Actions of seminal fluid signalling factors in the female reproductive tract and on pregnancy outcome

Danielle Jannette Glynn

Research Centre for Reproductive Health,
Discipline of Obstetrics and Gynaecology,
School of Paediatrics and Reproductive Health,
University of Adelaide, Adelaide,
Australia

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We used to think that if we knew one, we knew two, because one and one are two. We are finding that we must learn a great deal more about "and."

~Arthur Stanley Eddington

Some people walk in the rain, others just get wet.

~Roger Miller

The important thing is not to stop questioning. Curiosity has its own reason for existing. One cannot help but be in awe when he contemplates the mysteries of eternity, of life, of the marvellous structure of reality.

~Albert Einstein

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Abstract

The cytokine environment of early pregnancy is known to be a key determinant of the development of the pre-implantation embryo, and its subsequent implantation and growth. Factors in male seminal fluid have been identified as regulators of the expression of cytokines in the female tract of mice, humans and other mammalian species, with insemination eliciting a cascade of molecular and cellular events, reminiscent of a classic inflammatory response. In humans, perturbations in seminal fluid signalling have been proposed to predispose to pathologies of pregnancy including implantation failure, recurrent miscarriage and pre-eclampsia. Seminal transforming growth factor-beta (TGFβ) is identified as one key molecule present in seminal fluid responsible for inducing the female post-mating cytokine response in mice. Research in humans however, has shown the seminal TGFβ content of fertile versus infertile couples to be similar, while the content of other known seminal constituents such as interferon-gamma (IFNγ), correlate with reproductive success. This project aimed to investigate the nature of active factors present in seminal fluid in mice, and their interactions in regulating the uterine cytokine environment during early pregnancy, utilising a variety of in vitro and in vivo experimental strategies. Further, the effect of perturbation in the peri-conception cytokine environment on short and long term pregnancy and postnatal outcomes was investigated.

Evaluation of uterine fluids from estrous and mated mice showed a marked upregulation of a number of cytokines following mating, including granulocyte macrophage colony stimulating factor (GM-CSF), interleukin-6 (IL-6) and the chemokine KC (rodent IL-8 homologue). Increased production of factors such as GM-CSF and subsequent generation of a receptive uterine environment is thought to be crucial for optimal embryo development and placentation. It has previously been shown that seminal factors such as TGFβ contribute to the uterine post-mating inflammatory response, however other moieties present in seminal fluid, for instance cytokines induced in response to infection such as IFNγ or products from the mucosal microflora, may also play a regulatory role. Using uterine epithelial cells cultured in vitro, it was shown that a variety of immune modulators including the cytokines TGFβ and IFNγ, as well as bacterial products, gram negative lipopolysaccharide (LPS) and gram positive lipoteichoic acid (LTA), can alter basal cytokine production. IFNγ, a pro-inflammatory cytokine secreted by activated natural killer cells and T-cells, is known to interfere with TGFβ signalling in other contexts. Independently TGFβ, LPS and LTA stimulate GM-CSF production while differentially regulating IL-6 and KC production. Conversely IFNγ inhibits GM-CSF production, without effecting IL-6

or KC. Pair wise combinations of TGFβ, LPS and LTA resulted in additive stimulation of GM-CSF, while addition of IFNγ to cultures in conjunction with any of these molecules downregulated GM-CSF and KC stimulation. These in vitro studies indicate factor-specific interactions between seminal fluid constituents and highlight the complex nature of seminal fluid signalling. Consequently we propose that the relative ratio of seminal signalling factors is likely to be more important than the absolute concentration of various regulators, in determining the optimal female reproductive tract response.

Using the mouse as an in vivo model, I have in addition demonstrated that LPS and LTA instilled into an estrous uterus can elicit cytokine production comparable to that observed following insemination. Further, these studies have shown that IFNγ instilled into the uterus of a recently mated mouse can reduce the post-copulatory GM-CSF and KC surge. However administration of IFNγ had no effect on near term pregnancy outcomes including fetal or placental weights, fetal crown-rump length, or implantation or resorption rates. The 'developmental origins of adult disease hypothesis' proposes the idea that the early uterine environment encountered by the conceptus contributes toward the risk of metabolic disorders in adulthood, hence a long term study of progeny conceived after IFNγ administration was also undertaken. Neo-natal outcomes, such as birth weight, litter size and gestation length were unaltered, as was growth trajectory to 22 weeks of age. Adult metabolic markers, glucose tolerance, organ weight, muscle weight, adiposity and systolic blood pressure were not affected by the perturbation of peri-conceptual cytokine parameters.

This work has examined the potential regulatory role of a number of seminal fluid signalling agents in directing the post-mating cytokine response, and has furthermore shown the relatively resilient nature of the early cytokine environment to subtle perturbation. Delineating the identity and roles of seminal fluid factors in early pregnancy brings us closer to an understanding of the key physiological events of early pregnancy and assists in identifying potential risk factors for human pregnancy pathologies.

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 in the text.

I further grant my consent to the University of Adelaide to make this thesis available for loan and photocopying once accepted for the degree.

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Publications arising from these and related studies

1. SA Robertson, JJ Bromfield, **DJ Glynn**, DJ Sharkey, MJ Jasper.
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2. DJ Glynn and SA Robertson (in preparation)
   Role of LPS and LTA in regulation of the post-mating inflammatory response in mice.

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   Inhibitory effect of IFNγ on seminal fluid signalling in mice.

4. DJ Glynn and SA Robertson (in preparation)
   Perturbation of early cytokine environment influences fetal programming in mice.

Patent

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- DJ Glynn and SA Robertson
  "SEMINAL FACTORS AND UTERINE EPITHELIAL RESPONSIVENESS TO TGFβ"
  Australian Society for Medical Research (South Australian Division) Annual Meeting.

- DJ Glynn and SA Robertson
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  "DANGEROUS MALE PARTNERS"
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  36th Annual Conference of the Society for Reproductive Biology, Perth, Australia. (Abs. 287)

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Abbreviations

A	Adenine
Ab	Antibody
BMP	Bone morphogenic protein
Bp	Base pairs
BSA	Bovine serum albumin
C	Cytosine
cAMP	Cyclic adenosine monophosphate
cDNA	Complimentary DNA
Ct	Cycle threshold
DAB	Diaminobenzidine tetrachloride
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
DPBS	Dulbecco's PBS
DTH	Delayed-type hypersensitivity
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacteic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbancy assay
FCS	Fetal calf serum
FSH	Follicle stimulating hormone
G	Guanine
GM-CSF	Granulocyte-macrophage colony-stimulating factor
hCG	Human chorionic gonadotrophin
HLA	Human leukocyte antigen
HRP	Horse radish peroxidase

ICSI	Intra-cytoplasmic sperm injection
IFN	Interferon
IL	Interleukin
IUGR	Intrauterine growth retardation
IVF	In vitro fertilisation
Kb	Kilobase pairs
kDa	Kilo-dalton
LAP	Latency associated protein
LCA	Leukocyte common antigen
LGL	Large granular lymphocytes
LH	Luteinizing hormone
LIF	Leukaemia inhibitory factor
LPS	Lipopolysaccharide
LTBP	Latent transforming growth factor β binding protein
mAb	Monoclonal antibody
MCP	Monocyte chemotactic protein
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MMP	Matrix metalloproteinase
MQ	Milli-Q
mRNA	Messenger RNA
NK	Natural killer
°C	Degrees celsius
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGE	Prostaglandin
PSA	Prostate specific antigen

RNA	Ribonucleic acid
RNAse	Ribonuclease
rpm	Revolutions per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS	Sodium dodecyl sulphate
T	Thymine
TGF	Transforming growth factor
TIMP	Tissue inhibitor of metalloproteinase
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TSP-1	Thrombospondin-1
U	Uracil
v/v	Volume per volume
VIA	Video image analysis
w/v	Weight per volume
WHO	World Health Organisation

Chapter 1

Literature review

1.1 INTRODUCTION

The process of mammalian reproduction begins with insemination which initiates the formation, development, implantation and growth of the embryo in a receptive uterine environment. As well as providing male gametes, insemination elicits a cascade of molecular and cellular events within the female reproductive tract that is reminiscent of a classic inflammatory response. In mice, the female response to insemination induced by the interaction between female tract cells with soluble factors in seminal plasma, and similar changes are now being described in the human cervix [1]. The introduction of Assisted Reproductive Technologies (ART), such as *in vitro* fertilization (IVF), clearly demonstrates natural insemination and hence semen exposure is not necessary to produce a viable pregnancy. However studies in humans and in animal models have shown that the quality and success of pregnancies conceived in such a manner are significantly increased if the female tract is exposed to semen during the peri-conception period [2-11].

Natural insemination exposes the female reproductive tract to active molecules in seminal plasma, which act to elicit secretion of a number of pro-inflammatory cytokines and chemokines. These include granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-6 (IL-6). These cytokines and chemokines then cause an influx of leukocytes into the endometrium [1, 12, 13]. The cytokine response is tightly controlled in both a spatial and temporal sense. It is thought not only to play an integral role in activating the immunological changes that lead to the recognition and generation of tolerance toward paternal antigens introduced at insemination and shared by the conceptus, but also in endometrial remodelling facilitating optimal implantation, placental and fetal development. Recent studies in mice have shown impaired post-natal growth and altered adult metabolic status in progeny conceived in the absence of seminal plasma, hence indicating a role for seminal plasma in establishing an environment allowing achievement of optimal pregnancy and progeny outcomes [14].

Seminal transforming growth factor-beta (TGFβ) has been recognised as a key active signalling molecule in seminal fluid. However, recent research in humans has shown that factors other than TGFβ may be important since the TGFβ content of fertile versus infertile couples is similar, while the content of other seminal regulators such as interferon-gamma (IFNγ) are altered [15]. IFNγ is a pro-inflammatory cytokine known to modulate TGFβ-stimulated responses *in vitro* in a variety of cell

lineages [16-18]. It remains unclear whether this is the case in the female reproductive tract in vivo and what, if any, physiological relevance increased seminal fluid IFNγ holds for reproductive outcome.

This chapter will outline the current understanding of the maternal immune response to insemination with discussion focusing on the identity and potential regulatory role of factors introduced at insemination in optimising pregnancy and fetal outcomes.

1.2 CYTOKINES IN THE UTERUS

1.2.1 Cytokines – introduction

Cytokines are a group of small (generally < 30kDa) glycoproteins secreted by both immune and non-immune cells in response to a variety of stimuli. The principle role of cytokines is to act as intercellular chemical messengers. Due to their short half-lives, this usually occurs in an autocrine or a paracrine manner. Over one hundred cytokines have now been identified and these can broadly be classified into families on the basis of the receptor and signal transduction pathways they utilize, and the effects they elicit in target cells. Cytokines have been identified as being important regulators of fertility, having roles in ovulation, decidualisation, embryo development and implantation, placentation and the onset of labour.

Cytokines are secreted into an extracellular milieu in an antigen non-specific manner; hence the specific nature of cytokine biological activity must be controlled at an alternative level. Indeed, specificity is in part dependent upon tightly regulated cytokine receptor expression on target cells. Typically cytokine receptors are heteromeric complexes, composed of a low affinity cytokine specific α subunit and the β subunit in association with cytoplasmic signal transduction molecules. Cytokine activity is further regulated by the presence of receptor antagonists, decoy receptors and binding proteins. The signals conferred by a cytokine bound to its receptor lead to regulation of activation, differentiation, proliferation and migration of many cell lineages as well as secretion of immune molecules including other cytokines, chemokines and antibodies. This regulatory activity is executed in a variety of forms including synergism, antagonism, redundancy and pleiotrophy. Cytokines acting in concert to evoke an effect greater than the sum of the individual effects are described as having a synergistic relationship and conversely, inhibition of the activity of one cytokine by another is a form of negative regulation, or antagonism. While there is high degree of specificity in cytokine signalling, it is common for a number of cytokines to elicit a similar response in similar environments, illustrating the

phenomenon of redundancy. Redundancy may be a product of common intra-cellular signalling pathways or alternatively via cross-talk between distinct signalling pathways. Contrasting with redundancy is pleiotrophy where a single cytokine is capable of inducing a diverse range of effects.

Numerous human and animal studies have shown the differential expression of a range of cytokines and their receptors in the cycling and early pregnant uterus. Accumulating evidence suggests cytokines play a crucial role not only in facilitating cyclic endometrial remodelling but also in mediating communication between maternal tissues and the embryo. Cytokine and cytokine receptor knockout mice have proved useful tools in examining the functional role of cytokines in early pregnancy, demonstrating the bidirectional signalling between embryo and endometrium to be vital for optimal embryo development and implantation. Emerging data suggests TGFβ, IL-6, GM-CSF and leukaemia inhibitory factor (LIF) to be of particular interest and these will be discussed further.

1.2.2 Transforming growth factor β (TGFβ)

TGFβ, a member of the TGFβ superfamily of cytokines, is involved in extracellular matrix formation, inhibition of cell proliferation, development and differentiation, activation and suppression of macrophages. Members of the TGFβ superfamily share up to 80% amino acid sequence homology and include factors such as activin, the growth and differentiation factors (GDFs), the bone morphogenic proteins (BMPs) and inhibin. There are five known isoforms of TGFβ, three of which are present in mammals, TGFβ_1, β_2 and β_3 [19, 20]. While the three mammalian isoforms of TGFβ are encoded by separate genes on different chromosomes, their sequence and structural homology are extensive, from 64 to 82% [21-24]. Across species there is even greater conservation of homology of up to 91% affording cross species activity [25-27]. This initially lead investigators to believe them to have redundant functions however this was dispelled following identification of isoform specific expression. Additionally, functional analysis and receptor binding affinity studies demonstrated differential binding affinities correlating with potency of function [28, 29]. Furthermore, studies in mice deficient in individual TGFβ isoforms display differential phenotypes suggesting functionally distinct roles of the isoforms at least in development. Deviation from the expected Mendelian ratio at weaning is evidence of embryonic lethality in TGFβ_1 homozygous null mice, while those homozygotes that are born live succumb to a wasting disorder approximately three weeks after birth [30-32]. This phenotype is characterised by massive inflammation and tissue necrosis, particularly in the heart and stomach. TGFβ_1 heterozygotes are unaffected. In contrast to the extreme inflammatory phenotype of TGFβ_1 null mice, TGFβ_2 null mice exhibit a wide range of developmental defects involving the endocrine,

reproductive, cardiovascular, skeletal, sensory, nervous, renal and digestive systems [33-36]. However, TGFβ_2 homozygotes die shortly before or within hours of birth due to the cardiovascular defects [34]. Unlike the TGFβ_1 heterozygotes, which are phenotypically normal, TGFβ_2 heterozygotes exhibit hyperplasia in a number of tissues from the endocrine and reproductive systems [33]. Similar to TGFβ_2 nulls, TGFβ_3 null mice die within hours of birth [37]. The exact cause of the TGFβ_3 neonatal lethality is yet to be described, however newborn pups exhibit gasping and become cyanotic shortly after birth and have been reported to have abnormal airways. In addition to the respiratory defect, TGFβ_3 null mice exhibit a cleft palate phenotype [37].

TGFβ is translated as a multi subunit pre-proprotein complex, composed of a 12.5 kDa protein destined to be the bioactive molecule and a 65 kDa protein known as latency-associated peptide (LAP) [38, 39]. Prior to secretion these subunits are cleaved and non-covalently linked to latent TGFβ binding protein (LTBP), to form high molecular weight latent complexes [40]. TGFβ bioactivity is achieved following secretion and dissociation from the latency components of the complex, and subsequent formation of the 25 kDa homodimer. Exposure to any one of a variety of agents facilitates activation. Including in vivo association with proteases, binding to a growth factor receptor or thrombospondin [40-43], or in vitro by increasing temperature, or by acidic or alkaline pH [40]. Exogenous LAP has been demonstrated to inhibit the biological activity of the three mammalian isoforms of TGFβ in vitro and in vivo. This, together with the finding that endogenous LAP is constitutively present in most tissues indicates a potential regulatory role for LAP [44].

1.2.2.1 TGFβ receptors and signalling

TGFβ homodimers are known to signal via binding to and activation of membrane bound heteromeric receptor complexes predominantly composed of TGFβ type I, type II and type III receptors (TβR-I, TβR-II, TβR-III - betaglycan) (Figure 1.1) [45-52]. Ligand/receptor interactions exhibit differential affinity between TGFβ isoforms and receptors. TGFβ_1 or β_3 bind with high affinity to TβR-II elicit TβR-I recruitment and subsequent activation via trans-phosphorylation by TβR-II, and employment of the serine/threonine kinase activity known to be responsible for TGFβ signal transduction [53]. TGFβ_2, on the other hand has only low affinity for TβR-II and so must rely on the presence of accessory receptors to aid in functional receptor binding, a role thought to be fulfilled by membrane bound betaglycan
[51, 54]. Betaglycan has recently been identified as both a soluble and membrane bound proteoglycan receptor capable of binding TGFβs [51, 52]. It is known to enhance TGFβ/receptor interactions [55]

probably via presentation of bound TGFβ dimers to the signalling receptors particularly in the case of $TGF\beta_2$, but its mode of action is still unknown. However, the soluble form has been shown to antagonise TGFβ signalling by sequestering the ligand away from the signalling receptors [56] and hence may have a regulatory role.

Endoglin shares some sequence homology with betaglycan and is known to bind TGFβ isomers with differing affinity in association with TβR-I and TβR-II [57]. Endoglin is thought to be involved in modulating interaction between TGFβ dimers and the signalling receptors [57], as is the case for betaglycan mentioned above. Another TGFβ receptor, bone morphogenic membrane bound inhibitor (BAMBI), is known to associate with the signalling TGFβ and BMP receptors [58], inhibiting formation of receptor complexes and hence receptor activation and signalling. Thus BAMBI, a pseudo-receptor, has a negative regulatory role in TGFβ family signalling.

TGFβ signalling is predominately affected by the SMAD signalling pathway. Whereby ligand/receptor interaction evokes auto (TβR-II) and trans (TβR-I) phosphorylation, and the subsequent recruitment and phosphorylation of membrane anchored SMADs (SMAD2/3) [59]. Phosphorylation releases SMAD2/3 from membrane anchorage in order to permit association with Co-SMAD, SMAD4 [60, 61]. Together this hetero-oligomeric unit is translocated to the nucleus [45, 47, 48, 50, 62-64] and interacts with other transcriptional regulators to enhance DNA binding in promoter regions, SMAD binding elements (SBE), of TGFβ responsive genes [64-73]. A regulatory negative feedback loop has been identified for the TGFβ signalling pathway. Translocated SMADs bind SBEs in the promoter region of inhibitory SMAD (I-SMAD), SMAD7. SMAD7 is known to block SMAD2/3 phosphorylation hence activation of the signalling pathway [49, 50, 62, 63, 74-76].

1.2.2.2 Modulators of TGFβ signaling

TGFβ signalling is modulated by many factors including tumor necrosis factor-alpha (TNFα), IFNγ and endotoxin with differential effects depending on the cellular AND microenvironmental context. TNFα has been shown to inhibit TGFβ signalling in human embryonic kidney 293, hepatoma HepG2 cells and in dermal fibroblasts [71, 77], while acting synergistically to kill Schwann cells and to upregulate GM-CSF expression in retinal pigment epithelial cells [78, 79]. In vitro cultures of Peyer's Patch lymphocytes co-incubated with TGFβ and endotoxin exhibit synergistic stimulation of IgA production [80], yet Bitzer et al simultaneously demonstrated endotoxin inhibition of TGFβ signalling via activation of SMAD7 gene expression [72]. IFNγ addition to TGFβ stimulated cultures of retinal

pigment epithelial cells or monocytes rapidly downregulates GM-CSF secretion and cell adhesion properties respectively [17, 18, 78, 81].

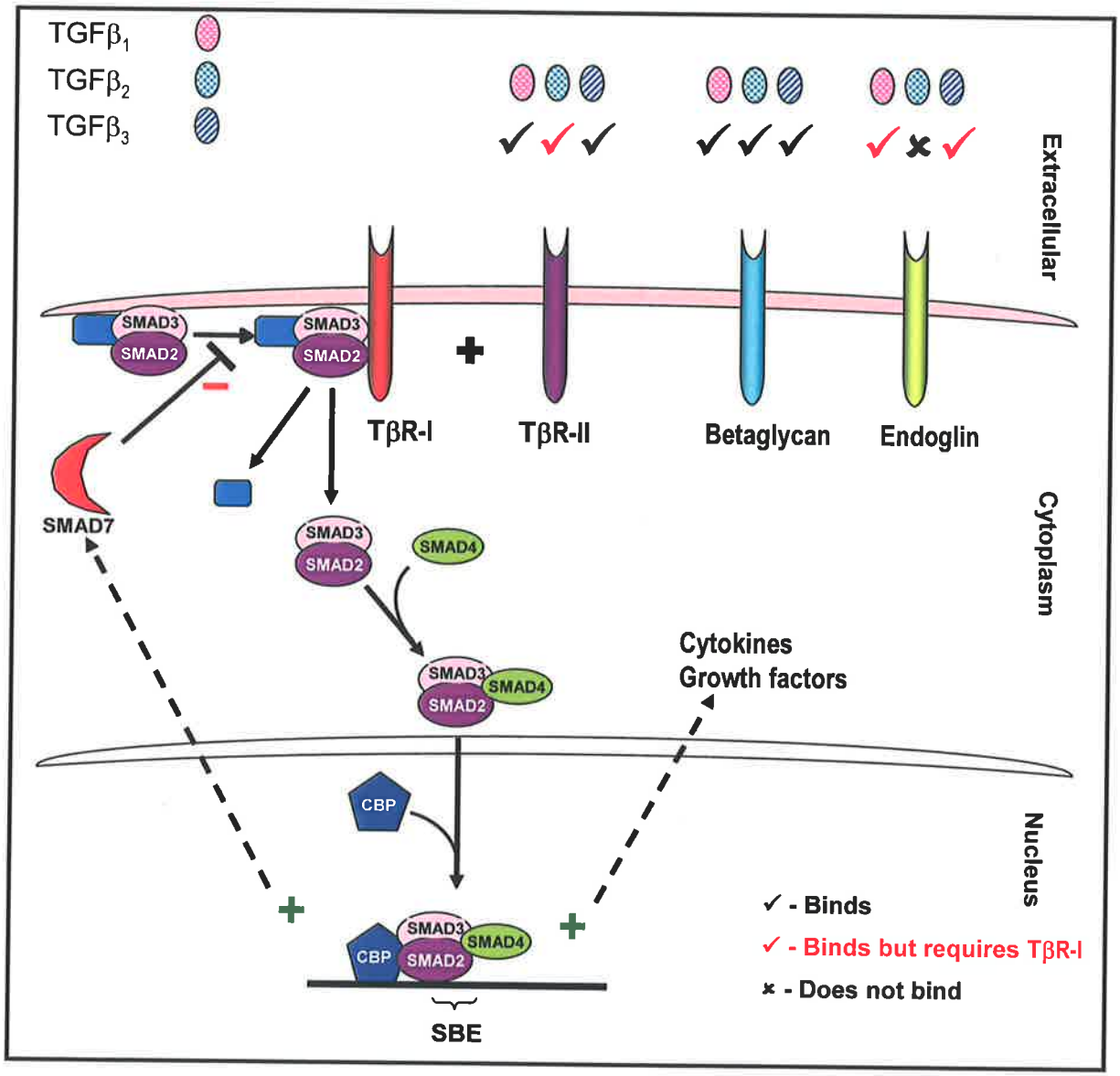


Figure 1.1 Schematic of TGFβ binding to its main receptors and signal transduction

1.2.2.3 *TGFβ in the cycling uterus*

The three TGFβ isoforms have previously been shown to be expressed in various compartments of the female reproductive tract and are instrumental in endometrial remodelling, menstruation, decidualisation, embryo development and implantation [82]. In the cycling female tract of humans mRNA expression and protein production of the three isoforms have been detected in temporally and spatially distinct patterns. $TGF\beta_1$ and $TGF\beta_2$ are predominantly found in the luminal and glandular epithelium and to a much lesser extent in the stroma with $TGF\beta_1$ also detected in the uterine luminal fluid. The converse is true for $TGF\beta_3$ which is almost exclusively detected in the endometrial stroma [83-85]. Cyclic endometrial TGFβ production has been demonstrated, increasing during the proliferative phase to maximal detection in the mid secretory phase in parallel with increasing progesterone, then plummeting with progesterone withdrawal at about days 26-28 of the menstrual cycle [83, 86, 87]. Similarly in mice, endometrial TGFβ expression is maximal following ovulation and during the peri-implantation period [88-90]. Examination of mouse oviductal TGFβ production also indicates specific localisation patterns of the isoforms. $TGF\beta_1$ is exclusively associated with the glandular epithelium, $TGF\beta_2$ is present in this region as well as in smooth muscle, whereas $TGF\beta_3$ is only found in the smooth muscle [91]. TGFβs are thought to contribute to the rapid continual morphological and functional changes in the endometrium during the human menstrual and murine estrous cycles via their proliferative, differentiative, angiogenic and immunomodulatory actions.

1.2.2.4 *TGFβ in the pregnant uterus*

Early pregnancy requires exquisite molecular and cellular coordination in a spatial and temporal manner in order to effect optimal embryo development and implantation. Uterine, oviductal and embryonic regulation of TGFβ expression is reported to be tightly controlled, with investigations demonstrating TGFβ and TβRI & II expression by the embryo from oocyte fertilisation until at least implantation in mice [92]. Previously described post-ovulation oviductal TGFβ expression and concurrent embryonic TβRI & II is suggestive of a role for TGFβ in embryo development. This was confirmed by subsequent in vitro and knockout studies in mice, showing improved blastocyst growth upon TGFβ addition and in-utero and post-natal lethality respectively [93-97]. During the peri-implantation period the three isoforms of TGFβ are produced in the endometrium of both humans and mice in the stroma, myometrium and luminal and glandular epithelium together with the signalling receptors [90-92, 98]. Apoptosis is one of the characteristic features of the implantation site. Studies by Kamijo et al [99] employing an in vitro model of mouse implantation have provided evidence in

support of the role of embryonic TGFβ in implantation via its apoptotic effects on endometrial epithelial cells.

TGFβs have been localised to the extracellular matrix in first trimester decidua associated with decorin, a binding protein thought to act as a reservoir for TGFβ [100]. TGFβ is thought to be involved in placental development firstly by restricting cytotrophoblast outgrowth via upregulation of tissue inhibitors of metalloproteinases (TIMPs) and secondly by promoting differentiation of invasive trophoblasts into non-invasive giant cells [101].

1.2.3 Granulocyte-macrophage colony-stimulating factor (GM-CSF)

GM-CSF is a secreted 22 – 23 kDa glycoprotein in mice and humans belonging to the family of hematopoietic cytokine growth factors or colony stimulating factors (CSFs). While exhibiting similar actions under some circumstances, CSFs have been shown to be functionally distinct, operating via separate specific receptors [102]. In vitro studies initially identified the proliferative and differentiative effects of GM-CSF on myeloid stem and progenitor cells to produce macrophages and granulocytes [103-105], subsequent investigations have shown it to also be a potent neutrophil activator in mice and humans and a regulator of leukocyte adhesion molecules [102, 106, 107].

GM-CSF is expressed in a regulated manner in a variety of cell types including fibroblasts, smooth muscle, endothelial, epithelial cells, chondrocytes and monocytes. A number of studies have shown GM-CSF production uterine luminal and glandular epithelial cells to be regulated in vivo by steroid hormones in humans, mice and sheep [108-110] as well as by cytokines including TGFβ, TNFα, IL-1β, LPS and IFNγ in retinal pigment or uterine epithelial cells *in vitro* [12, 78, 111].

1.2.3.1 GM-CSF in the cycling uterus

Human studies have shown the major source of GM-CSF in the non-pregnant endometrium to be luminal and glandular epithelial cells during the mid-secretory phase and to a lesser extent the proliferative phase of the menstrual cycle [108, 112]. While endometrial GM-CSF receptor expression, in particular the low affinity alpha sub-unit, exhibits the same temporal pattern as the cytokine, the spatial distribution differs with maximal expression observed in the endothelium, stroma and endometrial leukocytes [108, 113]. The spatial and temporal expression of endometrial GM-CSF and its receptors is suggestive of a role in regulating leukocyte recruitment and activation into the endometrium and implication for a preparatory role for endometrial embryo receptivity. Studies

investigating other regions of the human female reproductive tract have also detected high levels of GM-CSF in the cervical mucosa, secreted by cervical epithelium. In the ovary GM-CSF is produced by the surface epithelium, theca interna and leuteal cells during the early and mid leuteal phase. Whereas in the oviduct, GM-CSF is produced by epithelial cells from the mid-proliferative to mid-secretory phase of the menstrual cycle [114-117]. Together these studies demonstrate the steroid hormone dependent cyclicity of female reproductive tract GM-CSF expression and production. GM-CSF receptor expression in these tissues is also steroid dependent but is spatially distinct from that of the cytokine. For example maximal expression in the ovary is observed in the theca externa, and this together with the temporal expression pattern is consistent with a role in follicular growth and development and ovulation.

Correlating with observations in humans, murine uterine epithelial cells have been demonstrated to be a potent source of steroid regulated GM-CSF peaking at estrus in the non-pregnant uterus, ovary and oviduct [109, 118, 119]. Experiments utilising GM-CSF null mutant mice have described perturbations in estrous cycle length, progesterone secretion, adhesion molecule expression and follicle maturation possibly via regulation of leukocyte populations associated with follicle development and corpus luteum in the ovary [120, 121].

1.2.3.2 GM-CSF in the pregnant uterus

Mice are known to be intra-uterine ejaculators hence the main tissue contacted by semen in the murine female reproductive tract is the uterus. While no detectable level of GM-CSF is found in male reproductive glands, Robertson et al [122] have described a 20-fold increase in uterine GM-CSF content following mating with intact males in mice. Supernatants from cultures of uterine epithelial cells recovered from mated (day 0.5pc) mice also indicate an endometrial source. Furthermore, mating with intact, vasectomised or seminal vesicle deficient males found the seminal vesicle fluid component of semen to contain the GM-CSF stimulating factor [122, 123]. Subsequent in vitro experiments identified seminal TGFβ as being the molecule primarily responsible for the post-mating endometrial GM-CSF surge [12]. Proposed targets of GM-CSF action in the pregnant mouse uterus are endometrial leukocytes, the conceptus and the placenta. Firstly, the infiltrate of endometrial leukocytes recruited following mating are potential targets of GM-CSF activity, since instillation of recombinant GM-CSF into an estrous uterus has been shown to elicit leukocyte infiltration reminiscent of that seen following natural mating [124]. However contrary to this, results from studies conducted in GM-CSF null mutant mice exhibited leukocyte recruitment similar to that observed following GM-CSF replete matings although MHC class II expression was reduced, possibly indicating the phenomenon of

cytokine redundancy [125, 126]. A second GM-CSF target in the pregnant uterus is thought to be the pre-implantation embryo, since from oocyte fertilization to at least the blastocyst stage embryos have been shown to express the low affinity α receptor sub-unit.

Additionally the pre-implantation embryo is responsive to GM-CSF as shown in vitro by an increased rate of development and glucose uptake [127]. Indeed retarded blastocyst development and reduced blastomere numbers observed in embryos generated in GM-CSF null mutant mice is recoverable following culture in GM-CSF supplemented media [127]. Additionally embryos cultured in this environment demonstrate improved fetal viability and growth achieved by increased placental function [128]. A third target of GM-CSF activity during gestation is placental trophoblasts, with proliferative and differentiative effects on trophoblast cells in vitro, consistent with a role in promoting placental function in vivo [129]. Indeed, GM-CSF knock-out mice exhibit reproductive anomalies including a defect in the structure of the placental labyrinth associated with compromised growth trajectory in pups [125].

Contrasting with the mouse model, the initial site of interaction between semen and the human female reproductive tract after ejaculation is the cervix and vagina. Recent human studies have shown significant upregulation of cervical GM-CSF expression following unprotected intercourse compared to condom protected and abstinent controls accompanied by leukocyte infiltration similar to the post-mating influx observed in the murine uterus [130]. Additionally, in vitro experiments employing primary and immortalised cervical cells exposed to seminal plasma or recombinant TGFβ resulted in increased GM-CSF production correlating with the mouse model [130]. In women, GM-CSF is produced by tissues of maternal and fetal origin including first trimester decidua, trophoblasts and endometrial epithelial cells in the pregnant uterus. Receptor expression has been identified in embryos from first cleavage to at least blastocyst formation as well as in first trimester cytotrophoblast and extravillous trophoblasts [113, 131, 132]. These studies provide evidence in humans for similar targets of GM-CSF activity in reproduction as those demonstrated in mouse models. In vitro experiments have disclosed differentiative effects of GM-CSF on human placental cells, including cytotrophoblasts, directing the formation of the syncytiotrophoblast and stimulating production of placental lactogen and chorionic gonadotrophin [133]. Embryotrophic effects of GM-CSF on human 2-4 cell cultured embryos include improved rate of blastocyst development and developmental competence, indicating a role for oviductal GM-CSF in promoting pre-implantation embryo development [134].

1.2.4 Interleukin–6 (IL-6)

IL–6 is a secreted 26kDa cytokine belonging to the IL-6 family of cytokines known to exhibit a large degree of pleitropy and redundancy. Members of this cytokine family include LIF, IL-6, IL-11, cardiotrophin-1 (CT-1), cardiotrophin-like cytokine (CTC), oncostatin M (OSM) and ciliary neurotrophic factor (CNTF), all of which are comprised of a similar helical structure and signal via a common high affinity β-subunit receptor, gp130, with their own specific α-sub-unit receptor. The IL-6 cytokine family has numerous functions not only regulating inflammation and immunity but also including significant roles in embryonic development, infertility, haematopoiesis and cellular regeneration.

IL-6, initially known and identified as B cell growth factor (BCGF) for its proliferative and differentiative effects on B cells, has subsequently been demonstrated to induce the acute phase response via C-reactive protein, differentiation of myeloid precursors and stimulation T-cell cytotoxic activity [135-138]. Monocytes, endothelial cells, fibroblasts, epithelial cells and recently obese adipose tissue have been recognised as sources of IL-6 [139]. Regulation of IL-6 production and signalling is important for maintenance of normal immune function. Excess local or systemic production of IL-6 has been associated with autoimmune diseases including rheumatoid arthritis, lupus and Crohns disease as well as in multiple myeloma malignancies [140-143]. Competitive binding by the soluble gp130 receptor and negative feed back loops via SOCS proteins have been shown to regulate IL-6 signalling [144, 145].

1.2.4.1 IL-6 in the cycling uterus

IL-6 is produced in the human ovary, oviductal and follicular fluid, cervical mucosa and endometrium maximally during the mid-secretory phase of the menstrual cycle in the glandular and luminal epithelium [115, 146-148]. Whereas the IL-6R is expressed constitutively throughout the cycle predominantly in the endometrial glands [147]. One proposed biological function of IL-6 in the human is the self limited angiogenesis observed during the normal menstrual cycle in the ovary and decidua, while dysregulation of the angiogenic stimuli is associated with ovarian and cervical cancers [149, 150].

Mouse studies have shown similar expression patterns of IL-6 in the cycling female reproductive tract, with maximal expression and secretion in the uterus in the pro-estrous/estrous stages in vivo. More specifically IL-6 is secreted by uterine and glandular epithelial cells under the control of ovarian steroid hormones [151, 152]. The vascularisation role of IL-6 in the human ovary have also been

demonstrated in the murine model, with IL-6 directly upregulating expression of the angiogenic factor VEGF [153]

1.2.4.2 IL-6 in the pregnant uterus

Analysis of uterine lavage show a massive 250-fold increase in luminal IL-6 content following mating in mice which is thought to be partially responsible for post-mating leukocyte infiltration [152, 154]. This peak in the early initial response to insemination declines by the third day of pregnancy, with a second surge identified on days 5 to 6 following implantation on day 4 [155]. The mouse blastocyst is a source of and is responsive to IL-6. This has been demonstrated in vitro by detection in culture supernatants and inhibition of blastocyst adhesion to a laminin matrix. This may implicate a role for IL-6 aiding in the unfettered passage of the embryo through the oviduct and into the uterus [156, 157]. Murine endometrial IL-6 receptor expression is low until implantation when there is a marked increase in luminal epithelial expression, followed by a sudden change in the localization of the receptor with strong expression in the stroma on days 5 and 6 of pregnancy [158]. The differential temporal and spatial receptor expression may indicate distinct roles of the epithelium and stroma in the implantation process. Regulation of IL-6 activity at the feto/maternal interface is essential to positive pregnancy outcomes as a high level of production is associated with spontaneous abortion in mice hence detection of soluble IL-6 receptor expression in the decidua may implicate it as having a regulatory role [159, 160].

Recent studies in humans have shown a 2.6 fold increase in cervical IL-6 expression following unprotected intercourse in contrast to no change or a decrease following either abstinence or condom protected intercourse [130]. Subsequent in vitro studies employing primary or immortalised cervical cell culture have shown human seminal plasma and TGFβ to be potent stimulators of IL-6 production [130]. Decidual IL-6 production is enhanced during the first trimester of human pregnancy and is thought to be involved in tissue remodelling associated with placentation [161], while in vitro studies using human placental explants have demonstrated IL-6 signalling plays a role in stimulating trophoblast hCG production, essential for early pregnancy conceptus survival [162]. As with mice, human embryos are responsive to and benefit from oviductal IL-6 exposure [163]. Increased cervical production of IL-6 during pregnancy was initially correlated with the risk of pre-term labour, however further investigations have shown this phenomenon to be associated with imminent labour at any point of gestation and is known to be affected via enhanced leukocyte recruitment and cervical ripening [164-166]. While high levels of serum IL-6 are observed in women suffering recurrent miscarriage, examination of endometrial biopsies do not differ in their IL-6 expression however a reduction in the

regulatory soluble gp130 has been observed and may indicate a mechanism of excess IL-6 bioavailability [167, 168].

1.2.5 Leukaemia inhibitory factor (LIF)

LIF, a 45kDa glycoprotein, was independently identified by a number of groups and has a diverse range of functions and targets with demonstrated roles in haematopoiesis, neuropoiesis, metabolism and acute phase responses [169-172].

1.2.5.1 LIF in the cycling uterus

Localisation of LIF expression in the female reproductive tract has been described in the luminal and glandular epithelium, oviductal epithelium, follicular fluid and cervical secretions predominantly in a cycle dependent manner. Oviductal LIF secretion is constitutive and does not fluctuate with the female hormone cycle. Endometrial epithelial cell expression and secretion of LIF is maximal during the mid-luteal and late-secretory phase of the menstrual cycle (days 19-26) in humans and at estrous in mice but is only detectable in cervical mucosa at menses [173-176]. The differential timing of maximal LIF expression and production by the endometrium in these species indicates distinct control mechanisms, where human expression is influenced by the post-ovulatory surge of progesterone and murine expression is associated with the physiological increase in oestradiol levels at estrous [175]. Further to hormone regulation, a number of cytokines have been identified as having a potent stimulatory effect (e.g. IL-1, IL-4, TNFα, TGFβ, platelet-derived growth factor and epidermal growth factor) or interestingly a significant inhibitory effect (e.g. IFNγ, IL-12 and IFNα) on LIF expression and production [177, 178].

1.2.5.2 LIF in the pregnant uterus

Maternal expression of LIF is maximal at the time of implantation in both mice and humans, day 4 and days 5-6 post-fertilisation respectively, localised to the luminal and glandular epithelium [174, 179, 180]. Furthermore, embryos not only possess the functional capacity to respond to LIF stimulation i.e. receptor expression, but in fact have been demonstrated to do so in vitro displaying improved blastocyst development [180-182]. Investigations using LIF null mice have demonstrated an absolute requirement for maternal LIF for successful implantation. This is not due to embryonic defect but specifically due to implantation failure, since embryos generated from LIF knockout matings implant and develop successfully when transferred to LIF sufficient recipients [183]. In contrast, embryos

generated following mating between LIF receptor null mice have the capacity to implant and develop to term but are not viable after delivery due to the multi-system defects observed including reduced bone density, neuronal development and metabolic perturbations [184]. Temporal uterine luminal epithelial LIF expression in concert with human embryo LIF receptor expression indicates a similar role of LIF in human implantation as that in the mouse [181]

1.2.6 Keratinocyte-derived chemokine (KC)

KC, a member of the alpha (CXC) chemokine family, is a produced as a 96 amino acid residue precursor protein containing a secretory signal that is cleaved to produce the mature 68 aa isoform. KC is the murine counterpart of human IL-8 and growth-related-oncogene (GRO) [185]. KC and IL-8 are secreted by fibroblasts, leukocytes and endothelial cells. KC elicits its potent neutrophil chemoattractant and activator function by binding to and signalling through the functional receptor CXCR2, while IL-8 is a chemoattractant for both neutrophils and T lymphocytes in a concentration dependent manner.

Based on the pattern of KC expression in a number of inflammatory disease models, KC appears to have an important role in inflammation. KC has been found to be involved in monocyte arrest on atherosclerotic endothelium [186, 187] and may also play a pathophysiological role in Alzheimer's disease [188].

1.2.6.1 KC in the uterus

There have been a limited number of studies examining KC production and potential roles in the murine female reproductive tract. Wood et al [189] detected KC mRNA in the uterus throughout pregnancy with two obvious peaks. The first peak is observed during the post-mating inflammatory response, the second coincides with placental development, however whether KC mRNA peaks correspond with an increase in functional protein was not evaluated.

In the cycling human uterus, IL-8 is produced by luminal and glandular epithelial cells in a biphasic manner, with peaks in expression occurring in the early to mid proliferative phase and late secretory phase [190]. Endometrial IL-8 has also been detected in the perivascular cells in the non-pregnant and pregnant uterus and is thought to be one factor responsible for neutrophil accumulation in the endometrium and decidua, purportedly for a role in angiogenesis [190-192] In the pregnant uterus, IL-8 is reported to be produced in the placenta and chorio-decidual tissue, as well as

in the cervix where it is temporally related to neutrophil influx and subsequent cervical ripening near term [193-195].

There have been many studies investigating the role oft IL-8 in the pathogenesis of endometriosis. Peritoneal fluid contains high levels of IL-8 which correlates with severity of endometriosis. In vitro experiments have suggested that IL-8 has a role in ectopic cell adhesion to fibronectin, proliferation and angiogenesis, not only via leukocyte recruitment but additionally directly in a paracrine manner [196].

1.3 LEUKOCYTE POPULATIONS IN THE UTERUS

The female reproductive tract is a mucosal organ, providing protective immunity from potential pathogens while simultaneously permitting invasion and temporary residence of a foreign body, the semi-allogenic conceptus. As with other mucosal organs, the female reproductive tract is home to a heterogeneous population of leukocytes regulated by a range of cytokines, complete with lymphatic drainage. However, it has the distinction of not having secondary lymphoid organs. Despite this, the female tract is capable of eliciting both antigen specific humoral and cell mediated immune responses to infectious agents and antigens introduced at insemination [197]

Many aspects of the menstrual and estrous cycle of mammals are reminiscent of inflammation [198, 199]. During the human menstrual cycle both the cellular and extracellular components of the endometrium undergo extensive remodelling under the control of ovarian hormones. The population of leukocytes increases from approximately 5-10% of total endometrial cells in the early proliferative phase to 40-45% in the pre-menstrual phase in humans [200]. This cyclic regulation suggests hormonal control, particularly progesterone; however, neither resident nor circulating leukocytes express progesterone receptors [201-204], hence control must be via an indirect mechanism [204, 205]. This premenstrual influx is most likely orchestrated by progesterone receptor positive endometrial cells secreting chemoattractant molecules in response to decreased progesterone production [206, 207]. Leukocytes are not only implicated in the tissue remodelling and degradation indicative of menstruation but also in the mechanisms underlying endometrial repair following menses [208].

1.3.1 T Lymphocytes

T cell sub populations are partially designated by the presence of one of two types of accessory molecules, CD4 or CD8; CD4 – T helper (Th) or CD8 – cytotoxic T cell (Tc). Traditionally T helper cells are categorised according their cytokine secretory profile, which relates to effector function. Th1 cells mediate cell mediated immunity by activating CD8+ cells and macrophages via secretion of pro-inflammatory cytokines including TNFα and β, IL-2 and IFNγ. Th2 cells direct an antibody mediated response against extra-cellular antigens through production of interleukins 4, 5, 6, 10 and 13 while Th3 cells suppress Th1 and other immune cell responses by producing TGFβ [209]. However, due to emerging evidence demonstrating both CD4+ and CD8+ cells have the capacity to produce these distinct cytokine profiles, the current terminology is type 1 and type 2 T lymphocytes, where type 1 cells promote a cell mediated immune response, and type 2 cells promote a humoral response through targeting B-cells [210].

CD4+ and CD8+ cells are present throughout the menstrual cycle in the human vagina, cervix and endometrium of women [211, 212] and in the estrous cycle of mice [213-215]. The number of cells in these tissues remains relatively constant throughout the cycle indicating recruitment of these cells is ovarian steroid hormone independent [204, 205, 212]. The cytolytic activity of these cells in the lower female reproductive tract is indicative of a role in the protective response to pathogens [211]. Endometrial T lymphocytes predominantly appear in lymphoid aggregates in both the human and the mouse with the latter having a noticeably reduced occurrence by comparison [216]. Following natural insemination in women a transient increase in cervical CD8+ T cell populations is observed [130, 217]. The paucity of T-lymphocytes in the endometrium lead Vassiliadou and Bulmer to propose that T lymphocytes have little or no role in implantation and embryo survival [218]. However, subsequent studies have demonstrated a temporal increase in peripheral blood T cells from gestational week 9-10 with maximal numbers present in the second trimester thought to be responsible for maternal immunological recognition of pregnancy [219]. Investigations using rodent models have shown a similar pattern of transient increase in T cell populations in the uterine draining lymph nodes. It has been proposed that the seminal plasma component of seminal fluid that is most likely responsible for the immuno-supression towards paternal alloantigens and not sperm [220].

Two sub-populations of T cells are present at the fetal-maternal interface, type 1 cytokine producing (γ/δ) T cells and type 2 cytokine producing (α/β) T cells. Experiments in abortion prone mice have described the protective effect of in vivo deletion of (γ/δ) T cells and conversely the

detrimental effect of depleting (α/β) T cells [221]. Given both of these populations are present during normal pregnancy and that T cell deficient scid mice have successful pregnancies, this indicates that the relative ratio of these cells in the proximity of the fetal-maternal is paramount. Supporting this proposition is the observation of a diminished type 2 cytokine profile in endometrial T cells recovered from women suffering recurrent miscarriage, with the skewing towards type 1 profile at the fetal-maternal interface, thought to mediate the fetal loss [178].

Helper and cytotoxic T cells are not the only populations relevant to pregnancy, recently another subset of T cells, T regulatory (T_{reg}) cells, have been identified as having roles in mediating immunological tolerance and preventing autoimmunity in humans and mice [222-224]. T_{reg} cells are identified by expression of cell surface molecules CD4 and CD25 (CD4+CD25+) and additionally the lineage specific transcription factor, Foxp3 (CD4+CD25+Foxp3+) [225]. Transplantation studies in mice have shown CD4+CD25+ cells possess an innate capacity to regulate transplantation and that this ability may be amplified 10-fold following a "priming" event [223]. Treg cells have been suggested to play a role in regulating the maternal immune response to the semi-allogenic conceptus. CD25 depletion results in complete pregnancy loss following allogeneic mating in mice and observations in women experiencing spontaneous abortion have identified decreased decidual CD25+ cells compared to those women undergoing elective termination [226]. Together these studies suggest that decidual CD4+CD25+ cells contribute to maternal tolerance of the semi-allogenic conceptus and hence to the maintenance of pregnancy.

1.3.2 Natural Killer cells

Natural killer (NK) cells are large granular lymphocytes that lack B or T cell membrane bound receptors. NK cells contribute to early innate immunity prior to B and T cell activation by producing cytokines, predominantly IFNγ, and through cytotoxic activity against some tumour and virus infected cells. They do this by monitoring alterations in cell surface MHC I molecules via their NK receptor. NK cells express receptors for the Fc region of soluble antibodies hence following antibody binding, virally infected or altered self cells will be targeted by NK cytotoxic activity.

Uterine NK (uNK) cells are distinct from those found in circulation by their differential expression of the cell surface molecules CD56 and CD16. Most (90%) circulating NK cells express low levels of CD56 ($CD56^{dim}$) and are positive for CD16 (CD16+) – hence are designated $CD56^{dim}$ CD16+. In contrast, uNK cells predominantly express high levels of CD56 and are negative for CD16

(CD56 ^{bright} CD16) [227-229]. Additionally, circulating CD56 ^{bright} CD16- NK cells are agranluar whereas uNK cells CD56 ^{bright} CD16- are particularly granular, a phenotype thought to be linked with the involvement of these cells in controlling placentation [228].

Natural killer cells have been identified in the human uterus at low levels during the proliferative phase of the menstrual cycle but are mobilised and recruited from the spleen in the periovulatory period. Vigorous proliferation occurs in the decidua and stroma during the late secretory phase, so that uNK cells comprise up to 40% of total cells in the stroma at this time [230, 231]. Furthermore, uNK cell apoptosis has been proposed to be a predictor of commitment to undergo menstruation versus decidualisation [231, 232]. Mobilisation and uterine homing of CD56 ^{bright} CD16- NK cells is proposed to be effected indirectly by progesterone and estrogen upregulation of vascular adhesion pathways for CD56 ^{bright} CD16- NK cells. Additionally indirect progesterone action is thought to contribute to uNK proliferation by stimulation of endometrial stroma and lymphocyte cytokine production [233-236]. In contrast to humans, the appearance of uNK in mice does not occur naturally in the absence of decidualisation. Instead these cells proliferate vigorously at each implantation site following embryo attachment, where they are situated between the placenta and myometrium on the mesometrial side to form a structure – termed the mesometrial lymphoid aggregate of pregnancy. uNK cells are present in large numbers at the feto-maternal interface for the first half to two thirds of pregnancy. By parturition their numbers are very low, probably due to cell death and placental separation [237, 238]. Experiments in NK deficient mice have indicated an essential role for uNK cells in supporting decidual health, facilitating uterine arterial structural changes toward high volume conduits and placental development [239]. Despite this, however pregnancy is achievable in the absence of uNK cells. Histochemical studies have provided some evidence in support of an association between perturbed uNK cell populations and recurrent miscarriage in women, identifying an increased ratio of endometrial CD56^{dim} CD16+ compared to CD56 ^{bright} CD16- NK cells [240-243].

1.3.3 Macrophages

Macrophages are differentiated circulating monocytes originating from bone marrow derived myeloid cells. As part of a functional innate immune response, macrophages contain numerous transmembrane receptors that permit them to recognise and phagocytose both endogenous and exogenous antigenic particles, present processed antigen to activate T cells, and produce intracellular antibacterial and cytolytic modulators including reactive oxygen and nitrogen species, pro- and anti-inflammatory cytokines and chemotactic actors. In addition to their immunological activity,

macrophages play a prominent role in angiogenesis and tissue remodelling through secretion of extra-cellular matrix (ECM) proteases including plasminogen activator, matrix metalloproteinase-9, MT1-MMP and metalloelastase [244]. They also have been implicated in the process of transdifferentiation, whereby recently recruited tissue monocytes can differentiate into endothelial cells and contribute to vessel formation [245, 246].

Macrophages have been identified at a constant density of approximately 10% in the endometrium of virgin female mice throughout the estrous cycle and are of an immunosuppressive phenotype [247, 248]. However, the even distribution observed during met-estrus and diestrus is altered and cells accumulate in the sub-epithelial stroma region during proestrus and estrus [126, 247, 249-251]. Since neither resident nor circulating leukocytes express progesterone or estrogen receptors, this cyclic regulation must be effected indirectly [204]. Indeed, under the control of ovarian steroid hormones, uterine epithelial cells differentially secrete several cytokines and chemokines, including CSF-1, GM-CSF, TNFα and IL-6 [252]. These molecules are known for their ability to recruit macrophages as well as influencing their survival, differentiation and function [252]. Murine uterine macrophages are thought to be involved in the cyclic uterine tissue remodelling observed through estrogen regulated secretion of matrix metalloproteinases including MMP-9 [253]. Studies in humans indicate the presence and abundance of uterine, vaginal and cervical macrophages throughout the menstrual cycle [254-256]. Endometrial macrophage numbers increase over the menstrual cycle with a marked increase initially during the proliferative phase increasing to be maximal in the perimenstrual period [200, 244, 255-257]. These increases are thought to be attributable to both an influx of newly recruited macrophages from the blood and an expansion of populations already present. Uterine macrophages are thought to contribute to the phenomenon of menstruation by participating in extra cellular matrix breakdown through secretion of proteases such as plasminogen activator, matrix metalloproteinase-9, membrane type 1 matrix metalloproteinase (MT1-MMP) and metalloelastase [244].

In the mouse, the uterine macrophage population increases 2- to 3-fold during the post-mating inflammatory response. This is thought to be mediated by estrogen primed uterine epithelial cell upregulation of chemokines following exposure to seminal vesicle fluid components in the ejaculate [123, 258]. Activated uterine macrophages are evenly distributed throughout the endometrium during the peri-implantation period, however by day 5 of gestation are scarce in the primary decidua having been redistributed to the secondary decidua [259, 260]. By day 15 of murine pregnancy, activated macrophages return to the decidual tissue of the endometrium [261]. Endometrial macrophages are

thought to not only to facilitate implantation and placental development but also to contribute to uterine quiescence during pregnancy by temporally increasing production of iNOS, the limiting enzyme for production of the sooth muscle relaxant - nitric oxide, until just prior to term [262, 263]. Additionally migration of macrophages into the cervix and an increase in uterine and cervical macrophage MMP-9 production near term indicates a potential role in cervical ripening and parturition [264]. Macrophages present in the uterus following delivery contribute to the healing and regeneration of the uterine epithelium [265].

Similarly in humans cervical macrophage numbers increase transiently following insemination and are thought to be primarily involved in clearing post-insemination debris and limiting transfer of potential pathogens higher into the reproductive tract [217, 266]. Unlike the mouse model, human endometrial macrophages remain relatively constant throughout pregnancy representing approximately 10-15% of the total cellular component of the decidua, associated with the cytotrophoblast and fetal membranes [267-269]. At term approximately 100% of decidual macrophages are activated and thought to contribute to parturition by increasing TNFα secretion which subsequently increases protease production leading to decidual and cervical ECM breakdown [270-273].

In addition to physiologic roles in the decidua, macrophages have been identified as contributing to the immunological environment of the pregnant uterus. The immunological relationship between the mother and the fetus is a bi-directional communication determined on the one hand by fetal antigen presentation and on the other hand by recognition of and reaction to these antigens by cells of the maternal immune system. Significant evidence now exists showing that appropriate immunological recognition of pregnancy is important for the maintenance of gestation, and that inappropriate recognition of fetal antigens might result in pregnancy pathologies. Through all stages of pregnancy human and mouse cytotrophoblasts secrete high levels of the type2 immune deviating cytokine, IL-10, known to suppress production of type1 cytokines such as IFNγ [274, 275]. Secretion of IL-10 by cytotrophoblasts has the capacity to "switch off" decidual macrophages. These immune cells have also been identified as a source of decidual IL-10, suggesting that maternal tolerance towards the semi-allogeneic conceptus at the feto-maternal interface might be in part maintained by decidual macrophages [276].

