provided, develop to perform specific roles in different organs of the body. In the ovary they are potentially the source of secreted inflammatory factors that complementary research has demonstrated can have significant effects on aspects of ovarian function. It may be speculated that macrophages are critical regulators of ovarian function that infiltrate growing follicles as 'resting' cells capable of stimulating follicle growth or apoptosis. Following the LH surge these cells are classically activated to promote ovulation and then following ovulation promote inflammatory resolution and tissue restructuring in the CL (Figure 1-9). The role of macrophages in other tissues and organs has been elucidated via specific macrophage depletion, knockout of genes essential to macrophage development and isolation of these cells from the organ of interest followed by analysis of their capabilities and characteristics *in vitro*. To date no attempts have been made to deplete or isolate the ovarian macrophage population, enabling the examination of their role *in vivo* and characteristics *in vitro*. This review demonstrates that an opportunity exists to establish the importance of macrophages in the ovarian tissue, to reveal their immunological attributes and to begin

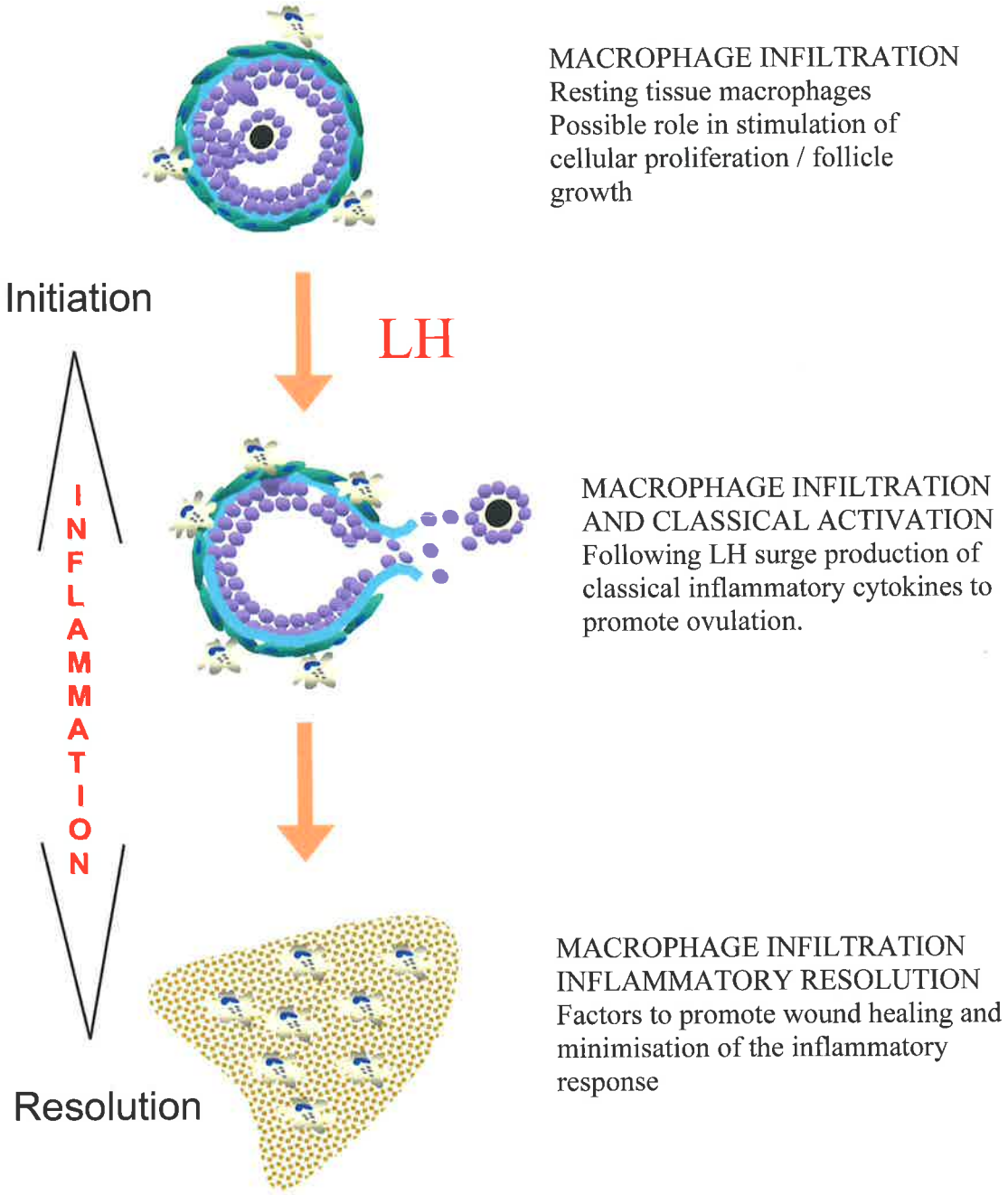


Figure 1-9 The Potential Roles Of Macrophages In Ovarian Function. Macrophages are located in the ovarian stroma and theca of antral and preovulatory follicles. Following the LH surge they infiltrate the follicle in a manner similar to that seen in classical inflammatory reactions. Further infiltration of macrophages into the CL occurs after follicular rupture for inflammatory resolution

to examine the characteristics of these cells in the ovarian tissue. This research hopes to more clearly define the role of macrophages in the murine ovary. The information obtained may assist in the understanding of how the processes of follicular growth, ovulation and CL formation and regression, in the mouse ovary are regulated. They may be extended to other mammalian species. Through the manipulation of the ovarian cytokine environment and/or ovarian macrophage function this work may have further implications in the understanding and treatment of some cases of unexplained infertility and/or polycystic ovary syndrome (PCOS), or in the regulation of human reproduction and the development of new contraceptions.

1.8.2 HYPOTHESIS

Ovarian macrophages, defined by the expression of the F4/80 and Ia antigens, play an essential role in regulating normal ovarian function through the production of inflammatory mediators across the different stages of the reproductive cycle.

1.8.3 GENERAL AIM

To determine if ovarian macrophages play a crucial role in regulating normal ovarian function and examine their functional and secretory characteristics, in an attempt to clarify the specific actions of these cells in the ovarian tissue.

1.8.4 SPECIFIC AIMS

1.8.4.1 Aim 1 - To deplete the macrophage population from the normal mouse ovary and determine at which times in the cycle these cells are of critical importance.

Microscopic lipid spheres or 'liposomes' containing clodronate, which when released into the cytoplasm induce macrophage apoptosis, have been used successfully to deplete the macrophage population of various organs [258]. Mature animals will have their ovarian cycles synchronised and be primed to ovulate simultaneously with PMSG/eCG and hCG. Liposomes will then be injected into the bursal cavity, which surrounds the murine ovary, on various days of the stimulated ovarian cycle. This will result in the death of the ovarian macrophage population at different times during the stages of follicular or CL development. The effect of the absence of an ovarian macrophage population on follicular growth, ovulation and cycle length will determine the importance of these cells in these phases of the ovarian cycle.

1.8.4.2 Aim 2 - To isolate viable ovarian macrophages from the ovarian tissue and examine basic immune functions.

Ovarian macrophages are present within the ovarian tissues and to extract them the tissue structure must be dissociated. Various dissociation methods have been utilised for tissues of different composition. The optimal conditions for ovarian dissociation and macrophage recovery will be determined. The purity and viability of the cells will be determined, isolated cell numbers correlated to previous studies, and the basic immune functions of phagocytosis and stimulation of T cell proliferation examined in these cells.

1.8.4.3 Aim 3 - To show that ovarian macrophages produce cytokines involved in ovarian function, and to show that this profile is unique and varies across the stages of the estrous cycle.

Various cytokines, which macrophages in the ovary have the ability to produce, have been found *in vitro* to influence ovarian function. To determine which of these cytokines are produced by macrophages in the ovary, immature female mice will be stimulated to ovulate with hCG and PMSG/eCG, and cells expressing specific macrophage markers will be isolated from the ovarian tissue and normal peritoneal cells at several time points in the ovarian cycle. From these isolated cells mRNA will be extracted for the analysis of cytokine content by quantitative RT-PCR techniques. This will allow comparison of the amount and type of cytokines produced by these cells during late follicular growth, ovulation and early CL growth and regression.

Chapter Two

2 THE DEPLETION OF MACROPHAGES IN THE MURINE OVARY BY INTRABURSAL INJECTION OF CLODRONATE (CL_2MDP) LIPOSOMES

2.1 INTRODUCTION

The previous chapter introduced and developed the concept that at ovulation ovarian macrophages may be a critical source of inflammatory cytokines that promote follicular rupture. It was noted that macrophages residing in peripheral tissues are derived from blood borne monocytes that differentiate in response to local cytokine and other microenvironmental signals to assume a functional phenotype specific to the requirements of the host tissue. Their roles in these tissues may include the phagocytosis and degradation of foreign organisms, tissue remodelling and the regulation of local immune and inflammatory responses. Studies examining the precise functions of these cells within the ovary have not been done.

The distribution of macrophages within the ovary has been reported to vary across the reproductive cycle in parallel with fluctuations in gonadotrophic and ovarian steroid hormones. In several species macrophages have been shown to be most abundant within the theca of the follicle during follicular development [53, 259, 260], in atretic follicles [63, 64] and in the CL [51, 66, 69]. In mice, immunohistochemical studies using macrophage-reactive antibodies such as Mac-1, F4/80 and CD18 have shown the presence of macrophages in the theca, stroma and CL as well as in association with atretic follicles [175]. These variations in location and numbers during the different stages of the cycle suggest that macrophages may participate in the tissue restructuring and inflammatory-like processes that occur in the normal adult ovary. Many studies have provided substantial evidence that factors produced by macrophages, such as IL-1β and TNFα can have stimulatory effects on ovulation [191, 261] and act to regulate steroid synthesis in cultured follicles [206, 262] or isolated granulosa and theca cells [229, 263, 264].

Together, these studies provide indirect evidence that implicate the ovarian macrophage population in the regulation of follicle development, ovulation and the formation and regression of the CL. Although studies conclusively showing a direct role for these cells are

currently lacking the reproductive characteristics of the csfm^{op}/csfm^{op} mouse, which has a null mutation in the gene encoding colony stimulating factor-1 (CSF-1), a cytokine that regulates both the development and differentiation of the macrophage lineage, provide convincing evidence to support this. These animals have few macrophages within the ovary, a significantly impaired ovulation rate and an extended estrous cycle [63] suggesting that the ovarian macrophage population is important in the normal functions of the adult cycling ovary. However, since macrophages may not be the exclusive target of CSF-1 in the ovary, it cannot be concluded that local macrophage deficiency is the primary cause of ovarian dysfunction in csfm^{op}/csfm^{op} mice. To clearly determine if macrophages play an essential role in ovulation it is necessary to remove macrophages specifically from normal ovarian tissue and study the resultant effects on ovulation and ovarian cyclicity. This may be achieved by local injection of CLi to the ovary.

Liposomes are synthetic phospholipid spheres that have been used extensively to target a variety of aqueous compounds to the macrophages present in different organs of the body. Following in vivo administration, liposomes are phagocytosed by macrophages, where upon the liposomal membranes are degraded by lysosomal phospholipases and the enclosed compound is released into the cytoplasm of the cell. The accumulation of CLi in the cytoplasm of target macrophages leads to cell death through the apoptosis pathway [265]. Macrophages in the liver and spleen can be depleted within 24 hours following a single intravenous injection of CLi and the population is not restored for 2 weeks thereafter [266]. Macrophages in the testis and peritoneal cavity have also been depleted following direct injection of CLi into the testis [267, 268] and peritoneum [269] respectively. It was therefore the aim of the study presented in this chapter to show the presence of macrophages in the ovary is essential for normal ovulation by the depletion of the macrophage population from normal mouse ovaries using intrabursal injection of CLi and examination of the resultant

effects on ovarian function. It was anticipated this would establish whether or not ovarian macrophages are important regulators of the ovulatory cascade.

2.2 MATERIALS AND METHODS

2.2.1 ANIMALS AND OVULATION INDUCTION

All animals were handled in accordance with The Australian Code of Practise for the Care and Use of Animals for Scientific Purposes and experiments were approved by the ethics committees of both The University of Adelaide and The Queen Elizabeth Hospital. Adult 8-11 week old C57Bl6 black female mice (from The University of Adelaide Central Animal House Facility) were maintained under controlled conditions (14 hour light : 10 hour dark cycle) with free access to food and water. The estrous cycles of the mice were synchronised with an intraperitoneal (ip) injection of 20 μg LHRH agonist (Des-Gly^{10} [D-Ala^{6}] LHRH ethylamide from Sigma Aldrich, St Louis, MO) at 0900 hours on day -4. At 1200 hours on day -2 animals were primed with a subcutaneous (sc) injection of 5IU of eCG (Folligon® from Intervet, Boxmeer, Holland) and ovulation was stimulated 48 hours later (1200 hours on day 0) with an ip injection of 5IU of hCG (Pregnyl® from Organon, Oss, Holland) (Figure 2-1). Ovulation occurred 12-15 hours after the hCG injection.

2.2.2 INTRABURSAL INJECTION TECHNIQUE

Animals were anaesthetised using a mixture of fluorothane, nitrous oxide and oxygen gases. Anaesthesia was then maintained throughout surgery with a continuous stream of the same gas mixture regulated by a Midget anaesthetic machine (CIG, Aust.). A single midline dorsal incision was made, followed by two small incisions into the peritoneum directly above the fat pad of both left and right ovaries. Each ovary was externalised through the respective incision and the intrabursal injection was performed under microscopic magnification by inserting a 30G needle through the ovarian fat pad into the ovarian bursa. In each animal

approximately 10µl of either clodronate liposomes (CLi) (containing dichloromethylene diphosphonate, CL_2MDP, a gift from Boehringer Mannheim GmbH, Mannheim, Germany) saline liposomes (SLi) (containing saline solution) or saline solution alone was delivered to both ovaries. All liposomes were prepared and supplied by N. van Rooijen as previously described [270]. Ovaries were returned to the peritoneal cavity and the wound was sealed with a single Autoclip® wound clip (from Becton Dickinson, Franklin lakes, NJ). Animals were initially placed in separate cages under a radiant heat source until normal behavioural activity was resumed and then housed separately under standard conditions until oocyte retrieval and tissue collection. Further details in Appendix 1.

2.2.3 Treatment Groups and Oocyte Retrieval

Intrabursal injections were carried out as described above on either day -3 or day -1 (Figure 2-1). In the first experiment, two groups of mice (n=8 in each) received either (a) CLi in one ovary and saline solution in the other (CLi/S group) or (b) CLi in one ovary and saline solution in the other (SLi/S group). In a second experiment, mice received CLi in both ovaries (CLi/CLi group, n=7) or saline solution in both ovaries (S/S, n= 8 for day -1 and n= 6 for day -3). On the morning of day 1 following ovulation, animals were anaesthetised with avertin (15µl/g), blood samples were taken via heart puncture and animals were killed by cervical dislocation. Ovaries and oviducts were recovered and the numbers of ovulated oocytes in the ampulla region were counted. In an additional experiment, animals treated as described above were allowed to proceed to the subsequent 'natural' ovulation following the induced ovulation. The day of estrus in these animals was monitored by daily vaginal smear tests [33] and on the morning when 100% cornified epithelial cells were detected, indicating metestrus -1 (ME-1), blood samples were taken, animals were killed and the number of ovulated oocytes in the ampulla region of the oviduct were counted. Further details in

Appendix 1. Progesterone levels were measured in serum samples using an automated chemiluminescence system (Ciba-Corning, Medfield, MA) with assay parameters as previously described [271].

2.2.4 Collection of Ovarian Tissue

Recovered ovaries were dissected free of fat and connective tissue and snap frozen in OCT (ornithine carbarnyl transferase, Tissue-Tek tissue freezing medium from Miles Inc. Elkhart, IN) at either the preovulatory stage, 1 hour prior to expected ovulation, or on day 1 post ovulation, at the time of oocyte collection (Figure 2-1). To establish that liposomes are able to penetrate the ovary, liposomes with the DiI fluorochrome (the lipophilic long-chain carbocyanine Di-I ($DiIC_{18}$ (3), Molecular Probes, USA) incorporated into the lipid bilayer of the liposome were injected into the bursa (n=2 per time point) and ovaries were recovered from animals killed 2, 4, 6, 12, 24 and 48 hours after surgery. Ten-micrometer sections from these ovaries were then examined using an Olympus Vanox microscope equipped for epifluoresence. Under conditions of green excitation (546nm) the Di-I in the liposomes fluoresces red.

2.2.5 Ovarian Morphology and Immunohistochemistry

Six-micrometer serial sections were cut from both ovaries of each mouse resulting in at least 140 consecutive sections from each ovary. Every 9th and 10th section was stained with haematoxylin and eosin (H&E) for morphological examination by light microscopy. Follicle size was measured in the H&E sections using video image analysis (VIA) software (Leading Edge Pty Ltd, Marion, S.A.) and adjacent sections containing preovulatory follicles

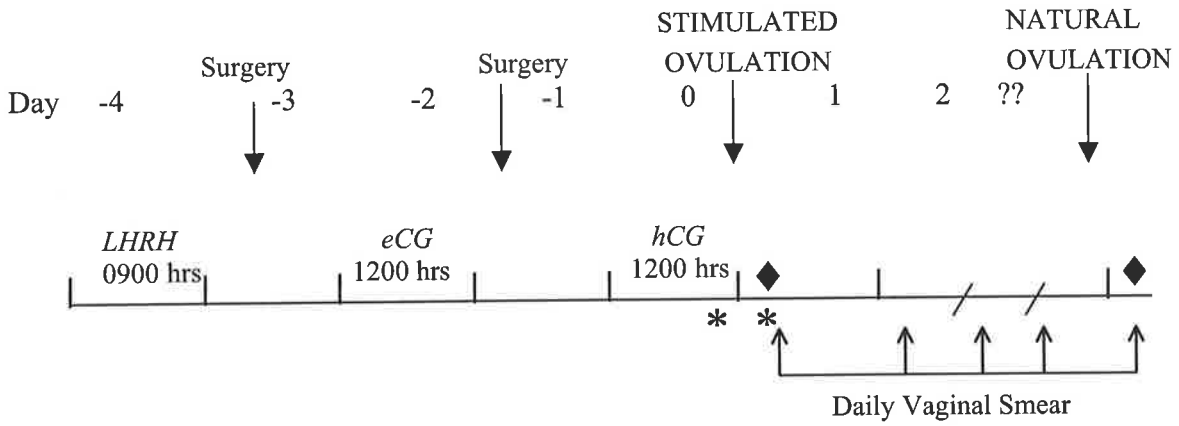


Figure 2-1 Schematic Diagram Of The Protocol For Ovulation Induction. The time of injection of gonadotrophins (LHRH, eCG, hCG), intrabursal injection (surgery), tissue collection (∗) and, oocyte retrieval and blood collection (♦) are indicated.

(>400μm) were stained with primary antibodies reactive with the anti-MHC II antigen (Ia) TIB120 (reactive with activated macrophages and dendritic cells, from American Type Culture Collection, Rockville MD), F4/80 (reactive with a surface glycoprotein specific to macrophages [113] and FA/11 (reactive with macrosialin [272]) both kindly supplied by S. Gordon, University of Oxford. Sections were fixed in 96% alcohol (4°C for 10 minutes) and then incubated with primary antibody from culture supernatant diluted in phosphate buffered saline (PBS, Gibco, BRL, Life Technologies, Grand Island, NY) containing 10% normal mouse serum (NMS) and 1% bovine serum albumin (BSA) (Boehringer Mannheim, Indpls., IN) (PBS-NMS) (Ia (1:200), F4/80 (1:10), FA/11 (1:600)) at 4°C for 3 hours, washed in PBS and then incubated with biotinylated-rabbit-anti-rat secondary antibody (from DAKO, Carpinteria, CA) diluted in PBS-NMS (1:300) at 4°C for 2 hours. Following another PBS wash, sections were incubated with avidin-horseradish-peroxidase (from DAKO, Carpinteria, CA) diluted in PBS-NMS (1:400) and enzyme was then visualised using Sigma-Fast DAB tablets (from Sigma Aldrich, St Louis, MO). Uterus and spleen were used as positive control tissues and negative controls included sections incubated without primary antibody or with irrelevant monoclonal antibodies. The area of positive stain in each section was evaluated by VIA and expressed as % positivity (area brown positive stain/area total stain x 100). Three thecal regions in each preovulatory follicle and six stromal regions in each ovary were counted and the mean % positivity value for each follicle and ovarian stroma was calculated. Further details in Appendix 1.

2.2.6 STATISTICS

A paired Students t-test was used to evaluate differences in the numbers of ovulations between the ovaries of animals within the CLi/S treatment group as well as differences in the numbers of preovulatory follicles present in the ovaries of these animals at the time of

ovulation and post ovulation. A one-way ANOVA with Tukey-Kramer multiple comparisons was used to examine the differences in ovulation rates between treatment groups and the length of the different stages of the estrous cycle in animals from the CLi/CLi and S/S groups. An unpaired Students t-test was used to compare cycle length and serum progesterone levels in animals from the CLi/CLi and S/S groups.

2.3 RESULTS

2.3.1 EFFECT OF CLODRONATE LIPOSOME TREATMENT ON GONADOTROPHIN STIMULATED OVULATION

To determine the effect of administration of CLi on ovulation rate, mice were killed 9 hours after ovulation following treatment on either day -1 or day -3 prior to ovulation (Figure 2-2). No significant difference in ovulation rate between CLi treated and the contralateral saline solution treated ovaries was observed in animals from the CLi/S group when treatment was administered on day -1 (number of oocytes ovulated, mean ± SE = 7 ± 1.7 and 6.4 ± 0.9 in between CLi treated and saline solution treated ovaries respectively). This ovulation rate did not differ significantly from that of animals in the S/S group, (6.5 ± 1.3 and 5.8 ± 0.9 in left and right saline solution treated ovaries respectively). However a significant reduction in the ovulation rate in CLi treated ovaries was observed in animals from the CLi/S group when treatment was administered on day -3 ($p<0.05$, Figure 2-2a). No significant difference was seen in the ovulation rate in saline liposome treated or saline solution treated ovaries in the SLi/S group when treatment was administered on day -3. A large reduction in the ovulation rate of both CLi treated ovaries was seen in the CLi/CLi group, while the ovulation rate in the S/S group was not diminished (Figure 2-2b). When these results were pooled to give a single mean value for all saline solution treated or CLi treated ovaries, a significant reduction in ovulation rate was found in CLi treated ovaries (number of oocytes ovulated = 8.5± 1.2 and 3.7± 0.8 in Sand CLi treated ovaries respectively, $p< 0.05$). Progesterone levels measured in serum samples from animals in which treatment was administered on day -3 showed no significant differences between any treatment groups, including those in which ovulation was affected (S/S= 32.5 ±5.7 nmol/L; SL/S = 39.6 ±4.3nmol/L; CLi/CLi = 34.7 ±10.4nmol/L).

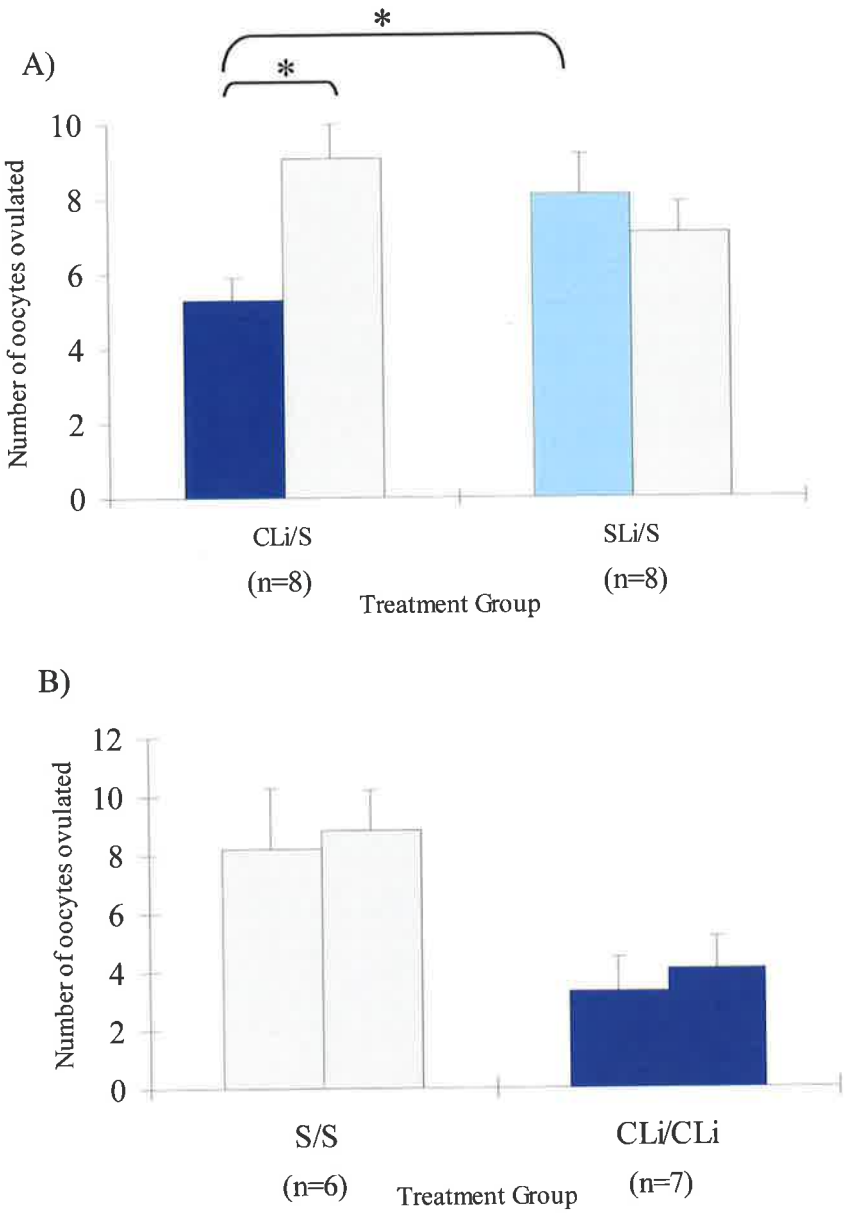


Figure 2-2 The Effect Of Clodronate Liposome Treatment On Ovulation Rate.

Data are mean (± SEM) number of oocytes ovulated after intrabursal administration on day -3 of (a) clodronate liposome/saline (CLi/S) and saline liposome/saline (SLi/S) groups and (b) S/S and CLi/CLi groups. When results were pooled to give a single mean value for all S-treated or CLi-treated ovaries, a significant reduction in ovulation rate following CLi-treatment was detected ($p < 0.05$) (S treated ovaries = 8.5± 1.2; CLi-treated ovaries =3.7± 0.8). In both graphs ■ = S-treated ovaries, ■ = SLi-treated ovaries and ■ = CLi-treated ovaries. * indicates significantly different to saline solution treated ovaries ($p<0.05$).

2.3.2 Effect of Clodronate Liposome Treatment on The Ovarian Macrophage Population

To establish that liposomes were able to penetrate the ovarian epithelium from beneath the bursa and enter the ovarian tissue, liposomes containing the fluorescent marker DiI were injected into the bursal cavity and animals were killed at several time points after injection. In sections from ovaries collected two hours after injection, bright fluorescence could be seen in the stroma and thecal regions of the follicles in the ovary but not the granulosa or antrum (Figure 2-3). Four hours after fluorescent liposome injection the fluorescence was less bright and six hours later the fluorescence appeared significantly diminished. No significant difference in fluorescence intensity could be seen between untreated and fluorescent liposome treated tissue at later time points.

