Cytokines and programming the pre-implantation embryo

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Abstract

As the pre-implantation embryo traverses the female reproductive tract, it experiences fluctuations in the composition of the surrounding maternal environment, including the availability of nutrients, growth factors and cytokines. In particular, the cytokine milieu surrounding the early embryo is pivotal in programming optimal embryo development. The pre-implantation embryo is sensitive to a range of perturbations such as maternal diet or *in vitro* culture. These and other insults influencing the maternal environment including infection, stress and environmental toxins may in part act via impact on oviduct and uterine cytokine synthesis. However the effect of maternal perturbation to inflammation or infection, on the embryo and the role of cytokines in mediating this is not fully elucidated. The studies described in this thesis employed an *in vivo* mouse model of maternal systemic inflammation with the pro-inflammatory bacterial lipopolysaccharide (LPS), where a pro-inflammatory cytokine response was elicited on days 2.5 and 3.5 post coitum (pc), prior to implantation. This model was studied in wildtype C57Bl/6 (*Il10* $^{+/+}$) mice and mice with a null mutation in the *Il10* gene (*Il10* $^{-/-}$) were studied to investigate the effects of maternal deficiency in the anti-inflammatory cytokine IL-10 during LPS treatment.

We demonstrated that the altered cytokine signals resulting from a low level pro-inflammatory LPS challenge (0.5 μg/mouse) in the pre-implantation period elicit changes in the embryo developmental trajectory that in turn alter fetal growth and delay postnatal growth in the male progeny from LPS-treated mothers. As LPS did not directly impact development of the embryo at low and moderate doses, this result appears to reflect indirect effects of LPS mediated via the maternal tract. This is consistent with data from day 3.5 pc oviduct and uterus tissues which revealed increased mRNA expression of pro-inflammatory cytokines including *Il6*, *Tnfa* and *Il12b* following maternal LPS treatment.

Peri-conceptional low dose LPS treatment in *Il10* $^{+/+}$ and *Il10* $^{-/-}$ mice revealed that the number of viable fetuses and fetal weight were both significantly reduced after LPS treatment, particularly in the *Il10* $^{-/-}$ mice. Embryo transfer was then utilised to investigate the mechanism by which LPS acts on the embryo, where day 3.5 pc embryos from donors treated with 0.5 μg LPS or PBS on days 2.5 and 3.5pc were transferred into day 2.5 pc pseudopregnant Swiss female recipients. The effect of maternal LPS treatment on fetal and placental development was seen to be maintained even after embryo transfer, suggesting that any effects of altered cytokine expression in embryos are exerted during cleavage stages before embryo recovery from donors.

In addition, postnatal investigation of male and female progeny derived from control PBS and LPS-treated $Il10^{+/+}$ and $Il10^{-/-}$ females from birth until 19 weeks of age showed that maternal LPS treatment constrains postnatal growth in male progeny regardless of maternal $Il10$ genotype, compared to male progeny from PBS-treated mothers. While the adult male progeny from LPS-treated $Il10^{+/+}$ and $Il10^{-/-}$ mothers did not display changes in fat mass compared to their PBS-treated control counterparts, the combination of maternal LPS treatment and maternal IL-10 deficiency resulted in greater fat mass accumulation in the adult male progeny from LPS-treated $Il10^{-/-}$ mothers compared to adult male progeny from LPS-treated $Il10^{+/+}$ mothers.

In addition, we investigated the effects of maternal systemic inflammation during the pre-implantation period on the response to LPS challenge during adulthood. Male progeny from LPS-treated $Il10^{-/-}$ mothers had a dampened response in LIF cytokine following a 100μg/kg LPS challenge at 18 weeks of age.

This study implies a role for cytokines as mediators of programming the embryo during the pre-implantation period, with altered responses in the event of maternal systemic inflammation impacting on later fetal and postnatal development. The anti-inflammatory cytokine IL-10 acts to protect the embryo from the adverse programming effects of exposure to LPS during the pre-implantation period, with absence of IL-10 resulting in altered postnatal phenotype and particularly fat mass accumulation in the male progeny during adulthood. It appears likely that the absence of IL-10 in the maternal environment delays the clearance of adverse pro-inflammatory cytokines induced during an inflammatory challenge, resulting in prolonged exposure of the embryo to circulating pro-inflammatory cytokines in the maternal tract, supporting a cytokine-mediated mechanism. These studies provide additional evidence for a role of cytokines in embryo sensing of environmental conditions, and indicate that IL-10 is a key regulator of this communication pathway.

Declaration

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Publications arising from this thesis

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Abbreviations

ANOVA	analysis of variance
ART	assisted reproductive technology
β-actin	beta actin
βc	beta common
BMI	body mass index
BMK1	big MAPK1
BMP	bone morphogenetic protein
BSA	bovine serum albumin
CD4+	cluster of differentiation 4 positive T cell
CD8+	cluster of differentiation 8 positive T cell
DAMPs	damage-associated molecular patterns
DEXA	dual-energy X-ray absorptiometry
DNA	deoxyribonucleic acid
DNMT	DNA methyl transferases
DOHaD	developmental origins of health and disease
E.coli	*Escherichia coli*
EGF	epidermal growth factor
ErbB1	epidermal growth factor receptor
ErbB4	v-erb-a erythroblastic leukemia viral oncogene homolog 4 (avian)
ERK	extra-cellular-signal-regulated kinase
F1	first generation
F2	second generation
FAS	apoptosis stimulating fragment
FGF	fibroblast growth factor
FOAD	developmental origins of adult diseases
G-CSF	granulocyte colony-stimulating factor
GD	gestational day
GH	growth hormone
GM-CSF	granulocyte-macrophage colony-stimulating factor
GM-Rα	GM-CSF specific alpha subunit

HAT	histone acetyltransferase
H_2O_2	hydrogen peroxide
Hegf1	human epidermal growth factor 1
HMGB1	high-mobility group protein B1
HSP	heat shock protein
i.p.	intra-peritoneal
ICM	inner cell mass
ICSI	intracytoplasmic sperm injection
IFNγ	interferon gamma
IGFBP1	insulin-like growth factor binding protein 1
IGFBP2	insulin-like growth factor binding protein 2
IGFBP3	insulin-like growth factor binding protein 3
IGF-I	insulin-like growth factor 1
IGF-II	insulin-like growth factor 2
IGF-IR	insulin-like growth factor 1 receptor
IgG1	immunoglobulin G, subclass 1
IGR-IIR	insulin-like growth factor 2 receptor
IL-10	interleukin 10
Il10 $^{-/-}$	interleukin 10 deficient
IL-10R	interleukin 10 receptor
IL-12α	interleukin 12, alpha
IL-12β	interleukin 12, beta
IL-15	interleukin 15
Il15 $^{-/-}$	interleukin 15 deficient
IL-1α	interleukin 1, alpha
IL-1β	interleukin 1, beta
IL-6	interleukin 6
IL-6Rα	interleukin 6 receptor alpha
IP-10	interferon gamma-induced protein 10
IUFD	intrauterine fetal death
IUGR	intrauterine growth restriction
IVF	*in vitro* fertilisation

KC	keratinocyte chemo-attractant
kDa	kilo Dalton
LE	luminal epithelium
LIF	leukocyte inhibitory factor
Lif $^{-/-}$	leukocyte inhibitory factor deficient
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemotactic protein-1
M-CSF	macrophage colony-stimulating factor
MIP-1α	macrophage inflammatory protein 1 alpha
MIP-1β	macrophage inflammatory protein 1 beta
MS	multiple sclerosis
mRNA	messenger RNA
miRNA	microRNA
mtDNA	mitochondrial DNA
MyD88	myeloid differentiation primary response 88
NFKB	nuclear factor kappa-light-chain-enhancer of activated B cells
NO	nitric oxide
O_2^-	superoxide
PBS	phosphate buffered saline
pc	post coitum
PCR	polymerase chain reaction
PI3K	phosphoinositide 3-kinase
piRNA	Piwi-interacting RNA
qPCR	quantitative polymerase chain reaction
RA	rheumatoid arthritis
RANTES	regulated on activation, normal T cell expressed and secreted
rmIL-10	recombinant mouse interleukin 10
ROI	region of interest
ROS	reactive oxygen species
SAPK	stress-activated protein kinase
SEM	standard error of the mean

siRNA	small interfering RNA
STI	sexually transmitted infection
TE	trophectoderm
TGFβ	transforming growth factor beta
Th1	type 1 T helper
Th17	type 17 T helper
Th2	type 2 T helper
TIRAP	toll-like receptor adapter protein
TLR	toll-like receptor
TLR2	toll-like receptor 2
TLR4	toll-like receptor 4
TNFα	tumour necrosis factor alpha
TNFαRc	tumour necrosis factor alpha receptor
Tollip	toll interacting protein
TRAIL	TNF-related apoptosis-inducing ligand
T_{reg}	regulatory T cell
VAS	vasectomised
X-ray	X-radiation

Chapter 1

Review of literature

1.1. INTRODUCTION

The peri-conceptional phase, which in broad terms spans the pre-implantation and peri-implantation period where embryo formation occurs and implantation commences, is critical for pregnancy success and fetal outcome. In recent years, increasing attention has been paid to the early embryo and the maternal environment in terms of long term consequences for maintenance of gestation, birth and later life health (Fazeli, 2008). In humans, the majority of early embryos perish before implantation and only approximately 60% of embryos that implant survive beyond the second week. This implies that there is a high rate of development failure and/or that highly selective quality control mechanisms operate at this early time.

Cytokines are critical regulators of both maternal receptivity and embryo implantation competence. There are now thousands of published studies showing that cytokines contribute to embryo development, implantation, trophoblast invasion and placental development. Cytokine synthesis in the female reproductive tract is regulated mainly by ovarian steroid hormones and is further modulated by introduction of male seminal fluid and the presence of micro-organisms. The cytokine signalling network is complex and extensive and its modulation can occur through various pathways due to external environmental factors such as infection, diet and stress. This may provide a mechanism through which the embryo can detect and adapt to its altered surroundings via sensing and responding to this cytokine environment. Cytokines may also be involved in quality control mechanisms that select and support some embryos, while causing the demise of others.

This chapter will summarise the current understanding of the events during the peri-conceptional period and discuss their possible modulation by perturbations in the maternal environment. Many of the processes occurring in the peri-conceptional period appear to be influenced by cytokines and other maternal factors in the female tract. As a consequence, disruption of the early maternal environment may have negative effects on programming future development of the pre-implantation embryo, resulting in detrimental outcomes for both establishing pregnancy and for offspring in later life.