1.3.4 Dendritic cells

Dendritic cells are considered the most specialised of antigen presenting cells. With subsets arising from myeloid and lymphoid precursors, these cells are capable of directing T cell immune responses towards a type2 or type1 phenotype respectively. Dendritic cells constitutively express high levels of MHC class II together with co-stimulatory molecules, required for naïve Th cell activation, hence are powerful T cell activators. Dendritic cells are present in most locations throughout the body including the skin, gut, kidney, heart, lung, liver and lymphoid tissue. Peripheral dendritic cells take up and process antigen, exiting via the lymphatics to the draining lymph nodes, where they present processed antigen in the context of MHC II to cognate CD4+ cells with high efficiency [277].

Dendritic cells have been identified in the vagina, cervix and uterus in mice and humans [256, 278-280]. Female reproductive tract dendritic cell populations do not fluctuate during the menstrual cycle humans but recently have been shown to be hormone regulated in mice [256, 280, 281]. Studies in mice have shown exogenous antigen sampling in the vagina and cervix by resident dendritic cells and subsequent migration to draining lymph nodes, indicating a role as sentinels in the recognition of potential pathogens and paternal antigens introduced at insemination [280]. Following insemination, the number of uterine dendritic cells rises in both mice and humans and remains at this elevated level throughout pregnancy [12, 256]. The role of dendritic cells during pregnancy is yet to be elucidated, however their spatial distribution in the decidua amongst a large population of NK cells in the presence of the invading trophoblast may indicate bidirectional communication between the innate and adaptive arms of immunity [282].

1.3.5 Neutrophils

Like macrophages, neutrophils are bone marrow derived myeloid cells. Neutrophils have lobed nuclei and large cytoplasmic granules that are released, degranulated, following activation. Neutrophils are phagocytic cells recruited into tissues from circulation, to the site of inflammation via a chemotactic gradient produced by factors such as complement, blood clotting factors and cytokines. Neutrophils are usually the first immune cells recruited to the site of inflammation and have an important role in responding to intracellular invaders under the direction of cytokines and chemokines such as GM-CSF and IL-8 (KC in the mouse) [283, 284].

In the cycling human uterus neutrophils are absent until the pre-menstrual and menstrual phase, when they accumulate in the luminal and glandular epithelium and constitute up to 15% of total endometrial cells [257, 285, 286]. The role of neutrophils in the human uterus during the perimenstrual phase is firstly the production and secretion of proteases including MMPs which target components of extracellular matrix for degradation [244, 257]. Secondly, one source of IFNγ secretion in the endometrium at this time has been identified as local neutrophils, the effect of which is activation of proximal macrophages enabling contribution toward endometrial breakdown and clearance of sloughed tissue [287-289].

The lower genital tract of mice is cyclically populated with neutrophils, the highest numbers are observed at diestrus and proposed to be involved in protection from potential pathogens [290]. Following insemination in mice, uterine neutrophil numbers increase significantly and significant transepithelial migration into the luminal cavity is evident. However they only remain abundant until day 2 of gestation when there is a dramatic decline prior to implantation [258]. Mating experiments with intact, vasectomised or seminal vesicle deficient male mice indicate a role for the seminal vesicle fluid component of semen in their recruitment [123]. While previous studies have identified the importance of seminal TGFβ, in eliciting cytokine secretion and macrophage recruitment following mating, the precise factors responsible for the post-mating neutrophil influx remain to be identified [12]. The short-lived influx of these cells may indicate an acute role in clearance of sperm, debris and potential pathogens introduced at mating. After this time neutrophils are rarely seen throughout the endometrium, with the exception of a number shown to be intimately associated with implantation sites [258, 259]. It has been shown that these cells remain restricted to the necrotic region of the trophoblast invading front possibly phagocytosing debris in this area [291]. Neutrophils are present in the cervix at the end of pregnancy, however Timmons et al [292] excluded a role for neutrophils in cervical ripening following observations of the temporal migration and activation of these cells in the cervix near term in mice. Additionally, this study demonstrated no effect on timing or success of parturition following depletion of neutrophils prior to term. Alternatively these cells may be important in post-partum-cervical remodelling [292]. Investigations in women have demonstrated a similar pattern of neutrophil recruitment and regression in the cervix following insemination [293]. However in contrast to findings in mice where the seminal vesicle fluid component appears to be the dominating component, neutrophil influx in the human cervix is reliant upon the presence of sperm [217].

1.4 IMMUNOLOGICAL RESPONSE TO MATING AND THE CONCEPTUS

The uterine environment after insemination resembles a 'classical' inflammatory response observed in other immunocompetent tissues with semen triggering expression of pro-inflammatory cytokines, chemokines, and activation and influx of leukocytes into the uterus. Yet despite this inflammatory state the semi-allogenic conceptus evades maternal immuno-rejection. The mechanism underlying this unique tolerance of a genetically disparate entity for the duration of pregnancy is still not clear. This section will discuss the post-mating immune response and the current understanding of its role in generation a permissive maternal environment for pregnancy.

1.4.1 Post-mating inflammatory response

Inflammation involves leukocyte extravasation, induced by upregulation of endothelial adhesion molecules, in response to chemokine and cytokine signals. A number of pro-inflammatory cytokines and chemokines are released into the uterine lumen following exposure to semen. Post-mating surge of factors including GM-CSF, IL-1, IL-6, RANTES and TNFα have been linked with endometrial leukocyte infiltration [152, 154, 155]. The intercellular signalling and immune deviating properties of these molecules in the microenvironment of the murine uterus have been explored in vivo [294, 295] and in vitro with respect to pregnancy outcome [152, 155]. The post-mating inflammatory state persists for
1-2 days declining to basal levels until implantation. One consequence of this is an increase in cytokines that benefit embryo growth, development and passage through the oviduct to the uterus [156, 157]. More detailed descriptions of individual cytokine actions in the pregnant uterus are found above.

The initial population of invading leukocytes in the uterus after mating include neutrophils, macrophages and dendritic cells [258, 296, 297]. Neutrophils are characteristically among the earliest cells present at the site of inflammation this is the case after mating. The function of the marked infiltration of neutrophils into the uterus following seminal fluid exposure in humans [298] and mice [299] appears to be primarily in clearance of potential pathogens and paternal antigens introduced during intercourse.

Following neutrophils, antigen presenting cells including macrophages and dendritic cells are recruited into the endometrium in the mating response. Instillation of recombinant GM-CSF shows the

key role of this cytokine in mediating macrophage and dendritic cell recruitment [252, 300]. Macrophages adopt two distinct phenotypes in a temporal fashion during pregnancy. The first is an activated form, allowing clearance of potentially pathogenic/damaging material from the uterus in preparation for accommodation of an embryo, and facilitation of paternal antigen processing and presentation [301-303]. A second population is immunosuppressive in nature, capable of inhibiting lymphocyte activation and is thought to have a role in preventing rejection of the semi-allogenic conceptus [276].

Other leukocytes including natural killer (NK) cells and T lymphocytes are thought to contribute to the immune tolerance necessary for successful pregnancy but whether their abundance of phenotypes are affected by seminal fluid has not been conclusively shown. The micro environment following mating results in NK cells stimulating expression of growth promoting cytokines [304] and preventing cytolytic activity [305-307]. Low numbers of uterine NK cells are deleterious to placentation and fetal growth and development [308], while reinstatement of uterine NK populations leads to an improvement in these parameters [239]. During early pregnancy the phenotype of T-lymphocytes in draining lymph nodes becomes skewed to Th2/Th3. However the precise mechanisms leading to immune tolerance are not defined, and whether mating has a causal role in this immunological skewing remains to be shown [178, 309].

1.4.2 Pre- and peri-implantation events in murine pregnancy

It is proposed that successful mammalian pregnancy is in part dependent upon pre and peri-implantation events in the uterus. The post-mating inflammatory response causes recruitment of leukocytes thought to contribute to endometrial preparation and receptivity for implantation. The precise mechanism and contribution of semen to these events is still being delineated. Animal mating studies, generating pregnancies in the absence of seminal vesicle fluid, or in cytokine knockout mice are supportive of role for the seminal vesicle induced post-mating inflammatory response in eliciting a cascade of molecular and cellular events promoting endometrial receptivity [123, 125, 126, 310].

The endometrium in most mammals is receptive to blastocyst attachment and implantation for only a very short time during the estrous/menstrual cycle. This is referred to as the "window of receptivity" or "implantation window" and occurs in three phases; 1) embryo apposition and attachment, 2) endometrial decidualisation and 3) trophoblast invasion. In mice the window of implantation lasts for

only 24 hours where as in humans the duration of the window of implantation is 5 days, usually days 20 – 24 of the menstrual cycle [311].

Following estrogen priming and under the influence of progesterone, uterine epithelial cells upregulate expression of adhesion molecules in parallel with down-regulation or cleavage of long chain glycoprotein anti-adhesion molecules, MUC1 [312]. Long MUC1 molecules are thought to extend further into the lumen than uterine adhesion molecules, hence posing a physical barrier for embryo attachment. In vitro studies using human tissues and embryo attachment models have provided evidence in favour of endometrial MUC1 cleavage possibly by metalloproteases directed by TNFα [313]. Furthermore the human blastocyst induces enzymatic cleavage at the implantation site [314]. In contrast, murine endometrial MUC1 is downregulated by progesterone prior to blastocyst apposition, additionally failure to achieve this inhibits blastocyst attachment [315].

Decidualisation is characterised by significant endometrial tissue remodelling and differentiation to form morphologically distinct decidual cells as well as angiogenesis. Decidualisation promotes embryo implantation and survival and occurs naturally during the menstrual cycle in women while requiring the presence of an embryo in mice [316]. The post-insemination influx of leukocytes including macrophages and uNK cells are thought to contribute to extracellular matrix remodelling of the endometrial environment in preparation for implantation through secretion of MMPs [259, 260, 308]. Activated uterine macrophages are also implicated in uterine angiogenesis, since immuno-localisation of these cells and that of the angiogenic agent, vascular endothelial growth factor (VEGF) exhibit similar spatial patterns [317]. Embryo survival is supported firstly by protection from maternal immuno-rejection by the decidua, namely uNK cells and macrophages, and secondly maintenance of the CL and hence its progesterone production by decidual secretion of prolactin.

Implantation in mice, after generation of the decidua, involves a rapid progression from embryo apposition and attachment to complete decidual envelopment. The uterus plays a functional role in implantation, closing down on the blastocyst bringing it into close proximity of the luminal epithelium permitting attachment. At the attachment site embryonic cells, trophoblasts, degrade the epithelium and the decidua invaginates, enveloping the embryo [318]. Human implantation differs from the mouse in that rather than decidual invagination, the competent blastocyst trophoblast cells invade between luminal epithelial cells. Trophoblast invasion of maternal tissue in these species is necessary for anchorage, nutrient and waste exchange and needs to be tightly controlled. Shallow invasion in humans is associated with pre-eclampsia, whereas excessive invasion into the myometrium potentially leads to placental accreta, which can result in uterine rupture. Macrophages and uNK cells are

thought to play a regulatory role in controlling trophoblast invasion (see sections 1.3.2 and 1.3.3 above).

1.4.3 Immune Tolerance

The ability of a fetus to implant and flourish in the maternal uterus defies rules generally accepted for traditional transplantation immunology. The genetic composition of the fetus, originating equally from maternal and paternal gametes, places it at risk of destruction by the maternal immune system, as it expresses both sets of transplantation antigens and should therefore be recognised as foreign. However, rejection does not generally ensue, as is evidenced by mammalian evolutionary success. To prevent rejection, a temporary state of immunological tolerance towards the embryo must be established within the maternal system without loss of protection from pathogenic organisms. In 1953, Medwar delivered a lecture in which he defined the paradox of fetal allo-graft survival in a genetically disparate, immunologically competent woman, as follows:

"The immunological problem of pregnancy may be formulated thus: how does the pregnant mother contrive to nourish within itself, for many weeks or months, a foetus that is an antigenically foreign body?" [319].

By way of explanation, Medawar proposed the following hypotheses:

"The reasons why the foetus does not habitually provoke an immunological reaction from its mother may be classified under three headings: (a) the anatomical separation of foetus from mother; (b) the antigenic immaturity of the foetus; and (c) the immunological indolence or inertness of the mother".

This section will address our current understanding of reproductive immunology in the light of Medawar's hypotheses.

1.4.3.1 "Anatomical separation of foetus from mother"

Prior to ovulation, the oocyte acquires a thick glycoprotein covering – the zona pellucida. The zona pellucida is required both for fertilisation, as it elicits the acrosomal reaction in sperm, and for protection from maternal cytotoxic T lymphocytes and NK cells [320-322]. In humans the zona pellucida is shed 5 days after fertilisation revealing a layer of trophoblast cells responsible for

attachment and implantation into maternal tissues. Throughout gestation the trophoblast layer completely encompasses the conceptus, and so more precisely the allograft at the feto-maternal interface is the outer trophoblast layer. Trophoblasts express non-classical MHC class I molecules known to inhibit macrophage and NK cell cytotoxicity, hence paralysing leukocytes at the feto-maternal interface, behaving to some extent as a barrier [323-326]. Non-classical MHC expression is also a strategy of evading detection by the maternal immune system, employed not only by the fetus but also commonly by many viruses. In spite of this, transgenic mouse experiments conducted by Tafuri et al [327] demonstrate maternal immunological recognition of and a transient state of tolerance toward the fetus. This possibly indicates that contact between the mother and fetus is not restricted to the placental maternal-fetal interface. Indeed studies in humans and mice have demonstrated a more intimate and systemic interaction, with fetal cells present in the maternal circulation of women lasting not only for the duration of pregnancy but for decades afterwards [328-330]. Furthermore, both human and animal studies demonstrate maternal recognition of paternal antigens at sites distant to the uterus [331-333]. These studies together demonstrate an incomplete barrier exists between mother and fetus.

1.4.3.2 "Antigenic immaturity of the foetus"

Cell surface MHC antigens are the molecular mechanism by which the immune system determines self from non-self and mediates an immune response accordingly, for example in the case of organ transplantation from a genetically distinct donor. As mentioned above the conceptus is known to express non-classical MHC class Ib antigens as well as classical MHC class Ia antigens. Since the conceptus is a genetic fusion of maternal and paternal origin, the MHC antigens it expresses are alien to the mother.

Trophoblastic MHC class I pattern of expression is unique to this tissue. In humans, expression of key cell surface molecules involved in antigen presentation to cytotoxic T cells is down regulated. Constitutive expression of these classical MHC class Ia molecules, human leukocyte antigen-A (HLA-A) and HLA-B is replaced with that of non-classical MHC class Ib molecules, HLA-G and HLA-E, and induction of classical HLA-C [334-336]. NK cells are known to express inhibitory receptors for HLA-C, –E and –G and furthermore, association of NK cells with these molecules on trophoblasts inhibits the cytotoxic actions of these leukocytes towards the allograft [337, 338]. Whether a similar mechanism is employed by mouse trophoblasts has yet to be determined, but non-classical MHC

molecules bc1/2 and Qa-1 are expressed and may function in an analogous manner to HLA-G and HLA-E [339].

MHC class II molecules are not expressed on trophoblast cells from successful pregnancies. IFNγ induced MHC class II expression is reliant on expression of MHC class II transactivator (CIITA). The CIITA gene contains an IFNγ responsive promoter (PIV), which in trophoblasts is hypermethylated, preventing expression of CIITA and consequently inhibiting MHC class II expression [340]. Mouse models demonstrate a detrimental effect following induction of MHC class II in the placenta, resulting in spontaneous abortion. Human studies add to these findings, showing placental cells from recurrent miscarriage can express MHC class II molecules whereas tissue from normal pregnancies do not [341].

Scientific investigation has challenged Medawar's second hypothesis, by providing evidence that the fetus is not immunologically immature, rather in fact expresses HLA molecules that are recognised by maternal leukocytes. However it is clear that the pattern of expression is highly specialized and restricted largely to non-polymorphic MHC.

1.4.3.3 "Immunological inertness of the mother"

Pregnancy does not render the mother susceptible to infection, unable to reject allogenic transplants nor unresponsive to immunization. Hence the hypothesis that the maternal immune system is inert during pregnancy cannot be upheld. Instead, investigations now suggest that an immunologically tolerant state to fetal antigens is actively generated within the mother. However it is clear that high levels of progesterone together with the abundant secretion of type2 cytokines from the placenta in pregnancy can exert a generalised immune suppression or skewing of the immune response [342, 343].

Medawar's hypotheses of anatomical separation, fetal antigenic immaturity and maternal immune inertness can not, therefore, be upheld. Instead we now believe that active immune tolerance mediated by Treg cells acting in concert with other antigen non-specific suppressive mechanisms is responsible for survival of the antigenetically dissimilar fetus.

Studies in mice, conducted by Tafuri et al [327], have illustrated maternal T cell acquisition of a transient state of tolerance towards paternal antigens during pregnancy. Further experiments have demonstrated that this paternal antigen specific tolerance is initiated at insemination whereby paternal

antigen priming results in permissive skin or tumour allografts of paternal origin or rejection of allograft from a third party at sites distant to the reproductive tract [331, 344, 345]. However, exposure to paternal antigen alone is not sufficient to generate tolerance and Beer and Billingham were the first to propose a role for seminal plasma in the acquisition of maternal tolerance to paternal antigens introduced at insemination [345].

This state of antigen specific immunological tolerance is thought to be affected by altered T cell response to recognition of paternal antigens and has led to the proposal of a pregnancy-related skewing in the maternal immune environment towards tolerogenic type2/type3 and away from protective/rejective type1. The direction of an immune response is dependent upon the cytokine environment in which antigen presenting cells, dendritic cells and macrophages, first encounter the antigen. Dendritic cells are known to populate the female reproductive tract, sample the environment and traffic to the draining lymph nodes [280]. Dendritic cells will then either stimulate or tolerize T lymphocytes, depending on their own activation state. Seminal components and uterine cytokine production, including GM-CSF and IL-10, following insemination may be contributing factors in balancing the type2/type3 – type1 immune balance. Reconstitution of abortion prone mice with GM-CSF or IL-10 is protective against pregnancy loss, whereas in humans high levels of the Th1 cytokine, IFNγ, is found in women experiencing recurrent miscarriage [346].

1.5 SEMINAL PLASMA

Semen consists of cellular and molecular components derived from the gonads, male accessory sex glands and the genitourinary tract. The cellular component of semen comprises not only spermatozoa but also leukocytes and epithelial cells shed from the genitourinary tract. The World Health Organisation defines normal sperm concentration, normozoospermia, in the ejaculate of men to be 20 – 250 million sperm/mL and normal leukocyte concentration to be below 1 million/mL [347]. In mice an estimate of 6 – 7 million has been proposed as the sperm content per ejaculate, based on counts in fluid retrieved from mated uteri [348].

The liquid portion of semen, seminal plasma, is composed of secretions primarily from the seminal vesicles, with smaller contributions from the epididymis and prostate [349]. The constituents of seminal plasma include moieties such as prostaglandins, polyamines, zinc compounds, metabolic enzymes, hormones and cytokines. Traditionally seminal plasma is thought of as the fluid vehicle for sperm delivery and survival in the female reproductive tract at insemination. Increasingly the role of

seminal plasma in eliciting an inflammatory response and other changes in the female tract is evident [252, 331]. The focus of this section will be on the inflammatory and immuno-modulatory roles of seminal plasma in reproduction.

1.5.1 The role of seminal plasma in post-insemination inflammation

Insemination induces pro-inflammatory cytokine production by the female reproductive tract, reminiscent of a classical inflammatory response. Seminal plasma interaction with the female tract is implicated in generating this transient inflammatory state, through activating synthesis of cytokines such as GM-CSF, IL-6, CSF-1, IL-1α and RANTES [252, 331]. In mice approximately 66% of seminal plasma is derived from the seminal vesicle glands [349]. It is the seminal vesicle component that has been identified as important for initiating the post-mating inflammatory response. Investigations in mice have shown that mating with males from which seminal vesicles have been surgically removed fails to elicit induction of uterine proinflammatory cytokines [123]. Additionally, Choudhuri and Wood demonstrated that induction of these pro-inflammatory cytokines is independent of sperm since mating with either intact or vasectomised males elicits similar responses [310]. Subsequent mouse studies have isolated seminal vesicle TGFβ as a key contributing factor in triggering up-regulation of uterine cytokine GM-CSF [12]. Uterine epithelial cell cytokine secretion following insemination results in the influx of leukocytes into the endometrium. In mice, the most abundant leukocytes initially are neutrophils but their presence is short lived and they disappear by the second day of gestation [258]. A large population of macrophages is recruited to the endometrium following mating, together with other antigen presenting cells, dendritic cells, and these cells persist for at least 48 hours before declining in number by the time of embryo implantation on day 4 pc [12, 123, 256, 258].

While these animal studies implicate seminal plasma as having a regulatory role in post-mating inflammation, the advent of Assisted Reproductive Technologies (ART) has shown that in many animals and in women seminal plasma is not a necessity for establishing successful pregnancies since this can be achieved using in vitro fertilisation and embryo transfer, bypassing female tract exposure to seminal plasma. However studies in several species have shown mating in the absence of seminal plasma not only impairs the ability of sperm to fertilise oocytes but also affects pregnancy outcome. These effects cause changes in fetal development that result in perturbed postnatal growth and metabolic parameters in progeny [5, 6, 14, 350].

1.5.2 Immunomodulation by seminal plasma

Seminal plasma has been shown to possess immune deviating properties that may extend beyond the requirement for safe passage of sperm traversing the female tract. Seminal plasma factors, specifically seminal vesicle derived TGFβ, interacts with female reproductive tract epithelial cells to elicit production of pro-inflammatory cytokines (see 1.4 and 1.5.1) [331]. This cascade of cytokines not only mediates leukocyte recruitment and activation but may also be involved in the events leading to hyporesponsiveness in type1 and enhanced type2 immunity supportive of optimal pregnancy success [331, 351]. Early experiments in mice demonstrated that allo-mated females had a reduced ability to reject skin grafts of paternal origin [344]. Furthermore, Beer and Billingham identified the critical role of seminal plasma in allograft tolerance, when females exposed to whole semen but not sperm alone also exhibited a reduced ability to reject skin grafts of paternal origin [345]. These experiments suggest that antigen exposure in isolation is not sufficient to generate tolerance, but requires also an appropriate cytokine environment in which antigen is encountered. A role for seminal TGFβ in contributing to maternal tolerance of paternal antigens is consistent with the immune deviating role of this cytokine in other mucosal tissues such as the eye and the lung [352, 353].

1.5.3 Antigenic nature of semen

The leukocytes and genitourinary tract epithelial cells present in semen all express paternal antigens that are introduced into the female tract at insemination. These paternal antigens transferred at insemination are also expressed by the conceptus at the feto-maternal interface on trophoblast cells [299]. Major histocompatibility complex (MHC) class I and class II transplantation antigens are expressed by mouse and human sperm and conceptus [354-358], together with minor histocompatibility antigen H-Y and trophoblast-lymphocyte cross-reactive antigens (TLX) [359, 360].

The role of MHC antigens was initially investigated in experiments described above examining allogeneic skin grafts of paternal origin in mated female mice [344, 345]. This concept was expanded using inbred strains of mice that differ only in their MHC class I haplotype. Following mating between intact or sterilized Balb/k female and intact Balb/c male mice, Balb/c tumour cells injected into females were 'tolerated' and grew into measurable tumours, whereas the same tumour cells injected into virgin Balb/k females are rejected [331]. Furthermore tolerance of paternal antigens on tumour cells is not achieved when mating occurs with seminal vesicle deficient males (Robertson, unpublished). These experiments demonstrate exposure to paternal antigens in the presence of seminal vesicle fluid

generates immunological tolerance to these antigens but does not require the presence of a conceptus or an ensuing pregnancy. Paternal antigens present in seminal fluid appear to be capable of eliciting an immune response in the female after insemination. Resident antigen presenting cells (APC), including dendritic cells and macrophages, in the female tract are known to phagocytose and process antigens introduced into the tract [280]. Additionally, temporal enlargement of the uterine draining lymph nodes following mating in mice has been described however paternal antigen containing APC trafficking to these lymph nodes is yet to be investigated.

This may be significant in humans where restricted exposure to whole semen due to the use of barrier contraceptives or short duration of cohabitation is associated with pathologies of pregnancy with an immunological etiology [361].

1.5.4 Immune regulatory molecules in seminal plasma

Over the last forty years the immune deviating properties of seminal plasma have been investigated and the current understanding is described above. Studies aiming to identify seminal factors with a potential role in post-mating inflammation and immune deviation have followed. The presence and fluctuation of a plethora of immuno-regulatory molecules have been described in human seminal plasma of proven fertile and infertile men including TGFβ, IFNγ, IL-1, IL-2, IL-6, IL-8 and TNFα [146, 362, 363]. Recently a multiplex detection system has been employed to show a similar cytokine profile in murine seminal plasma [364]. Significant correlations between perturbed cytokine concentration and infertility have been demonstrated. Increased levels of IL-6, IL-8, IL-2 and IL-11 are associated with abnormal sperm parameters [365-369], while increased IL-11 [370], G-CSF, IL-1α and TNFα [369] are linked with leukocytospermia. Sperm production is negatively correlated with the level of seminal IFNγ [371], while other semen parameters are negatively associated with TNFα and IL-8 [368, 372].

The genitourinary tract is a mucosal organ similar to that of the gastrointestinal and respiratory tracts. Mucosal surfaces are known to both facilitate a symbiotic relationship between non-pathogenic (commensal) organisms and their host as well as acting as a protective barrier against pathogenic organisms [373-376]. While the upper female reproductive tract is considered to remain sterile, except following mating, the lower female tract mucosa is constantly exposed not only to whole bacteria but also their products [377-379]. These include potent pro-inflammatory cell wall components lipopolysaccharide (LPS) and lipoteichoic acid (LTA) from gram negative and positive bacteria

respectively. Bacteria are also abundant in seminal fluid, examination of boar and human semen has revealed the presence of LPS which may contribute to the female immune response to mating when they are introduced during the natural course of insemination [130, 380].

1.5.4.1 Seminal Transforming growth factor β (TGFβ)

TGFβ is present at high concentrations in seminal plasma and is produced in the seminal vesicles and prostate mice (74 ng/mL) and humans (238 ng/mL) respectively [12, 381]. The immune deviating capacity of TGFβ has been explored in other tissues and has been shown to be a potent factor in the generation of tolerance [382-384]. Furthermore, seminal TGFβ in both mice and humans is a major contributor to the immunosuppressive effects of seminal plasma [381, 385]. TGFβ instilled into the murine uterine lumen stimulates GM-CSF secretion and leukocyte recruitment reminiscent of the postmating inflammatory response [12]. It is not known whether the TGFβ in seminal plasma can account for the full range of cytokines, including IL-6, and chemokines induced in the murine uterus. This is possible since TGFβ is a potent stimulator of IL-6 in a primary human lung fibroblasts culture system [386].

Seminal TGFβ is a combination of both active and latent forms, with 25% being active. However 70% of TGFβ recovered from the uterine lumen of mice following insemination is biologically active [12]. This activation after ejaculation is most likely the result of the acidic environment of the lower female reproductive tract together with enzymes present in seminal plasma or secreted into the luminal compartment from female tract cells [387].

1.5.4.1.1 Modulators of TGFβ in semen

TGFβ has been identified as having a pivotal role in instigating the maternal tolerogenic immune response to mating, however examination of human seminal plasma has shown firstly that TGFβ levels do not fluctuate significantly over time and secondly that there is no difference in TGFβ concentrations found in the semen of proven fertile and infertile men [130]. In contrast a higher proportion of males from couples suffering recurrent miscarriage exhibited detectable to high concentrations of seminal IFNγ than those proven fertile. Other groups have identified altered concentrations of various immune regulators in seminal plasma from infertile men (defined as showing abnormal sperm count by WHO criteria), including endotoxin, IFNγ and TNFα [15, 362, 388-391]. These findings together with in vitro experiments described in 1.2.2.2, demonstrate the potential modulatory effect of these moieties on

TGFβ stimulated activity and may suggest a regulatory role for these molecules in TGFβ induced maternal tolerance.

1.5.4.2 IFNγ

IFNγ, a pleiotropic pro-inflammatory Th1 cytokine, is a member of the interferon family of glycoproteins [392]. Interferons were initially identified by their ability to interfere with viral replication. To date there have been two types of interferons identified, type I and type II. Type I interferons include alpha, beta and tau, whereas IFNγ is the only member of the type II group. IFNγ is primarily secreted by activated T-cells and natural killer cells and is involved in immunomodulatory functions beyond its defence against intracellular and viral activity, including chemotactic agent for neutrophils and eosinophils by inducing expression of intercellular adhesion molecule-1 (ICAM-1) and MHC class II and activation of macrophages and natural killer cells [393, 394].

1.5.4.2.1 IFNγReceptors and Signalling

The IFNγ receptor complex is comprised of an alpha and a beta subunit. The alpha subunit, otherwise known as IFNγ receptor 1 (IFNγR1 or IFNGR1), contains the high affinity ligand binding domain of the complex but is unable to elicit the biological activities of this cytokine [395]. The beta subunit, initially identified as an accessory factor (AF-1) and later named IFNγ receptor 2 (IFNγR2 or IFNGR2), together with IFNγR1, is obligatory for cellular responsiveness to IFNγ [396].

Janus kinase family members JAK-1 and JAK-2, constitutively associated with IFNγR1 and IFNγR2 respectively, are intracellular signalling molecules activated following ligand binding and are required for IFNγ responsiveness [397, 398]. JAK-1 and JAK-2 are then auto- and trans phosphorylated and subsequently phosphorylate a recognition sequence for a member of the **s**ignal **t**ransducer and **a**ctivator of **t**ranscription family, STAT-1, on IFNγR1 [397, 399, 400]. STAT-1 phosphorylation results in the formation of a homodimer, gamma activation factor (GAF) [401]. The GAF dimer translocates to the nucleus where it binds to gamma activation site (GAS) response elements within the promoter region of responsive genes culminating in targeted gene expression [402, 403].

IFNγ responsive genes exceed 200 in number and include suppressor of cytokine signalling -1 (SOCS-1), class II transactivator (CIITA) and interferon regulatory factor family members (IRFs) [145]. SOCS-1 is a negative regulator of IFNγ signalling and is discussed in detail below. CIITA is a

transcription factor required for inducible MHC II expression following stimulation of cytotoxic T lymphocytes (CTLs) [404]. IRFs, particularly IRF-1, in conjunction with STAT-1 are essential not only for basal levels of MHC class I expression but also for IFNγ induced expression [405].

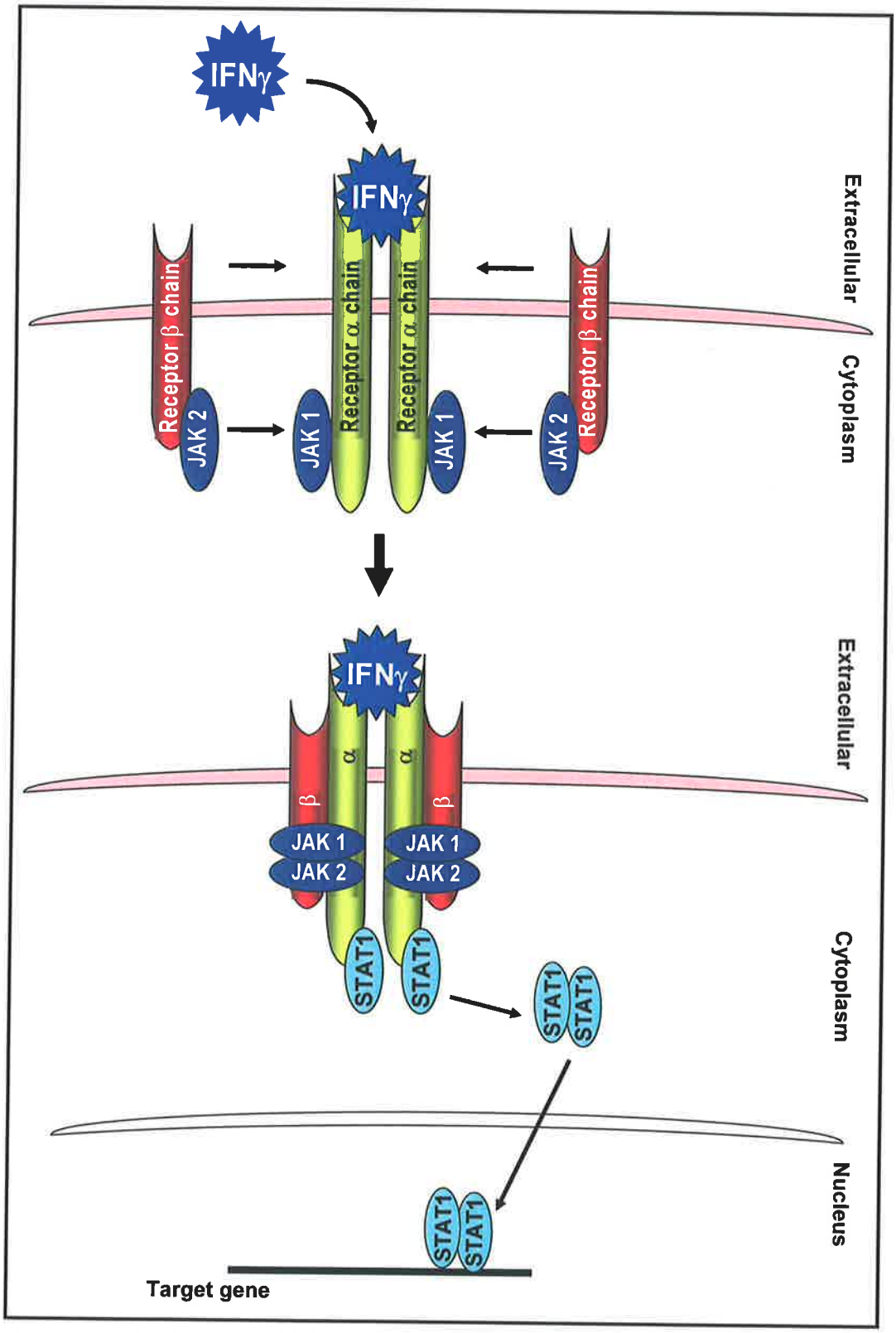


Figure 1.2 Schematic of IFNγ binding to its main receptors and signal transduction

1.5.4.2.2 *Regulation of IFNγ signalling*

SOCS-1 is upregulated by IFNγ and binds to and inhibits JAK-2 phosphorylation and activation, in turn preventing STAT-1 phosphorylation and inhibiting IFNγ targeted gene expression [406, 407]. SOCS-1 is thus an integral component of a negative feedback mechanism, in place to modulate cellular responsiveness to IFNγ [408]. Investigations using SOCS-1 knockout mice (SOCS-$1^{-/-}$) identified a neonatal lethal phenotype exhibiting low body weigh, necrotic liver, macrophage and granulocyte organ infiltration and depletion of lymphocytes [409-411]. This lethality can be overcome by daily injection of anti-IFNγ antibodies, demonstrating its necessary role in controlling IFNγ signalling [412]. These studies demonstrate the deleterious effects of excessive/uncontrolled IFNγ activity. In addition to the SOCS-1/IFNγ negative feedback loop, regulation of IFNγ signalling may be mediated through interference with receptor phosphorylation hence restriction of STAT-1 binding and phosphorylation. Unphosphorylated STAT-1 ubiquitination targets it for proteosome degradation [397, 410, 413, 414].

1.5.4.3 *IFNγ Antagonists*

A number of IFNγ antagonists have been identified including the neuroendocrine mediator thyrotropin (Thyroid-Stimulating Hormone – TSH), TGFβ, β-lactam benzylpenicillin, soluble virus encoded IFNγ receptors and a family of lipid lowering agents – statins.

Thyrotropin (TSH) is a 28 kDa glycoprotein produced and secreted by pituitary derived thyrotrope cells [415] in response to an increase in circulating thyrotropin-releasing hormone (TRH) concentrations. TSH and its receptor (TSHR) are key mediators in the control of thyroid function including directing iodine uptake and utilization, promotion of thyroid growth and anti-apoptotic protection for thyroid cells. Park et al [416] delineated the mechanism underlying TSH inhibition of IFNγ activity, whereby following TSH exposure rat thyroid cells upregulate two SOCS family members, SOCS–1 and –3 [416]. SOCS-1 and –3 reduce STAT-1 and JAK-1 phosphorylation required for IFNγ signalling, significantly inhibiting activation of an IFNγ target, intercellular adhesion molecule (ICAM)-1 [417].

Two of the three mammalian isoforms of TGFβ, β_1 and β_2, are known to down regulate MHC class II expression induced by IFNγ [418, 419] and further Armendariz-Borunda et al [420]

demonstrated HLA-DR expression stimulated by IFNγ was significantly reduced following TGFβ$_1$ exposure. This may indicate a regulatory mechanism limiting T-cell activation in an immune response.

Research into penicillin allergy research lead Brooks et al [421] to examine interactions between the prototypic β-lactam antibiotic and IFNγ using an array of immuno- and bio-activity assays. Evidence suggested that penicillin binds directly to IFNγ, inhibiting immunodetection and biological activity.

Successful infection of target cells and survival of virus within a host is dependent to a degree on the ability of the virus to avoid or manipulate host defences. IFNγ is a key mediator of anti-viral activity in vertebrates and so identification of a virally encoded soluble IFNγ receptor (vIFNγR) was not surprising. The rabbit myxoma virus was the first to be identified as having the ability to inhibit IFNγ bioactivity via its secreted T7 protein [422]; subsequently it was identified that this protein contributed to its virulence. Following this discovery numerous orthopoxviruses have been recognised as containing similar sequences, including camelpox, cowpox, vaccinia and ectromelia virus [423]. The acutely species specific nature of IFNγ it is somewhat surprising given that the soluble vIFNγR products are reported to have broad species activity not only restricted to mammals but extended to chickens [424].

Hypercholesterolemia is routinely treated with 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, or statins. Statins are a group of molecules shown to have serum lipid-lowering properties, significantly reducing morbidity and mortality associated with cardiovascular disease. Since Kobashigawa et al [425] initially described the beneficial effect of Pravastatin therapy on heart transplant outcomes, a series of investigations into the immunomodulatory effects of related statins has ensued. MHC-II expression is tightly controlled by the transcriptional coactivator, class II transactivator (CIITA). IFNγ induced MHC-II production is via stimulation of the CIITA promoter IV. Statin family members; atorvastatin, pravastatin, lovastatin and simvastatin have been shown to exert their anti-IFNγ activity by inhibiting activation of the CIITA promoter IV hence MHC-II expression [426]. The benefits observed in heart or kidney transplant patients undergoing statin therapy together with in vitro studies provides support for the use of these drugs as therapeutic immunosuppressive agents [427]. These drugs are potentially useful for preventing organ transplant rejection and also for pathologies where IFNγ induced MHC-II expression/T-cell activation may play an integral role such as in autoimmune disease states including type 1 diabetes and rheumatoid arthritis.

1.6 PATHOLOGIES OF PREGNANCY AND FETAL PROGRAMMING

Described in various sections above are the conditions required for normal endometrial maintenance, turnover and preparation for embryonic receptivity, implantation and growth, as well as direction of the maternal immune response to paternal antigens introduced during intercourse and hence to the semi-allogenic conceptus. Generation and maintenance of a type 2 immune response to sexually introduced alloantigens is critical for optimal placental and embryonic growth and development. Perturbations at any point in the reproductive cycle can lead to infertility or impaired pregnancy outcome. Recurrent spontaneous abortion, implantation failure, pre-eclampsia and intra-uterine growth retardation are some pathologies of pregnancy hypothesised as having an immunological basis [428-430].

1.6.1 Pregnancy pathologies

Cytokines play an integral role in endometrial regulation, so identifying links between altered uterine cytokine profiles and specific reproductive conditions may lead to an understanding of the pathophysiological mechanisms behind some forms of infertility. A skewed type 1 cytokine profile is implicated in pregnancy loss and pre-eclampsia [431]. Changes in other cytokines may also be important. LIF is essential for successful blastocyst implantation in the mouse model [183], perhaps indicating a potential physiological role for LIF in implantation in humans. Indeed low uterine luminal fluid concentrations of LIF correlates with unexplained early pregnancy loss in humans [178, 432].

Similarly, implantation failure has been demonstrated in IL-11Rα null mice [433] due to defective decidualisation. Experiments in endometrial and chorionic tissues from humans show a significant reduction in IL-11 production by decidual and chorionic samples from women with a diagnosed anembryonic pregnancy compared to those with a normal pregnancy [434]. Together the animal and human studies suggest decreased IL-11 production/signalling during decidualisation and implantation might contribute to implantation failure and or early pregnancy loss. However further investigation is needed to clarify if altered IL-11 production is a cause or a consequence of these pathologies.

IL-6 is normally up-regulated during the menstrual/estrous cycle in humans and mice. However a significant reduction in endometrial IL-6 mRNA expression during these menstrual phases has been shown in women suffering recurrent miscarriage [435, 436]. This is consistent with observations of increased resorption rates in IL-6 -/- mice (Robertson, abstract 2000).

GM-CSF has been shown to increase the developmental competence of in vitro derived embryos in mice, humans and cattle [111, 127, 134, 437]. Experiments using GM-CSF null mice show impaired blastocyst formation, increased rates of resorption and peri-natal mortality with fetal growth restriction. While this indicates pregnancy is possible without GM-CSF, there is substantial evidence supporting an optimising role especially in embryo development. This finding is further substantiated by results obtained by Chaouat et al [438], where exogenous administration of GM-CSF to abortion prone CBAxDBA/2 mice reduced resorption rates and increased fetal and placental weights.

Darmochwal-Kolarz et al [439] examined the relative expression of Th1 and Th2 cytokines expressed by peripheral blood lymphocytes in women with a normal and pre-eclamptic pregnancy. In pre-eclamptic women the expression of IL-2 was significantly increased compared to normally fertile control women. Indicating a skewed Th1/Th2 immune response away from tolerogenic type 2 towards one resembling a type 1 response. A study of women experiencing either multiple implantation failures or recurrent spontaneous abortion demonstrated a similar Th1 skewed cytokine profile as in the previous study, when compared to women with normal pregnancies [440].

Alteration in the proportion and or function of different leukocytes present in the endometrium during pregnancy has also been indicated in sub-optimal pregnancy outcomes. Arck et al [221] reported the detrimental effect of excessive $\gamma\delta$ T cells in the endometrium on pregnancy survival in mice and conversely that immunological acceptance of the embryo is dependent on the presence of $\alpha\beta$ T cells. Depletion of $\gamma\delta$ T cells was shown to decrease abortions in the abortion-prone CBA x DBA/2 model, where depletion of $\alpha\beta$ T cells in the same model results in a 100% abortion rate. The direction of T lymphocyte activation and differentiation elicited by antigen presenting cells (APC) is thought to primarily be driven by the specific cytokine microenvironment in which the APC was first exposed to the antigen. Preferential recruitment of $\gamma\delta$ T cells (Th1) is thought to be mediated via upregulation of endothelial adhesion molecule, P-selectin, the ligand for which is principally expressed on Th1 lymphocytes [441]. It is not clear how P-selectin is regulated.

Studies in mice have shown that NK cells play a role in placental growth, development and maintenance through the local secretion of IFNγ [442]. IFNγ expression and activity during pregnancy requires a high degree of regulation. Since studies in knockout mice demonstrate that a deficiency of this cytokine or inhibition of its signalling pathway leads to altered NK cell numbers and morphology in the uterus [443]. Furthermore, decidual necrosis and inadequate arterial remodelling was evident [444]. The morphological features observed in these studies were reminiscent of those seen in

pre-eclamptic women. It could be postulated that women suffering pre-eclampsia have a defect in either NK cell recruitment or function, or at some point in IFNγ production or signalling pathway. Similarly, detrimental outcomes are observed following exogenous administration of IFNγ during murine pregnancy with embryotoxic antibody production by the mother subsequently leading to poor fetal development or abortion as well as maternal haematological disorders [445, 446].

Clearly the processes involved in reproduction are multifaceted and tightly controlled in the 'normal' situation. Given the complexity of the mechanisms involved it is understandable, or even expected that this balance is highly susceptible to perturbation resulting in a sub-optimal pregnancy or infertility.

1.6.2 Fetal programming

The section above describes the current understanding of the implications of a perturbed uterine cytokine environment on acute pregnancy outcomes, such as miscarriage, pre-eclampsia and intra-uterine growth restriction. There is a growing body of evidence suggesting that in utero perturbations that do not necessarily result in an acute maternal or fetal pathology may contribute to life long increased susceptibility to coronary and metabolic disorders in progeny. Epidemiological studies conducted by David Barker showed significant correlations between low birth weight and increased risk of developing high blood pressure and ischemic heart disease in adulthood [447-449]. Subsequent studies have shown not only is low birth weight a risk factor for these conditions but more precisely, low birth weight coupled with an increased rate of weight gain after 2 years of age [450]. This phenotype appears to be a predictor of developing insulin resistance, abdominal obesity and spontaneous hypothyroidism in adulthood as well as high blood pressure and coronary heart disease [451-453].

Maternal nutrition, placental function, pregnancy pathologies and maternal stress are factors thought to influence fetal programming by altering in utero nutrient or oxygen availability or steroid exposure. In utero exposure to these stressors effects organ size and development, hormone/endocrine axes (hypothalamic-pituitary-adrenal-axis) and autonomic nerve development, which lead to the clinical signs of hypertension, insulin resistance and obesity. Together these symptoms are the defining features of what is now termed the Metabolic Syndrome predisposing to increased risk of cardiovascular disease.

In an evolutionary context, fetal stressors such as nutrient availability and steroid exposure could be seen as a mechanism of signalling to the developing fetus, relaying information on the likely post-natal environmental conditions. Communication in this manner may encourage fetal development optimal for survival under harsh post-natal conditions, known as the 'thrifty' phenotype. However, the current climate of excess nutritional availability and sedentary lifestyle is incompatible with the thrifty phenotype, hence development of the prototypic symptoms of the Metabolic Syndrome. Animal studies are able to consistently reproduce this phenotype using interventions such as restricted maternal nutrient intake or restriction of placental growth [454, 455].

The molecular and biochemical processes responsible for fetal programming remain to be unravelled. There is growing evidence to suggest that maternal nutrition can influence fetal DNA methylation patterns and histone modifications, in a process known as epigenetic regulation. Other stressors including embryo culture ex vivo, exposure to toxic metabolites or growth factor deprivation may exert similar effects [125, 456-458]. Proposed targets of epigenetic regulation that may effect fetal growth include nitric oxide synthase (NOS) and ornithine decarboxylase (ODC). NOS is responsible for the synthesis of nitric oxide (NO), which in turn is controls placental blood flow to the fetus and hence the availability of nutrients and oxygen [459]. Polyamines are synthesised by ODC and are important in DNA and protein synthesis [460, 461]. Together NO and polyamines are essential in placental and fetal growth and development. The significance of these entities has been demonstrated in animal models where NO inhibition results in fetal growth restriction [462] and polyamine deficit reduces placental size and fetal growth [461]. Additionally IUGR in humans is associated with significantly reduced circulating NO in both mother and fetus [463].

1.7 SUMMARY

Generation of an appropriate maternal immune response to paternal antigens expressed by the conceptus is essential for optimal pregnancy outcome. Seminal fluid may have a role in inducing immune tolerance to male transplantation antigens prior to embryo implantation. Embryo development, implantation, endometrial receptivity, placentation and optimal fetal growth all rely on the regulated expression of a multitude of cytokines and other molecular signals in a tightly controlled spatiotemporal manner. The literature to date indicates an important role for semen in generating maternal immune environment conducive to optimal embryo implantation and development.

Human studies demonstrate links between perturbations in the immune and cytokine axis and various pathologic states in reproduction, including implantation failure, recurrent miscarriage and pre-eclampsia. The maternal immune response may also contribute to fetal programming in utero with its life-long implications for progeny health. Numerous studies in animal models have shown direct causal links between dysregulation of many molecular and cellular events involved in the cascade towards pregnancy and compromised pregnancy outcome. However it remains unclear how these perturbations arise, whether they are environmental or genetic in nature or of maternal or paternal origin.

We propose that moieties present in seminal fluid can promote generation of an immunological and cytokine environment that facilitates implantation, and that this process may be dysregulated in some individuals. This might occur when the abundance of seminal fluid signalling agents is abnormal, or when inhibitory factors capable of perturbing the maternal immune or cytokine response are present in seminal fluid. The studies described in this thesis aim to examine the interaction between regulatory factors present at insemination and the maternal uterine response and to investigate the implications of these interactions for pregnancy success and progeny health.

1.8 HYPOTHESES

The experiments described in this thesis aim to address the following hypotheses:

- A range of factors present in seminal fluid interact with uterine epithelial cells at insemination to regulate the post-mating inflammatory response.

- Perturbations of the peri-conception uterine environment will have a negative impact on the ensuing pregnancy outcome and progeny health.

1.9 AIMS

The experiments described in this thesis are aimed to investigate:

- The regulatory role of signalling agents in seminal fluid on cytokine production by murine uterine epithelial cells *in vitro*.

- The impact of seminal fluid signalling agents on cytokine and leukocyte parameters in the murine estrus uterus *in vivo*.

- The significance of altered peri-conception uterine cytokine environment on pregnancy outcome.

- The significance of altered peri-conception uterine cytokine environment on post-natal parameters, progeny growth and metabolic markers.

Chapter 2

Materials and Methods

2.1 ANIMALS

Mice used in these studies were purchased from the University of Adelaide Laboratory Animal Services and were used in accordance with ethics approval obtained from the University of Adelaide Ethics Committee (approval number M-61-04). Mice were housed at the Medical School Animal House in a pathogen free facility under a 12 h light - 12 h dark cycle with food and water available ad libitum. CBA x C57Bl/6 F1 (CBA F1) female mice aged 8-12 weeks were used for the majority of *in vitro* experiments unless otherwise specified. C57Bl/6 female mice aged 8-12 weeks were used for the majority of *in vivo* experiments unless otherwise specified. C3H/HeJ females were used for some *in vitro* and *in vivo* experiments. Estrus was determined by examining vaginal smears (see below for detail). Male BALB/c mice up to the age of 12 months were housed individually and used in all matings. Day 1 of pregnancy was determined by observation of a vaginal plug following mating with an intact or vasectomised male or by observing sperm in a vaginal smear after mating with a seminal vesicle deficient male (kindly provided by Dr John Bromfield).

In order to ascertain estrous cycle stage of female mice, 20 µL of sterile PBS was gently pipetted into the vagina and immediately removed. The lavage was transferred onto a microscope slide and examined, unstained, under a phase contrast microscope (Nikon TMS, Japan). Vaginal sears were classified as proestrus, estrus, metestrus-1, metestrus-2 or diestrus according to the cellular content of the smear as described previously [464] (see Table 2.1).

Stage	Cellular characteristics of vaginal smear
Proestrus	E – E C or E C L – E C
Estrus	E C - C++
Metestrus-1	C++ (clumped)
Metestrus-2	C++E L++ (large clumps)
Diestrus	E L (+/- mucus)

Table 2.1 Changes in the cellular characteristics of mouse vaginal lavage during the estrous cycle. E - epithelial cells, C - cornified epithelial cells, L - leukocytes, + - many cells, ++ - very many cells

Surgery for vasectomy or seminal vesicle gland removal from male BALB/c mice was conducted as described previously [331]. Briefly, males undergoing either procedure were anaesthetised and the abdominal cavity exposed via a small lateral incision. Wounds were sutured using 5/0 silk suture on a cutting needle (Ethicon, Sydney, Australia). Seminal vesicle glands were carefully excised after ligation 5/0 silk suture (Ethicon) at the proximal tubule with, leaving the coagulating glands intact, to produce seminal vesicle deficient (SVX) males. SVX males were mated three times in order to clear the tract of any residual seminal vesicle fluid. To generate vasectomised (VAS) males, the vas deferens was bisected by quarterisation following ligation with 5/0 silk suture at the proximal tubule. As with the SVX males, VAS males were mated three times to clear the tract of residual sperm. The inability to generate a pregnancy following a fourth mating was confirmation of a successful vasectomy.

Seminal vesicle fluid was collected from stud males of proven fertility. Secretions from the seminal vesicle glands were gently extruded and solubilised in 6 M guanidine HCl (~100 µL fluid recovered per gland). The fluid was then desalted by Sephadex G-25 chromatography. Solubilized seminal vesicle fluid was applied to a 5 mL Sephadex G-25 desalting column (Pharmacia, Uppsala, Sweden). Five millilitres of RPMI was passed through the Sephadex column and the eluted material was collected in 0.5 mL fractions which were stored at -80°C until required for addition to uterine epithelial cell culture.

2.2 IN VITRO CULTURE OF PRIMARY MURINE UTERINE EPITHELIAL CELLS

2.2.1 General

All *in vitro* experiments were carried out using aseptic techniques. Cultures were incubated at 37°C in 5% CO_2 and monitored for cell adherence, growth and to confirm absence of contamination using a phase contrast microscope (Nikon TMS, Japan).

2.2.2 Culture Medium

RPMI – 1640 (JRH-CSL, Victoria, Australia) was supplemented with 5% or 10% fetal calf serum (FCS) (JRH-CSL) as specified, 5 x 10^{-5} M β-mercaptoethanol, 2 mM L-glutamine, 60 µg/mL penicillin-G and 100 µg/mL streptomycin (RPMI-FCS). RPMI-FCS was used to culture FD5/12 [465] cells and enriched uterine epithelial cells. Experiments involving IFNγ were conducted in the absence of penicillin-G (RPMI-FCS -P).

In some experiments RPMI–1640 was supplemented with 0.5% FCS, 1% Nutridoma-SP (Roche Diagnositics, Mannheim, Germany), 5 x 10^{-5} M β-mercaptoethanol, 2 mM L-glutamine, 60 μg/mL penicillin-G and 100 μg/mL streptomycin (RPMI-SP). RPMI-SP was used to culture 7TD1 [466] cells.

2.2.3 Recombinant cytokines, antibodies and bioactive molecules.

E. coli derived recombinant mouse TNFα, recombinant mouse rmIFNγ, recombinant mouse GM-CSF, CHO cell derived recombinant human rhTGFβ$_1$ and rhTGFβ$_2$ were purchased from R&D Systems (Minneapolis). *Salmonella typhimurium* and *E. coli* derived lipopolysaccharide (LPS), *B. subtilis* lipoteichoic acid (LTA) and Thyrotropin (TSH) were purchased from Sigma Aldrich (St. Louis, MO). *E. coli* derived recombinant human rhIL-6 was purchased from Roche Diagnositics and Simvastatin was purchased from Wako Pure Chemicals (Japan). Recombinant human rhTGFβ$_3$ (lyophilised drug product 903) and the gel solvent for reconstitution, hydroxypropylmethlycellulose (hypromellose gel) were provided by GroPep Pty Ltd, (Thebarton, Australia).