To determine whether CLi treatment was effective in depleting macrophages from the ovary and to determine whether the observed reduction in ovulation rate was correlated with any decrease in thecal macrophage numbers, ovarian sections were incubated with antibodies specific for the macrophage antigens Ia, F4/80 and macrosialin. Macrophages reactive with all of these antibodies were present in all ovarian sections (Figure 2-4,2-5). A comparison of the abundance of macrophages between the two ovaries from mice in the CLi/S groups treated on either day -1 or day –3 indicate that CLi treatment had no apparent effect on the mean stromal positivity of any of the macrophage antigens (Figure 2-6C,D). In the theca of preovulatory follicles a reduction in macrophage numbers was seen for all antigens and this was statistically significant for the macrosialin$^+$ macrophages ($p< 0.05$, Figure 2-6B) in animals administered CLi on day –1 and both the macrosialin$^+$ and Ia$^+$ macrophages in animals administered CLi on day –3. ($p<0.03$, Figure 2-6A).

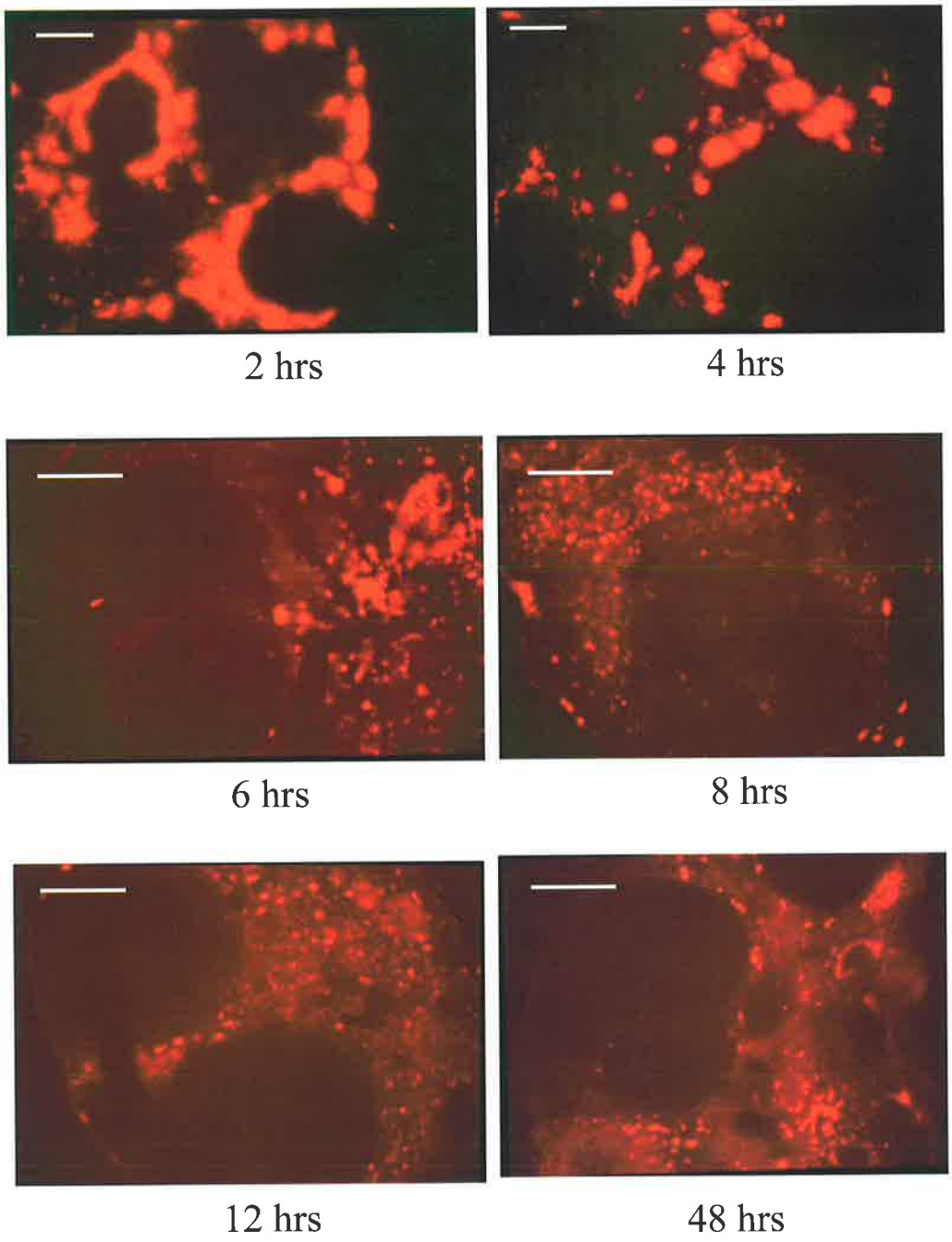
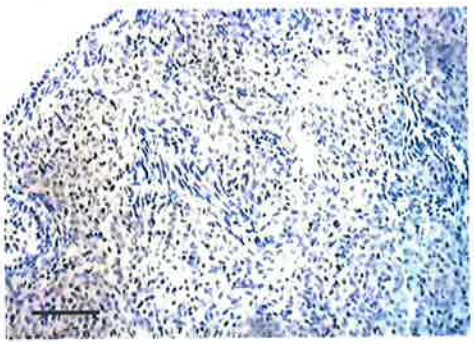


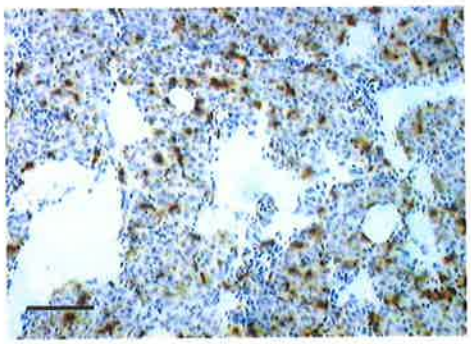
Figure 2-3 Ovarian Distribution Of Fluorescent DiI Liposomes Following Intrabursal Injection. Tissue sections were examined 2, 4, 6, 12, 24 and 48 hours following intrabursal injection of liposomes containing the fluorescent marker DiI. (Bars in each image represent 200μm).

Figure 2-4 Immunohistochemical Localisation Of Macrophages In The Stroma Of Ovarian Tissue Sections. Sections were cut from the preovulatory ovaries of animals in the group receiving intrabursal injection of clodronate liposome (CLi) (b, d, f)/saline (S) (C, E, G), on day –3 in which ovulation was reduced. (A) negative control, no primary antibody; (B, C) macrosialin^{+} macrophages in the stroma of the ovary; (D, E) F4/80^{+} macrophages in ovarian stroma and (F, G) Ia^{+} macrophages in the stroma. Bars in each figure represent 100μm.

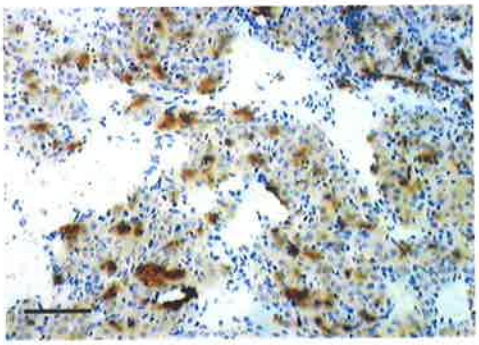
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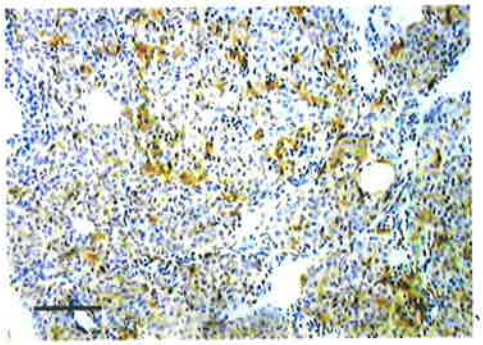
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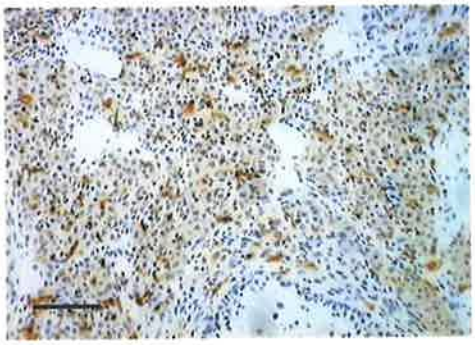
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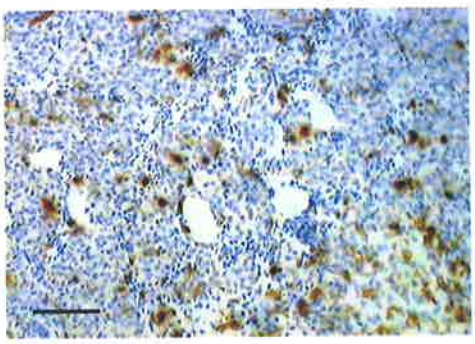
D)



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F)



G)

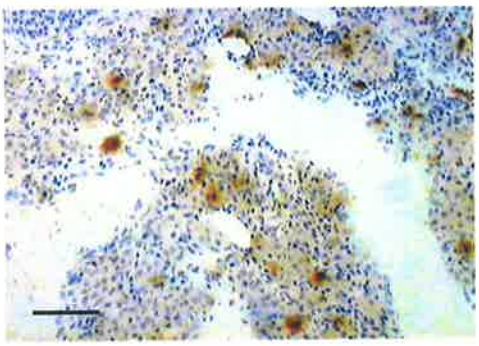
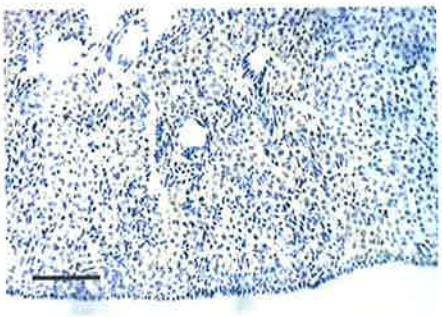
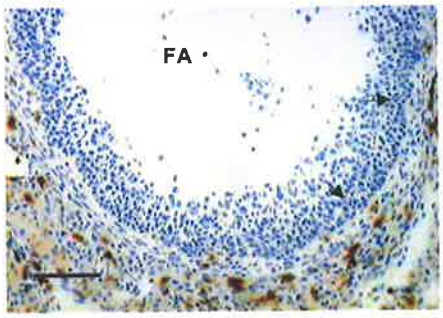


Figure 2-5 Immunohistochemical Localisation Of Macrophages In The Theca Of Ovarian Follicles. Sections were cut from the preovulatory ovaries of animals in the group receiving intrabursal injection of clodronate liposome (CLi) (B, D, F) and saline (S) (C, E, G), on day –3 in which ovulation was reduced. (a) negative control, no primary antibody; (B, C) macrosialin^{+} macrophages in the theca; (D, E) F4/80^{+} macrophages in theca and (F, G) Ia^{+} macrophages in the theca. Arrows indicate macrophages in the theca of the follicles, FA =follicular antrum, O = oocyte. Bars in each figure represent 100μm.

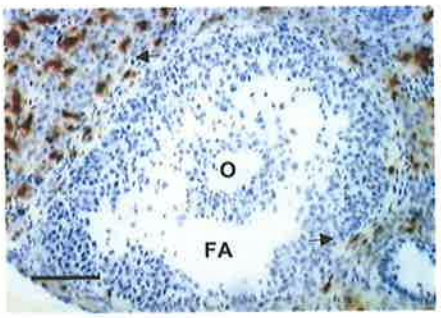
A)



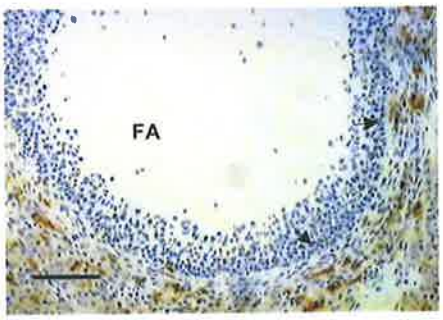
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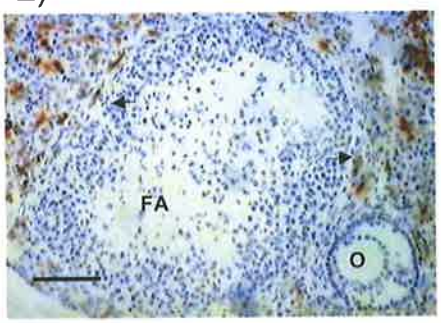
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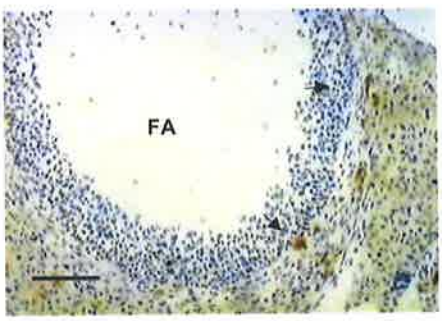
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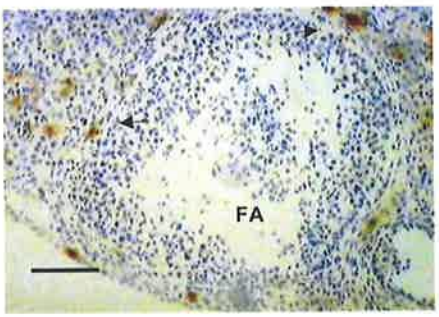
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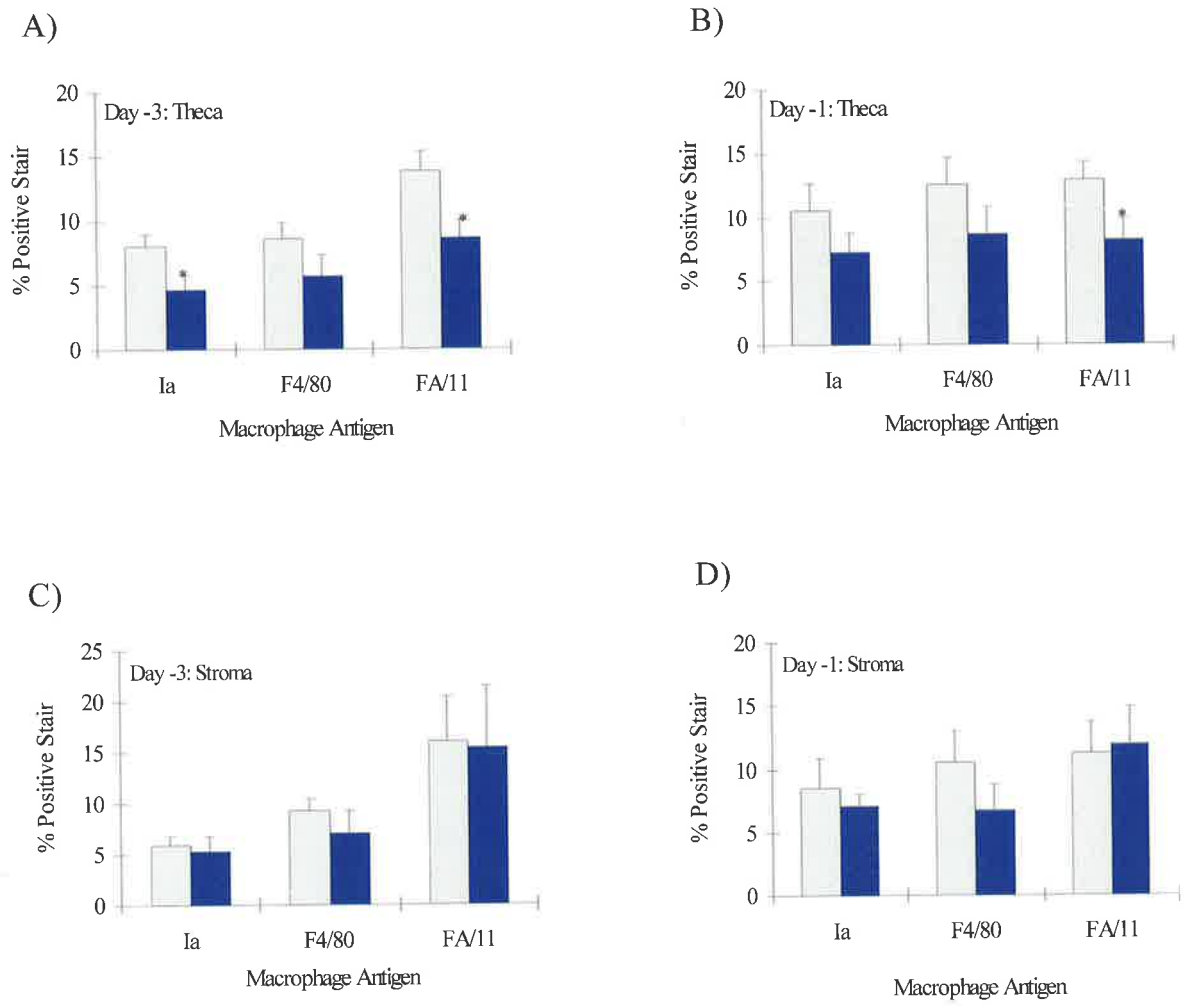


Figure 2-6 The Effect Of Clodronate Liposome Treatment On The Number Of Macrophages In The Ovarian Theca And Stromal Compartments. Data are mean (± SEM) percentage of positive stain (macrophages) for the antigens Ia, F4/80 and macrosialin (FA/11). Tissue is from mice in the clodronate liposome/saline (CLi/S) treatment group and graphs show mean positivity in the theca (B) and stroma (D) of ovaries following intrabursal injection of either S (, n= 19 follicles, in 4 animals) or CLi (, n= 9 follicles, in other ovary of same 4 animals) on day -1 or in the stroma (C) and theca (A) of ovaries following intrabursal injection of either S (, n = 21 follicles, in 4 animals) or CLi (, n=16 follicles, in other ovary of same 4 animals) on day -3. * indicates significantly different to saline solution-treated ovaries ($P<0.05$).

2.3.3 Effect of Clodronate Liposome Treatment on Ovarian Tissue Morphology

To determine whether the reduction in ovulation rate seen following CLi treatment on day -3 was due to the prevention of follicular rupture or a reduction in the number of preovulatory follicles that develop, the total number of follicles of preovulatory size (diameter >400 um) and corpora lutea were counted in H&E sections of both the ovaries from 4 animals in the CLi/S group. When ovaries were collected following ovulation there appeared to be fewer corpora lutea and more preovulatory sized, unruptured follicles in the CLi treated ovaries than the S treated ovaries, although these differences were not significant (Table 2-1). In ovaries collected immediately prior to ovulation there appeared to be fewer preovulatory sized follicles and more corpora lutea in the CLi treated ovaries than the S treated ovaries, although no significant differences were detected.

Ovarian Treatment and collection time	Ovulation rate	No. of Follicles >400μm	No. of Corpora lutea
Post Ovulation			
S-treated	12.5 ± 2.6	2 ± 0.7	13 ± 1.8
CLi-treated	7.8 ± 1.8	4 ± 1.6	9.3 ± 1.4
Prior to Ovulation			
S-treated	-	6.5 ± 1.8	6.8 ± 1.8
CLi-treated	-	5.3 ± 1.9	7.8 ± 1.6

Table 2-1 The Effect Of Clodronate Liposome Treatment On The Number Of Preovulatory Follicles And Corpora Lutea. Data are mean (±SEM) number of preovulatory follicles and corpora lutea counted in each ovary of animals in the CLi/S group treated on day –3. n=4 in each group. S-treated = saline solution treated ovaries, CLi-treated = clodronate liposome treated ovaries.

2.3.4 Effect of Clodronate Liposome Treatment on Subsequent Natural Ovulation

Since CLi treatment appeared to affect the numbers of follicles developing to the preovulatory size, we sought to determine the consequences of this for the next ovulatory event. In all animals allowed to progress to the next natural ovulation after treatment, ovulation rates were reduced in comparison to those of the stimulated cycle. No significant differences were detected regardless of the treatment group (CLi/SLi, SLi/SLi, CLi/CLi) or the time point of treatment (day-1 or day-3) (Table 2-2). However it was noted that some animals receiving CLi treatment appeared to take longer to reach the subsequent natural ovulation. In animals treated on day -1 no significant differences in cycle length were found between any of the treatment groups regardless of treatment, although the cycle length was extended beyond the expected 4-5 days in all of these animals (Table 2-2). In animals treated on day -3 in the CLi/S and CLi/CLi groups, cycle length was significantly extended over that in the SLi/SLi group (Table 2-2). Further breakdown of the cycle in this affected group revealed a significant delay in the metestrus-2 / diestrus (ME-2/DE) stage (Figure 2-7). The progesterone levels in serum obtained following ovulation were not significantly different between the treatment groups displaying delayed ME-2 / DE stage (CLi/CLi = 7.1 ± 1.8nmol/L; S/S =4.9 ± 1.3nmol/L).

	Treatment Group S/S	SLi/SLi	SLi/CLi	CLi/CLi
Subsequent Ovulation Rate				
IB injection on Day -1	(n=6)	n=6)	(n=8)	-
	3 ±0.8	4.2 ±0.7	2.8 ±0.7	-
	2.8 ±1.1	4.0 ±1.2	2.6 ±0.8	-
IB injection on Day -3	(n=10)	(n=5)	(n=7)	(n=6)
	3.4 ±0.6	3.8 ±1.1	2.9 ±0.6	3.3 ±1.0
	3.2 ±0.4	4.2 ±1.6	2.0 ±0.9	4.5 ±1.3
Cycle Length (days)				
IB injection on Day -1	6.6 ±1.4	7.2 ±1.3	7.4 ±2.6	-
IB injection on Day -3	5.2 ±0.6	3.4 ±0.4	6.2 ±0.7*	7.5 ±1.3*

Table 2-2 The Effect Of Clodronate Liposome Treatment On Subsequent Natural Ovulation And Cycle Length. Data are the mean (±SEM) ovulation rate from each ovary and mean cycle length of animals in the different treatment groups. IB= intrabursal injection. Mice in the S/S group received saline solution in both ovaries, mice in the SLi/SLi group received saline liposomes in both ovaries, mice in the SLi/CLi group received saline liposomes in one ovary and clodronate liposomes in the other, mice in the CLi/CLi group received clodronate liposomes in both ovaries. * indicates significantly different to SLi/SLi ($p<0.01$).

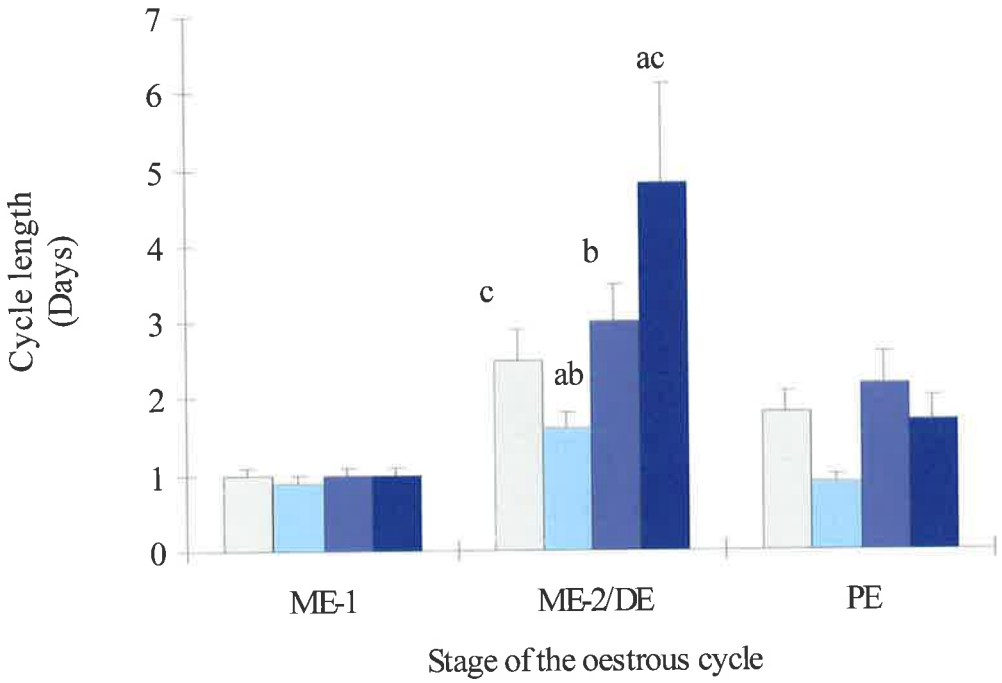


Figure 2-7 The Effect Of Clodronate Liposome Treatment On Progression Through The Subsequent Estrous Cycle. Data are mean (± SEM) number of days spent in each stage of the estrous cycle for animals treated on day -3. ■ = animals in the S/S group (n=5), ■ = animals in the SLi/SLi group (n=5), ■ = animals in the CLi/SLi group (n=8) and ■ = animals from the CLi/CLi group (n=6). Stages of the cycle are represented as follows; ME-1 denotes metestrus-1, ME-2/DE denotes the combined metestrus-1/diestrus stages and PE denotes proestrus. Bars with the same letter are significantly different to each other ($P<0.05$).

2.4 DISCUSSION

Liposomes containing clodronate have been used extensively to target macrophage cells in various organs within the body. In the current study, we have employed clodronate-filled liposomes to achieve a moderate but significant reduction in macrosialin and MHC-Class II (Ia) $^{+}$ macrophages in the ovarian theca. This depletion was found to be associated with impaired ovarian function, as measured by the number of oocytes ovulated, and the duration of the subsequent estrous cycle. The strongest results were obtained when CLi were injected on day -3, 84 hours prior to ovulation. The reduction in ovulation rate seen following CLi treatment at this time, during the early stages of the follicular cycle, can be fully attributed to the clodronate contained within the liposomes since no reduction in ovulation rate was seen in the SLi/S group. The corresponding immunohistochemistry data show that administering treatment at this time point does not significantly affect the number of macrophages in the ovarian stroma, while within the theca of preovulatory follicles there were significant reductions in both Ia^{+} and $macrosialin^{+}$ macrophages. This suggests that the detrimental effect on ovulation rate might be the consequence of a perturbation in the thecal macrophage population.

Examination of follicle numbers in the ovaries of this treatment group immediately prior to and following ovulation suggests that the growth of follicles to the preovulatory size, as well as follicular rupture, may be compromised by CLi treatment. This finding concurs with studies in the CSF-1 deficient $csfm^{op}$/ $csfm^{op}$ mice where severe reduction in macrophage numbers in the ovary is associated with significantly fewer antral and mature follicles present at the proestrus stage [273]. The difference in the extent of the effect in the two experimental systems might be related to the difference in the relative severity of the macrophage depletion achieved. Mice treated with CLi 3 days prior to ovulation were also found to have delayed progression through the ME2/DE stage of the subsequent cycle. This stage is characterised by high

numbers of macrophages within the ovary when compared to other stages of the cycle [64]. Interestingly this stage of the ovarian cycle appears to be particularly susceptible to perturbations in macrophage population, as both GM-CSF null mice (which have a reduced number of stromal Ia^+ cells in the ovarian stroma) and $csfm^{op}$/ $csfm^{op}$ mice, exhibit delays in the DE stage of the estrous cycle [63].