1.2. MOUSE PRE-IMPLANTATION EMBRYO DEVELOPMENT

The mouse pre-implantation embryo develops *in vivo* as a free floating entity without any direct cell-cell contact with the epithelial cells lining the maternal reproductive tract for approximately 4 days before implanting into the endometrial lining of the uterus (Figure 1). The progression of a single cell zygote to a differentiated blastocyst is controlled both by maternal and embryonic factors. The embryo moves continuously through the reproductive tract, initially traversing the oviduct and then passing into the uterus approximately 72 hours after fertilisation, at the time of compaction at the morula stage. The embryo experiences a changing fluid environment as it traverses the tract, with fluctuations in the physiochemical composition and the availability of nutrients and growth factors. The progression from the zygote to the 2-cell embryo initiates the expression of the embryonic genome as opposed to maternal regulation in the zygote stage via cellular products laid down during oogenesis, such as transcriptional machinery and an abundance of maternally derived mRNA species.
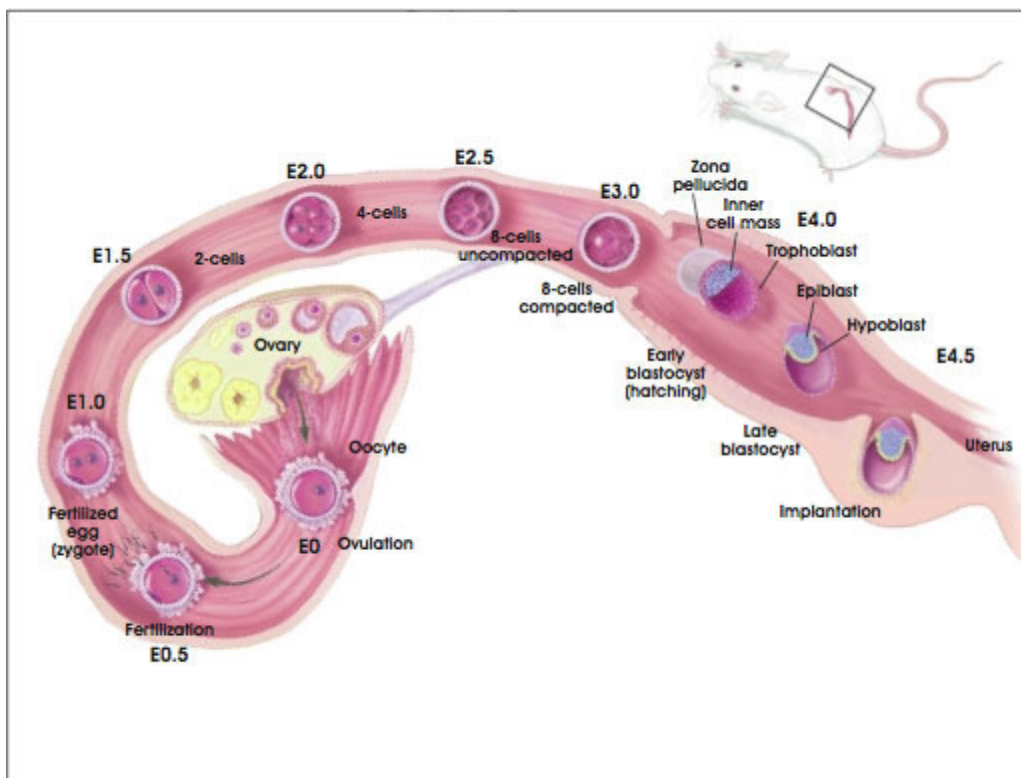
Figure 1.1   Development of pre-implantation embryo in mice from embryonic day 0 (E0) to embryonic day 5 (E5) (NIH, 2001).

The embryo is dependent on secretions from the oviduct and the uterus for nutrients during its development. Its cellular activities such as mitosis, gene expression, metabolism and apoptosis are influenced by the availability of metabolic substrates and signals produced by the cells of the reproductive tract. The secreted signals influence the cleavage of the fertilised ovum, compaction and morula formation, then cavitation and the formation of the blastocyst. During its development, the cells within the embryo differentiate into two different types of embryonic cells, the inner cell mass (ICM) cells which later form the fetus and the trophectoderm (TE) cells which later form the placenta. Blastocyst implantation is a complex phenomenon which involves a series of interactive processes which require the participation of both the conceptus and the maternal tissues. Thus, successful blastocyst development, implantation and pregnancy require a supportive maternal tract milieu and an effective maternal to embryo communication.

1.3. CYTOKINE RECEPTOR SIGNALLING PATHWAYS IN PRE-IMPLANTATION EMBRYO

The pre-implantation embryo is known to express cytokine receptors and also secrete a number of cytokine ligands prior to implantation, many of which have beneficial effects on embryo development (Harvey et al., 1995a, Hardy and Spanos, 2002). Cytokines have been implicated in the regulation of embryo development during the pre-implantation period. Cytokines function through binding of cognate receptor proteins which trigger phosphorylation and activation of intracellular signalling proteins to ultimately cause changes in gene expression, metabolism and other intracellular events linked with cell survival and differentiation. Many studies have shown that different cytokines act through one or more signalling pathways including the MAPKinase, JAK/STAT and PI3K/AKT pathways in the pre-implantation embryo. Some redundancy occurs as some cytokines can activate multiple signalling pathways, for instance G-CSF can activate both MAPK and JAK/STAT pathways in trophoblastic cells (Marino and Roguin, 2008).

1.3.1. Mitogen-activated protein kinase (MAPK) pathway

The mitogen-activated protein kinase (MAPK) pathways are major components of pathways controlling embryogenesis, cell differentiation, cell proliferation and cell death (Pearson et al., 2001). The MAPK superfamily of proteins consists of four separate signalling cascades: the c-Jun N-terminal kinase or stress-activated protein kinase (JNK/SAPK) (Woodgett et al., 1996), the extra-cellular-signal-regulated

kinase (ERKs) (Boulton and Cobb, 1991), the ERK5 or big MAPK1 (BMK1) (Zhou et al., 1995) and the p38 MAPK group of protein kinases (Han et al., 1994). The MAPK pathway mediates mitogenic signal transduction in most cell types. It is stimulated by fibroblast growth factor (FGF) in adult somatic cell lines for cell differentiation and proliferation (Lovicu and McAvoy, 2001). In mouse pre-implantation embryo, FGF is necessary for the maintenance of placental trophoblast cell division (Chai et al., 1998).

The entire MAPK pathway is present in the mouse pre-implantation embryo in mRNA, protein and phosphoprotein level (Wang et al., 2004b). The p38 MAPK cascade is reported to be activated in response to many types of environmental stress and pro-inflammatory cytokines (Ono and Han, 2000). Targeted inactivation of the p38 MAPK α gene results in embryonic lethality on day 10.5 as this gene is essential for placental morphogenesis (Adams et al., 2000). Natale et al. reported that the active p38 α/β MAPK signalling is required for development from the 8- to 16- cell stage to the blastocyst stage and the inhibition of this pathway *in vitro* caused complete loss of filamentous actin in 8- to 16- cell embryos (Natale et al., 2004, Paliga et al., 2005). SAPK/JNK mRNA transcripts were found to be expressed in mouse pre-implantation embryos, suggesting an important role as mediators of stress-induced responses that can cause embryo loss (Zhong et al., 2004).

1.3.2. Janus kinase/Signal transducer and activator of transcription (JAK/STAT) pathway

Janus kinases (JAK) are a family of four tyrosine kinases (JAK1, JAK2, JAK3 and TYK2) that associates with cytokine receptor chains and mediate signalling by phosphorylating tyrosine residues on various proteins in the pathway and including themselves and the receptor chains, and signal transducer and activator of transcription (STAT) factors (Leonard, 2001). STATs bind to the phosphorylated tyrosine residues on the receptors and dimerise and translocate to the nucleus following phosphorylation by JAKs, where they bind to DNA and either initiate or inhibit transcription. There are seven member of the STAT family (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6), some of which can be activated by several cytokines (Table 1.1, adapted from (Mitsuyama et al., 2001)). However, different STAT molecules have non-redundant biological roles. For instance, STAT1 and STAT2 mediate interferon signalling while STAT3 and STAT4 mediate IL-12 signalling, essential for the differentiation of Th1 helper cells (Mitsuyama et al., 2001). STAT3 and STAT5A/B are responsible for mediating a broad range of cytokines and this was shown by the lethal phenotypes in mice deficient in either STAT3, STAT5A or STAT5B (Leonard and O'Shea, 1998).

STAT5A/B protein expression was reported to be present in the nucleus of early 1-cell to blastocyst stage in mouse pre-implantation embryo and reduced embryo development was observed following culture with JAK inhibitor, suggesting that STAT5 regulates pre-implantation development by mediating signals from cytokines (Nakasato et al., 2006). STAT target genes that regulate cell survival and proliferation such as B-cell lymphoma 2 (Bcl2) family members, survivin, cyclin D1 and myc (Lai and Johnson, 2010). LIF acts through the LIFR/gp130 and activates STAT3, an important regulator of mouse embryonic stem cell self-renewal. However, binding of LIF to LIFRβ/gp130 can also activate the MAPK and PI3K pathways. Activation of these pathways is essential components for the regulation in biological responses in embryonic stem cells (Graf et al., 2011).

Table 1.1   Cytokines and JAK/STAT pathways

Cytokine	JAK	STAT
IFNα/β	JAK1, TYK2	STAT1, STAT2
IFNγ	JAK1, JAK2	STAT1
IL-2	JAK1, JAK3	STAT3, STAT5
IL-3	JAK1, JAK2	STAT5
IL-4	JAK1, JAK3	STAT6
IL-5	JAK1, JAK2	STAT5
IL-6	JAK1, JAK2, TYK2	STAT1, STAT3
IL-7	JAK1, JAK3	STAT5
IL-10	JAK1, TYK2	STAT1, STAT3
IL-12	JAK2, TYK2	STAT3, STAT4
IL-13	JAK1, JAK3	STAT6
IL-15	JAK1, JAK3	STAT3, STAT5
GM-CSF	JAK1, JAK2	STAT5
G-CSF	JAK1, JAK2	STAT3

Adapted from (Mitsuyama et al., 2001)

1.3.3. Phosphatidylinositol-3 kinase (PI3K)/Protein Kinase B (AKT) pathway

Phosphatidylinositol-3 kinase (PI3K) is a lipid kinase family characterised by the ability to phosphorylate the inositol ring 3'-OH group in inositol phospholipids (Fruman et al., 1998). Class I PI3K are heterodimers composed of a catalytic subunit (p110) and an adaptor/regulatory subunit (p85). This class is further divided into the subclass IA, which is activated by receptors with protein tyrosine kinase activity (Receptor Protein Tyrosine Kinase, RPTK), and the subclass IB, which is activated by receptors coupled with G proteins. The substrate for class I PI3Ks is phosphatidylinositol-4,5-bisphosphate (PI-4,5-P2) to generate the second messenger phosphatidylinositol-3,4,5-trisphosphate (PI-3,4,5-P3). Three isoforms of catalytic subunit p110, named α, β and γ, and seven adapting protein generated by alternative splicing of three genes (p85α, p85β and p55γ) are known for PI3K class IA (Fruman et al., 1998). The PI3K pathway is involved in cell growth, differentiation, proliferation, motility, survival and intracellular trafficking. It is recognised as one of the major anti-apoptotic pathway operating in cells, turned on in response to activation of a wide range of trophic signals including soluble growth factors and attachment to extracellular matrix (Cantley, 2002). A major component of survival signal provided by the activation of PI3K is mediated by the protein kinase B (AKT) family of protein serine/threonine kinases, AKT1, AKT2 and AKT3 also known as PKBα, PKBβ and PKBγ (Osaki et al., 2004).

The PI3K pathway is present in cells within the early embryo (Li et al., 2007) and is activated by embryotrophic ligands such as PAF, insulin, IGF-1 and TGFα (Lu et al., 2004, Navarrete Santos et al., 2008, Kawamura et al., 2005). Inhibition of PI3K or AKT was reported to reduce embryo development *in vitro* and this inhibition could be partially reversed through the addition of exogenous PAF to the culture medium (Li et al., 2007). The transcription of the cell survival gene *Bcl2* in the 2-cell embryo, is dependent on PAF receptor and PI3K (Jin and O'Neill, 2011) and activation of the PI3K/AKT pathway is required to keep transformation related protein 53 (TRP53), a cellular stress transcription factor, in a latent state in the embryo (Jin et al., 2009).

1.4. CYTOKINES

Cytokines are low molecular weight regulatory glycoproteins secreted by white blood cells and various other cells in the body in response to a number of stimuli. Cytokines bind to specific receptors on target cells, triggering specific signal transduction pathways. In general, cytokines act in short range, where they exert autocrine and paracrine actions. Cytokines exhibit characteristics including pleiotropy,

redundancy, synergy, antagonism and cascade induction which together permit them to regulate target cell activity in a coordinated and interactive way. These attributes of cytokines would explain how embryo development can be promoted and/or inhibited by exposure to cytokines in the female reproductive tract. A wide range of *in vitro* studies have shown that cytokines facilitate the competence of embryos for normal fetal growth and pregnancy success (Harvey et al., 1995b, O'Neill et al., 1989, Sjoblom et al., 2005).