2.2.4 Uterine Epithelial Cell Culture

Uteri were harvested from estrous mice and epithelial cell cultures were prepared as previously described [152]. Briefly, uteri were excised, placed in cold PBS, trimmed of fat, mesentery and blood vessels, and slit length-wise. Pooled uteri were subjected to a 0.5%-trypsin + 2.5%-pancreatin digest for 45 min at 4°C followed by 45 min at 37°C. An equal volume of RPMI-FCS or RPMI-FCS -P was then added and the uteri passaged several times up and down a plastic transfer pipette. The liberated epithelial cells were pelleted at 1100 rpm and washed in RPMI-FCS or RPMI-FCS -P. Cell cultures prepared by this method are comprised of approximately 75% epithelial cells and 25% stromal cells [109].

Epithelial cells were resuspended at 4 x 10^5 cells/mL in RPMI-FCS or RPMI-FCS-P and 100 or 500 μL aliquots were plated in 96-well or 4-well mulitdishes (Nunc, Roskilde, Denmark) respectively as indicated. Cells were incubated for 4 hrs at 37°C in 5% CO_2, to permit adherence, then treatment or media alone was added in duplicate. Treatment-containing media was replaced with fresh media following overnight (16 hrs) incubation. Culture supernatants were collected after a further 24 hrs incubation, centrifuged at 1100 rpm to remove any cellular material and stored at -20°C until assay.

Adherent cells were quantified following Rose Bengal dye uptake (0.25% in PBS, 5 minutes at room temperature) (Faulding, Adelaide, Australia) and cell lysis in 1% SDS by measuring absorbance at 570 nm in a Bio-Rad microplate reader (Bio-Rad Laboratories, CA).

2.2.5 Cytokine ELISAs

Uterine epithelial cell culture supernatants were assayed for GM-CSF, IL-6 and KC immunoactivity in cytokine specific sandwich ELISAs (R&D Systems, Minneapolis).

2.2.5.1 GM-CSF ELISA

The GM-CSF ELISAs were conducted according to the manufacturer's instructions. Briefly, following an overnight incubation with a rat anti-mouse GM-CSF monoclonal antibody in a 96-well maxisorp ELISA plate (Nunc, Roskilde, Denmark) and blocking, standards, controls and samples were then added. An affinity purified polyclonal goat anti-mouse biotinylated antibody was added and quantified by the chromogenic reaction produced by incubation with streptavidin-HRP conjugate and substrate. Absorbance was measure on a Benckmark™ microplate reader (Bio-Rad Laboratories, Hercules, US) at 450nm. The graphing software, SigmaPlot 10, was used to generate standard curve using a 4-parameter logistic equation from which GM-CSF content in samples was calculated. The sensitivity of this assay described by the manufacturer is 1.8 pg/mL with an inter assay variation of 10%.

2.2.5.2 IL-6 ELISA

The IL-6 ELISAs were conducted according to the manufacturer's instructions as outlined in 2.2.5.1 above. The sensitivity of this assay described by the manufacturer is 1.6 pg/mL with an inter assay variation of 22%.

2.2.5.3 KC ELISA

The KC ELISAs were conducted according to manufacturers' instructions as outlined in 2.2.5.1 above. The sensitivity of this assay described by the manufacturer is 2 pg/mL with an inter assay variation of 8%.

2.2.5.4 *Cytokine bioassays*

In some experiments, GM-CSF and IL-6 bioactivity in supernatants was measured in cytokine specific bioassays using the GM-CSF dependant cell line FD5/12 [465] and IL-6 dependent cell line 7TD1 [466] respectively.

2.2.5.5 *GM-CSF Bioassay*

Duplicate serial dilutions of culture supernatants were incubated with $2x10^3$ FD5/12 cells in 96 well multidishes (Nunc, Roskilde, Germany) in a total volume of 200 µL, at 37°C in 5% CO_2 for two days. The cells were then pulsed for six hrs with 50 µL of 20 µCi/mL ^{3}H-thymidine (Amersham, Arilington Heights, IL) and harvested onto glass fiber paper by a PHD cell harvester (Cambridge Technology, Inc.). A Beckman LS 600LL liquid beta scintillation counter was used to measure radioactivity. A twelve point standard curve, beginning at 4 ng/mL, was included in every assay. Regression analysis in graphing software, SigmaPlot 10, was used to fit a 5-parameter sigmoidal equation to data points in the standard curve. This equation was then used to calculate sample values.

2.2.5.6 *IL-6 Bioassay*

Serial dilutions of culture supernatants were incubated in duplicate with $2x10^3$ 7TD1 cells in a total volume of 200 µL, in 96 well multidishes at 37°C in 5% CO_2 for four days. 25 µL of AlamarBlue (Astral Scientific, Gymea, Australia) was then added to each well and incubation was continued for four hrs. The absorbance of the colorimetric reduction of AlamarBlue was measured on a Bio-Rad microplate reader at 570 nm with a reference wavelength of 595 nm. A sixteen point standard curve, beginning at 4 ng/mL, was included in every assay. Regression analysis in the graphing software, SigmaPlot 10, was used to fit a 4-parameter sigmoidal equation to data points in the standard curve. This equation was then used to calculate sample values.

2.2.6 Real time RT-PCR

2.2.6.1 *Tissue Collection.*

Uterus, kidney and liver were collected from estrous mice. Approximately one third of uterine tissue collected was snap frozen, intact whole uterus (W Ut), in liquid nitrogen and stored at -70°C, while the remainder was digested (see 2.2.4 above) to generate enriched uterine epithelial cells (UEC) and residual stroma (RS).

2.2.6.2 RNA Extraction.

RNA was extracted from tissue in RNAzol B (Tel-Test, Friendswood, Texas) followed by chloroform treatment and centrifugation at 13 000 rpm for 15 min. Addition of 2 volumes of cold 97% ethanol to the decanted aqueous phase precipitated total cellular RNA which was then stored at -20°C.

2.2.6.3 DNase Treatment.

Samples were pelleted at 14 000 rpm for 30 min, washed twice in 70% ethanol, dried for 30 min then resuspended in MQ H_2O. Following addition of 5 µL of 10 U/µL DNase I (Roche, Basel, Switzerland), 1 µL RNase Inhibitor (Roche) and 25 µL of 5 x DNA buffer, samples were incubated for 1 hr at 37°C. 125 µL saturated phenol and 125 µL of chloroform and isoamyl alcohol (24:1) were added and the tubes centrifuged at 13 000 rpm for 10 min. The aqueous RNA phase was decanted to a clean tube and 2.5x sample volume of 100% ethanol and 2 M sodium acetate added and stored at -20°C until reverse transcription.

2.2.6.4 Reverse Transcription.

A Beckman Du530 Spectrophotometer (Beckman Coulter, USA) was used to quantify RNA at 260 nm. RNA was then reverse transcribed using random hexamers (Geneworks, Adelaide, Australia) and Superscript II enzyme kit (Invitrogen, Carlsbad, California, USA) according to the manufacturer's instructions.

2.2.6.5 Real Time PCR.

Primers for genes of interest, including IFNγ receptor 1 (IFNGR1) and receptor 2 (IFNGR2), TGFβ type 1 and type 2 receptors (TGFR1, TGFR2), LPS receptors CD14 and TLR4 and LTA receptor TLR2 were designed using Primer Express (Applied Biosystems) and NCBI online facilities and were purchased from GENSET OLIGOs (Lismore) (see Table 2.2). Pre-existing housekeeper gene primers for β-actin and 18S mRNA were used. The PCR reactions were completed on an ABI Prism 5700 Sequence Detection System and software (Applied Biosystems). A three stage program was used where Stage 1- 50°C for 2 min, Stage 2- 95°C for 10 min initial denature, and Stage 3 – 40 cycles of (15 sec at 95°C then 1 min at 60°C).

PCR product identity was confirmed by sequencing carried out by Molecular Pathology IMVS (Adelaide, Australia) after amplicon size confirmation by 2% agarose electrophoretic gel analysis, excision and purification using Qiagen MinElute PCR Purification Kit (Valencia, USA). Briefly, PCR product DNA in solution was bound to a binding column during centrifugation, washed then eluted in 10 μL of Tris-Cl

Target	Primer - Position	Amplicon size	Genbank Accession Number
IFNGR1	Forward – 562 5'-AGAGAGCCAGGGAACCATGTT	116 bp	GI: 6652953
	Reverse – 677 5'-TTTCGACCGTATGTTTCGTATGTAG		
IFNGRβ	Forward – 776 5'-CCTGTCACGAAACAACAGCAA	119 bp	GI: 545841
	Reverse – 798 5'-TGCCTCCGCCAGGCTGCA		
TGFR1	Forward – 1196 5'-CTGACATCTATGCAATGGGCTTAG	78 bp	GI: 6678322
	Reverse – 1273 5'-GATAGTCTTCATGGATTCCACCAA		
TGFR2	Forward – 377 5'-GCTGTGCAAGTTTTGCGATGT	74 bp	GI: 6678324
	Reverse – 450 5'-TGATGCTGCAGTTGCTCATG		
CD14	Forward – 16 5'-TTCAGAATCTACCGACCATGGA	156 bp	GI: 6753331
	Reverse – 171 5'-AATTGAAAGCGCTGGACCAA		
TLR4	Forward – 93 5'-CTTGAATCCCTGCATAGAGGTAGTT	111 bp	GI: 10946593
	Reverse – 203 5'-CTCAGATCTATGTTCTTGGTTGAAGAA		
TLR2	Forward – 253 5'-GAATGCATCACCGGTCAGAA	127 bp	GI: 6755812
	Reverse – 379 5'-GCGTTTGCTGAAGAGGACTGT		
β-Actin	Forward - 182 5'-CCGTGGGCCGCCCTAGGCACCA	170 bp	GI: 6671508
	Reverse - 351 3'-CACGCAGCTCATTGTA		
18S	Forward & Reverse Commercially produced, Ambion	315 bp	Ambion

Table 2.2 Primer Sequences for Real Time RT-PCR

2.3 *IN VIVO* ADMINISTRATION OF IMMUNE MODULATING MOITIES

All animals undergoing invasive procedures were anaesthetised with an i.p. injection of 20 mg/mL Avertin (2,2,2 tribromoethanol, Sigma Aldrich, St Louis, MO), at a dose of 0.015 mL/g (body weight). Mice were monitored and kept warm on a heating pad until recovery from anaesthesia. One of three injection routes were used to administer treatments, uterine lumen injections via transcervical (TC) or intrauterine (IU) route, or intraperitoneal (IP) injections.

2.3.1 Transcervical Injection

A process to administer a 30 μL volume TC was devised. This involved initially marking the level where 30 μL of sterile MQ was drawn up into a tomcat catheter attached to a disposable 1 mL syringe, after which an additional 30 μL was drawn up and the level marked (total 60 μL). This gives the first and second marks mentioned below. The catheter was loaded to the second mark (60 μL) with treatment. An auroscope with paediatric attachment was lubricated and inserted into the vagina to visualise the cervix. The magnifier was removed and a rigid dilation catheter was inserted through the cervical opening. The dilation catheter was removed and the treatment catheter inserted, and treatment injected until the first mark (30 μL deposition). The catheter and auroscope were slowly withdrawn and recovery permitted.

TC treatment administration of recently mated female mice required removal of the copulatory plug prior to auroscope insertion. This was achieved by gently separating the plug away from the vaginal wall with curved forceps. However, until approximately 0900 h on the morning following mating the plug was bound tightly to the vaginal wall preventing removal without causing significant trauma. Hence TC treatment administration to newly mated mice was delayed until 0900 h when plug adhesion had decreased, making removal possible.

2.3.2 Intrauterine Injection

Administration of a 30 μL volume via an IU injection involved making an incision (~ 8mm long) was made through the dorsal skin. The peritoneal lining near the ovary fat pad was visualised and secured and a second small incision made. The ovarian fat pad was then visualised and secured, the uterus exteriorised and secured with surgical clips enabling 15 μL treatment via a 50μL Hamilton syringe to be administered. The uterus and ovary were then replaced into the peritoneal cavity and the mouse rotated 180° to repeat the procedure on the other side. Once correct uterine and ovarian

replacement was observed the wound was closed with a sterile 9mm wound clip (Becton Dickinson, Sparks, USA).

2.4 ESTROUS AND DAY 0.5pc MEASURES

2.4.1 Estrous Outcomes

Estrous cycle stage was determined by examining vaginal smears (detailed in 2.1 above). Treatment was administered at 0800-1000 h either by TC, IU or IP injection (see 2.3.1, 2.3.2 above). Mice were then killed by cervical dislocation 8 hrs post-treatment. Uterine luminal fluid was flushed from each horn in RPMI-FCS (500 μL) and stored at -20°C for later analysis of GM-CSF, IL-6 and KC by ELISA. Uterine horns were excised and inserted upright and parallel in O.C.T. Tissue Tek (Miles Scientific, Elkhart, IN) and stored at -80°C for immunohistochemical studies.

2.4.2 Day 0.5pc Outcomes

Male and female animals were co-housed between 2200 h and 0000 h, then females were checked for the presence of a plug at 0400 h. With the aim of administering treatments as close as practicable to the time of mating, IU (see 2.3.2 above) and IP treatments were administered at 0400 h and TC treatments were administered at 0900 h (see 2.3.2 above). Mice were then killed by cervical dislocation 8 hours post-treatment. Uterine luminal fluid was flushed from each horn in RPMI-FCS (500 μL) and stored at -20°C for later analysis of GM-CSF, IL-6 and KC by ELISA. Uterine horns were excised and inserted upright and parallel in O.C.T. Tissue Tek (Miles Scientific, Elkhart, IN), and stored at -80°C for immunohistochemical studies.

2.4.3 Immunohistochemistry

Frozen uterine tissue was cut into seven micron (7 μm) sections which were melted onto glass slides. Slides were air dried for 1 hour then sealed in boxes together with silica beads (Ajax Chemicals, Auburn, NSW). When required for staining, slides were removed from the -80°C freezer and equilibrated to room temperature.

Uterine sections were fixed in 96% ethanol for 10 min at 4°C, then rinsed three times in phosphate buffered saline (PBS - pH 7.4, osmolarity 283). Sections were then blocked in PBS + 1% BSA (also used as dilution buffer for all dilutions) for 2 min to prevent non-specific binding. Primary

monoclonal antibodies (see Table 2.3) were diluted, where required, in dilution buffer with the addition of 10% normal mouse serum and 60 µL was applied. Duplicate noon-serial sections of each tissue were stained with each antibody. Negative controls included slides incubated with dilution buffer alone, without the primary antibody or slides incubated with irrelevant isotype-matched rat anti-mouse mAbs. Slides were then incubated in a humidified chamber at 4°C overnight. The next morning sections were again washed three times in PBS and blocked in 1% BSA for 2 min. Rabbit anti-rat biotinylated secondary antibodies (60 µL) (DAKO, Denmark) were applied to sections at 1:300 in dilution buffer and incubated for a further 2 hrs at 4°C. Sections were then rewashed in PBS, blocked in 1% BSA and incubated with 60 µL Avidin-HRP conjugate (DAKO, Denmark) at 1:400 in dilution buffer for 45 min at 4°C. SigmaFAST DAB tablet set (diaminobenzidine tetrachloride - 0.7 mg/mL and urea hydrogen peroxide - 0.67 mg/m with 60 mM Tris buffer dissolved in MQ H_2O) (Sigma, Castle Hill, AU) was employed to develop HRP staining following application to the sections and incubation for 10 min at room temperature. Haematoxylin (Sigma, Castle Hill, AU) was used to counterstain tissue sections which was followed by dehydration in absolute ethanol, drying and mounting with Depex (BDH Laboratory Supplies, Toronto, CA). Stained slides were stored at room temperature until viewing and analysis.

Video image analysis (VIA) software (Video Pro 32) (Leading Edge Pty. Ltd, Blackwood, AU) was used to quantify the density of positive (DAB) staining in the uterine tissue sections where indicated. Prior to each session of data collection, the VIA system was calibrated using a standard field of tissue. The mean area of positive staining (% positivity) in 10 medium power fields (x 20 objective) from each horn in two non-serial sections of endometrial stroma was expressed as a percentage and was calculated as:

$$\%\ \text{positivity} = \frac{\text{DAB stained area}}{\text{total (hematoxylin + DAB) stained area}} \times 100$$

Monoclonal antibody	Antigenic specificity	Reactive cell lineages	Dilution
TIB122 (in house hybridoma)	CD45 (LCA, Ly-5)	pan leukocytes	Neat supernatant
F4/80 (clone CI:A3-1) (Serotec)	F4/80	macrophage	1:200 (final-5 µg/mL)
RB6-8C5 (in house hybridoma)	Ly-6G	Granulocytes, neutrophils	Neat supernatant

Table 2.3 The antigenic specificities and cell lineage specificities of the rat anti-mouse mAbs used for the immunohistochemical analysis of uterine tissue.

2.5 DAY 18 PREGNANCY OUTCOMES

Male and female animals were co-housed between 2200 h and 0000 h, then females were checked for the presence of a plug at 0400 h. With the aim of administering treatments as close as practicable to the time of mating, IU (see 2.3.2 above) and IP treatments were administered at 0400 h and TC treatments were administered at 0900 h (see 2.3.1 above). To control for the administration time difference, an IU and IP treatment group at 0900 h were included as well. Females recovered and continued pregnancies to day 18. At 0900-1200 h on day 18 of pregnancy females were killed by cervical dislocation. Upon sacrifice the whole uterus was removed and the number and location of viable fetuses and resorptions was documented. Each conceptus was dissected from the uterine tissue, the fetal membranes were removed, the fetuses and placentas were separated, weighed and crown-rump length and girth were measured.

2.6 LONG TERM PROGENY OUTCOMES

Male and female animals were co-housed after 1500 h and then females were checked for the presence of a plug at 0900 h the next morning, at which time TC treatments were administered (see 2.3.1 above). Following treatment, females were housed individually or in pairs, with expected delivery date one week apart so that litters could easily be attributed to specific dams. Females were checked 12 hourly after day 18 until delivery. The length of gestation and the number of live and dead pups was recorded. Pups were weighed at 12 – 24 hrs, 3 days, 7 days and then weekly until 22 weeks of age. At 3 weeks of age the pups were weaned, coded by ear punching, separated by gender and housed accordingly. At 22 weeks of age up to 2 males and 2 females from each litter were randomly selected for blood pressure measurement, glucose tolerance test and post-mortem full body compositions performed. An outline of procedures carried out at 22 weeks of age is depicted below in Figure 2.1 below.

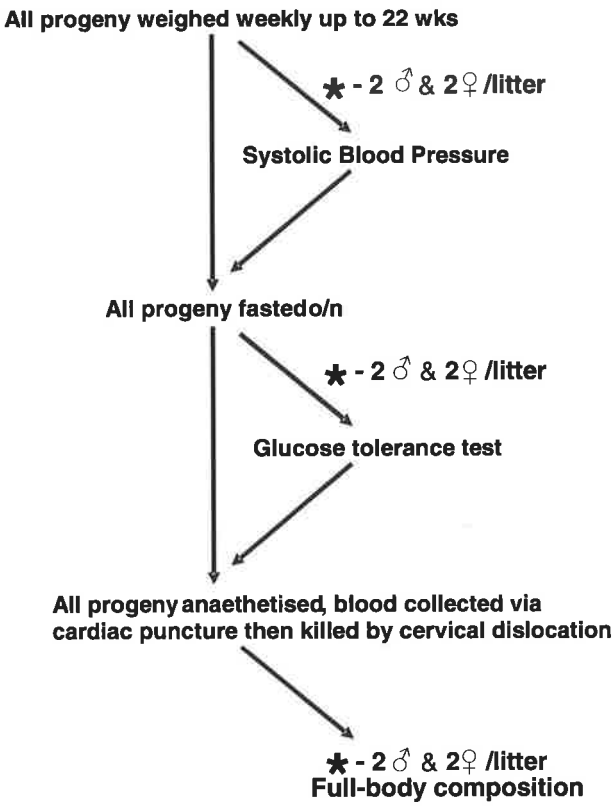
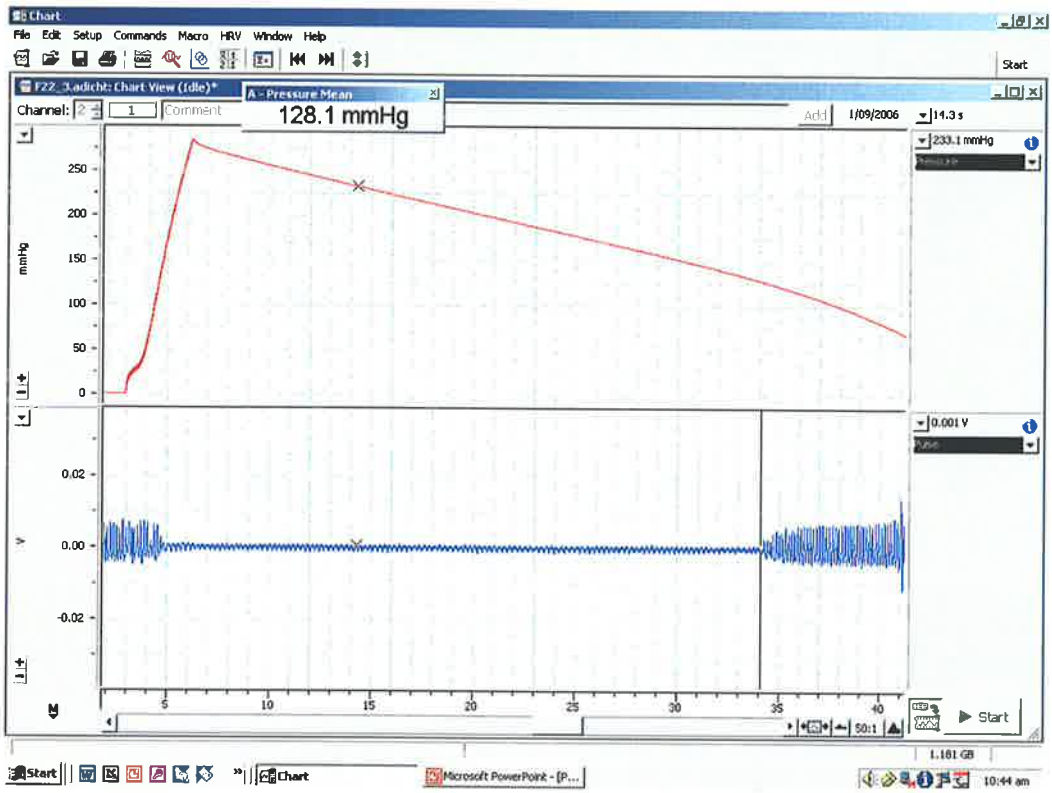


Figure 2.1 Flow Chart of Progeny Outcome Measures at 22 weeks.

2.6.1 Systolic Blood Pressure Measurement

Systolic blood pressure was measured using PowerLab 4/30 and ML125 NIBP Controller and MLT125/M pulse transducer and tail cuff (ADInstruments, Colorado Springs, USA) and Chart 5 for Windows software. This system is used to determine a systolic blood pressure measure by recording caudal artery pulse while occluding the artery with the tail pressure cuff. Pressure and pulse measures are displayed and the pressure at which the pulse returns after occlusion is ascertained (see Figure 2.2 (A) below). Briefly, the protocol employed involved placing individual mice into a ventilated restrainer and warming to 32°C on a heat pad where they were allowed to acclimatise for 30 mins. The tail cuff was placed at the proximal end of the tail with the pulse transducer positioned directly after the cuff on the ventral surface of the tail directly below the caudal artery. Another 5 mins for settling was allowed and then recording commenced, pressure cuff was inflated and measurements were taken. Amplification of the mouse caudal artery pulse is required, leaving it vulnerable to interference from animal movement (see Figure 2.2 (B) below).

A



B

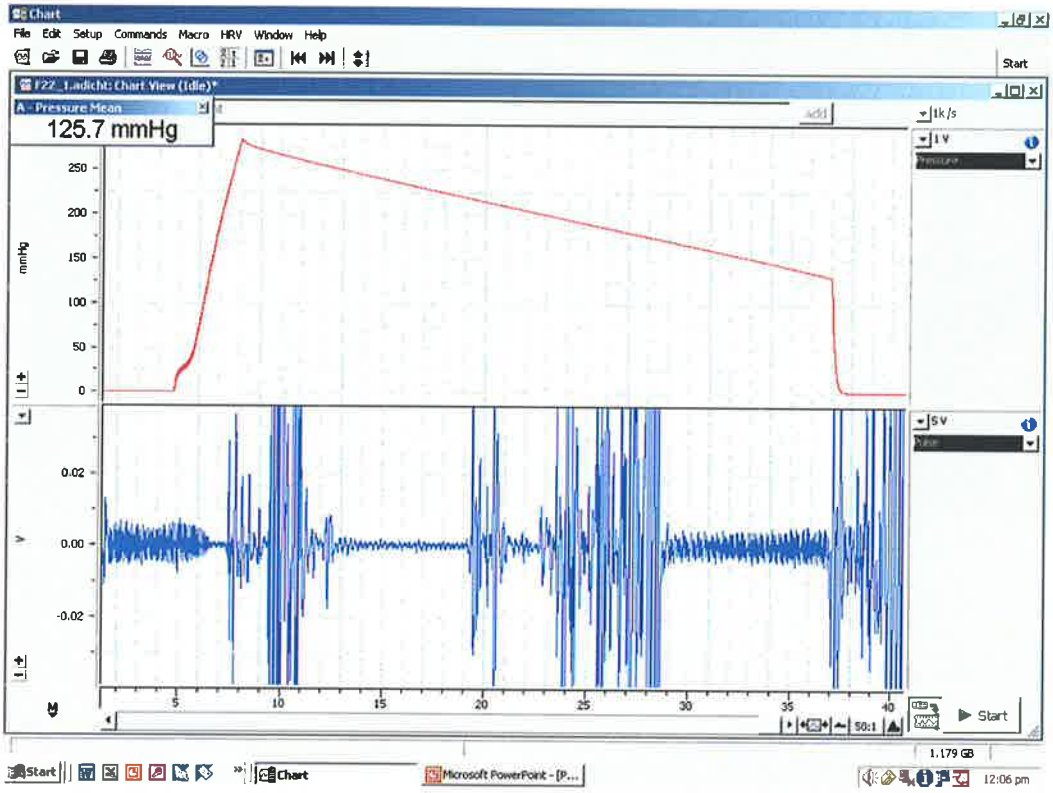
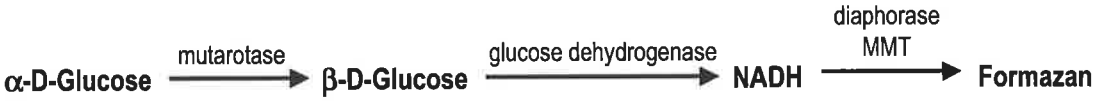


Figure 2.2. Systolic blood pressure plots (A) - Good caudal artery occlusion and pulse resumption allowing accurate determination of systolic blood pressure. (B) – Good caudal artery occlusion with movement interference.

2.6.2 Glucose Tolerance Test

Following overnight fasting, mice were restrained and the tail vein was nicked with a sterile scalpel blade. Blood was collected by capillary action directly into HemoCue B-Glucose Microcuvettes and read immediately in a HemoCue B-Glucose Analyzer (Hemocue, Anelholm, Sweden). This procedure relies on an enzymatic whole blood glucose dehydrogenase reaction within the cuvette followed by photometric quantification in the analyser, according to the pathway;



The formazan is then measured using a photometric method at 660 nm and 840 nm with the readout given in mmol/L. Mice were injected i.p. with 1 mg/g D-Glucose (250 mg/mL in sterile PBS) immediately after obtaining basal blood glucose levels (mM) on the morning following fasting (T0). Blood collection and readings were repeated 30 and 60 mins after D-Glucose injection (T30 and T60 respectively).

2.6.3 Serum metabolic parameters

All progeny were anaesthetised with Avertin and whole blood collected via cardiac puncture. Blood was placed on ice until centrifugation at 10 000 rpm, serum was collected and stored at –80°C. Serum was used to measure free fatty acids, serum glucose, triglycerides and cholesterol in a an automated COBAS MIRA system and leptin, insulin and adiponectin were measured via specific RIAs.

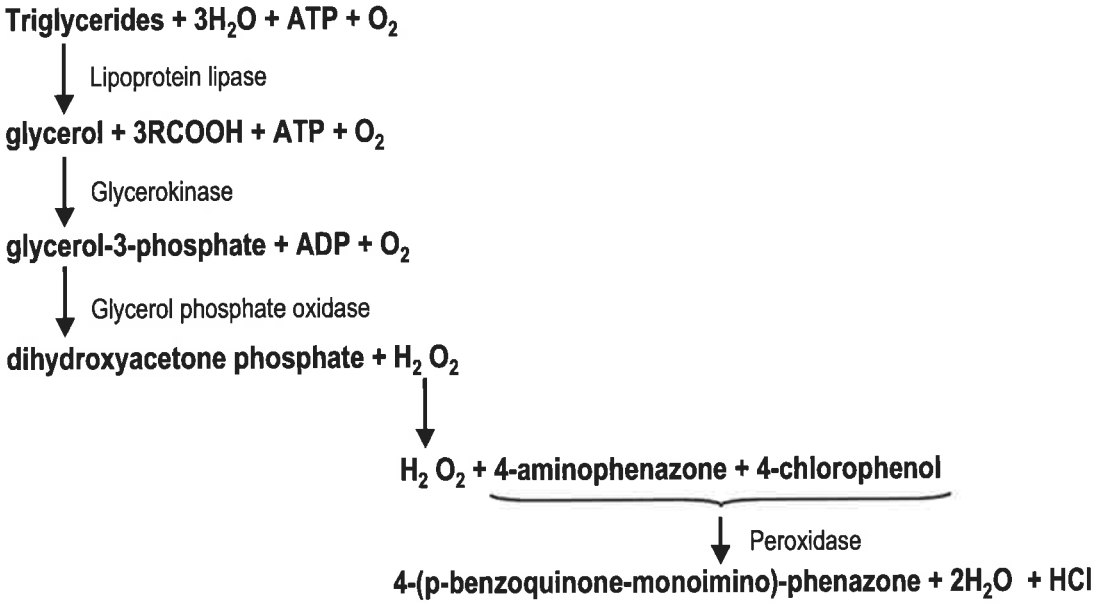
2.6.3.1 Serum Glucose

Fasting serum glucose was measured using the Gluco-quant Glucose HK assay kit in an automated COBAS MIRA sample system using C.f.a.s. calibrator standards and Precinorm U and Precipath U internal controls (Roche Diagnostics, Sydney, Australia). The principle of this assay is based on the hexokinase method [467, 468];

The NADPH produced at the end of this reaction is directly proportional to the glucose concentration and is measured photometrically. The intra-assay coefficient of variance (c.v.) for this assay was < 2% and the inter-assay c.v. was 1.05%.

2.6.3.2 *Triglycerides*

Fasting serum triglycerides were measured using the Triglycerides GPO-PAP assay kit in an automated COBAS MIRA sample system using C.f.a.s. calibrator standards and Precinorm U and Precipath U internal controls (Roche Diagnostics, Sydney, Australia). The principle of this assay is based on the hydrolysis and oxidation of triglycerides to dihydroxyacetone phosphate and hydrogen peroxide [469];

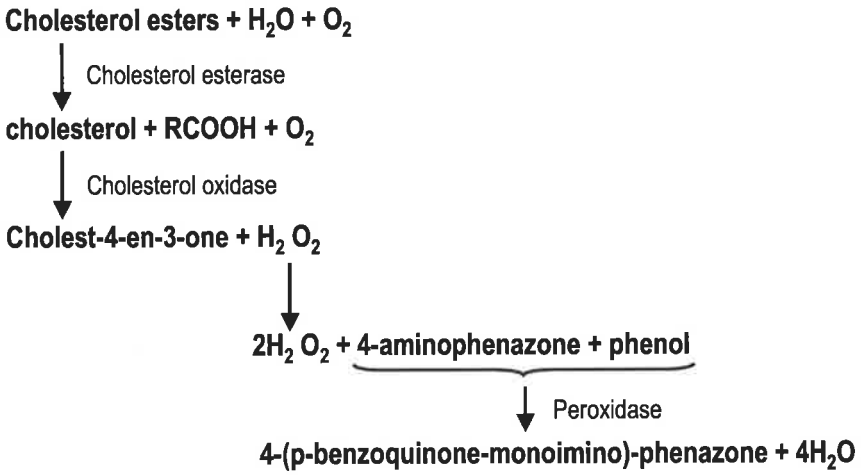


The red chromogenic change following the Trinder endpoint reaction [470] i.e. the reaction of hydrogen peroxide and 4-aminophenazone and 4-chlorophenol, is directly proportional to the concentration of triglycerides and is measured photometrically. The intra-assay c.v. for this assay was < 1% and the inter-assay c.v. was 0.26%.

2.6.3.3 *Cholesterol*

Fasting serum cholesterol were measured using the Cholesterol CHOD-PAP assay kit in an automated COBAS MIRA sample system using C.f.a.s. calibrator standards and Precinorm U and Precipath U internal controls (Roche Diagnostics, Sydney, Australia).

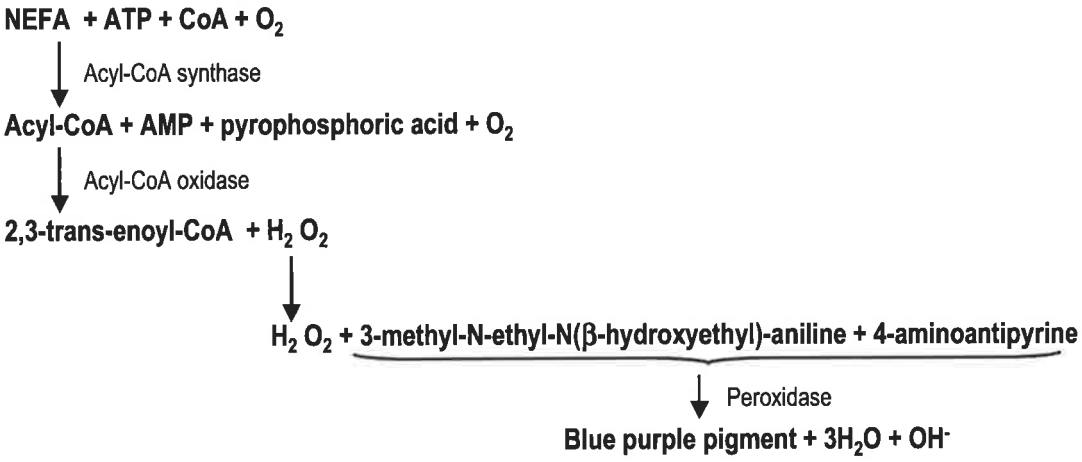
The principle of this assay is based on the enzymatic cleavage and oxidation of cholesterol ester to Δ-cholestenone phosphate and hydrogen peroxide [471, 472];



The red chromogenic change following the Trinder endpoint reaction [470] i.e. the reaction of hydrogen peroxide and 4-aminophenazone and phenol, is directly proportional to the concentration of cholesterol and is measured photometrically. The intra-assay c.v. for this assay was < 1% and the inter-assay c.v. was 0.58%.

2.6.3.4 Free fatty acids

Fasting serum free fatty acids were measured using the NEFA C (ACS-ACOD method) assay kit in an automated COBAS MIRA sample system using C.f.a.s. calibrator standards and Precinorm U and Precipath U internal controls (Roche Diagnostics, Sydney, Australia). The principle of this assay is based on the enzymatic conversion and oxidation of non-esterified fatty acid (NEFA) to 2,3-trans-enoyl-CoA and hydrogen peroxide;



The chromogenic change is directly proportional to the concentration of free fatty acids and is measured photometrically. The intra-assay c.v. for this assay was < 9% and the inter-assay c.v. was < 5%.

2.6.3.5 *Insulin*

Serum insulin was measured in a Sensitive Rat Insulin RIA Kit (LINCO Research, Missouri, USA). The sensitivity of this assay is 0.02 ng/ml of rat insulin and is 100% cross reactive with human, pig, sheep, hamster and mouse insulin but not with similar molecules such as IGF-I. RIAs were carried out according to the manufacturer's instructions. The principle of this assay is based on competitive binding of the radio-labelled tracer, ^{125}I-insulin, and unlabelled antigen (insulin in the serum sample) to a constant concentration of antibody. A Wallac 1261 Multigamma Gamma Counter was used to quantify precipitated radioactivity and serum insulin was calculated using a standard curve from known concentrations of insulin provided in the kit. The manufacturer has determined that this assay has an intra-assay c.v. of < 6% and inter-assay c.v. of < 11%.

2.6.3.6 *Adiponectin*

Serum adiponectin was measured in a mouse specific adiponectin RIA Kit (LINCO Research, Missouri, USA). The sensitivity of this assay is 1 ng/ml of mouse adiponectin and is cross reactive with human and rat adiponectin but not with similar molecules such as human C1q. RIAs were carried out according to the manufacturer's instructions. The principle of this assay is based on competitive binding of the radio-labelled tracer, ^{125}I-adiponectin, and unlabelled antigen (adiponectin in the serum sample) to a constant concentration of antibody. A Wallac 1261 Multigamma Gamma Counter was used to quantify precipitated radioactivity and serum adiponectin was calculated using a standard curve from known concentrations of adiponectin provided in the kit. The manufacturer has determined that this assay has an intra-assay c.v. of < 5% and inter-assay c.v. of < 9%.

2.6.3.7 *Leptin*

Serum leptin was measured in a mouse specific leptin RIA Kit (LINCO Research, Missouri, USA). The sensitivity of this assay is 0.2 ng/ml of mouse leptin and is < 50% cross reactive with human and rat leptin but not with similar molecules such as IGF-1. RIAs were carried out according to the manufacturer's instructions. The principle of this assay is based on competitive binding of the radio-labelled tracer, ^{125}I- leptin, and unlabelled antigen (leptin in the serum sample) to a constant concentration of antibody. A Wallac 1261 Multigamma Gamma Counter was used to quantify precipitated radioactivity, serum leptin was calculated using a standard curve from known concentrations of leptin provided in the kit. The manufacturer has

determined that this assay has an intra-assay c.v. of < 12% and inter-assay c.v. of < 15%.

2.6.4 Post Mortems and Full Body Compositions

Up to two male and two females from each litter underwent a full-body composition analysis at post-mortem having a number of fat depots, muscles and organs dissected out for weighing and fixation. Organs collected and weighed included: brain, thymus, heart, liver, spleen and kidneys. Reproductive organs collected and weighed included: ovaries, uterus, testicles, epididymis and seminal vesicles. Central fat depots collected and weighed included: parametrial fat, perirenal fat, retroperitoneal fat and epididymal fat. Muscles collected and weighed included: quadriceps, gastrocnemius, bicep and tricep. Tissues were fixed in 4% paraformaldehyde overnight and stored for future analysis.

2.7 STATISTICAL ANALYSIS

Statistical models to analyse *in vivo* experimental outcomes where repeated measures were taken on tissues, fetuses or progeny from a single experimental unit (i.e. the mother) were constructed using a linear Mixed Model with fixed and random effects, covariates and interactions. Consultation with a biostatistician assisted with model development as well growth trajectory analysis (Mr Thomas Sullivan, Department of Public Health, University of Adelaide). Data were analysed using SPSS 12.0 software (SPSS Inc., Chicago, USA). The model design defined mothers as the subjects who received treatment, with fixed factors including treatment group, litter size (some measures) and gender, while end progeny weight was included as a covariate. Factors and covariates were analysed systematically both individually and in combination to assess interactions. Outcomes compared as proportions or percentages were analysed using χ^2 test. Single outcome measures from an experimental unit were analysed using a one way ANOVA.

Analysis of i*n vitro* data employed non-parametric Kruskall Wallis and Mann-Whitney U test since goodness-of-fit, or normality tests generally showed lack of normal distribution. Statistically significant differences between treatment groups were attributed when $p < 0.05$.

Chapter 3

Effect of seminal fluid factors on uterine epithelial cell cytokine production

3.1 INTRODUCTION

The female reproductive tract is a mucosal organ, comprised of a number of compartments including the vagina, cervix and uterus. These tissues are each lined by epithelial cells, which form a physical barrier providing protection from potential pathogens. During insemination in mice, ejaculate is deposited into the uterus, along with cellular debris and micro-organisms. This contrasts with humans where the vagina and cervix of the lower tract is major site of semen exposure. The female reproductive tract epithelium has been shown to play a central role in the post-mating inflammatory response in a number of species, including mice, humans and several other animal species. The female tract mucosa is thought to contribute to the generation of an appropriate immune response to paternal antigens introduced at insemination, in a similar manner to that observed in the gut mucosa towards food antigens or in the lung towards airborne antigens.

Seminal plasma is no longer merely considered simply as the fluid vehicle for sperm delivery and survival in the female reproductive tract at insemination. There is mounting evidence indicating a role for seminal plasma in eliciting an inflammatory response in the female tract and in directing molecular and cellular changes in the pre-implantation uterus. Studies in mice suggest that the changes elicited by seminal plasma in the uterus are important for recovering reproductive tract sterility after insemination, and also may be directed towards providing an optimal environment for embryonic growth and development.

Experiments in mice using neutralising antibodies have identified a seminal vesicle fluid cytokine, TGFβ, as being at least in part responsible for eliciting the post-mating inflammatory response [12]. Instillation of recombinant TGFβ into an estrous mouse uterus upregulates GM-CSF production to a similar degree as that seen following mating [12]. This also leads to leukocyte recruitment reminiscent of that following natural insemination, with the exception of neutrophils which were not recruited in response to TGFβ. Similarly instillation of recombinant GM-CSF into an estrous uterus demonstrates a pattern of uterine macrophage and eosinophil but not neutrophils recruitment akin to that observed following mating [124, 473]. Since uterine TGFβ treatment stimulates GM-CSF production yet is not able to elicit the neutrophil recruitment and trans-epithelial migration observed following mating, we propose that there are other regulators present in seminal fluid that elicit this response.

Recently in mice mRNA expression of another immune regulating molecule KC, has been identified as being increased following mating [189]. The chemokine KC is the murine counterpart of

human IL-8 and growth-related-oncogene (GRO) [185]. KC and IL-8 are known to be potent neutrophil chemo-attractants and activators. Additionally, recent studies in humans have shown IL-8 mRNA is increased in the cervix following intercourse and IL-8 secretion by cervical epithelial cells in culture following exposure to seminal plasma but not following TGFβ exposure [130, 474].

Immune regulators other than those produced by male reproductive glands may be introduced into the mouse uterus during the natural course of insemination. As mentioned above the female reproductive tract, as with the male reproductive tract, is a mucosal organ. The mucosa is known to facilitate a symbiotic relationship between non-pathogenic (commensal) organisms and their host. Whole commensal bacterial cells are present in the mucosa, and their products including cell wall components lipopolysaccharide (LPS) and lipoteichoic acid (LTA) can also be detected. Furthermore, the physical events of mating and the intromission of male fluid can introduce commensal and potentially pathogenic bacteria from the male tract and the lower female tract into the uterus [377-379]. LPS and LTA are potent pro-inflammatory molecules, which in high levels or in an inappropriate environment are known to be responsible for septic shock. LPS and LTA signal through distinct members of the Toll-like Receptor (TLR) superfamily that recognise pathogen-associated molecular patterns (PAMPs). LPS signalling is elicited by binding to membrane bound receptors CD14 and TLR4 and the accessory molecule MD2. LTA, on the other hand binds to the membrane bound TLR2. Both of these signalling pathways can result in upregulation of various cytokines and chemokines, including GM-CSF, IL-6 and KC in intestinal, respiratory, hepatic and genitourinary tract epithelial cells in humans and rodents [111, 475-480]. Therefore, we hypothesis that LPS and/or LTA may have a role in regulating the post-mating inflammatory response when they are introduced from the lower female tract and/or the male tract during the natural course of insemination.

The experiments described in this chapter aim to investigate the contribution of male signalling factors that may be active at insemination in generating the post-mating inflammatory response in the murine uterus. Initially uterine luminal fluid from mated female mice was collected and evaluated for secretion of GM-CSF, IL-6 and KC. To investigate whether seminal vesicle fluid (SVF) could mimic the post-mating uterine cytokine profile, an in vitro cell culture system was established, where uterine epithelial cells were harvested from estrous mice and incubated with SVF collected from stud males. Supernatants were assayed for GM-CSF, IL-6 and KC content by commercial cytokine specific ELISA. TGFβ has previously been shown to increase uterine epithelial cell GM-CSF secretion. Recombinant $TGF\beta_1$, $TGF\beta_2$, and $TGF\beta_3$ were added to cells in these experiments to investigate whether they also altered IL-6 and/or KC production. Bacterial LPS and LTA addition investigated the potential role of these moieties in regulating uterine epithelial cell GM-CSF, IL-6 and KC production. Additionally

IFNγ, the inhibitory cytokine detected in human semen and is known to downregulate human uterine epithelial cell GM-CSF production, was also investigated. Lastly, pairwise combinations of these molecules were added to cell cultures to examine the interaction between them.

3.2 UTERINE CYTOKINE PRODUCTION FOLLOWING MATING

Previous studies in mice conducted by our group identified seminal vesicle fluid as the predominant component of the ejaculate responsible for eliciting uterine GM-CSF production following mating [12]. Recent research in humans has shown not only GM-CSF and IL-6 secretion to be upregulated in the female tract following insemination but also the chemokine, IL-8 [130]. To further investigate the relative contribution of male reproductive tract components toward modulating the murine uterine cytokine profile following mating, uterine flushings were collected from mated female mice to assess secretion of GM-CSF, IL-6 and KC.

Female C57Bl6 mice were placed with intact, vasectomised (vas-) or seminal vesicle deficient (sv-) Balb/c males in the late afternoon. Successful mating was determined at 0900h the following morning by observation of a vaginal plug following mating with an intact or vasectomised male or by observing sperm in a vaginal smear after mating with a seminal vesicle deficient male. Mated females were killed by cervical dislocation 0800h – 1000h. Uterine flushings were collected in RPMI-FCS (500 μL/horn) and stored at -20°C for analysis of GM-CSF, IL-6 and KC by ELISA (see Chapter 2).

Mating with intact males resulted in a 6-fold increase in uterine GM-CSF production compared to estrous controls ($p=0.036$) (see Figure 3.1 A). Importantly no such increase was seen following mating with sv- or vas- males ($p=1.00$ and $p=0.72$), where GM-CSF was detectable in uterine fluid from only a single female (vas-). This differential response to mating with intact, sv- or vas- resulted in 84% and 83% less uterine GM-CSF content in the latter two groups compared to that collected following intact mating ($p=0.043$ and $p=0.074$ respectively). It was clear from this experiment however, that while mating with intact males elicited a significant increase in uterine GM-CSF production, the response was variable and was observed in only 3 out of 8 mice.

Mating with intact males resulted in a 120-fold increase in uterine IL-6 production ($p< 0.001$) (see Figure 3.1 B). Mating with vas- males elicited a 28-fold increase in uterine IL-6 production, while IL-6 content of uterine fluid from females following mating with sv- males showed no change ($p=0.003$ and $p=0.661$ respectively). The increases in uterine IL-6 content following mating with sv- or vas- males

was 97% and 78% less than that recorded following intact matings (p=0.001 and p=0.003).

Mating with intact males elicited a 7-fold increase in uterine KC production following mating (p< 0.001) (see Figure 3.1 C). Uterine KC content following sv- or vas- mating showed marginal elevation, 2.0- and 2.9-fold respectively (p=0.345 and p=0.028). Intact mating was a much more powerful stimulator of uterine KC production than were sv- and vas- matings, with 71% and 42% less KC produced respectively (p=0.043 and p=0.169).

3.3 SEMINAL VESICLE FLUID STIMULATED UTERINE EPITHELIAL CELL CYTOKINE SECRETION

The results from the previous experiment confirm previous findings that have shown an increase in luminal fluid GM-CSF and IL-6 content following mating and further demonstrate a similar increase in KC content. To further investigate the seminal fluid regulators of epithelial cell cytokines, an in vitro system was used to investigate the ability of seminal vesicle fluid to induce GM-CSF, IL-6 and KC secretion by primary uterine epithelial cells.

Briefly, primary uterine epithelial cells were harvested by enzymatic digestion from uterine tissue of estrous CBA F1 female mice. The purity of enriched epithelial cells prepared as per the protocol detailed in 2.2.4 was ~75%. Following a 4 hr incubation to permit adherence, seminal vesicle fluid (10%) was added to the culture and incubated for 16 hrs. Media was replaced then collected 24 hrs later, immediately centrifuged to remove cellular debris then stored at -20°C until assay for GM-CSF, IL-6 and KC. Remaining adherent cells were quantified by Rose Bengal uptake.

Baseline GM-CSF secretion, 4.5 pg/mL/10^5 cells, was above the minimum detectable limit of the assay (2.7 pg/mL/10^5 cells). Incubation with seminal vesicle fluid elicited an increase of at least 39.8-fold in GM-CSF production by uterine epithelial cells compared to untreated control (see Figure 3.2 A).

In this experiment there was no effect on basal IL-6 (341pg/mL) secretion by uterine epithelial cells in response to incubation with seminal vesicle fluid (see Figure 3.2 B).

Uterine epithelial cell basal KC production (1297 pg/mL) was completely ablated by all fractions of seminal vesicle fluid (see Figure 3.2 C).

Seminal vesicle fluid fraction 1 (F1) from both males displayed a consistent effect on uterine epithelial cell cytokine secretion, however as can be seen in Figures 3.2A and 3.2B there was marked variability in the activity of fractions 2 – 5 (F2 – F5).

3.4 TGFβ MODULATED UTERINE EPITHELIAL CELL CYTOKINE SECRETION IN VITRO

Recent studies have shown a differential regulatory role for seminal fluid TGFβ in modulating the human cervical cytokine environment [130]. In contrast to increasing GM-CSF and IL-6 production, IL-8 secretion is decreased by rTGFβ in primary and immortalised human cervical epithelial cells [130]. To further investigate the effect of TGFβ isoforms on cytokine secretion by mouse uterine epithelial cells, cells were harvested from estrous CBA F1 mice and incubated for 16 hrs with 1.25, 5 or 20 ng/mL of TGFβ_1, β_2 or β_3. Supernatants were replaced and collected 24 hrs later and assayed for GM-CSF, IL-6 and KC content by commercial ELISAs.

Uterine epithelial cell secretion of GM-CSF was significantly increased when cells were cultured in the presence of TGFβ_1, β_2 or β_3 and was dose dependent (see Figure 3.3 A). Addition of 1.25, 5 or 20 ng/mL TGFβ_1 resulted in a 2.1-, 2.4- and 2.4-fold increase in GM-CSF production respectively compared to the control ($p<0.001$, $p<0.001$ and $p=0.004$). While the response to TGFβ_2 was also dose dependent, the magnitude of increased GM-CSF production was less than that observed following the addition of TGFβ_1 with 27%, 75% and 100% increases in response to increasing doses respectively ($p=0.054$, $p<0.001$ and $p=0.093$). TGFβ_3 addition to uterine epithelial cells elicited a similar but marginally higher GM-CSF response than that seen following TGFβ_1 addition with 85%, 2.1- and 2.8-fold increases to 1.25, 5 or 20 ng/mL respectively compared to control ($p<0.001$, $p<0.001$ and $p<0.001$).

In contrast to GM-CSF upregulation by all three TGFβ isoforms, there were small but significant decreases in IL-6 secretion by uterine epithelial cells exposed to TGFβ which were not dose dependent (see Figure 3.3 B). IL-6 production was decreased by 18%, 11% and 13% following addition of TGFβ_1 at concentrations of 1.25, 5 or 20 ng/mL respectively ($p=0.046$, $p=0.096$, $p=0.096$). Similar responses were seen following TGFβ_2 and TGFβ_3 addition with 17%, 22% and 23% ($p=0.046$, $p=0.010$, $p=0.012$) decreases and 23%, 18%, 11% ($p=0.010$, $p=0.022$, $p=0.096$) decreases in IL-6 production respectively.

KC production by uterine epithelial cells was significantly reduced in a dose dependent manner in response to TGFβ (see Figure 3.3 C). Addition of 1.25, 5 or 20 ng/mL TGFβ_1 to the culture media of uterine epithelial cells resulted in a 35%, 43% and 43% decrease in KC secretion ($p=0.005$, $p=0.004$,

p=0.001). Similarly TGFβ_2 exposure lead to 8%, 23% and 47% decreases in KC production (p=0.317, p=0.024, p=0.002). TGFβ_3 was also shown to be a potent inhibitor of epithelial cell KC production with 38%, 42% and 45% decreases compared to controls (p=0.002, p=0.002, p=0.001).

3.5 EFFECT OF BACTERIAL LPS AND LTA ON UTERINE EPITHELIAL CELL GM-CSF, IL-6 AND KC SECRETION IN VITRO

The male and female reproductive tracts are mucosal organs similar to those of the gastrointestinal and respiratory tracts. Mucosal surfaces are known to both facilitate a symbiotic relationship between non-pathogenic (commensal) organisms and the host as well as acting as a protective barrier against pathogenic organisms. Hence the mucosa is constantly exposed to whole bacteria and their products. These include potent pro-inflammatory cell wall components lipopolysaccharide (LPS) and lipoteichoic acid (LTA) from gram negative and positive bacteria respectively. We hypothesised that LPS and/or LTA may have a role in modulating the female tract cytokine response to mating when they are introduced from both the lower female tract and the male tract during the course of mating and insemination.

3.5.1 Effect of bacterial components on CBA F1 uterine epithelial cell cytokine secretion

Uterine epithelial cells were harvested from estrous CBA F1 female mice and incubated for 16 hrs with 0, 2, 20 or 200 ng/mL *E.coli* LPS or 0, 0.1, 1 or 10 μg/mL *B.subtilis* LTA. Supernatants were replaced and collected 24 hrs later and assayed for GM-CSF, IL-6 and KC content by commercial ELISAs.

The addition of 2, 20 or 200 ng/mL LPS to uterine epithelial cells resulted in 67%, 4-fold and 7- fold increases in GM-CSF secretion respectively (see Figure 3.4 A), compared to the control (p=0.038, p=0.013, p=0.038). Similarly a 2-fold increase was observed following 10 μg/mL of LTA treatment (p=0.01). The GM-CSF response to both bacterial products was dose dependent.

IL-6 secretion by uterine epithelial cells was significantly inhibited by the addition of LPS or LTA. E.coli LPS at 2, 20 or 200 ng/mL resulted in a 19%, 10% and 41% (p=0.023) decreases respectively (see Figure 3.4 B). Similarly B.subtilis LTA at 0.1, 1 or 10 μg/mL brought about 36% (p=0.034), 35% and 42% reductions in IL-6 production. The response to either bacterial product appeared to be dose dependent, however as a consequence of variation between duplicates, some larger decreases were not significant.