CLi treatment at the stage of late follicular development (day -1) did not affect the numbers of oocytes ovulated. This suggests that macrophages may be less critical for the events immediately preceding ovulation or that the extent of depletion achieved was not sufficient to have a detectable effect on the process. In support of the latter the effect of liposome treatment on macrophages is dependant on both tissue penetration by the liposomes and the rate of macrophage phagocytosis. It has been suggested that splenic macrophages expressing different markers are depleted at different rates [265] and the immunohistochemistry results presented here also support this concept in the ovary. Therefore it may take more than 24 hours for the full extent of clodronate liposome treatment to occur in the macrophages of the murine ovary.

Although numerous authors have shown significant effects of macrophages on progesterone production *in vitro* [174, 238, 239] [229], the levels of peripheral blood progesterone measured in this study were not altered by thecal macrophage depletion. This implies that the cells we have targeted are not essential for the maintenance of systemic steroid levels. This is in agreement with the finding that the reduced number of macrophage cells in $csfm^{op}$/ $csfm^{op}$ mice does not correlate with any change in the levels of progesterone produced during the luteal phase [63].

Although our results show that CLi treatment is clearly influencing ovarian function and altering macrophage numbers, complete depletion of the ovarian macrophage population, as has been seen elsewhere with CLi treatment, was not achieved. This may be due to either

insufficient or uneven distribution of CLi through the ovarian tissue and might have been improved if it was possible to administer a larger volume of CLi under the ovarian bursa. Alternatively, it is plausible that a high turnover of macrophages in the ovary may result in rapid replacement of those cells that are killed by CLi treatment. Clearly the treatment regime employed in the study had some non-specific effect on ovarian function since all animals treated on day -1 had extended cycles regardless of treatment type. This may to be due to the proximity of anaesthesia and surgery to the ovulatory event, and interference of the drugs used with hypothalamic function or the tissue remodelling that occurs during this process. We have found in other experiments that these procedures have significant effects on ovulation when carried out close to the initiation of this event (unpublished observations). We have been unable to determine the duration of the effect of macrophage depletion on the numbers of oocytes ovulated, as the ovulation rate in the subsequent natural ovulation was much reduced in comparison to the ovulation rate of the first cycle regardless of the treatment type. This is probably due to either the hyper-stimulation regime employed to induce the first ovulatory event exhausting the pool of growing follicles, or the physiological stress related to the surgery carried out.

It is interesting that macrophages in the vicinity of the thecal layer surrounding developing follicles appear to be preferentially targeted by CLi treatment. The reasons for this are not clear. The physical proximity of the developing follicle to the ovarian surface (and hence the bursal cavity where the clodronate was administered) might be a contributing factor and it may also be speculated that the composition of the extracellular matrix in this region is more conducive to diffusion of liposomes than the more dense collagenous structures of the ovarian stromal matrix. The results also indicate that even within the thecal compartment CLi treatment may deplete macrosialin$^+$ and to a lesser extent Ia$^+$ macrophages in preference to F4/80$^+$ macrophages. This suggests that there is heterogeneity amongst the ovarian macrophage

population and implies that some subsets might be more phagocytically active or otherwise more susceptible to the effect of CLi treatment. In view of the association of macrosialin with the phagocytic process it might be speculated that strong macrosialin expression is indicative of high phagocytic activity and perhaps a greater capacity to take up liposomes from the extracellular milieu.

In conclusion, we have shown that local CLi treatment can be used to achieve a reduction in ovarian macrophage numbers. The technique has been successfully employed in the current study to generate data that suggests thecal macrophages have a role in stimulating the growth of follicles to the preovulatory stage of development and in achieving follicular rupture. Further studies to define the phenotype and cytokine profile of the various macrophage populations residing in the ovary will help to elucidate the precise mechanisms through which they participate in regulating the different ovarian processes.

Chapter Three

3 The Numbers and Characteristics of Murine Ovarian Ia and F4/80 Positive Macrophages Isolated during the Gonadotrophin -Stimulated Reproductive Cycle

3.1 INTRODUCTION

The previous chapter presented direct experimental evidence that ovarian macrophages are important for normal follicular development ovulation, however exactly what key functions these cells perform in the ovary is not yet understood. Ovarian macrophages have the potential to produce a large number of different cytokines including IL-1β and TNFα which are known to significantly affect steroidogenesis [202, 237], cellular proliferation [203, 274, 275], vascularisation [197, 276] and apoptosis [171, 178] occurring in the ovarian tissue (reviewed by Terranova et al [277]). To allow more in depth studies into the roles of these cells, they must be isolated with high purity from the other ovarian cell types. Thus far studies examining macrophages in the ovary have, utilising immunohistochemical techniques, simply recognized and quantified these cells through their expression of surface molecules specific to the monocyte/macrophage lineage [64, 278-280]. Some studies have gone further investigating the effects of the depletion of ovarian macrophages, by adherence to plastic dishes, on the activities of ovarian dispersates [278]. Still more specifically, selected mRNA transcripts produced by $CD11b^{+}$ cells of the adherent population from ovarian dispersates [281] have also been studied. To date, no attempt to purify murine ovarian macrophages directly, using specific macrophage antibodies, from an ovarian digest has been reported.

The isolation of mononuclear cell types from murine tissues other than the ovary has been successful and is routinely performed in immunological studies. Blood monocytes, peritoneal and alveolar macrophages can all be isolated with relative ease using density gradient centrifugation and peritoneal or bronchi alveolar lavage respectively. However, the isolation of cells from solid tissues, such as the ovary, requires dissociation of the tissue mass into a single cell suspension before purification of the desired cell type can be performed. Collagenase is most commonly used for tissue digestion and has been used to isolate cells from many other murine organs including gut [282], liver [283], spleen [284], testis [134,

137] and uterus [143, 147]. It has also been used in combination with trypsin or dispase to isolate cells from heart and kidney, [285] or intestine [286] respectively. Ovarian dissociations have been performed in previous studies with the purpose of examining activities of thecal-interstitial cells [287], luteal cells [288] or of the ovarian dispersate as a whole [289, 290]. The methods employed vary, with ovarian tissue dissociation lengths of both 45 and 90 minutes reported [281, 289-291]. The specific isolation of $CD11b^+$ cells from luteinised mouse ovaries more recently reported by Tadros et al [281] gives no indication of either the numbers of animals required or cells recovered.

It was the aim of this study to develop and describe a technique to isolate murine ovarian macrophages. This will allow further studies of the characteristics of these macrophages and lead to a better understanding of the contribution these cells make to ovarian function.

3.2 METHODS

3.2.1 ANIMALS AND GONADOTROPHIN STIMULATION

SV129/Ola mice were maintained in a 14L: 12D light: dark cycle with food and water available *ad libitum*. All experiments were approved by animal ethics committees of both The Queen Elizabeth Hospital and The University of Adelaide and animals were used in accordance with The Australian Code of Practise for the Care and Use of Animals for Scientific Purposes. Unless otherwise stated, reagents were purchased from Sigma Aldrich Chemical Co., St Louis, MO, USA. Adult animals were used at 7-9 wks of age. The first ovulatory cycle was stimulated in immature animals aged 25-29 days with 5 IU of eCG (Intervet, Boxmeer, Holland) in 0.1ml PBS (Gibco BRL, Life Technologies, Grand island, NY, USA) with 0.1% BSA (Fraction V) at 1200 hours on day –2 followed by 5 IU of hCG (Pregnyl®, Organon, Oss, Holland) 48 hours later on day 0 to stimulate ovulation approximately 12 hours later. Animals were killed by cervical dislocation at 0900h on day -1, day 0 am, day 1 and day 2 as well as 6 hours post hCG administration (day 0pm). Following the collection of peritoneal macrophages by peritoneal lavage with 2.5mls Hanks Balanced Salt Solution (HBSS) with 0.35 g/L sodium bicarbonate ($NaCO_3$), 5mM Ethylenediamine tetra-acetic acid and 0.01% Sodium Azide (HBSS/EDTA/Az) ovaries were collected from groups of 8-10 animals for digestion and subsequent isolation of macrophages.

3.2.2 DISSOCIATION CONDITIONS

Peritoneal cells collected from four adult animals in HBSS/EDTA/Az were centrifuged (300G) and 1 x 10^6 re-suspended in 2mls of a 1mg/ml Collagenase, 25U/ml Deoxyribonuclease I (DNase I) solution made up in RPMI medium 1640 (Gibco, BRL, Life Technologies, Grand Island, NY, USA) with 2 g/L sodium bicarbonate ($NaHCO_3$) and 10%

heat-inactivated fetal calf serum (FCS) (Trace, Victoria, Australia) and cultured at either room temperature (RT) or 37°C for 90 minutes in siliconised (Coatasil Glass Treatment Solution, Labchem, APS Ajax Fine Chem, NSW, Australia) glass or plastic beakers. Cells were then washed in 10mls of HBSS/EDTA/Az and centrifuged (200G) at 4°C for 10 minutes. The cell pellet was re-suspended in 1ml of HBSS/10% FCS and the numbers of recovered live cells counted using trypan blue exclusion. This was repeated four times and the percentage of cells that were recovered live for each condition compared statistically using a One-Way Anova.

3.2.3 Dissociation of Ovaries

To optimize the ovarian dissociation conditions, ovaries from three adult mice aged 7-9 weeks were collected, dissected free of the surrounding bursa and fat, weighed and minced finely with vannas spring scissors under a dissecting microscope. The resulting tissue pieces were cultured for either 45 or 90 minutes in 1mg/ml collagenase (Sigma C9891) and 0.025mg/ml DNase made in each of the following media bases;

1) RPMI with 2 g/L Na HCO3 and 10%FCS (RPMI/FCS);

2) Hepes buffered (Hepes sodium salt 3.25 g/L and Hepes acid salt 2.98 g/L) alpha Minimum Essential Medium (αMEM, Life Technologies, Grand Island NY, USA)) with 2.2 g/L $NaHCO_3$(HMEM1) (This media lacks glutathione that can inhibit enzyme activity).

3) Hepes buffered αMEM with 2.2 g/L $NaHCO_3$ and 5 mM Calcium Chloride ($CaCl_2$). (H-MEM) (The latter ensures maximal activation of the collagenase enzyme).

For every 20mg of ovarian tissue, 1ml of enzyme solution was added. All digests were done at RT with gentle shaking in siliconized glass beakers. The resulting cell suspension was filtered through a 70 μm cell strainer (Falcon®, Becton Dickinson Labware, NJ, USA). The filtered cells were washed twice in HBSS/EDTA/AZ and centrifuged (200G) at 4°C and then

re-suspended in 1 ml HBSS/10% FCS for counting of live and dead cells. The ovarian tissue remnants remaining in the cell strainer were washed back into the beaker with 3mls of ice cold HBSS/EDTA/AZ and incubated for a further 20 minutes at 4°C. The resulting cell suspension was filtered, washed once in HBSS/EDTA/AZ and centrifuged (200G) at 4°C before being re-suspended in 500µl of HBSS/FCS for counting of live and dead cells. Ovarian digestion under each condition was repeated four times and the total number of cells per milligram and percentage of live cells isolated was compared statistically using a One-Way Anova. Other macrophage isolation methods attempted are described in Appendix 2.

3.2.4 Labelling of Ovarian macrophages

Digested cells were re-suspended in 1 ml HBSS/10%FCS and 500µl of cells were incubated with 500µl of either anti-MHC class II antigen (Ia) (TIB120 from American type culture collection, MD, USA) or F4/80 (supplied by S. Gordon, University of Oxford) hybridoma supernatant, in glass silicon coated tubes (Vacutainer®, Becton Dickinson, NJ, USA) for 1 hour at 4°C with gentle rocking. Unbound antibody was removed by washing with HBSS/EDTA/Az followed by centrifugation (200G) at 4°C. The pellet was re-suspended in 3ml of HBSS/EDTA/Az over-laid onto 3ml of FCS and centrifuged (300G) at 4°C. Cells were re-suspended in 1ml of HBSS/10%FCS and added to prepared panning plates.

3.2.5 Antibody Panning

Bacterial grade 36mm petri dishes (Sarstedt, NC, USA) were incubated for 15 hours at 4°C with 10mg/ml anti-rat IgG (Calbiochem®, Merck KgaA, Darmstadt, Germany) in PBS, washed with 5 ml PBS then cultured with PBS-10%FCS for 20 minutes at 4°C. Plates were

washed again with 5 ml of PBS and the labeled cell suspension then added. The plates were then incubated for 2 hours with gentle rocking at 4°C. Unbound negative cells were then washed from the surface of the plate with 10ml HBSS/10%FCS. The numbers of cells bound in 10 randomly selected fields were counted using an inverted phase contrast microscope and the total numbers of cells bound per plate calculated for all the time points listed in 3.2.1. Cell numbers were compared statistically using a One-Way Anova.

3.2.6 VIABILITY

Bound cells isolated before (day 0am) and after ovulation (day 1) were stained for 10 minutes with Hoechst (10 µl/ml) and propidium iodide (PI) (15 µl/ml) in HBSS/10%FCS, wet mounted and examined under an Olympus Vanox (AHBT3) fluorescent microscope. The numbers of live (blue) and dead (red) cells were counted in randomly selected fields at each time point and expressed as the percentage of total cells.

3.2.7 COLLECTION OF CELLS

3.2.7.1 Adherent Ovarian Cells

Animals were primed as in 3.2.1 and ovaries collected 24 hours post hCG. Ovaries were dissociated in digest solution made with a H-MEM media base. Cells collected were washed, added to 36mm petri dishes (Sarstedt, NC, USA) and cultured for 2 hours at 37°C in RPMI/FCS. Non-adherent cells were washed off with HBSS/FCS and plates placed at 4°C for 10 minutes with cold 0.25% trypsin in HBSS/EDTA/Az. Cells were then sheared off the plates with forceful pipetting and washed in HBSS/EDTA/FCS before leukocyte antigen staining. This was only performed once.

3.2.7.2 Granulosa Cells

21-26 day old mice were administered 5IU eCG, 48 hours later animals were killed by cervical dislocation. Ovaries were removed and dissected free of fat and the bursa. In a petri dish preovulatory follicles were then ruptured using fine forceps and 30G needle (Becton Dickinson, NJ, USA). Granulosa cells released into the plate were collected in separate petri dish in hepes buffered M199 with polyvinyl alcohol 3mg/10ml. Cells were washed with HBSS/EDTA/Az and then re-suspended in 500µl HBSS/EDTA/Az. Aliquots in triplicate of 50,000 and 100,000 cells were made. To lyse the cells 400ul tri Tri-reagent™ was added and left for 5 minutes before continuing with mRNA isolation.

3.2.7.3 Isolated Macrophages

Ovarian Ia^+ and $F4/80^+$ macrophages were collected as described (3.2.3-3.25) on day 0 am and day 1 of the gonadotrophin stimulated cycle.

3.2.8 Leukocyte Antigen Expression

Populations obtained were fixed in 4% para-formaldehyde (PFA) in saline for 10 minutes and then washed in HBSS/EDTA/Az. Positive cells bound to plates were then incubated 20 minutes with 10% normal rat serum in HBSS/EDTA/Az while negative cells or cells in suspension were centrifuged at (200G) 4°C. NSE staining was also attempted, but unsuccessful (Appendix 4).

3.2.8.1 LCA

All cells were then washed again in HBSS/EDTA/Az and incubated at for 60 minutes at RT with R-phycoerythrin (R-PE) conjugated rat anti-mouse CD45 (leukocyte common antigen, LCA) monoclonal antibody (Becton Dickinson, NJ, USA) at 1:25 dilution.

3.2.8.2 F4/80:Ia Mix

Cells were resuspended in 200µl of PBS-10%FCS and then incubated for 30 minutes at RT with 100µl of F4/80:Ia mix, made by combining 200 µl $F4/80^+$ hybridoma supernatant with 200 µl Ia^+ hybridoma supernatant. Unbound antibody was washed off with PBS-10%FCS and cells centrifuged at (200G) 4°C. Resuspended cells were then incubated with FITC conjugated anti-rat IgG (1:100) for 30 minutes at 4C. Unbound antibody was washed off with HBSS/EDTA/Az and cells were wet mounted in saline with Hoechst (1:25000) and examined under Olympus Vanox (AHBT3) fluorescent microscope. The numbers of positive (RPE-red cells or FITC-green cells) and negative cells in each population were counted and expressed as the percentage of total cells (Hoechst-blue cells).

3.2.9 Isolation of messenger RNA and generation of complementary DNA

Total RNA was isolated from the cell lysate as recommended by the Tri reagent™ manufacturers (mRNA isolation using Qiuagen® RNeasy kits did not yield sufficient mRNA to work with (Appendix 3)). Briefly, RNA was extracted with chloroform and precipitated overnight with isopropanol. The pellet was washed in 70% ethanol dissolved in RNase free water and DNase treated with RQ1 RNase-Free DNase (0.05U/µl) (Promega, Madison, USA), and RNasin Ribonuclease inhibitor (1 U/µl) (Promega, Madison, USA) at 37°C for 90 minutes. RNA was then re-extracted with 1:1 phenol-chloroform: isoamylalcohol and precipitated overnight with 2 M sodium acetate: 100% ethanol. The pellet was washed in 100% ethanol and re-suspended in 20µl RNase free water. Reverse transcription was performed as described in Invitrogen Superscript ™II RNase H reverse transcriptase (Invitrogen, CA, USA) product information. The mRNA from isolated ovarian macrophages was measured prior to reverse transcription using a Ribogreen RNA Quantitation Kit

(Molecular Probes, Eugene, OR, USA). Messenger RNA standard curves were generated as per the manufacturers instructions and concentration in samples calculated from this curve using Fluorostar software (Appendix 1). This allowed for each ovarian macrophage sample the reverse transcription of a volume containing the same amount of total RNA (40ng) while for granulosa cells 10µl of each sample of RNA was reverse transcribed. All reverse transcription was carried out in a total volume of 20µl using 250ng of random primers in the presence of 40U of RNase OUT Recombinant Ribonuclease Inhibitor (Invitrogen, CA, USA)(Appendix 1). The generated cDNA samples were then diluted 1:4 for analysis using quantitative RT-PCR.

3.2.10 Luteinising Hormone and Follicle Stimulating Hormone Receptor mRNA Expression

3.2.10.1 Primers

Primers for LH receptor (LHr) and FSH receptor (FSHr) were designed using NCBI sequences and Primer Express (Applied Biosystems, Australia). Products generated by the designed primers were isolated and sequenced (Appendix 1), before use in an ABI Prism 5700 sequence detection system (Applied Biosystems, Australia). It was established that both primers had the same efficiency by plotting the log of the dilution against the CT value to create a line and the slopes of each line were compared statistically (LHr, FSHr).

3.2.10.2 Quantitative RT-PCR

Quantitative analysis of mRNA levels was carried out using an ABI Prism 5700 sequence detection system (Applied Biosystems, Australia). 3 µl of sample was added to 17 µl of SYBR green master mix containing 0.5µM of each primer. Positive and negative controls were included on every plate. Analysis of mRNA levels in each sample was done in

triplicate and thus for each gene of interest three separate real-time RT-PCR plates were analysed and threshold levels (CT value) adjusted according to identical granulosa mRNA (G) positive controls included on every plate. Samples with cycle threshold values of 37 or greater were regarded as negative.

3.2.11 Macrophage Conditioned Media

Ovarian macrophages were isolated as described in 3.2.3-3.2.5 48 hours post eCG (day 0am) and 24 hours post hCG (day 1) on three separate occasions. Isolated bound cells were cultured at 37°C for 24 hours in 800µl of RPMI with 10%HI-FCS, L- glutamine (150 µg/ml)(ICN Biochemiclas, USA) and Penicillin (100 U/ml) Streptomycin (100 µg/ml) solution (CSL Biosciences, Australia) (RPMI-medium) at 5%CO_2 in air. Media was collected, centrifuged to remove debris and stored at –80°C for future assay.

3.2.12 Progesterone Analysis

Progesterone levels were measured in macrophage conditioned media using a radioimmunoassay kit (Diagnostic Systems Laboratories, Webster, TX) as described previously [292]. This kit has a lower detection limit of 1nmol/L. This assay was introduced as measurements can be done on much smaller sample volumes than the assay used in Chapter 2 (2.2.3)

3.2.13 Phagocytic Assessment

Isolated ovarian and peritoneal macrophages obtained by antibody panning were cultured in RPMI/10% FCS for 90 minutes to allow adherence of cells to the petri dish.

Fluorescent beads (Fluoresbrite® Polysciences Inc PA, USA) were then added (1:100 dilution) and cells cultured for a further 2 hours at 37°C. Un-ingested beads were washed off with 10 ml warm RPMI/FCS10% and the number of macrophages that had internalized 8 or more beads (in an attempt to differentiate between uptake and attachment) was assessed and expressed as a percentage of total cells (more than 400 cells were counted per replicate). An additional group isolated prior to ovulation and cultured in the presence of hCG (0.05 IU/ml) was also included. This was repeated four times at each time point, percentages were arcsin transformed to satisfy ANOVA criteria and then compared using a One -Way ANOVA.

3.2.14 PHA-STIMULATED SPLENOCYTE PROLIFERATION

Spleens were removed from three adult female mice (>7weeks of age) and placed in cold Hepes buffered RPMI (H-RPMI), minced with vannas spring scissors, homogenized in a sterile glass homogeniser, filtered through a cell strainer and washed in H-RPMI. Red blood cells were removed by flash lysis with 900µl of milli Q water followed immediately by 100µl of 10X PBS. Adherent cells were removed by incubation in 20mls RPMI/10%FCS at 37°C in a 60mm petri dish (Falcon®, Becton Dickinson, NJ, USA) for 1 hour. Non-adherent cells, or splenic lymphocytes, were collected and washed in H-RPMI and then resuspended in RPMI medium. These cells were then added at a concentration of 10^5 cells per well to 130µl macrophage conditioned media from either ovarian or peritoneal macrophages and cultured with phytohaemagglutnin (PHA)(10ug/ml) in 96 well plates in duplicate, for 48 hours. Tritiated thymidine (H^3-thymidine, 1 µCi/ml) (ICN Biochemicals, USA) was then added in RPMI-medium and cells cultured a further 24 hours. Cells were then harvested using a Tomtec Harvester 96 and incorported H^3-thymidine was quantified using a Wallac microbeta

scintillation counter. Results are expressed as percentage of the mean counts of PHA stimulated proliferation and were compared statistically using a One-Way ANOVA.

3.3 RESULTS

3.3.1 OPTIMISATION OF CONDITIONS FOR OVARIAN MACROPHAGE RECOVERY

As the macrophage population in the ovary comprises less than 10% of the total tissue (evident in immunohistochemistry studies [68, 293]) small cell losses would result in severely depleted numbers available for purification. Therefore we have carried out experiments to optimize conditions of the tissue dissociation and obtain the maximum numbers of ovarian macrophages.

3.1.1.1 *Effect Of Siliconised Glassware*

No significant differences were found in the percentage of live peritoneal macrophages retrieved from plastic beakers regardless of incubation temperature (Table 3-1). The use of siliconised glass beakers resulted in significantly more live cells recovered as the temperature decreased and at 4°C a significant difference in cells recovered was found between the use of plastic or siliconised glass beakers.

3.1.1.2 Effect Of Temperature

The collagenase enzyme is optimally active at 37°C, however digestion at this temperature regardless of the digest vessel used results in substantial cell losses due to adherence, evident as no significant differences were seen in the numbers of dead cells present (data not shown). Reducing the dissociation temperature will inhibit the physical ability of macrophages to adhere to surfaces as will the use of a siliconised glass vessel but will limit activity of the collagenase enzyme, which is optimally active at 37°C, and thus prevent tissue dissociation. Given that no significant difference was found between the percentage of live cells recovered from siliconised glass beakers at 4°C or 24°C (Table 3-1) subsequent digests were carried out at room temperature in siliconised glass beakers as a

	Incubation temperature		
Digest Vessel	**4°C**	**24°C**	**37°C**
Plastic	$34^{a} \pm 5.7$	37.3 ± 14.6	17 ± 11.4
Siliconised Glass	$66.5^{ac} \pm 8.8$	$54^{b} \pm 12.5$	$18^{bc} \pm 10.2$

Table 3-1 The Mean Percentage Of Live Peritoneal Cells Recovered From Each Digest Vessel. Values are mean ± SEM. A total of 1×10^{6} live peritoneal cells were added to each vessel in 1ml RPMI/1mg collagenase/25U Dnase1/10%FCS and incubated at the temperatures indicated for 90 minutes. (For all experiments n=4). Values with the same letter are significantly different (^{a} $p<0.01$, ^{bc} $p<0.02$).

compromise between optimal enzyme activity and the loss of macrophages due to adhesion.

3.1.1.3 Effect Of Digest Length And Media Base

Ovaries from adult mice were used to determine the optimal dissociation length and the most favourable media base to use. Utilizing a shorter digest period of 45 minutes as opposed to 90 minutes did not significantly alter the total number of live cells recovered from ovarian tissues in each media base. In fact, performing the digest for 90 minutes increased the variation in the numbers of live cells recovered (Table 3-2). The use of αMEM with added $CaCl_2$ 5 mM (αMEM/$CaCl_2$) to optimally activate the collagenase enzyme increased the numbers of live cells recovered per milligram of tissue in the 90 minute digest compared to αMEM and RPMI/FCS. A significant increase in live cell numbers was seen in tissue digested in αMEM/$CaCl_2$ compared with RPMI/FCS for the 45 minute digest period. For all subsequent experiments the digests were performed for 45 minutes at room temperature in αMEM/$CaCl_2$.

3.3.2 Ovarian Macrophage Cell Numbers Across the Gonadotrophin-Stimulated Cycle

Following the attainment of a viable, single cell population, attempts were made to specifically isolate the macrophage population at various time points during the gonadotrophin-stimulated cycle for further analysis of their characteristics. All ovarian digests were performed with collagenase/DNase in αMEM/$CaCl_2$ at room temperature. In addition, following the post digest washing step the cells were resuspended in 3 ml of HBSS/EDTA/Az, layered over 3 ml of FCS and centrifuged (300G) at 4°C, to aid in the removal of dead cells. Many peritoneal cells were found to bind to the panning plates at all time points and with each experimental replication.

		Media base of digest		
		RPMI/10%FCS	**αMEM**	**αMEM+$CaCl_2$**
Length of Digest				
45 min	cells/mg ($x10^3$)	9.7± 4.2[a]	23.1±3.7	24.6±3.2[a]
% live		26.7±6.5[b]	41.8±5.9	54.6±6.6[b]
90 min	cells/mg ($x10^3$)	23.5 ± 13.8	48.2±11.4	51.4±18.4
% live		33.4±5.9	29±2.1	38.1±4.3

Table 3-2 The Number And Percentage Of Live Cells Recovered Per Mg Of Ovarian Tissue Dissociated. Values are mean ± SEM. Each media base was tested for digest of ovarian tissues for either 45 or 90 minutes at 24°C. (n = 4 for all groups). The same letter indicates significant differences ($P < 0.02$).

Negative controls, where plates were not coated with anti-rat IgG, displayed no non-specific binding of cells. At the time points examined prior to ovulation, low numbers of cells per mg of tissue digested bound to the panning plates (Figure 3-1). After ovulation had occurred there was a significant increase in the numbers of Ia^+ (day 1) cells isolated per mg of tissue digested at which point there significantly more Ia^+ than $F4/80^+$ cells were isolated. On day 2 post ovulation there was a significant increase in the number of $F4/80^+$ cells isolated and a further increase in the numbers of Ia^+ cells isolated with no significant difference between the numbers of Ia^+ and $F4/80^+$ cells isolated.