Cytokines secretion by oviduct cells is one important pathway by which these cells support survival and development in embryos. The oviduct has been highlighted to contribute to embryo development, as demonstrated by experiments using *in vitro* culture of embryos with explanted oviducts in rodents (Biggers et al., 1962, Minami and Iritani, 1993). Co-culture with human ampullary cells was found to improve the quality of human embryos (Bongso et al., 1989). Similarly, co-culture with oviductal cells has been reported to improve implantation rates in a human IVF trial (Yeung et al., 1996) as well as improve mouse embryo development (Liu et al., 1995).

Embryos from a range of mammalian species express cytokine receptors and also secrete a number of cytokine ligands, many of which have beneficial effects on embryo development (Hardy and Spanos, 2002). Attributes such as redundancy and synergy are important in explaining the effects of cytokine exposure on embryo development, as gene knockout studies have shown that the lack of a specific cytokine or growth factor usually has subtle effects on embryo development (Ingman and Jones, 2008). In recent years, knockout studies and animal models have contributed to revealing the reproductive functions of many cytokines. The specific roles for individual cytokines are discussed below.

1.4.1.   Anti-inflammatory cytokines and pro-inflammatory cytokines

The Th1/Th2 paradigm of anti-inflammatory and pro-inflammatory cytokines produced by Th1 (Type 1 T helper) and Th2 (Type 2 T helper) cells has been central to explaining how the maternal immune system accommodates pregnancy as proposed by Chaouat and Wegmann (Wegmann et al., 1993). Helper T cells are CD4+ T lymphocytes that have an important role in determining the nature of the adaptive immune response. Th1 cytokines are known to be pro-inflammatory while Th2 cytokines are known to be anti-inflammatory. There is a correlation between the balance of Th1 cytokines and Th2 cytokines

with pregnancy success. Th1 phenotype cells promote cellular immunity and cause inflammatory responses whereas Th2 phenotype cells promote humoral responses to cause antibody mediated responses. This proposed concept implies that Th2 cytokines are essential for pregnancy success. Although many studies agreed with this idea, this concept was found to not extend to the implantation process since at this early time of establishing pregnancy, many pro-inflammatory agents are also involved. Furthermore, more studies have unveiled that Th1 cytokines are also involved in various aspects of establishing pregnancy. Tumour necrosis factor alpha (TNFα) has been shown to play a role in ovulation by inducing prostaglandin and nitrogen oxide production to promote follicle rupture (Machelon and Emilie, 1997). Peaks of interferon gamma (IFNγ) have been found on days 7 and 9 of gestation, which correlates to the period of the ectoplacental cone trophoblast differentiation into the spongio- and labyrinthine-trophoblast populations of the mature placenta (Athanassakis et al., 2000). A more advanced understanding is now evolving whereby a range of cytokines, both pro- and anti-inflammatory, have different roles in different aspects of the range of female reproductive events.

1.4.2. Cytokines promoting pre-implantation embryo development

1.4.2.1. Granulocyte-macrophage colony-stimulating factor (GM-CSF)

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a haematopoietic cytokine which was originally identified as a regulator of the proliferation, differentiation and activation of myeloid haematopoietic cells (Ruef and Coleman, 1990). GM-CSF is a 23kDa glycoprotein and its receptor consists of a GM-CSF specific alpha (α) subunit (GM-Rα) which confers low-binding affinity and a high affinity beta common (βc) subunit which does not bind to GM-CSF itself but forms a high affinity complex when associated with the ligand coupled GM-Rα (Gearing et al., 1989). During early pregnancy, GM-CSF is produced by epithelial cells lining the oviduct and the uterus (Giacomini et al., 1995, Zhao and Chegini, 1994), and is regulated by oestrogen in the cycling uterus. Its expression correlates with the time of fertilisation and embryo development and implantation (Zhao and Chegini, 1994). GM-CSF mRNA is further up-regulated in the uterus following exposure to seminal fluid at mating (Robertson and Seamark, 1992).

Sjoblom et al. have shown that human embryos express the GM-Rα subunit but not the βc subunit. A neutralising antibody targeting GM-Rα was found to block GM-CSF effects in human embryos but βc

neutralising antibody had no inhibitory effect (Sjoblom et al., 2002). GM-Rα mRNA was found to be expressed in all pre-implantation embryo stages but GM-CSF mRNA expression itself was not evident in embryos of any pre-implantation developmental stage (Robertson et al., 2001). Absence of GM-CSF expression in embryos indicates that maternal GM-CSF secreted from uterine epithelial cells acts in a paracrine manner on the pre-implantation embryo (Robertson et al., 2001). Mice with a null mutation in the *Csf2* gene encoding GM-CSF (*Csf2* $^{-/-}$ mice) have 25% smaller litter size linked with impaired blastocyst development, whereby *Csf2* $^{-/-}$ embryos have fewer cells in both the trophectoderm and inner cell mass at blastocyst stage (Robertson et al., 2001).

In vitro experiments showed that GM-CSF acts as an embryotrophic factor with survival-promoting effects on the ICM of both mouse and human embryos grown *in vitro* (Robertson et al., 2001, Sjoblom et al., 2002). The most evident effect of GM-CSF in blastocysts is the increase in the total number of blastomeres. Differential staining has shown that GM-CSF particularly affects the ICM (Sjoblom et al., 2002). Similar findings of GM-CSF improving blastomere development in mice was reported but only when human serum albumin was absent from the culture media (Karagenc et al., 2005). Exposure to GM-CSF during the post-thaw period was reported to enhance the re-expansion of the blastocoele (Papayannis et al., 2007). In humans, GM-CSF was found to elicit approximately a two-fold increase in the proportion of embryos reaching the blastocyst stage and this was accompanied by a faster rate of development, an increase in the number of blastomeres particularly in the ICM, and more frequent progression to hatching and implantation (Sjöblom et al., 1999). Enhanced embryo development during the pre-implantation period was also reported in a randomised study by Shapiro et al. (Shapiro et al., 2003). Studies have shown a significantly improved clinical pregnancy and survival rate in human ART programs when GM-CSF is used as a culture medium supplement (Kim et al., 2001a, Ziebe et al., 2013).

1.4.2.2. Leukaemia inhibitory factor (LIF)

Leukaemia inhibitory factor (LIF) is a 45-56kDa secreted glycoprotein that has multiple functions on various culture systems and is a member of the interleukin-6 (IL-6) family of cytokines (Auernhammer and Melmed, 2000). LIF was initially identified and characterised by its ability to induce macrophage differentiation of the myeloid leukaemia cell line M1 (Hilton et al., 1988). LIF has multiple effects on a

variety of cell types and tissues which includes the induction of acute phase response proteins in hepatocytes and also regulating adipocyte formation.

LIF is produced by the luminal epithelium and epithelial glands in the mouse uterus and its expression is up-regulated from the ovulation period until implantation (Bhatt et al., 1991). After implantation and decidua formation, LIF expression is down-regulated (Bhatt et al., 1991). LIF mRNA was also shown to be expressed in the oviduct and uterus of mice (Robertson et al., 1993). LIF was reported to be expressed in human hydrosalpingeal fluid (Strandell et al., 2004). In pre-implantation embryos, LIF has been found to be expressed in the trophectoderm cells of day 3.5 mice blastocysts (Murray et al., 1990) and LIF was reported to increase the hatching rate and outgrowth of blastocysts cultured from the 8-cell stage onwards (Robertson et al., 1991). LIF receptors (LIFR) are reported to be expressed only at the blastocyst stages in both mice and humans (Nichols et al., 1996, Sharkey et al., 1995, Charnock-Jones et al., 1994). LIF is the first cytokine discovered to have a non-redundant function in reproduction, which cannot be compensated by other cytokines. It was shown to be essential for embryo implantation success as LIF deficiency results in implantation failure and infertility of female *Lif* $^{-/-}$ mice (Stewart et al., 1992). Pregnancy can be rescued in *Lif* $^{-/-}$ mice by injecting LIF on the day of implantation and the lack of implantation is due to the absence of maternal LIF cytokine as LIF deficient embryos are still able to implant successfully in wild-type mice (Stewart et al., 1992). Human oviduct co-culture experiments was reported to express LIF (Barmat et al., 1997).

1.4.2.3. Insulin-like growth factor I and II (IGF-I and IGF-II)

The insulin-like growth factor family consists of the ligands, IGF-I and IGF-II as well as their respective receptors, IGF-I receptor (IGF-IR) and IGF-II receptor (IGF-IIR). IGF-I expression is regulated by growth hormone (GH) and it solely binds to the IGF-IR. On the other hand, IGF-II binds strongly to the IGF-IIR but it also binds weakly to the IGF-IR. IGF-I and IGF-IR was reported to be expressed in the fallopian tube/oviduct in humans and rodents (Lighten et al., 1998, Pfeifer and Chegini, 1994, Dalton et al., 1994, Carlsson et al., 1993). IGF-II was also reported to be expressed in the rodent oviducts (Shao et al., 2007, Zhang et al., 1994). IGF-I was found to promote pre-implantation embryo development, cell number and metabolism in many species such as mouse (Brison and Schultz, 1997), rabbit (Herrler et al., 1998), bovine (Palma et al., 1997) and human (Spanos et al., 2000). Human embryos express IGF-I receptors (IGF-IR) during the pre-implantation period but not the IGF-I ligand (Lighten et al., 1997). Both

IGF-IR and IGF-IIR has been shown to be expressed in the mouse and human pre-implantation embryo (Rappolee et al., 1992, Smith et al., 1993, Lighten et al., 1997). IGF-II was also found to be expressed by the mouse pre-implantation embryo (Rappolee et al., 1992). Addition of IGF-I to the culture medium was found to promote blastocyst development rate and increase the inner cell mass cells in mouse embryos (Harvey and Kaye, 1992b). IGF-II was found to increase protein synthesis within TE cells in mice (Rappolee et al., 1992).

Knockout studies have shown that the absence of *Igf1, Igf2* or *Igf1r* results in significantly reduced birth weight and this is associated with neonatal lethality (Baker et al., 1993, Liu et al., 1993). IGF-I knockout mice were also found to exhibit postnatal lethality, growth retardation and infertility (Liu et al., 2000). The use of a bi-transgenic mouse model showed that IGF-I plays a significant role in programming growth as well as sustaining postnatal development and reproductive function in both sex (Stratikopoulos et al., 2008). In contrast, IGF-II was found to be a major modulator of fetal and placental growth (Constancia et al., 2002) and a recent study has reported on placental programming of anxiety in the adult offspring from IGF-II knockout mice (Mikaelsson et al., 2013). Culture of human pre-implantation embryos with IGF-I was found to enhance embryo development (Lighten et al., 1998). Lai et al. detected concentrations of insulin-like growth factor binding protein 1, 2 and 3 (IGFBP1, IGFBP2 and IGFBP3), which improve binding of IGF to target cells, in media from mouse embryos cultured in the presence and absence of human oviductal cells (Lai et al., 1996).

1.4.2.4. Heparin-binding EGF-like growth factor (HB-EGF)

Heparin-binding EGF-like growth factor (HB-EGF) is a member of the EGF family. HB-EGF was identified as a mitogen for fibroblasts and smooth muscle cells (Nishi and Klagsbrun, 2004). In the uterine luminal epithelium, HB-EGF is expressed in transmembrane and in soluble forms (Raab et al., 1996), suggesting a role as a juxtacrine and paracrine signalling mediator. HB-EGF has been highlighted as an early molecular marker for embryo-maternal uterine epithelium communication during the implantation process as an *in vitro* study by Das et al. reported that the expression of HB-EGF in the luminal epithelium (LE) is induced by the blastocyst in the early process of implantation and that the expression of HB-EGF is not hormone dependent as there was no expression of HB-EGF observed in LE region not adjacent to blastocyst and in pseudopregnant females (Das et al., 1994). In humans and mice, HB-EGF has been reported to be expressed in the oviduct (Sun et al., 2006, Dalton et al., 1994).