Uterine epithelial cell KC secretion increased by 90% and 2.9 fold in response to 20 or 200 ng/mL (p=0.035) LPS respectively (see Figure 3.4 C). The response to LTA was less strong yet markedly increased by 60% when 10 μg/mL (p=0.055) was added to the culture media. Again the response to these moieties was dose dependent.

3.5.2 Effect of bacterial components on C3H/HeJ uterine epithelial cell cytokine secretion

C3H/HeJ mice are a strain of mice known to be hyporesponsive to bacterial LPS due to a mutation in the gene encoding the membrane bound LPS receptor, TLR4. In order to investigate the specificity of LPS modulated uterine epithelial cell cytokine secretion, uterine epithelial cells were harvested from estrous C3H/HeJ mice and incubated with 0, 2, 20 or 200 ng/mL *E.coli* LPS or 0, 0.1, 1 or 10 μg/mL *B.subtilis* LTA as described above.

In contrast to CBA F1 cells, C3H/HeJ cells were completely non-responsive to *E.coli* LPS with regard to GM-CSF, IL-6 and KC secretion (see Figure 3.5 A-C). Strain differences were highlighted by the differential basal level of GM-CSF and IL-6 production by C3H/HeJ uterine epithelial cells, being 50% and 78% less than that of CBA F1 cells and higher KC production (42%) higher. However these differences failed to reach significance due to variability between wells.

C3H/HeJ uterine epithelial cells significantly upregulated GM-CSF production after addition of 1 or 10 μg/mL LTA by 50% and 4-fold. KC secretion was increased by 11% - 2-fold following addition of 1 or 10 μg/mL LTA respectively. Again these differences were not significant due to variability between wells. IL-6 production did not alter in the presence of LTA. C3H/HeJ response to LTA was dose dependent and similar in degree to that observed in CBA F1 cells (see Figure 3.5 D-F).

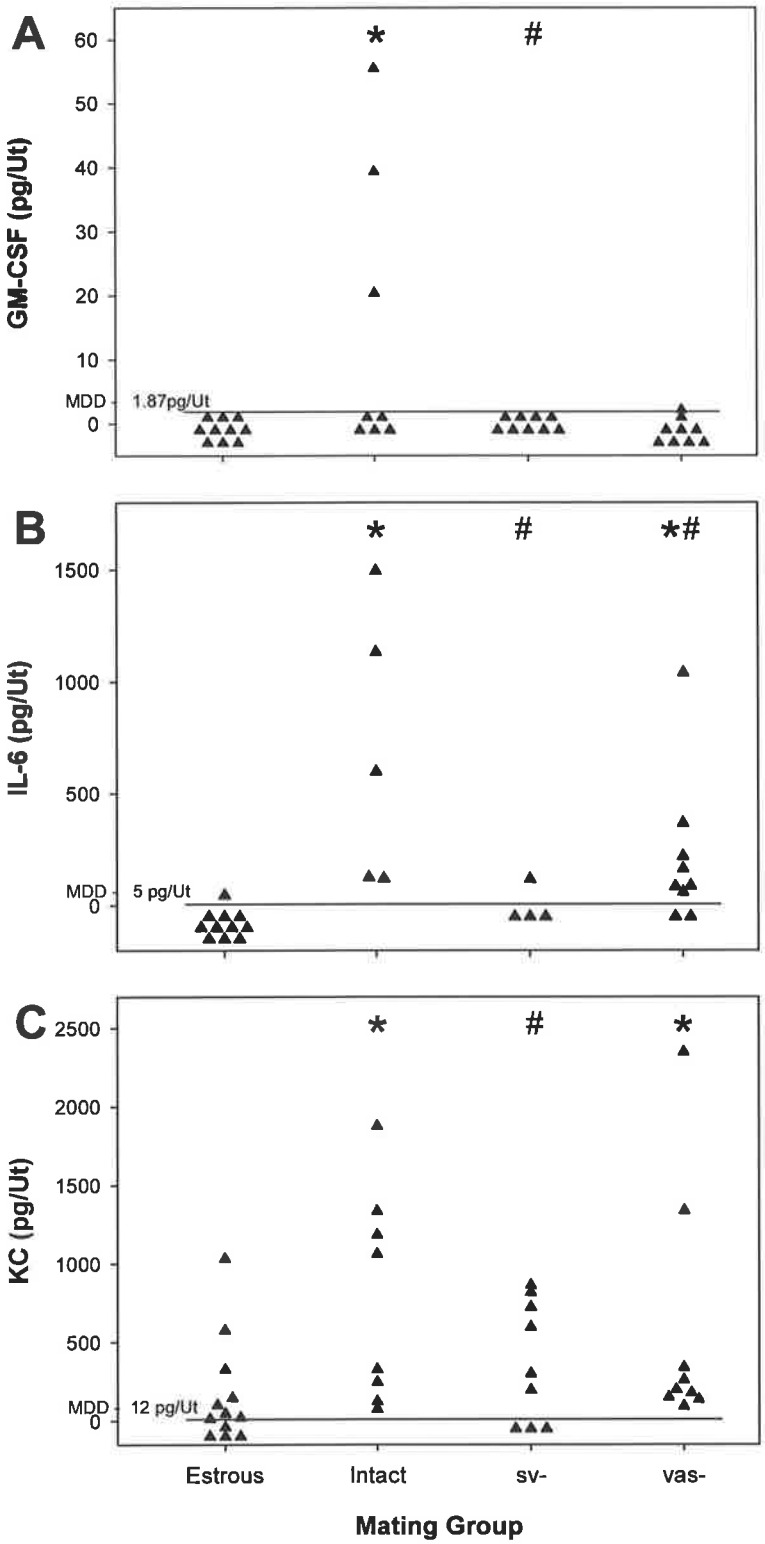


Figure 3.1 Effect of mating on cytokine content of uterine luminal fluid. Cytokine content of uterine flushings was assessed eight hours following mating with intact, seminal vesicle deficient (sv-) or vasectomised (vas-) males. (A) GM-CSF, (B) IL-6 and (C) KC were measured by ELISA. Individual animals are represented as a single mark on the graph (▲). Kruskal-Wallis test and Mann-Whitney tests were used to determine significant differences. ✱- $p < 0.05$ compared to Estrous, #-$p<0.05$ compared to Intact mating.

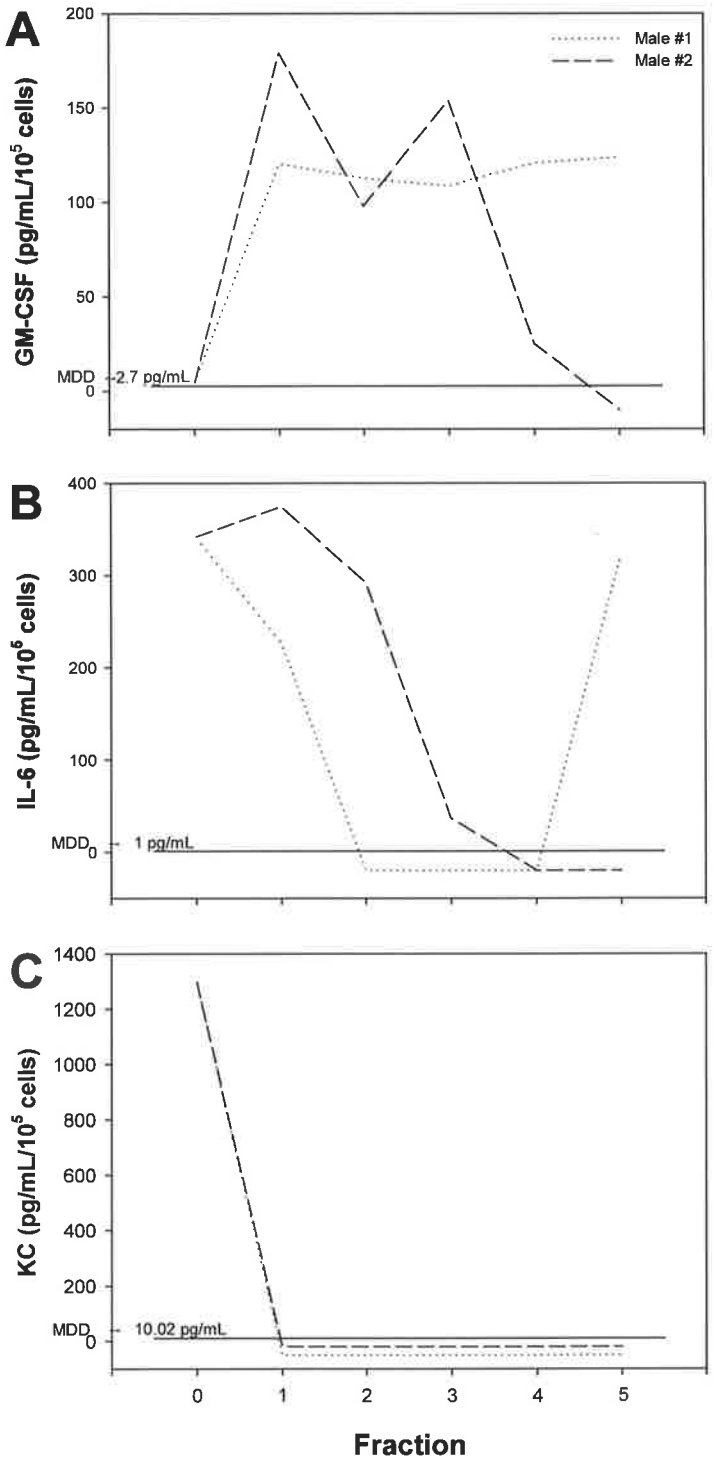


Figure 3.2 Effect of seminal vesicle fluid on uterine epithelial cell GM-CSF, IL-6 and KC secretion. Uterine epithelial cells were cultured with culture media alone (0), or fractions 1-5 of seminal vesicle fluid from two individual mice (Male #1, Male #2). Supernatants were collected 24 hrs following replacement of treatment media and assayed for GM-CSF (A), IL-6 (B) and KC (C) by cytokine specific ELISAs. Data was normalised to the number of viable uterine epithelial cells remaining at the end of the culture period and expressed as pg/mL/10^5 cells. These data are representative of three replicate experiments. MDD = Minimum Detectable Dose.

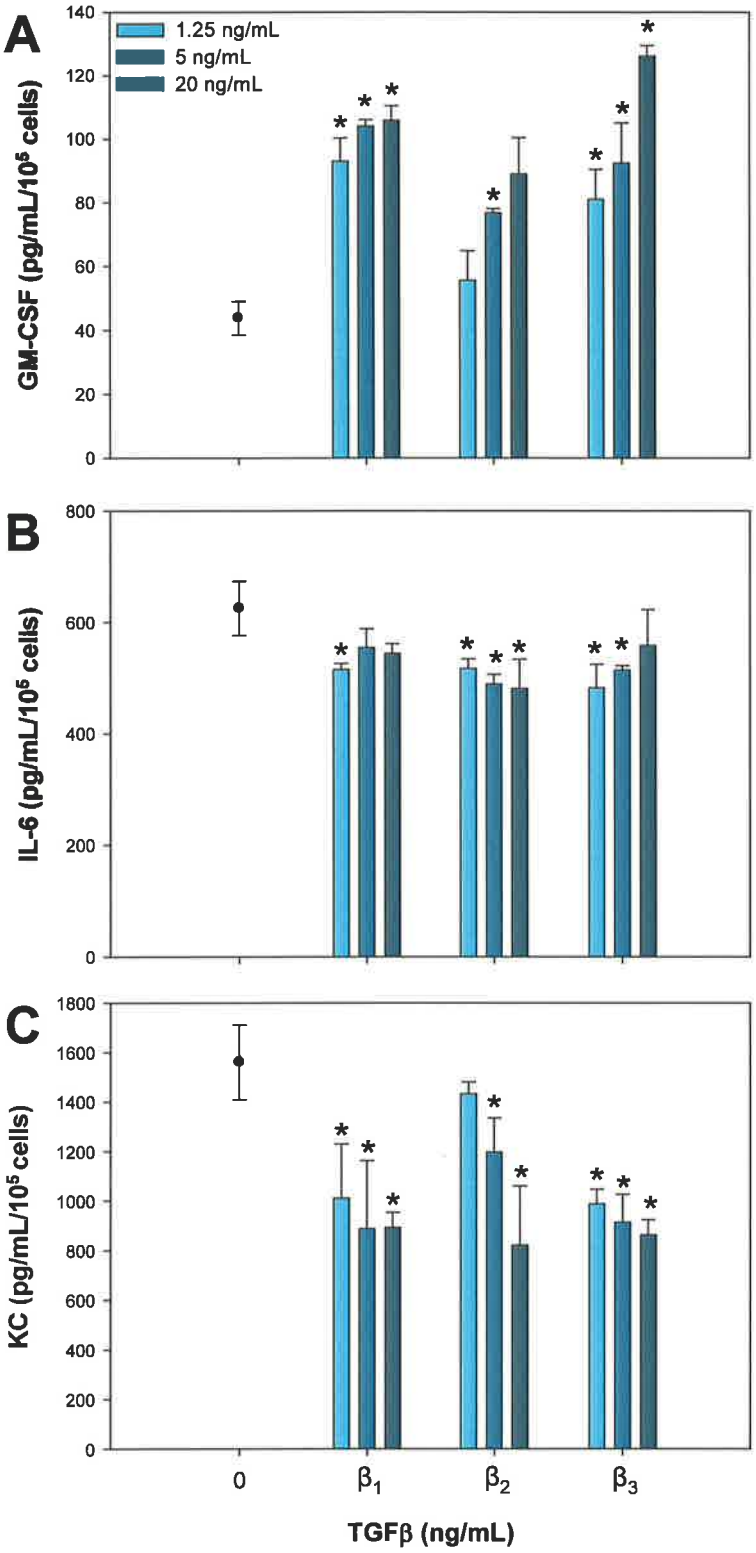


Figure 3.3 Effect of recombinant TGFβ1, β2 and β3 on uterine epithelial cell GM-CSF, IL-6 and KC secretion. Uterine epithelial cells were cultured with culture media alone (0) or recombinant TGFβ1, TGFβ2 or TGFβ3 at concentrations of 1.25, 5 or 20 ng/mL Supernatants were collected 24 hrs following replacement of treatment media and assayed for GM-CSF, IL-6 and KC by cytokine specific ELISAs. Data was normalised to the number of viable uterine epithelial cells remaining at the end of the culture period and expressed as pg/mL/10^5 cells (A) GM-CSF, (B) IL-6 or (C) KC. All measurements are the mean ± SD of duplicate wells for each treatment. These data are representative of three replicate experiments. ✱ $P < 0.05$.

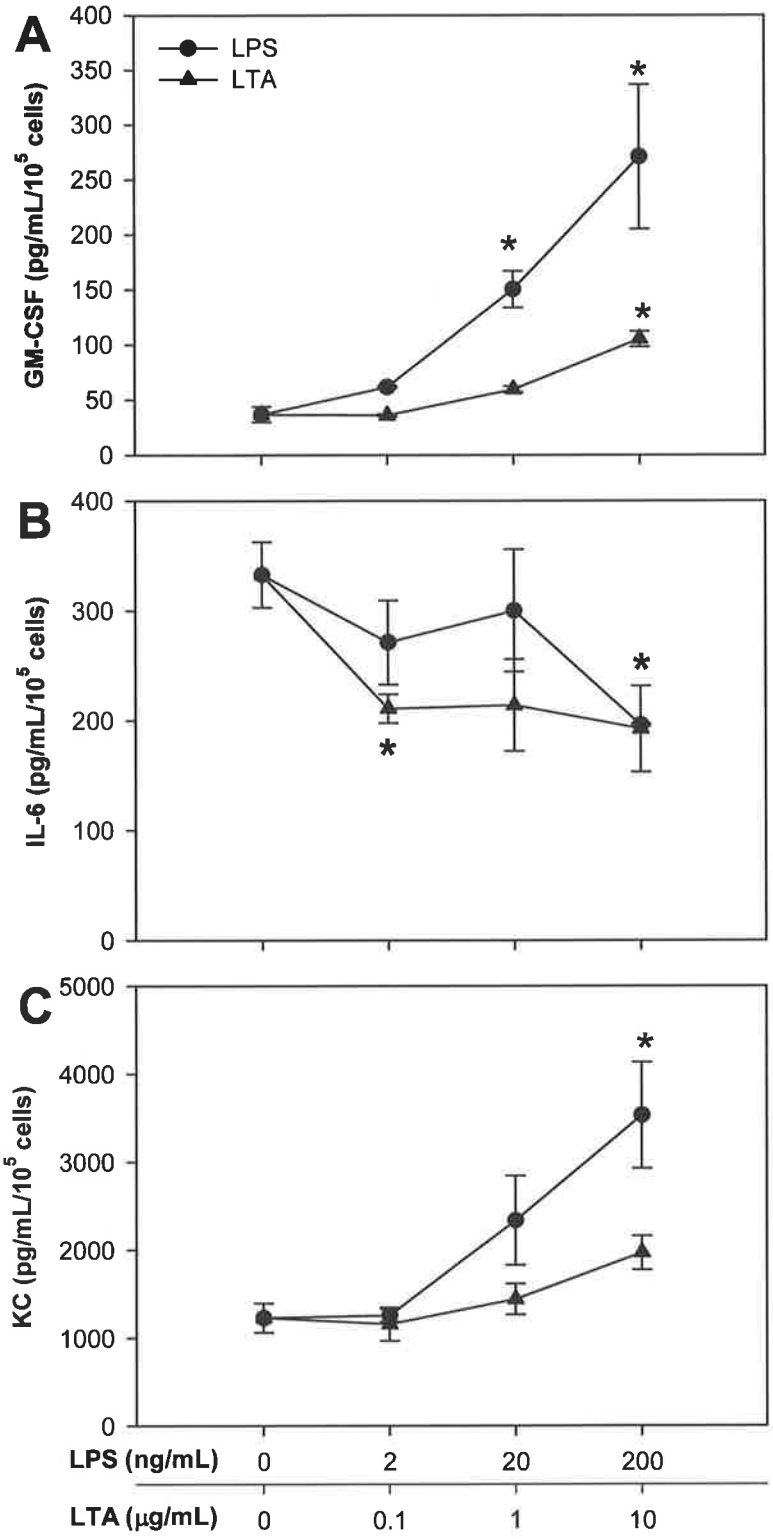


Figure 3.4 Effect of bacterial components on uterine epithelial cell GM-CSF, IL-6 and KC secretion. Uterine epithelial cells were cultured with *E.coli* LPS (●) at 2, 20 or 200 ng/mL or *B.subtilis* LTA (▲) at concentrations of 0, 0.1, 1 or 10 mg/mL. Supernatants were collected 24 hrs following replacement of treatment media and assayed for GM-CSF, IL-6 and KC by cytokine specific ELISAs. Data was normalised to the number of viable uterine epithelial cells remaining at the end of the culture period and expressed as pg/mL/10^5 cells (A) GM-CSF, (B) IL-6 or (C) KC. All measurements are the mean ± SD of duplicate wells for each treatment. These data are representative of three replicate experiments. $P < 0.05$, ✱- compared with control - media only.

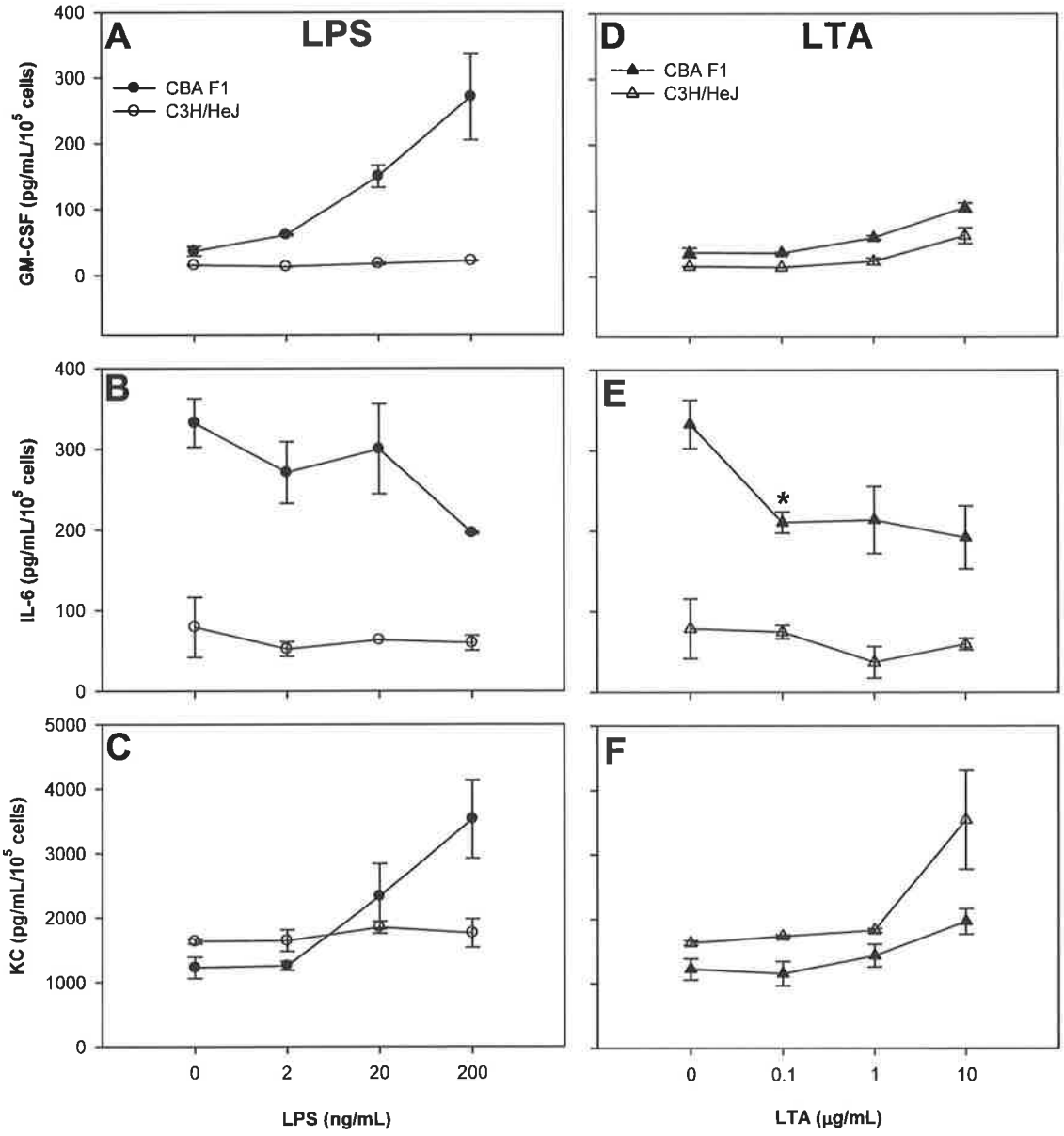


Figure 3.5 Effect of bacterial components on C3H/HeJ uterine epithelial cell GM-CSF, IL-6 and KC secretion. C3H/HeJ uterine epithelial cells were cultured with *E.coli* LPS (○) at 0, 2, 20 or 200 ng/mL or *B.subtilis* LTA (△) at 0, 0.1, 1 or 10 mg/mL. (For comparison CBA F1 data from Figure 3.4 above is included *E.coli* LPS (●) and *B.subtilis* LTA (▲)). Supernatants were collected 24 hrs following replacement of treatment media and assayed for GM-CSF, IL-6 and KC by cytokine specific ELISAs. Data was normalised to the number of viable uterine epithelial cells remaining at the end of the culture period and expressed as pg/mL/10^5 cells (A) GM-CSF, (B) IL-6 or (C) KC. All measurements are the mean ± SD of duplicate wells for each treatment. These data are representative of three replicate experiments. $P < 0.05$, ★- compared with control – media only.

3.6 THE EFFECT OF TGFβ AND LPS OR LTA ON UTERINE EPITHELIAL CELL GM-CSF, IL-6 AND KC SECRETION IN VITRO

The previous experiments demonstrated that TGFβ as well as bacterial LPS and LTA individually are capable of eliciting cytokine responses by female reproductive tract cells. It is foreseeable that during insemination commensal bacteria and or their products may be introduced into the female tract in the context of seminal TGFβ. To investigate whether there is any interaction between TGFβ and these moieties, uterine epithelial cell cultures were established and incubated with recombinant $TGF\beta_1$ 0.5 and 5 ng/mL alone or in combination with either *E.coli* LPS (0, 3.125, 12.5, 50, 200 or 800 ng/mL) or *B.substilis* LTA (0., 1 or 10 μg/mL) for 16 hrs. Supernatants were replaced and collected 24 hrs later and assayed for GM-CSF, IL-6 and KC content by commercial ELISAs.

3.6.1 Interaction between TGFβ and *E.coli* LPS

As in the previous experiments, GM-CSF production was increased in response to TGFβ ($p=0.036$ and $p=0.037$) and LPS alone ($p=0.011$, $p=0.03$, $p=0.023$, $p=0.012$ and $p=0.025$). Upon addition of 0.5 ng/mL TGFβ there was a 45% to 2.7-fold increase in GM-CSF production. There was an additive but not synergistic effect of LPS at this TGFβ dose, with the exception of the highest concentration of LPS, where there was an antagonistic effect (see Figure 3.6 A). However when 5 ng/mL TGFβ was added in the presence of LPS, the additive effect was lost with no significant increases above that observed for TGFβ alone.

IL-6 secretion was significantly decreased following TGFβ ($p=0.021$) or LPS administration alone ($p=0.003$ and $p=0.023$). The interaction between 0.5 ng/mL TGFβ and LPS was additive, with decreases from 22% down to 55% following addition of 0 to 800 ng/mL LPS (p-0.04) (see Figure 3.6 B).

Consistent with previous experiments described in this chapter, TGFβ inhibited and LPS increased KC production by uterine epithelial cells, however this did not reach statistical significance. Addition of 0.5 ng/mL TGFβ, together with LPS reduced LPS stimulation of KC secretion by uterine epithelial cells by 50% - 70% (see Figure 3.6 C). Similarly, 5 ng/mL TGFβ inhibited LPS stimulated KC production by 66% - 75%. The observed differences were not statistically significant.

3.6.2 Interaction between TGFβ and *B.subtillis* LTA

As in the previous experiments, GM-CSF production was increased in response to both TGFβ (p=0.001) and LTA alone (p=0.007). Addition of 5 ng/mL TGFβ alone resulted in a 2.4-fold increase in GM-CSF production. This effect was additive but not synergistic in the presence of LTA where there were 2.4-fold, 86% and 62% (p=0.017) increases to 0.1, 1 and 10 ng/mL LTA respectively (see Figure 3.7 A).

In this experiment IL-6 secretion was not significantly altered following TGFβ or LTA administration alone, due to the large amount of variation between wells. However, addition of 5 ng/mL TGFβ reduced IL-6 production by 32% (p=0.053), in the presence of 0.1, 1 and 10 ng/mL LTA there were decreases of 41%, 57% and 41% respectively (see Figure 3.7 B).

Consistent with previous experiments described in this chapter, TGFβ inhibited (p=0.059) and LTA increased (p=0.055) KC production. However statistical significance was not reached, again most likely due the amount of variation between wells. Addition of 5 ng/mL TGFβ reduced KC production by 43% and in the presence of 0.1, 1 and 10 ng/mL LTA respectively there were decreases of 32, 51 and 38% respectively (see Figure 3.7 C).

3.7 THE COMBINED EFFECT OF LPS AND LTA ON UTERINE EPITHELIAL CELL GM-CSF, IL-6 AND KC SECRETION IN VITRO

Experiments conducted in 3.5 above demonstrated the ability of both Gram +ve LTA and Gram –ve LPS bacterial components to individually alter the cytokine secretion profile of uterine epithelial cells in culture. In a natural insemination scenario it is reasonable to expect these products to be present concurrently. In order to asses the joint effect of LPS and LTA, uterine epithelial cell cultures were established and 0, 2, 20 or 200 ng/mL *E.coli* LPS was added to wells containing 0, 0.1, 1 or 10 μg/mL *B.subtilis* LTA for 16 hrs. Supernatants were replaced and collected 24 hrs later and assayed for GM-CSF, IL-6 and KC content by commercial ELISAs.

GM-CSF dose dependent responses to LPS and LTA individually were representative of those seen in 3.5 and 3.6 above with up to 7.2- and 2.6-fold increases respectively (p=0.032 and p=0.004). The joint administration of these molecules did not elicit additive effects, i.e. GM-CSF production significantly increases from 25 pg/mL/10^5 cells to 40, 100 and 180 pg/mL/10^5 cells in response to 2, 20

or 200 ng/mL LPS and remains unchanged regardless of the concentration of LTA added (see Figure 3.8 A).

In this experiment IL-6 secretion was not significantly altered following LPS or LTA administration independently or in combination. However, there was a trend towards reduced IL-6 production in the presence of 1 and 10 ng/mL LTA respectively (see Figure 3.8 B).

LPS and LTA individually elicited dose dependent responses in KC production which were similar to those seen in 3.5 and 3.6 above with up to 2.1-fold and 71% increases respectively ($p=0.027$ and $p=0.076$). The concurrent addition of these molecules to cultured cells resulted in an additive effect on KC secretion. Following the addition of 2 or 20 ng/mL LPS KC production increased in a dose dependent manner to 0.1, 1 and 10 μg/mL LTA but generally failed to reach significance. However with 200 ng/mL LPS there was no greater KC elicited in response to LTA (see Figure 3.8 C).

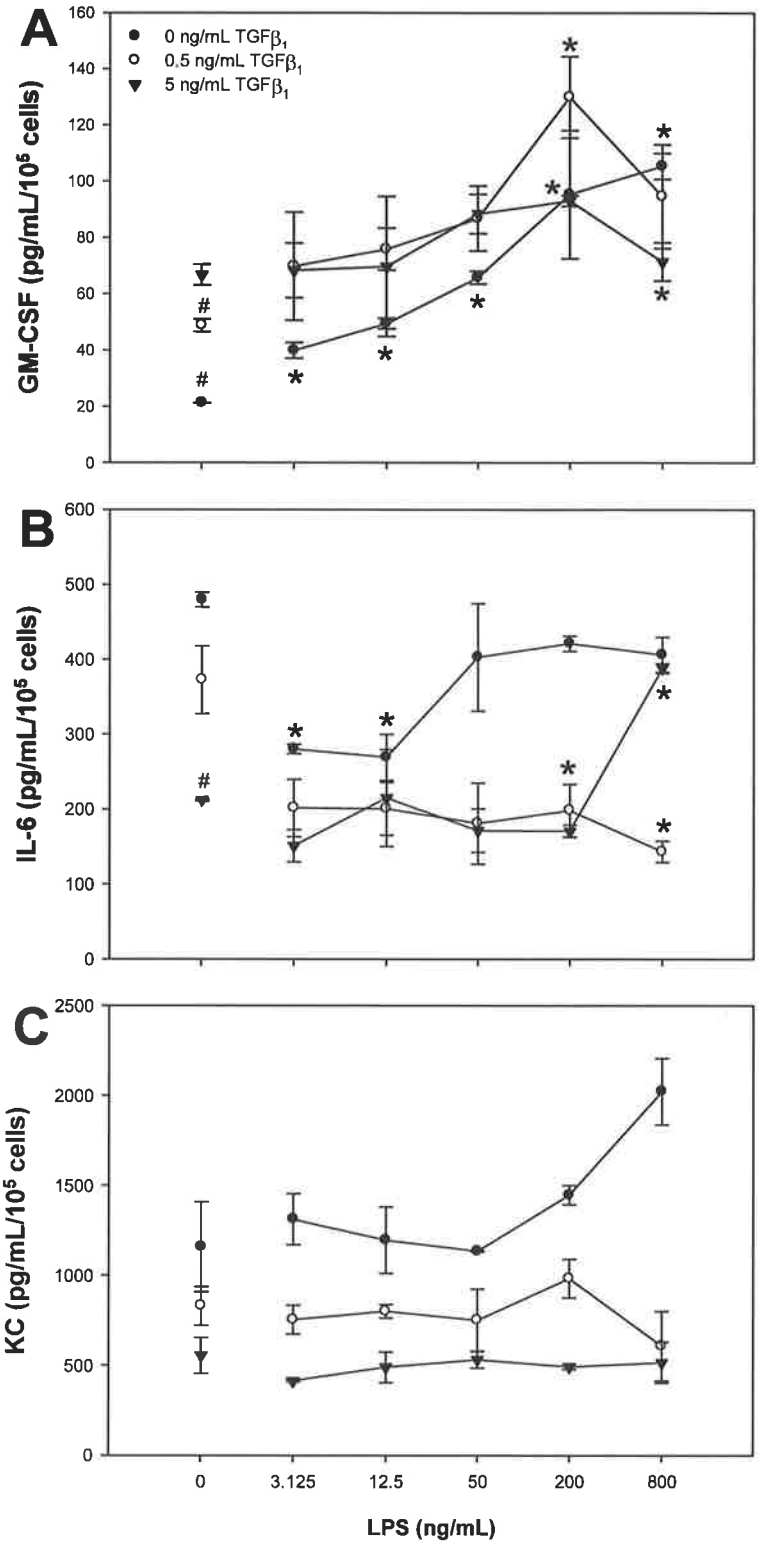


Figure 3.6 Effect of recombinant TGFβ and E.coli LPS on uterine epithelial cell GM-CSF, IL-6 and KC secretion. Uterine epithelial cells were cultured 0 ng/mL (●), 0.5 ng/mL (○) or 5 ng/mL (▼) TGFβ1 together with *E.coli* LPS (3.125, 12.5, 50, 200 or 800 ng/mL). Supernatants were collected 24 hrs following replacement of treatment media and assayed for GM-CSF, IL-6 and KC by cytokine specific ELISAs. Data was normalised to the number of viable cells at the end of the culture period and expressed as pg/mL/10^5 cells (A) GM-CSF, (B) IL-6 or (C) KC. Measurements are the mean ± SD of duplicate wells for each treatment and are representative of three replicate experiments. $P < 0.05$ ✱-compared to TGFβ without LPS. # - compared to control – media alone.

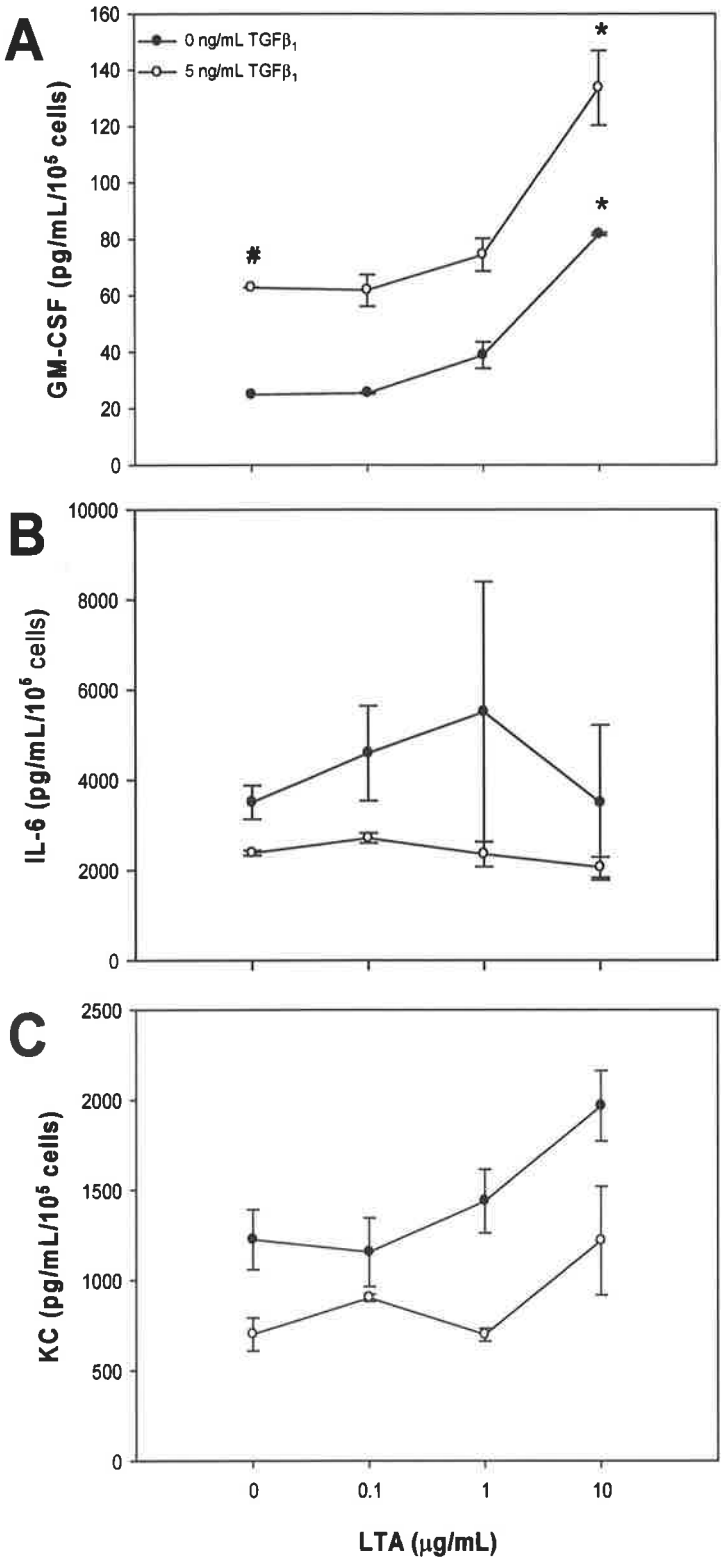


Figure 3.7 Effect of recombinant TGFβ and *B.subtilis* LTA on uterine epithelial cell GM-CSF, IL-6 and KC secretion. Uterine epithelial cells were cultured with 0 ng/mL (●) or 5 ng/mL (○) TGFβ1 together with *B.subtilis* (0.1, 1 or 10 mg/mL). Supernatants were collected 24 hrs following replacement of treatment media and assayed for GM-CSF, IL-6 and KC by cytokine specific ELISAs. Data was normalised to the number of viable uterine epithelial cells remaining at the end of the culture period and expressed as pg/mL/10^5 cells (A) GM-CSF, (B) IL-6 or (C) KC. All measurements are the mean ± SD of duplicate wells for each treatment and are representative of three replicate experiments. $P < 0.05$, ✱ - compared to TGFβ without LTA, # - compared to control – media alone.

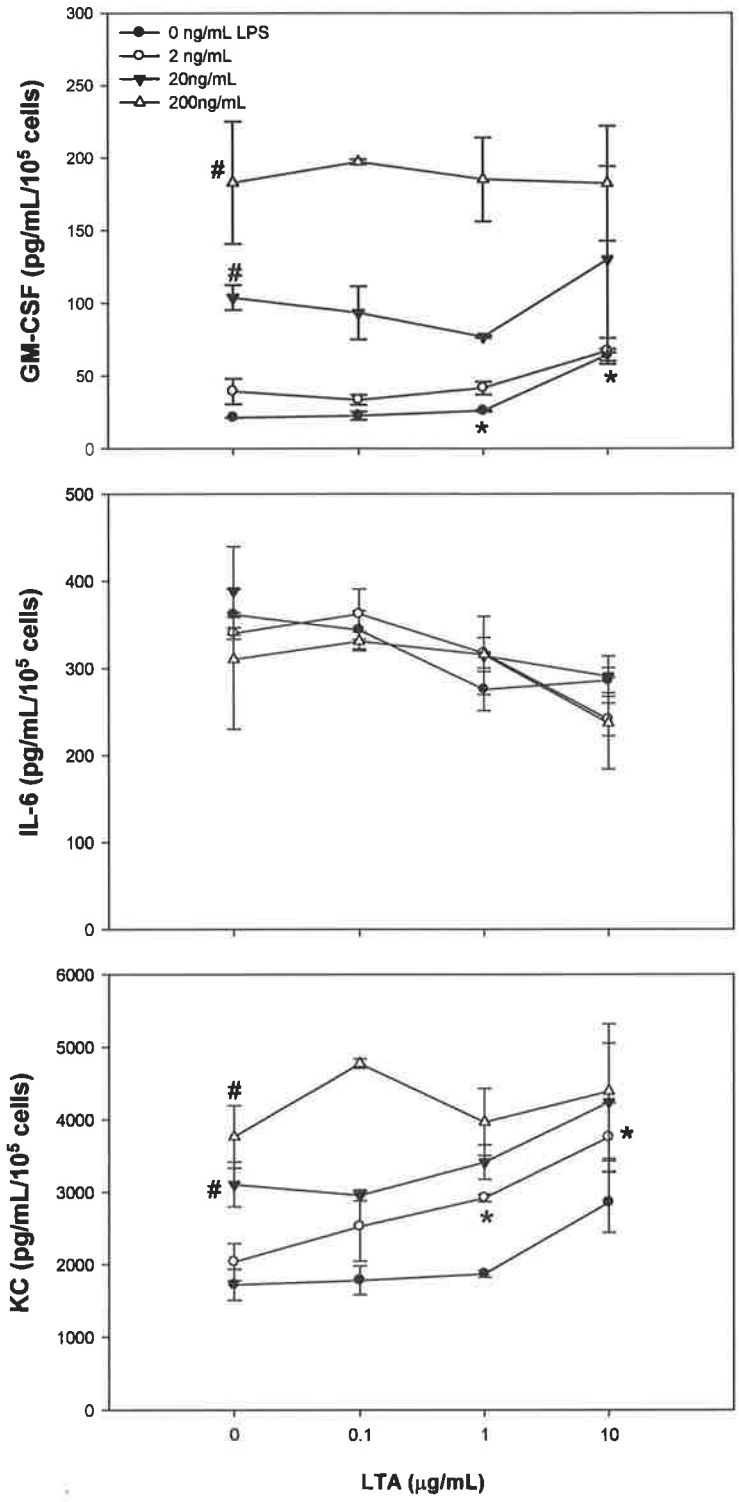


Figure 3.8 Combined effect of *E.coli* LPS and *B.subtilis* LTA on uterine epithelial cell GM-CSF, IL-6 and KC secretion. Uterine epithelial cells were cultured with 0 ng/mL (●), 2 ng/mL (O), 20 ng/mL (▼) or 200 ng/mL (△) *E.coli* LPS together with *B.subtilis* LTA 0, 0.1, 1 or 10 mg/mL. Supernatants were collected 24 hrs following replacement of treatment media and assayed for GM-CSF, IL-6 and KC by cytokine specific ELISAs. Data was normalised to the number of viable uterine epithelial cells remaining at the end of the culture period and expressed as pg/mL/10^5 cells (A) GM-CSF, (B) IL-6 or (C) KC. All measurements are the mean ± SD of duplicate wells for each treatment and are representative of three replicate experiments. $P< 0.05$, ✱ - compared to TGFβ without LTA, # - compared to control – media only.

3.8 THE EFFECT OF IFNγ ON GM-CSF, IL-6 AND KC SECRETION BY PRIMARY UTERINE EPITHELIAL CELLS IN VITRO

IFNγ, a pro-inflammatory type 1 cytokine, has been detected in both human and pig semen [15, 369, 371, 481] (O'Leary unpublished) and has been shown to interfere with TGFβ stimulated GM-CSF production in a diverse range of cell lineages [18, 78, 81]. To investigate the effect of IFNγ addition on basal and TGFβ stimulated cytokine production by uterine epithelial cells, cells were incubated with either $TGF\beta_1$ or IFNγ alone or in combination. $TGF\beta_1$ at 0, 0.5 and 5 ng/mL were added to cultures with either 0, 0.03, 0.15, 0.6, 2.5, 10 or 40 ng/mL of IFNγ and incubated for 16 hrs. Supernatants were replaced and collected 24 hrs later and assayed for GM-CSF, IL-6 and KC content by commercial ELISAs.

TGFβ addition to the cells elicited similar increases in GM-CSF production shown earlier in Figure 3.3 A. The presence of IFNγ alone, at increasing concentrations resulted in significant inhibition of GM-CSF secretion of up to 88% (p=0.005). Addition of IFNγ together with TGFβ demonstrated an interaction between these cytokines, with IFNγ significantly down regulating 0.5 or 5 ng/mL TGFβ stimulated GM-CSF production, by up to 65% and 60% respectively (see Figure 3.9 A) (p=0.002 and p=0.018).

This experiment again showed the inhibitory effect of TGFβ in decreasing IL-6 production by uterine epithelial cells (see Figure 3.9 B), which was significant at a TGFβ dose of 5 ng/mL (p=0.001). There was a trend towards IFNγ overcoming 0.5 ng/mL TGFβ inhibition of IL-6 but this did not reach statistical significance (see Figure 3.9 B). However, IFNγ overcame the inhibitory effect observed on IL-6 production following the addition of 5 ng/mL of TGFβ and indeed, was restored to basal levels at 10 – 40 ng/mL IFNγ (p=0.046 and p=0.042).

Consistent with previous experiments described in this chapter, TGFβ inhibited KC production, while IFNγ had no significant effect. Co-incubation with TGFβ and IFNγ did not alter the inhibitory effect exerted by TGFβ (see Figure 3.9 C).

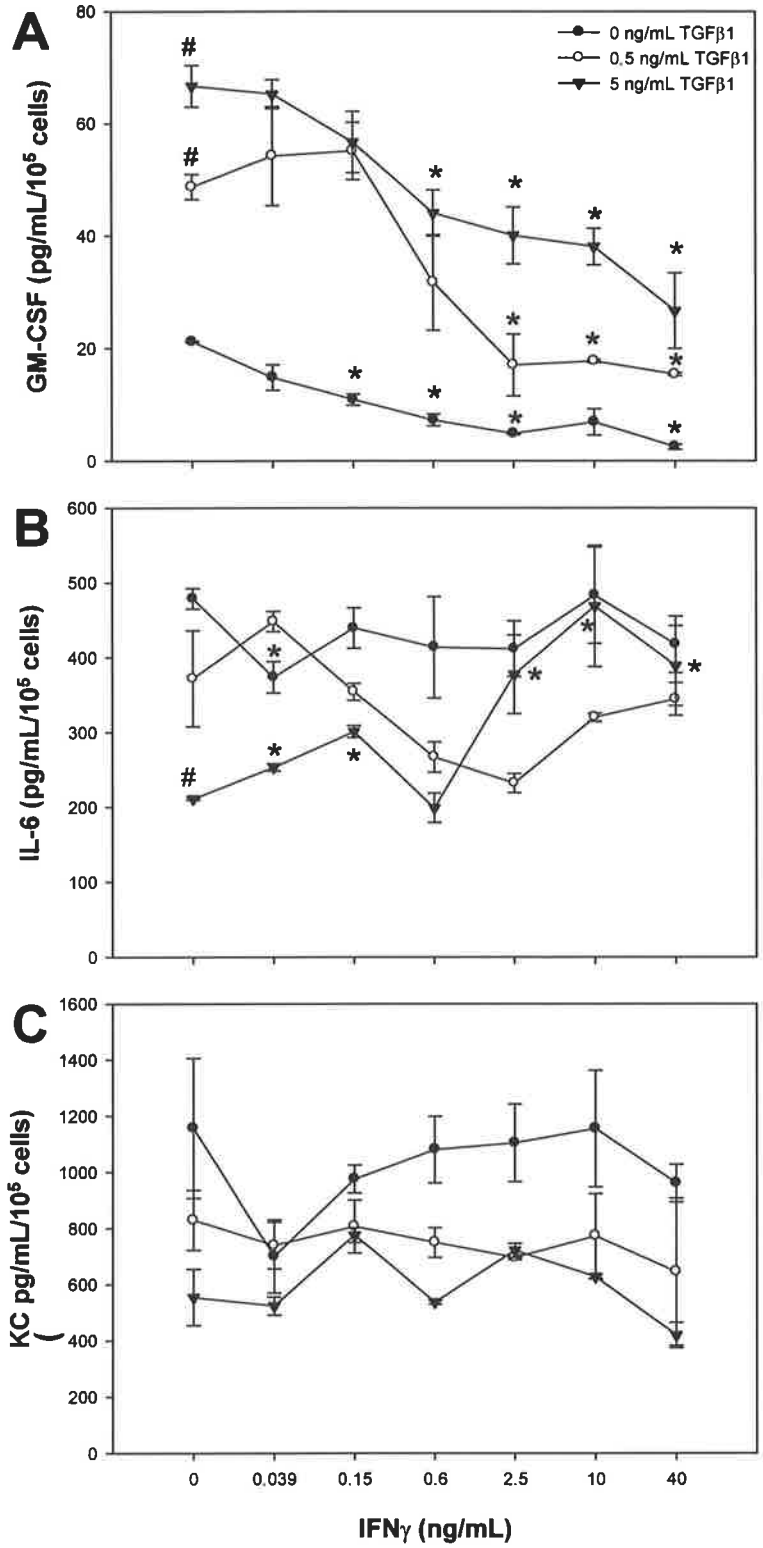


Figure 3.9 Effect of IFNγ and TGFβ on uterine epithelial cell cytokine production. Uterine epithelial cells were cultured with 0 ng/mL (●), 0.5 ng/mL (○) or 5 ng/mL (▼) TGFβ1 together with 0, 0.03, 0.15, 0.6, 2.5, 10 or 40 ng/mL IFNγ or TGFβ1 together with IFNγ. Supernatants were collected 24 hrs following replacement of treatment media and assayed for GM-CSF, IL-6 and KC by cytokine specific ELISAs. Data was normalised to the number of viable uterine epithelial cells remaining at the end of the culture period and expressed as pg/mL/10^5 cells (A) GM-CSF, (B) IL-6 or (C) KC. All measurements are the mean ± SD of duplicate wells for each treatment. These data are representative of three replicate experiments. $P < 0.05$, ✱ - compared to TGFβ without IFNγ, # - compared to control – media only.

3.9 THE EFFECT OF IFNγ AND BACTERIAL LPS AND LTA ON UTERINE EPITHELIAL CELL GM-CSF, IL-6 AND KC SECRETION IN VITRO

Experiments earlier in this chapter demonstrate regulatory effects of LPS or LTA on uterine epithelial cells in culture (see 3.5 above) as well as the ability of IFNγ to inhibit basal and TGFβ stimulated GM-CSF production (see 3.8 above). To investigate whether IFNγ can alter the LPS or LTA generated cytokine profile of uterine epithelial cells, cells were incubated with 0, 0.4, 4 or 40 ng/mL IFNγ in combination with 0, 2, 20 or 200 ng/mL LPS or 0, .01, 1 or 10 μg/mL LTA for 16 hrs. Supernatants were replaced and collected 24 hrs later and assayed for GM-CSF, IL-6 and KC content by commercial ELISAs.

3.9.1 Interaction between IFNγ and *E.coli* LPS

Consistent with earlier findings of LPS upregulation of GM-CSF production (see 3.5 above), uterine epithelial cells increased GM-CSF secretion by 2.0-, 6.2- and 10.6-fold in response to 2, 20 or 200 ng/mL LPS alone ($p=0.068$, $p<0.001$ and $p=0.003$) (see Figure 3.10 A). Similarly IFNγ alone inhibited GM-CSF production up to 72% compared to control ($p=0.013$). The addition of IFNγ at increasing concentrations in the presence of 2, 20 or 200 ng/mL LPS caused significant inhibition of GM-CSF secretion up to 54%, 68% and 65% respectively and was dose dependent ($p=0.044$, $p<0.001$ and $p=0.062$).

In this experiment IL-6 secretion was not altered following LPS or IFNγ treatment independently or in combination (see Figure 3.10 B).

LPS elicited a dose dependent increase in KC production which was comparable to those seen in 3.5 and 3.6 above with up to 3.2-fold increases ($p=0.005$ and $p=0.012$). Increasing doses of IFNγ in the presence of 20 or 200 ng/mL LPS caused significant inhibition of KC secretion by up to 36% and 66% respectively ($p=0.002$ and $p=0.012$) (see Figure 3.10 C).

3.9.2 Interaction between IFNγ and *B.subtilis* LTA

Supporting the preceding findings, addition of LTA to uterine epithelial cells increased GM-CSF secretion up to 3.3-fold in response to LTA alone ($p=0.007$) (see Figure 3.11 A). Similarly IFNγ alone inhibited GM-CSF production up to 76% compared to control ($p=0.001$). GM-CSF production was

unchanged by the addition of 0.1 μg/mL LTA but significant 58% – 75% reductions were observed when cells were co-cultured with 4 and 40 ng/mL IFNγ (p=0.022 and p=0.002). While not statistically significant, there was a 25% increase in GM-CSF secretion in response to 1 μg/mL LTA followed by a 46% decrease when co-cultured with 40 ng/mL IFNγ (p=0.023). Most significant was the 3.3-fold increase in uterine epithelial cell production of GM-CSF in response to 10 μg/mL LTA and subsequent 44% and 56% decreases observed after the addition of 4 and 40 ng/mL IFNγ (p=0.021 and p=0.023).

IL-6 secretion was not altered in this experiment whether uterine epithelial cells were cultured with LTA or IFNγ independently or in combination (see Figure 3.11 B).

KC production was increased by 60%, in response to 10 μg/mL LTA, similar to the result shown in section 3.5 above (see Figure 3.11 C). This failed to reach statistical significance due to the amount of variation between wells. There was no significant effect of the addition of IFNγ at any concentration.

3.10 UTERINE EPITHELIAL CELL RECEPTOR EXPRESSION

The results described in this chapter show uterine epithelial cell responsiveness to a variety of immune modulators, including TGFβ, LPS, LTA and IFNγ. In order to confirm that uterine epithelial cells have the molecular capacity to respond to these seminal fluid constituents, real time reverse transcription – PCR (RT-PCR) was employed to evaluate uterine epithelial cell expression of mRNAs content for TGFR1 & R2 (TβR-I and TβR-II), TLR2 & 4, CD14 and IFNGR1 & R2 mRNA. RNA was isolated from uterine epithelial cells harvested from CBA F1 female mice, reverse transcribed and amplified in an ABI Prism 5700 Sequence Detection System using primers designed in Primer Express (see Chapter 2).

Uterine epithelial cell expression of TβR-I & II, TLR2 & 4, CD14 and IFNGR1 & R2 was confirmed by real time RT-PCR using targeted primers. PCR products generated were run on a 2 % agarose gel together with pUC19 ladder in order to visualize and estimate the size of amplicons (see Figure 3.12). Primers against mIFNGR1, mIFNGR2, CD14, TβR-I, TβR-II, TLR2 and TLR4 amplified fragments of 116bp, 119bp, 156bp, 78bp, 74bp, 127bp and 111bp respectively, which corresponded to the expected sizes.