3.3.3 Viability and Purity of Isolated Macrophages

Viability was confirmed using two DNA binding dyes, Hoechst's and propidium iodide. Cells were found to be >90% viable using H/PI staining both before (day 0am) and after (day 1) ovulation. In the populations isolated using antibody panning the expression of the LCA antigen was then used to determine purity. This antigen is expressed uniquely by cells of the haematopoietic lineage [294]. Cells bound to the panning plate were found to be 95-98% LCA positive while the negative fraction, washed from the panning plate, was only 2-6% positive. This indicates minimal non-specific binding of non-leukocyte ovarian cells types. Both LHr and FSHr mRNAs were expressed in all the isolated ovarian macrophage samples, but not the peritoneal macrophage samples. This implies either ovarian macrophages express low levels of mRNA for both LHr and FSHr or some contamination of the isolated cells with granulosa cells. To determine if the levels of message for the gonadotrophin receptors measured correspond to that which would be seen in granulosa cells contaminating at the levels suggested by LCA staining (2-5%), granulosa cells were collected and a curve of known numbers of granulosa cells against quantitative RT-PCR threshold cycle number (CT) was constructed (Figure 3-2).

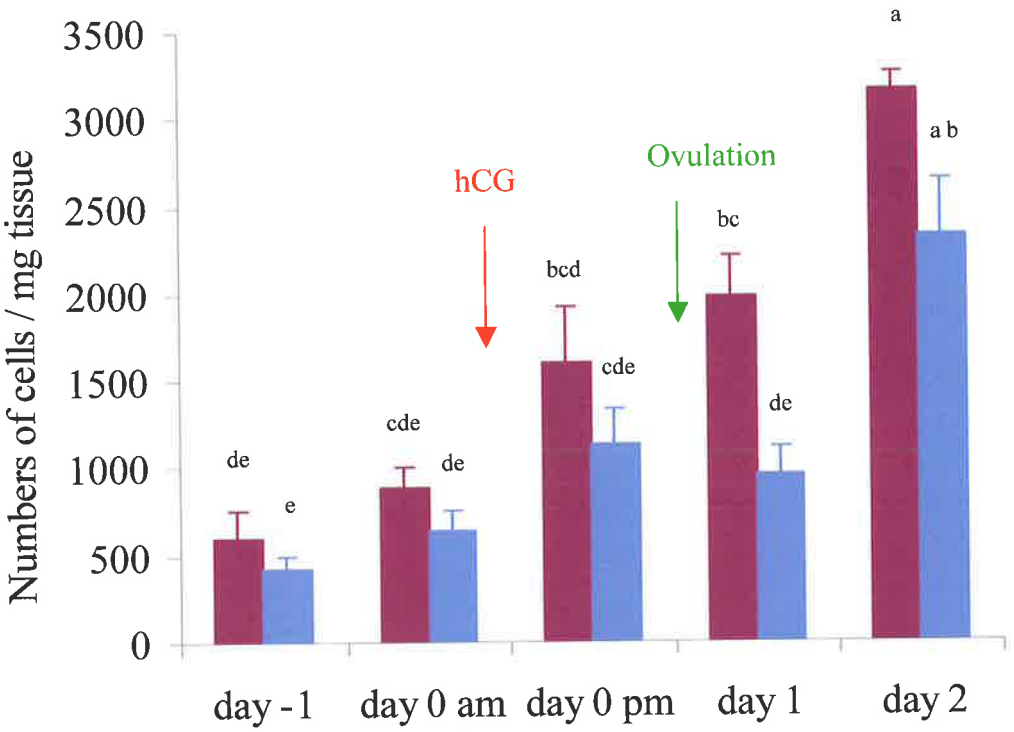


Figure 3-1 The Numbers Of Ovarian Ia (■) And F4/80 (■) Positive Cells Isolated Per Milligram Of Tissue Digested. Values are mean (± SEM). n = 3-4 for each time point. Day -1 = 24h post eCG, day 0am = 48h post eCG, day 0pm = 6h post hCG, day 1 = 24h post hCG, day 2 = 48h post hCG). Bars with the same letter are not significantly different (for all differences $p < 0.02$).

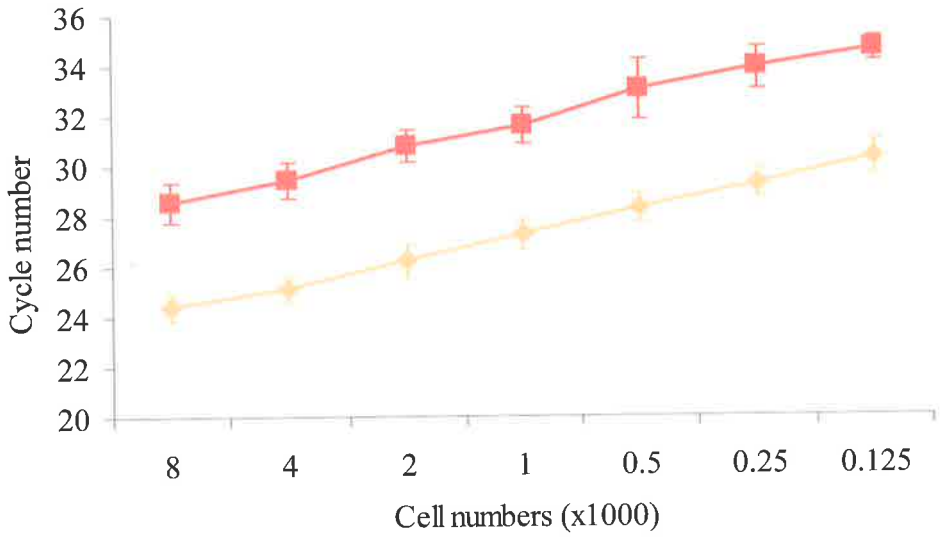


Figure 3-2 The Threshold Cycle (CT) Numbers For LHr and FSHr mRNA in Known Numbers Of Granulosa Cells. Values are the mean CT ± SE, n = 3 at each point. Messenger RNA was isolated from samples of granulosa cells containing known numbers cells (x axis). The levels of mRNA for luteinising hormone receptor ( ) and follicle stimulating receptor ( ) were measured in these samples (y-axis).

This was then used to equate the CT values of both FSHr and LHr measured in the ovarian macrophage samples to the possible numbers of contamination granulosa cells. Minimal numbers of contaminating cells were found (Table 3-3). Moreover, to confirm minimal contamination we found that levels of progesterone in media conditioned for 24 hours by peritoneal or ovarian macrophages collected both before and after ovulation were not detectable (data not shown, <1nmol/L) further indicating negligible contamination with granulosa cells.

In addition, an analysis of LCA expression performed on the adherent and non-adherant cells from an ovarian digest, an accepted method of macrophage isolation and used extensively by others to obtain ovarian macrophages for further studies, show that the purity of the antibody panning population obtained in this study far exceeds that of the ovarian adherent cells. Adherent ovarian cells were found to be only 33 % LCA and 30% $F4/80^+$:Ia^+while the non-adherent population were less than 3% positive for either LCA or $F4/80^+$:Ia. The antibody panning positive cells are 95% LCA.

3.3.4 Phagocytic Capability of Ovarian Macrophages

To measure the phagocytic capabilities of ovarian macrophages, cells bound to the panning plates were cultured with fluorescent beads. All isolated ovarian and peritoneal macrophage populations were found to exhibit phagocytic ability. The percentage of cells able to phagocytose beads increased significantly following ovulation (Figure 3-3). There was no significant difference between the phagocytic capacity of isolated $F4/80^+$ and Ia^+ cells. Isolated peritoneal cells were approximately 50% phagocytic regardless of the collection time point (data not shown). Since fewer cells were phagocytic prior to ovulation than after, isolated macrophages were cultured with hCG to determine if this hormone directly stimulated phagocytosis, but this did not result in any significant change in the percentage of

	LHr CT value	Estimated Contaminating Cell Numbers	FSHr CT value	Estimated Contaminating Cell numbers
day 0 am	29.02	50	34.7	125
	28.75	125	32.8	500
Ia^+	*26.41*	*2000*	*29.4*	*4000*
	30.18	500	33.6	500
Average	**28.6 =**	**200 or 1.1%**	**32.6 =**	**500 or 2.7%**
day 0 am	29.3	250	33.7	250
	32.45	<125	32.9	500
$F4/80^+$	*27.26*	*1000*	*29.6*	*2000*
	29.14	500	33.7	250
Average	**29.5 =**	**125 or 0.4%**	**32.4 =**	**500 or 0.2%**
day 1	30.1	125	35.9	<125
	29.78	250	36	<125
Ia^+	31.69	<125	33.2	250
	31.06	<125	--	0
Average	**30.6 =**	**<125 or 0.1%**	**35 =**	**<125 or 0.1%**
day 1	29.6	250	34.6	125
	29.44	250	33.9	250
$F4/80^+$	31.94	<125	32.9	750
	31.84	<125	35.6	<125
Average	**30.7 =**	**<125 or 0.2%**	**34.25 =**	**<250 or 0.4%**

Table 3-3 Threshold Cycle (CT) Values For Luteinising Hormone (LHr) And Follicle Stimulating Hormone (FSHr) Receptors mRNA In Ovarian Macrophages Samples And Estimated Contaminating Cell Numbers. Each CT value represents the LH or FSH receptor levels measured in the mRNA from macrophages isolated from the ovaries of 10 animals in a single experiment. The average contaminating cell number is estimated by using the CT value to read the cell numbers from Figure 3-3. The percentage is calculated using the average cell contaminating numbers from this table and the average total numbers of cells isolated per time point using the antibody panning method (data used in Figure 3-2)

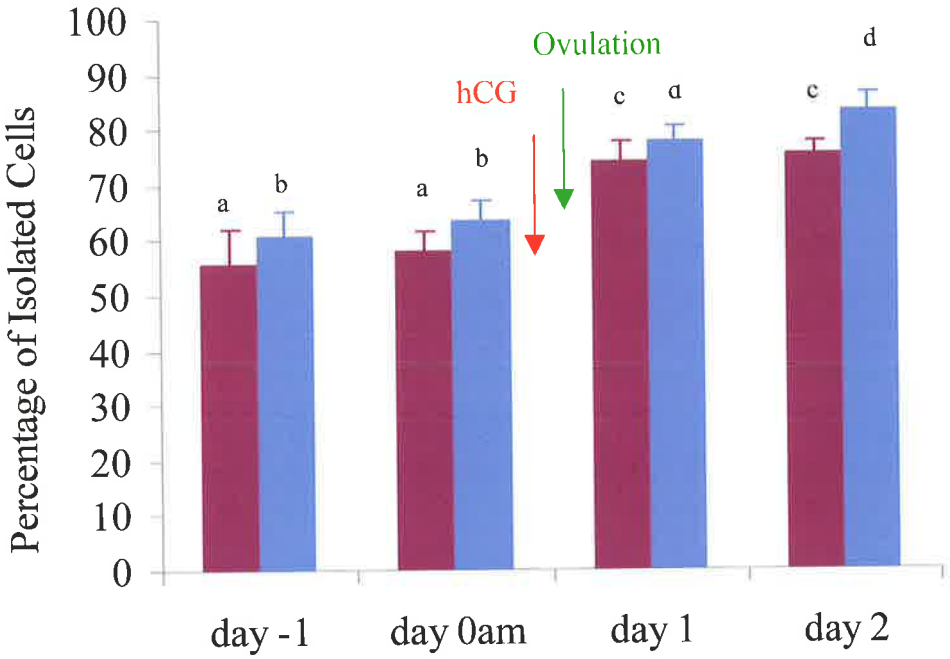


Figure 3-3 The Percentage Of Isolated Ovarian Ia (■) And F4/80 (■) Positive Cells That Exhibit Phagocytic Capacity. Values are mean ± SE, n = 4 for each time point, 400 cells counted in each experiment. day -1 = 24h post eCG, day 0am = 48h post eCG, day 1 = 24h post hCG, day 2 = 48h post hCG. At each time point there was no significant difference between the percentage of Ia^+ or $F4/80^+$ cells with phagocytic capacity. For all Ia^+ cells or all $F4/80^+$ cells bars with the same letter are not significantly different (all differences $p < 0.04$).

phagocytic cells (Day 0 Ia^+; –hCG = 57.8 ± 4%, + hCG = 54.7 ± 4.5%; Day 0 $F4/80^+$; –hCG = 63.4 ± 3.8%, + hCG = 69.5 ± 1.9%)

3.3.5 Effect of Macrophage-Conditioned Media on PHA-Stimulated Splenocyte Proliferation

To determine if ovarian macrophages participate in the regulation of T cell proliferation, macrophage-conditioned media was added to PHA-stimulated, adherence-purified splenocytes. Limited proliferation of splenocytes was seen in the absence of PHA, whilst the addition of PHA significantly stimulated T cell proliferation to a level designated to be 100% (Figure 3-4). The proliferation stimulated by PHA alone was not significantly different to proliferation produced by conditioned media from any of the ovarian macrophage populations. In addition, no significant differences were seen between proliferation induced by media from Ia^+ or $F4/80^+$ cells, either prior to or following ovulation. Conditioned media from Ia^+ and $F4/80^+$ peritoneal cells were found to significantly inhibit PHA-stimulated proliferation.

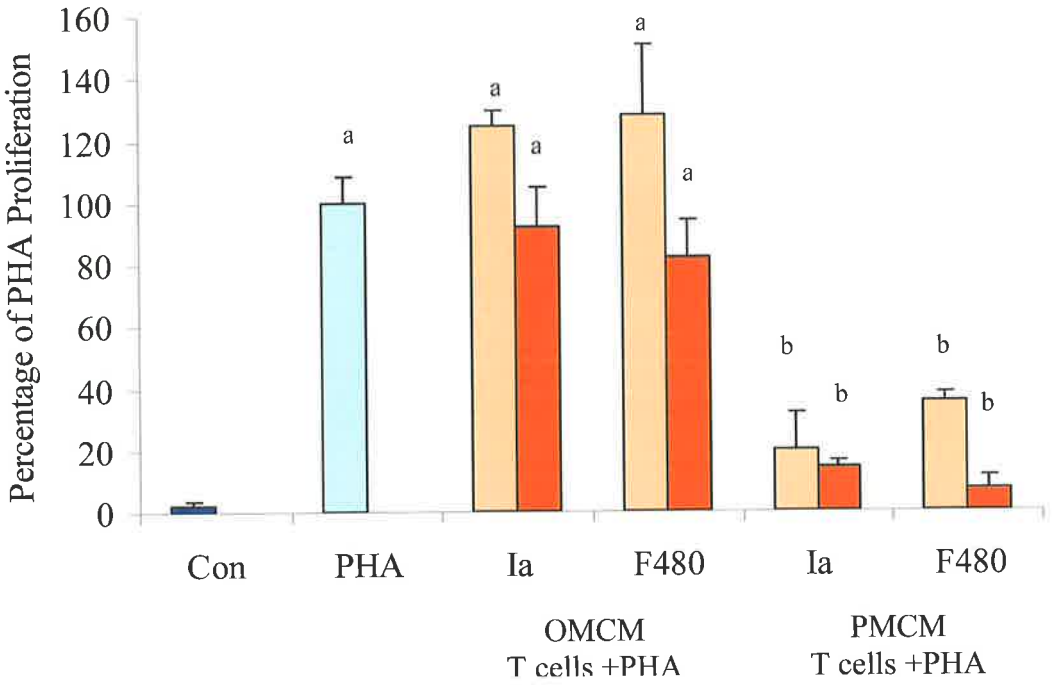


Figure 3-4 The Percentage Of PHA-Stimulated T Cell Proliferation Induced By Macrophage Conditioned Media. Values are mean ±SE. Proliferation was induced by PHA (□) alone or in combination with ovarian macrophage conditioned media (OMCM) or peritoneal macrophage conditioned media, (PMCM) from Ia^+ or $F4/80^+$ cells, before (□) and after (■) ovulation. (n = 3 for each time point) Con (■) = control, or cells cultured in media alone. Same letter indicates not significantly different. (For all significant differences $p < 0.02$).

3.4 DISCUSSION

This study has described a method for the isolation of the ovarian macrophage population to purity levels not previously achieved for this tissue type. We have also described some basic characteristics of these isolated cells.

We initially set out to ensure the ovarian dissociation minimised the loss of adherent cell types, in this first critical step of the isolation procedure. This was achieved by carrying out the dissociation at 24°C, in combination with changing the media base from RPMI, which contains small amounts of reduced glutathione that inhibits collagenase activity, to α-MEM, which has no reduced glutathione, with chloride ions added to optimally activate the enzyme. We also found, that using a shorter digest period (45 minutes and 90 minutes are both reported in the literature) did not significantly alter the numbers of total live cells isolated per milligram of tissue.

The dissociation conditions described ensure that the highest possible numbers of live cells for further purification procedures are obtained. It is interesting to note that the enzyme Dispase can significantly alter the surface phenotype of lymphocytes [295] while the collagenase enzyme itself has been reported to inhibit macrophage adherence through disruption of surface antigen expression [157]. Conversely, it has more recently been shown that tissue digestion with the enzyme collagenase type IA, as used in this study, does not have such significant detrimental effects on leukocyte surface antigen expression [296].

We then proceeded to examine the isolated ovarian macrophage population, and have shown it to be a viable and defined population suitable for further analysis. The data demonstrating high prevalence of LCA antigen and minimal LHr or FSHr expression combined with the absence of progesterone production by these cells show firstly, that the Ia and F4/80 antibodies are indeed binding to and isolating the anticipated macrophage cell types

and secondly, that contaminating cells are minimal. This is further supported by our previous work in ovarian tissue sections showing that the F4/80 and anti-Ia antibodies used in the isolation do not bind to thecal and granulosa cell layers [68, 293]. Also, work in other fields shows the markers are expressed exclusively on macrophage cells [113, 279] and not other leukocyte types. Unfortunately, attempts to enzymatically or mechanically remove the cells from the panning plates for further verification of purity FACS analysis, was not successful or resulted in the death of the majority of the population respectively, and consequently this type of analysis could not be carried out here.

Following these experiments we went on to isolate the ovarian macrophage population at different time points during gonadotrophin-stimulated immature mice. The isolated macrophages are derived from whole dissociated ovaries and thus we cannot distinguish between macrophages isolated from the stroma, theca, atretic follicles or CL, of the ovary. This problem is limited by the use of gonadotrophin-primed immature animals rather than adult mice where CL's from previous ovulations would contribute to the macrophage pool. The administration of eCG establishes a cohort of follicles at the same stage of development, limiting the presence of atretic follicles, so that cells isolated at the various time points are predominantly of thecal or corpora luteal origin. Changes in the total numbers of macrophages isolated per milligram of tissue substantiate immunohistochemical observations in various species, showing increases in numbers of these cells in, the theca as ovulation approaches, and during formation of the CL [49, 53, 54, 59, 64, 297]. Following ovulation (Day 1) the total number of Ia^+ cells increased significantly above the numbers of $F4/80^+$ cells. The F4/80 antigen is expressed on newly recruited monocytes/macrophages and its expression can be down regulated on some activated macrophages [113] or on macrophages exposed to high concentrations of cytokines [298]. This implies that following ovulation there are significantly more activated macrophages than newly recruited macrophages present in the ovary. This is

supported by previous work suggesting greater numbers of Ia^+ cells than F4/80 $^+$ cells are present in the ovarian stroma and theca of follicles approaching ovulation [68]. Interestingly, it was also found that GM-CSF deficiency reduced the numbers of Ia^+ or activated cells present in the stroma of ovaries examined and the estrous cycles of these animals was extended. A similar dysfunction of the estrous cycle was seen in macrophage depleted mice (Chapter 2 [293]). Together these studies, suggest that an influx of Ia^+ cells may be essential in the regulation of luteal function or regression. On day 2 after ovulation the numbers of Ia^+ and $F4/80^+$ cells are not significantly different, with both populations increasing significantly to numbers greater than that seen on day 1. This corresponds with further invasion of the CL with newly recruited macrophages and their subsequent activation. It has been suggested elsewhere that macrophages have both luteotrophic and luteolytic affects [299] and there is evidence that ovarian macrophages in the CL can be luteotrophic, with the ability to stimulate progesterone production [229, 300] or luteolytic, with roles in the regulation of luteal regression [301, 302]. These conflicting actions of macrophages could perhaps be attributed to the activation status of these cells at differing times of the estrous cycle. In agreement with this, studies in other fields show that the effects of steroids on macrophage functions [117, 118] and conversely, the effects of macrophages on granulosa cells [174, 238] may be vary depending on the source or state (elicited or resident) of the macrophages. It would therefore be of interest to examine additional markers of macrophage activation in this population to give an insight into any changes in the activation status of the ovarian macrophage population across the gonadotrophin-stimulated cycle.

The low phagocytic capacity of the isolated ovarian macrophages was unexpected since it is considered a feature of the macrophage lineage. It has however, been shown elsewhere that ovarian macrophages have a reduced phagocytic capacity when compared to testicular macrophages [303], while in other tissues such as the brain, microglial cells express markers of

the macrophages lineage, but do not exhibit phagocytic activity in early stages of activation [161]. It should be noted that microglial cells isolated from the brain have been shown to be only 50% phagocytic when cultured with non-apoptotic cells but this rates increases to 100% if apoptotic cells are used [304]. Since ovarian macrophages also reside in a complex tissue mass, they may also fall into a phagocytic category similar to these cells. The increase in phagocytic activity post ovulation corresponds to the time at which profuse tissue reorganisation and remodeling is occurring, and thus it seems reasonable that these cells would become activated and involved in the clearance of tissue debris and apoptotic cells created by these processes. We have also shown here that the addition of hCG to ovarian macrophages *in vitro* does not stimulate a change in the percentage of phagocytic cells. This change must therefore be initiated by other means, such as changes in ovarian steroid levels. Both progesterone and oestradiol have been shown to influence macrophage and monocyte phagocytosis [119, 120, 123] Macrophage phagocytic capacity can also be manipulated by changes in cytokine levels or simply by the presence of apoptotic cell debris and/or extracellular matrix fragments generated following ovulation. The phagocytic capacity of the peritoneal population was lower than expected even considering that resident peritoneal macrophages, as used in this study, have a lower phagocytic ability than elicited/activated macrophages. It has also been reported that phagocytic activity of cells can be reduced by exposure of the cells or animals to periods of low temperatures [305, 306] and the peritoneal cells in this study were held at four degrees for a period longer than that for the ovarian cells.

Another main function of macrophages is their interaction with T cells and subsequent stimulation of an immune response. We found that ovarian macrophage conditioned medium does not significantly alter PHA-stimulated splenocyte proliferation. Our results indicate that the isolated Ia^+ and $F4/80^+$ cells do not produce significant levels of immunomodulatory molecules. Hence at the time points that we have studied, the regulation of T cell proliferation

within the ovary may not be an important function of these cells. In the cycling ovary, few T cells are been localized in the ovarian tissue until luteinisation, when increases have been reported [51, 53]. Interestingly, it has been reported that at luteolysis bovine luteal cells themselves express MHC II [255, 307] and can stimulate T cell proliferation [256], although, macrophages were not depleted from these luteal cell populations. This suggests that during CL regression the macrophages present are actively invoking an immune response- presenting antigens and activating T cells, while our results suggest that prior to ovulation and in the early CL macrophages are not actively regulating T cell proliferation. Alternatively, the apparent lack of response in our study could be due to low concentration of immunomodulatory factors as a results of low numbers of isolated cells, or the lack of direct T cell/macrophage contact. Macrophages isolated from the uterus [147] and testis [135] have both been shown to inhibit PHA-stimulated splenocyte proliferation in assays in which the macrophages were in direct contact with the splenocytes, rather than utilizing macrophage supernatant only as in this study. The mitogen stimulated proliferation assay used in this study will determine only if the supernatant contains any soluble components capable of stimulating or inhibiting splenocyte proliferation. If it were possible to isolate higher numbers of ovarian macrophages to perform cell co-culture experiments a different result may be observed. In contrast to our ovarian macrophage results, the resident peritoneal macrophage population was found to significantly inhibit PHA stimulated T cell proliferation suggesting that these cells are producing significant amounts of inhibitory molecules. It has been reported elsewhere that peritoneal cells will inhibit mitogen stimulated T cell proliferation [308] and there is ample evidence that this can occur through the production NO and/or prostaglandins by activated cells [308-310].

In summary, we have for the first time described a method for isolating ovarian macrophages. We found that these cells are more phagocytic post-ovulation than prior to ovulation and conditioned culture supernatant from these cells has no influence on PHA-

induced splenocyte proliferation. The utilization of this method to isolate ovarian macrophages will allow further examination of the cellular and molecular characteristics of these cells during folliculogenesis, ovulation and luteinisation.

Chapter Four

4 The Expression of inflammatory mediators by the Macrophage Population in the Ovary of the Gonadotrophin-Stimulated Mouse

4.1 INTRODUCTION

The previous chapters of this thesis have demonstrated that ovarian macrophages are necessary for normal ovarian functions and identified a method suitable for the isolation of ovarian macrophages from the other prevailing ovarian cell types. Our results have implicated these cells in follicular growth, ovulation and corpus luteum regression, and suggest these isolated cells display irregular immune functions. No research examining the direct contributions of these cells to the ovarian cytokine environment has previously been performed even though various inflammatory cytokines, that are commonly produced by macrophages elsewhere in the body, have been shown to have significant effects on follicle growth/atresia [311-314] ovulation [61, 191, 205] and isolated granulosa and thecal cell types [202, 232, 241, 315].

In this study we have selected several cytokines of interest to measure in the isolated ovarian macrophage population, in particular the classical inflammatory macrophage products interleukin-1 beta (IL-1β), nitric oxide (NO) and tumour necrosis factor alpha (TNFα) as well as selected anti-inflammatory molecules.

Nitric oxide (NO), an established product of classical inflammatory macrophages [208] has been shown by our group [60, 61] and others [212-214] to be a critical component in the ovulatory cascade. It is produced by the enzyme nitric oxide synthase (NOS) of which there is two reported varieties - inducible (iNOS) and constitutive (cNOS). Jablonka-Shariff et al demonstrated expression of iNOS in the theca and stroma of immature and preovulatory ovaries while after ovulation iNOS staining was restricted to the outer layers of the CL and became more widespread in the degenerating CL [211]. The NO molecule itself has been postulated to have a role in vascular permeability and dilation [217, 218], the regulation of follicular and luteal cell apoptosis [171, 219-221], and the inhibition of leukocyte adhesion to endothelium [316]. It has also been shown to have significant effects on both prostaglandin

production, through activation of the COX enzymes in the ovary [222, 223] and other tissues [224, 225], and progesterone production by luteinized granulosa cells [223, 236, 317].

There are numerous studies supporting roles for TNFα in the regulation of many different aspects of ovarian function including the ovulatory event [207, 261], apoptosis, steroidogenesis and proliferation within both ovarian follicles and corpus luteum (reviewed in [318]). In the ovary, expression of TNFα has been demonstrated in 'macrophage like' cells of the theca and corpus luteum [251, 252]. It has been suggested by others that macrophages may be a source of this cytokine in the ovary [319-321].