Its signalling is known to be important in human implantation (Paria et al., 2001). The primary receptors for HB-EGF are the ErbB1 and ErbB4 from the EGF tyrosine kinase receptor family, which was shown to be expressed on the trophectoderm of day 4 normal blastocysts (Lim and Dey, 2009). HB-EGF is expressed in both the blastocyst and the uterus during implantation, suggesting an auto-induction loop in the regulation of this cytokine during implantation (Hamatani et al., 2004). HB-EGF was also found to be regulated in the mouse endometrium by LIF (Song et al., 2000). HB-EGF, encoded by the *Hegf1* gene promotes embryonic growth through EGF receptors expressed on blastocyst cell surface (Wang et al., 2000). HB-EGF has been shown to be a potent growth factor for enhancing the development of human IVF-derived embryos to blastocysts (Martin et al., 1998). *In vitro* studies have shown that soluble HB-EGF stimulates proliferation, zona-hatching and trophoblast outgrowth in mouse blastocysts (Das et al., 1994). In a study by Xie et al., the normal 'window of implantation' was reported to be altered in *Hbegf* $^{-/-}$ mice which limits and delays implantation in mice, resulting in compromised pregnancy outcome (Xie et al., 2007).

1.4.3. Cytokines inhibiting pre-implantation embryo development

1.4.3.1. Tumour necrosis factor alpha (TNFα)

TNFα is a pleiotropic cytokine exhibiting pro-inflammatory functions, eliciting a number of different cellular responses (Baker and Reddy, 1998). TNFα is synthesised as a 26kDa cell surface precursor (Birkland et al., 1992), later cleaved into a 17kDa protein (Mohan et al., 2002). Uterine macrophages and natural killer (NK) cells are the primary source of TNFα in the reproductive tract. Expression of TNFα was reported in oviductal epithelial cells of mice and humans (Hunt et al., 1993, Bedaiwy et al., 2005, Strandell et al., 2004). TNFα stimulates translocation of the nuclear factor kappa B (NFκB) transcription factors to the nucleus, subsequently triggering transcription of various inflammatory mediators such as IL-6 and IL-1β (Iwamoto and Konicek, 1997, Malinin et al., 1997).

TNFα is known to exert detrimental effects on pre-implantation embryo development. *In vitro* culture experiments have shown that addition of TNFα at the concentrations of 25 ng/ml or higher for 24 hours increases the percentage of dead blastomeres in mouse blastocysts (Fabian et al., 2007). Previous studies have shown that the synthesis of TNFα is up-regulated in the uterus of the pregnant diabetic rat (Pampfer et al., 1995). The pre-implantation embryo development was found succumb to increased

apoptosis when rats blastocyst were exposed to TNFα *in vitro* (Pampfer et al., 1997b). Soluble TNFα production was found be higher in diabetic female rat and this results in embryo exposure to high levels of TNFα exposure (Pampfer et al., 1997b). Mouse pre-implantation embryos were found to express the p60 form of the TNFα receptor (TNFαRc) (Pampfer et al., 1994b), presumably starting from the morula to blastocyst transition as there was no TNFαRc expression found during the morula stage (Lachapelle et al., 1993).

TNFα administration to mice in early pregnancy was also found to impair implantation or reduce litter size in mice and rats (Chaouat et al., 2004). Treatment with TNF inhibitors of women with a history of recurrent spontaneous abortion was found to increase live birth rates (Winger and Reed, 2008), suggesting that inflammation or other factors leading to elevated TNFα are mechanisms contributing to fetal loss. A study looking at the effects of growth factors on embryos exposed to TNFα showed that insulin-like growth factor 1 (IGF-I) and 2 (IGF-II) was protective against the inhibitory effects caused by TNFα (Glabowski et al., 2005).

1.4.3.2. Interferon gamma (IFNγ)

IFNγ is a pro-inflammatory cytokine secreted in the uterus during early pregnancy. It plays important roles in diverse cellular processes, including activating innate and adaptive immune responses, inhibiting cell proliferation and inducing apoptosis (Boehm et al., 1997). Studies in mice show that a localised and timely synthesis of IFNγ by uterine natural killer (NK) cells contributes to normal placental development and pregnancy outcome (Ashkar et al., 2000). IFNγ plays critical roles that include initiation of endometrial vasculature remodelling, angiogenesis at implantation sites, and maintenance of the decidual component of the placenta in normal rodent pregnancies (Murphy et al., 2009). However, IFNγ has been found to inhibit secretion of the GM-CSF that promotes blastocyst growth and differentiation (Robertson et al., 1992), suggesting that in the pre-implantation period IFNγ is detrimental towards the maintenance of pregnancy. High doses of IFNγ administered to mice was shown to prevent implantation while administration of IFNγ after implantation was found to increase the rate of resorption (Chaouat et al., 1990, Martal et al., 1997). The IFNγ receptor was found to be expressed on mouse oocytes and pre-implantation embryos (Truchet et al., 2001). High levels of IFNγ were observed in the serum of women suffering from spontaneous miscarriage (Jenkins et al., 2000). Earlier studies have

reported IFNγ as an embryo inhibitory factor in mouse embryo culture detectable in serum or activated lymphocytes and macrophages from women with recurrent abortions (Haimovici et al., 1988, Hill et al., 1992).

1.4.4. Other important cytokines

1.4.4.1. Interleukin-10 (IL-10)

Interleukin-10 (IL-10) is a pleiotropic cytokine secreted by leukocytes and somatic cells with well characterised anti-inflammatory and immune-deviating properties (Moore et al., 2001). Activated Th2 cells are the primary source of IL-10. IL-10 acts to participate in a negative feedback loop to reduce inflammation (Moore et al., 2001). IL-10 acts to terminate inflammatory responses and limit inflammation-induced pathology by inhibiting synthesis of TNFα and other pro-inflammatory cytokines in macrophages and monocytes. IL-10 inhibits proliferation and cytokine synthesis of Th1 cells. It also regulates tolerance in mucosal and epithelial tissues. IL-10 was shown to modulate resistance to inflammatory stimuli such as lipopolysaccharide (LPS) by down-regulating pro-inflammatory cytokine levels in the uterus and placenta (Robertson et al., 2006). It has also been shown to be a critical determinant of resistance against preterm labour (Robertson et al., 2006).

The balance of cytokines in the milieu present in the reproductive tract is vital for pregnancy success as having a poor quality embryo may lead to many physiological or pathological outcomes for the offspring in later life. Elevated levels of pro-inflammatory cytokines were found to limit implantation, resulting in the rejection of the fetus and recurrent spontaneous abortion in women (Winger et al., 2011). On the other hand, elevated levels of anti-inflammatory cytokines were found to promote implantation and elicit tolerance of the fetus, resulting in pregnancy success. The balance between anti-inflammatory IL-10 & pro-inflammatory TNFα appears to be involved in regulating the immune response towards pregnancy (White et al., 2004).

IL-10 has been proposed to be a major player in suppressing endometrial inflammatory responses during pregnancy. *Il10* $^{-/-}$ mice were observed to have differences in placental structure and function compared to wild-type mice when both genotypes were under identical environmental conditions (Roberts et al., 2003). The current literature shows that the primary role for IL-10 in pregnancy is in

protecting the fetus from inflammatory challenges such as those induced by pathogens. *Il10 $^{-/-}$* mice are more susceptible to fetal loss and pregnancy complications such as preterm labour (Robertson et al., 2006) when challenged with an inflammatory stimulus such as LPS. It was also found that in the absence of any inflammatory challenge IL-10 was not essential for maternal immune tolerance or successful pregnancy but it is a determinant of growth trajectory *in utero* and after birth (White et al., 2004).

1.4.4.2. Interleukin-6 (IL-6)

Interleukin-6 (IL-6) is a multifunctional cytokine which was first reported in 1986 (Hirano et al., 1986) that regulates various events in the immune system and has some redundant function with IL-11 and LIF. IL-6 exhibits both pro-inflammatory and anti-inflammatory characteristics, playing important roles in acute and chronic inflammation and autoimmunity (Ishihara and Hirano, 2002). IL-6 is secreted as a 26kDa protein that binds to the IL-6Rα (Taga and Kishimoto, 1997). IL-6 was reported to be expressed in the oviduct of humans and pigs (Bedaiwy et al., 2005, Jiwakanon et al., 2010). IL-6 secreted by endometrial epithelial cells was found to be associated with improved blastocyst development and implantation rates (Dominguez et al., 2010). IL-6 has been reported to be present in the culture media 24 hours following IVF treatment (Austgulen et al., 1995). Addition of IL-6 to the culture media was also reported to increase cell number and reduce apoptosis in mouse blastocysts (Shen et al., 2009). A recent study has shown that ovarian stimulation reduces IL-6 secretion in mouse and human pre-implantation embryos (Yu et al., 2012). In late gestation, the decidua is richly populated with leukocytes compared with the non-decidualised uterine tissue and high levels of pro-inflammatory cytokine mRNAs are expressed, increasing as parturition approaches (Osman et al., 2006). IL-6 knockout mice are fertile (Kopf et al., 1994), although they were found to have reduced fertility, low implantation rates and increase rate of miscarriage in mid-gestation (Robertson et al., 2000). Serum IL-6 was shown to be elevated in patients with recurrent abortions (Zenclussen et al., 2003). However, IL-6 mRNA expression was found to be reduced in the endometrium of women with recurrent miscarriage (Jasper et al., 2007). There is a relationship between pro-inflammatory cytokines and preterm birth or fetal loss (Robertson et al., 2006). Serum, uterine and placental TNFα and IL-6 levels were found to be elevated in *Il10 $^{-/-}$* mice in response to LPS compared to wild-type mice. Administration of recombinant mouse IL-10 (rmIL-10) to *Il10 $^{-/-}$* mice was found to decrease TNFα and IL-6 responses to LPS and to attenuate fetal loss. Human oviduct co-culture experiments was also reported to express IL-6 (Barmat et al., 1997).

1.4.5. Cytokines as immune modulators in protecting pregnancy

Cytokines have been primarily defined as modulators of the immune system and the balance in their relative abundance has been shown to impact the susceptibility of pregnancies to pathologies such as fetal growth restriction, miscarriage and preterm delivery caused by inflammatory stimuli, in mice (Robertson et al., 2007, Robertson et al., 2006). Cytokines such as IL-10 and TNFα with anti-inflammatory and pro-inflammatory properties respectively are involved in regulating the balance in the immune response towards pregnancy (White et al., 2004). However, their functions are not restricted to immune cell regulation as they are also critical to the success of the reproductive process by direct and indirect signalling on non-hemopoietic cells in the reproductive tract and gestational tissues. Comprehensive studies often in cytokine null mutant mice have unravelled the functions of specific cytokines in reproduction (Ingman and Jones, 2008) and have revealed evidence for cytokines such as GM-CSF or TNFα having either positive or negative influence on the pre-implantation embryo development which may lead to later pregnancy success or failure (Sharkey, 1998). This is important as poor quality embryo development may lead to many physiological or pathological outcomes in the offspring in later life.