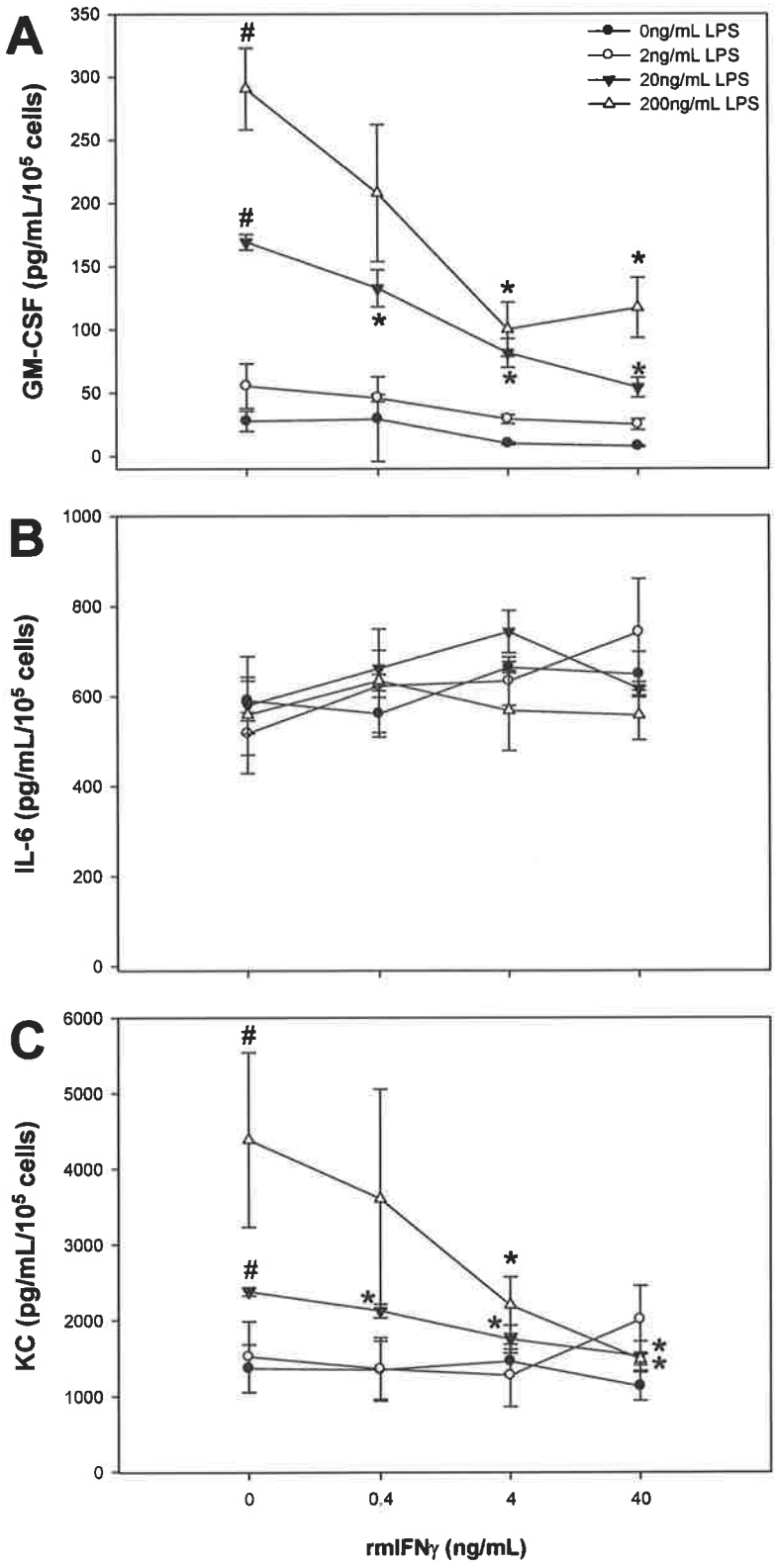


Figure 3.10 Interaction between IFNγ and *E.coli* LPS on uterine epithelial cell GM-CSF, IL-6 and KC secretion. Uterine epithelial cells were cultured with 0 ng/mL (●), 2 ng/mL (○), 20 ng/mL (▼) or 200 ng/mL (△) *E.coli* LPS together with 0, 0.4, 4, or 40 ng/mL IFNγ. Supernatants were collected 24 hrs following replacement of treatment media and assayed for GM-CSF, IL-6 and KC by cytokine specific ELISAs. Data was normalised to the number of viable uterine epithelial cells remaining at the end of the culture period and expressed as pg/mL/10^5 cells (A) GM-CSF, (B) IL-6 or (C) KC. All measurements are the mean ± SD of duplicate wells for each treatment and are representative of three replicate experiments. $P < 0.05$, ★ - compared to LPS without IFNγ, # - compared to control – media only.

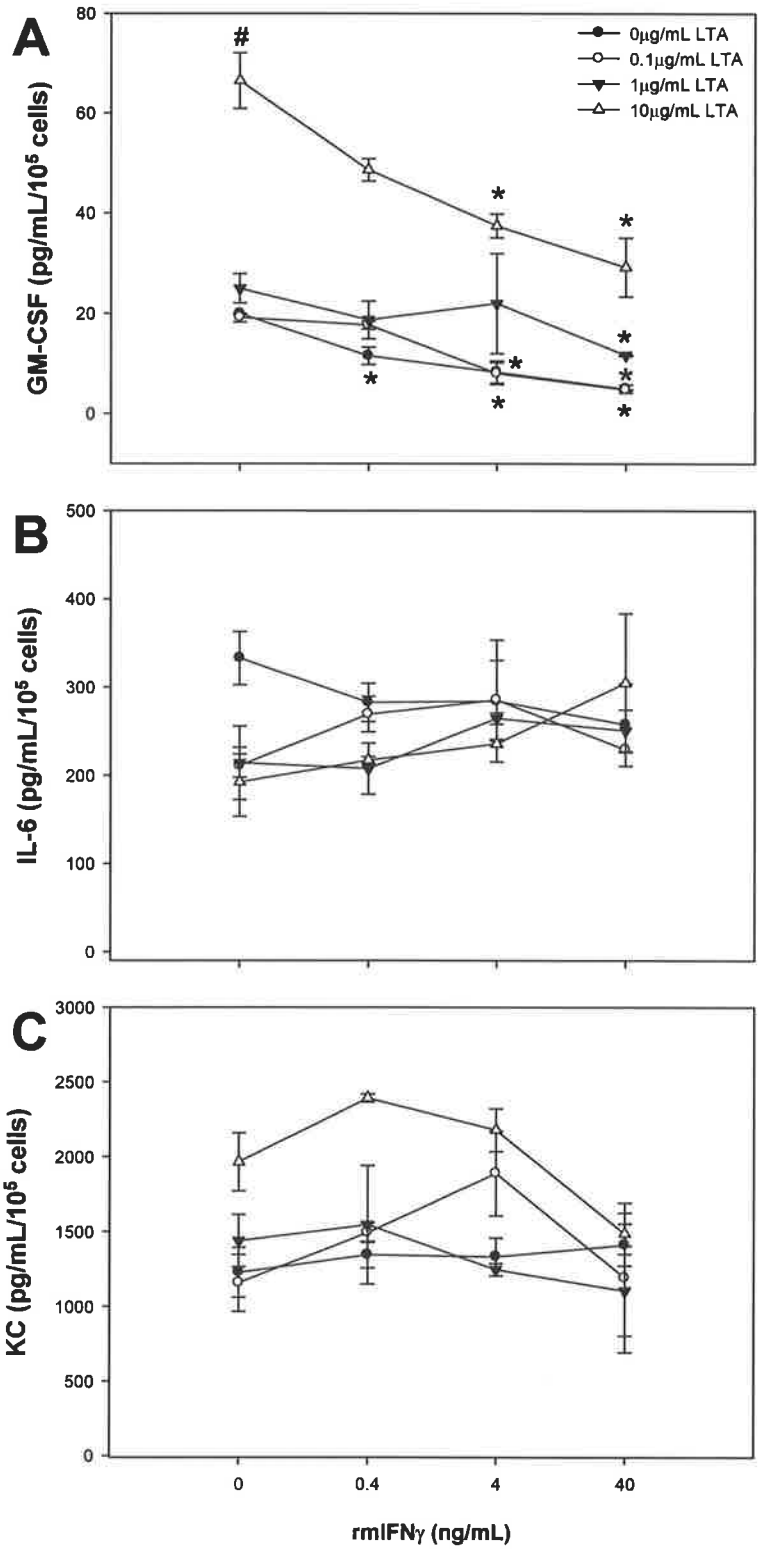


Figure 3.11 Interaction between IFNγ and *B.subtilis* LTA on uterine epithelial cell GM-CSF, IL-6 and KC secretion. Uterine epithelial cells were cultured with 0 ng/mL (●), 0.1 ng/mL (○), 1 ng/mL (▼) or 10 mg/mL (△) *B.subtilis* LTA together with 0, 0.4, 4, or 40 ng/mL IFNγ. Supernatants were collected 24 hrs following replacement of treatment media and assayed for GM-CSF, IL-6 and KC by cytokine specific ELISAs. Data was normalised to the number of viable uterine epithelial cells remaining at the end of the culture period and expressed as pg/mL/10^5 cells (A) GM-CSF, (B) IL-6 or (C) KC. All measurements are the mean ± SD of duplicate wells for each treatment and are representative of three replicate experiments. $P < 0.05$, ★ - compared to LTA without IFNγ, # - compared to control – media only.

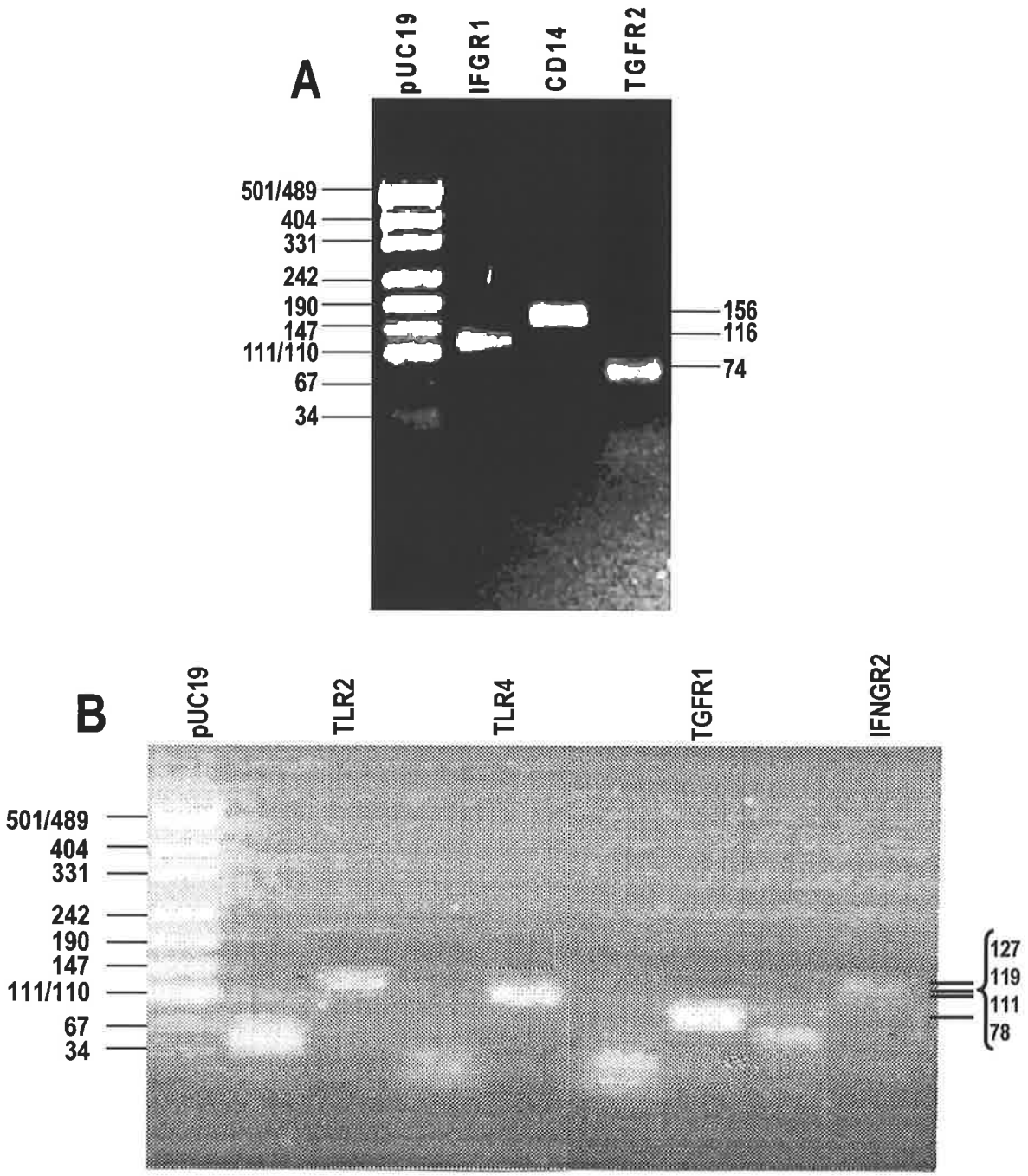


Figure 3.12 Uterine epithelial cell receptor expression. Real time RT-PCR was used to confirm uterine epithelial cell (A) TGFR2, IFNGR1, CD14 and (B) TLR2 & 4, TGFR1 and IFNGR2 expression. PCR products were electrophoresed on an agarose gel together with pUC19 ladder (bp left) enabling amplicon size determination (bp right).

3.11 DISCUSSION

The experiments described in this chapter were designed to further understand the role that factors present in seminal fluid play in regulating the post-mating inflammatory response in the murine uterus. The results shown here demonstrate the complex nature of the uterine epithelial cell cytokine profile and its differential regulation by a variety of seminal fluid constituents (summarised in Table 3.1). These experiments have shown that uterine GM-CSF is increased following mating with an intact male and that IL-6 and KC are similarly increased. Post-mating increases in uterine GM-CSF, IL-6 and KC content measured in these experiments were not due to their deposition into the female tract at the time of mating. Previous studies have demonstrated that male accessory gland secretions contain low levels of IL-6 (5 pg/mL) and KC (40 pg/mL) representing 1% and 10% respectively of that measured here in mated uterine luminal fluid and no detectable GM-CSF [122, 364]. Additionally, mating in the absence of seminal vesicle fluid (SVF) fails to induce this cytokine response, indicating a pivotal role for SVF in its induction. This is consistent with previous studies in mice showing that indeed SVF is required for inducing the increase in GM-CSF production by uterine epithelial cells following mating [12].

Table 3.1 The effect of seminal vesicle fluid and constituent factors on pro-inflammatory cytokine expression from uterine epithelial cells.

		GM-CSF	IL-6	KC
In vivo	Mating	↑↑↑	↑↑↑	↑↑↑
	Mating –sv	↔	↑	↑
	Mating -vas	↔	↑	↑
In vitro	SVF	↑↑↑	↔	↓↓↓
	$TGF\beta_{1-3}$	↑↑	↔	↓
	LPS	↑↑↑	↓	↑↑
	LTA	↑↑	↓↓	↑
	IFNγ	↓	↔	↔
	$TGF\beta_1$ & LPS	↑↑↑	↓	↓
	$TGF\beta_1$ & LTA	↑↑	↓	↓
	LPS & LTA	↑↑↑	↔	↑↑
	IFNγ & $TGF\beta_1$	↓	↔	↓
	IFNγ & LPS	↓	↔	↓
	IFNγ & LTA	↓	↔	↔

SVF was found to markedly increase uterine epithelial cell GM-CSF production in culture. The increase observed in vitro was proportionately larger relative to the untreated control compared to the increase seen in vivo. This finding is consistent with previous studies in the mouse and human where GM-CSF production is upregulated in response to seminal vesicle fluid [12, 130]. However, in contrast the magnitude of the increase following mating has previously shown to be greater than that achieved in vitro, here the converse is true [12].

Addition of recombinant TGFβ to uterine epithelial cells elicited stimulation of GM-CSF production similar to increases in response to seminal vesicle fluid. There was some evidence that the stimulatory activity of TGFβ might be isoform specific, where TGFβ_1 and β_3 had a marginally higher stimulatory effect on GM-CSF production than that of β_2 at the same dose. This could be explained by differential expression pattern of the TGFβ receptors by the uterus. TGFβ_1 and TGFβ_3 bind with high affinity to the type 2 TGFβ receptor, Tβ-RII, whereas TGFβ_2 has low affinity for this receptor and requires co-localisation of the type 3 TGFβ receptor, Tβ-RIII (betaglycan) to elicit a high affinity interaction [54, 55]. This study confirmed Tβ-RI and Tβ-RII expression in the estrous uterine epithelium via RT-PCR, however this was not quantified. Nor was a study undertaken to evaluate the effect of estrous cycle on receptor expression. Jones et al [482] have identified Tβ-RIII (betaglycan) expression and localisation in the human endometrial stroma and luminal epithelium; however it remains to be shown whether this is the case in the murine uterus. While one study has demonstrated estrogen withdrawal increases Tβ-RII [483] expression in the mouse uterus, to date no studies have examined endometrial TGFβ receptor expression throughout the estrous cycle in the mouse. This type of investigation would contribute to understanding regulatory mechanisms of TGFβ signalling in the cycling uterus. A recent study in rats has shown weak Tβ-RI and Tβ-RII expression throughout the estrous cycle, with an increase following mating and then a decrease prior to implantation [484]. In contrast to this, human studies have shown Tβ-RII endometrial expression to be maximal in the proliferative stage of the menstrual cycle, occurring highest in epithelial cells [86].

It is well documented that the female genitourinary tract supports a healthy population of bacteria, generally of the gram positive *Lactobacillus* species [373, 374]. The commensal microflora compete with pathogenic bacteria that may appear transiently especially in mice that are sexually active. Hence the female genitourinary mucosa is a constant source of the pro-inflammatory bacterial cell wall components LPS (gram negative) and LTA (gram positive). In addition LPS has been detected in human and boar semen [130, 380]. In agreement with previous studies, incubation of uterine

epithelial cells with LPS or LTA evoked dose dependent increases in GM-CSF production [111]. This experiment demonstrates the potent stimulatory effect of LPS compared to LTA, requiring approximately 1000-fold less to elicit comparable GM-CSF secretion by uterine epithelial cells. The relative LPS and LTA potencies shown here are consistent with previous studies using human gingival fibroblasts [485]. Uterine epithelial cell expression of the LTA and LPS receptors was confirmed with TLR2, TLR4 and CD14 mRNAs respectively detected by RT-PCR consistent with the biochemical capacity of these cells to respond to LPS and LTA. The role of TLR4 in eliciting this response was confirmed by employing the use of LPS-hyporesponsive TLR4 mutant mice, C3H/HeJ. LPS was unable to stimulate GM-CSF production by C3H/HeJ uterine epithelial cells, where as LTA stimulation was unaffected and comparable to that seen in CBA F1 mice. Incubation of uterine epithelial cells with a combination of LPS and LTA did not elicit GM-CSF production higher than that seen by incubation with LPS alone. This means that in vivo, LPS from gram negative bacteria is likely to be a physiologically more important regulator of epithelial GM-CSF production than LTA from gram positive bacteria.

TGFβ and bacterial components in combination demonstrate additive effects on uterine epithelial cell GM-CSF production but did not act in synergy. The exception to this was at the highest dose of LPS in the presence of TGFβ where there was an inhibitory effect. This might be explained by upregulation of a TGFβ inhibitory molecule, Smad7, in response to high concentrations of LPS, as demonstrated previously in fibroblasts [72].

Human studies have shown IFNγ can be present in human semen at varying concentrations within the normal fertile population. Furthermore, IFNγ is detected more frequently in semen from the male partners of couples experiencing recurrent miscarriage [130]. Experiments described here show IFNγ significantly reduces basal and TGFβ stimulated uterine epithelial cell secretion of GM-CSF. This result is consistent with findings from human studies showing IFNγ inhibition of human the cervical cell response to TGFβ stimulation [130]. IFNγ has been shown to inhibit a variety of TGFβ stimulated effects in different cell lineages. It is thought that the IFNγ signalling molecule STAT1 induces expression of a TGFβ inhibitory molecule Smad7 [486]. Similarly, we have demonstrated reduced LPS and LTA stimulated GM-CSF secretion by uterine epithelial cells in the presence of IFNγ. GM-CSF as with many other cytokines, including IL-6 and KC, have a number of regulatory binding sites in the 5' proximal promoter region of the gene, including AP-1 and $NF_{\kappa}B$. Investigations in astrocytes have shown that IFNγ inhibits transcription of one of the LPS stimulated AP-1 subunits, c-Jun [487], which might explain the reduction we observe here. However the mechanism behind reduced LTA stimulation in the presence of IFNγ is not clear. Uterine epithelial cell expression of IFNγ receptors, IFGR1 and

IFGR2, was confirmed by RT-PCR correlating with their ability to respond to IFNγ.

SVF had no effect on basal levels of IL-6 secretion by mouse uterine epithelial cells. This was surprising, since mating experiments show that the absence of seminal vesicles abrogates the post-mating IL-6 surge. However, it is possible that the sperm and seminal vesicle components of semen together act synergistically to elicit uterine IL-6 production. Alternatively, the IL-6 inducing components of seminal vesicle fluid might not be active in vitro due to physical loss or lability during SVF recovery. This finding is in contrast to studies in humans where seminal plasma elicits IL-6 mRNA and protein production in endometrial and cervical cells or splenic lymphocytes in vitro [130, 488, 489], and in animal studies where boar seminal plasma increases mouse spleen cell secretion of IL-6 in vitro [490]. Addition of recombinant TGFβ to uterine epithelial cells inhibited IL-6 secretion regardless of the isoform tested. This result is not consistent with recent human studies showing significant increases in IL-6 production by primary and immortalised cervical cells as well as lung fibroblasts following incubation with TGFβ in vitro [130, 491]. This difference may be indicative of differences in signalling pathways between these species or differences in seminal fluid preparation. Alternatively, differential contribution of active moieties from other male tract glands, for example the coagulating or prostate glands, might occur since the in vitro human experiments utilize whole seminal plasma while the mouse experiments utilize seminal vesicle fluid in isolation.

Bacterial LPS or LTA reduced basal uterine epithelial cell secretion of IL-6 in a dose dependent manner. This result contradicts previous findings where LPS or LTA incubated with human cervical or hepatic cells markedly increases IL-6 production [130, 475, 492]. As expected, LPS incubated with uterine epithelial cells harvested from TLR4 mutant, C3H/HeJ mice, had no effect on IL-6 secretion. LTA addition to C3H/HeJ uterine epithelial cell cultures did not alter IL-6 production which was surprising in the light of down regulation in CBA F1 cells and given that LTA signals via TLR2, but not TLR4. However there were significant strain differences in basal IL-6 production, where C3H/HeJ cells secreted approximately 80% less IL-6 than CBA F1 cells, potentially accounting for diminished IL-6 responsiveness by these cells. Combined LPS and LTA did not alter IL-6 production however there was a trend toward reduced secretion at high LTA concentrations.

TGFβ and LPS added in combination to uterine epithelial cell cultures together exhibited additive but not synergistic reduction of IL-6 production. An unusual feature was the ability of high concentrations of LPS to overcome TGFβ inhibition of IL-6 production, although this may be explained by LPS upregulation of a TGFβ inhibitory molecule, Smad7, as mentioned above.

Addition of IFNγ to uterine epithelial cell cultures did not alter basal IL-6 production. There was an indication of possible IFNγ reversal of TGFβ inhibition of IL-6 production but the effect did not statistical significance. This is a surprising result given the marked interference of IFNγ with TGFβ stimulation of GM-CSF however it may be indicative of divergent intracellular signalling cascades required for differential cytokine responses. Interestingly, this result is consistent with recent human data showing no effect of IFNγ on TGFβ regulated IL-6 production [130]. There was no interaction between IFNγ and LPS or LTA on IL-6 uterine epithelial cell production.

Mating with sv- males showed that the absence of seminal vesicle fluid reduced the post-mating KC surge, suggesting that a component of seminal vesicle fluid is a required to induce KC production in vivo. In surprising contrast to the in vivo mating experiments, SVF completely ablated KC production by mouse uterine epithelial cells in vitro. This shows that KC inducing factors present in vivo are not present or inactivated in the in vitro experiments. As with IL-6 inducing factors in seminal fluid, explanations for the in vivo and in vitro disparity include inactivation of seminal vesicle fluid constituents, or important contributions of factors not originating in the seminal vesicle. This result is contradictory to human studies where human plasma elicits an increase of IL-8 (human ortholog of KC) by human cervical epithelial cells [130, 493]. Recombinant TGFβ reduced basal uterine epithelial cell KC secretion by up to 48%. This concurs with recent human studies showing significant decreases in IL-8 production by primary and immortalised cervical cells as well as lung fibroblasts following incubation with TGFβ [130, 491]. However the effect of TGFβ on IL-8/KC production appears to be cell lineage specific, since upregulation is achieved in human renal tubular cells, while downregulation occurs in human endothelial cells and mouse macrophages [494-496]. The mechanisms underlying TGFβ regulation of IL-8/KC production remain to be elucidated. However, the -133 to +44 of the 5' flanking region, containing AP-1, $NF_{\kappa}B$ and NF-IL6 binding sites, is known to be essential for TGFβ stimulation of IL-8 in the human [497]. The reduction in KC production following TGFβ addition to uterine epithelial cells was not as complete as the ablation following SVF exposure, suggesting that TGFβ on its own may not explain the inhibitory effect. However whether any additional regulatory factors are active in vivo is not clear, although it is possible that the SVF extraction method activates an inhibitory factor that normally remains inactive during natural mating or conversely deactivates a stimulatory factor.

LPS and LTA increased uterine epithelial cell secretion of KC dose dependently. These results are reflective of previous findings in mice where LPS or LTA in vitro and in vivo elicit large increases in KC production [498, 499]. A combination of LPS and LTA exhibited an additive effect on KC production

at lower levels of LPS but no additional KC secretion was observed at the highest LPS concentration above that stimulated by LPS alone. C3H/HeJ uterine epithelial cell KC production remained unaltered following LPS exposure, showing the requirement for TLR4 in the KC response to LPS. Expectedly, LTA addition to C3H/HeJ uterine epithelial cell cultures increased KC production clearly demonstrating differential signalling requirements of these bacterial components.

TGFβ reduces basal and LPS stimulated KC production by uterine epithelial cells. LPS was not able to overcome the inhibitory effect of TGFβ at any of the concentrations used in this study. The mechanism behind TGFβ inhibition of LPS stimulated KC has been investigated in mouse macrophages and is thought to be via destabilisation of KC mRNA by p38 MAPK [500]. TGFβ reduction of basal KC production by uterine epithelial cells was returned to basal levels following the addition of higher concentrations of LTA. LTA was able to overcome TGFβ inhibition of KC production in a dose dependent manner that paralleled the KC response to LTA in the absence of TGFβ. Together these results strongly suggest that LPS and LTA account for the induction of KC in the mouse uterus after mating. In order to draw firm conclusions regarding this role, mating experiments utilizing LPS or LTA receptor hyporesponsive or null mice would be useful in providing further insight into the contribution of these factors in vivo.

IFNγ did not alter basal, TGFβ inhibited or LTA stimulated KC production by uterine epithelial cell cultures. However, KC secretion by cells stimulated with LPS was reduced by up to 66% following co-culture with IFNγ.

The results described in this chapter emphasize the differential contribution of moieties present at insemination towards the post-mating inflammatory response in the mouse uterus. The in vitro experiments described here indicate that the marked increase in post-mating uterine GM-CSF content may be contributed to by soluble seminal and bacterial components and furthermore this stimulation is inhibited by IFNγ. The increase in post-mating uterine IL-6 content was unable to be accounted for by individual seminal or bacterial components in these in vitro experiments. Similarly, the marked increase in uterine KC production following mating was not explained by the addition of soluble seminal components-indeed they were shown to downregulate KC secretion in vitro. Interestingly however, bacterial components exhibit a potent stimulatory effect that again was subject of inhibition by IFNγ. These results demonstrate not only the role of seminal factors in generating post-mating inflammation, but also the potential role of commensal bacteria. The data from cross titration experiments conducted in this chapter indicate that the relative concentration of active moieties present at insemination is

possibly of more importance than the absolute abundance of any one factor in generating a maternal environment conducive for optimal pregnancy outcomes. Given the differential regulatory nature of factors examined here, it would be interesting to identify additional seminal factors that may contribute to the post-mating cytokine response. Furthermore, investigation of the intracellular mechanisms responsible for eliciting interactions between factors observed here may provide targets for interventions in a clinical setting. Furthermore, the use of in vitro models is a first step in attempting to understand the more complex in vivo model and care must be taken when interpreting these results and applying them to the physiological situation, since the full functionality and composition of the seminal fluid deposited in the female reproductive tract is difficult to reproduce in vitro.

Chapter 4

Effects of in vivo administration of TGFβ or LPS on uterine cytokine production and leukocyte recruitment

4.1 INTRODUCTION

Experiments in the previous chapter identified the differential regulatory effect of factors present in seminal fluid or in the female reproductive tract on uterine epithelial cell cytokine secretion in vitro. Additionally, evidence was found to suggest that seminal factors and moieties present in the female tract interact to regulate epithelial cell cytokine production.

In vivo studies in mice have revealed the importance of the seminal vesicle fluid component of semen in eliciting the post-mating uterine cytokine surge responsible for endometrial leukocyte recruitment [12, 123-125, 152, 331, 473]. While seminal TGFβ has been identified as a key contributor in inducing uterine GM-CSF synthesis and macrophage recruitment following mating in mice, it fails to elicit neutrophil recruitment into the endometrium [12]. These studies indicate a role for factors other than seminal TGFβ in stimulating the influx of neutrophils into the endometrium following mating. As described in Chapter 3, the male and female reproductive tracts are mucosal organs, inhabited by populations of commensal bacteria as well as their degraded cell wall components, LPS and LTA. Since moieties are known to be potent immune regulators, we therefore propose that they may be introduced into the upper female tract during insemination and contribute to activating the post-mating inflammatory response.

The experiments described in this chapter aim to investigate the contribution of bioactive moieties present at insemination, identified in Chapter 3, in generating the post-mating inflammatory response in the murine uterus in vivo. These experiments required treatments to be administered via intra-uterine injection. Initially, investigations focussed on defining an appropriate route and timing of treatment administration. To assess these variables uterine luminal fluid was collected and assayed for GM-CSF as an indicator of uterine inflammation. Conventionally surgical exteriorisation of the uterine horns and injection via puncture of the uterine wall with a needle is the method used to administer intra-uterine injections (surgical intra-uterine injection). A less invasive alternative was also investigated, which involved accessing the cervix via the vagina and passage of a soft vinyl catheter across the cervical opening allowing administration into the uterine lumen (transcervical administration). Uterine epithelial cells are known to be differentially receptive to stimuli in an estrous cycle dependent manner. Hence having established an appropriate route of treatment, the timing of treatment administration with respect to ovulation was also examined.

To investigate the effect of immune regulators, identified in Chapter 3, uterine luminal fluid from CBA F1 and C57Bl/6 female mice treated with intra-uterine recombinant human TGFβ in a matrix solvent, or LPS or LTA were collected and assayed for GM-CSF, IL-6 and KC content. Additionally the ability of these regulators to stimulate leukocyte infiltration into the endometrium was investigated using immunohistochemical techniques. Furthermore, the contribution of LPS or activation of its receptor in stimulating uterine pro-inflammatory cytokine secretion and leukocyte influx following mating was examined in wildtype C57Bl/6 mice, or C3H/HeJ mice which have a non-functional TLR4 rendering them LPS hyporesponsive. Luminal fluid cytokine content and inflammatory cell recruitment was also evaluated in uterine tissue of estrous and mated wildtype C57Bl/6 and C3H/HeJ mice to provide in vivo control data.

4.2 EFFECT OF ROUTE AND TIMING OF INTRA-UTERINE TREATMENT ADMINISTRATION

Administration of treatment directly into the uterine lumen via transcervical catheterisation was an appealing alternative to traditional surgical approaches, where the skin and peritoneal cavity are breeched, traumatised and potentially exposed to infectious micro-organisms. The experiments described below aimed to investigate firstly dispersal of treatment administered transcervically, secondly the effect of cervical catheterisation on uterine cytokine content and thirdly the timing of administration with respect to ovulation.

4.2.1 Distribution of transcervically administered treatment

To investigate treatment dispersion in the uterus of treatment administered transcervically, estrous female mice were anesthetised then bromophenol blue dye was injected into the uterine lumen via transcervical catheterisation. One hour later mice were sacrificed and the uterus was examined for distribution of staining.

Uteri were uniformly stained blue from the cervix to the oviductal junction in 4 out of 5 females treated transcervically with blue dye (Figure 4.1). Treatment in the fifth mouse most likely leaked back out the cervix and out of the vagina as there was blue staining evident around the vaginal opening.

4.2.2 Transcervical vs surgical intra-uterine treatment administration

To examine whether the physical events of catheterisation or surgical treatment have an impact on uterine cytokine content, estrous mice were either left untreated, sham treated or injected with 30 µL PBS via catheterisation or surgery. Sham treatment included needle puncture through the uterine wall for the surgical route or catheter insertion through the cervix for the transcervical route. Treatment was administered between 0900 – 1000 h and mice were sacrificed at 1500 - 1800 h. Uterine luminal fluid was collected and assessed for GM-CSF and IL-6 content by commercial ELISAs.

GM-CSF within uterine fluids was significantly increased following catheterisation in both sham and PBS injected groups by 26- and 8-fold respectively ($p=0.026$ and $p=0.026$) compared to the untreated control (Figure 4.2 A). No change in GM-CSF content was seen following sham surgery, or surgery with PBS injection (Figure 4.2 B).

There was no effect of sham surgery or surgery with PBS injection on uterine IL-6 concentrations

(Figure 4.2 C). The large error bar in the PBS group is due a single outlier data point.

On the basis of these results, we initially used surgical administration of treatments rather than transcervical administration, since the latter appeared to cause greater changes in cytokine synthesis.

4.2.3 Optimal estrous cycle stage for surgical treatment administration

To investigate the optimal time to administer intra-uterine treatments via the surgical route, daily vaginal smears were performed on female CBA F1 mice to determine estrous cycle stage. Treatments [30 μL PBS or 30 μL rhTGFβ$_1$ (50 ng)] were administered 4 hrs prior to ovulation (pro-estrous) (where ovulation is expected to occur at 0000 h), at 1000 h on the day after ovulation (early estrous) or at 2000 h on the day after ovulation (late estrous). Uterine luminal fluid was collected at 1800 h (for 1000 h administration) or at 0900 h the next morning (for 2000 h administration) following treatment and assessed for GM-CSF content using commercial ELISAs. Three control groups were used including sham surgery (needle insertion through uterine wall) conducted at the pro-estrous time point with uterine luminal fluid collected as indicated above. Uterine fluid was also collected at 1000 h, from mice day 0.5 pc after mating. Estrous uterine fluid was collected from untreated mice at 1000 h on the day after ovulation.

As with the previous experiment there was no change in uterine GM-CSF production following sham surgical injection treatment. Consistent with previous studies there was a statistically significant increase in uterine GM-CSF production following mating compared to the estrous and sham controls, 2.4- and 2.5-fold respectively (p=.0.004 and p=0.027) (Figure 4.3). Early estrous was the only time point at which TGFβ instillation elicited a significant 2.1-fold increase in GM-CSF production compared with control mice receiving PBS (p=0.029). However, at this time point only 2 of 4 mice responded to TGFβ treatment.

Based on this result, the early estrous time point was judged to be the most sensitive to treatment application, and was utilized in subsequent experiments.

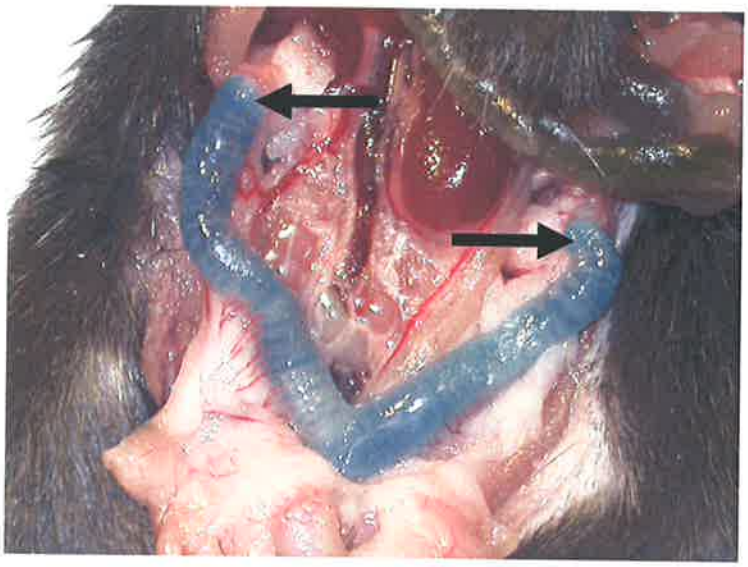


Figure 4.1 Dispersion of transcervically administered dye. Blue dye was administered via transcervical catheter into the murine uterine luminal cavity and was distributed evenly along the full length of both uterine horns (arrows) where the mouse was killed one hour after dye administration.

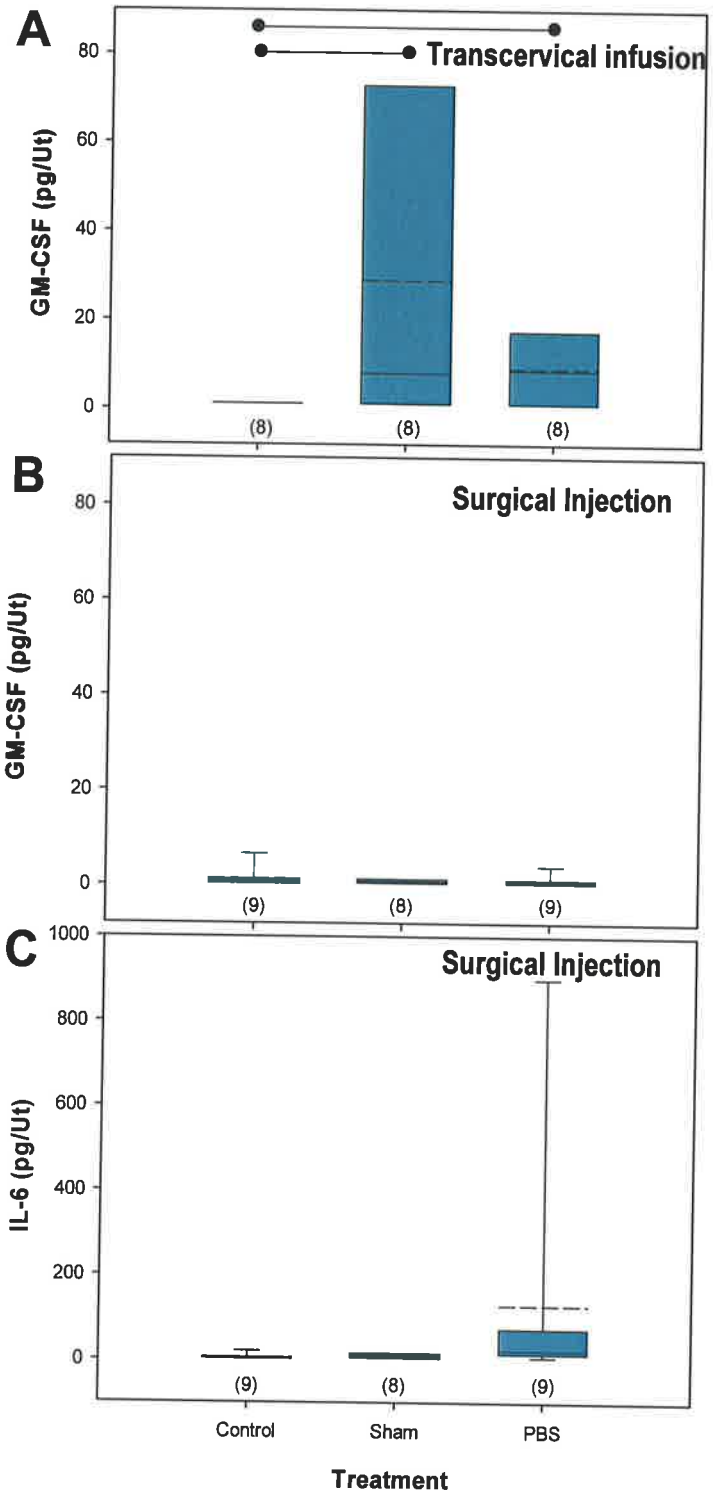


Figure 4.2 Effect of cervical catheterisation on uterine cytokine production. Cytokine content of uterine flushings was assessed eight hours after sham treatment or administration 30μL PBS in estrous mice. Uterine GM-CSF content following transcervical catheterisation (A), uterine GM-CSF (B) and IL- 6 (C) content following surgical administration were measured by ELISA. The number of mice in each group is shown in brackets. The upper and lower edges of the box and whisker plots indicate the 75th and 25th percentile respectively, while the internal solid and dashed lines are the median and the mean respectively. Error bars signify the 5th and 95th percentiles. Kruskal-Wallis and Mann-Whitney tests were used to determine statistically significant differences between treatment groups represented as dot-ended lines, significance is $p<0.05$.

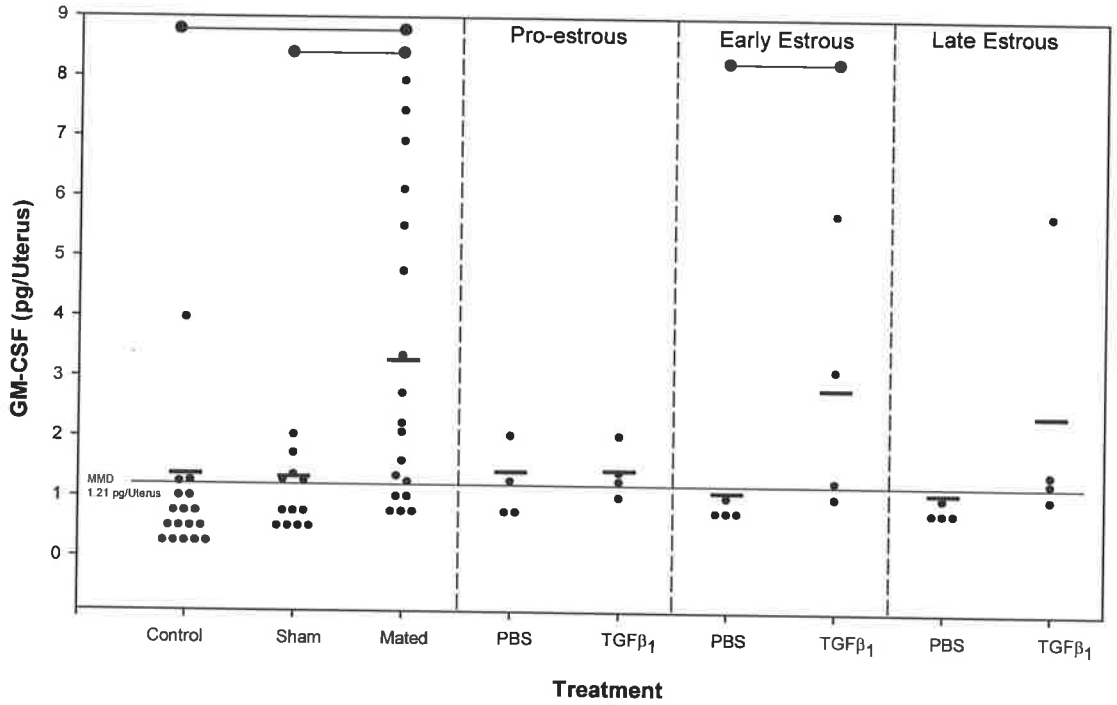


Figure 4.3 Optimal timing for surgical intra-uterine treatment. GM-CSF content of uterine luminal fluid was assessed 8 hrs or 13 hrs after surgical intra-uterine injection alone or with delivery of 30μL PBS or rhTGFβ (50ng). Treatment was administered at 2000 h prior to ovulation (Pro-estrous), 1000 h after ovulation (Early estrous) or 2000 h after ovulation (Late estrous). Data from individual animals are represented as symbols (●) with the dash (—) being the group mean. Kruskal-Wallis and Mann-Whitney tests were used to determine statistically significant differences between treatment groups represented as dot-ended lines, statistical significance is $p < 0.05$. MDD – is the minimum detectable dose for this assay.

4.3 BIOAVAILABILITY OF TGFβ3 IN HYDROXYPROPYLMETHYLCELLULOSE GEL

As a result of previous work by our laboratory showing significant increases in uterine GM-CSF production following instillation of up to 40 ng rhTGFβ1, candidate drug formulation PV903 was made available by our commercial collaborator, GroPep Limited. This study was designed in collaboration with GroPep to evaluate the in vivo and in vitro techniques for assessment of the bioavailability and efficacy of recombinant human TGFβ3 (lyophilised drug product 903) reconstituted in hydroxypropylmethlycellulose (hypromellose gel solvent).

4.3.1 Bioavailability of rhTGFβ3 in hypromellose gel *in vitro*

Initially, to investigate the bioavailability and efficacy of rhTGFβ3 in hypromellose gel a uterine epithelial cell culture system was employed. Briefly, uterine epithelial cells were harvested from estrous CBA F1 mice, 400 μL aliquots of cell suspension were pipetted into a 24 well plate and allowed to adhere for 4 hours. Trans-well inserts were placed into wells containing cells and then media +/- rhTGFβ3 or hypromellose gel +/- rhTGFβ3 was added to the inserts. Fresh media replaced treatments after overnight incubation and was collected following a further 24 hrs incubation. GM-CSF content was assessed by cytokine specific bioassay using FD5/12 cells (Chapter 2).

GM-CSF production by uterine epithelial cells was significantly increased in response to TGFβ3 similarly whether in media or in gel, 2.6- and 2.8-fold respectively compared to the relevant controls ($p=0.05$ and $p=0.05$) (Figure 4.4). However TGFβ3 formulated in hypromellose gel elicited 55% less GM-CSF compared to that produced in response to TGFβ3 in culture media ($p=0.05$).

4.3.2 Bioavailability of rhTGFβ3 in hypromellose gel *in vivo*

Subsequent to the previous experiment, assessment of *in vivo* bioavailability of TGFβ3 in hypromellose gel was commenced. Briefly, female CBA F1 mice were either mated or smeared daily to identify estrous then allocated to one of eight treatment groups; untreated estrous, mated day 0.5 pc, sham intra-uterine injection, PBS injection, TGFβ1 in PBS injection, TGFβ3 in PBS injection, gel injection or TGFβ3 in gel injection. Treatments were administered by surgical intra-uterine injection at the 'early estrous' time point described in 4.2.3 above and harvested 8 hrs later. Uterine luminal fluid was collected and stored at -20°C for assessment of GM-CSF and IL-6 content. Uterine tissue was

frozen in OCT for immunohistochemical analysis of endometrial leukocyte abundance.

4.3.2.1 rhTGFβ_3 stimulated uterine cytokine production

Uterine GM-CSF production following mating increased by 96% compared to the estrous control, but was not a statistically significant change due the presence of an individual outlier (▲ hBL) (Figure 4.5 A). None of the TGFβ treatments elicited a significant GM-CSF response with respect to the relevant control. There was a trend for TGFβ_3 in PBS to elicit an increase in GM-CSF production 24% greater than that observed following TGFβ_1 administration but this did not reach significance.

As expected, IL-6 production was significantly increased following mating compared to the estrous and sham controls by up to 17-fold (Figure 4.5 B). The significant 6-fold increase following PBS instillation, compared to the sham control is attributable to a single outlier (△ eB3).

Outliers marked as ▲ hBL, △ eB3, ■ dB1R2 and ◆ bB31 have been removed from the data set used to generate box plots, but are included in analysis described above. Removal of outliers from statistical analysis only affects the level of significance when comparing GM-CSF content in control v mated groups, which becomes significant (p=0.03). It is interesting to note that while ▲ hBL and △ eB3 were outliers in both GM-CSF and IL-6 measures, ■ dB1R2 and ◆ bB31 were only outliers in either one of GM-CSF or IL-6 respectively.

4.3.2.2 rhTGFβ_3 stimulated uterine F4/80 positive cell recruitment

Uterine tissues embedded in OCT were sectioned (7μm) and stained by standard immunohistochemical techniques with the macrophage reactive antibody, F4/80. Video image analysis was used to quantify F4/80 positive staining. There was no difference in this experiment in F4/80 positivity between the untreated estrous and mated groups (Figure 4.6).

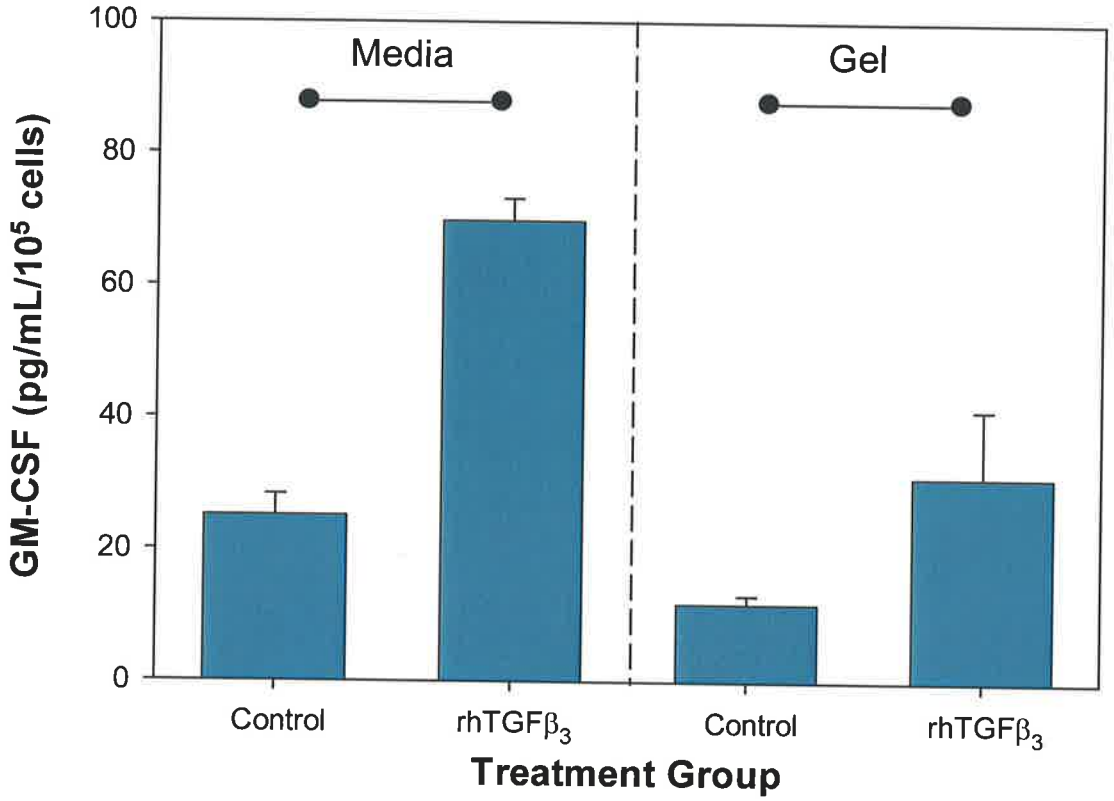


Figure 4.4 Bioavailability of rhTGFβ_3 in hypromellose gel in vitro. Murine uterine epithelial cells were incubated with culture media alone or with 50 ng/mL rhTGFβ_3 (Media) or with hypromellose gel alone or with 50 ng/mL rhTGFβ_3 (Gel). Supernatants were collected 24 hrs following replacement of treatment media and assayed for GM-CSF by cytokine specific bioassay. Data was normalised to the number of viable uterine epithelial cells remaining at the end of the culture period and expressed as pg/mL/10^5 cells. All measurements are the mean ± SD of triplicate wells for each treatment. Statistically significant differences are identified by dot-ended lines where $p < 0.05$.

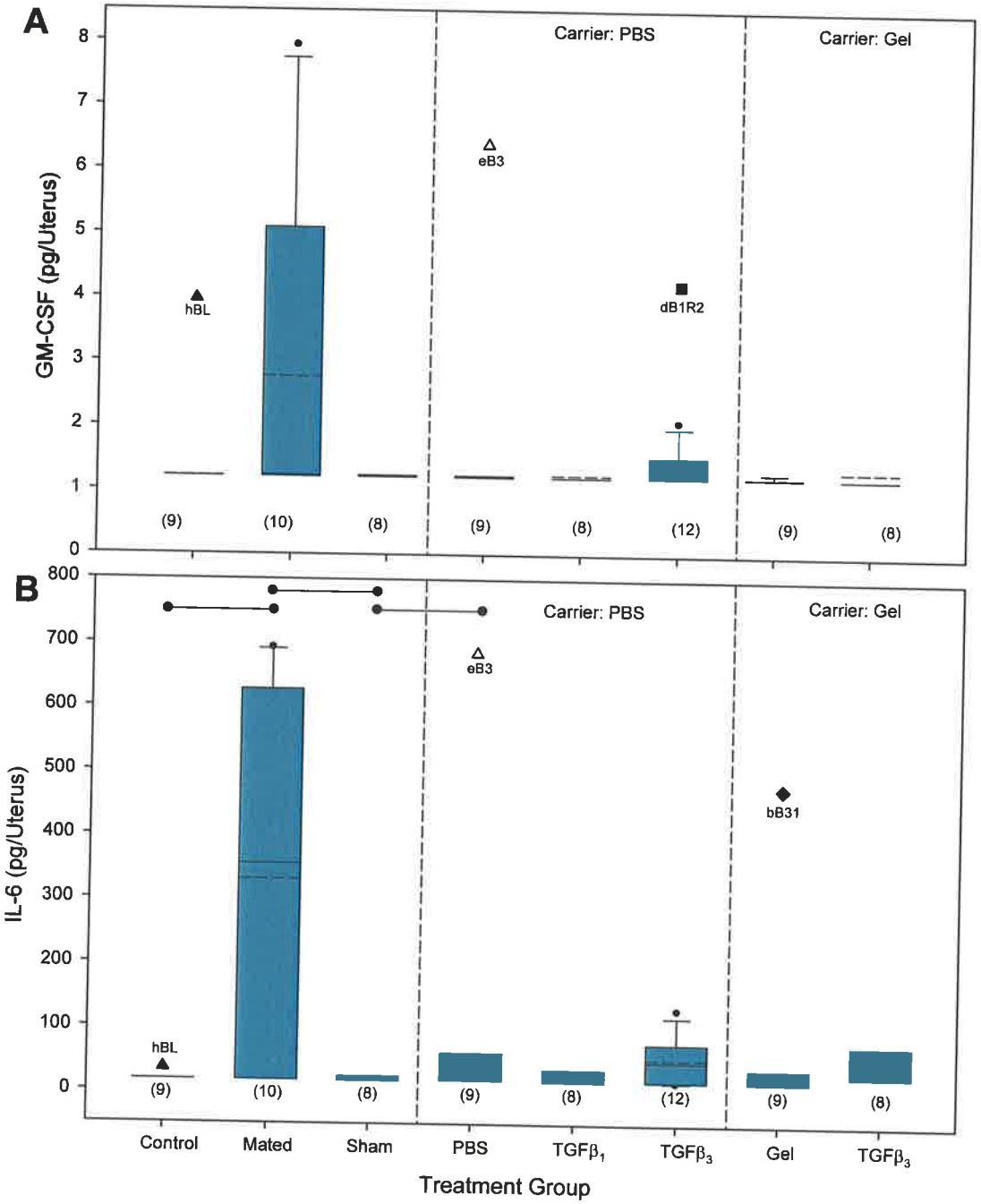


Figure 4.5 Biological activity of rhTGFβ3 administered via intra-uterine injection. Cytokine content of uterine fluid was assessed 8 hrs after ovulation, mating, sham surgery or intra-uterine injection of PBS alone or with rhTGFβ or Gel alone or with rhTGFβ. Uterine GM-CSF (A) and IL- 6 (B) content were measured. The number of mice in each group is shown in brackets. The upper and lower edges of the box and whisker plots indicate the 75th and 25th percentiles respectively, while the horizontal solid and dashed lines are the median and the mean respectively. Error bars signify the 5th and 95th percentiles. Kruskal-Wallis and Mann-Whitney tests were used to determine statistically significant differences between treatment groups represented as dot-ended lines, statistical significance is $p < 0.05$.