IL-1β has been reported to stimulate ovulation in the rat *in vitro* [191, 193] while *in vivo* administration of interleukin–1 receptor antagonist (IL-1ra) inhibits ovulation [322]. It has been postulated that IL-1β directly on nitrite production [61, 171, 215, 216] steroidogenesis [206] or collagenase activity [323, 324] and vascularisation [197]. IL-1ra is expressed in the granulosa and thecal layers of human preovulatory follicles and the regressing corpora lutea [186] while cultured granulosa cells and theca cells have been reported not to express IL-1ra mRNA [325]. In the rat ovary IL-1ra expression has more recently been reported to be confined to the granulosa cell compartment with some staining in the oocytes [194]. IL-1ra is a protein that significantly inhibits or down regulates the inflammatory actions of IL-1β and in a classical inflammatory response the pattern of expression of this protein is delayed but comparable to the expression of IL-1β.

$TGF\beta_1$, a member of the TGF superfamily, is a pluripotent growth factor with established roles in processes important in ovarian function such as the regulation of cell apoptosis [326], proliferation, steroidogenesis, wound healing [102] and immune responses [327]. The $TGF\beta_1$ protein is expressed in the theca of the follicle in the pig, mouse and rat [173, 312, 328]. Increases in thecal staining following human chorionic gonadotrophin (hCG) administration have been reported although the granulosa of the follicle also expresses $TGF\beta_1$ protein [329].

In addition intrabursal injection of $TGF\beta_1$ into the mouse ovary has been shown to significantly inhibit follicular rupture [330] and although naturally cycling $TGF\beta_1$ knockouts have reduced ovulations and an extended estrous cycle [331], ovulation is normal following gonadotrophin stimulation.

IL-10, produced predominantly by macrophages and Th_2 cells, is a potent anti-inflammatory cytokine through its inhibitory effects on the proliferation and cytokine expression of T cells. It also has significant effects on macrophages and dendritic cells, inhibiting differentiation and antigen presentation as well as cytokine expression (reviewed in [332, 333]. Studies examining the expression of this cytokine in the ovary are limited. The protein has been detected in human follicular fluid [334, 335] and is present in human mononuclear/luteal cell co cultures [336]. IL-10 mRNA has also been demonstrated in day 10-20 rabbit corpus luteum [337] and been found in cancerous ovarian cell types [338, 339].

Using the method described in Chapter 3 to isolate ovarian macrophages the purpose of this study was to determine if ovarian macrophages are producing cytokines that regulate ovarian function, by analysing the expression of mRNA and protein for molecules with established roles in ovarian function and immune regulation.

4.2 METHODS

4.2.1 ANIMALS

SV129 animals were obtained from the University of Adelaide Animal services and maintained in the Queen Elizabeth Hospital. Unless otherwise stated reagents were purchased from Sigma Aldrich Chemical Co., St Louis, MO, USA. The first ovulatory cycle was stimulated in immature animals of 25-27 days of age with 5IU eCG (Intervet, Boxmeer, Holland) in 0.1ml PBS (Gibco BRL, Life Technologies, Grand island, NY, USA) with 0.1% BSA (Fraction V) on day –2, followed 48 hours later on day 0 by 5IU hCG (Pregnyl®, Organon, Oss, Holland) to stimulate ovulation. Groups of 8-12 gonadotrophin primed animals were killed by cervical dislocation at 0900 hours on day 0am, day 1 and day 2 with an additional group killed 6 hours post hCG administration (day 0pm). Following the collection of peritoneal macrophages by peritoneal lavage with 2.5mls Hanks Balanced Salt Solution (HBSS) (with sodium bicarbonate (0.35g/L) 5mM Ethylenediamine tetra acetic acid (EDTA), 0.01% Sodium Azide (Az)) ovaries were removed for digestion and subsequent isolation of macrophages.

4.2.2 MACROPHAGE MESSENGER RNA ISOLATION AND MEASUREMENT

Ovarian macrophages were isolated on prepared antibody panning plates as devised in Chapter 3, Section 3.3.1 on three separate occasions from groups of 8-12 animals. Ovaries were digested in a collagenase/DNase solution, single cells were then incubated with either the anti-Ia or F4/80 antibody supernatants and then added to anti-rat IgG-coated plates. Immediately following washing of the unbound cells from the panning plate with HBSS/10% FCS, 400µl Tri reagent™ was added to the plate and cells lysed for 5 minutes. Any remaining cells were then scraped from the plate into the lysis solution with cell scraper. Samples were

stored at –80 °C until commencement of purification of RNA. Total RNA was isolated from the thawed cell lysate as recommended by the Tri reagent™ manufacturers and described in Chapter 3, section 3.2.9. 2ul of each resultant RNA sample was put aside for RNA measurement. Total RNA was measured in all samples prior to reverse transcription using a Ribogreen RNA Quantitation Kit (Molecular Probes, Eugene, OR, USA)(Appendix 1). Standard curves were generated as per the manufacturers instructions. A high standard curve (detecting 6.25-800ng RNA) was used to measure diluted (1:100) peritoneal macrophage samples and a low standard curve (detecting 1.6-100ng/ml RNA) was used to measure neat ovarian macrophage mRNA samples. All samples were measured in singleton on one plate in a BMG microplate reader and values were calculated from standard curves generated by Fluorostar software. The measurement of these concentrations allows us to use the same amount of RNA for each reverse transcription reaction by adding the appropriate volume of each sample.

4.2.3 Reverse Transcription and Quantitative RT-PCR

A total volume of 20µl was used for each RT reaction which was performed as described in Invitrogen Superscript ™II RNase H reverse transcriptase (Invitrogen, CA, USA) product information (Appendix 1). For each sample, a volume containing 40ng of total RNA was reverse transcribed using 250ng of random primers in the presence of 40U of RNase OUT Recombinant Ribonuclease Inhibitor (Invitrogen, CA, USA). Both no RNA template (no RNA) and no superscript enzyme (no SS) controls were also included. The generated cDNA samples were then diluted 1:4 to give a total of 80µl of sample for analysis using quantitative PCR. 1µg of RNA from each of spleen (SP), lymph node (LN), and granulosa cells was also reverse transcribed for use as positive controls and to minimize plate-to-plate variability.

Specific primers for murine cytokines of interest and potential housekeeping genes were designed using NCBI sequences and Primer Express (Applied Biosystems) or obtained as indicated in Table 4-1. Primer efficiencies between the target genes and housekeeping gene were determined to be the same by serial dilution of spleen or granulosa cell cDNA and quantitative RT-PCR analysis. The log of the dilution was plotted against the CT value to create a line and the slopes of each line were compared statistically. The products generated were also purified and the sequence confirmed (Flinders University DNA Sequencing Core Facility, SA) before being used for analysis of mRNA levels in isolated macrophage RNA samples (Appendix 1). Quantitative analysis of mRNA levels was carried out using an ABI Prism 5700 sequence detection system (Applied Biosystems, Australia). 3µl of sample was added to 17µl of SYBR green master mix containing 0.5µM of each primer. Positive (LN, SP and GC) and negative (no SS, no RNA) controls were included on every plate. Analysis of cytokine mRNA levels in each sample was done in triplicate and thus for each cytokine of interest three separate real-time PCR plates were analysed and threshold levels (CT value) adjusted according to identical splenic RNA (SP) and lymph node RNA (LN) positive controls included on every plate. Samples with cycle threshold values of 37 or greater were regarded as negative. The house-keeper genes 18S and HPRT were assessed for suitability by confirming consistent expression in all samples across the murine stimulated cycle, between cell source (ovary or peritoneal) and isolated cell type (Ia^+ or $F4/80^+$) in relation to the amount of total RNA reverse transcribed. Quantitative RT-PCR results for each of the target genes were normalized to the levels of the house-keeper gene and then expressed as the fold change in mRNA levels from the control group, nominated for each gene as the average for the ovarian Ia+ day 0am group and gene. All results were log transformed to normalize the data and then analysed by One-Way Anova across both Ia^+ or $F4/80^+$ cell types and ovary or peritoneal cell source (Appendix 1).

TARGET GENE	FORWARD 5'-3'	REVERSE 5'-3'	SIZE (BPS)	GENBANK ACCESSION NO.
Interleukin-1-beta (IL-1β)	Start 69 TGAAGTTGACGGACCCCAAA	Start 169 TGATGTGCTGCTGCGAGATT	60	NM_008361
Interleukin-10 (IL-10)	Start446 GGCGCTGTCATCGATTTCTC	Start 532 CTTGGTCTTGGAGCTTATTAAAATCA	87	NM_010548
Interleukin-1-receptor antagonist (IL-1ra)	Start 183 CCTTCAGAATCTGGGATACTAACCA	Start 295 CACCATGTCTATCTTTTCTTCTAGTTTGA	58	NM_031167
Tumour Necrosis Factor-alpha (TNF-α)	Start 236 CCAGGCGGTGCCTATGTCT	Start 358 TACTCTTCAAGGGTTTACCGG	82	NM_013693
Transforming Growth Factor-beta-1 (TGF-β_1)	Start 1162 CCCGAAGCGGACTACTATGCT	Start 1238 GTTTTCTCATAGATGGCGTTGTTG	77	NM_011577
iNOS (Supplied by Dr W Ingman)	Start CATCAGGTCGGCCATCACT	Start CGTACCGGATGAGCTGTGAA	86	NM_010927
Hypoxanthine guanine phosphoribosyl transferase (HPRT)	Start 43 CTTCCTCCTCAGACCGCTTTT	Start 136 AACCTGGTTCATCATCGCTAATC	94	NM_013556
18S (Supplied by Dr A Harvey)	Start 56 AGAAACGGCTACCACATCCAA	Start 119 CCTGTATTGTTATTTTTCGTCACTACCT	91	AF176811

Table 4-1 Details Of The Primers Used In This Study. For each gene of interest the quantitative reverse transcription polymerase chain reaction primer sequences, product sizes in base pairs (bps), and Genbank Accession numbers for the gene sequences used in primer design, are specified.

4.2.4 CULTURE OF ISOLATED MACROPHAGES

Cells were isolated using the antibody panning technique on three separate occasions from groups of 8-12 animals at the same four time points across the stimulated reproductive cycle as those used for the RNA collection. Following the removal of the unbound cells the numbers of cells remaining were counted in ten 20x fields of view. Cells were then cultured in 800µl αMEM with L- glutamine (150µg/ml)(ICN Biochemicals, USA) and Penicillin (100U/ml)/Streptomycin (100µg/ml) solution (CSL Biosciences, Australia) at 5 %CO_2 for 24 hours. Further details in Appendix 5. Media was collected, ultra-spun to remove any debris, and then stored in 180µl aliquots at –80°C until assay. For each time point conditioned media was collected from three separate experiments.

4.2.5 MEASUREMENT OF SECRETED OVULATORY MEDIATORS

Quantikine® kits for the measurement of the ovulatory mediators IL-1β, TNFα, NO and IL-10 were purchased from R&D Systems Inc. (Minneapolis, MN, USA). Assays were performed as recommended by the manufacturer, except in the case of IL-1β and IL-10 in which the initial incubation of samples on the plate was carried out overnight (15 hours) at 4°C (Appendix 1). The specifications of these assays are provided in Table 4-2. Results were expressed per 1000 cells cultured. Data was log transformed and a One-way ANOVA was used to detect any significant changes

	INTERLEUKIN-1 -BETA	TUMOUR NECROSIS FACTOR ALPHA	NITRIC OXIDE	INTERLEUKIN-10
Detection limit	3 pg/ml	5pg/ml	1μMol/L	4pg/ml
Intra-assay precision	20.2 pg/ml = 4.4	5.6 pg/ml = 9	20.9 μmol/L = 5.3	2.5 pg/ml = 9.3
% CV's	249 pg/ml = 1.5	23 pg/ml = 4.3	68.5 μmol/L = 1.2	29 pg/ml = 5
Inter-assay precision	19.6 pg/ml = 6.1	5.4 pg/ml = 9.2	18.7 μmol/L = 7	1.7 pg/ml = 6.5
% CV's	258 pg/ml = 2.8	32.5 pg/ml = 6.1	68.2 μmol/L = 3.3	31pg/ml = 5.3
Cross reactivities	Murine IL-1β (r and n) 1% r human IL-1β 4% r rat IL-1β	Murine TNF-α (r and n) 78% r rat TNFα	NA	Murine IL –10 (r and n) 0.4% r rat IL-10

Table 4-2 Specifications Of The Assays Used To Detect Inflammatory Mediators In Ovarian Macrophage Conditioned Media.

All information is extracted from the product information booklets provided with the Quantikine® assay kits by R&D systems (MN, USA). r = recombinant, n = natural.

4.3 RESULTS

4.3.1 ISOLATED MACROPHAGE RNA

The amount of total RNA isolated at each time point varied enormously. For ovarian cells isolated with the anti-F480 hybridoma supernatant the mean total RNA per experimental group (cells from the ovaries of 8-12 animals) was day 0am = 109.9ng± 22.4, day 0pm = 202.7ng ± 13.4, day 1 = 201ng ± 93.8, day 2 = 282ng ± 90.9. For cells isolated with the anti-Ia antibody supernatant day 0am = 62ng ± 42.5, day 0pm = 201.1ng ± 40, day 1 = 193ng ± 74.6, day 2 = 422ng ± 64.2. These differences reflect the variations in the numbers of macrophages present in and therefore isolated from the ovary. At each time point many peritoneal cells were isolated resulting in large amounts of peritoneal cell RNA varying from 2.μg to 11.6μg.

4.3.2 PRIMER EFFICIENCIES AND HOUSE-KEEPER VALIDATION

To enable the valid use of the △△CT method for evaluation gene expression it must first be established that the expression of the nominated house-keeper gene is not influenced by the experimental treatments. Secondly, the primer sets used must be shown to prime with the same efficiency within the PCR reaction. The potential house-keeper genes HPRT and 18S were both found to be consistently expressed in cells isolated across the stimulated cycle with no significant difference in the level of mRNA expression across the treatment groups (Table 4-3). HPRT was chosen as the house-keeper for this work since it was less variable and the CT values were closer to those expected for the target genes. All primer sets were found to amplify at the same efficiency as the HPRT house-keeper (Table 4-4).

Target Gene *Tissue Source*	**Isolated Cell Type**	**Day of the Gonadotrophin Stimulated Cycle** **day 0 am**	**day 0 pm**	**day 1**	**day 2**
HRPT	Ia+	26.6 ± 0.5	26.2 ± 0.2	26.2 ± 0.4	25.2 ± 0.1
Ovarian	F480+	26.9 ± 0.8	26.3 ± 0.2	26.3 ± 0.5	25.6 ± 0.3
HPRT	Ia+	27.9 ± 1	27.3 ± 0.2	27.3 ± 0.4	27.3 ± 0.3
Peritoneal	F480+	27.4 ±0.5	27.5 ± 0.5	26.3 ±0.2	27.1 ±0.2
18S	Ia+	16.8 ± 0.7	15.6 ± 0.1	16.5 ± 0.5	15.4 ± 0.1
Ovarian	F480+	18.1 ± 1.6	15.8 ± 0.3	16.8 ± 0.7	15.6 ±0.3
18S	Ia+	17.1 ± 0.8	16.2 ± 0.3	16.5 ± 0.5	16.2 ± 0.3
Peritoneal	F480+	16.4 ± 0.4	16.5 ± 1	16.3 ± 0.5	15.9 ± 0.2

Table 4-3 House-Keeper Genes And Their Threshold Cycle Values Across The Gonadotrophin Primed Cycle. The mean (± SE) threshold cycle (CT) values for the potential house-keeper genes HPRT (hypoxanthine guanine phosphoribosyl transferase) and 18S (18S subunit ribosomal RNA) in cDNA generated from 40ng of total RNA from each sample collected across the gonadotrophin stimulated cycle. No significant differences in CT value were detected between the Ia^+ and $F480^+$ cells types of cells from each tissue source or between the same cell types from the different tissue sources.

Target Gene	Slope	Standard Error	P Value
HPRT	3.164	0.02	---------
TGFβ1	3.42	0.12	0.065
IL-1β	3.133	0.4	0.948
TNFα	3.481	0.42	0.446
IL-1ra	2.68	0.5	0.366
IL-10	3.067	0.47	0.862
iNOS	3.38	0.26	0.579

Table 4-4 The Data Utilised To Establish Equivalent Primer Efficiencies. The slope and standard error for each primer set calculated from a curve generated by serial dilutions of splenic (SPL) cDNA and the P value when calculated slopes are compared statistically to the slope of the designated house-keeper gene HPRT (hypoxanthine guanine phosphoribosyl transferase). No significant differences were detected between the target gene primer sets and the house-keeper gene primers.

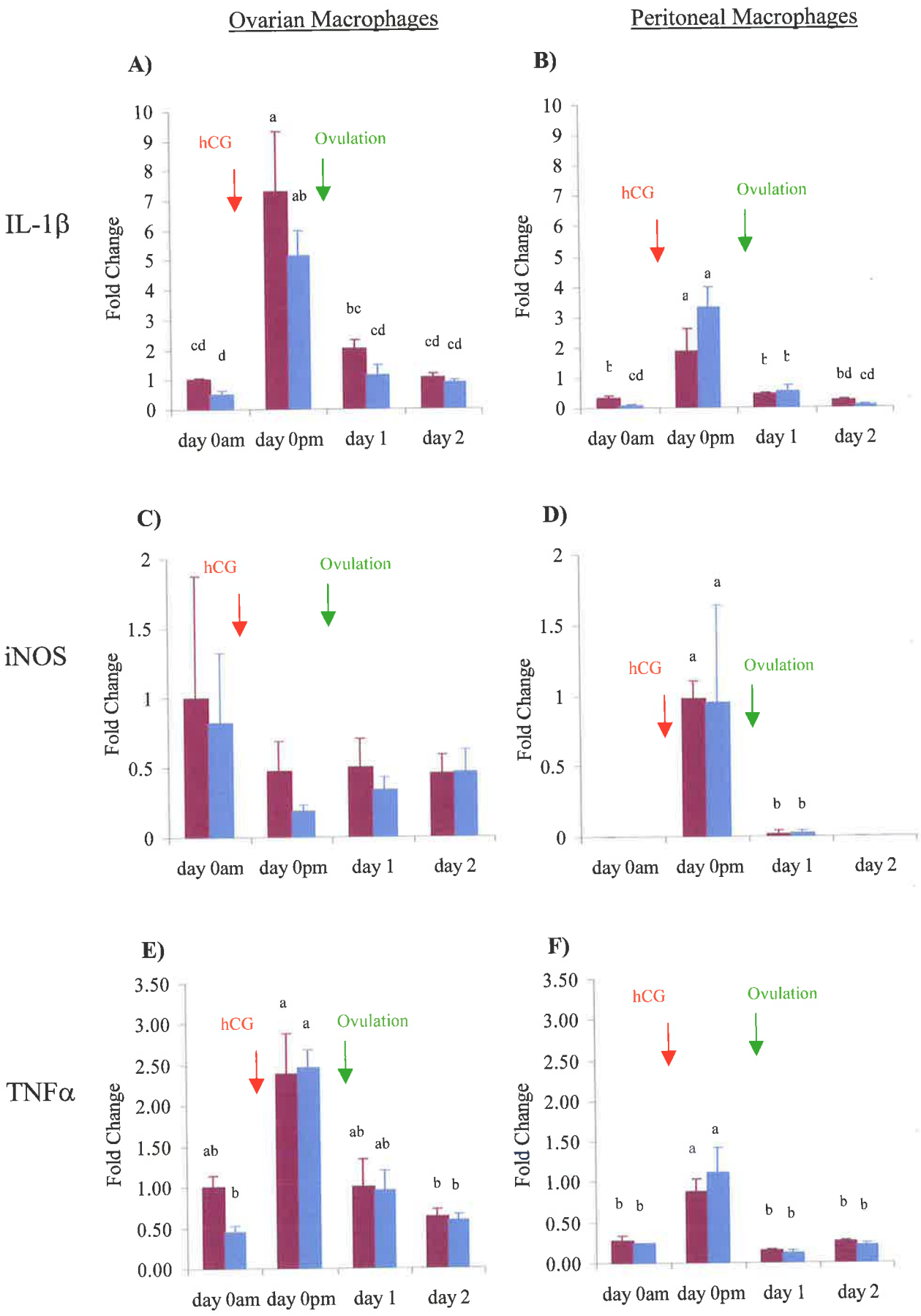
4.3.3 Expression of Cytokine mRNA in Ovarian Macrophages

We have examined the changes in expression of mRNA encoding the inflammatory molecules IL-1β, TNFα, iNOS, and the anti-inflammatory molecules IL-1ra, IL-10 and TGFβ1, in ovarian and peritoneal macrophages across the gonadotrophin-stimulated reproductive cycle. The expression of mRNA for the classical inflammatory molecules IL-1β, iNOS and TNFα was detected in all samples regardless of cell type isolated or tissue source.

IL-1β mRNA levels increased significantly following hCG administration in both ovarian and peritoneal cell types returning to pre-hCG levels on day 1 (24 hours post hCG, Figure 4-1A, B). Isolated ovarian Ia^+ cells contained significantly more IL-1β mRNA than isolated Ia^+ peritoneal cells at all time points while isolated ovarian $F480^+$ cells contained significantly more IL-1β mRNA than isolated $F480^+$ peritoneal cells on day 0am and day 2 only. No significant changes in ovarian expression of iNOS mRNA were seen. iNOS mRNA was only detected in peritoneal cells following hCG administration and these levels were significantly higher than those in ovarian $F480^+$ cells at the same time-point ($p<0.006$) (Figure 4-1E, F). TNFα mRNA increased following hCG administration in ovarian and peritoneal cell types and this increase was significant in all cases except the for the ovarian Ia^+ cells. TNFα mRNA levels returned to pre-hCG levels on day 2 (24 hours post hCG). Isolated ovarian $F480^+$ cells contained significantly more TNFα mRNA than peritoneal $F480^+$ cells on day 1 only. Isolated Ovarian Ia^+ cells contain significantly TNFα mRNA more than peritoneal Ia^+ cells on day 0am and day 1.

The expression of mRNA for the anti-inflammatory molecules IL-1ra, IL-10 and TGFβ1 was detected in all ovarian samples but only some the peritoneal samples. IL-1ra mRNA levels increased following hCG administration in ovarian and peritoneal cell types

Figure 4-1 Quantitative RT-PCR Results Showing Changing mRNA Levels Of The Inflammatory Mediators Interleukin-1-Beta, Tumour Necrosis Factor-Alpha And Inducible Nitric Oxide Synthase. Expression of mRNA for the inflammatory molecules interleukin-1-beta (IL-1β)(A,B), inducible nitric oxide synthase (iNOS) (C,D) and tumour necrosis factor-alpha (TNFα) (E,F) in isolated murine ovarian (A,C, E) and peritoneal (B,D, F), Ia^+ (■) and $F4/80^+$ (■) cells isolated at different times during a gonadotrophin primed cycle. For each target gene in both graphs the control group with the designated value of 1 is the ovarian day 0am Ia^+ group. Within each individual graph bars with the same letter are not significantly different (for all significant differences $P<0.05$). Ovarian $F4/80^+$ cells contain more IL-1β mRNA than peritoneal $F4/80^+$ cells on day 0am and day 2 ($P<0.003$), more iNOS mRNA than peritoneal cells on day 0pm and day 2 only ($P<0.02$) and more TNFα mRNA than peritoneal $F4/80^+$ cells on day 0am and day 1 ($P<0.026$). Ovarian Ia^+ cells contain more IL-1β mRNA than peritoneal Ia^+ cells at all time points ($P<0.003$), significantly more iNOS mRNA than peritoneal cells at all times points except day 0pm ($p<0.01$) and more TNFα mRNA than peritoneal Ia^+ cells on and day 1 only ($P<0.026$).


Ovarian Macrophages
Peritoneal Macrophages
A)
B)
C)
D)
E)
F)
IL-1β
iNOS
TNFα
Fold Change
hCG
Ovulation
day 0am
day 0pm
day 1
day 2

although this was not significant for ovarian Ia^+ cells (Figure 4-2A, B). Levels returned to pre hCG levels on day 1. The isolated ovarian cells, both Ia^+ and $F4/80^+$, contained significantly more IL-1ra mRNA than the corresponding isolated peritoneal cells at all time points except day 0pm. No significant changes across the stimulated cycle were seen in ovarian mRNA for IL-10 or TGFβ1. In peritoneal cells IL-10 mRNA was only detected transiently following hCG administration on day 0pm while TGFβ1 mRNA was constitutively expressed (Figure 4-2C, D, E, F). On day 0pm when IL-10 transcripts were present in peritoneal samples isolated ovarian $F4/80^+$ cells contained significantly more IL-10 mRNA than the corresponding peritoneal cells. No significant differences were found for TGFβ1 across the cycle, between Ia^+ and $F4/80^+$ cell types from the same tissue or between ovarian or peritoneal cell types isolated with the same antibody.

4.3.4 Soluble Mediators Secreted By Ovarian Macrophages

We went on to examine the secreted concentrations of selected inflammatory molecules which have established roles in ovarian function (IL-1β, TNFα and NO) or for which little data pertaining to function in the ovary has been reported (IL-10). Variable numbers of total cells were isolated and subsequently cultured at each time point, ranging from around 2.1-2.7 x 10^4 on day 0 am, up to 7 x 10^4 (day 1 Ia^+ and day 2 for $F4/80^+$), or 1 x 10^5 day 2 Ia^+. Thus results were expressed as a function of the numbers of cultured cells. Surprisingly, cultured ovarian $F4/80^+$ cells did not produce detectable amounts of secreted IL-1β protein while cultured ovarian Ia^+ cells produced detectable amounts of only when isolated following hCG administration (Figure 4-3A). IL-1β protein was detectable in all peritoneal cell conditioned media and was not significantly different to concentrations measured in

Figure 4-2 Quantitative RT-PCR Results Showing The Changing mRNA Levels Of The Anti-Inflammatory Mediators Interleukin-1-Receptor Antagonist, Interleukin-10 And Transforming Growth Factor -Beta-1. Expression of mRNA for the anti-inflammatory molecules interleukin-1 receptor antagonist (IL-1ra) (A,B), interleukin-10 (IL-10) (C,D) and transforming growth factor-beta 1 (TGFβ1) (E,F) in isolated murine ovarian (A,C,E) and peritoneal (B,D,F) Ia^+ (■) and $F4/80^+$ (■) cells isolated at different times during a gonadotrophin primed cycle. For each target gene in both graphs the control group with the designated value of 1 is the ovarian day 0am Ia^+ group. Within each graph bars with the same letter are not significantly different (for all significant differences $P<0.05$). Within each graph, bars with the same letter are not significantly different (for all significant differences $P<0.03$). No significant differences were found in TGFβ1 mRNA levels. Ovarian $F4/80^+$ cells produce significantly more IL-1ra mRNA than peritoneal $F4/80^+$ cells at all time points except day 0pm ($P<0.032$) and more IL-10 mRNA than peritoneal $F4/80^+$ cells at all time points ($P<0.001$). Ovarian Ia^+ cells contain more IL-1ra mRNA than peritoneal Ia^+ cells at all time points except day 0pm ($P<0.001$) and more IL-10 mRNA than peritoneal Ia^+ cells on day 0am only ($P<0.003$).