1.5. CYTOKINE ACTION IN CYCLING AND PREGNANT UTERUS

The cytokine milieu in a pregnant uterus varies from the non-pregnant cycling uterus. A study by Orsi et al. in 2006 reported on 18 different cytokines measured throughout the estrous cycle, pregnancy and post-partum period in mice (Orsi et al., 2006). Orsi reported that most cytokines, including IL-1α, IL-1β, IL-6, IL-10, IL-12, GM-CSF, TNFα and IFNγ were detected throughout all stages. While modest variation in cytokine profile was observed when comparing estrous to early pregnancy, a greater increase in cytokine levels were observed during mid and late gestation which continues to the post-partum stage, particularly for IL-1β, IL-2, IL-6, IL-10, IL-12, TNFα and IFNγ (Orsi et al., 2006). The post coitum stage was characterised by a reduction in IFNγ and IL-12 concentrations and a rise in KC and G-CSF levels (Orsi et al., 2006), which was likely due to mating-induced immunomodulation. This shift signals the establishment of pregnancy and is consistent with suppression of detrimental cytokines since IFNγ is associated with fetal abortion while IL-12 is known to elicit TNFα production (Lentsch et al., 1996, Mattsson et al., 1992). In a more detailed study utilising a 23-multiplex immunoassay of the

uterine fluid from naturally cycling estrus mice, G-CSF was found to be elevated 173-fold compared to serum levels, which indicates this cytokine is prominent together with the other colony-stimulating factors in the uterine fluid at this stage (Orsi et al., 2007). Other cytokines including IL-1α, eotaxin, IL-6 and KC were also found to be higher in uterine fluid compared to serum (Orsi et al., 2007). IFNγ expression is reported to be stage-dependent in a cycling uterus, where it is strongest during the estrous phase and limited to the luminal and glandular epithelial cells. IFNγ expression was reported to be stage-dependent in both cycling and pregnant uteri, where IFNγ expression was predominant during estrus in the cycling uterus while IFNγ expression was found to be is prominent at early (gestation day 6-10) and late (gestation day 18) stages in a pregnant uterus (Platt and Hunt, 1998). LIF is also another main player in the early pregnant uterus. Peak levels of LIF expression in the uterus coincides with the window of implantation in mice (Bhatt et al., 1991).

1.6.   ENVIRONMENTAL REGULATION OF EMBRYO DEVELOPMENT

Maternal environmental effects are a critical factor in embryo development, with the embryo exhibiting responsiveness to maternal factors secreted by the oviduct and the uterus. These factors may include signalling factors such as hormones, cytokines and growth factors as well as nutritional components, stress factors and oxygen availability. Disruption of any of those signals can affect the development of the embryo which may lead to infertility, pregnancy pathologies or postnatal growth disturbances. Furthermore, there is evidence the embryo can adapt its developmental program and gene expression depending on environmental factors. There is increasing evidence that *in vivo*, cytokines and growth factors originating from the oviduct and uterus under the influence of ovarian steroid hormones (Kaye et al., 1992, Pampfer et al., 1991) are important mediators of signalling between the embryo and the endometrium, acting to promote or limit cell division, gene expression, implantation competence and post-implantation development in embryos.

The growth and the development of the pre-implantation embryo are influenced by the surrounding cytokine milieu as it traverses the maternal tract. As embryos expresses cytokine receptors, it is sensitive to changes in cytokine levels and several cytokines exert different effect on cell number and viability, gene expression and developmental competence (Robertson et al., 1994, Sharkey et al., 1995). Many *in vitro* studies involving exogenous cytokine supplementation to embryo culture have

revealed that cytokines such as GM-CSF, LIF, IGF-I and IGF-II act to promote blastocyst development (Sjöblom et al., 1999, Stewart et al., 1992, Harvey and Kaye, 1992a, Rappolee et al., 1992) while others such as TNFα and IFNγ inhibit embryo development (Pampfer et al., 1997b, Hill et al., 1987). The synthesis of these signalling factors can be altered in the event of maternal infection, inflammation, diet and other stressors. Thus, the cytokines profile within the oviduct and uterus may be a mechanism by which the embryo senses and adapts to changeable environmental parameters.

1.6.1. Factors regulating cytokine production

Cytokine production can be regulated by environmental factors that fluctuate in daily life and then the cytokine profile in the reproductive tract might be viewed as reflecting the internal and external environment of the mother. Some of these include infection and inflammation, nutrition and lifestyle factors such as exercise, smoking, drugs and alcohol consumption. These factors regulate the cytokine levels in the body and may promote or disrupt the production of particular cytokines that are important for the pre-implantation embryo development or pregnancy success. As the pre-implantation embryo is surrounded and fully exposed to cytokines and growth factors while it matures in the reproductive tract, any minor changes to the cytokine environment would affect the embryo and could potentially influence the embryo to adapt through altered gene expression or through epigenetic mechanisms.

Even minor changes in cytokine profile may program embryo growth and development to adapt and better survive in the specific environment. However, when the offspring is born, these adaptations may physiological or pathological difficulties that may compromise survival and health after birth. This may be particularly evident when the programming influence does not match the environmental reality experienced after birth. However, the mechanism of cytokines programming the embryo and embryo programming at the pre-implantation stage is unknown and has yet to be studied extensively.

Cytokines can presumably impact imprinting pathways as cytokine signalling can alter expression of methylases and other genes controlling the epigenome in various cell lineages (Samson et al., 2011, Zelinkova et al., 2012, Whitaker et al., 2013), although this has not been studied to date in embryos.

1.6.1.1. Assisted Reproductive Technology (ART)

Assisted reproductive technologies or ART, are methods used to overcome infertility and achieve pregnancy by artificial or partially artificial means. Common methods used in ART include in vitro fertilisation (IVF) and intracytoplasmic sperm injection (ICSI). However, some studies have shown that pregnancies conceived by assisted reproduction have an increased risk of pregnancy disorders and birth defects (Hansen et al., 2005). ART children are found to be at risk of genetic disorders as a result of DNA damage, low birth weight and preterm birth (Reefhuis et al., 2009). Studies in mice have shown that IVF techniques coupled with embryo transfer can increase or decrease placental size depending on the specific culture medium and the gestational age at assessment (Tanaka et al., 2001, Khosla et al., 2001, Ogawa et al., 2003, Palmieri et al., 2008). Manipulation using ART methods appears to induce cellular stress in embryos and may cause imprinting defects.

Women taking IVF treatments undergo ovarian stimulation using hormone-manipulating medications that interfere with normal hormone levels and therefore affect normal function of reproductive tissues, which in turn could disrupt local cytokine production. Several studies have indicated that stimulation prior to IVF causes endometrial abnormalities which appear to adversely affect implantation rates (Mirkin et al., 2004, Valbuena et al., 1999, Devroey et al., 2004). A recent study has shown a reduction in IL-6 expression levels in both human and mouse blastocysts following ovarian stimulation (Yu et al., 2012). This is similar to superovulating mice for *in vitro* experiments, which has been found to affect endometrial receptivity and delay embryonic and fetal development (Van der Auwera and D'Hooghe, 2001, Ertzeid and Storeng, 2001, Ertzeid and Storeng, 1992). Other studies have also shown ovarian stimulation to alter global gene expression profile in mouse blastocysts (Zhang et al., 2010) and affect imprinted gene expression in the placenta (Fortier et al., 2008).

1.6.1.2. Infection and inflammation

Infection is one of the factors that regulate cytokine production in the body. Infections can result in either a local or systemic response. Many studies have associated periodontal disease as a risk factor for pregnancy. Periodontitis, which causes a systemic immune response, is associated with poor pregnancy outcomes such as low birth weight and preterm birth (Lopez et al., 2002). One study has shown that experimental periodontitis in rats results in females experiencing higher local and systemic concentrations of pro-inflammatory cytokines (Bain et al., 2009). A preliminary study on mothers with

early and established periodontal diseases such as periodontitis and gingivitis showed that the body size of newborns decreases with the severity of the maternal periodontal condition (Shirmohammadi et al., 2009). Asthma is another common chronic illness that causes complications in pregnancy, with a prevalence of 8-13% worldwide (Kurinczuk et al., 1999). There is increasing evidence that asthma can adversely impact pregnancy outcomes, resulting in low birth weight, preterm labour, preeclampsia and caesarean section (Bracken et al., 2003, Murphy et al., 2005).

As mothers with existing infectious diseases have high levels of pro-inflammatory cytokines circulating in their immune system, this may prove to be a risk factor particularly if they have an established reproductive tract or systemic infection when conceiving. This could result in high levels of pro-inflammatory cytokines circulating at the reproductive tract. A recent study has shown that maternal infection, particularly urinary tract infection during pregnancy is associated with childhood asthma in the offspring (Collier et al., 2013). Chorioamnionitis has also been linked with preterm birth and was found to promote recurrent childhood wheezing, asthma, allergy and eczema in the offspring (Kumar et al., 2008, Rocha, 2013).

1.6.1.3. Diet

Diet is a factor that is often implicated in the link between pregnancy and fetal programming. Maternal obesity often results in low birth weight babies which have complications in later life (Ramsay et al., 2002). Obesity was found to be associated with changes in pro-inflammatory and immune-modulatory cytokine levels during pregnancy (Madan et al., 2009). A recent study by Hallam et al. revealed that maternal high-protein diet predisposes to increased fat mass in the resulting adult female offspring in rats (Hallam and Reimer, 2013). In a recent review, the effects of obesity on oocyte and embryo quality was highlighted where oocyte fertilisation rate was observed to decreased in obese women (BMI>30) (Robker, 2008). Embryo quality was found to be significantly reduced when compared between obese women and women of healthy weight (Carrell et al., 2001, Metwally et al., 2007).

Some studies have investigated the effect of maternal diet on pre-implantation embryo development where the mothers were given a control or low protein diet. One study looked at the embryos collected from different stages of development (from 2-cell to blastocyst stage), and malnourished mice were

found to produce fewer fertilised embryos with retardation in development to the blastocyst stage (Munoz and Bongiorni-Malave, 1979). In rats on low protein diets, the pre-implantation embryos were found to have significantly lower cell numbers in the ICM (during early blastocyst stage) and both TE and ICM (during late blastocyst stage) caused by slow embryo development (Kwong et al., 2000).

1.6.1.4. Stress

In recent years, stress has been found to be a critical factor that interferes with reproduction (O'Hare and Creed, 1995). It affects implantation and fetal growth, and can in turn lead to abortion occurring in women experiencing stress (Arck et al., 1995). Spontaneous abortions are one of the most common adverse reproductive outcomes in women. Social or environmental factors that increase stress perception are known to be important causes of spontaneous abortion. Studies have found that negative life events such as job stress increases the risk of medical disorders and induces spontaneous abortions in women with chromosomally normal embryos (Neugebauer et al., 1996). Stress is well known to alter the immune system and since abortions are linked with altered immune function (Khansari et al., 1990), this may be due to changes in reproductive tract cytokine production.

Maintenance of early pregnancy is mediated by hormones and endocrine-immune interactions. Progesterone is a stress-responsive hormone and its production is suppressed in the event of stress (Blois et al., 2004, Szekeres-Bartho et al., 2001). Progesterone is essential for implantation and maintenance of pregnancy and in part this may be mediated by actions of progesterone in regulating cytokines and immune cells. Several studies have revealed that insufficient progesterone results in abortions in rodents (Deanesly, 1973, Wiebold et al., 1986), while administration of progesterone derivative dydrogesterone can protect from stress-induced fetal loss (Joachim et al., 2003). It has been postulated that stress may exert effects on reproduction through shifting the immune response from T helper 2 (Th2) to T helper 1 (Th1) dominance during pregnancy in humans and mice. Exposure of pregnant mice to a stressor during the peri-implantation period was shown to induce activation of T cells, mast cells and macrophages and to result in increased secretion of TNFα (Arck, 2001). Prenatal stress has recently been shown to increase pro-inflammatory cytokine expression which exacerbates the inflammatory response to LPS in the male offspring (Diz-Chaves et al., 2013). Prenatal hypoxia-induced oxidative stress has also been found to be associated with behavioural alterations in the adult progeny (Sab et al., 2013).

1.6.1.5. Other factors

Other lifestyle factors that may affect cytokine production include factors such as alcohol consumption, smoking, drugs and medicine usage during pregnancy or before conception. Pregnant women are advised against drinking alcohol, smoking or taking drugs or certain medications in pregnancy as many studies have shown that these agents can endanger the health of the unborn baby. Alcoholic mothers are known to give birth to babies with fetal alcohol syndrome (FAS) and exposure to alcohol as early as during the pre-implantation period has been linked with later fetal death (Abel, 2006). *In vitro* studies have shown that brief exposure to ethanol alters gene expression in mouse blastocysts by inducing intracellular calcium signalling (Rout et al., 1997). Another study where ethanol was given to Swiss male mice before mating with untreated females was found to decrease the number of implanting embryos, suggesting sperm-associated effects leading to failure of blastocyst development or implantation (Rao et al., 1994).