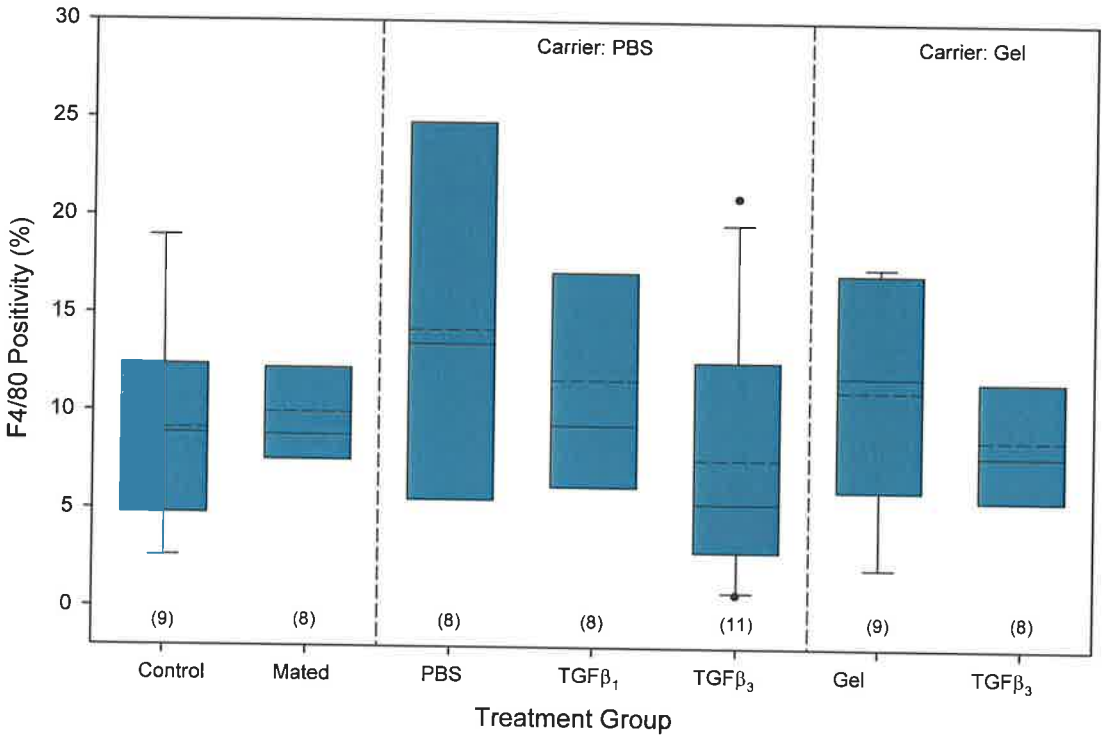


Figure 4.6 Effect of TGFβ3 on uterine F4/80 positivity. F4/80 positive staining of OCT fixed uterine sections was assessed 8 hrs after ovulation, mating or intra-uterine injection of PBS alone or with TGFβ or Gel alone or with TGFβ. The number of mice in each group is shown in brackets. The upper and lower edges of the box and whisker plots indicate the 75th and 25th percentiles respectively, while the solid and dashed horizontal lines are the median and the mean respectively. Error bars signify the 5th and 95th percentiles. Kruskal-Wallis and Mann-Whitney tests were used to determine statistically significant differences between treatment groups, $p<0.05$.

4.4 ROLE OF BACTERIAL LPS AND LTA IN UTERINE CYTOKINE PRODUCTION AND LEUKOCYTE RECRUITMENT

In Chapter 3 (3.5) an *in vitro* cell culture system was used to show the regulatory effect of bacterial LPS and LTA on uterine epithelial cell secretion of GM-CSF, IL-6 and KC. This experiment sought to investigate the potential roles of these agents in regulating uterine cytokine secretion and leukocyte recruitment in an *in vivo* model.

4.4.1 Effect of bacterial LPS and LTA on uterine cytokine production

To investigate the potential role of LPS and LTA in modulating the uterine cytokine environment, carrier alone (RPMI + 1% BSA) , 50 ng *E.coli* LPS or 2.5 μg *B.subtilis* LTA was administered to the uterine lumen of estrous female C57Bl/6 mice using surgical delivery. Uterine luminal fluid was collected eight hours post surgery for analysis of GM-CSF, IL-6 and KC content. Uterine tissue was embedded and frozen in OCT for immunohistochemical analysis.

Uterine GM-CSF content was increased by 80% and 2.1 fold following intra-uterine injection with LPS or LTA respectively compared to the carrier control, however this did not reach statistical significance (Figure 4.7 A). LPS and LTA stimulation of GM-CSF production by the uterus was very similar in magnitude. LPS and LTA stimulated GM-CSF production 3.5- and 4.2-fold greater than that elicited by natural mating but the increase was not statistically different, due the amount of variation within groups. There was no increase in GM-CSF production following injection with carrier alone compared to estrous control.

Intra-uterine injection with carrier alone did not alter IL-6 production, whereas LPS or LTA administration elicited a 2.3- and 2.3- fold increase in uterine IL-6 production compared to the carrier control. These increases were not statistically significant (Figure 4.7 B). LPS and LTA stimulation of IL-6 production by the uterus was very similar in magnitude. LPS or LTA intra-uterine injection elicited 30% less uterine IL-6 than observed following mating, but the difference was not statistically significant.

Luminal KC content was increased by 74% and 80% following intra-uterine administration of LPS (600 ± 255) or LTA (618.8 ± 279) respectively compared to injection with carrier alone (344 ± 190), but the increase failed to reach statistical significance (Figure 4.7 C). LPS and LTA stimulation of KC production by the uterus was very similar in magnitude. However, LPS or LTA treatments elicited only 68% of the uterine KC production measured following mating (1890 ± 743), with a statistically

significant difference between LPS and mating (p=0.033). There was no change in KC production following injection with carrier alone compared to estrous controls.

Outliers marked as ▼a#4.R3, ■ a2.6, ▲ d1.5 and △ d4.9 are individually identified on the graphical representation of the data and are included in statistical analysis described above. Statistical analysis was also conducted after removal of these four outliers. Removal of outliers resulted in significant increases in IL-6 and KC production following LTA treatment compared to measures following mating (p=0.004 and p=0.018 respectively).

4.4.2 Effect of bacterial LPS and LTA on uterine leukocyte recruitment

Previous studies have shown that TGFβ instilled into an estrous uterus mimics the post-mating inflammatory response with regard to both the increase in GM-CSF production and macrophage infiltration, but not neutrophil recruitment. In vivo (4.4.1 above) and in vitro (Chapter 3 – 3.5, 3.6, 3.7 and 3.9) results show significant upregulation of uterine cytokine production following LPS or LTA treatment, particularly KC production. KC is known to be a potent neutrophil chemoattractant and activator. To investigate whether LPS or LTA have the capacity to recruit neutrophils into the uterine tissue, uteri from the experiment described above was cut into 7μm sections and stained with the anti-neutrophil antibody RB6. Macrophage recruitment was also investigated by staining with the anti-macrophage antibody F4/80. Stained slides were blinded and a semi-quantitative analysis conducted, considering intensity and location of staining, where + - weak sparse staining, ++ - moderate staining increasing inward toward the lumen, +++ - strong staining with dense populations throughout the stroma concentrating towards the lumen. Neutrophil trans-epithelial migration was determined by counting the number of RB6 positive cells crossing the uterine luminal epithelium (Table 4.1).

The degree of RB6+ neutrophil recruitment into the endometrium and alignment along the basal surface of the luminal epithelium was similar in the carrier, LPS and LTA injected groups (Figure 4.8 D, E, F). The number of neutrophils migrating across the epithelium into the lumen following uterine LPS or LTA administration was 20% and 2.3-fold higher respectively compared to the carrier control, but this was not statistically significant. Positive neutrophil staining in the treated uterine tissue appeared to be higher than that seen in the untreated estrous uterus but less than that observed following mating (Figure 4.8 B, C).

LPS administration resulted in a pronounced increase in F4/80 positive macrophage infiltration into the endometrium compared to carrier treated controls (Figure 4.9). The spatial distribution throughout the tissue was similar to that seen following mating.

Table 4.1 Summary of IHC analysis of uterine tissue following LPS or LTA treatment in C57Bl/6 mice
Data is given as mean ± SD and was analysed using Kruskal-Wallis and Mann-Whitney tests. ✚ - weak, ✚✚ - moderate and ✚✚✚ - strong uterine staining.

	RB6 +ve Uterine Neutrophils	RB6 +ve Trans-epithelial Neutrophils	F4/80 +ve Macrophages
Day 0.5pc	✚✚	23.6 ± 7.3	✚✚✚
Carrier	✚	14.0 ± 6.3	✚
LPS	✚	16.7 ± 6.3	✚✚
LTA	✚	31.9± 18.1	✚

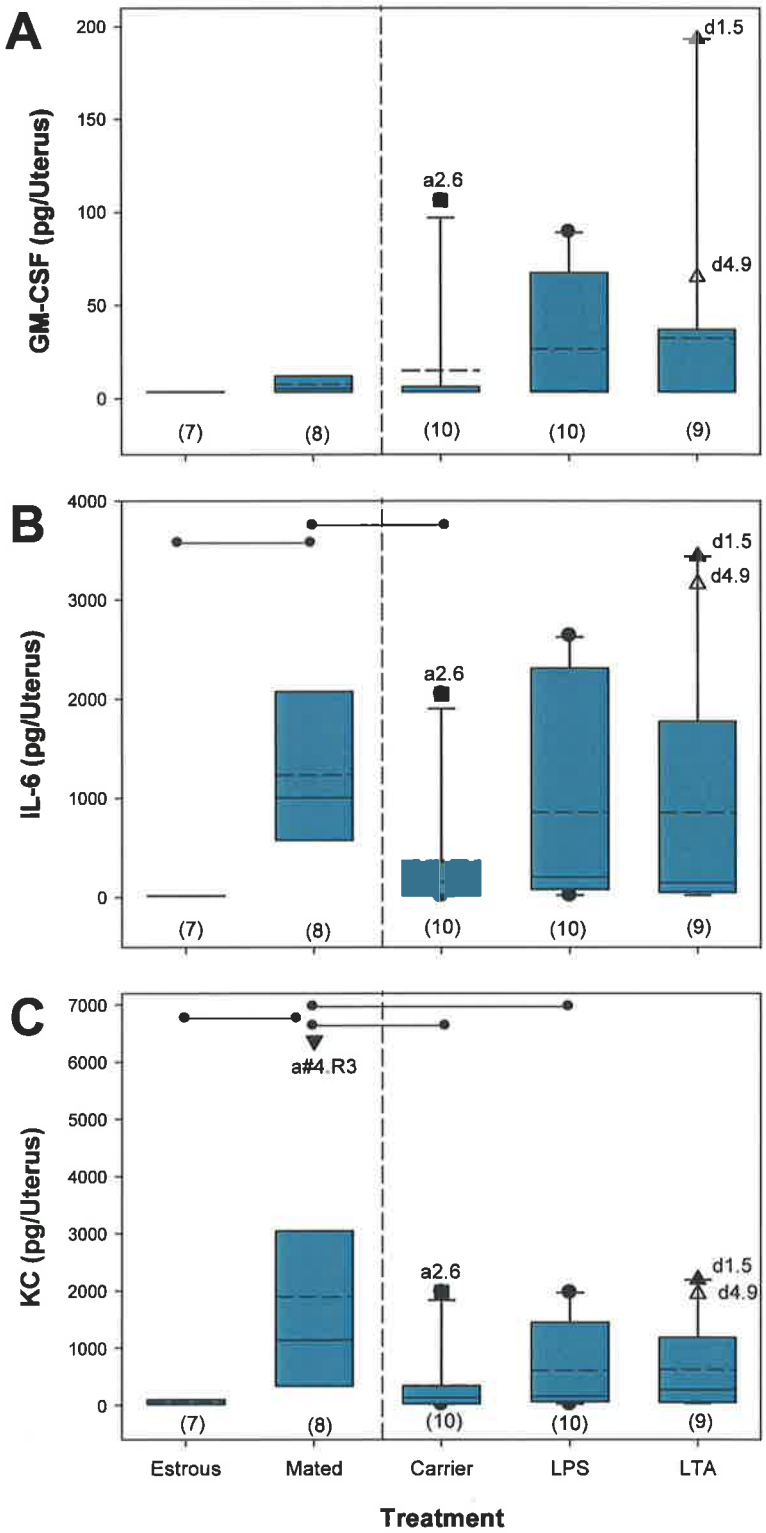


Figure 4.7 Effect of bacterial components on uterine cytokine production. Estrous C57Bl6 female mice were injected with carrier, 50ng *E.coli* LPS or 2.5mg *B.subtilis* LTA into the uterine lumen. Uterine fluid was collected 8 hrs post treatment and assayed for GM-CSF (A), IL-6 (B) and KC (C) content. The number of mice in each group is shown in brackets. The upper and lower edges of the box and whisker plots indicate the 75th and 25th percentiles respectively, while the solid and dashed horizontal lines are the median and the mean respectively. Error bars signify the 5th and 95th percentiles. Kruskal-Wallis and Mann-Whitney tests were used to determine statistically significant differences between treatment groups represented as dot-ended lines, $p<0.05$.

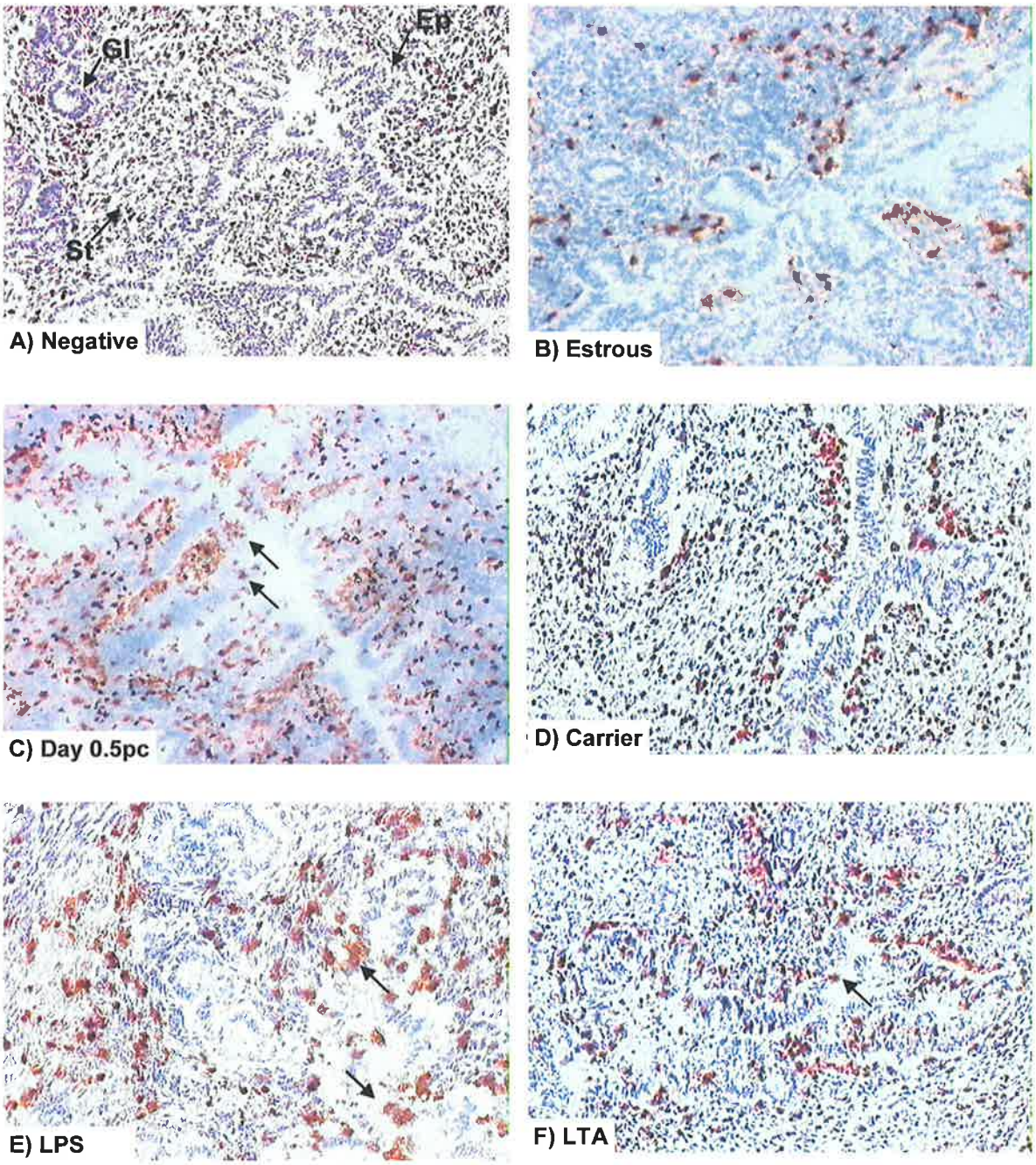


Figure 4.8 Effect of bacterial components on uterine neutrophil recruitment. Uterine tissue sections were obtained from female C57Bl6 mice following no treatment (B), mating (C) injection with carrier alone (D), LPS (E) or LTA (F) and were incubated with diluent (A) or monoclonal antibody Rb6.
Ep; Epithelium, Gl; Glandular epithelium, St; Stroma, → - neutrophil undergoing transepithelial migration. All images captured at the same magnification (x 20).

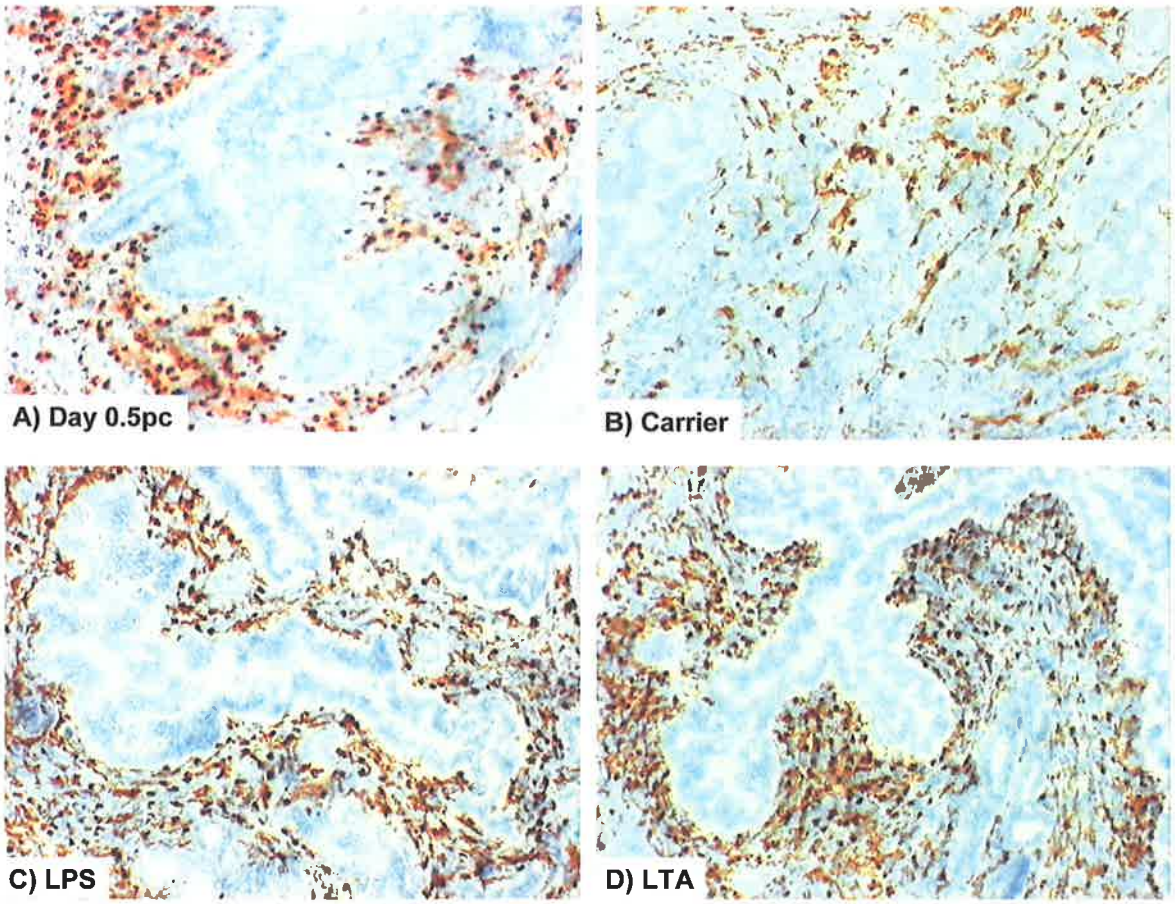


Figure 4.9 Effect of bacterial LPS and LTA on uterine F4/80 positive cell recruitment. Uterine tissue sections were obtained from female C57Bl6 mice following mating (A), injection with carrier alone (B), LPS (C) or LTA (D) and were labelled with a monoclonal antibody specific for F4/80 antigen (MCA497). All images captured at the same magnification (x 20).

4.5 EFFECT OF MATING ON UTERINE CYTOKINE PRODUCTION AND NEUTROPHIL RECRUITMENT IN C3H/HEJ MICE

As detailed above, seminal TGFβ did not mimic the effects of seminal fluid in inducing neutrophil infiltration into the endometrium or trans-migration of neutrophils across the uterine luminal epithelium. While neutrophil recruitment into the uterus is less following LPS or LTA instillation compared to that following mating, these treatments were capable of eliciting trans-epithelial migration. TLR4 defective C3H/HeJ mice were used to further investigate the potential role that LPS introduced at insemination may have in regulating the post-mating cytokine environment. Uterine luminal fluid was collected from estrous or day 0.5 pc C3H/HeJ female mice and assayed for GM-CSF, IL-6 and KC. Uterine tissue was frozen in OCT for immunohistochemical analysis.

4.5.1 Uterine cytokine production following mating in C3H/HeJ mice

There were significant 9.2-, 45- and 5.5-fold increases in uterine GM-CSF, IL-6 and KC content in C3H/HeJ females compared to estrous controls following mating with Balb/c male mice ($p=0.001$, $p=0.001$ and $p=0.008$) (Figure 4.10 A, B and C).

This chapter has included experiments investigating the effect of mating in a variety of mouse strains, measuring at least GM-CSF and IL-6 as well as KC in some cases. It is interesting to compare uterine cytokine production following mating in C3H/HeJ, C57Bl/6 and CBA F1 mice. Mating elicited a significantly greater GM-CSF response from the uteri of C3H/HeJ mice than that of C57Bl/6 or CBA F1 (Figure 4.7A and Figure 4.5A) mice when compared to their own estrous controls (9.2- and 6-fold and 96% respectively). An absolute (pg/ut) comparison shows a significant 6.8- and 18.3-fold higher GM-CSF response in C3H/HeJ (mean ± SEM pg/ut) (51.6 ± 14.5 pg/ut) mice versus C57Bl/6 (7.6 ±1.8 pg/ut) and CBA F1 (2.8 ± 0.8 pg/ut) mice ($p = 0.001$ and $p<0.001$).

The fold increase in uterine KC production following mating in C3H/HeJ was similar to that seen in C57Bl/6 mice (Figure 4.7C). The absolute (pg/ut) increase was also similar in C3H/HeJ (2188 ± 693 pg/ut) mice and C57Bl/6 mice (1890 ± 75 pg/ut).

Mating elicited a 45-fold increase in uterine IL-6 production in C3H/HeJ mice, less than the 120-fold increase seen in C57/Bl6 mice, yet notably higher than the 17-fold increase in uterine IL-6 production by CBA F1 mice (Figure 4.7B and Figure 4.5B). Absolute uterine IL-6 production following mating in C3H/HeJ (592 ± 70 pg/ut) was reduced by 52% compared to C57Bl/6 (1231 ± 292 pg/ut)

mice, yet 79% higher than that measured in CBA F1 (330 ± 89 pg/ut) mice, although this was not statistically significant (p=0.074 and p=0.075).

4.5.2 Uterine leukocyte recruitment following mating in C3H/HeJ mice

The extent of neutrophil infiltration in estrous C3H/HeJ uterine tissue is markedly higher than that seen in estrous C57Bl/6 uterus but similar to that observed following mating in C57Bl/6 mice (Figure 4.11). There was no difference between estrous and mated C3H/HeJ mice with respect to the amount of neutrophil positive staining or spatial distribution within the tissue. The most notable difference between estrous and mated uterine tissues was the number of neutrophils undergoing transepithelial migration after mating. Uteri from mated C3H/HeJ mice had higher numbers of neutrophils entering the uterine lumen from the endometrial stroma via the luminal epithelium (mean ± SEM) (21.8 ± 3.5 cells/ut) compared to that observed in estrous C3H/HeJ uteri (7.0 ± 5.3 cells/ut) (p=0.038). Similarly there was a trend towards an increase in the number of neutrophils crossing the luminal epithelium into the luminal cavity in the uteri of C57Bl/6 mated mice (23.5 ± 7.3 cells/ut) compared to C3H estrous mice (6.98 ± 5.3 cells/ut) but this was not statistically significant (p=0.07).

Similar to RB6+ neutrophil staining, F4/80 reactive endometrial macrophages were abundant in uterine tissue from both estrous and mated C3H/HeJ mice and further were indistinguishable from mated C57Bl/6 uteri (Figure 4.12).

Table 4.2 The effect of strain on post-mating uterine cytokine production.
Data was analysed using Kruskal-Wallis and Mann-Whitney tests. a b represent statistical differences between groups, $p<0.05$. ✚ - weak, ✚✚ - moderate, ✚✚✚ - strong and ✚✚✚✚ - very strong increases in cytokine content of uterine luminal fluid compared with estrous controls of the same genotype.

	GM-CSF	IL-6	KC
Day 0.5pc CBA F1	✚ [a]	✚ [a]	-
Day 0.5pc C57Bl/6	✚✚ [b]	✚✚✚✚ [b]	✚✚
Day 0.5pc C3H/HeJ	✚✚✚✚ [c]	✚✚ [ab]	✚✚✚

Table 4.3 Summary of immunohistochemical analysis of uterine tissue following mating in C3H/HeJ mice
Data is given as mean ± SD and was analysed using Kruskal-Wallis and Mann-Whitney tests. a b c represent statistical differences between groups, $p<0.05$. ✚ - weak, ✚✚ - moderate and ✚✚✚ - strong uterine staining.

	RB6 +ve Neutrophils	RB6 +ve Trans-epithelial Neutrophils	F4/80 +ve Macrophages
Estrous C57Bl/6	✚	13.9 ± 6.3 [ac]	✚✚
Day 0.5pc C57Bl/6	✚✚	23.6 ± 7.3 [abc]	✚✚✚
Estrous C3H/HeJ	✚✚	7.0 ± 5.3 [a]	✚✚
Day 0.5pc C3H/HeJ	✚✚✚	21.8 ± 3.5 [bc]	✚✚

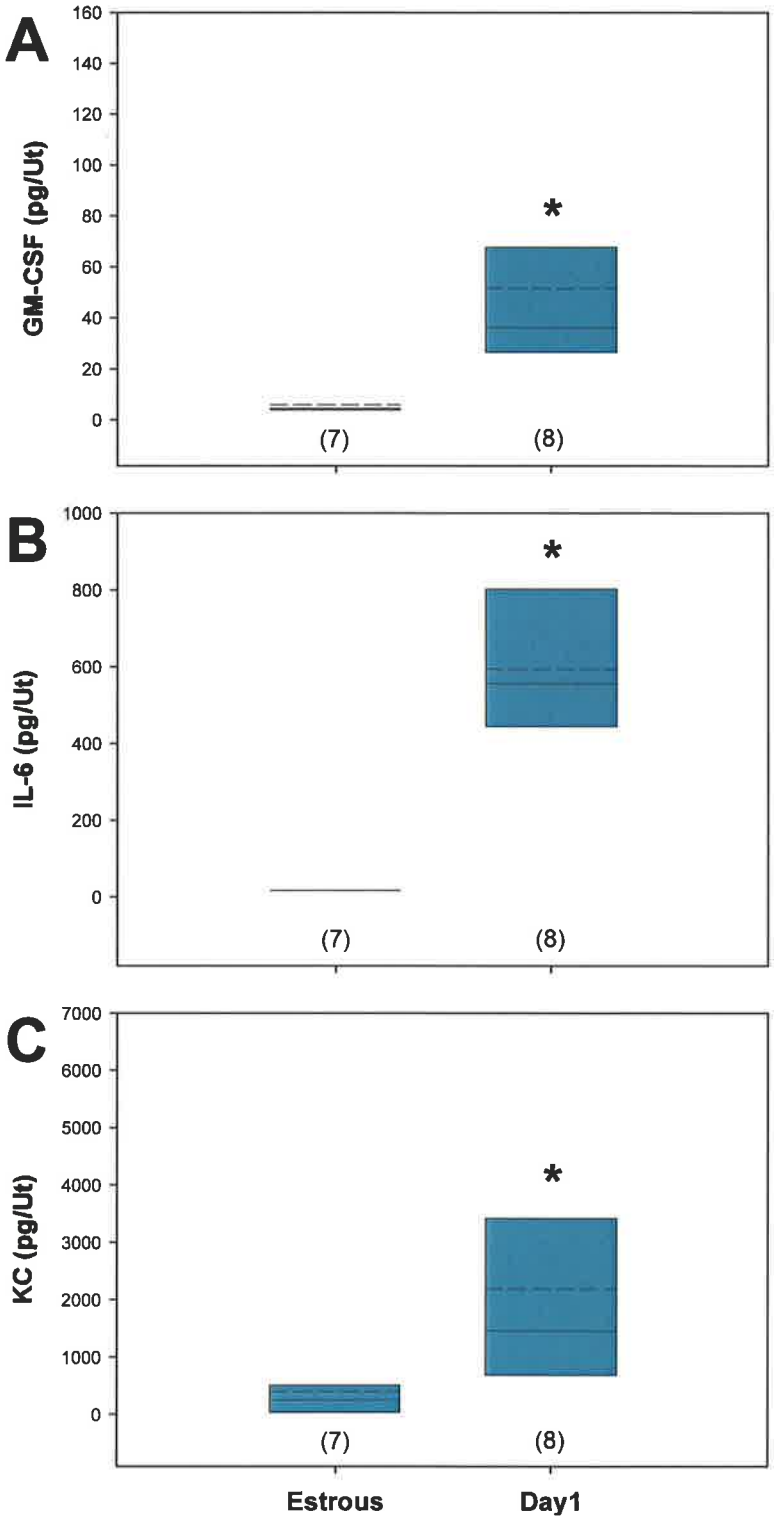


Figure 4.10 Effect of mating on uterine cytokine production C3H/HeJ mice. Uterine luminal fluid was collected from estrous and mated C3H/HeJ female and assayed for GM-CSF (A), IL-6 (B) and KC (C) content. The number of mice in each group is shown in brackets. The upper and lower edges of the box and whisker plots indicate the 75th and 25th percentiles respectively, while the solid and dashed horizontal lines are the median and the mean respectively. Error bars signify the 95th and 5th percentiles. Kruskal-Wallis and Mann-Whitney tests were used to determine statistically significant differences between treatment groups - ✱, $p < 0.05$.

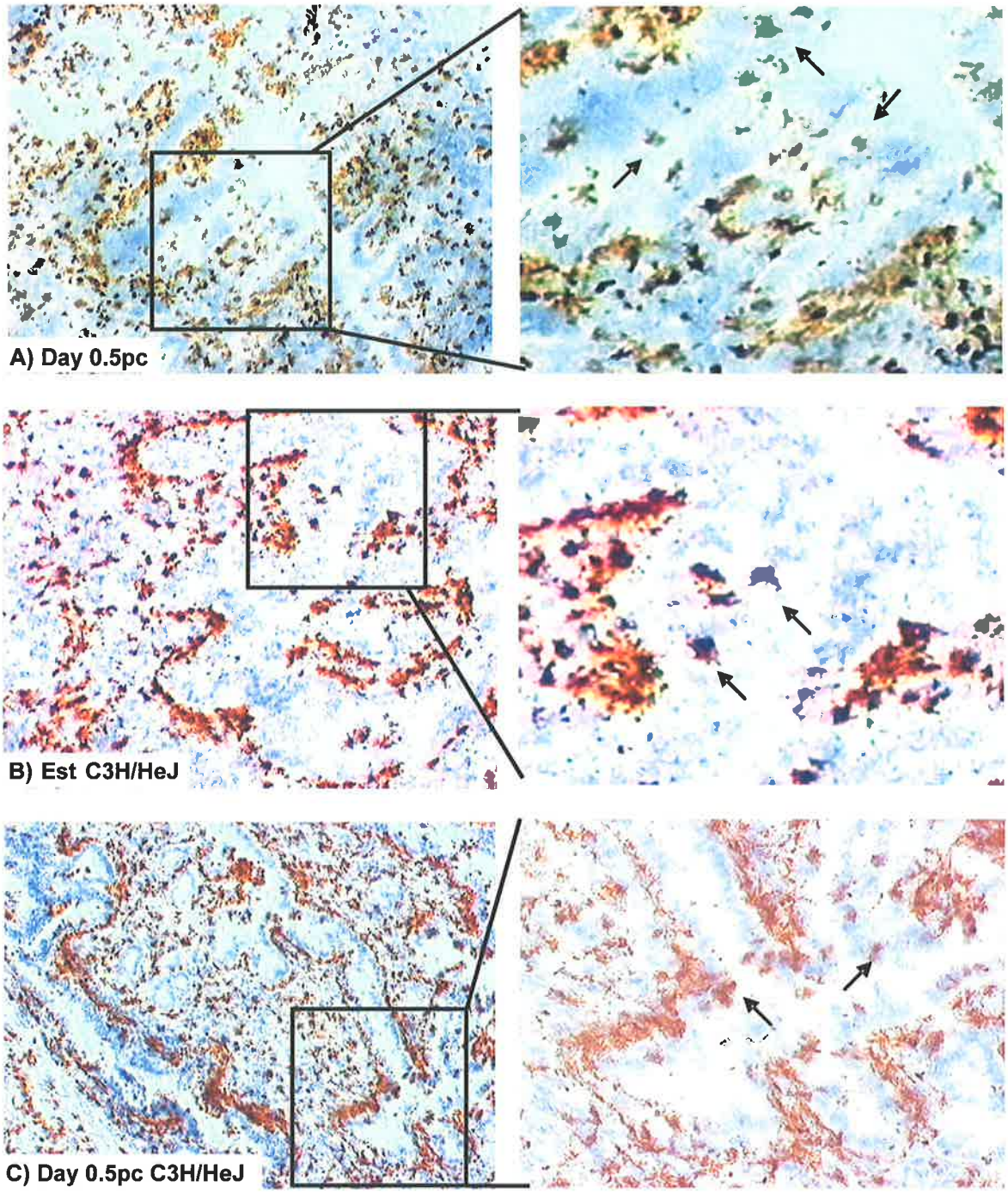
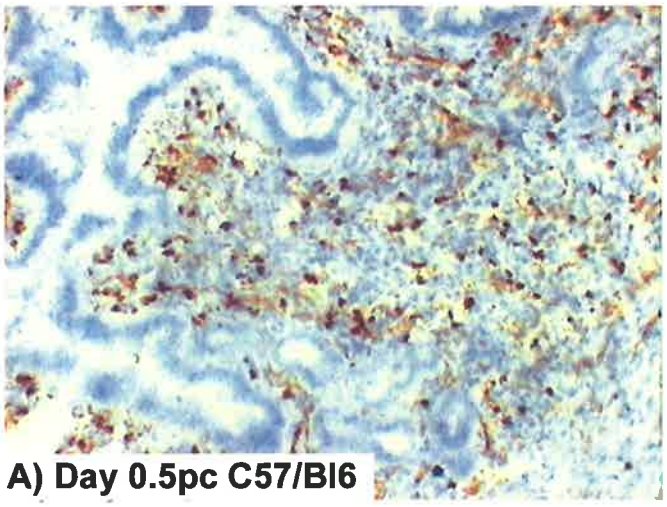
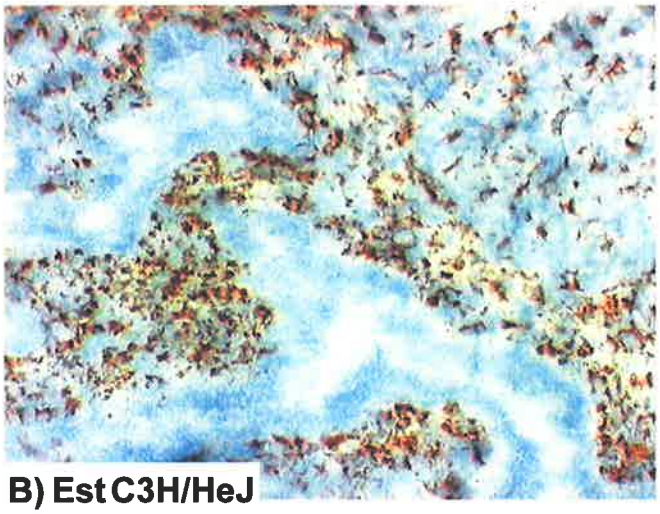


Figure 4.11 Effect of mating on uterine neutrophil recruitment in C3H/HeJ mice. Uterine tissue sections were obtained from day 0.5 pc C57Bl6 female mice (A), Estrous (B) or day 0.5 pc (C) C3H/HeJ mice and were labelled with monoclonal a antibody specific for RB6 neutrophil antigen. ➙ indicates intra-epithelial RB6 positive neutrophils. All images on the left captured at the same magnification (x 20).





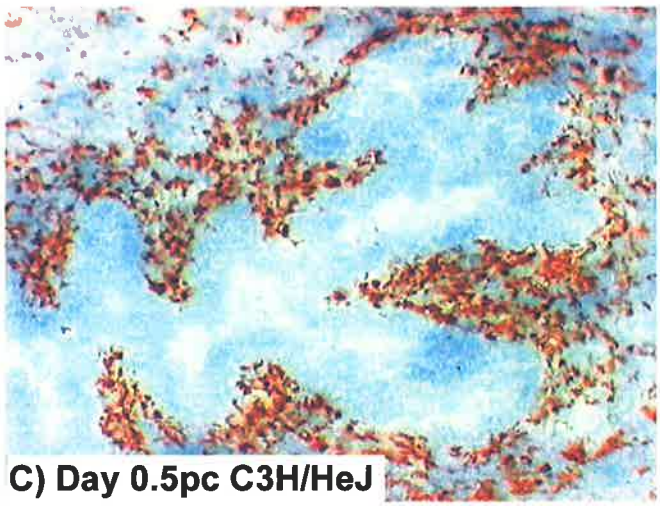


Figure 4.12 Effect of mating on uterine F4/80 positive cell recruitment in C3H/HeJ mice. Uterine tissue sections were obtained from day 0.5 pc C57Bl6 female mice (A), Estrous (B) or day 0.5 pc (C) C3H/HeJ mice and were labelled with a monoclonal antibody specific for F4/80 antigen (MCA497). All images captured at the same magnification (x 20).

4.6 DISCUSSION

The experiments described in this chapter were undertaken to investigate the in vivo effect of factors identified in the previous chapter as potential regulators of the post-mating inflammatory response in the murine uterus. The results have shown that regulation of uterine pro-inflammatory cytokine production and leukocyte recruitment is complex and likely requires multiple regulatory factors acting in concert. The ability of TGFβ, as well as bacterial LPS and LTA to stimulate uterine cytokine production and leukocyte recruitment in vivo when administered into an estrous mouse uterus was examined. LPS and LTA were found to stimulate marked increases in uterine GM-CSF, IL-6 and KC production as well as RB6 and F4/80 cell positivity. Thus LPS and LTA are likely to be active contributing factors in the physiological response to insemination. TGFβ, on the other hand was unable to stimulate uterine GM-CSF or IL-6 production or F4/80 positive cell recruitment. This is contrary to previous studies showing an increase in GM-CSF production in response to uterine TGFβ instillation, similar to that measured following mating [12].

Table 4.4 The effect of mating and potential seminal fluid regulators on pro-inflammatory cytokine expression and leukocyte recruitment in the mouse uterus. $TGF\beta_1$ or $TGF\beta_3$ were administered in aqueous medium or in hypromellose gel, and LPS and LTA were administered in aqueous medium. ✚ - weak, ✚✚ - moderate and ✚✚✚ - strong uterine staining. ↔ no change from basal, ↑- small, ↑↑- moderate, ↑↑↑- strong and ↑↑↑↑ very strong increases in cytokine content of uterine luminal fluid. NA – not assessed.

		GM-CSF	IL-6	KC	RB6	Epithelial RB6	F4/80
In vitro	$TGF\beta_3$ in medium	↑↑↑	NA	NA			
	$TGF\beta_3$ in gel	↑	NA	NA			
In vivo CBAF1	Mating	↑	↑	NA	NA	NA	✚
	$TGF\beta_1$ in medium	↔	↔	NA	NA	NA	✚
	$TGF\beta_3$ in medium	↔	↔	NA	NA	NA	✚
	$TGF\beta_3$ in gel	↔	↔	NA	NA	NA	✚
In vivo C57Bl/6	Mating	↑↑	↑↑↑↑	↑↑↑	✚✚	↑↑↑	✚✚✚
	LPS in medium	↑↑↑↑	↑↑	↑	✚	↑↑	✚✚
	LTA in medium	↑↑↑↑	↑↑	↑	✚	↑↑↑	✚
C3H/HeJ	Mating	↑↑↑	↑↑↑	↑↑↑	✚✚✚	↑↑↑	✚✚

TGFβ_3 applied to uterine epithelial cells was found to stimulate GM-CSF production to a similar degree to that described in the previous chapter however the efficacy of TGFβ was significantly reduced when applied in hypromellose gel at the same dosage. Hypromellose gel is a hydrophilic matrix used to maintain sustained and controlled release of a pharmaceutical agent to prolong its therapeutic effect as opposed to a single dose of drug in a non-matrix solvent. This result was unexpected but may be explained by examining the stability of TGFβ_3 in this gel or alternatively by considering the effect of the gel on drug delivery. For example a 50 ng dose in a hypromellose gel solvent may release 10 ng/h thus exposing cells in culture (1 mL) to a concentration of 10 ng/mL, with increased release over time not necessarily cumulative given the labile nature of TGFβ. In contrast cells receiving a 50 ng dose in a non-matrix solvent are exposed to 50 ng/mL instantaneously. This mode of treatment has been applied to transdermal, intra-nasal and ophthalmic drug delivery routes with a high degree of success [501-503]. However each of these routes of drug administration has required identification of enhancing agents to promote transport of drug to the site of action. For example glycols or non-ionic surfactants have been added to hypromellose gel to assist transdermal transport of lidocaine [501]. Similarly, increased mucosal residence of gel treatment is achieved by the addition of Carbopol ® to gel formulations for intra-nasal administration [502].

Development of an experimental strategy for in vivo administration of intra-uterine treatments was undertaken in order to optimise route and timing of uterine administration of treatments. Transcervical catheterisation and injection was an attractive alternative to traditional surgical techniques that require exteriorizing the uterus to allow injection through the uterine wall, as it is less invasive and reduces the risk of exposure to infectious agents. Prolonged exposure of the luminal epithelium to transcervically administered treatments was demonstrated. However passage of a catheter across the cervix stimulated cytokine production, equivalent to that following insemination with an intact male (Figure 3.1 and Figure 4.2). This may have been due to the mechanical stimulation encountered during catheterisation being similar to that at mating. Stimulation was not evident following mating with seminal vesicle deplete mice showing that mechanical stimulation associated with coitus is to elicit the inflammatory response. However passage of a catheter across the cervix could comprise a more intense stimulus than that induced by the male. There may be other factors present contributing to the uterine cytokine profile in addition to the mechanical stimulation at mating or catheterisation. It is possible that stimulatory factors were unintentionally introduced into the uterine lumen during catheterisation, by a similar mode as at mating. Such factors could include bacterial agents LPS and LTA. This effect was not observed following surgical intra-uterine injection, hence surgical intra-uterine injection was used in subsequent experiments.

Uterine epithelial cells in culture are known to be differentially responsive to stimuli in an estrous cycle dependent manner [109]. Examination of effect of cycle stage in vivo revealed intra-uterine rhTGFβ treatment elicited greatest GM-CSF production when it was administered by early estrous, on the morning following ovulation. Uterine GM-CSF responsiveness to rhTGFβ at this time point was reflects that measured following mating and as such was used in subsequent experiments.

Uterine luminal GM-CSF content was reproducibly increased following mating in the three strains of mice examined in this chapter, CBA F1, C57Bl/6 and C3H/HeJ. There was a large amount of variation between individuals in any given group which often affected statistical significance. The increases measured in these experiments were not due to deposition of GM-CSF into the female tract at the time of mating as it has previously been demonstrated that male accessory gland secretions contain no detectable GM-CSF [122, 364]. This data is consistent with previous studies in mice, where GM-CSF mRNA and protein production are increased following insemination [122, 126]. Furthermore recent in vitro and in vivo investigations in humans demonstrate upregulation of GM-CSF mRNA and protein following semen exposure [130]. The post-mating increase in GM-CSF is thought to contribute to recruitment and activation of leukocytes into the endometrium. Recombinant GM-CSF instillation into estrous uteri has been shown to recruit macrophages, neutrophils and dendritic cells [126]. However mating experiments in GM-CSF null mice exhibit endometrial leukocyte recruitment similar to that seen in GM-CSF replete mice [125]. Additionally GM-CSF possesses embryotrophic properties, clearly demonstrated in in vitro and in vivo experiments where GM-CSF knockout embryos supplemented with exogenous GM-CSF exhibited increased glucose uptake and enhanced proliferation and/or viability of blastocysts [127, 128].

Mating stimulated marked increases in uterine IL-6 production in C57Bl/6, CBA F1 and C3H/HeJ mice in these experiments. The individual variability within the strain groups again reduced statistical significance of differences observed. The surge in uterine IL-6 following mating is not due to deposition of IL-6 from the ejaculate. Seminal vesicle fluid has previous been shown to contain low levels of IL-6 (5 pg/mL) representing 1% of that measured in the post-mating uterine luminal fluid [364]. These findings are consistent with previous studies in mice [152, 154] and recent studies in humans [130]. The importance of IL-6 production by the uterus in response to mating is thought to be in enhancing antigen presentation by maternal uterine immune cells [295].

These studies show for the first time that uterine KC protein content in C57Bl/6 and C3H/HeJ mice is dramatically increased after mating. This increase is not due to KC deposition into the uterine

lumen during insemination. Seminal vesicle fluid is known to only contain up to 10% (40 pg/mL) of the KC detectable in the mated uterus [364]. This result is consistent with previously described increases in uterine KC mRNA in early pregnancy in mice [189]. Prior to the current study, a role for seminal fluid in regulating KC had not been identified. However the KC stimulating factor present in seminal fluid remains to be identified. Recent data from human studies have shown similar increases in cervical IL-8 (human equivalent to KC) mRNA expression following exposure to semen [130]. The role for KC in the mouse uterus following mating remains to be investigated. However, KC is known to be a potent neutrophil chemoattractant and activator and further, the uterine neutrophil population is known to increase significantly in response to mating [258]. The large increase in luminal KC content following mating may therefore contribute to uterine neutrophil recruitment.

Interestingly there were differences between strains in the magnitude of uterine cytokine production in response to mating. Strain differences in the inflammatory response described here have also been demonstrated in mouse models of allergy and inflammation [504-506]. Notwithstanding the results presented here and the previously described differential inflammatory responses between mouse strains, the strains in the current study are equally fertile. Hence mechanisms must exist to cope with a range of inflammatory environments at the outset of pregnancy.

Consistent with previous studies showing macrophage responsiveness to components of semen, the number of F4/80 positive macrophages in the uterus increased following mating in this study [123, 258]. Again there were obvious strain differences with C57Bl/6 and C3H/HeJ exhibiting a marked influx of macrophages and interestingly, the poor response in CBA F1 mice in these experiments correlated with smaller increases in inflammatory cytokine production observed after mating in this strain. The influx of macrophages into the endometrium following mating is thought to be involved in tissue remodelling and endometrial vascularisation required for optimal implantation and placental development. A similar transient influx of macrophages into the human cervix has been described in humans, where roles in debris and pathogen clearance following insemination have been postulated [217, 266].

Mating elicited marked increases in the neutrophil population of endometrial tissues compared to estrous controls in C57Bl/6 mice. This was not the case in C3H/HeJ mice, where RB6 staining for neutrophils was similar between estrous and mated mice. The most remarkable difference following mating in both these strains of mice was the number of neutrophils undergoing transepithelial migration, entering the uterine luminal cavity. This observation is consistent with early studies in mice and rats showing polymorphonuclear cell infiltration within hours of mating [507, 508]. The influx of neutrophils

following mating is transient, lasting only 2 days in the mouse [258]. The role of these leukocytes following mating is thought to be in clearance of debris, non-fertilising sperm and potential pathogens introduced at insemination. This migration is likely to be the result of the post-mating increase in luminal chemotactic factor content, KC. However other chemokines might contribute to regulating this response.

The results in C3H/HeJ mice suggest that the pro-inflammatory cytokine surge and leukocyte influx following mating is independent of the LPS signalling receptor TLR4. The normal KC production and neutrophil response in C3H/HeJ mice might be interpreted as evidence that LPS is not necessary for a normal post-mating inflammatory response. However studies in other tissues caution against this interpretation. The abundance of neutrophils in C3H/HeJ mice compared to other strains has been described previously in a variety of experimental models. Interestingly, intraperitoneal LPS administration stimulates more prolific neutrophil recruitment in LPS hyporesponsive C3H/HeJ mice than in the related responsive strain C3H/Han [509]. Similarly C3H/HeJ mice exhibit a higher degree of neutrophil infiltration in response to ozone treatment or *Candida albicans* treatment when compared to C57Bl/6 or TLR4-/- mice respectively [510, 511]. Together these previous studies indicate a potential TLR4 independent pathway mediating LPS stimulated neutrophil recruitment.

Recombinant human TGFβ instilled into an estrous CBA F1 uterus did not alter GM-CSF content of uterine luminal fluid in this study. The lack of GM-CSF stimulation by TGFβ in the experiment described here was not due its inactivity since the same batch of cytokine stimulated GM-CSF responses by CBA F1 uterine epithelial cells in vitro (Chapter 3 and 4.3.1). This result was surprising since Tremellen et al have previously published studies demonstrating 10 – 40 pg/mL rhTGFβ elicits an increase in GM-CSF production equivalent to at least half of that observed following mating [12]. However the previous study used Balb/c x C57Bl/6 female mice, whereas the current study used CBA x C57Bl/6 female mice. For reasons outlined above, strain differences between in vivo experiments may explain the contradictory results observed. Similarly, uterine IL-6 production was not altered by intra-uterine administration of rhTGFβ in this experiment. This was not surprising as the in vitro studies described in Chapter 3 showed small but significant decreases in IL-6 production following TGFβ exposure.

Uterine TGFβ treatment did not result in an influx of F4/80 positive macrophages in this experiment. Previous studies in mice have demonstrated a 2.5-fold increase in F4/80 positive staining in the uterus following rhTGFβ administration compared to carrier injection alone [12]. There was no correlation between GM-CSF or IL-6 levels and F4/80 staining. However given the absence of GM-

CSF and IL-6 induction, it is perhaps not surprising macrophage responses were not seen in this experiment.

Instillation of bacterial LPS or LTA into an estrous uterus elicited marked increases in luminal GM-CSF content, ~ 4-fold higher than those measured following natural mating. This finding together with the in vitro experiments described in Chapter 3 provide good evidence that LPS and/or LTA might contribute to induction of the post-mating inflammatory response. Introduction of commensal bacteria and/or components into the upper female reproductive tract at insemination may thus contribute positively to pregnancy outcomes, since GM-CSF synthesis is linked with improved embryo development. Chaouat et al have shown that GM-CSF treatment of abortion-prone mice (CBA/J x DBA/2) increases fetal survival from 53% to 92% [438]. Similarly, pre-insemination uterine LPS exposure in pigs does not detrimentally affect fertility or fetal outcomes; on the contrary, it enhances fetal growth [512].

Uterine IL-6 and KC production increased following instillation of LPS or LTA but did not reach the levels measured following mating. Considering these data together with in vitro results obtained in Chapter 3 (Figure 3.2 and 3.3), where seminal vesicle fluid had no effect on uterine epithelial cell IL-6 production and TGFβ inhibited IL-6 production, it may be possible that commensal bacterial products introduced at insemination contribute to the natural post-mating inflammatory response. This might explain why seminal vesicle fluid recovered aseptically did not increase KC and IL-6 production in vitro. However, it is possible that agents from other male accessory glands are responsible for stimulating IL-6 production. Similarly, KC production by uterine epithelial cells in vitro is ablated by seminal vesicle fluid and reduced by TGFβ and may be augmented by the presence of bacterial LPS or LTA in the female tract at insemination. Indeed in vitro results in Chapter 3 (Figure 3.6 and 3.7) demonstrate the ability of LTA but not LPS to overcome TGFβ inhibited KC production by uterine epithelial cells.

LPS and LTA administration to estrous uteri resulted in a marked influx of F4/80 positive macrophages into the endometrium compared to untreated controls. The spatial distribution of the positive staining in treated animals was similar to that seen following mating, again supporting the hypothesis that bacterial LPS and LTA contribute to the post-mating inflammatory response. Similarly, RB6 positive neutrophils were more abundant in LPS and LTA treated uteri than untreated controls and similar to that of mated mice. Of interest was the ability of LTA to elicit transepithelial migration of neutrophils to a similar degree of that observed following mating and 2-fold greater than that achieved by carrier alone or LPS treatment.

Studies in women have shown the microflora of the vaginal mucosa is comprised primarily of gram positive lactobacilli strains [513]. To date the genitourinary tract microflora has been considered important for its ability to protect the host from colonisation by potentially pathogenic organisms. This is achieved by competitive binding to the mucosal surfaces as well as secretion of biosurfactants preventing pathogen adhesion, bacterial growth inhibitors such as acids, bacteriocins and hydrogen peroxide and coaggregation factors that prevent pathogen spread [514]. However, the data presented here may indicate an additional role for male or female genitourinary tract microflora. LTA from gram positive lactobacilli may contribute to the postmating inflammatory response in the uterus by upregulating cytokines responsible for neutrophil recruitment.