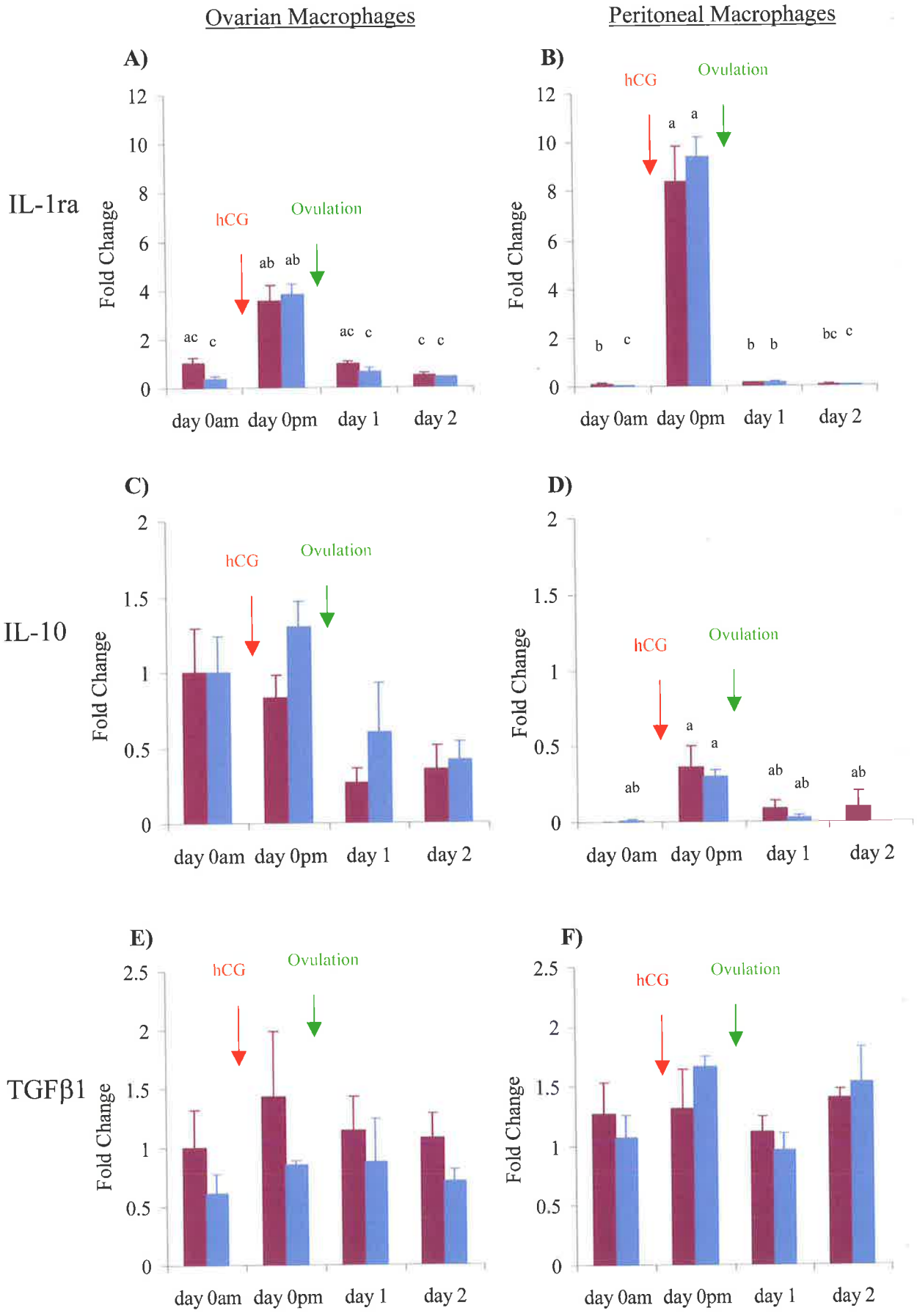
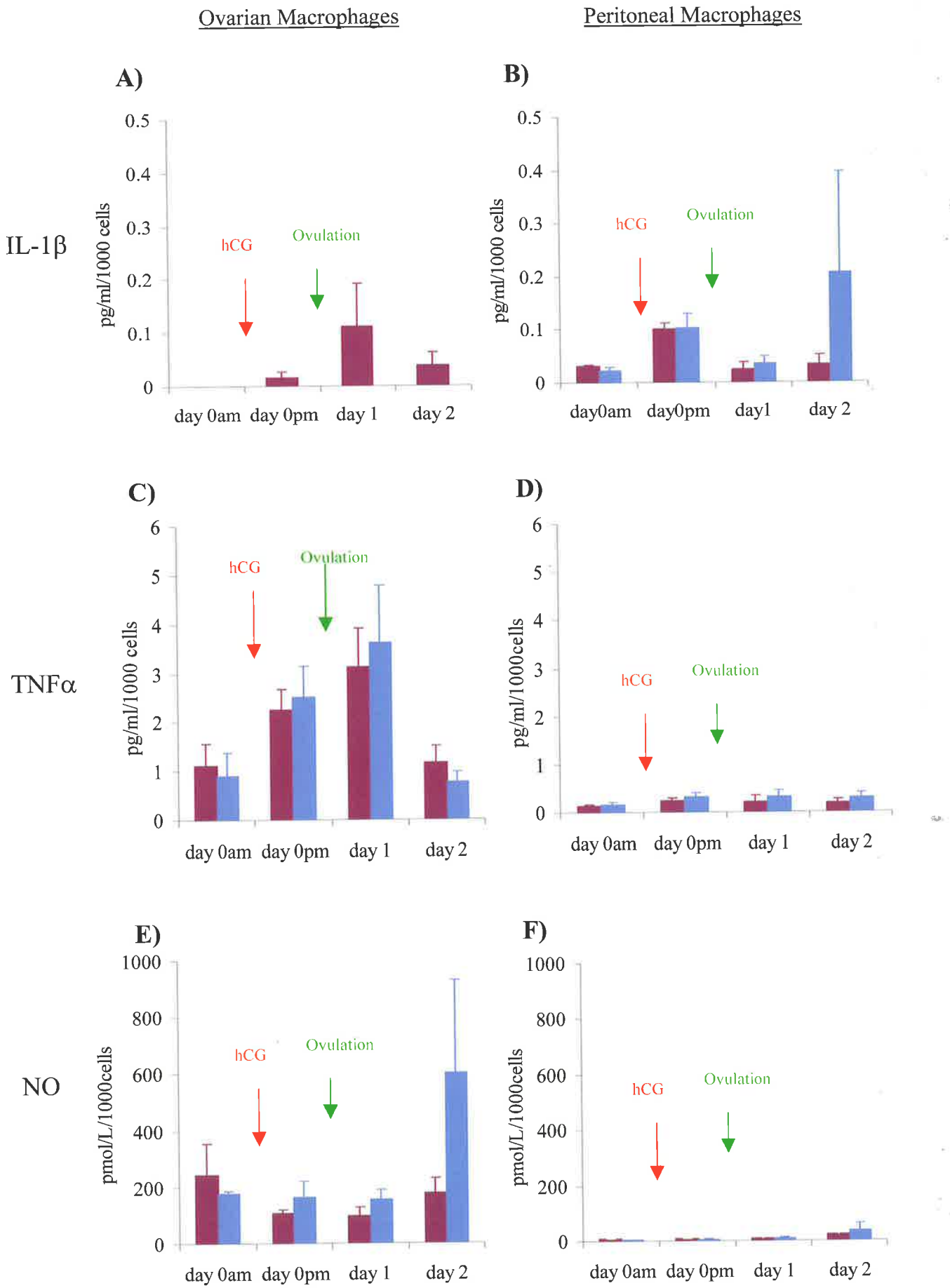

Ovarian Macrophages
Peritoneal Macrophages
A)
B)
C)
D)
E)
F)
IL-1ra
IL-10
TGFβ1
Fold Change
hCG
Ovulation
day 0am
day 0pm
day 1
day 2
ac c
ab ab
ac c
c c
b c
a a
b b
bc c
ab
a a
ab ab
ab

Figure 4-3 The Concentrations Of Inflammatory Mediators Measured In Macrophage Conditioned Media. Interleukin-1-beta (IL-1β) (A,B) and tumour necrosis factor alpha (TNFα) (C,D) protein concentrations in medium conditioned by 24hr culture of ovarian (A,C) or peritoneal (B,D) Ia^+ (■) or $F4/80^+$ (■) cell types isolated at times during the gonadotrophin stimulated cycle indicated in on the x axis. No IL-1β production was detected in any of the ovarian $F4/80^+$ cells or in Ia^+ cells prior to hCG administration. Where IL-1β protein was detected, no significant difference in IL-1β production across the cycle or between the ovarian and peritoneal Ia^+ cells was found. No significant differences in TNFα or NO concentrations were detected across the cycle or between the ovarian Ia^+ and $F4/80^+$ cell types. Ovarian Ia^+ cells produce significantly more TNFα and NO than peritoneal Ia^+ cells at all comparable times ($P< 0.04$). Ovarian $F4/80^+$ cells produce significantly more TNFα than peritoneal $F4/80^+$ cells at all points except day 2 ($P<0.02$).


Ovarian Macrophages
Peritoneal Macrophages
A)
B)
C)
D)
E)
F)
IL-1β
TNFα
NO
pg/ml/1000 cells
pmol/L/1000cells
hCG
Ovulation
day 0am
day 0pm
day 1
day 2
day0am
day0pm
day1

ovarian macrophages. Ovarian and peritoneal cells were both found to secrete detectable concentrations of TNFα (Figure 4-3C, D) however, no significant differences were detected across the stimulated cycle, even though it appeared concentrations were increased on day 0pm and day 1. Ovarian cells were found to produce significantly more TNFα than isolated peritoneal cells at all times except on day 2 where conditioned media from ovarian $F4/80^+$ cells contained protein concentrations not significantly different to that in conditioned media from peritoneal $F4/80^+$ cells. NO (Figure 4-3E, F) and IL-10 (Figure 4-4A, B) could be detected in all samples from both ovarian and peritoneal conditioned media. Ovarian cells were found to produce significantly more IL-10 protein and NO than peritoneal cells. Despite a small increase in IL-10 concentration in ovarian macrophage conditioned media on day 1 post ovulation, no significant changes in IL-10 or NO concentrations were detected across the cycle.

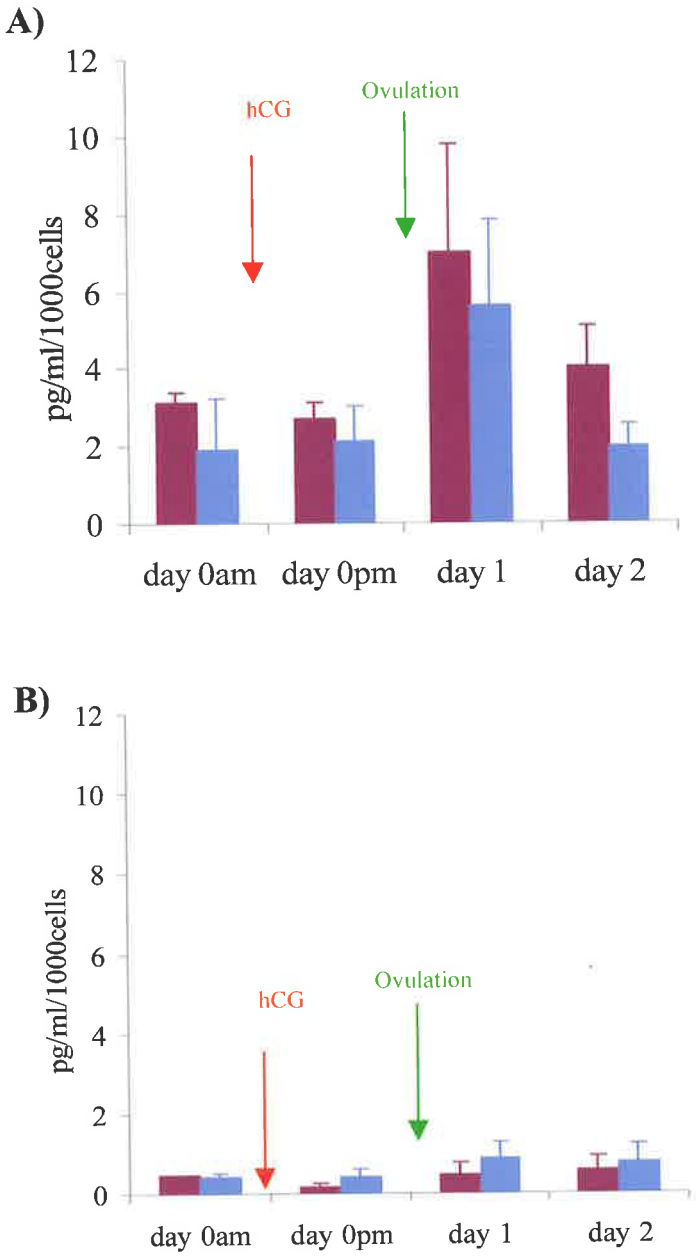


Figure 4-4 The Concentration Of Interleukin-10 Measured In Macrophage Conditioned Media. Interleukin-10 (IL-10) concentrations in medium conditioned by 24hr culture of ovarian (A) or peritoneal (B) Ia^+ (■) or $F4/80^+$ (■) cell types isolated during the gonadotrophin stimulated cycle at times indicated on the x-axis. No significant differences in IL-10 production were detected across the cycle or between the ovarian Ia^+ and $F4/80^+$ cells or the peritoneal Ia^+ and $F4/80^+$ cell. Ovarian cells did produce significantly more IL-10 than peritoneal cells ($P< 0.005$) at all time points.

4.4 DISCUSSION

This study has examined for the first time the changing levels of mRNA and protein expression of both inflammatory and anti-inflammatory molecules in isolated ovarian macrophages. At the times points studied, isolated ovarian macrophages produced mRNA for the inflammatory molecules, IL-1β, the enzyme iNOS, which generates NO, and TNFα. Corresponding active proteins were detected in conditioned culture media although the concentraions measured did not always reflect those anticipated as a result of the mRNA measurements. A cytokine mRNA and protein profile for murine ovarian macrophages, different to that seen in peritoneal macrophages, has been constructed in an attempt to further clarify the potential roles of these cells within the ovary.

A significant 3 fold increases in IL-1β mRNA leading up to ovulation has also been reported in whole rat ovary [185], using RNase protection assays, followed by analysis with Image Quant Software. The fold changes we have measured are much more dramatic than these, with a 5-7 fold increase in IL-1β mRNA. This may be a reflection of either the increased sensitivity of measurement using the quantitative RT-PCR method, or the increased purity of the IL-1β mRNA expressing population. This implies that, post hCG, macrophages may be a major contributor to the levels of ovarian IL-1β. Analysis of the conditioned culture media for mature IL-1β protein using the commercially available Quantikine kits, revealed that the amount of active secreted protein was virtually undetectable. This demonstrates that at the time points studied, the substantial increases in the levels of transcript could not be shown to be translated into mature protein. The regulation of the production of mature IL-1β is a complex well regulated process that has been reported to require two stimulating steps, a priming step and a processing step (reviewed by Bruns et al, [340]). The priming step initiates transcription and translation of the inactive proform of IL-1β that then remains intracellular,

while the secondary signal promotes processing of intracellular proIL-1β and secretion of the mature IL-1β protein The inactive proIL-1β is not accurately detected by the ELISA kit [341] used in this study and it could be hypothesized that ovarian macrophages are producing this form of IL-1β. If secreted this precursor protein could be converted extracellularly to active IL-1β, by enzymes such as caspase-1, or IL-1β converting enzyme, and matrix metalloproteinases (MMP's) -2,-3 and –9 [342], located within the ovarian tissues [343, 344]. However this does seem unlikely since the proIL-1β is found predominantly intracellularly, and has reported to be the principal secreted IL-1β form only in activated peritoneal cells under certain stimulatory conditions [345]. It should also be noted that it has been suggested that extracellular proIL-1β is derived predominantly from dying cells [346]. The results presented in this study suggest that at ovulation ovarian macrophages are stimulated to produce IL-1 mRNA and may contain intracellular pro-IL-1β but these cells do not appear to be a significant source of secreted mature IL-1β protein. Such a phenomenon has been observed in other systems ([347]; reviewed by Dinarello [348]). Furthermore, Simon, et al [188] concluded that although macrophage cells stained positive for IL-1β protein, luteal cells were thought to be the major source of this protein. Interestingly, the presence of macrophages has been shown to increase the amount of IL-1β mRNA expressed by granulosa cells [349], and therefore although ovarian macrophages may be required for maximal IL-1β production, they are probably not a major source. IL-1β concentrations measured produced ovarian macrophages are lower than those measured in resting blood monocytes and monocyte derived macrophages [350, 351] but comparable to those produced by isolated resting microglial cells [352]. The levels measured by bioassays in testicular macrophage cultures are reportedly low [137] and no differences between basal levels from these macrophages and cultured peritoneal cells were found.

The current study also shows, ovarian macrophages express iNOS mRNA and produce the NO molecule at consistent levels across the gonadotrophin-stimulated cycle. Elsewhere iNOS mRNA expression in the ovary has been reported to fluctuate. Levels in theca have been shown to decrease significantly 10 hours after hCG administration [353] while in contrast Zackrisson et al have reported an increase in iNOS 6 hours post hCG, with lower levels measured in the theca, stroma and CL, at other times of the gonadotrophin stimulated cycle [209]. Although the levels of iNOS mRNA and NO we have detected in ovarian macrophages do not change over the stimulated cycle, the numbers of macrophage themselves do vary and hence these cells may contribute to fluctuating iNOS mRNA levels and NO concentrations measured by others in the rodent ovary. In comparison to other macrophages those from the testis do not produce detectable concentrations of NO in culture [136] while resting microglial cells in culture produce comparable concentrations [354].

The levels of TNFα mRNA and protein in ovarian macrophages were found to increase following hCG administration, this increase was significant only for the mRNA levels measured, not the secreted protein concentrations. In the ovary mRNA and protein for this cytokine have both previously been demonstrated in regions of macrophage accumulation [251, 252], but studies examining levels of TNF mRNA expression across the reproductive cycle have not been done. The data presented in this study show significant variations in mRNA levels, and fluctuating protein production during the stimulated reproductive cycle. This, in combination with previous studies, suggests that ovarian macrophages may be a significant source of this cytokine in the murine ovary, stimulating ovulation, and CL steroidogenesis. The TNFα levels measured in isolated ovarian macrophages are comparable to those reported in microglial cells [354] while levels reported in resting blood monocytes cultures are either equivalent [350] or marginally lower [351]. Resting testicular macrophage production has been described as not significantly different to that seen in resting peritoneal

macrophages [137] implying they are lower than those seen in ovarian macrophages, while levels of this cytokine reported following LPS stimulation of testicular macrophages are comparable to those produced by resting ovarian macrophages [355].

Thus, as macrophages producing NO and TNFα infiltrate the pre-ovulatory follicle and CL in growing numbers detectable levels of mRNA and the gene products would similarly increase, and may play roles in the stimulation of steroidogenesis and ovulation. Ovarian macrophages may therefore be a source of NO and TNFα activities during the preovulatory period and in the CL. We suggest that ovarian macrophages may not be the predominant source of the inflammatory cytokine IL-1β prior to ovulation although further analysis of the IL-1β isoforms produced intra- and extra-cellularly by these cells is required to confirm this. Ovarian macrophages may directly contribute to IL-1β concentrations in the CL, as IL-1β can be detected in Ia^+ cells after ovulation, or may stimulate production of this cytokine by the granulosa/luteal cells within the ovary.

This chapter further demonstrates that ovarian macrophages also produce the anti-inflammatory cytokines IL-1ra, IL-10 and TGFβ1. Ovulation has been likened to an inflammatory event, displaying the typical features of vasodilation, leukocyte infiltration and cytokine production [29] As such, it was anticipated that anti-inflammatory molecules would be expressed predominantly post hCG administration or ovulation, where they would be key molecules in containing the inflammatory ovulatory event.

As expected, the levels of IL-1ra mRNA mirrored those of IL-1β, but initial levels of mRNA for this anti-inflammatory factor (day 0am) were significantly higher in ovarian cells than peritoneal cells, and since IL-1ra is not expressed in resting monocytes, [356] this indicates that, surprisingly, these isolated ovarian cells are activated prior to hCG administration. It should be noted that there are two reported forms of IL-1ra, a soluble isoform (which contains a hydrophobic leader sequence) and an intracellular isoform (without this leader sequence)

each is regulated by a different promoter [357]. The primers used in this study do not distinguish between these mRNA forms, although it is probably not of importance for two reasons. Firstly, using RNase protection assays it has been reported that in the rat ovary the sIL-1ra is more predominant than icIL-1ra [194]. Secondly, it is possible the icIL-1ra isoform is also secreted, since it is structurally very similar IL-1β and IL-1α, both of which are secreted by a non-classical mechanism. Secreted IL-1ra protein levels were not measured in this study.

Expression of TGFβ1 mRNA was found to be constitutive with no difference observed across the gonadotrophin-stimulated cycle. This study does not rule out the possibility that TGFβ1 concentrations may be manipulated by posttranscriptional regulatory pathways. Significant post-transcriptional regulation of this factor has been reported at the translational [358], protein activation (reviewed in [359, 360]) and receptor expression [361, 362] levels. The levels of TGFβ1 mRNA previously reported in the theca of the follicle may correspond to that of macrophages within the thecal region with increases in mRNA as the ovulatory event approaches corresponding to increased cell numbers.

The presence of IL-10 mRNA and protein prior to ovulation was not anticipated. The concentrations produced by ovarian macrophages are higher than those reported in resting blood monocyte [351] and microglial cell [352] cultures, being more comparable to concentrations observed in these cultures following LPS stimulation. Due to its anti-inflammatory properties, it was anticipated that this cytokine would be produced predominantly following the ovulatory event, or at the earliest following hCG administration. In direct contradiction with these expectations, we have shown production of both the mRNA and protein of this cytokine, in ovarian macrophages both before and after the ovulatory event. This, combined with evidence of a lack of active IL-1β production, discussed earlier, has significant implications in determining the purpose and activation state of macrophages in

the ovarian tissue. Ovarian macrophages are the most likely source of this cytokine in the ovary, since T cells, the other significant IL-10 producing cell type, are rare in ovarian tissues. Little research examining the presence or role of IL-10 in the ovary has been done, although there have been some reports that IL-10 may be involved in CL function [336, 337].

Fluctuations in the production of inflammatory molecule transcripts seen in isolated peritoneal cells were unexpected, and it was intended that these cells would be a resting resident macrophage population with which to compare the isolated ovarian macrophages. Further examination of this phenomenon led to us to speculate that this was caused by the presence of BSA in the hCG dose, which was included to stabilise the hormone in storage. Subsequent experiments performed in our lab have revealed that endotoxin levels (Limulus Amebocyte Lysate QCL-1000 kit (Cat #50-648U), Bio Whittaker, Walkersville MD 21793-0127) in this preparation are greater than 1EU/ml and if the BSA is excluded from the hormone preparation the elevation in the peritoneal transcripts no longer occurs, while the elevated levels remain in the ovarian cells. Therefore, these changes are as a result of a classical inflammatory response of the peritoneal cells to the injected hormone solution, which contains some endotoxin. Thus, at day 0 pm these cells may instead be considered a positive control for the characteristics of classical inflammatory macrophages. Ovarian cells were found to have a cytokine mRNA and protein profile significantly different to that of the isolated peritoneal cells, the most notable being the relatively large amounts of IL-10 produced by the ovarian cells.

By combining the results presented in this chapter, a cytokine profile of the ovarian macrophages present at the different stages of the gonadotrophin-stimulated cycle can be constructed (Figure 4-5). On day 0am, prior to hCG administration and ovulation, ovarian macrophages are activated, expressing all mRNA transcripts measured, except TGFβ1, at higher levels than isolated resting peritoneal cells. These macrophages are also actively

producing TNFα, NO and IL-10 molecules at concentrations significantly higher than the peritoneal cells. Following hCG administration transcripts for the cytokines IL-1β, TNFα and IL-1ra increase significantly in ovarian macrophages although these changes are not as dramatic as those increases seen in the classically activated peritoneal macrophages. This may be due to the already activated state of the ovarian macrophages, a reduced response in more activated cells has been reported elsewhere in other experimental systems, or to the type of response, with that of the peritoneal cells being a classical endotoxin response and that of the ovarian macrophages a response to factors induced in the ovary by the LH surge. Immediately post ovulation, the stimulated cytokine transcripts in the isolated ovarian macrophages return to preovulatory levels but production of the TNFα, NO and IL-10 molecules continues. TNFα and IL-10 concentrations both appear to be at their peak at this time point returning to preovulatory levels on day 2. These two cytokines have significant roles in the inhibition of macrophage activation and consequently T cell functions that may both be important during the early formation of the CL post ovulation.

Figure 4-5 Overview Of The mRNA And Protein Concentrations Measured In Isolated Ovarian Macrophages. No differences in the amounts of mRNA present in Ia^+ and $F4/80^+$ cells were detected at any time point, while the only significant difference in measured protein concentrations was in IL-1β, that was not detected in conditioned media from $F4/80^+$ cells. Those mediators in purple are inflammatory and those in blue are anti-inflammatory. Day 0am is 48hours post PMSG/eCG but prior to hCG, Day 0pm is 6 hours post hCG injection, day 1 is 24 hours post ovulation and day 2 is 48hours post ovulation.

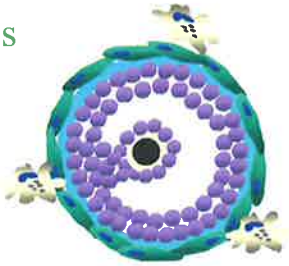
MACROPHAGE mRNA EXPRESSION

MACROPHAGE PROTEIN EXPRESSION

Ia$^+$ and F4/80$^+$ Cells

Ia$^+$ Cells

F4/80$^+$ Cells



day 0am

IL-1β +
TNFα +
iNOS +

IL-1ra +
TGFβ1 +
IL-10 ++

IL-1β –
TNFa +
NO +

IL-10 +

hCG

day 0pm

IL-1β +++
TNFα ++
iNOS +

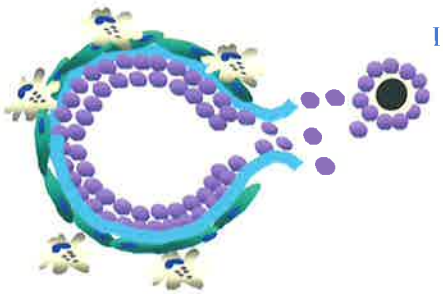
IL-1ra ++
TGFβ1 +
IL-10 ++

IL-1β ½ +
TNFa +
NO +

IL-10 +

IL-1β –
TNFa +
NO +

IL-10 +



day 1

IL-1β +
TNFα +
iNOS +

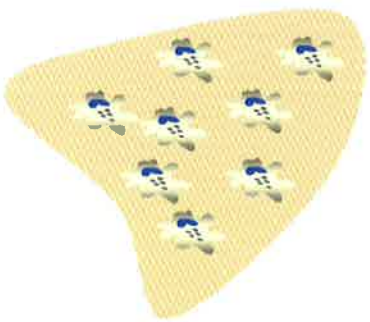
IL-1ra +
TGFβ1 +
IL-10 +

IL-1β+
TNFa ++
NO +

IL-10 ++

IL-1β –
TNFa ++
NO +

IL-10 ++



day 2

IL-1β +
TNFα +
iNOS +

IL-1ra +
TGFβ1 +
IL-10 +

IL-1β ½ +
TNFa ++
NO +

IL-10 ++

IL-1β –
TNFa ++
NO +

IL-10 ++

Traditionally, it is thought macrophages become activated by inflammatory stimuli, after which they regulated the immune response, killing pathogens and healing wounds. It is now apparent that, depending on the environmental stimuli, there are varying forms of activation. Three activation states have been recently described, based on several traits, including the secretory production of these cells, classical activation, alternative activation and type-II activation of macrophages [363]. Using these recently described states, and the cytokine analysis carried out in this thesis, it can be concluded that ovarian macrophages are not classically activated. They are constantly producing the cytokine IL-10 and their profile differs markedly from the classically activated peritoneal cells. As they are producing the inflammatory molecules, TNFα and NO (alternately activated macrophages do not produce this latter molecule, Table 4-5) it can be postulated that these cells are of, or related to, the type-II activated macrophage. This cell type has been described as a potent anti-inflammatory cell, which can preferentially induce a Th_2 adaptive immune responses and may be important in preventing autoimmune reactions [363].