Maternal smoking has been found to significantly inhibit the neonate's innate immune response to a variety of TLR ligands, affecting the production of antigen presenting cells (APC) derived cytokines. TNFα synthesis in response to Toll-like receptor 2 (TLR2) and Toll-like receptor 4 (TLR4) is attenuated in neonates exposed to tobacco smoking *in utero* (Noakes et al., 2006). Placental pathologies and reduced birth weight was associated with the lower oxygen-carrying capacity of smoking mothers and the consequence of nicotine and carbon monoxide from cigarette smoke (Jauniaux and Burton, 2007). High levels of cannabinoids, the principal biologically active component of marijuana, was found to inhibit blastocyst development, hatching and trophoblastic growth (Taylor et al., 2007).

Lastly, cytokine gene polymorphisms are also a factor regulating cytokine production and have been linked with variations between individuals in outbred populations on the level of cytokine production.

1.7. LIPOPOLYSACCHARIDE (LPS)

Lipopolysaccharide is a bacterial endotoxin produced by Gram-negative bacteria. LPS consists of a hydrophobic domain (lipid A), a core oligosaccharide and a distal polysaccharide (O-antigen) (Raetz and

Whitfield, 2002). LPS triggers cells to synthesise and release a cascade of inflammatory mediators which may progress to septic shock *in vivo* (Glauser et al., 1991). LPS triggers the activation of the innate immune system through induction of cytokine production when released from bacteria. LPS from *Escherichia coli (E.coli)* induces production of many pro-inflammatory cytokines such as TNFα, IL-1β and IFNγ in macrophages and many cell lineages (Hirschfeld et al., 2001). LPS is recognised by Toll-like receptors (TLRs). Studies have shown that TLR4 plays a crucial role in LPS-mediated signalling (Du et al., 1999) and mice deficient in TLR4 have a far less sensitive response to LPS (Poltorak and Xiaolong, 1998). LPS binding is facilitated by LPS-binding protein which binds it to CD14 on the target cell membrane and presents LPS to TLR4. This activates the intracellular signalling complexes comprised of MyD88, Toll/interleukin-1-receptor-domain-containing adaptor protein (TIRAP), Toll interacting protein (Tollip) and p85 (Golenbock and Fenton, 2001). In turn this leads to nuclear translocation of NFκB and transcription of cytokine-encoding genes, which results in cytokine production. LPS has been shown to elicit strong immune responses in mice and is often used to mimic for Gram-negative infection, including intrauterine infection. Intrauterine or systemic treatment with LPS has been found to elevate pro-inflammatory cytokines, induce preterm labour (Robertson et al., 2006) as well as miscarriage and intrauterine growth restriction (IUGR) depending upon the time of gestation at which it is administered (Aisemberg et al., 2010, Arce et al., 2012, Robertson et al., 2007).

1.7.1. LPS effect on cytokines

The presence of LPS causes an inflammatory response and up-regulates TNFα and IFNγ levels in the body. TNFα is secreted and produced by macrophages and uterine epithelial cells while IFNγ is secreted and produced by T cells and NK cells. However as there are few T cells and NK cells within the oviduct and the uterus in non-pregnant and early pregnant mice, the pro-inflammatory TNFα is the main LPS-induced cytokine produced in the reproductive tract. In response to the LPS challenge, anti-inflammatory mediators, including IL-10 production is induced in order to terminate or dampen this inflammatory response. As mentioned before, maternal exposure to LPS results in increased TNFα, IL-1β and IL-6 levels in the placenta. These cytokines may cross the placenta and enter the fetal circulation. It was postulated that their occurrence may target and further activate fetal immune cells, resulting in the production of more cytokines within the fetus itself (Ashdown et al., 2006, Pfeffer, 2003). These changes in the cytokine environment surrounding the fetus can result in altered fetal growth and development. A recent study has found that IL-15 is required for maximal LPS-induced abortion (Lee et

al., 2013), indicating that IL-15 or IL-15 dependent NK cells may play a role in mediating LPS-induced resorption. Lee et al. demonstrated that mice deficient in the IL-15 cytokine (*Il15 $^{-/-}$ mice) are deficient in NK cells and are more resistant to LPS-induced abortion. This observation was supported by another study involving IL-15 transgenic mice, where over-expression of IL-15 leads to increased susceptibility to LPS-induced sepsis and decreased survival (Yajima et al., 2004).

1.7.2. Inflammatory response to LPS in pregnancy

LPS has been associated with adverse developmental outcome which includes intrauterine fetal death (IUFD) and IUGR. Pregnancy is a major challenge to the maternal immune system as both the mother and fetus have to be protected from any pathogenic attack. The immune system must also prevent immune-mediated rejection of the semi-allogenic fetus either by suppression or alteration of the maternal immune system to enable tolerance to be acquired to the fetus. Wegmann proposed that pregnancy requires a switch from the inflammatory Th1 immune response to a protective Th2 profile (Wegmann et al., 1993), while more recent studies have implicated regulatory T cells (Treg cells) as having a key role (reviewed in (Guerin et al., 2009)).

Maternal infection during pregnancy represents one common form of inflammatory stress that has long term consequences for the fetus. LPS has been shown to induce permanent neuroendocrine changes via release of cytokines IL-1β, IL-6 and TNFα following prenatal (Gotz et al., 1993) and neonatal exposure (Shanks et al., 1995). Apart from an inflammatory response, maternal exposure to LPS can elicit other changes in both mother and fetus such as fever, *in utero* hypoxia, hypertension and oxidative stress (Cambonie et al., 2004, Coumans et al., 2005, Dalitz et al., 2003), and is linked with fetal loss, preterm birth and IUGR (Robertson et al., 2006, Xu et al., 2006a). Presumably, LPS mediates its effects through increased production of pro-inflammatory cytokines or NK cells which can directly kill placental trophoblasts or otherwise impair placental function. It has been shown that maternal exposure to LPS increases the level of TNFα, IL-1β and IL-6 in the maternal blood (Chen et al., 2005) and in the placenta (Ashdown et al., 2006). A study by Vizi et al. demonstrated that pregnancy shifts the cytokine response to LPS (Vizi et al., 2001), with a substantial change in cytokine profile consisting of an increase in pro-inflammatory cytokines and decrease of IL-10 production. Pregnancy was also found to enhance LPS-induced mortality (Vizi et al., 2001). Pregnancy loss induced by LPS can be alleviated by

maternal administration of immunoregulatory cytokine IL-10 or by blocking TNFα activity after treatment with etanercept (Enbrel) in rodents (Renaud et al., 2011, Robertson et al., 2007).

1.8. PREGNANCY COMPLICATIONS

Pregnancy complications common in women include miscarriages, preeclampsia, IUGR and preterm birth. Pregnancies displaying signs of infections are characterised by elevated levels of pro-inflammatory cytokines including IL-1β, IL-6, IL-8 and TNFα (Christiaens et al., 2008). Inflammation has been implicated as the mechanism responsible for spontaneous preterm birth due to the presence of pro-inflammatory cytokines in the gestational tissues (Gotsch et al., 2008). Gotsch et al. reported increased concentrations of anti-inflammatory IL-10 in amniotic fluid of women delivering preterm and proposed that IL-10 plays a role in dampening inflammation, postulating that modulation of inflammation using anti-inflammatory cytokines or other factors can limit fetal injury and alleviate adverse fetal outcome (Gotsch et al., 2008).

1.9. EARLY ORIGINS OF ADULT DISEASE AND FETAL PROGRAMMING

Perturbations in the *in utero* environment have been linked with altered metabolic status and increased risk of disease in adult life. A study by Barker and his colleagues in 1989 first defined a clear association between low birth weight in humans and the development of adult hypertension (Barker et al., 1989). Since then a series of epidemiological studies have shown associations between low birth weight and the onset of adult diseases including hypertension and extending to obesity, type II diabetes mellitus, heart disease, kidney disease and the insulin resistance syndrome (reviewed in (Barker, 1998)). The relationship between birth weight and the development of adult disease has been termed the "fetal origins of adult disease". The fetal origin hypothesis or the "Barker hypothesis" proposes that *in utero* influences permanently alters the embryo or the fetus, resulting in low birth weight and that this in turns results in reduced development and chronic diseases in adult life. This concept is also termed fetal programming. The *in utero* maternal environment is a critical factor that controls fetal growth potential. Maternal undernutrition, through experimental approaches or food shortage, like that of the Dutch famine during World War II, has been correlated with low birth weight and subsequent changes in endocrine, metabolic and reproductive parameters in these offspring later in life, as well as predisposition to certain cancers (Roseboom et al., 2001, Kind et al., Desai et al., 2004, Robinson et al.,

1985, Elias et al., 2004, Elias et al., 2005a, Elias et al., 2005b, Kind et al., 1999, Belobrajdic et al., 2003, Kwong et al., 2000, Symonds, 2007).

Offspring experiencing a perturbed *in utero* environment may not show overt health problems during postnatal development. However, during adulthood, different growth and metabolic parameters can be revealed. The lifestyle patterns of affected individuals which can include risk factors such as smoking, alcohol, drugs and lack of exercise may exacerbate changes in endocrine and metabolic parameters, further modifying disease patterns in adult life. Many animal models now exist describing the development of metabolic syndromes using either maternal feed restriction early in pregnancy or by reducing the nutrient supply to the fetus via uterine blood vessel occlusion (Hayashi and Dorko, 1988) or placental insufficiency (Kind et al., 1995). A number of these animal models also exhibit placental insufficiencies associated with reduced nutrient transport to the fetus, suggesting alteration of placental development which subsequently impacts nutrient supply to the fetus (Roberts et al., 2001a, Roberts et al., 2001b). Exposure to air pollutants such as carbon monoxide, sulphur/nitrogen oxides and tobacco smoke during fetal development has been associated with adverse outcomes such as low/very low birth weight, preterm birth and intrauterine growth restriction (Wang and Pinkerton, 2007). Several studies in animal models and humans have implied that events occurring during the prenatal and early life play a role in the pathogenesis of diseases in adult life (Seckl, 1997). Different stressors during a particular developmental stage may result in changes in behavioural and hormonal responses to stress in adulthood.

1.9.1. Mechanisms of programming

The molecular mechanisms underpinning fetal programming are not clearly defined. Studies have only recently begun to uncover the possible mechanisms leading to permanent changes in short and long term physiology and pathophysiology. The possible mechanisms involve epigenetics, mitochondria and reactive oxygen species (ROS).

1.9.1.1. Epigenetic changes

The term 'epigenetic' refers to stable and heritable alterations in gene expression that are independent of physical changes to the DNA sequence. One of the most extensively studied methods of epigenetic

regulation is DNA methylation. DNA methylation is associated with gene expression or imprinting. Genomic imprinting is one of the examples of epigenetic regulation where one of the two parental alleles is repressed or 'silenced' by epigenetic modifications while the other is maintained in its active state. This method of regulation is entirely dependent on whether the gene is inherited from the maternal or paternal genome. At early stages of embryonic development, epigenetic modification of the conceptus can be influenced by environmental factors. Imprinted genes play important roles in embryo growth and development and placenta function (Fortier et al., 2008). Many imprinted genes have epigenetic marks laid down during oocyte growth and may be susceptible to epigenetic disruption following hormonal stimulation or changes. Some studies have shown that regulation of some imprinted genes can also be affected later in development by environmental factors. It was demonstrated that enzymes involved in the regulation of methylation and demethylation are controlled by dietary intake and folate availability (van Engeland et al., 2003). Maternal nutrition during fetal and placental development was found to be a factor that can influence the extent and pattern of epigenetic modification (Cooney et al., 2002). Superovulation has also been reported to affect growth and development of the early embryo, but its effects are not described in post-implantation embryos. A recent finding suggests that manipulating the pre-implantation embryo by superovulation alters the expression of imprinted genes in the mid-gestation of the mouse placenta (Fortier et al., 2008). Whether the epigenome of the mouse embryo is affected by local cytokine environment is unknown, but experiments in other cell lineages suggest this may be possible. Mishra et al. have shown that overexpression of pro-inflammatory IL-15 in human large granular lymphocytes results in chromosomal instability and DNA hypermethylation while El-Omar et al. showed that overexpression of IL-1 in human monocytes is associated with increased risk of gastric cancer (El-Omar et al., 2000, Mishra et al., 2012).