The results described in this chapter show similarities as well as marked differences in the post-mating inflammatory response between different strains of mice with regard to uterine cytokine secretion and leukocyte recruitment. While the magnitude of differences for some out comes measured were very large, the fact remains that these strains of mice are all fertile. The phenomenon of cytokine redundancy may be a mechanism by which organisms can tolerate variability in absolute cytokine production. Here, uterine GM-CSF, IL-6 and KC secretion were examined, which does not represent the complete post-mating cytokine profile in the murine uterus. Previous studies have also shown a post-mating increase in uterine mRNA for other cytokines including IL-1 and TNFα and chemokines RANTES, MIP-1α, MIP-1β and MCP-1 [154, 155, 250]. These signalling molecules may contribute toward modulating leukocyte recruitment and activation following insemination, so it would be informative to include post-mating uterine measures of them in order to compare the relative abundance of these factors in different strains of mice. The mating experiments conducted here are consistent with studies examining the effect of semen exposure on cytokine expression and leukocyte recruitment in the human cervix [130].

Chapter 5

Effect of IFNγ present at insemination on the uterine inflammatory response and reproductive outcomes

5.1 INTRODUCTION

Experiments in Chapter 3 demonstrate the inhibitory effect of recombinant IFNγ on uterine epithelial cell secretion of GM-CSF. Both basal GM-SCF secretion as well as TGFβ stimulated GM-CSF and KC secretion were substantially reduced in vitro with very low concentrations of IFNγ. This raises the question of whether IFNγ present at insemination has the capacity to inhibit uterine cytokine secretion during the post-mating inflammatory response in vivo, and whether this would in turn influence leukocyte recruitment and implantation rate or pregnancy outcomes.

Recent studies in humans examining signalling moieties in seminal plasma of fertile and infertile couples have shown that male partners in couples experiencing recurrent miscarriage have a higher incidence of seminal IFNγ detectable at levels > 3pg/mL [130]. Additionally the ratio of seminal IFNγ relative to TGFβ content is altered in infertile groups. A previous study has shown increased IFNγ is associated with altered sperm parameters, however the work in our group indicates that high IFNγ can occur even in the absence of altered sperm parameters [371]. Studies in mice have previously demonstrated the detrimental effect of IFNγ on fetal resorption rates in abortion prone mice in a gestational age dependent manner, however whether there is a similar effect during the peri-conceptual period remains to be investigated [438]. In vitro results in humans [130] and those presented here in Chapter 3 demonstrate the potent inhibitory effect of IFNγ on TGFβ stimulated epithelial cell cytokine secretion. This in vitro data together with the higher likelihood of IFNγ positive seminal plasma samples from infertile couples may indicate an adverse effect of seminal IFNγ on the female immune response to male seminal fluid and conceptus antigens. The mode by which IFNγ may be affecting these outcomes could be the consequence of its potent type I immune deviating activities including activating antigen presenting cells and promoting type I cellular differentiation via upregulation of the transcription factor T-bet and inhibition of NFκB.

It is reasonable to hypothesise that dysregulation of the post-mating inflammatory response in the female reproductive tract may lead to altered pregnancy or fetal outcomes. There are several lines of evidence demonstrating that long term effects on fetal growth and metabolic function can be induced by insults at critically sensitive periods of development. This phenomenon, referred to as fetal programming, is influenced by placental function, genetic, maternal and environmental factors and the interaction between these components [515-517]. The peri-implantation period of embryo development is highly susceptible and effects on both the embryo and the uterus contribute to influencing long term progeny outcomes. Changes consistent with the metabolic syndrome phenotype can be elicited in

animal models following perturbations in the early embryonic environment or fetal nutrient deprivation due to either maternal nutrient or placental restriction similar to those seen in human epidemiological studies [454, 455, 458]. One proposed mechanism by which these factors instigate change is via altering DNA methylation patterns in pre-implantation embryos [518-521]. Several methylated genes are known to be key regulators of fetal growth and development [522, 523]. The environment of the oviduct ideally facilitates early embryo development through optimal growth factor and metabolic substrate availability and removal of potentially toxic metabolic waste [524, 525]. Early exposure to a suboptimal environment can be detrimental to the long term health of progeny, without acute signs of reproductive dysfunction such as embryonic loss or implantation failure. Embryo culture and transfer experiments in mice and livestock species together with epidemiological studies in humans clearly show increases in low and very low birth weight offspring, birth defects and deficits in cognitive function [522, 523, 526, 527]. Furthermore animal ART studies have described the impact of oocyte fertilization and/or embryo culture conditions on long-term health of progeny. Such effects include altered growth trajectories and adult weights, predisposition toward obesity, altered metabolic profiles and insulin resistance and increased systolic blood pressure [456, 458, 528-533].

The experiments in this chapter aimed to investigate the effect of IFNγ present at insemination on pregnancy and progeny outcomes. Initially, to investigate the effect of IFNγ on the post-mating inflammatory response, uterine luminal fluid was collected from mated female mice following intra-uterine injection of recombinant IFNγ and assayed for GM-CSF, IL-6 and KC content. The effect of IFNγ on post-mating leukocyte infiltration into the endometrium was then investigated using conventional immunohistochemical techniques. Additionally, the effect of IFNγ present in the peri-conception period on implantation rate, pregnancy and post-natal progeny outcomes was examined. Near term pregnancy parameters including pregnancy and resorption rates and fetal and placental measures were recorded. Post-natal and adult growth of progeny was measured together with endocrine metabolic markers, glucose tolerance, systolic blood pressure and post-mortem tissue weights.

5.2 EFFECT OF IFNγ ON DAY0.5pc UTERINE CYTOKINE SECRETION AND LEUKOCYTE RECRUITMENT

In Chapter 3, *in vitro* experiments showed down regulation of uterine epithelial cell GM-CSF production following culture with recombinant mIFNγ. In order to assess the effect of exogenous recombinant IFNγ on the post-mating inflammatory response in vivo, female C57Bl/6 mice were mated IFNγ was instilled into the uterine cavity. Intra-uterine treatments with 50 ng IFNγ were administered via surgery and injection (iu) at either 0400 h or by infusion via transcervical catheter (tc) at 0900 h on the morning of visualisation of a copulatory plug (day 0.5pc). Control groups included mice given carrier alone or mice mated and not treated. All treatments were given in a 30 μL volume. Mice were sacrificed eight hours post-treatment and uterine luminal fluid was collected for cytokine analysis. Uterine tissue was frozen in OCT for sectioning and immunohistochemical analysis.

5.2.1 Effect of IFNγ on post-mating uterine cytokine secretion

The post-mating surge in uterine GM-CSF was inhibited following surgical injection or transcervical instillation of 50 ng IFNγ by 69% and 91% respectively compared to the corresponding carrier controls (p=0.021 and p=0.026 respectively) (Figure 5.1 A). This result is consistent with *in vitro* data shown in Chapter 3.

Uterine IFNγ treatment via transcervical infusion following mating caused a 44% decrease in luminal IL-6 secretion but this failed to reach statistical significance when compared to carrier treatment (Figure 5.1 B). However statistical significance was reached when compared to the mating alone control group (p=0.011) with IL-6 production reduced by 61%. There was no effect on IL-6 secretion following IFNγ treatment via surgical injection however there was a statistically significant 2.2 fold increase in response to the carrier alone control group (p=0.038).

Uterine luminal KC secretion, following mating and subsequent surgical or transcervical IFNγ treatment, was reduced by 37% and 55% however statistical significance was not reached when compared to carrier administration (p=0.059 and p=0.178) (Figure 5.1 C). However IFNγ treatment significantly reduced uterine KC production by 67% compared to the untreated mated control group (p=0.003).

Outlier data points have been identified for graphical representation but were included in all

statistical analysis described above. There was one animal displaying outlying data for GM-CSF and KC measures. Statistical analysis was also conducted on data excluding outliers but this only affected statistical significance of KC outcomes. Surgical IFNγ treatment reduced uterine KC production compared to the carrier control group (p=0.025) and transcervically administered carrier reduced uterine KC production compared to the untreated mated control group (p=0.009).

5.2.2 Effect of IFNγ on post-mating leukocyte recruitment

To investigate whether intra-uterine injection of IFNγ into recently mated mice has the capacity to alter leukocyte recruitment into the uterine tissue, uteri from the experiment described above was cut into 7μm sections and stained with the anti-neutrophil antibody RB6 or the anti-macrophage antibody F4/80. Stained slides were blinded and a semi-quantitative analysis conducted, considering intensity and location of staining. The number of neutrophils in the intra-epithelial region of each section was also assessed. Surgical intra-uterine injection of IFNγ into a recently mated uterus did not affect the abundance of F4/80 macrophages compared to both untreated and carrier treated controls, (Table 5.1 and Figure 5.2 A-C).

Neutrophil recruitment into uterine tissue of mice surgically administered with carrier or IFNγ was equally reduced compared to untreated controls. Surgical intra-uterine IFNγ injection or carrier treatment similarly reduced the number of RB6 positive neutrophils located in the intraepithelial region compared to untreated controls (p=0.014 and p=0.014) (Table 5.1 and Figure 5.2 D-F).

Table 5.1 Summary of IHC analysis of uterine tissue following intra-uterine IFNγ injection after mating. Data is given as mean ± SD and was analysed using Kruskal-Wallis and Mann-Whitney tests. a b represent statistical differences between groups, p<0.05. ✚ - weak, ✚✚ - moderate and ✚✚✚ - strong uterine staining

	RB6 +ve Uterine Neutrophils	RB6 +ve Trans-epithelial Neutrophils	F4/80 +ve Macrophages
Day 0.5pc	✚✚	23.6 ± 7.3 [a]	✚✚✚
Carrier	✚	1.3 ± 0.4 [b]	✚✚✚
IFNγ	✚	1.2 ± 1.0 [b]	✚✚✚

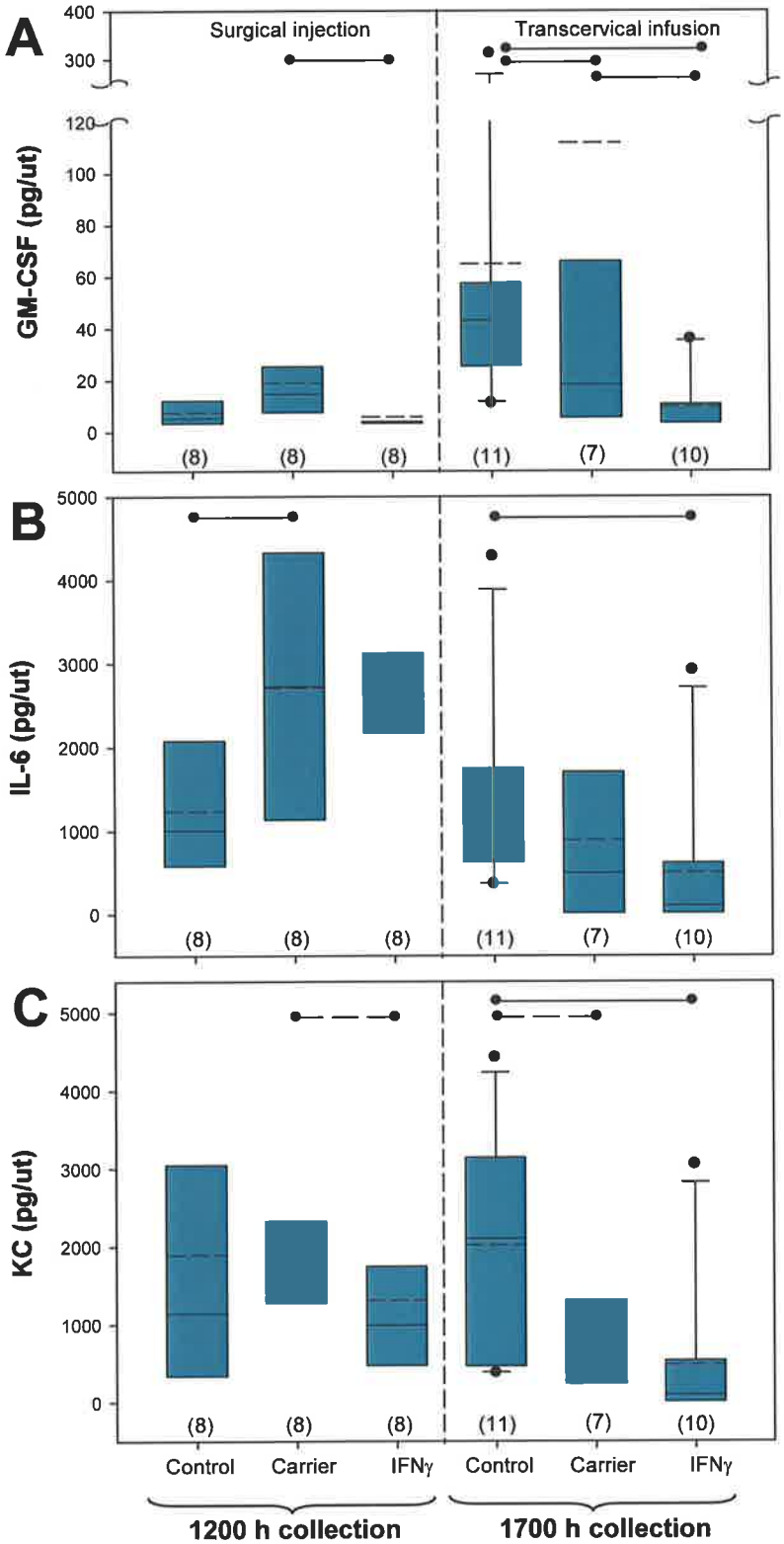


Figure 5.1 Effect of IFNγ treatment on uterine post-mating cytokine secretion. Uterine luminal fluid was collected eight hours after surgical or transcervical intra-uterine injection of carrier +/- IFNγ on day 0.5pc. Uterine GM-CSF (A), IL- 6 (B) and KC (C) were measured. The number of mice in each group is shown in brackets. The upper and lower edges of the box and whisker plots indicate the 75th and 25th percentiles respectively, while the solid and dashed horizontal lines are the median and the mean respectively. Error bars signify the 5th and 95th percentiles. Kruskal-Wallis and Mann-Whitney tests were used to determine statistically significant differences between treatment groups represented as solid dot-ended lines, $p<0.05$. Dashed dot-ended lines indicate significant differences identified following removal of outliers, $p<0.05$.

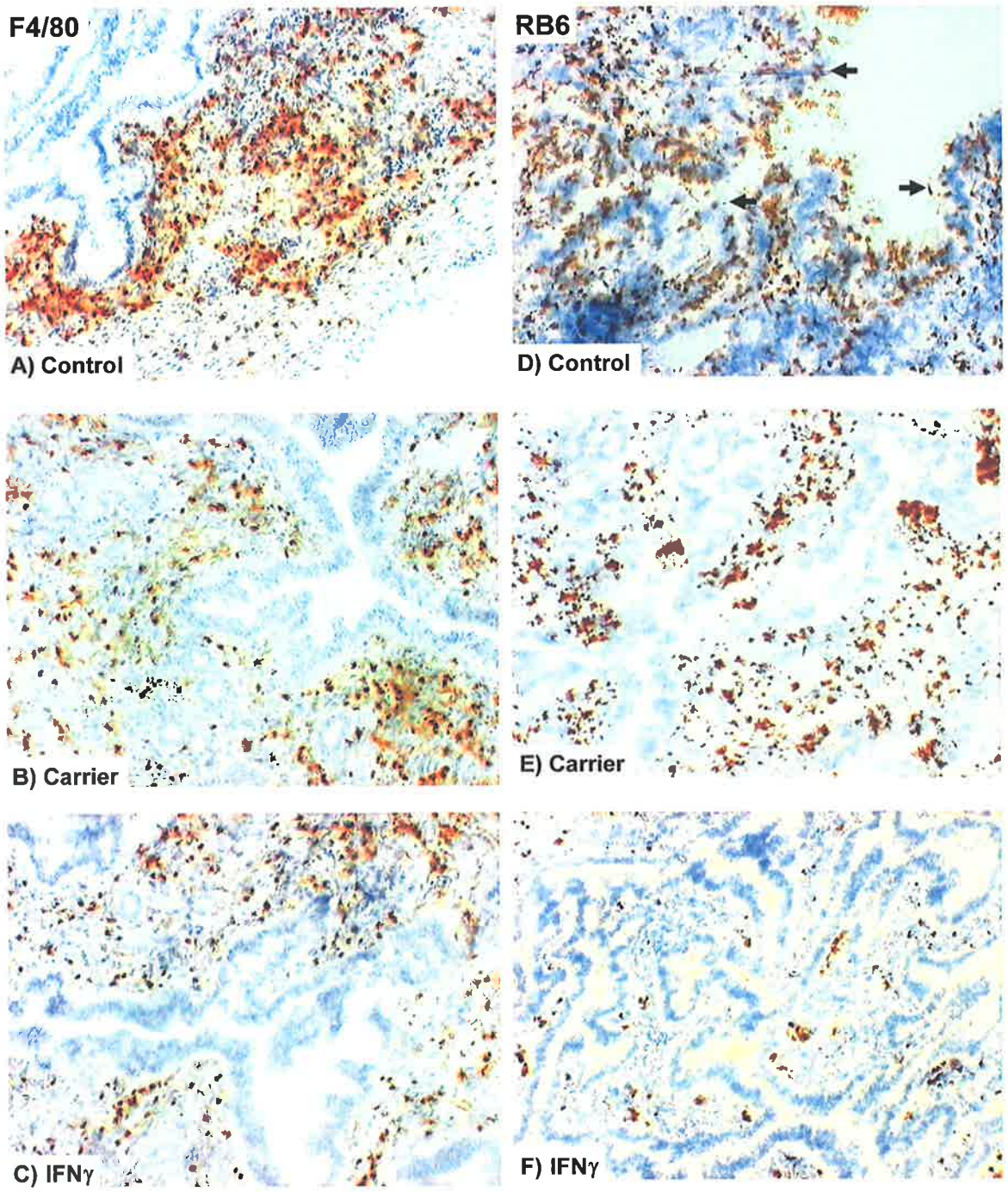


Figure 5.2 Effect of IFNγ exposure on uterine leukocyte recruitment. Uterine sections were obtained from female C57Bl6 mice following mating (A,D), surgical intra-uterine injection with carrier alone (B,E) or IFNγ (C,F) and were labelled monoclonal antibodies specific for the F4/80 antigen (MCA497) (A,B,C) or anti-neutrophil RB6 antigen (D,E,F). → indicates intra-epithelial RB6 positive neutrophils. All images captured at the same magnification (x 20).

5.3 THE EFFECT OF IFNγ ON GESTATION DAY 17.5 PREGNANCY OUTCOMES

The previous experiment showed perturbations in uterine cytokine production following IFNγ administration during the peri-conception period. To examine the extent to which IFNγ exposure alters implantation rates and near term (gd17.5) pregnancy outcomes, a second cohort of C57Bl/6 were mated and treated with 50 ng IFNγ. Treatments were administered via surgery and injection (iu) or transcervical catheter (tc) at either 0400 h or 0900 h respectively, on the morning of visualisation of a copulatory plug (day 0.5pc). Mice were sacrificed on gd17.5 and fetuses and placentas were collected as described in Chapter 2. Fetal development was assessed by measuring crown-rump length and fetal weight, and placental weights were obtained and used to calculate fetal: placental ratio. A total of 83 litters were assessed from 120 matings which generated 435 fetuses. Statistical analysis was conducted using a Chi-squared test or Oneway ANOVA as indicated to firstly examine whether there was an effect of intervention compared to untreated controls (combined surgical and transcervical modes of intervention) and secondly to examine whether there was a differential effect of individual modes of intervention, then thirdly to examine the effect of IFNγ treatment within each mode of intervention.

5.3.1 The effect of IFNγ on pregnancy parameters

Pregnancy rates (the number of plugged females with viable implantation sites) were reduced by 46% following any intervention ($p<0.001$). While there was no statistical effect of transcervical intervention on pregnancy rates, surgical intervention decreased pregnancy rates by 72% ($p<0.001$). Surgical intra-uterine injection reduced pregnancy rates by 60% - 80% with carrier alone at 0400 h ($p<0.001$) or IFNγ treatment at 0400 h and 0900 h ($p<0.001$ and $p=0.0017$) (Table 5.2). This reduction was commonly associated with and likely to be due to sub-lethal and lethal abscess formation in 36% – 55% of females receiving surgical injections. Presumably, this is a consequence of post-mating uterine fluid escaping into the peritoneal cavity via the injection puncture site. Intrauterine injections in unmated mice did not cause abscess formation (data not shown). There was no adverse effect of transcervical treatment with IFNγ on pregnancy rate (Table 5.3).

Litter size was reduced by 20% as a result of having any intervention compared to untreated controls ($p=0.021$). Surgical intervention reduced litter size by 45% compared to untreated controls ($p=0.001$). Transcervical intervention had no adverse effect on litter size. Intra-uterine treatments administered by surgery and injection reduced litter size by 24% – 51%, with a statistical reduction

following carrier injection (p=0.002) (see Table 5.2). There was no change in litter size as a result of transcervically administered IFNγ treatment (Table 5.3).

Fetal resorption rates were increased by 71% following any intervention compared to untreated controls (p<0.001). Increases of 67% and 72% were observed following either surgical or transcervical intervention respectively (p=0.008 and p=0.004). The rate of fetal resorption was altered by surgical intra-uterine injections of either carrier alone (p=0.0019) or IFNγ treatment (0400 h: p<0.001 and 0900 h: p<0.001) compared to untreated controls. However this was not in a consistent manner with carrier and 0400 h IFNγ treatments decreasing and 0900 h IFNγ treatment increasing resorption rates (Table 5.2). Transcervical carrier injection resulted in a 2.2 fold increase in resorption rate compared to untreated controls (p<0.001). Transcervical IFNγ treatment did not alter resorption rates (Table 5.3).

5.3.2 The effect of peri-conception administration of IFNγ on fetal and placental development

The outcomes measured as indicators of fetal and placental development were analysed using a linear mixed model analysis of variance with the mother being the subject, treatment group the fixed factor and viable litter size was a covariate. Litter size had a statistically significant effect on placental weights, fetal:placental weight ratios and on crown rump length. However there was no interaction between litter size and treatment group on any of the outcomes measured in this experiment indicating litter size affected each treatment group equally and so was removed as a covariate.

Fetal weight was reduced by 9% as a consequence of any intervention when compared to untreated controls (p<0.001). Surgical and transcervical interventions statistically decreased fetal weight by 8% and 10% respectively compared to untreated controls (p=0.019 and p<0.001). IFNγ treatments failed to elicit any effect when compared to the relevant carrier control (Table 5.4 and Table 5.5). Both transcervically administered carrier and IFNγ reduced fetal weight by 8% compared to untreated controls (p=0.002 and p=0.005). There was no effect of litter size on fetal weight across the treatment groups.

Placental weight was increased by 4% following any intervention compared to untreated controls (p=0.033). Surgical intervention increased placental weights by 10% compared to untreated controls (p=0.003), which was different to transcervical intervention which had no effect compared to controls. IFNγ did not elicit a significant change in placental weight by either route of intervention (Table 5.4 and Table 5.5).

Intervention resulted in a significant 13% reduction in fetal:placental weight ratios compared to the untreated control ($p<0.001$). Surgical and transcervical interventions decreased fetal:placental weight ratios by 16% and 12% respectively compared to untreated controls ($p<0.001$ and $p<0.001$). Transcervical administration of IFNγ did not affect fetal:placental weight ratios compared to the carrier control, however there was an 11% reduction when compared to untreated controls ($p=0.006$). Surgically applied IFNγ did not elicit any significant effect. There were reductions in the fetal:placental weight ratios following carrier injection by both surgical (18%) and transcervical (11%) interventions compared to the untreated controls ($p=0.003$ and $p=0.001$) (Table 5.4 and Table 5.5).

Crown-rump length was reduced by 2% following any intervention compared to untreated controls ($p=0.017$). Transcervical intervention reduced crown-rump length by 3% ($p=0.019$), while surgical intervention elicited no effect. Administration of IFNγ by either surgical or transcervical modes did not affect crown-rump length compared with the untreated and carrier treated control groups (Table 5.4 and Table 5.5).

Table 5.2 The effect of surgically administered IFNγ at insemination on pregnancy parameters at gd17.5.

Effect of group on Pregnancy Rate and Resorption Rate were analysed using a χ^2 test.
@ Effect of group on Litter size was analysed using a Oneway ANOVA followed by Post hoc Sidak t-test.
§ - indicates statistically significant effect of intervention compared to the untreated control group. Different alphabet superscripts indicate statistical differences between treatments, $p < 0.05$

	Treatment	Pregnancy Rate § % (Litters/Matings)#	Litter size @ § **Total** Viable Mean ± SEM (Pups/Litters)	Resorptions/ mouse § Mean ± SEM (%) #
Control	Mated	89 (32/36) [a]	**6.9 ± 0.3 (220/32)** [a] 6.0 ± 0.3 (191/32) [a]	0.91 ± 0.17 (13.2) [a]
Surgical Intra-Uterine Injection §	Carrier	36 (9/25) [b]	**3. 8 ± 0.6 (34/9)** [b] 2.9 ± 0.6 (26/9) [b]	0.89 ± 0.26 (23.5) [b]
	0400 h IFNγ	20 (3/19) [b]	**3.7 ± 2.2 (11/3)** [b] 3.7 ± 2.2 (11/3) [ab]	0.00 ± 0.00 (0) [c]
	0900 h IFNγ	18 (3/17) [b]	**7.0 ± 0.6 (21/3)** [ab] 4.7 ± 1.8 (14/3) [ab]	2.33 ± 1.45 (14.3) [b]

Table 5.3 The effect of transcervically administered IFNγ at insemination on pregnancy parameters at gd17.5.

Effect of group on Pregnancy Rate and Resorption Rate were analysed using a χ^2 test.
@ Effect of group on Litter size was analysed using a Oneway ANOVA followed by Post hoc Sidak t-test.
§ - indicates statistically significant effect of intervention compared to the untreated control group. Different alphabet superscripts indicate statistical differences between treatments, $p < 0.05$

	Treatment	Pregnancy Rate % (Litters/Matings) #	Litter size @ **Total** Viable Mean ± SEM (Pups/Litters)	Resorptions/ mouse § Mean ± SEM (%) #
Control	Mated	89 (32/36)	**6.9 ± 0.3 (220/32)** 6.0 ± 0.3 (191/32)	0.91 ± 0.17 (13.2) [a]
Transcervical Infusion	Carrier	81 (17/21)	**7.2 ± 0.2 (123/17)** 5.2 ± 0.8 (89/17)	2 ± 0.34 (24.4) [b]
	0900 h IFNγ	81 (17/21)	**6.7 ± 0.5 (114/17)** 5.5 ± 0.6 (94/17)	1.18 ± 0.32 (17.5) [ab]

Table 5.4 The effect of surgically administered IFNγ at insemination on fetal and placental development gd17.5.
All data is given as estimated marginal mean ± SEM and was analysed using Linear Mixed Model Analysis of Variance. § - indicates statistically significant effect of intervention compared to the untreated control group. Different alphabet superscripts indicate statistical differences between treatments, $p < 0.05$

	Treatment (Pups/Mothers)	Fetal Weight (mg) §	Placental Weight (mg) §	Fetal: Placental Ratio §	Crown-rump length (mm)
Control	Mated (191/32)	978 ± 18	115 ± 2 [a]	8.6 ± 0.2 [a]	22.2 ± 0.2
Surgical Intra-uterine Injection	Carrier (26/9)	912 ± 35	132 ± 7 [b]	7.0 ± 0.4 [b]	22.3 ± 0.4
	0400 h IFNγ (11/3)	922 ± 61	118 ± 6 [ab]	7.9 ± 0.7 [ab]	21.9 ± 0.6
	0900 h IFNγ (14/3)	862 ± 58	119 ± 6 [ab]	7.7 ± 0.6 [ab]	21.6 ± 0.6

Table 5.5 The effect of transcervically administered IFNγ at insemination on fetal and placental development gd17.5.
All data is given as estimated marginal mean ± SEM and was analysed using Linear Mixed Model Analysis of Variance. § - indicates statistically significant effect of intervention compared to the untreated control group. Different alphabet superscripts indicate statistical differences between treatments, $p < 0.05$

	Treatment (Pups/Mothers)	Fetal Weight (mg) §	Placental Weight (mg)	Fetal: Placental Weight Ratio §	Crown-rump length (mm) §
Control	Mated (191/32)	987 ± 20 [a]	115 ± 2	8.6 ± 0.2 [a]	22.2 ± 0.2
Transcervical Infusion	Carrier (89/17)	881 ± 21 [b]	118 ± 2	7.5 ± 0.2 [b]	21.6 ± 0.2
	0900 h IFNγ (94/17)	888 ± 22 [b]	117 ± 2	7.6 ± 0.2 [b]	21.6 ± 0.2

5.4 THE EFFECT OF IFNγ ON PROGENY GROWTH AND DEVELOPMENT

A third cohort of animals was mated and IFNγ administered during the peri-conception period in order to examine the impact on post-natal outcomes. The previous experiment demonstrated adverse outcomes for female mice receiving surgery and injection with morbidity and mortality rates of 23% and 26% respectively, hence the transcervical infusion mode of treatment was employed for the current experiment. Carrier or 50 ng IFNγ were administered by transcervical infusion to female C57Bl/6 mice at 0900 h on the morning of visualisation of a copulatory plug (day 0.5pc). Females were housed in pairs until weaning of pups at 3 weeks of age. Progeny were ear punched for identification purposes at weaning, housed according to gender and weighed weekly until 22 weeks of age. At 22 weeks of age blood pressure was measured, blood was collected for metabolic analysis and autopsy with full body composition analysis was conducted. A total of 120 matings gave rise to 80 litters generating 360 pups. Statistical analysis was conducted using a Chi-squared test, Oneway ANOVA or a Linear Mixed Model of Analysis as indicated to firstly examine whether there was an effect of intervention compared to untreated controls (intervention = combined transcervically administered carrier and IFNγ treatment groups) and secondly to examine the effect of IFNγ treatment within the mode of intervention.

5.4.1 Effect of peri-conception administration of IFNγ on pregnancy outcomes

Pregnancy rate (the number of plugged females proceeding to deliver viable pups) was not altered by intervention compared to the untreated control mice. Pregnancy rate was reduced by 20% and 12.5% following uterine IFNγ treatment compared to untreated and carrier treated controls respectively but this failed to reach significance (Table 5.6). Litter size at birth was reduced by 23% following intervention compared to the untreated controls ($p=0.018$). There was a 26% and 10% decrease in litter size at birth following treatment of mothers with IFNγ compared to untreated and carrier treated controls respectively but this did not reach statistical significance. There were no differences in gender ratios (male:female) following intervention or as a result of treatment. Progeny survival to weaning was reduced by 12% following intervention compared to the untreated controls ($p=0.01$). There was a 7% decrease and a 13% increase in progeny survival to weaning following treatment with IFNγ compared to the untreated and carrier treated control groups respectively ($p<0.001$ and $p=0.14$).

Table 5.6 Effect of peri-conception IFNγ treatment on pregnancy outcomes.

Effect of group on Pregnancy Rate, Gender Ratio and Survival to Weaning were analysed using a χ^2 test.
@ Effect of group on Litter Size was analysed using a Oneway ANOVA followed by Post hoc Sidak t-test.
§ - indicates statistically significant effect of intervention compared to the untreated control group. Different alphabet superscripts indicate statistical differences between treatments, $p < 0.05$

	Pregnancy Rate % (Litters/Matings)#	Litter size § Mean ± SEM (Pups/Litters) @	Gender Ratio Male : Female #	Survival to Weaning § % (Pups/Litters) #
Control	73 (41/56)	5.1 ± 0.3 (211/41)	1.00	97 (204/40) [a]
Carrier	67 (18/27)	4.2 ± 0.5 (72/18)	0.68	79 (57/16) [b]
IFNγ	58 (21/36)	3.8 ± 0.5 (77/21)	0.92	90 (69/18) [b]

5.4.2 Effect of peri-conception uterine exposure to IFNγ on post-natal growth of progeny

Statistical analysis of progeny growth showed a significant interaction between treatment and gender thus male and female body weight data was analysed separately. Exposure to IFNγ at conception increased the weight of 22 week old male progeny by 2.3% compared to the carrier treated control group (NS) and by 3.6% compared to the untreated control group (p=0.004) (Figure 5.3 A). Male progeny of IFNγ treated mothers were heavier than controls at all time points with the exception of the 16 week weight (Figure 5.3 B). There was no significant effect of either IFNγ or carrier treatment on female offspring with both groups consistently ~2% lighter at all time points following weaning until autopsy at 22 weeks (Figure 5.3 C).

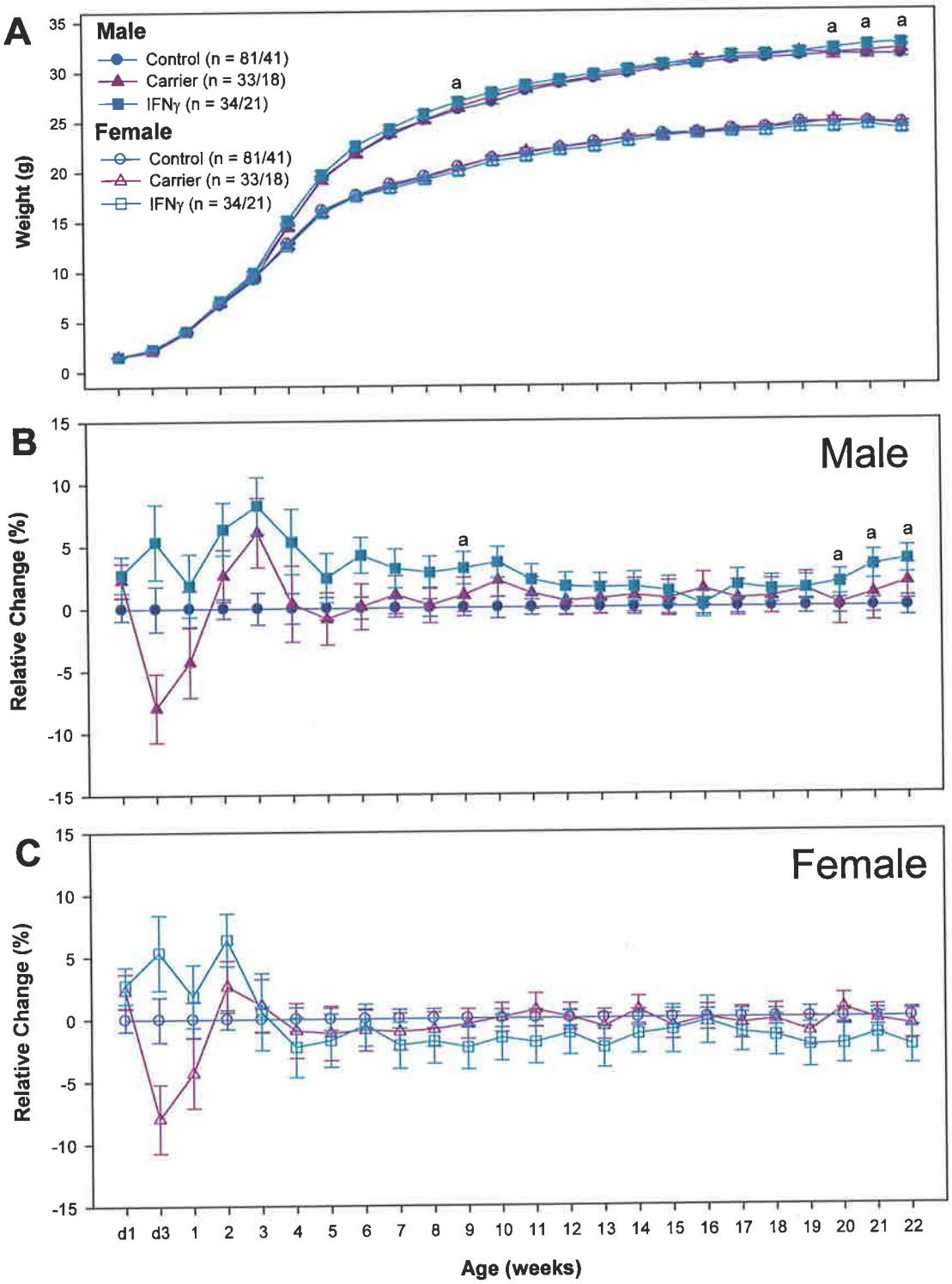


Figure 5.3 Effect of peri-conception IFNγ treatment on progeny growth. Progeny of mothers untreated (●) or treated with carrier (▲) or carrier plus IFNγ (■) on Day1 of pregnancy were weighed within 24hrs of birth (d1), post-natal day 3 (d3), 1 week and then weekly until 22 weeks of age. Absolute weight growth trajectory (A - where n = number of pups/number of litters) and weight relative to untreated control Males (B) and Females (C) are shown. Data is given as estimated marginal mean ± SEM. a – statistical significance IFNγ group compared to untreated control. Linear Mixed Model of Analysis was used to examine data, $p<0.05$.

5.4.3 Effect of peri-conception IFNγ treatment on body composition of progeny

Full body compositions were carried out on 177 progeny at 22 weeks of age. Organ, reproductive tissue, central adipose tissue and muscle weights were obtained to examine the effect of peri-conception uterine perturbation on progeny development. Absolute tissue weights were normalised to body weight for statistical analysis. There was no significant effect of intervention on any organ, fat or muscle weights compared to untreated controls. There were however significant effects of gender on these measures and furthermore, interactions between intervention and gender for some measures. There was no effect of either IFNγ or carrier treatment on any organ, fat or muscle weights compared to untreated controls. However, gender elicited a significant effect on all measures and interacted with treatment for some.

Male progeny generated following treatment with carrier or IFNγ did not exhibit altered organ, fat or muscle weights compared to untreated control progeny (see Table 5.7). IFNγ treatment did not significantly affect male progeny body composition. Although there were marked increases in muscle weights (range = 4 – 17%) and decreases in fat depots (range = 6.8 – 16%) compared to the carrier control group, these changes did not reach statistical significance.

Female progeny produced following treatment with carrier did not show significant changes in organ, fat or muscle weights compared to untreated controls (see Table 5.8). IFNγ treatment resulted in 21% reductions in both left and right ovary weights compared to untreated controls ($p=0.025$ and $p=0.039$). There were no significant effects on total adipose tissue or muscle mass.

Table 5.7 Effect of peri-conception IFNγ treatment on male progeny adult body composition.
Data is given as estimated marginal mean (tissue weight / body weight %) ± SEM and was analysed using Linear Mixed Model Analysis of Variance.
Number of progeny / number of litters are shown in parentheses. Central fat is the total weight of all adipose depots collected, similarly combined muscle is the total weight of all muscle collected.

	Control (n = 42/26)	Carrier (n = 19/16)	IFNγ (n = 24/17)
Brain	1.291 ± 0.023	1.229 ± 0.033	1.271 ± 0.030
Heart	0.467 ± 0.008	0.454 ± 0.011	0.463 ± 0.010
Lung	0.531 ± 0.008	0.503 ± 0.011	0.525 ± 0.010
Thymus	0.108 ± 0.005	0.101 ± 0.008	0.104 ± 0.007
Kidney (left)	0.617 ± 0.008	0.646 ± 0.012	0.621 ± 0.011
Kidney (right)	0.634 ± 0.009	0.666 ± 0.013	0.650 ± 0.012
Liver	3.697 ± 0.045	3.745± 0.065	3.702 ± 0.059
Spleen	0.230 ± 0.005	0.238 ± 0.007	0.226 ± 0.007
Testicle (left)	0.366 ± 0.004	0.361 ± 0.006	0.367 ± 0.005
Testicle (right)	0.378 ± 0.005	0.369 ± 0.007	0.380 ± 0.007
Seminal vesicle	0.659 ± 0.019	0.702 ± 0.028	0.692 ± 0.025
Epididymis	0.068 ± 0.002	0.067 ± 0.003	0.065 ± 0.003
Epididymal fat	1.389 ± 0.126	1.749 ± 0.183	1.487 ± 0.166
Retroperitoneal fat	0.352 ± 0.027	0.396 ± 0.039	0.331 ± 0.035
Peri-renal fat	0.162 ± 0.012	0.176 ± 0.017	0.164 ± 0.016
Bicep	0.052 ± 0.002	0.047 ± 0.003	0.055 ± 0.003
Tricep	0.412 ± 0.009	0.401 ± 0.014	0.420 ± 0.012
Quadricep	0.687 ± 0.023	0.667 ± 0.033	0.714 ± 0.030
Gastrocnemius	0.540 ± 0.017	0.532 ± 0.025	0.528 ± 0.023
Central fat	1.886 ± 0.158	2.361 ± 0.234	1.995 ± 0.208
Combined muscle	1.693 ± 0.042	1.645 ± 0.063	1.712 ± 0.056

Table 5.8 Effect of peri-conception IFNγ treatment on female progeny adult body composition.
Data is given as estimated marginal mean (tissue weight / body weight %) ± SEM and was analysed using Linear Mixed Model Analysis of Variance.
Number of progeny / number of litters are shown in parentheses. Different alphabet superscripts indicate statistical differences between groups, $p<0.05$. Central fat is the total weight of all adipose depots collected, similarly combined muscle is the total weight of all muscle collected.

	Control (n = 47/26)	Carrier (n = 21/16)	IFNγ (n = 24/17)
Brain	1.675 ± 0.022	1.653 ± 0.032	1.675 ± 0.030
Heart	0.453 ± 0.008	0.436 ± 0.011	0.450 ± 0.010
Lung	0.602 ± 0.008	0.593 ± 0.011	0.604 ± 0.010
Thymus	0.150 ± 0.005	0.163 ± 0.007	0.156 ± 0.007
Kidney (left)	0.538 ± 0.008	0.536 ± 0.012	0.546 ± 0.011
Kidney (right)	0.555 ± 0.009	0.557 ± 0.013	0.569 ± 0.012
Liver	3.675 ± 0.043	3.502± 0.064	3.664 ± 0.059
Spleen	0.282 ± 0.005	0.285 ± 0.007	0.269 ± 0.007
Ovary (left)	0.019 ± 0.001 [a]	0.017 ± 0.001 [a]	0.015 ± 0.001 [b]
Ovary (right)	0.019 ± 0.001 [a]	0.019 ± 0.001 [a]	0.015 ± 0.001 [b]
Uterus	0.352 ± 0.017	0.372 ± 0.025	0.353 ± 0.023
Parametrial fat	2.284 ± 0.172	2.373 ± 0.262	2.370 ± 0.233
Retroperitoneal fat	0.247 ± 0.025	0.235 ± 0.038	0.219 ± 0.035
Peri-renal fat	0.213 ± 0.012	0.216 ± 0.017	0.225 ± 0.016
Bicep	0.05 ± 0.002	0.047 ± 0.003	0.051 ± 0.003
Tricep	0.373 ± 0.009	0.375 ± 0.013	0.385 ± 0.012
Quadricep	0.650 ± 0.022	0.658 ± 0.033	0.678 ± 0.030
Gastrocnemius	0.517 ± 0.013	0.532 ± 0.024	0.528 ± 0.023
Central fat	2.699 ± 0.149	2.768 ± 0.223	2.767 ± 0.208
Combined muscle	1.586 ± 0.040	1.619 ± 0.060	1.647 ± 0.056

5.4.4 Effect of peri-conception IFNγ treatment on metabolic hormones and metabolites in progeny

To examine the consequence of conception in a perturbed uterine environment on progeny several endocrine hormones and metabolites known to be markers of metabolic status, including adiponectin, leptin, free fatty acids, triglycerides, cholesterol, insulin and glucose, were measured in plasma collected at 22 weeks of age from 264 offspring arising from 64 litters.

Transcervical intervention did not elicit a change in any metabolic marker compared to untreated controls. However there was an effect of gender and weight at autopsy on most metabolic marker measures. Female progeny had higher levels of adiponectin (2.6-fold, $p<0.001$) and leptin (2-fold, $p<0.001$). Male progeny had higher levels of triglycerides (11%, $p=0.022$), cholesterol (43%, $p<0.001$), glucose (15%, $p<0.001$) and leptin:adiponectin ratio (34%, $p<0.001$). Further, in some measures there was a significant intervention-gender interaction, meaning that intervention had a differential effect depending on gender. Intervention in female progeny resulted in leptin levels increasing by 3% whereas male progeny exhibited a 7% decrease ($p=0.033$). Glucose measures in female progeny decreased by 11% following intervention whereas those of male progeny increased by 4% ($p=0.013$). Leptin:adiponectin ratios decreased by 17% in female progeny following intervention whereas male progeny exhibited an 11% increase in this parameter ($p=0.02$).

There was no overall effect of IFNγ or carrier treatment on any metabolic marker assayed in this experiment when compared to untreated controls. Gender and weight at autopsy influenced metabolic parameters. Also examined were interactions between treatment, weight at autopsy and gender on metabolic parameters. There was a significant positive correlation between weight and cholesterol ($p=0.01$), triglycerides ($p=0.05$), glucose ($p=0.01$) and leptin:adiponectin ratio ($p=0.01$). There was a significant negative correlation between weight and adiponectin ($p=0.01$) and leptin ($p=0.01$). Male progeny of mothers treated with carrier had reduced adiponectin levels (35%) compared to untreated controls ($p=0.002$) (Table 5.9). Female progeny of mothers treated with carrier had increased adiponectin levels (14%) compared to untreated controls ($p=0.002$) (Table 5.10). These same female progeny showed a significant decrease in the leptin:adiponectin ratio (43%) compared to untreated controls ($p=0.02$). There were no significant effects of uterine IFNγ treatment compared to carrier treated controls in either male or female progeny with regard to any of the metabolic markers measured.

Table 5.9 Effect of peri-conception IFNγ treatment on male progeny metabolic markers.

Data is given as estimated marginal mean ± SEM and was analysed using Linear Mixed Model Analysis of Variance. Number of progeny / number of litters are shown in parentheses. Different alphabet superscripts indicate statistical differences between groups, $p<0.05$. § Indicates outcome affected by transcervical intervention. * Indicates number of progeny samples included in measure.

	Control (n = 51* or 68/31)	Carrier (n = 23/16)	IFNγ (n = 29/17)
Adiponectin (µl/ml) *	10.1 ± 1.1 [a]	6.6 ± 1.8 [b]	8.2 ± 1.9 [ab]
Leptin (ng/ml) *	4.5 ± 1.1	3.1 ± 1.8	3.2 ± 1.8
Free fatty acids (meq/L)	0.83 ± 0.08	0.65 ± 0.15	0.63 ± 0.14
Triglycerides (mmol/L) §	0.54 ± 0.05	0.47 ± 0.08	0.47 ± 0.08
Cholesterol (mmol/L) §	2.19 ± 0.09	2.15 ± 0.16	2.06 ± 0.16
Insulin (ng/ml) *	0.08 ± 0.05	0.09 ± 0.09	0.03 ± 0.09
Glucose (mmol/L) §	7.8 ± 0.4	7.3 ± 0.8	6.4 ± 0.7
Leptin: adiponectin ratio§	0.66 ± 0.13	0.67 ± 0.20	0.43 ± 0.2
Insulin: glucose ratio	0.01 ± 0.01	0.01 ± 0.01	0.004 ± 0.011

Table 5.10 Effect of peri-conception IFNγ treatment on female progeny metabolic markers.

Data is given as estimated marginal mean ± SEM and was analysed using Linear Mixed Model Analysis of Variance. Number of progeny / number of litters are shown in parentheses. Different alphabet superscripts indicate statistical difference between groups, $p<0.05$. § Indicates outcome affected by transcervical intervention. . * Indicates number of progeny samples included in measure.

	Control (n = 51* or 68/31)	Carrier (n = 32/16)	IFNγ (n = 31/17)
Adiponectin (µl/ml) *	18.1 ± 1.3 [a]	20.8 ± 1.6 [b]	18.7 ± 1.5 [ab]
Leptin (ng/ml) * §	13.8 ± 1.3 [a]	8.6 ± 1.6 [b]	9.5 ± 1.5 [b]
Free fatty acids (meq/L)	0.85 ± 0.07	0.86 ± 0.11	1.06 ± 0.12
Triglycerides (mmol/L)	0.52 ± 0.04	0.47 ± 0.06	0.53 ± 0.07
Cholesterol (mmol/L)	1.60 ± 0.08	1.46 ± 0.13	1.64 ± 0.13
Insulin (ng/ml) *	0.19 ± 0.06	0.12 ± 0.08	0.13 ± 0.07
Glucose (mmol/L) §	8.0 ± 0.38	8.2 ± 0.60	7.9 ± 0.63
Leptin: adiponectin ratio	0.79 ± 0.15	0.45 ± 0.18	0.53 ± 0.18
Insulin: glucose ratio	0.024 ± 0.008	0.020 ± 0.01	0.020 ± 0.01

5.4.5 Effect of peri-conception IFNγ treatment on glucose tolerance in progeny

To further investigate the significance of uterine IFNγ exposure in the peri-conception period on metabolic function in offspring, fasting blood glucose tolerance test was performed at 22 weeks of age on all progeny undergoing full body compositions (n= 177). Time points investigated were 0 (basal), 30 (T30) and 60 (T60) minutes after glucose administration, with statistical analysis performed on data represented as Area Under the Curve (AUC).

Basal blood glucose levels were consistent across the treatment groups and genders and the entire cohort had a mean fasting blood glucose level of 5.24 ± 0.19 mmol.

Transcervical intervention had no overall effect on progeny glucose tolerance measures compared to untreated controls. Neither gender nor weight at autopsy had an overall effect on glucose tolerance, however there were significant interactions between intervention, gender and weight at autopsy. Male progeny from mothers undergoing an intervention exhibited a 2.5% increase in total glucose AUC measures while female progeny had an 11% decrease in total glucose AUC measures compared to untreated controls (p=0.01).

There was no overall effect of treatment via transcervical intervention on progeny glucose tolerance measures compared to untreated controls. Again, neither weight at autopsy nor gender had a significant effect on glucose tolerance measures. However there were significant interactions between treatment, gender and weight at autopsy. Statistical analysis demonstrated that male progeny arising from mothers treated with carrier have significantly higher blood glucose measures at T30 (36%, p=0.03), T60 (44%, p=0.001) and Total AUC (41%, p=0.002) compared to untreated control progeny (Table 5.11). There was no significant effect of peri-conception IFNγ treatment on glucose tolerance of male progeny compared to either untreated or carrier treated controls.

In contrast to male progeny, there was no difference between glucose tolerance in female progeny of untreated and carrier treated controls (Table 5.12). There was no significant effect of peri-conception IFNγ treatment on glucose tolerance in female progeny compared to either untreated or carrier treated controls.

Table 5.11 Effect of peri-conception IFNγ treatment on male progeny glucose tolerance.

Data is given as estimated marginal mean (area under the curve) ± SEM and was analysed using Linear Mixed Model Analysis of Variance.
Number of progeny / number of litters are shown in parentheses. Different alphabet superscripts indicate differences between groups, $p<0.05$.

	Control (n = 42/26)	Carrier (n = 19/16)	IFNγ (n = 24/17)
T30 (AUC)	75 ± 11 [a]	109 ± 15 [b]	96 ± 19 [ab]
T60 (AUC)	117 ± 19 [a]	175 ± 33 [b]	140 ± 33 [ab]
Total (AUC)	191 ± 29 [a]	284 ± 52 [b]	236 ± 51 [ab]

Table 5.12 Effect of peri-conception IFNγ treatment on female progeny glucose tolerance.

Data is given as estimated marginal mean (area under the curve) ± SEM and was analysed using Linear Mixed Model Analysis of Variance.
Number of progeny / number of litters are shown in parentheses. Different alphabet superscripts indicate differences between groups, $p<0.05$. § Indicates outcome affected by transcervical intervention

	Control (n = 47/26)	Carrier (n = 21/16)	IFNγ (n = 24/17)
T30 (AUC) §	71 ± 13	69 ± 18	80 ± 17
T60 (AUC) §	103 ± 21	104 ± 30	116 ± 28
Total (AUC) §	174 ± 34	173 ± 48	196 ± 44

5.4.6 Effect of peri-conception IFNγ treatment on systolic blood pressure in progeny

To investigate the significance of peri-conception uterine IFNγ exposure on adult systolic blood pressure, systolic blood pressure was measured in progeny prior to fasting and full body composition analysis. Blood pressure measures of 161 offspring were obtained (n = male/female; Control n = 35/44, Carrier n = 17/20, IFNγ n = 22/23).

Statistical analysis showed no significant effect of intervention on progeny blood pressure compared to untreated controls. In this experiment uterine IFNγ treatment did not have a significant impact on progeny blood pressure. However blood pressure was increased by 24% in both male and female mice generated from mothers undergoing intra-uterine carrier treatment compared to untreated controls (both p=0.036). There was no effect of gender and no interaction between treatment and gender on blood pressure of progeny (Table 5.13). The mean cohort blood pressure measure was 109 ± 4 mmHg (estimated marginal mean ± SEM).

Table 5.13 Effect of peri-conception IFNγ treatment on progeny systolic blood pressure.

Data is given as estimated marginal mean (mmHg) ± SEM and was analysed using Linear Mixed Model Analysis of Variance. Number of males/ females are shown in parentheses. Different alphabet superscripts indicate differences between groups, p<0.05.

	Control (n = 35/44)	Carrier (n = 17/20)	IFNγ (n = 22/23)
Male	98 ± 7 ^{a}	120 ± 12 ^{b}	109 ± 12 ^{ab}
Female	95 ± 8 ^{a}	119 ± 10 ^{b}	115 ± 10 ^{ab}

5.5 DISCUSSION

The experiments described in this chapter were undertaken to investigate the effects of uterine IFNγ treatment on pregnancy outcomes. The experimental approach was to administer intra-uterine IFNγ within 4-8 hours of natural mating then mice were examined at day 0.5pc or pregnancies were allowed to progress until gd17.5 or term. Together the results from these experiments enabled determination of the effects of IFNγ present at insemination on the post-mating inflammatory response and on pregnancy and progeny outcomes compared to pregnancies without intervention or carrier treated controls.

In vitro experiments described in Chapter 3 demonstrated the stimulatory effect of factors introduced at insemination, including TGFβ, and bacterial LPS and LTA on uterine epithelial cell cytokine production. Also described in Chapter 3 was the inhibitory effect of IFNγ in blocking the effects of these factors on uterine epithelial cells. In this chapter, it has been demonstrated that intra-uterine IFNγ administration reduces post-mating GM-CSF, IL-6 and KC secretion and neutrophil recruitment into the endometrium. However this peri-conception perturbation did not cause any substantial adverse effect on pregnancy parameters or progeny outcomes.