We have shown that *in vivo* ovarian macrophages respond to the LH surge with significant changes in mRNA expression. Ovarian macrophages may be a primary source of ovarian TNFα and IL-10 and may contribute to ovarian levels of IL-1β and NO. They also express mRNA for TGFβ and IL-1ra. The transcripts these cells are producing show that they are not classical inflammatory cells but appear to be a more anti-inflammatory phenotype. Although, they may promote ovulation through the production of TNFα in the preovulatory follicle, their major roles are more likely to be in minimising tissue damage following the ovulatory event and, promoting tissue repair and/or apoptosis, in subsequent CL formation.

	Classical	Alternative	Type II
Activation signals	TNF, IFNγ	IL-4, glucocortioids	TL or Fc receptors,CD40
Secretory products	↑ **TNF**, ↑ IL-12, **IL-1**, IL-6	↑ **IL-1ra, IL-10**	↑ **IL-10, TNF**, IL-6
Markers	↑ MHCII, ↑ CD86 ↓ MR	↑ MR, ↑ SR, ↓ CD14,	Unique markers not available
Molecules	**NO**, O_2	-	**NO**, O_2
Chemokines	IP-10, MIP, MCP	AMAC-1	unknown
Role	Pro-inflammatory	Wound healing	Anti-inflammatory

Table 4-5 The Characteristics Of Three Described Macrophage Activation States. The cytokines measured in this thesis (highlighted in bold) can give an indication of the activation state of ovarian macrophages. The activating signals and the proposed roles in physiology are listed as well as the chief secretory products and currently detectable distinguishing cell surface markers. Adapted directly from Mosser, 2003 [363]. MR, mannose receptor; SR, scavenger receptor; TNF tumour necrosis factor; IL interleukin; MHC, major histocompatibility factor; MIP, macrophage inflammatory protein; MCP, macrophage chemoattractant protein; AMAC alternative macrophage activation associated CC chemokine; IP, interferon inducible protein.

Chapter Five

5 FINAL DISCUSSION

5.1 SUMMARY OF FINDINGS

At the commencement of these studies it was established that macrophages are present in ovarian tissues, with an influx of these cells at ovulation and during CL formation and regression. In addition, much indirect experimental evidence implicated these cells in the regulation of ovulation and CL function. It was hypothesised that ovarian macrophages are critical to normal ovarian function through the regulated secretion of various inflammatory mediators. It was speculated that blood monocytes infiltrate the ovary becoming resting resident cells, following the LH surge, these macrophages become activated producing classical inflammatory cytokines that promote follicular rupture. Following ovulation and in the developing CL, macrophages then produce factors important in tissue reorganisation/wound healing and finally, in the regressing CL they are required for the clearance of apoptotic cells.

The work presented in this thesis provides evidence that ovarian macrophages, in particular the Ia^+ population, are critical for normal ovulation and estrus cyclicity. Conditions for the isolation of maximum numbers of live macrophages from the murine ovary tissue were determined and these isolated cells shown to be viable, 98% pure and have varying phagocytic characteristics along with an inability to promote T cell proliferation. These cells were further shown to produce a unique array of both pro- and anti-inflammatory mediators the absence of which may account for the experimental affects observed by clodronate liposome treatment presented in Chapter 2. Combining the studies presented in this thesis a preliminary description of the characteristics of ovarian macrophages during the gonadotrophin primed cycle can for the first time be constructed.

Prior to ovulation, 50% of isolated ovarian macrophages were shown to be phagocytic (on day 0am). Many of these are probably found in the stroma of the ovary, with some located around the growing follicles. These thecal macrophages may be important for optimal

stimulation of follicle growth, as depleting these cells appears to reduce the numbers of follicles reaching the preovulatory size. These ovarian macrophages are producing TNFα, NO and IL-10 and although conditioned culture media from these cells appears to stimulate PHA-induced T cell proliferation, this was not statistically significant.

Following i*n vivo* administration of hCG (on day 0pm), phagocytic activity of the isolated macrophages remained the same but the expression of mRNA for TNFα, IL-1β and IL-1ra, were augmented. It was found that these mRNA levels were not always consistent with the levels of secreted mature protein these cells produce. TNFα was a significant product of these cells while the Ia+ population produced IL-1β at very low concentrations. TGFβ1, IL-10 and iNOS mRNA and protein concentrations were detectable but remained static. In the theca of preovulatory follicles these ovarian macrophages, in particular those expressing the Ia antigen, may be key regulators of normal follicular rupture.

Following the ovulatory event, the percentage of phagocytic Ia^+ and $F4/80^+$ ovarian macrophages increased to 80% (on day 1). Many of these are probably found in the early CL, and although these cells have less stimulatory influence on PHA stimulated T cells this effect was again not statistically significant. These cells appear to be important in progression through the postovulatory stages of the normal estrous cycle to the next ovulatory event. The mRNA levels for those cytokines that had responded to hCG administration fell to levels not significantly different to those prior to hCG administration. It appeared that IL-10 mRNA levels may be less but this was not significant. At this time point, cells were found to produce IL-10 and TNFα proteins at slightly but not significantly higher concentrations, than those prior to follicular rupture. NO concentrtions did not change, but Ia^+ cells began to produce detectable amounts of IL-1β.

It can be concluded from these results along with comparisons of the cytokine profile of the isolated ovarian macrophages with the peritoneal macrophages, that the former are not

classical inflammatory cells as anticipated, but display a cytokine profile more comparable to that of anti-inflammatory macrophages, as described by Mosser [363]. Based on these results, in particular the IL-10, the role of macrophages within the ovary is more complicated than hypothesised and it is necessary to produce a new model in which the primary role of these cells may be an anti-inflammatory one.

5.2 Evidence of Macrophage Heterogeneity in the Murine Ovary

It was suggested in Chapter 2 that the ovarian macrophage population may be heterogeneic, since macrosialin$^+$ (FA/11$^+$) and Ia$^+$ cells were significantly depleted by CLi treatment but F4/80$^+$ cells, although reduced in numbers, were not significantly depleted. This suggested a difference in the phagocytic activity of the different cell types, however such a difference between these isolated cell types was not observed in the analysis of phagocytosis employed in Chapter 3. In addition in Chapter 4, the cytokine analysis carried out did not reveal any significant differences in the expression any of the cytokine mRNA by the Ia$^+$ and F4/80$^+$ cells types at any time point examined. These results combined suggest that the Ia$^+$ and F4/80$^+$ cells are not significantly different to each other. Indeed, the only significant difference observed was the very low levels of IL-1β detected following hCG administration in conditioned media from the Ia$^+$ cells only. It does however seem important to note that in Chapter 2 it was a significant reduction in this cell type that resulted in significantly reduced ovulation rates. It may be that the small amount of mature IL-1β being secreted by these cells is of critical importance for this event. This suggests the local ovarian factors, such as steroids [119, 120, 123] or cytokines [364], dictate the activity and phagocytic capacity of the

recruited macrophages as a whole. Even though no differences were observed between the isolated Ia^+ and $F4/80^+$ cell types, whether differences in the characteristics of cells located in the different ovarian compartments exist cannot be concluded from these studies, although the differences in the depletion of ovarian macrophages populations in Chapter 2 sugest they may exist. It could be proposed that the markers used in this thesis to isolate ovarian macrophages are widespread in the ovarian macrophage populations and do not distinguish between the heterogeneous populations. This would mean that the populations examined in this thesis are of mixed characteristics. In light of this, the non-phagocytic macrophage population isolated at each time point, may be mature resident macrophage cell types located in the ovarian stroma, which was not significantly affected by CLi injection in Chapter 2, while the phagocytic cells could be located chiefly in the theca of the follicles and CL where significant reductions in macrophage numbers were seen following CLi treatment. In support of this, the increase in phagocytic cells following ovulation could be explained by the increasing numbers of new active macrophages recruited into the CL.

It follows that if thecal and luteal macrophage populations could be studied independently, changes in cytokine production across the reproductive cycle may be even more striking, although this would be very time consuming and required the sacrifice of many laboratory mice. It would be more practical to simply examine the distribution of additional distinguishing macrophage activation markers and cytokines in ovarian tissue sections.

5.3 Implications For Ovarian Macrophage Phenotype and Function

The protein profile of ovarian macrophages seen in the final chapter of this thesis suggests that ovarian macrophages are not a classically stimulated inflammatory cells but are more likely to be an alternatively activated cell type and the foremost role of these cells in the vary may be in minimising the inflammatory ovulatory event and regulating CL formation.

The later is supported by animals models in which regulators of macrophage functions have been disrupted resulting in extended estrous cycles [68, 331], as well as the work presented in Paper I, showing extended cycles potentially due to lack of activated macrophages to stimulate CL regression, while an important role for ovarian macrophages in the regulation of follicular growth and ovulation is also inferred. It is clear that the phagocytic Ia^+ macrophages located in the theca of the follicle, which were significantly depleted by CLi treatment in Chapter 2, may play an important role in the stimulating either follicular growth and/or the ovulatory event. The results in Chapter 4 (showing Ia^+ macrophages produce significant amounts of TNFα and NO prior to and following hCG administration, as well as minimal amounts of IL-1β following hCG administration), suggest this could occur through the production of either TNFα, IL-1β and/or possibly NO, which have all been shown elsewhere to stimulate follicular growth [220, 318] or follicular rupture [191, 213, 261]. The examination of ovarian morphology carried out in Chapter 2, although not conclusive, does suggest that this reduced ovulation rate is due to an inhibition of follicular rupture, further suggesting that thecal macrophage production of TNFα and/or IL-1β is critical in stimulating follicular rupture.

It is of particular interest that we have found the alternative activation state is present prior to the LH surge, suggesting the ovarian environment induces this state when macrophages initially infiltrate the ovarian tissue. There are diverse reported regulators of infiltrating macrophage function including the cytokines present in the tissue [127], the adhesion molecules drawing the cells into the tissue [365] and the structure of the extracellular matrix within the tissue [366, 367] or even the presence or absence of apoptotic cells[368]. It is primarily the production of the cytokine IL-10 and the minimal production of mature IL-1β by the isolated ovarian macrophages that compels this view of ovarian macrophage activation. IL-10 is an autoregulatory cytokine with numerous anti-inflammatory

roles and interestingly, it is thought to be an important mediator in maintaining peripheral tolerance [369]. It prevents the activation and migration of antigen presenting cells, inhibits T cell activation and cytokine production and is considered a promoter of local resolution of inflammation [370, 371]. More specifically it can inhibit the expression of co-stimulatory molecules CD80/CD86 on the surface of macrophages leading to immune tolerance through the inactivation or skewing of T cells [371]. In the ovary this cytokine may act to prevent the migration and maturation of macrophages, and destructive cytotoxic T cells, following the inflammatory ovulatory event. It follows that ovarian macrophages may be present to prevent 'immune surveillance' of the ovulated follicle. CL regression involving immune cells has been proposed to occur via one two mechanisms, immune mediated regression, or programmed cell death [233]. Both of these processes would require significant regulation by ovarian macrophages by divergent mechanisms. Firstly, for immune mediated CL regression macrophages would need to become classically activated expressing co-stimulatory molecules and subsequently promoting cytotoxic T cell activation leading to cell mediated regression of the CL. In support of this the production of the potent macrophage activator, IFNγ also occurs at CL regression [249, 372]. Secondly, for CL regression via programmed cell death, the anti-inflammatory ovarian macrophages may promote, local resolution of the tissue damage following ovulation, and the generation of regulatory T cells, restricting any inflammatory reaction and promoting apoptosis. Only further research into the cytokines and co-stimulatory molecules expressed by ovarian macrophages during the formation and regression of the CL, including during pseudopregnancy, will assist in determining which of these roles ovarian macrophages may have in CL function.

5.4 SUMMARY

Macrophages are critical to normal ovarian function. It is suggested that the local ovarian environment influences resident and infiltrating macrophages to develop into cells of the type II activated or anti-inflammatory state. These macrophages, present in the ovary prior to the LH surge and ovulation, maintain an immunosuppressive environment through the production of IL-10, preventing macrophage maturation, migration and interactions that generate destructive T cells. These cells may produce NO and TNFα to promote, follicle survival and growth (Figure 5.1). Following the LH surge, the cells infiltrating the theca of the follicle, in particular the Ia^+ population, are critical to follicular rupture probably through the production of TNFα and/or IL-1β which stimulate follicular rupture. In the early CL although these cells are more phagocytically active, they continue to produce IL-10 and TNFα. It is further speculated that macrophages may have one of two roles in CL regression – maturation and then stimulation of T cells cytotoxic towards the luteal cells, or maintenance of immune restriction and stimulation of programmed luteal cell apoptosis.

On the basis of work presented in this thesis it may be proposed that the major role of macrophages in the ovary is in the minimisation of the inflammatory-like reactions stimulated at ovulation, with contributions to follicular rupture through the production of TNFα and IL-1β, and potentially in subsequent survival and demise, of the CL.

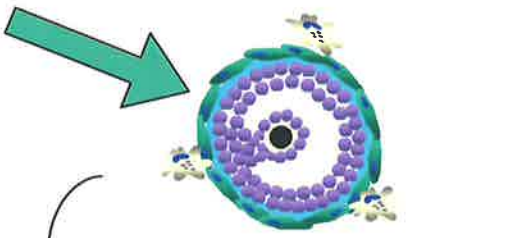
Figure 5-1 The Emerging Role Of Macrophages In Ovarian Function.

The studies presented in this thesis suggest that ovarian macrophages may be a critical source of TNFα and/or, IL-1β and NO required to stimulate ovulation and follicle growth. These cells also produce interleukin-10, at consistently high levels during the gonadotrophin-primed cycle, implying that they have an immunosuppressive role in the ovary. It has been speculated that at luteolysis these cells may become activated and promote corpus luteum demise through the activation of destructive T cells.

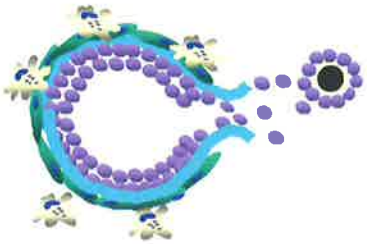
Ovarian environment induces anti-inflammatory ovarian macrophage phenotype

TNFα
NO
IL-1β??

Stimulate follicular growth and /or rupture

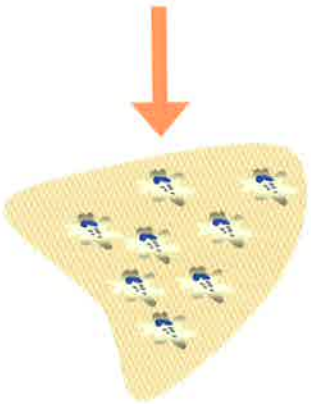


LH



IL-10

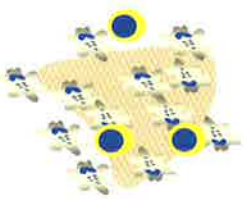
- •Prevents macrophage maturation/migration
- •↓IL-1β production
- •Inhibits CD4+ T cell migration
- •↓co-stimulatory molecule expression
- •?? maintains functional CL



??

PROPOSED ROLE IN CL REGRESSION

?? Macrophage Activation (IFNγ)
↑Co-stimulatory molecule expression
↓IL-10
↑ inflammatory molecules
Cytotoxic T cell invasion and activation



5.5 FURTHER STUDIES

The results of the studies presented in this thesis raise interesting questions regarding the phenotypes of the macrophages located in the murine ovary. Examining these questions will further clarify the roles these cells have in ovarian function.

Are The Macrophages In The Ovarian Compartments Heterogeneic?

- Studying the dual expression of the Ia^+ and $F4/80^+$ and other distinctive macrophage markers in digested ovarian tissues using flow cytometry, will determine the proportions of the populations that overlap and thus more clearly establish the presence or absence of heterogenic populations.
- Histological examination of macrophage activation markers, co-stimulatory molecules and cytokine mRNAs in ovarian tissue sections will further elucidate the activation status and heterogeneity of the localised ovarian macrophage populations across the reproductive cycle, including during pseudo-pregnancy were macrophages will probably play a primary role in CL survival and regression,.
- Isolation of macrophages from ovarian dissected follicles and corpora lutea of different stages of development would allow specific determination of the characteristics of these cell types but may be impractical.

Do Ovarian Macrophages Have Irregular Immune Functions?

- Further examination of the phagocytic activity of these cells before and after ovulation as this may be a critical role of these cells in the ovarian tissue. It would be of particular interest to analyse this activity in response to apoptotic cells, since these are relatively common in some follicles and during CL regression.
- Examination of the ability of cells isolated before and after ovulation to directly stimulate T cell proliferation including analysis of the cytokines and T cell phenotype

stimulated would enable further conclusions on this aspect of macrophage biology and its role in ovarian biology.

- How do these cells response to traditional stimulators of classical inflammatory responses. Macrophages in others tissue have been shown to exhibit altered responses to factors such as LPS. It would be of interest to determine if ovarian macrophages have a response similar to that seen in any other macrophage cell type.

What Is It That Induces The Ovarian Macrophage Phenotype And Do Alterations Of This Phenotype Lead To Ovarian Dysfunction ?

- Since obtaining ovarian macrophages is a difficult and 'animal expensive' process it would be useful if this phenotype could be generated by other means, such as culturing of peritoneal macrophages with factors from the ovarian environment or even generation of an ovarian macrophage cell line. The effects of various factors on ovarian macrophages and possibly ovarian function could then be studied.

Are Other Macrophage Derived Factors Involved In Ovulation?

- Finally to confirm the role of macrophages in stimulation of the ovulatory event via analysis of mRNA from macrophages at several time points more immediate to the ovulatory event using micro-array techniques. This will help determine if other genes, not considered in this study, are activated in these cells following the LH surge and therefore of interest for further studies. Results obtained must be interpreted carefully since it was found in thesis that ovarian macrophage mRNA levels do not always reflect active protein levels.

5.6 IMPLICATIONS

A greater understanding of the activation status and secretory profile of the ovarian macrophage will lead to better understanding of the mechanisms effecting normal ovarian function. The specialised secretion by ovarian macrophages of factors that stimulate specific functions at precise stages of the reproductive cycle could be a key component of reproductive function. Alterations or defects in ovarian macrophage phenotype or secretory profile may be found to have consequences that lead to ovarian dysfunction. Interactions between the immune, reproductive and metabolic systems are becoming increasingly evident with perturbations in one significantly influencing normal functions of the others. Clinically an understanding of ovarian macrophage biology may assist in the understanding and/or treatment of ovarian disorders such as the polycystic ovary syndrome (PCOS), ovarian autoimmune disease or cases of unexplained anovulation. Further, these cells may also become a potential target for manipulation in contraceptive development.

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APPENDIX 1 – GENERAL METHODS

OVARIAN INTRABURSAL INJECTION TECHNIQUE

Animals were anaesthetised using a mix of fluorothane, nitrous oxide and oxygen gases in a box. This was then maintained via regulated continuos delivery of the same gases with a Midget anaesthetic machine (CIG, Aust.). Mice were placed on their ventral side and swabbed generously with chlorhexidine. A small incision was made in the skin of the mid dorsal region above the ovarian fat pad on both left and right sides. Skin was resected from the wall of the peritoneum and then an incision made in the peritoneal wall above the ovarian fat pad. The fat pad and attached ovary was then carefully externalized through the incision and using a microscope a needle laden with liposome treatment or saline was inserted through the fat pad into the intrabursal space. 10µl of fluid was injected or until the bursa was visibly bloated. The needle was then slowly withdrawn and any leakage of fluid or bleeding noted. The ovary and fat pad were then gently returned to the peritoneum. Anaesthetic was applied to the wounds and the skin drawn together with a wound clip. Animals were placed in individual cages under a heat lamp until normal activity resumed, usually observed after 30 minutes.

VAGINAL SMEARING, BLOOD SAMPLE COLLECTION, OOCYTE COUNTING AND TISSUE FREEZING.

Vaginal smears were performed every day following surgery at 0900-1000 hours. Mice were held firmly to the bench with the left hand and the tail raised with the thumb and forefinger. The vagina was gently flushed with 10µl of sterile saline with a finepipette. Saline could be seen to become clouded with cells. Smears were placed on a microscope slide and observed on an inverted phase contrast microscope for stage of the cycle classified as described in Chapter 1, Table 1-1.

If ovulation had occurred, mice were anaesthetised with avertin and blood samples taken via heart puncture. This was performed by inserting a 26G gauge needle between the ribs on the animals left, through the lungs and into the ventricles of the heart. As much blood as possible was collected and the animal then killed by cervical dislocation. Ovaries with the

fat pad intact were removed from the animal and trimmed further under a dissecting microscope. The ampulla of the oviduct was examined for 'swelling' and oocytes flushed out from this region by piercing the bursa and squeezing the fluid, cumulus and oocytes from the oviductal lumen. Collected cells and oocytes were placed in hyaluronidase to loosen cumulus and enable accurate counting of oocytes. Ovaries were then placed into foil boxes filled with OCT and then placed in freezing isopentane immersed in liquid nitrogen. Blocks were stored at -80°C till use.

TISSUE STAINING AND VIDEO IMAGE ANALYSIS (VIA)

Tissue blocks were removed from the -80°C freezer and placed in the -20°C cryostat for 2 hours prior to cutting. Blocks were trimmed and 6μm sections cut through each entire ovary with all intact sections collected, two to a slide. This resulted in from 80 to 180 consecutive slides for each ovary. These were also stored at -80°C in sealed boxes with silica gel. Slides were allowed to cool to room temperature over night or at for least 5 hours.

Haematoxylin/Eosin (H/E) staining

To determine the number of preovulatory follicles every 5th section was fixed in alcohol and then stained with H/E, dehydrated and mounted in DPX mounting media (BDH) as described below:

- Haematoxylin 60 seconds
- Milli U wash
- Acid wash Very quick 5 second dip
- Milli U wash
- Tap water 2 minutes (check for blue stain under microscope if not dark re-stain, longer)
- Milli U wash
- 95% alcohol 5 minutes
- 100% alcohol 1 5 minutes
- Eosin-Y (Sigma) 30 seconds
- 100% alcohol 2 5 minutes
- xylene substitute 1 5 minutes
- xylene substitute 2 5 minutes

Each slide was examined under a Vanox microscope the CLs counted and the diameter of each follicle in each section measured using a Video Image Analysis (VIA, Leading Edge, Pty Ltd, S.A) enabling the identification of every follicle of preovulatory size in each ovary.

Specific Macrophage Staining

For each preovulatory follicle identified in H/E stained sections, neighbouring slides were selected, stained with macrophages specific antibodies and visualized with DAB. In each follicle three regions of the theca and stroma were analysed using the VIA software. For each follicle the average positivity was determined and for each animal the average of the all follicles was determined. The average positivity of each experimental group of animals (CLi or S treated) based on the results of 4 animals was then graphed.

- Slides were labelled and primary antibody aliquot thawed. (Allow ≅ 50µl per slide to calculate amounts of diluted antibody etc needed).
- Prepare 1L of PBS (Gibco)
- 50mls of PBS/ 1%BSA (0.5g BSA in 50ml PBS).
- 10%NMS/ 1%BSA/PBS (400µl NMS in 3,600µl of 1%BSA/ PBS).
- Sections were fixed with cold 96% ethanol, 10 minutes and then circled closely with grease pen.
- Slides were washed for 5 minutes in PBS, 3 times and then dipped in PBS/1% BSA/10%NMS.
- Sections were incubated with 200µl primary antibody, as hybridoma supernatant, diluted in 20µl NMS + 20µl PBS/ 1%BSA (rat-anti-mouse FA11, F4/80 or Ia), for 3 hours at 4°C in humidified container.
- Slides were washed for 5 minutes in PBS, 3 times and then dipped in PBS/ 1% BSA/10%NMS.
- Sections were incubated for 2 hours in an humidified container at 4°C with 4.8µl Biotin conjugated rabbit-anti-rat antibody (Dako, Carpentaria, CA) in 1496µl PBS/1% BSA/10%NMS (1:300).
- Slides were washed for 5 minutes in PBS, 3 times and then dipped in PBS/ 1% BSA/ 10%NMS.

- Sections were incubated for 40 minutes in an humidified container at 4°C with 3.3µl avidin-horseradish peroxidase enzyme reagent (Dako, Carpentaria, CA) in 1497µl PBS/1% BSA/10%NMS (1:400).
- Slides were washed for 5 minutes in PBS, 3 times and then incubated with DAB (1 tablet Sigma-Fast DAB + tablet Urea Hydrogen Peroxide + 5mls H_2O. Vortex until dissolved.)
- Filter DAB solution through a 0.45µm filter. Best results are obtained if used within one hour.
- Slides were incubated with DAB solution for 10 minutes on a tray lined with foil and absorbent paper.
- DAB was tapped off onto hypochlorite solution (100mls NaH_2O_2 + 100mls H_2O) soaked towel and slides rinsed in post-DAB PBS wash container.
- Slides were counterstained as described in 7.1.3.1 except the 30 second Eosin-Y step was omitted.

PRODUCTION OF ANTI-IA AND F4/80 MONOCLONAL ANTIBODIES FROM HYBRIDOMA CELL LINES

Thawing

- Place the cell aliquot in a 37°C water bath until just thawed. Rinse the ampoule with alcohol and transfer to a 10ml falcon tube.
- Add 1ml of RPMI-10%FCS drop wise with swirling then stand for 10 minutes.
- Add another 2mls in a similar fashion and allow to stand for 5 minutes.
- Centrifuge at 200G for 5 minutes, remove supernatant and then resuspend in ml RPMI-10% FCS.
- Make up to 10mls with RPMI-10%FCS and repeat spin.
- Resuspend pellet in 1ml RPMI-10%FCS and then add to 30mls RPMI-10%FCS in a culture flask. Incubate at 37°C, 5% CO_2 with a loose lid.

Growing

- After 4 days cells should be confluent. These hybridoma cells are free floating and do not adhere to the surface of the flask.
- Split the cells by adding 30mls RPMI-10% FCS and dislodge them with a few 'gentle' taps of the side of the flask.

- Remove 30mls containing dislodged cells and seed a new flask. Culture a further 4 days to confluence.

Collecting Ab and Freezing Cells

- Make 12mls freezing mix.

  60% - 7.2mls RPMI-10%
  20% - 2.4mls FCS
  20% - 2.4mls DMSO.

- Dislodge cells from the flask by a few 'gentle' taps of the side of the flask and collect all the media within the flask.
- Spin collected media at 200G.
- Collect and freeze (-20°C) the supernatant that contains the antibody.
- Resuspend the cell pellet in 6mls of RPMI-10%FCS and make up to 12mls with freezing media.
- Place 1ml aliquot's in cryotubes, place in –70°C freezer overnight and then transfer to liquid nitrogen straws.