1.9.1.2. Mitochondria

Mitochondria are membrane-bound organelles consisting of a permeable outer mitochondrial membrane where molecules up to 10kDa can pass and an impermeable inner membrane which contains all enzymes involved in the respiratory chain (Wilding et al., 2009). In oocytes and embryos, the selection of mitochondria for transmission to the offspring occurs through a genetic 'bottleneck' at the oocyte stage of development (Shoubridge and Wai, 2007). This mechanism appears to eliminate poor quality mitochondria and prevent them from being passed to offspring. Mitochondrial abnormalities have also been suggested as mechanisms affecting fetal programming, as the mitochondria are important for many metabolic processes such as glucose transport and other cellular metabolisms. Perturbations that

occur in the mitochondria will affect the cellular processes in the mitochondria and possibly affect resulting embryo viability or development. Defects in mtDNA are maternally inherited as mitochondria in the sperm are destroyed at the time of fertilisation. Studies have shown that mitochondrial abnormalities occurring due to environmental stressors such as *in vitro* embryo culture (McConnell and Petrie, 2004, Taylor et al., 2005). It has been shown that the mitochondrial malate-aspartate shuttle regulates mouse embryo nutrient consumption (Lane and Gardner, 2005) and disruption of the activity of this particular shuttle in mouse blastocysts results in impaired viability and fetal growth (Mitchell et al., 2009). The offspring of rats fed on a high fat diet were reported to not only have impaired glucose homeostasis, but also have a decreased mitochondrial number accompanied by a decrease in the expression of mitochondrial specific genes (Taylor et al., 2005).

1.9.1.3. Reactive oxygen species

Reactive oxygen species (ROS) are free radicals that are the by-product of normal physiological reduction-oxidation reaction carried by eukaryotic cells. ROS are involved in many cellular and physiological functions, including proliferation, differentiation and apoptosis (Covarrubias et al., 2008). Common ROS includes nitric oxide (NO), superoxide (O_2^-) and hydrogen peroxide (H_2O_2). Oxidative stress occurs when the ROS levels exceed threshold levels and begin to impair physiological functions. Several studies have linked IUGR and increased ROS in the fetus (Karowicz-Bilinska et al., 2002, Raab et al., 2009). More studies have reported that oxidative stress appears to cause long term impairment to the cardiovascular system. Offspring of nutrient-restricted rats developed hypertension due to increased oxidative stress in the heart as well as ROS-mediated damage in the heart (Franco Mdo et al., 2002). ROS was also found to be involved in LPS-induced intrauterine growth restriction and skeletal growth retardation (Xu et al., 2006b).

1.9.2. Consequences of programming

Long term consequences of fetal programming caused by nutritional or endocrine perturbations have been shown to lead to postnatal diseases in adult life. Current literature regarding the impact of maternal diet and lifestyle patterns during pregnancy on the offspring has unveiled much information on fetal programming. Various animal studies, particularly in rodents, have clearly demonstrated the implications of maternal dietary protein supplementation during pregnancy for programming of adverse

metabolic-related outcomes such as hypertension (Thone-Reineke et al., 2006), heart disease (Fernandez-Twinn et al., 2005) and diabetes (Petry et al., 2001). Although the implications of maternal diet supplementation before and during the time of conception is still unclear, several studies suggests that programming effects of the offspring's growth and health can be influenced during early pre-implantation embryo development in the uterus (Kwong et al., 2000). This has increased awareness that the pre-implantation period is an important stage of development (Boerjan et al., 2000) when perturbations may affect the capacity of the conceptus to receive nutrition or respond to environmental signals or stress.

The significance of programming in the peri-conceptional period is important as there are substantial implications for humans and animals. Assisted reproduction technologies (ART) have been introduced to overcome reproductive failure in humans and also to increase the number of offspring in livestock. However, IVF offspring have been found to show signs of impaired growth and development (D'Souza et al., 1997) as well as increased incidence of health problems during postnatal life (Kallen et al., 2010, Scherrer et al., 2012). There are many factors that can contribute to these adverse outcomes as ART conditions vary greatly from natural conception. One of the reasons might be that growth factors and cytokines that are natural in the body are not present during embryo development in ART techniques. The addition of the GM-CSF cytokine to the culture medium has been found to down-regulate protein and gene expression of apoptosis, heat shock and stress response genes in mouse and bovine embryos (Chin et al., 2009, Loureiro et al., 2011). Other consequences of programming such as genetic or pharmalogical ablation of specific embryotrophic ligands activating 1-o-phosphatidylinositol-3-kinase (PI3K) disrupts normal pre-implantation embryo development (O'Neill, 2008). LPS (Robertson et al., 2006, Shanks et al., 1995) and cytokines (Gotz et al., 1993, Sjoblom et al., 2005) are believed to be involved in programming the neuroendocrine and the autonomic nervous systems. Maternal obesity at conception is another factor that programs obesity in offspring (Shankar et al., 2008).

1.9.3. The role of cytokines in embryo programming

In the pre-implantation embryo, the cytokine milieu to which the embryo is exposed during the first four days of its life has the potential to influence embryo development. Additionally, embryo-derived cytokines act as messengers to send signals between the embryo and the maternal tissues. Any perturbation during the pre-implantation period might thereby cause adverse outcomes in later life as

the embryo is more vulnerable to developmental programming and its epigenome is easier to manipulate than in the fetus. One example of cytokine action on the pre-implantation is the cytokine GM-CSF, where pre-implantation embryo exposure to GM-CSF was observed to impact later fetal development in utero, as well as the viability of offspring and their growth trajectory postnatally (Sjoblom et al., 2005). A recent study has found that the maternal cytokine response to LPS during inflammation in the peri-conceptional period can program the pre-implantation embryo, leading to permanent, sex-specific changes in the offspring (Williams et al., 2011). Other examples include studies involving prenatal exposure to elevated IL-6 and TNFα, which result in obesity particularly in female adult offspring (Lambin et al., 2006, Dahlgren et al., 2001). However, the precise mechanism by which cytokines act to program later fetal development during the pre-implantation period is still unclear. Key questions are (1) the extent to which cytokines impact the embryo directly versus female reproductive tract receptivity to implantation, (2) the importance of epigenetic pathways in the embryo, and (3) whether programming acts directly within the developing fetus or indirectly through the placenta. Therefore more research is needed to understand how programming is elicited within the pre-implantation period, and particularly the role of cytokines as key signalling agents.

1.10. SUMMARY

There is substantial evidence showing the importance of cytokines in facilitating the pre-implantation embryo's competence for optimal development in pregnancy. The processes known to be influenced by cytokines in the reproductive tract including embryonic development, implantation and trophoblast invasion are critical for a successful pregnancy. The significance of cytokine perturbation during early pregnancy has not been thoroughly investigated. The cytokine milieu is speculated to contribute to altered gene expression with lasting consequences for development, or to epigenetic programming in pre-implantation embryos (Sjoblom et al., 2005). However, the extent of the effects of environmental insults on embryo development and fetal programming mediated via cytokines are still unclear, particularly as many relevant studies have involved cytokine perturbations *in vitro*, as opposed to *in vivo*. Thus, whether this can occur in the physiological situation to affect the embryo, placenta and offspring is unknown. Therefore, better knowledge of the cytokine environment that the pre-implantation embryo is exposed to and how this is affected by maternal stressors such as infection, inflammation or diet, or diseases such as diabetes, is required. For example, it might be that common factors such as these can alter the *in utero* cytokine environment and exert programming effects on embryos. If correct,

this might help explain how altered reproductive events are contributing to the high incidence of metabolic disorders in Western society. This knowledge will also support the advancement of cytokine-supplemented culture medium in ART technologies. The known and proposed role of cytokines during the pre-implantation period is illustrated in Figure 1.2.

1.11. HYPOTHESES

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

Perturbation to the cytokine environment experienced by the embryo *in vivo*

    i.   affects cell viability and implantation competence in the blastocyst;

    ii.   programs post-implantation fetal and placental development, and

    iii.   programs post-implantation growth and phenotype in later life.

1.12. AIMS

The experiments described in this thesis will address the following experimental aims:

- To identify the effects of systemic maternal LPS administration on embryo development *in vivo*

- To identify the effects of systemic maternal LPS administration and IL-10 genotype on fetal and placental development

- To investigate any direct effect of LPS and IL-10 on pre-implantation embryo development *in vitro*

- To identify the effects of systemic maternal LPS administration and IL-10 genotype on cytokine gene expression in the oviduct and uterus

- To utilise embryo transfer to investigate whether the effects of LPS-induced cytokine changes are mediated via effects on the pre-implantation embryo, uterine receptivity, or both

- To identify the effects of systemic maternal LPS administration and IL-10 genotype during fetal life on postnatal development after birth

- To identify the effects of systemic maternal LPS administration and IL-10 genotype on progeny immune response to LPS challenge
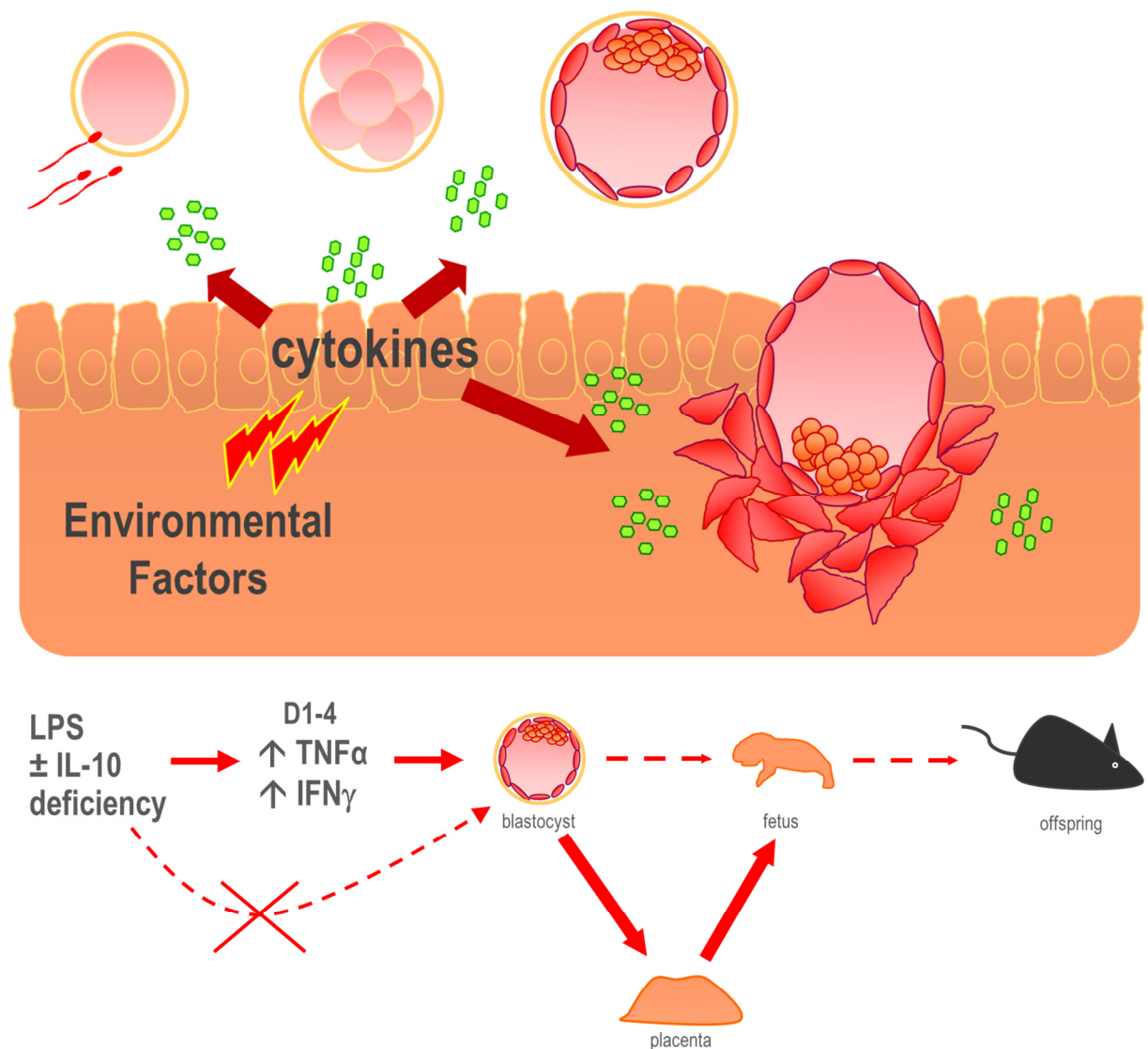
Figure 1.2   Schematic illustration of the proposed role of cytokines as mediators of environmental insult during the pre-implantation period.