High morbidity and mortality rates in recipients of surgically applied treatments were evident at gd17.5. The detrimental effects of surgical intervention were also reflected in poor pregnancy outcomes of those surviving without obvious morbidity. Unmated estrous female mice treated via surgery and injection, then observed for 17.5 days exhibited no morbidity or mortality seen with identical treatment in mated mice (data not shown). It therefore seems likely that luminal fluid from the mated uterus containing infectious agents can escape via the injection puncture sites and enter the peritoneal cavity, leading to abscess formation and in some cases death. While this detrimental outcome appears contrary to extensive reports of embryo transfer experiments carried out in mice, in fact the time of injection/embryo transfer is vastly different. The treatments administered here were conducted within approximately 4 hours of mating whereas embryo transfers into the uterus are made 3-4 days post-mating. By the time embryo transfer occurs, seminal debris and infectious agents introduced at insemination are cleared from the uterus hence uterine puncture does not result in the morbidity seen here. While there was no outward sign of morbidity at day 0.5pc, most likely due to the short interval since treatment, the longer term experiments suggest this mode of treatment in mated females may be a confounding factor. Due to the detrimental effect of the surgical intervention, only measures resulting from transcervical treatments will be discussed further.

Instillation of IFNγ into a recently mated uterus has been shown here to reduce post-insemination inflammatory cytokine secretion by the uterus. The reduction in uterine GM-CSF secretion as a result of IFNγ exposure in vivo is consistent with in vitro results described in Chapter 3. The consequences of reduced uterine GM-CSF secretion following insemination have been examined in pregnancies and progeny generated in GM-CSF null mice. Fetal viability from mid-gestation through to weaning is compromised in these mice and is thought to be primarily the result of defective blastocyst development [127]. While uterine macrophage, neutrophil and eosinophil numbers and distribution are unchanged in these mice, in the absence of GM-CSF these cells are less activated with reduced MHC expression and altered antigen-presenting function [125]. Interestingly uterine IL-6 and KC secretion were also significantly decreased following uterine IFNγ treatment, an effect which was only observed in vitro when combined with LPS stimulation. That an effect was observed in vivo supports the interpretation of a role for non-seminal vesicle derived factors in stimulating post-mating inflammation. The implications for pregnancy and progeny outcomes following conception in an environment of absent or low IL-6 or KC secretion have not been investigated to date.

The post- mating inflammatory cytokine surge and endometrial leukocyte recruitment has been proposed previously to have an important role not only in clearance of foreign material following insemination, but also in providing an optimal environment for embryo development [12, 127] as well as preparation of the endometrium for implantation [299, 331]. Mating experiments in mice have identified seminal vesicle fluid components as essential in eliciting the molecular and cellular changes observed following insemination [123]. The implications for pregnancy and progeny outcomes generated in the absence of seminal vesicle fluid have also been recently investigated. Studies conducted in our laboratory have demonstrated reduced pregnancy rates and viable litter size resulting from mating with seminal vesicle deficient males [14]. Additionally progeny generated in the absence of seminal vesicle fluid exhibit increased adult body weight, attributable to an increase in central fat depots. Altered metabolic profiles are also evident as a result of conception in this manner. Together with increased adiposity these changes are associated with insulin resistance in humans and animal models [458, 534-538]. In this context it was relevant to investigate the effects of perturbations of seminal plasma signalling in the female reproductive tract elicited by means less overt than ablation of seminal vesicles. Thus we investigated the effect of cytokine perturbation achieved by IFNγ instillation on pregnancy and progeny outcomes.

Measures at gd17.5 and term delivered progeny revealed unaltered pregnancy rates following maternal transcervical administration of IFNγ. There were notable decreases in pregnancy rates

following IFNγ treatment at both time points that were not statistically significant. However, interestingly when these data were combined and analysed together pregnancy rates were reduced by 16% following IFNγ treatment compared to untreated controls (p=0.041) but not when compared to carrier treated controls (p=0.434). Litter size was not altered at either time point as a result of IFNγ treatment or when data at both gd17.5 and term were analysed together. However rates of fetal resorption increased in response to IFNγ administration which was similar to that seen after carrier injection. Crown-rump length, fetal weights and consequently fetal:placental ratio measures were decreased as a result of intervention, and were equally evident after carrier and IFNγ treatment compared to untreated controls. Thus, despite the cytokine perturbations observed in the post-mating inflammatory response following IFNγ injection, there was no overt consequence for pregnancy evident in late gestation.

Despite decreases in fetal weights at gd17.5, there were no changes in birth or neonatal weights of progeny generated following intra-uterine IFNγ exposure compared to control progeny. Post-weaning growth of progeny was similar for treatment and control progeny until mid adulthood (20 weeks). At this time point male progeny from IFNγ treated mice became significantly heavier than untreated control and carrier treated progeny. Although peri-conception IFNγ treatment increased male body weight in adulthood, there was no change in progeny body composition as a result of maternal intra-uterine IFNγ treatment following insemination. Neither male nor female progeny derived from mothers exposed to intra-uterine IFNγ exhibited altered metabolic profiles, glucose tolerance or systolic blood pressure.

The extent to which intra-uterine injections are useful in understanding the role of uterine IFNγ exposure is confounded by the effect of the physical intervention on perturbing maternal post-mating inflammation and subsequent pregnancy and progeny effects. Interestingly, pronounced changes were observed in central fat, metabolic and blood pressure measures in progeny of mothers receiving intra-uterine carrier treatment. Male progeny exhibited significantly altered relative central fat depots, adiponectin levels, impaired glucose tolerance and increased blood pressure. While female progeny showed altered adiponectin and leptin levels (paradoxically, not in parallel) resulting in a perturbed leptin:adiponectin ratio as well as increased systolic blood pressure compared to untreated control progeny. This cluster of changes, seen particularly in the male progeny of mothers receiving intra-uterine carrier injection, is indicative of the metabolic syndrome associated with increased risk of developing type-2 diabetes and cardiovascular disease. These same measures in progeny from IFNγ treated mothers are almost identical to those of untreated control progeny. This may be interpreted to indicate that the physical stress of transcervical passage of a catheter, or perhaps dilution of

endogenous intrauterine cytokine with PBS, can program adverse outcomes in progeny. Furthermore, rather surprisingly, IFNγ may be contributing to a recovery mechanism following this intervention. One possible interpretation of this lies in the effect of intervention on local inflammatory parameters. It might be speculated that a physical insult to the cervix further upregulates inflammatory mediators beyond levels induced by natural mating. Perhaps through its ability to inhibit inflammatory mediators, IFNγ can limit the 'damage' caused by the physical intervention. It would be interesting to undertake more detailed analysis of inflammatory mediators to evaluate this.

In summary, experiments carried out in this chapter demonstrate that IFNγ can indeed alter the post-mating uterine cytokine profile, confirming conclusions drawn from the in vitro experiments. However this perturbation was insufficient to adversely affect pregnancy progression or long term progeny growth, metabolic profile or systolic blood pressure. In contrast, transcervical perturbation and instillation of PBS into the peri-conception uterus is sufficient to elicit changes in progeny reminiscent of those associated with the metabolic syndrome. Gene expression profile analysis and DNA methylation studies may be useful in investigating the molecular mechanisms in the embryo underlying these changes.

To further investigate the role of IFNγ present at insemination on pregnancy and progeny outcomes, development of alternative delivery method would be required. One proposed method is by establishment of a transgenic mouse, manipulated to secrete high levels of IFNγ into fluids released in the ejaculate. This could be achieved by insertion of the mouse IFNγ gene under the control of a prostate specific promoter. Initial steps in generating such a mouse have been successfully completed with a plasmid construct cloned and shown to stimulate secretion of immunodetectable protein by prostatic cancer cell lines in vitro (Appendix 1). Alternatively, down-regulating uterine post-mating cytokine production, similar to that observed following IFNγ treatment could be achieved using small interfering (si) RNA technology. This approach, applied systemically, would overcome potential confounding factors such as physical trauma to the cervix during catheterization, dilution of endogenous uterine cytokines following intrauterine treatment instillation and loss of uterine fluid following plug removal and catheterization.

Chapter 6

General Discussion and conclusions

6.1 GENERAL DISCUSSION AND CONCLUSIONS

This thesis examines the identity and role of the agents present in seminal fluid which act to elicit a molecular and cellular response in the female reproductive tract following insemination in mice. The characteristic changes induced by seminal fluid are reminiscent of a classical inflammatory response and contribute to optimal fertilization, embryo development, implantation and growth. Previous studies in mice have identified TGFβ as one of the active seminal agents acting to induce some aspects of the post-mating inflammatory response. However it is evident from these studies that there are additional factors that modulate the actions of TGFβ and contribute to the pro-inflammatory state of the female tract after insemination. These include agents of bacterial origin, LPS and LTA, and also include IFNγ potentially originating in tissues of the male reproductive tract. It was found that LPS and LTA enhance while IFNγ dampens the female reproductive tract inflammatory response. Furthermore, the extent to which negative regulators in semen which alter the peri-conception uterine environment can consequently influence pregnancy and progeny outcomes was examined and shown not to cause detectable adverse effects.

In Chapter 3, an in vitro cell culture system was used to investigate the contribution of various active molecules present at insemination in triggering the post-mating inflammatory response in mice. Previous studies in mice conducted in our laboratory have identified seminal TGFβ as a key active molecule within seminal vesicle fluid responsible for increased uterine GM-CSF secretion and macrophage recruitment [12]. A number of other pro-inflammatory cytokines and chemokines are secreted by the female reproductive tract in response to semen exposure in mice and humans [130, 152, 154, 155]. Together this led us to hypothesise that seminal TGFβ or other seminal factors may induce uterine secretion of a variety of cytokines in addition to GM-CSF. Initially assays of post-mating uterine lavages demonstrated not only the potency of whole semen in stimulating uterine cytokine secretion but also that the sperm and seminal fluid compartments both contribute to this response differentially. Furthermore, for the first time insemination was shown to increase uterine KC protein production, correlating with previously described mRNA increases seen following insemination in mice and humans [130, 189]. Interestingly, while GM-CSF secretion was increased in vitro, the increase in IL-6 and KC secretion could not be replicated in vitro. Possible explanations for this are that the active agents are removed or destroyed during seminal fluid collection or alternatively that IL-6 and KC production is stimulated by agents not derived from seminal plasma. The differential effects observed indicate that TGFβ, a seminal factor previously identified as the agent responsible for uterine GM-CSF production, is not the agent principally responsible for stimulation of IL-6 or KC by uterine epithelial

cells. This was confirmed when TGFβ evoked a similar differential cytokine profile in vitro to that elicited by seminal vesicle fluid, stimulating increases in GM-CSF but not IL-6 or KC. This suggests that multiple factors contribute to the cytokine profile observed following insemination in vivo. The finding that products from commensal microflora of the lower reproductive tract were able to stimulate cytokine production in a distinct pattern to that observed for TGFβ, and can interact with TGFβ to modulate the cytokine response, indicates a likely physiological role for these agents. Another factor detected in mouse seminal vesicle fluid, IFNγ, interacted with TGFβ and bacterial factors, in both cases reducing their effect on uterine epithelial cells. The cross-titration design of the experiments described herein demonstrates the activity and interaction of these agents is dependent on the relative concentrations of each molecule present. Not all female tract cytokine responses examined were explained by the active molecules investigated. Therefore it is necessary to investigate the identity of additional active seminal agents of both endogenous and exogenous origin likely to contribute to generating an environment conducive for optimal pregnancy success. It would be interesting to employ microarray analysis to obtain a global picture of the transcriptome induced in response to seminal fluid and to examine the effect of altering the relative concentration of constituents. To complement this strategy, the use of Luminex beads would allow measurement of numerous factors in one assay from a small sample volume, in uterine luminal fluid for example.

The differential regulation of uterine secretion of GM-CSF, IL-6 and KC (IL-8) in mice by seminal and reproductive tract factors mirrors that described in the human cervix. Human studies examining cytokine expression in the cervix following semen exposure during unprotected intercourse demonstrated upregulation of GM-CSF, IL-6 and KC (IL-8) similar to our results in mice [130]. Additionally, in vitro experiments using primary and immortalised human female reproductive tract cells identified the three mammalian isoforms of TGFβ as the predominant active agents in human seminal plasma, markedly inducing GM-CSF and IL-6 secretion [130]. IL-6 and IL-8 secretion in human in vitro studies has been demonstrated to increase in response to the most abundant prostaglandin in human seminal plasma, 19-OH PGE_1 [130]. Corresponding with our results, IFNγ has been shown to interact with TGFβ and potently reduce its activity in cervical epithelial cells [130].

Experiments in Chapter 4 utilised intra-uterine injections to investigate the contribution of active molecules present at insemination in triggering cytokine production and leukocyte recruitment reminiscent of the post-mating inflammatory response in mice. The current study failed to establish TGFβ stimulated GM-CSF secretion by uterine epithelial cells in vivo similar to that demonstrated in vitro. This finding differs from that of a previous study investigating the activity of seminal TGFβ in the

post-mating inflammatory response, where uterine TGFβ instillation led to GM-CSF secretion similar to that observed following mating [12]. It is likely that the difference in the strain of mice used could account for this, since inflammatory response to a given stimulus is strain dependant. Additionally, the protein carrier used in our in vivo studies was specifically a low endotoxin BSA, enabling a more precise measure of the contribution of TGFβ in isolation, since in vitro experiments in Chapter 3 demonstrate interactions between TGFβ and LPS. It is possible that low levels of contaminating LPS present either in the exogenous cytokine or endogenously in the female tract worked in combination with TGFβ to induce the in vivo response previously reported [12].

Our studies went further to investigate the potential role of reproductive tract agents, bacterial LPS and LTA which would be present in both the male and female reproductive tracts. These molecules were found to be potent stimulators of uterine cytokine secretion and leukocyte recruitment into the endometrium. Furthermore these active molecules evoked striking neutrophil transepithelial migration into the uterine lumen similar to that seen following insemination. Mutant mice lacking a functional LPS receptor (TLR4) were found to exhibit similar cytokine and leukocyte responses following insemination as that seen in wild type mice. This indicates that while LPS has the capacity to elicit a cytokine and leukocyte profile in the uterus similar to that observed after semen exposure, it is not obligatory to achieve the female response. Instead, it is likely to act physiologically to amplify the effects of endogenously synthesized male signalling agents.

In Chapter 5, transcervical treatment administration was employed to examine the impact of IFNγ exposure on pregnancy outcome and the postnatal growth and adult metabolic profile of progeny. Our results indicate that uterine exposure to IFNγ alters the uterine post-mating cytokine profile and neutrophil recruitment during the peri-conception period. However, the perturbed peri-conception uterine environment elicited by IFNγ did not alter pregnancy parameters or progeny outcomes, with the exception of reduced ovary size. Reproductive consequences of reduced ovary size in these female offspring were not examined however it would be informative to conduct such a study, examining estrous cyclicity and reproductive performance as well as ovarian morphology and follicle development. However, it must be noted that the capacity of this experiment to detect effects of IFNγ were limited due to the unexpected effects of the intervention (transcervical catheterisation) protocol. Interestingly, carrier treatment altered a number of measures including uterine secretion, resorption rates, some metabolic markers and systolic blood pressure which appeared to be 'normalised' following the addition of IFNγ. The early pregnancy parameter of uterine cytokine secretion appears to be an indicator of longer term effects on pregnancy and progeny observed in the carrier group. Our in vitro studies in

chapter 3 demonstrated the ability of IFNγ to dampen cytokine secretion. In the context of studies conducted in chapter 5, over-stimulation of uterine cytokines following carrier delivery by catheterisation is also dampened after treatment with IFNγ. In our studies IFNγ treatment inhibited cytokine synthesis to levels similar to those observed in the untreated group. With this in mind, it is not surprising then, that progeny from the carrier group with elevated uterine cytokine secretion in early pregnancy exhibited altered long term outcomes, whereas progeny from the IFNγ treated group with 'normalised' uterine cytokine secretion exhibited generally 'normalised' long term outcomes. This unexpected result may be of relevance to women undergoing medical procedures to the cervix during early pregnancy. IFNγ could potentially be used to 'normalise' the uterine environment and perhaps fetal outcomes if applied at the time of the procedure.

Surprisingly, results demonstrate marked effects on most pregnancy and progeny measures as a consequence of the transcervical administration intervention, which potentially masked any effect of IFNγ. Reduced fetal weights and increased placental size resulted in reduced fetal:placental weight ratios in carrier-treated progeny. While the structure and morphology of placentae were not examined in this study, altered fetal:placental ratios are indicative of altered placental function. Our results also indicate progeny viability is compromised since survival to weaning is reduced as a consequence of intervention. These progeny also exhibited an altered metabolic profile, glucose tolerance and systolic blood pressure associated with increased risk of developing coronary artery disease and type 2 diabetes in rodents and humans [456, 515, 517].

It is not clear how the intervention affected the events of early pregnancy to program these downstream consequences. In chapter 4 (Figure 4.2) we described the effect of transcervical catheterisation on the uterine cytokine milieu in the estrous mouse uterus. Transcervical catheterisation is a physical insult to the cervix which may mimic the mechanical trauma experienced during mating and indeed mating with an intact male or catheterisation elicits a similar level of uterine GM-CSF production (Figure 3.1 and Figure 4.2). However under similar mating conditions but instead with a seminal vesicle deplete mouse, there is no such stimulation of GM-CSF. This would indicate that factors other than mechanical stimulation at mating or catheterisation are necessary. A possible explanation may be that catheterisation provides a more severe stress or intense physical trauma to the tissue than mating. Alternatively, contaminating stimulatory factors could be introduced into the uterine lumen during catheterisation, in the same manner as at insemination. Potential candidates would include bacterial agents LPS and LTA. Given the findings on pregnancy outcome, it would now be sensible to examine the effect of removing the copulatory plug in conjunction with cervical

catheterization in early pregnancy. Indeed, a recent study examining markers of inflammation in the human cervix following Papanicolaou smears (Pap smear) demonstrated significant elevation of markers of cell mediated immunity (IL-12 p70 and TNFα) and T cell regulation (IL-10) [539], consistent with the possibility that physical trauma to the tissue can induce inflammatory cytokines.

A hallmark of the acute stress response to physical trauma is a functional perturbation in circulating hormone levels. Increases in cortisol, growth hormone and prolactin have been reported in conjunction with decreases in gonadotropin, gonadal steroid and thyroid hormone all of which are known to interact with the immune system promoting either Th1 or Th2 responses [540]. Interestingly, the effects of these hormones can be maximal up to 4 days post trauma, which in the context of our studies represents the entire pre-implantation period. Altering the uterine or the embryonic environment during early pregnancy has been demonstrated to have adverse consequences on the long term health of subsequent offspring [456, 458, 528-533]. While many studies have described changes in health and metabolic parameters of offspring, little is known about the cascade of events leading to these outcomes. Modifications to embryonic epigenetic programming leading to perturb placental development are commonly proposed as a potential mechanism. Our findings, together with ongoing research into the molecular mechanisms underpinning fetal origins of adult disease, may be relevant to human offspring particularly those arising from assisted reproductive technology (ART). ART procedures involve firstly a physical insult to the female tract at the time of oocyte pickup and at the time of embryo transfer, and secondly, involves hormonal manipulation through the process of ovarian hyperstimulation by exogenous gonadotrophins. Both of these interventions have been shown to induce an inflammatory state, indicated by high levels of circulating C-reactive protein, which is currently being investigated for its validity as a prognostic indicator of IVF success [541-544].

Interaction between different seminal signalling factors is thought to shape the precise nature of the molecular and cellular changes observed within the female reproductive tract therein following insemination (Figure 6.1). Since the cytokines synthesized in the female response are proposed to enhance fertilization, embryo development, implantation and growth and to promote immune tolerance of semen and the embryo, the extent to which this is affected would be influenced by the profile of male seminal plasma constituents. While the advent of assisted reproduction demonstrates pregnancy is achievable in the absence of seminal plasma, epidemiological studies in humans suggest the ensuing pregnancy and subsequent offspring may be compromised [2-4]. Conversely, exposure to seminal plasma has been shown to be protective from recurrent miscarriage and further, partner-specific cumulative exposure is associated with a reduced risk of pre-eclampsia [545-548]. Reduced pregnancy rates, implantation rates and fetal viability have been described in embryo transfer experiments in

mice and rats, where female recipients have not been exposed to seminal plasma [5, 7, 9]. Furthermore progeny derived from pregnancies where the female tract has not been exposed to seminal vesicle fluid have been shown to exhibit long term post-natal effects. Recent studies conducted in our laboratory in mice, have demonstrated altered post-natal growth and metabolic profile progeny conceived in such a manner [14].

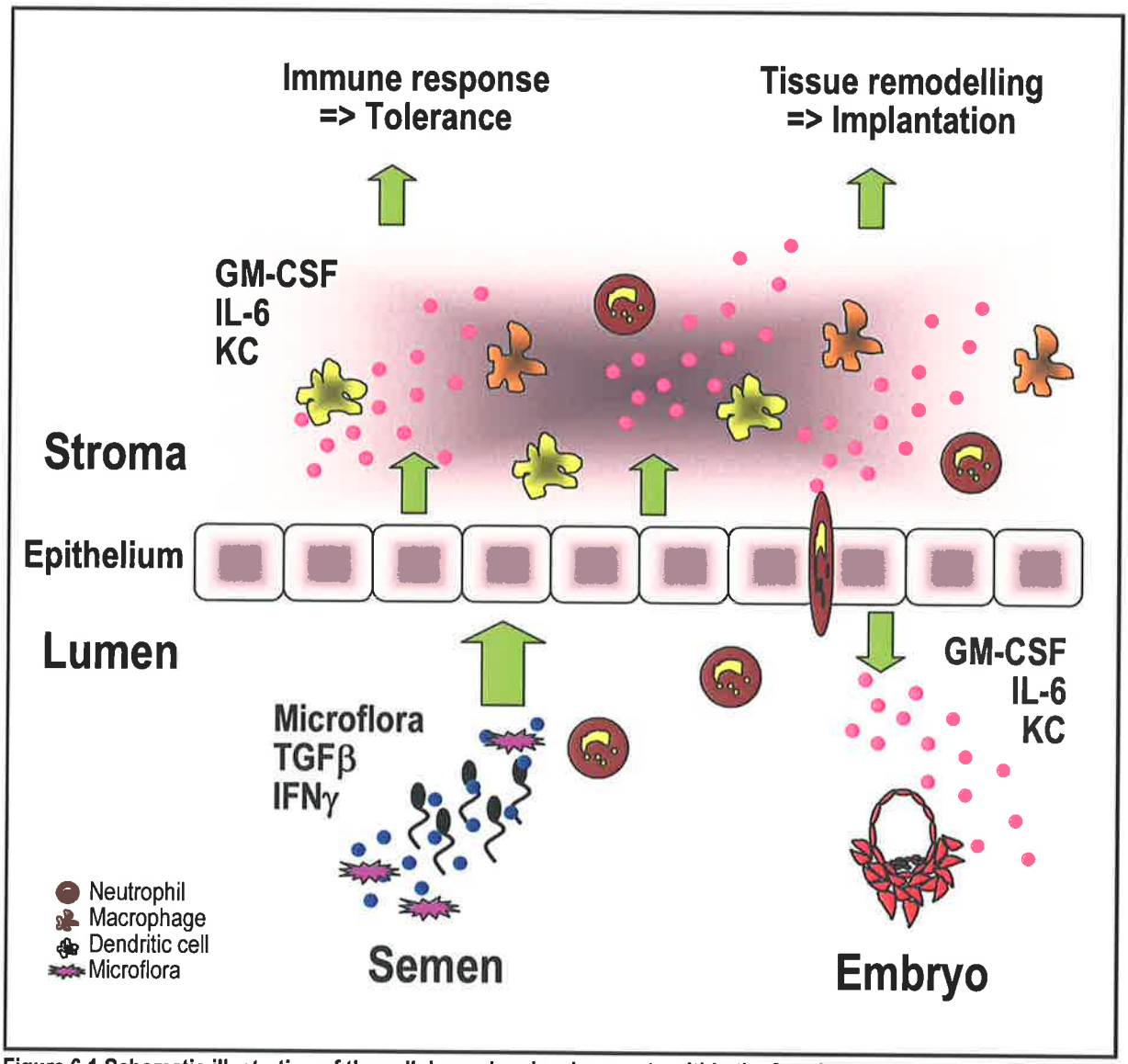


Figure 6.1 Schematic illustration of the cellular and molecular events within the female reproductive tract following mating. Factors introduced at insemination including seminal TGFβ and IFNγ together with microflora, target uterine epithelial cells and activate secretion of the pro-inflammatory cytokines. These cytokines not only lead to the recruitment of inflammatory leukocytes important for establishment of pregnancy and generating immune tolerance towards the conceptus and also act as growth factors for the developing conceptus.

Semen exposure elicits secretion of a number of cytokines into the uterine lumen as well as expression in the oviduct of mice, many of which are attributed with embryotrophic effects, contributing to viability, proliferation and differentiation in the embryo [82]. Uterine GM-CSF targets the pre-implantation embryo promoting blastocyst formation, glucose uptake and blastomere viability in the mouse [127]. Other cytokines, including IL-6 and LIF are also increased following insemination and implicated in optimal pregnancy outcomes [156, 180-182, 488]. Commensal microflora, in conjunction with seminal factors, may contribute to producing a post-mating uterine cytokine milieu optimal for embryo development. Our studies have shown that changes in the relative concentrations of factors in seminal fluid, including those synthesized endogenously in the male and those derived from bacterial microflora can alter cytokine and growth factor production by uterine epithelial cells. Specifically, increased levels of IFNγ dampens both TGFβ stimulated GM-CSF secretion and LPS stimulated GM-CSF and KC secretion. Perturbations in the post-insemination cytokine and growth factor profile encountered by the pre-implantation embryo are associated with impaired fetal and placental development, progeny growth and adult health [128]. Previous studies have demonstrated that altered seminal constituents or ex vivo embryo culture can induce altered fetal and progeny outcomes. Our study has added to the knowledge base on the programming effects of early environment by showing that female reproductive tract trauma (transcervical catheterisation) experienced in the peri-conception period changes the cytokine environment encountered by the developing embryo, and further that this perturbation alters the long term health of progeny.

Stimulation of pro-inflammatory cytokines by the endometrium following insemination in mice induces an influx of leukocytes into the stroma, luminal epithelium and luminal cavity. These inflammatory cells contribute to the phagocytic clearance of potential pathogens introduced during insemination together with other cellular debris including non-fertilising sperm. Additionally, these cells are thought to contribute to endometrial remodelling in preparation for implantation via secretion of cytokines and extra-cellular matrix degrading enzymes [549]. In particular, macrophages and neutrophils have been implicated in tissue remodelling, angiogenesis and altering luminal epithelial cell expression of adhesion and anti-adhesion molecules responsible for coordinating apposition, attachment and implantation of the embryo [262, 317, 550] (M Jasper and S Robertson unpublished data). The studies described in this thesis have shown LTA to be a potent inducer of transepithelial neutrophil migration in the uterus, indicating a potential role for commensal microflora of the reproductive tract in enhancing this facet of the female inflammatory response to mating.

Studies in mice have shown that leukocytes recruited into the endometrium can also activate the

maternal immune response. Dedicated antigen presenting cells, macrophages and dendritic cells, phagocytose paternal antigens introduced into the female tract at mating then migrate to the draining lymph nodes resulting in activation of maternal immune responses to these antigens. Evidence of enlarged uterine draining lymph nodes and lymphocyte activation typify this immune response [551-553]. Mating experiments have demonstrated the absolute requirement for seminal vesicle fluid as opposed to sperm in activating the maternal immune response [553]. While the maternal immune system recognises paternal antigens in semen, rather than aggressive immune response to these antigens a state of functional immune tolerance is established. This is important as a hostile response would consequently be detrimental to pregnancy since the resulting conceptus shares these same paternal antigens [299]. The abnormal immune response associated with recurrent miscarriage and pre-eclampsia may be the result of an inappropriate immune response to paternal antigens at insemination.

The three facets of the post-insemination uterine environment are described above and include 1) early embryonic cytokine and metabolic milieu, 2) endometrial tissue remodelling and angiogenesis and 3) the maternal immune response to the semi-allogenic conceptus. Each of these contribute to optimal implantation, placentation and progeny health. Adequate placental development early in pregnancy is essential for optimal function when faced with the demands of the fetus. Previous studies in humans and in animal models have demonstrated the short and long term detrimental effects of restricted placental function on maternal, pregnancy and fetal outcomes.

The studies described in this thesis have expanded our current knowledge of the cellular and molecular events that occur within the female reproductive tract following insemination. Firstly, our in vitro experiments have demonstrated that the relative ratios of signalling agents present in seminal fluid have a profound impact on the pro-inflammatory cytokine profile of uterine epithelial cells. Secondly, our in vivo experiments describe perturbed uterine cytokine profile in the presence of altered relative ratios of signalling agents and after physical trauma/manipulation to the abdominal/reproductive tissue. And thirdly, our in vivo studies have shown a negative impact on the long term health of progeny conceived following physical insult to the female reproductive tract. Together the results described here may be physiologically relevant to human health, for example couples experiencing forms of infertility with an immunological aetiology as well as those undergoing assisted reproductive treatment. To evaluate the extent to which our findings can be extrapolated to women, it would be useful to undertake experiments to investigate the effects of the physical interventions of laparoscopy on uterine cytokine synthesis by obtaining endometrial biopsies and uterine lavages.

To further examine the contribution of microfloral signalling in the female tract on post-insemination uterine cytokine secretion, leukocyte influx and activation and embryo development, mice with null mutations in signalling receptors for these factors could be employed. Oviductal tissue and fluid could be collected and assayed for growth factor expression and secretion and oocytes fertilised in these tracts could be cultured and assessed for developmental competence. These studies would give an indication as to the relative contribution of endogenous populations of microflora or contaminating pathogens to the cytokine profile of the post-insemination female tract and implications for embryo health. In conjunction with null mutant mice studies, a panel of bacterial products from species commonly found to populate both the male and female reproductive tracts could be compiled and examined for their ability to differentially stimulate female tract cytokine secretion and leukocyte recruitment. In particular, it would be interesting to evaluate bacteria known to have probiotic effects in other mucosal tissues, such as lactobacilli, versus pathogenic bacteria including those that cause STDs such as chlamydia, gonorrhoea etc.

Many studies have described the beneficial effects of probiotic treatment on female reproductive tract health [374, 513, 514, 554-557]. Perhaps there is a potential application for oral or local probiotic administration in regulating the murine uterine cytokine profile. Studies to investigate the effects of probiotic bacteria and cytokine parameters as well as how this may affect embryo viability and development and pregnancy outcomes are warranted.

It is apparent that a more detailed understanding of role of regulatory factors present at insemination is required. Identification of and investigation into the role of the complex array of agents contributing to the inflammatory response in the female reproductive tract and how this may impact on pregnancy and fetal outcomes is necessary. For example the peptide hormone relaxin, present in human semen [558], is known to possess chemotactic-like properties [559]. Furthermore, recent studies have shown relaxin stimulates MMP production by macrophages, endometrial and vascular endothelial cells [559-562]. Together this may indicate a contributory role for seminal relaxin in the cellular and molecular changes observed following insemination. Other seminal candidates for investigation of potential role in signalling between male and female reproductive tracts include cytokines such as RANTES and eotaxin. Both RANTES and eotaxin have been measured at high concentrations in mouse seminal plasma and are known to recruit eosinophils and neutrophils [364]. In addition to a potential role in endometrial leukocyte recruitment, seminal RANTES may have both an immunomodulatory role in antigenic selection of sperm prior to ejaculation as well as in the female tract [563].

While negative regulators of the post-mating inflammatory response present in mouse and human semen were investigated in the studies described here, the confounding effect of intervention prevented definitive evaluation of the physiological consequences of perturbing levels of signalling factors, TGFβ and IFNγ. To further investigate the role of such factors in altering the uterine cytokine environment, alternative methods of female tract exposure need to be investigated. Generation of transgenic mice may be a useful tool. Expression of factors under the control of a male tract specific promoter and secretion into seminal fluid would permit delivery into the female tract at the time of insemination without external intervention. Assessment of uterine cytokine/growth factor production and leukocyte infiltration, as well as pregnancy and resorption rates could be carried out.

Although results from animal studies cannot be directly to extrapolated to humans, these studies provide insights to guide formulation of research questions regarding the mechanisms underlying forms of human infertility with an immunological basis, such as recurrent miscarriage and pre-eclampsia. Furthermore, these results highlight the possible long term health risks for offspring conceived via ART, involving physical insult to the female reproductive tract during the peri-conception and pre-implantation period. Lastly, these studies may present possible targets worthy of further investigation for therapeutic interventions, in particular where the composition of the genital tract microflora is unbalanced or where relative concentration of seminal signalling agents is perturbed.

Appendix A

Generation of a prostate specific IFNγ expression plasmid.

A.1 PROSTATE SPECIFIC IFN-GAMMA EXPRESSION CONSTRUCT

The aim of this study was to create a plasmid construct that expresses mouse IFNγ under the control of a prostate specific promoter, with the intention of ultimately using the construct to generate a transgenic mouse. Upon maturity, the male transgenic mice would be expected to over express IFNγ in a prostate specific manner and secreted IFNγ protein would be contained within the ejaculate. Deposition of ejaculate containing high levels of IFNγ into the female tract at the time of mating would allow experiments to analyse the effect of IFNγ in the ejaculate on subsequent pregnancy outcome. This approach would avoid the confounding effects of interventions associated with surgical or transcervical delivery of exogenous IFNγ at a critically vulnerable time in early pregnancy.

A.1.1 Cells, Media and Reagents

Lauria Broth (LB) - 10 g Tryptone (Becton Dickinson, Sparks, USA), 5 g Yeast Extract (Becton Dickinson, Sparks, USA), 5 g NaCl, 1 mL 1 M NaOH was combined with MQ H_20 to a final volume of 1 L then autoclaved and used under aseptic conditions. Ampicillin was added to LB immediately prior to use, to a final concentration of 0.1 mg/mL.

LB-Agar - was prepared using LB described above with the addition of 1.5% Agar (Becton Dickinson, Sparks, USA) following autoclaving. LB-AGAR plates were prepared by melting pre-prepared LB-AGAR, adding ampicillin to a final concentration of 0.1 mg/mL then decanting into 85 mm petri dishes.

SOB Medium - 20 g Tryptone, 5 g Yeast Extract, 0.5 g NaCl was combined with MQ H_20 to a final volume of 1 L then autoclaved, stored and used under asceptic conditions. 10 mL 1 M $MgCl_2$ and 10 mL 1 M $MgSO_4$ were added prior to use.

SOC Medium - was prepared using SOB Medium described above with the addition of glucose to a final concentration of 0.02 M immediately prior to use.

Prostatic cancer cell lines PC-3, LN-Cap and DU-145 and pancreatic cancer cell line Panc-1 (kindly provided by Prof. Wayne Tilley, IMVS and University of Adelaide, Australia) were grown to confluence in RPMI-FCS (see Chapter 2). During transfections phenol-red free (PRF) – RPMI together with 5% charcoal stripped fetal calf serum (PRF-RPMI-CSFCS) was used to support these cell lines.

XL1-Blue Supercompetent Cells were purchased from Stratagene (USA) for transformations and used according to the manufacturer's instructions (see below).

A.1.2 Plasmid Transformation

An SK-PB/sv40t vector containing a composite prostate specific promoter and insulator element, ARR_2PBi [564], was kindly donated by Ass Prof Fen Wang (Institute of Biosciences and Technology, Texas, USA) subject to Material Transfer Agreement with Prof RJ Matusik. Murine IFNγ cDNA was provided in the PFB-X plasmid [565], courtesy of Prof Ian Ramshaw (Australian National University, Canberra, Australia). An androgen receptor expression construct, pCMV-AR3.1, kindly provided by Prof. Wayne Tilley (IMVS and University of Adelaide, Australia), was co-transfected into cell lines to gain androgen responsiveness.

Transformations were carried out using XL1-Blue cells in SOC Medium according to the manufacturer's instructions. Briefly, 2 ng of plasmid was added to XL1-Blue cells and incubated on ice for 30 min. The transformation mixture was then heat pulsed for 45 seconds followed by a further 2 min incubation on ice. Pre-warmed SOC media was added and the mixture incubated at 37°C for 1 hr with shaking. Up to 200µL of transformation mixture was spread on LB-Agar plates (see above) and incubated at 37°C overnight. The presence of colonies on selective plates indicated successful transformation. Single colonies were picked and grown in LB overnight at 37°C with shaking.

A.1.3 Plasmid Purification and Identification

Plasmids were purified from overnight cultures using either a DNA Miniprep or Midiprep System (Promega, Madison, USA) according to the manufacturer's instructions. Briefly, overnight cultures were pelleted, lysed then protease treated. The lysate was cleared via centrifugation then decanted to a DNA binding column for further centrifugation. The binding column was washed then DNA eluted in nuclease-free water. Plasmid concentration was calculated from the optical densities (OD) obtained using a Beckman Du530 Spectrophotometer (Beckman Coulter, USA) such that:

DNA (ng/µL) = OD @ 260nm x 50 x diln factor

Primers were designed (see Table A.1), using Primer Express (Applied Biosystems), at each end of known sequences to extend past the promoter or cDNA region into the vector to confirm DNA identity and to obtain vector sequence, hence allowing identification of restriction enzyme sites.

Primers and plasmids were diluted to 5 μM and 100 ng/μL respectively and sent to Southpath and Flinders Sequencing Facility (Flinders Medical Centre, Bedford Pk, Australia) for sequencing. Sequence identity was confirmed online via NCBI, then aligned and entered into NEBcutter online tool for restriction enzyme mapping (BioLab, New England).

Table A.1 Primer Sequences for Cloning.

Name	Primer Sequence	Template Sequence
5'IFNgplasmid/gene	TCCTCCTGCGGCCTAGCT	Mouse IFN-gamma cDNA GI: 26354598
3'IFNgplasmid/plas	CGTGGCAGTAACAGCCAGAA	Mouse IFN-gamma cDNA GI: 26354598
5'IFNgplasmid/plas	AGCTGTTGCCGGAATCCA	Mouse IFN-gamma cDNA GI: 26354598
arr2pb-5'- #1	TGTCAATGTCTGTGTACAACTGCC	ARR_2PB sequence (provided by Prof RJ Matusik)
arr2pb-3'	TGCAGAGTAGCTTCCTGCTTTTTATA	ARR_2PB sequence (provided by Prof RJ Matusik)
arr2pb-5'- #2	TAGCATCTTGTTCTTAGTCTT	ARR_2PB sequence (provided by Prof RJ Matusik)
insulator-5' - #1	CACGGGATCGCTTTCCTCTG	Chicken HS4 insulator GI: 54303678
insulator-3' - #1	TCTGCAGGCTCAAAGAGCA	Chicken HS4 insulator GI: 54303678
insulator-5' - #2	CAAAGCCCCCAGGGA	Chicken HS4 insulator GI: 54303678
insulator-3' - #2	GGGAGGGACGTAATTAC	Chicken HS4 insulator GI: 54303678

A.1.4 Restriction enzyme digests and ligation

Enzymes and compatible buffers used in restriction enzyme digests were provided by Dr Darryl Russell (University of Adelaide, Australia), including EcoRI (G/AATTC), XbaI (T/CTAGA), Buffer H and All-Phor-One Buffer (Roche Diagnostics, Mannheim, Germany), EcoRV (GAT/ATC), BamHI (G/GATCC), Buffer E and Multicore Buffer (Promega, Madison, USA), Klenow (*E.coli* DNA Pol I, Pharmacia), calf intestinal phosphatase (CIP) (New England Biolabs, Massachusetts, USA) and MinElute Gel Extraction Kit (Qiagen, Valencia, USA). Restriction enzyme digests were conducted at 37°C for 1 hour following the addition of 1 μg plasmid to 1 μL restriction enzyme/s, 5 μL 10x buffer and water up to a final volume of 50 μL.

Following mIFNγ cDNA (insert) excision from PFB-X with EcoRI, Klenow and dNTPs were added and incubated for 20 min at room temperature, to create blunt ends on the insert preceding purification and further digestion with BamHI. ARR_2PBi (vector) underwent a simultaneous double digest with EcoRV and BamHI followed by CIP treatment at 37°C for 30 min. Double digestion in this manner permitted directional insertion of the cDNA into the vector. After separation on a 1% agarose gel, insert (~707 bp) and vector (~4.4 kb) (Figure A.1) DNA was extracted using a Gel Extraction Kit, according to the manufacturer's instructions. Briefly, the DNA band was visualised using UV light, excised from the gel then dissolved in the solubilisation and binding buffer. DNA was then bound to a binding column during centrifugation, washed then eluted in 10 μL of Tris-HCl.

Ligation of insert into the vector (construct - ARR_2PBi-mIFNγ) using T4 DNA Ligase (New England Biolabs, Massachusetts, USA) was conducted at room temperature for 1 hr prior to overnight incubation at 16 °C. Following transformation into XL1-Blue cells and overnight expansion (see A.1.2 above), the construct was purified from culture using a Midiprep kit (see A.1.3 above). In order to confirm ligation of insert into the vector, an aliquot was subjected to double digest using BamHI and XbaI followed by electrophoresis on a 1% agarose gel. Expected band sizes of ~2.3 Kb, 1.6 Kb and 1.2 Kb showing a successful ligation were obtained. In contrast, a vector alone control yielded bands of 2.3 Kb, 1.2 Kb and 0.89 Kb (Figure A.2).

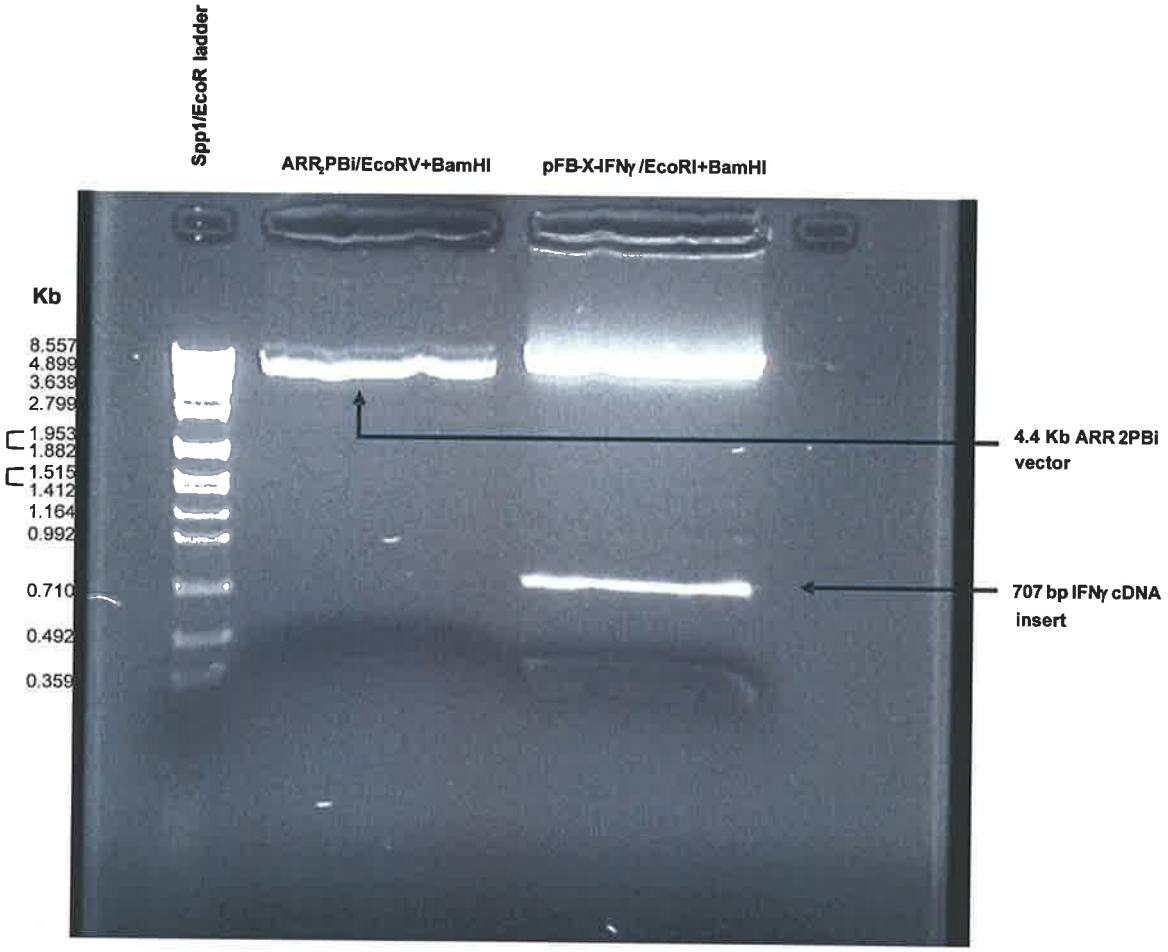


Figure A.1 Gel purification of vector and insert restriction digests. EcoRV/BamHI digested vector (ARR2PBi - 4.4 Kb) and EcoRI/BamHI digested insert (mIFNγ cDNA - 707bp) were electrophoresed on a 1% agarose gel for purification prior to ligation.

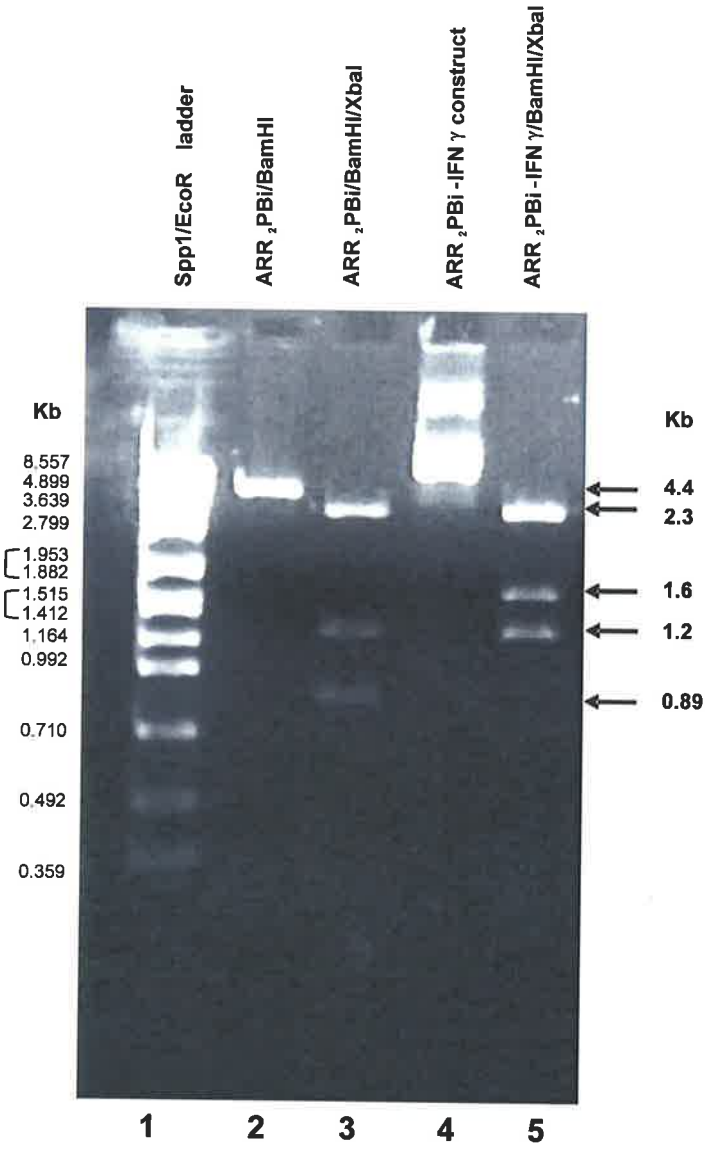


Figure A.2 Ligation of mIFNγ cDNA insert into ARR2PBi vector. BamHI/XbaI double restriction digest of the ligation product and electrophoresed on a 1% agarose gel to confirm successful insertion of mIFNγ cDNA into ARR2PBi vector. Vector only digest yielded products 2.3 Kb, 1.2 Kb and 0.89 Kb (track 3), while ligation reaction digests yielding products 2.3 Kb, 1.6 Kb and 1.2 Kb (track 5) confirm successful ligation.

A.1.5 Expression of ARR_2PBi-mIFNγ construct

The final construct was transfected into prostatic cancer cell lines PC-3, LN-Cap and DU-145 and pancreatic cancer cell line Panc-1 using Fugene6 (Roche Diagnositics, Mannheim, Germany) according to the manufacturer's instructions. Briefly, confluent cell lines were trypsinized and aliquotted into Nunc 24 well mulitdishes (Nunc, Roskilde, Denmark) at 1.78 x 10^5 cells/well on the afternoon prior to transfection and were incubated overnight. The transfection complex was prepared by incubating 0.6 μL/well Fugene6 with serum free PRF-RPMI for 5 min, then adding 0.2 μg/well of construct and plasmid containing androgen receptor cDNA and incubating for a further 30 min. PRF-RPMI-CSFCS replaced RPMI-FCS media on cell lines prior to incubation with 20 μL/well of transfection complex for 6 hrs. Following an 18 hr incubation in the presence of 10^{-8} M dihydrotestosterone (DHT), supernatants were collected and assayed in a commercial mouse IFNγ ELISA (R&D Systems, Minneapolis) according to the manufacturer's instructions (see Chapter 2) (Figure A.3). High levels of IFNγ (750 pg/mL) was detected in the SN of transfected PC-3 cells and other transfected cell lines secreted 100-200 pg/mL. This compared with SN from untransfected or unstimulated cell lines which did not contain any detectable IFNγ (< 28 pg/mL). An indication of transfection efficiency was provided by transfection with a green fluorescent protein expression construct, pcDNA-GFP. Evaluation of cells by fluorescence microscopy after counterstaining with 4'-6-Diamidino-2-phenylindole (DAPI), kindly provided by Dr Darryl Russell (University of Adelaide, Australia), showed an efficiency of 10-20%.

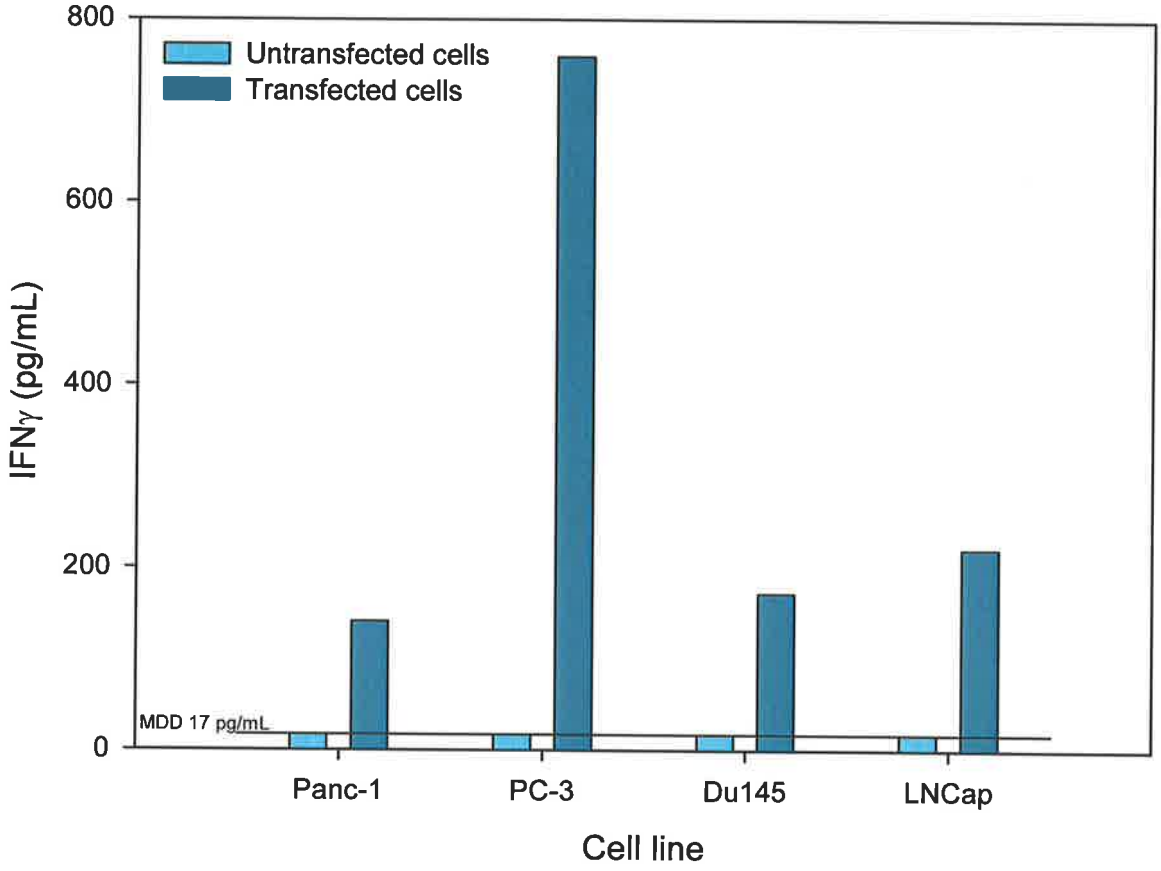


Figure A.3 Production of mouse IFNγ following stimulation of ARR2PBi-mIFNγ by DHT in cell lines. Prostatic cancer cell lines PC-3, LN-Cap and DU-145 and pancreatic cancer cell line Panc-1 (control) were untransfected or co-transfected with AR and ARR2PBi-mIFNγ. Six hours following transfection the cells were incubated with 100mM DHT for 18 hrs, supernatants were then collected and assayed for IFNγ in a commercial ELISA. MDD – minimum detectable dose for this assay.

A.2 DISCUSSION

The results described above demonstrate successful introduction of the mouse IFNγ cDNA downstream of the ARR_2PBi prostate specific promoter, transient transfection into prostatic cancer cell lines and induction of IFNγ secretion following exposure to DHT. This provides the first step towards generating a mouse transgenic for IFNγ expression in the male reproductive tract. However several steps would need to be completed to achieve this aim. Firstly, while we were able to detect mIFNγ protein by immunoassay, it would be important to next assess the functionality of the secreted product. To achieve this, a primary mouse uterine epithelial cell bioassay would be designed to examine the effect of addition of supernatants from DHT stimulated transfected cells on uterine epithelial cell cytokine production. This would be compared to recombinant mIFNγ activity in the same assay. The final stage in this study would involve production of transgenic mice, which could be approached in one of two ways. The first approach would require performing a restriction digest to excise the ARR_2PBi-IFNγ fragment and injecting approximately 200 copies into the male pronucleus of fertilised mouse oocytes, which would then be transferred to psuedopregnant female mice. The second approach would utilise lentiviral technology, entailing cloning our construct into a lentiviral gene transfer vector, packaging it into viral particles and injecting the lentiviral particles into the perivitelline space (the space between the oocyte and the zona pelucida). Once transgenic lines were established, experiments aimed at investigating the role of high levels of seminal IFNγ present at insemination on pregnancy and offspring outcomes would be commenced.

Chapter 7

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