T CELL PROLIFERATION ASSAY METHOD

Cell Collection

- Place four spleens in 8-10ml of HBSS.
- Tease the spleens apart with forceps/scissors, homogenise and pass through a 70μm cell strainer.
- Spin suspension at 200G for 10 minutes.
- Resuspend and lyse RBC by flash lysis. To cells add 0.9ml MQ, swirl and follow quickly with 100μl of 10x saline.
- Incubate at RT with Ia and F4/80 antigens for 30 minutes.
- Wash and then culture at 37°C for 1 hour in RPMI with (10% FCS) complement enriched guinea pig serum.
- Non-adherent cells are collected, washed in RPMI/FCS and then passed through cell strainer. Cells passing through are collected and washed for PHA assay. Count and then resuspend to $1x10^6$/ml.

T Cell Proliferation Assay

- Add 10^4 spleen cells (in 10μl) per well in 130μl conditioned media or plain media.

- Culture with 10μg/ml PHA (total 1.5μg) per well added in 10μl. Therefore make 150μg/ml and add 10μl per well.
- Culture for 48 hours at 37°C, 5% CO_2.
- Pulse with 1μCi/ml and culture for 24 hours.
- Harvest.

RNA QUANTIFICATION WITH A MOLECULAR PROBES RIBOGREEN® RNA QUANTITATION KIT

Measurements of ovarian samples were carried out using a modified low-range assay able to detect detecting concentrations from 0-100ng/ml. For peritoneal and ovarian samples beyond the range of the low curve a modified high curve was used. Peritoneal samples were prepared by dilution of 1:100 in TE assay buffer and ovarian samples were assayed without dilution. For each sample prepared 2μl was diluted in 98μl TE water and then 100μl of Ribogreen reagent added immediately before measurement. Fluorescence was measured using a BMG microplate reader with Fluorostar software to generate curves and calculate sample values. The stock ribosomal RNA standard provided is 100μg/ml and the Ribogreen quantitation reagent is diluted for use in the TE assay buffer provided at 1:2000 for the low curve and 1:200 for the higher curve. For the lower curve rRNA stock was diluted 1:2 to 10μg/ml and then 20μl diluted rRNA was added to 980μl TE water and the curve prepared as in Table A1-1. For the higher curve 14.4μl rRNA stock added to 885.6μl TE water and the curve prepared as in Table A1-2.

Volume of TE buffer (µl)	Volume RNA stock (200ng /ml) (µl)	Volume of Ribogreen (µl)	RNA concentration (ng/ml)
0	100	100	100
25	75	100	75
50	50	100	50
75	25	100	25
87.5	12.5	100	12.5
93.7	6.3	100	6.3
96.9	3.1	100	3.1
98.4	1.6	100	1.6
100	0	100	blank

Table A1-1 Details Of The Set-Up Of The Low mRNA Standard Curve.

Volume of TE buffer (µl)	Volume RNA stock (1.6mg/ml) (µl)	Volume of Ribogreen (µl)	RNA concentration (ng/ml)
0	100	100	800
50	50	100	400
75	25	100	200
87.5	12.5	100	100
93.7	6.3	100	50
96.9	3.1	100	25
98.4	1.6	100	12.5
99.2	0.8	100	6.25
100	0	100	blank

Table A1-2 Details Of The Set-Up Of The High mRNA Standard Curve.

REVERSE TRANSCRIPTION METHOD

- For a 20µl reaction volume add the following to an RNAse free tube:

1µl	random primers (250ng/µl)
?µl	volume containing 40ng RNA sample
1µl	10mM dNTP mix (10mM dATP,aGTP,dTTP and dCTP, pH 7.4)
12- (?+2) µl	water (to make up to 12µl)

- Heat to 65°C for 5 minutes then quick chill on ice.
- Quick spin to collect contents then add

4µl	5X first strand buffer
2µl	0.1 M DTT
1µl	RNAseOUT (40U/ml)

- Mix gently and incubate at 25°C for 10 minutes.
- Incubate at 42°C for 2 minutes. Chill on ice.
- Add 1µl (200IU) of superscript pipette up and down.
- Incubate for 50 minutes at 42°C.
- Inactivate by incubation at 70°C for 15 minutes and then quick chill on ice.

METHOD OF EXTRACTION OF CDNA FOR SEQUENCING

All primers were designed using the Primer Express program and mRNA sequences were downloaded from the NCBI Entrez Nucleotide website. It was confirmed that the primers produced only one product and this product was then sequenced at the NATA accredited Flinders University DNA Sequencing Core Facility prior to the use of designed primers in real-time PCR.

PCR reaction

For each primer pair two PCR reactions of 50µl were carried out.

Primer 1	2µl
Primer 2	2µl
10x PCR buffer	5µl
10mM dNTPs	2µl
Hot star Taq	0.2µl
CDNA	2µl
PCR water	36.8µl

Samples were placed in a minicycler and subject to

15 minutes 95°C
1 minute 95°C
1 minute 60°C
1 minute 72°C
6 minutes 72°C
} Cycled 39 times

PCR products from each reaction were combined, mixed with loading buffer (20µl) and run on a 1.5% agarose gel (1.3 mg agarose, 75ml 0.5 TBE, 3µl Ethidium bromide - Large wells for the 100µl samples were created by taping together adjacent lane prongs) immersed in 0.5X Tris Borate EDTA at 80mV for 40 minutes.

cDNA extraction

A Qiagen QIAquick Gel Extraction Kit (using a microcentrifuge) was used to extract the cDNA from the agarose gel as described in the handbook.

- Cut the Band from the agarose gel with a clean scalpel. Trim off excess gel.
- Place slice into a pre-weighed tube and add three volumes of buffer QG to 1 volume of gel.
- Incubate at 50°C for 10 minutes, with regular mixing, until the gel has dissolved.
- Add one gel volume of isopropanol and mix. Add sample to a Qiagen spin column over a 2 ml collection tube. Centrifuge for 1 minute.
- Add 0.5ml of buffer QG to column, stand for 5 minutes and then centrifuge for 1 minute.
- Place column over a 1.5ml microcentrifuge tube.
- Add 30µl of elution buffer directly to the membrane, allow to stand for 1 minute and then spin for 1 minute.

The sequencing facility requires 0.05 pmol/µl for the sequencing reaction and thus it is necessary to determine the total amount of cDNA product. This was done by running 1µl and 2µl samples of purified cDNA on a 2% agarose gel with a low DNA mass ladder (Life Technologies) (80mV, 45 minutes) to get an estimate for ng of DNA. Based on the known product length (bp) the correct concentration was calculated:

- To calculate the ng/ µl required

  0.05 pmole/µl = KB x 32.45 (KB is length in kilobases)

- To work out dilution of cDNA product

  (ng/DNA product) ÷ (KB x 32.45)

QUANTITATIVE REAL TIME PCR CALCULATIONS

Unknown mRNA levels were calculated using the real-time PCR CT values as follows: Each sample was normalised by subtracting the CT value for the house-keeper gene (HPRT) for that sample from the CT value of the gene of interest (ΔCt). All the values from the reference group, designated the arbitrary value of 1 (day 0am Ia), were averaged (value *a*). This value was then subtracted from the normalised unknown samples (ΔΔCt) and since the PCR reaction is an exponential, levels calculated as $2^{\Delta\Delta Ct}$. These resulting values were averaged, with those for the reference group (*a1*) being very close to 1. The value *a* is altered until the value *a1* is equal to 1. The rest of the results are then averaged and the resultant values represent a of fold change in mRNA levels relative to the mRNA levels present in the group designated as 1.

METHODS FOR THE IMMUNOASSAY KITS (R&D SYSTEMS)

- Reagents, standards and controls for each assay were prepared as indicated in Table A1-3.
- Microplates were removed from the foil pouches.
- 50µl of the appropriate assay diluent was added to each well.
- 50µl of sample, control or standard, was added in duplicate to the appropriate wells.
- The plate was mixed by gentle tapping, and sealed with the adhesive strip provided.
- Samples were incubated in the plates for the following times:

  IL-1β and IL-10, 15 hours at 4°C,
  TNFα, 2 hours at RT.

- Wells were aspirated and washed with wash buffer using a squirt bottle.
- The wash was repeated three times, ensuring complete removal of wash buffer from the wells after each wash.
- Plates were blotted on clean, absorbent paper towels.
- 100µl of the appropriate conjugate was added to each well.

- The plate was then mixed by gentle tapping, sealed with an adhesive strip, and incubated for 2 hours at RT.
- Wells were aspirated and washed as above.
- 100µl of substrate solution was added to each well and incubated for 30 minutes protected from light in a box wrapped in foil.
- 100µl of stop solution was added to each well and the plate was mixed by tapping.
- The optical density of each well was measured within 30 minutes at both 450nm and 570nm using a microplate reader and Microplate Manager Software from Bio-Rad Laboratories, Inc.
- The readings at 570nm were subtracted from the readings at 450nm to correct for optical imperfections in the plate.
- The duplicate readings were averaged and the average OD of the zero samples subtracted from each value.
- A curve of mean absorbance was constructed and values for each sample were calculated from this curve.

	IL-1β	TNFα	IL-10
Control	Reconsituted in 1ml deionized water		
Conjugate	0.5ml added to 11ml conjugate diluent		
Wash buffer	25ml wash buffer concentrate to 625ml demonized water		
Substrate	5ml each of colour reagents A and B mixed and protected from light, used within 15 minutes.		
Assay diluent	RD1-14	RD1W	RD1-14
Stock Standard	IL-1β standard reconstituted in 5ml calibrator diluent RD5T	TNFα standard reconstituted in 2ml calibrator diluent RD5Z	IL-10 standard reconstituted in 5ml calibrator diluent RD5T
Standard curve	200µl standard serially diluted in calibrator diluent RD5T (500pg/ml – 3.9pg/ml)	200µl standard serially diluted in calibrator diluent RD5Z(1500pg/ml-23.4pg/ml)	200µl standard serially diluted in calibrator diluentRD5T (1000pg/ml- 7.8pg/ml)
Zero Standard	diluent RD5T	diluent RD5Z	diluent RD5T

Table A1-3 Details Of Reagent Preparation For The Cytokine Immunoassays.

METHOD FOR THE MEASUREMENT TOTAL NITRIC OXIDE

(R&D Systems)

Reaction buffer was made by diluting 30ml of the reaction buffer concentrate with 270ml of deionized water.

NADH reagent was made by reconstituting one vial provided with 1ml deionized water. Mix gently for 3 minutes, keep on ice. Immediately prior to use 900µl NADH reagent was diluted with 1.8ml of deionized water and kept on ice.

Nitrate reductase was reconstituted with 1ml of the provided nitrate reductase storage buffer, briefly vortexed and then left on the bench for 15 minutes. The vortex was repeated and reductase left again on the bench for 15 minutes. This was kept on ice for the duration of the assay. Immediately before use, 360µl nitrate reductase was mixed with 1440µl of reaction buffer, placed on ice and used within 15 minutes.

Samples were filtered through a 10,000 MW Amicon MicroconÒ centrifuge filter unit (YM-10, Millipore Australia, Sydney, Australia) and analysed as recommended by the manufacturer.

The highest standard was made from the 1000µmol/L standard; 100µl into 900µl reaction buffer. Subsequent standards were made by serial dilution in reaction buffer (500µl: 500µl) to give a curve ranging from 100µmol/L to 3.12µmol/L.

- 200µl of reaction buffer was added to the blank wells.
- 50µl of reaction buffer was added to the zero standard wells. Standards or samples were added to the remaining wells in duplicate.
- 25µl of NADH was added to all standards and samples.
- 25µl of nitrate reductase was added to all standards and samples.
- The plate was mixed by tapping, covered with the adhesive strip provided and incubated for 30 minutes at 37°C.

- 50µl of Griess reagent I was added to all wells except the blanks, followed by 50µl of Griess reagent II to all wells except the blanks.
- The plate was mixed by tapping and then incubated at RT for 10 minutes.
- The optical density of each well was measured at 540nm.
- Duplicate results were averaged, the blank average subtracted from each well and the mean absorbance plotted against the total nitrite concentration.
- Values for each sample were calculated from this curve.

APPENDIX 2 - OVARIAN MACROPHAGE ISOLATION

Numerous attempts were made to isolate ovarian macrophages using two immunoaffinity methods; Minimacs columns (Miltenyi Biotec, Bergisch Gladbach, Germany) and Dyna beads (Dynal, Oslow, Norway). In each case cell numbers were extremely low and contamination with ovarian debris was evident, even when peritoneal cells could be adequately isolated, the ovarian cell populations isolated in this manner were not suitable for our further studies. In our hands the antibody panning technique proved to be most suitable for our studies. Below are the protocols that were attempted:

MINIMACS

- Ovaries were digested as described in the main body of the thesis (3.3.1).
- Cells were incubated at 4°C for one hour with 500µl of either F4/80 or anti-Ia hybridoma supernatant.
- Cells were diluted in 10mls HBSS/EDTA/Az and then spun over 3mls FCS.
- Cells were resuspended in degassed HBSS/Gent 100µl and live cells counted.
- Cells were cultured for 20 minutes at 4°C with Minimacs goat anti-rat beads (20% or 20µl, and 10% or 10µl).
- Labelled cells were added to a Minimacs separation column on magnet 500µl at a time, with washing through with an extra 1ml degassed HBSS/Gent.
- Separation column was then removed from the magnet and negative cells washed through with 1ml of degassed HBSS/Gent.
- Cells were then washed in HBSS/EDTA/Az, resuspended in 1ml and then incubated for 15 minutes with anti-rat FITC (1% or 10µl).
- The antibody was washed off and the cells resuspended in 100µl of HBSS/EDTA/Az. 10ml of each of the positive and negative fractions were then examined under the microscope.

DYNA BEADS

- Ovaries were digested as described in the main body of the thesis (3.3.1).

- Cells were incubated at 4°C for one hour with 500ml of either F4/80 or anti-Ia hybridoma supernatant.
- Macrophages comprise 10% ($1x10^5$ cells) or less of ovarian digest (average $1x10^6$ cells), the equivalent to 16 beads per macrophage (ratio recommended by the manufacturer) is therefore, $1.6x10^6$ beads or 4µl beads). 8µl ($3.2x10^6$ beads) of anti-rat Dynabeads were washed twice with 500ml PBS by resuspending beads in saline, placing them on the magnet and removing saline with a pipette, then repeating. Beads were also washed once in alpha MEM. Finally, beads were resuspended in 100µl of alpha MEM and placed on ice.
- Cells were resuspended in 350µl of alpha MEM/FCS and then incubated on ice, shaking with 50µl (total of $1.6x10^6$ beads or $4x10^6$ beads/ml) of Dynabeads for 50 minutes.
- Cell preparations were placed on the magnet, beads and bead-bound cells were allowed to migrate to the magnet and the supernatant removed.
- Beads and bead-bound cells were then resuspended and applied to the magnet again.
- Cells were then washed in HBSS/EDTA/Az, resuspended in 1ml and then incubated for 15 minutes with anti-rat FITC (1% or 10µl).
- The antibody was washed off and cells resuspended in 100µl of HBSS/EDTA/Az. 10ml of each of the positive and negative fractions were then examined under the microscope.

APPENDIX 3 – MESSENGER RNA EXTRACTION METHODS

A complete set of ovarian and peritoneal macrophage samples, consisting of 53 samples – three repeats of isolation of Ia^+ and $F4/80^+$ cells from groups of 8 animals at 4 time points across the stimulated reproductive cycle, was collected and the RNA isolated using the Qiagen RNeasy Mini Kit method recommended for isolation of RNA from very small amounts of starting material.

QIAGEN RNEASY® MINI KIT RNA ISOLATION METHOD

- Briefly lysate buffer from the kit was added directly to the isolated cells on the panning plate.and then collected and frozen at –80°C for later RNA isolation.
- Thawed lysate was mixed with 70% ethanol and added to a spin column.
- The spin column was washed with 700µl RW1 buffer and spun for 15 seconds at 10,000RPM.
- The spin column was treated with 80µl DNase solution (10µl DNAse stock: 70µl RDD buffer) for 15 minutes and washed with 350µl RW1 buffer and spun for 15 seconds at 10,000 rpm.
- The spin column was further washed with 500µl RPE buffer and spun for 15 seconds at 10,000 rpm, this wash was then repeated.
- The spin column was incubated for 20 minutes with RNAse free water and then centrifuged 1 minute 10,000 to collect RNA.

Levels of RNA were measured in these samples using a Ribogreen Quantitation Reagent Kit. Levels were found to be extremely low and variable (Table A2-1) and it was decided to increase the numbers of animals in each group to 10, abandon the day-1 group, and try the TriReagent® method of isolation instead. Some direct comparisons between the Qiagen and TriReagent® isolation methods were done. Peritoneal cells were collected washed and counted. RNA was isolated using either the Qiagen kit method or the TriReagent® method alone or TriReagent® spiked with glycogen from cells designated as high (80 000 cells) or low (20 000cells) numbers .

	mRNA OBTAINED FROM ISOLATED OVARIAN MACROPHAGE CELL TYPES			
STAGE OF STIMULATED CYCLE	Ia positive Cells		F4/80 positive Cells	
	Mean (mg/ml)	SE	Mean (mg/ml)	SE
Day-1	0.57	0.3	0.2	0.2
Day 0am	0.17	0.1	0.6	0.5
Day 0pm	1.28	0.6	0.56	0.3
Day1	2.34	0.4	1.7	0.7
Day2	10.4	3.2	9.8	4

Table A2-1 The Amount Of RNA Measured In Ovarian Macrophages Samples Following RNA Isolation Using The Qiagen Rneasy® Mini Kit RNA Isolation Method.

TRIREAGENT® (TR) RNA ISOLATION METHOD

RNA Extraction

- Samples were thawed on ice and spun at 14,000 rpm, 4°C for 10 minutes.
- Supernatant was transferred to a fresh tube and the sample left on ice for 5 minutes.
- 250µl of chloroform/500µl of TR (fume hood) was added, vortexed briefly and left on ice for 15 minutes.
- Samples were spun at 14,000 rpm, 4°C for 10 minutes and the upper (aqueous) layer transferred to a fresh tube.
- 1µl of glycogen & 25µl isopropanol (per 500µl TR) was added, mixed and the samples were left on ice for 5 minutes.
- Samples were spun at 14,000 rpm, 4°C for 10 minutes and the supernatant transferred to a fresh 0.6ml tube with 225µl isopropanol (/500µl TR).
- RNA was precipitated overnight at -80°C.

DNase Treatment

- RNA was pelleted by centrifuging for 30 minutes at 14,000rpm, 4°C.
- The supernatant was discarded and the pellet washed with 500µl of ice cold 75% ethanol.
- Samples were spun at 14,000 rpm, 4°C for 10 minutes and the supernatant discarded
- The RNA pellet was dried by inverting the tube on the bench. (~15-20 minutes)
- After drying the pellet was resuspended in 79.5µl of PCR grade water, vortexed briefly and then allowed to dissolve at room temperature (~20 minutes).

DNase solution (make up on ice)	per tube
10x DNase Buffer	10µl
RNAsin (40U/µl)	2.5µl
DNase 1 (1U/µl)	5µl

- 17.5µl of DNase solution was added to RNA sample on ice, follwed by a vortexing.
- Samples were incubated at 37°C for 90 minutes.
- 100µl of room temperature phenol (water saturated) & 100µl of chloroform: isoamyl alcohol (24:1) was added and samples vortexed.
- Samples were spun at 14,000 rpm, 4°C for 10 minutes.
- Supernatant was transferred to a fresh tube
- RNA was re-extracted by adding 100µl of chloroform: isoamyl alcohol, vortexing and then spinning at 14,000 rpm, 4°C for 10 minutes.
- Supernatant was transferred to a fresh tube RNA precipitated O/N at -80°C with 10µl 2M NaOAc (0.1X vol of aqueous phase) & 250µl of ice cold 99.7-100% ethanol (2.5x vol of aqueous phase).

RNA Recovery

- The RNA was pelleted by centrifuging for 30 minutes at 14,000 rpm, 4°C.
- The supernatant was discarded and the pellet washed with 500µl of ice cold 75% ethanol.
- Samples were spun at 14,000 rpm, 4°C for 10 minutes and the supernatant discarded.
- The RNA pellet was then dried by inverting tube on bench. (~15-20 minutes)
- Resuspend pellet in 20µl of PCR grade water and leave for 30 minutes to dissolve.

The RNA levels were measured in all samples using the Ribogreen assay. For both the high and low cell numbers cases the spiked Trireagent® method gave the greatest yield of mRNA (Table A2.2). The Qiagen kit was better than Trireagent® alone at high cell numbers but both

were equivalent at low cell numbers. Samples in Chapter 4 were collected from across the cycle for a second time with increased numbers of animals in each group and using the spiked Trireagent® method.

	RNA ISOLATION METHOD		
CELL NUMBERS	Qiagen	Tri Reagent	Tri Reagent + glycogen
2 x104 (low)	2.9 ± 0.4	2.5 ± 0.3	6.2 ± 0.7
5 x104 (high)	14.9 ± 1.1	10.1 ± 1.1	19.1 ± 0.9

Table A2-2 The Amount Of RNA Isolated From Known Numbers Of Cells Using Various Methods. Values are mg/ml ± SE.

APPENDIX 4 – NON-SPECIFIC ESTERASE STAINING METHODS

To confirm the purity of the isolated macrophage population experiments to stain macrophages bound to the panning plates for non-specific esterase were carried out. Staining was attempted by two different methods. Unfortunately neither method was successful on either the peritoneal or ovarian cells present on the panning plates.

METHOD FROM YAM ETAL (19)

Fixing Cells

- Cells can be stored unfixed at room temp for at least two weeks without affecting the enzyme activity.
- Fix cells in the following solution, adjusted to pH 6.6.

  20mg Na_2HPO_4
  100mg KH_2PO_4
  30ml H_2O
  45ml acetone
  25ml Formalin (37%)
- Fix for 30 seconds only at 4°C, wash in three changes of water and air-dry for 10-30 minutes before staining.

- Make up the following solutions:

  M15 phosphate buffer (stock solution)
  1 4.73g Na_2HPO_4 in 500mls RO water.
  2 4.54g KH_2PO_4 in 500mls RO water.
  Add 21.7mls of 2 to 78.3mls of 1. Adjust to pH 7.4 with phosphates. Na_2HPO_4 will increase pH, KH_2PO_4 will decrease pH. Filter and store at 4°C.

  α-Naphthyl acetate (stock solution)
  Mix 20mg of α-naphthylacetate per 1ml ethylene glycol monomethyl ether.

Hexazoitzed pararosanilin

Mix equal volumes of pararosanilin-HCl solution and 4% sodium nitrite (0.4g/10mls) solution for 1 minute immediately before use.

Staining Mixture and Procedure

- Mix 1.8mls of hexatozoitzed pararosaniline with 1.5ml of α-naphthyl acetate and 26.7mls M/15 buffer.
- pH to 6.1 with NaOH and filter before use.
- Incubate slides/cells with this mixture at room temperature for 45 minutes.
- Wash with RO water.
- Counter stain with 1% methyl green for 1-2 minutes.
- Wash and allow to dry.
- Mount in permount and examine. Activity is seen as dark green granules in the cytoplasm.

SIGMA NSE STAINING KIT

Ensure glassware is cleaned of all detergent residues by washing coplin jars and other glassware with dilute bleach and then wash thoroughly with deionised water as any residue can affect enzyme activities. Ensure all reagents are warm as weak reactions may result if the correct temperature is not achieved.

- Fix slides for 1 minute in freshly made Citrate-acetone-methanol fixative at room temperature.

Citrate-Acetone-Methanol Fixative

18mls of citrate dilute solution (1 part citrate concentrate with 9 parts water. pH to 5.4.)
27mls ACS grade acetone
5ml methanol.
Discard after 8 hours.

- Wash in deionised water and air dry for at least 20 minutes.
- To 50ml of warm (37°C) Trizmal dilute buffer solution add (with constant stirring) 1 capsule of Fast blue RR salt.

Trizmal Buffer

1 part Trizmal 7.6ml concentrate to 9 parts deionised water pH to 7.6

- When the capsule is completely dissolved add 2ml of fresh α-naphthyl acetate solution.

  α-Naphthyl Acetate Solution

  Dissolve one capsule of α-naphthyl acetate in 2mls of ethylene glycol monomethyl ether. Use immediately.

- Stir this yellow turbid solution for 30 seconds but do not filter.
- Place specimens in staining solution and incubate at 37°C for 30 minutes and protect from exposure to light with foil.
- Remove slide from stain and rinse in deionzed water for 3 minutes.
- Counter stain with Mayers Haematoxylin for 5-10 minutes and rinse in tap water.
- Air dry and examine for positive stain. Use only aqueous mounting media if a cover slip is required. Sites of activity appear as black granules.

APPENDIX 5- VIABILITY OF CULTURED MACROPHAGES

At the onset of this project it was anticipated that granulosa and thecal cells would be cultured with macrophage conditioned media to determine the influence of ovarian macrophages on the ovarian cell types. Initial experiments revealed that the macrophage culture media itself (RPMI/10%FCS/100U/ml Penecillin/100g/ml streptomycin/2mMGlutamine/0.25M β–Mercaptoethanol) was influencing granulosa cell proliferation, FCS was identified as inhibiting granulosa cell proliferation (Figure A4.1), and it was considered desirable to remove this from the culture media.

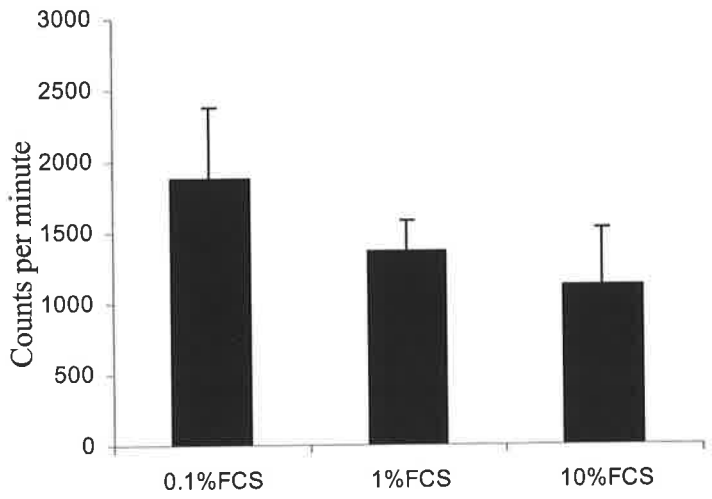


Figure A5-1 Proliferation of Cultured Granulosa Cells.
Proliferation {as counts per minute) of granulosa cells stimulated by cultured media with varying concentrations of fetal calf serum (FCS).

Experiments were undertaken to select a media that would give a comparable number of viable macrophages without the addition of FCS. M199 media was found to be a suitable substitute (Table A4-1).

	Media Type used		
Isolated cell type	RPMI+FCS (n=2)	RPMI –no FCS (n=4)	M199 (n=3)
Ia+	87 ± 2.8	42.8 ± 8.7	82 ± 6.6
F4/80+	84 ± 7.8	38 ± 7.9	80 ± 3.4

Table A5-1 Percentage of Viable cells in Isolated Ovarian Macrophage Populations. The isolated cells were cultured in the indicated media overnight and the percentage of viable cells assessed by staining with Hoechsts and propidium iodode.