The illustration depicts the proposed mechanisms following maternal perturbation to LPS, an inflammatory stimulus during the pre-implantation period. Maternal systemic inflammation is hypothesised to result in embryo exposure to elevated levels of pro-inflammatory cytokines in the maternal reproductive tract, which are expected to indirectly affect embryo development. Exposure to adverse pro-inflammatory cytokines during the pre-implantation period may program later changes in fetal and placental development or postnatal development during adult life. Also hypothesised is that in the absence of the anti-inflammatory IL-10 cytokine, the effect of programming due to maternal perturbation is more adverse than in wild-type mice.

Chapter 2

Materials and methods

2.1. ANIMALS AND SURGERIES

2.1.1. Mice

Il10 null mutant mice were generated by targeted mutation of the *Il10* gene in 129/Ola embryonic stem cells, propagated on a C57Bl/6 background (*Il10* $^{-/-}$) (Kuhn et al., 1993). Null mutant status was confirmed in *Il10* $^{-/-}$ mice by PCR of DNA extracted from blood or tail tissue of adult mice (Figure 2.1). PCR primers diagnostic for the *Il10*-null mutation and the neomycin insertion cassette were as previously reported (Kuhn et al., 1993). *Il10* $^{-/-}$ mice and control C57Bl/6 mice (*Il10* $^{+/+}$) were bred in the University of Adelaide Medical School Animal House. CBA x C57Bl/6 F1 (CBA F1) mice and Swiss females were obtained from the University of Adelaide Central Animal House.
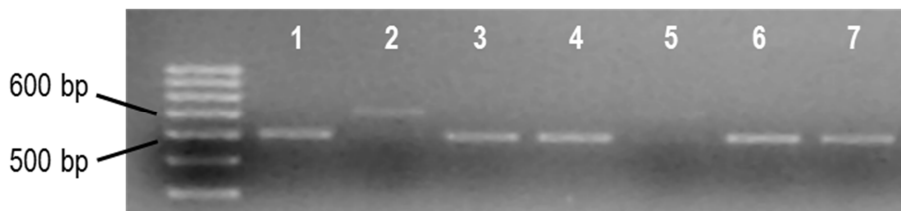
Figure 2.1 Gel image of PCR genotyping.

Genotype of *Il10* $^{-/-}$ mice was confirmed by PCR where single band at 500 bp denotes null mutants while double bands at 500 and 600 bp denotes heterozygote mice.

All mice used in these studies were housed in the University of Adelaide Laboratory Animal Services at the Medical School Animal House in a specific pathogen-free facility. Food and water were provided *ad libitum* and a 12:12 h light-dark cycle was maintained within the enclosure. *Il10* $^{-/-}$ mice received broad-spectrum antibiotics (Oxymav 100: 100 g/kg oxytetracycline hydrochloride; Mavlab, Queensland, Australia) in autoclaved drinking water twice weekly at a concentration of 2 mg/ml to prevent colitis. Animals were maintained and used in experiments under the NHMRC Australian Code of Practice for the care and use of animals for scientific purposes.

All female mice were virgin prior to the onset of any experiment. All *in vivo* experiments utilised females between 8 and 10 weeks of age at the time of treatment. *In vitro* experiments utilised embryos from pre-pubertal CBA F1 females at 3 weeks of age. *Il10* $^{+/+}$ males were utilised for *in vivo* experiment and CBA F1 males (> 8 weeks in age) were utilised as stud males to generate embryos for *in vitro* experiments.

2.1.2. General procedures

All surgical instruments were sterilised by autoclaving, or were submerged in 70% ethanol (ANALAR, Melbourne, Australia) prior to use. For anaesthesia, mice received an intra-peritoneal (i.p.) injection of 15 μl/g body weight of 2% Avertin (tribromoethanol; Sigma-Aldrich, St. Louis, USA), Surgical incisions were closed using 9mm MikRon® wound clips (Becton Dickinson, Sparks, USA), which were removed 2 weeks following surgery. Following anaesthesia, mice were placed into cages kept on a 37ºC heating pad until the following morning. When mice regained consciousness, they were injected subcutaneously with carprofen analgesia (Rimadyl; Pfizer, New York, USA). Carprofen was administered at 0.05 mg/10 g of body weight.

2.1.3. Matings

Adult naturally cycling female mice were housed with a proven-fertile stud male. Both *Il10* $^{-/-}$ females and *Il10* $^{+/+}$ females were mated with *Il10* $^{+/+}$ studs and were checked each morning between 0900h and 1100h for the presence of a vaginal copulatory plug (2:1 female to male ratio). The day of sighting of a vaginal plug was day 0.5 of pregnancy (0.5 pc) and the females were separated from the males.

2.1.4. Lipopolysaccharide (LPS) treatment

Plugged females were allocated to three cohorts to allow the effects of peri-implantation lipopolysaccharide (LPS) (*Salmonella typhimurium*; Sigma-Aldrich) administration to be evaluated at different pregnancy time points; day 3.5 pc, day 17.5 pc and at parturition for offspring assessment. Within all cohorts, both *Il10* $^{+/+}$ and *Il10* $^{-/-}$ females were allocated to two treatment groups, a control (PBS) and LPS treated group. Mice were injected i.p. with LPS at 0.5 μg dose on both day 2.5 pc and day 3.5 pc. Additional doses of LPS at 2.5 μg, 12.5 μg, and 62.5 μg were used for preliminary *in vivo* experiments to evaluate the effect of LPS administration on embryo development *in vivo*. All LPS for injection was administered in 200 μl PBS + 0.1% BSA, from pre-prepared aliquots stored frozen at -20ºC.

A second group of pregnant *Il10* $^{+/+}$ and *Il10* $^{-/-}$ mice was given an additional treatment of a soluble TNFα receptor, etanercept (Enbrel®) (Wyeth, Baulkham Hills, Australia). Plugged females were given

three injections on both day 2.5 pc and day 3.5 pc. First i.p. injection was 100 μg etanercept in 200 μl PBS +0.1% BSA at 0900 h, followed by 0.5 μg LPS or PBS (as control) at 1100 h and the mice were injected again with 100 μg etanercept later at 1500 h. The effect of LPS with and without etanercept administration was evaluated at day 17.5 pc.

2.1.5. Vasectomies and Seminal Vesicle Removal

Male *Il10* ^{+/+} mice underwent vasectomy as previously described (Robertson et al., 1996). Briefly, mice were anaesthetised and a small lateral incision was made to expose the abdominal cavity. To generate vasectomised (VAS) males the vas deferens were ligated with 5/0 silk suture (Ethicon, Sydney, Australia), then bisected using scissors. Wounds were then sutured using 5/0 silk suture. Two weeks post-surgery vasectomised male mice were caged with normal cycling females to assess their ability to fertilise females. Mated females, detected by the presence of a vaginal plug, were observed for signs of successful pregnancy to determine if studs were fertile.

2.1.6. Embryo transfer

Il10 ^{+/+} females were mated to males to generate embryo donor females and Swiss female were mated to VAS males to generate pseudopregnant recipient females. Donor females were treated with either control PBS or 0.5 μg LPS. Embryos at the blastocyst stage were transferred from day 3.5 pc donors into day 2.5 pc recipients. Donor females were sacrificed by cervical dislocation and the uterus was collected and blastocysts were flushed out of both uterine horns. The blastocysts were washed briefly in MOPS handling media. The recipient female was anaesthetised, shaved in the dorsal region, and swabbed with 70% ethanol. A small longitudinal dorsal incision was made, followed by a retroperitoneal incision above the ovarian fat pad. The ovary, oviduct and the beginning of the uterus was pull out by grabbing the fat pad and clipped to the back of the mouse. Blastocysts were loaded into the transfer pipette and were transferred into each uterine horn near the uterine-oviductal junction and the reproductive tract tissue was returned to the peritoneal cavity with minimal physical manipulation of any part of the tract. The procedure was repeated on the other side and the skin was closed using wound clips.

2.2. IN VITRO EMBRYO CULTURE

2.2.1. Superovulation

CBA F1 females were primed at 1230 h with 5 IU equine chorionic gonadotropin (PMSG; Folligon, Intervet, Victoria, Australia) injected intra-peritoneally (i.p) followed 46-48 hours later with 5 IU human chorionic gonadotropin (hCG; Chorulon, Intervet) and then placed 1 female per cage with males. Females were checked for the presence of a copulatory plug the next morning and were separated from males immediately.

2.2.2. Growth media

Culture media used for embryo culture were G1.2 and G2.2 sequential culture media and MOPs handling media (prepared in house according to published formulations) (Gardner et al., 2004). See Table 7.1 for G1.2 and G2.2 media formulation and 7.2 for MOPS media formulation. Human serum albumin (HSA) (Vitrolife AB, Sweden) was added at 5 mg/ml to G1.2 media and 10 µg/ml to G2.2 media. Recombinant mouse IL-10 (rmIL-10) (R&D Systems, Minneapolis, MN) was added at 1 ng/ml to both G1.2 and G2.2 culture media. LPS (*Salmonella typhimurium*; Sigma-Aldrich) was added at 25 µg/ml, 2.5 µg/ml, 250 ng/ml and 25 ng/ml to both G1.2 and G2.2 culture media.

2.2.3. Embryo collection and culture

Superovulated mice were sacrificed by cervical dislocation at 1200 h on day 1 of pregnancy and the oviducts were excised. Single cell zygotes were recovered from the ampullary region of each oviduct and the cumulus cells removed by incubation in 500 U/ml hyaluronidase for several minutes (Sigma-Aldrich). Embryos were then washed in MOPS handling media before being allocated randomly to 4 treatment groups; a control group was cultured in G1.2 media only and 3 treatment groups were cultured in G1.2 media with 25 ng/ml LPS, 1 ng/ml rmIL-10, or both LPS and rmIL-10, from day 1 to 3. Embryos were then transferred to respective G2.2 media (control and treatment groups) from day 3 to 5.

Prior to culture, G1.2 and G2.2 sequential culture media was equilibrated at 37°C in 5% oxygen and 6% CO_2. Embryos were cultured in 20 µl droplets of culture media under mineral oil (Sigma-Aldrich). Pulled glass Pasteur pipettes (Chase Instruments, New York, USA) were used to place 10 embryos into each

droplet of equilibrated culture media. Embryo development was assessed by standard morphological criteria daily (Hogan et al., 1986) and on the morning of day 5, blastocysts were used for cell number analysis by differential staining. A preliminary dose response experiment using LPS (25 ng/ml – 25 μg/ml) was performed first to ensure the LPS dose used for treatment *in vivo* experiment did not exert direct detrimental effects towards embryo development *in vitro*.

2.2.4. Differential staining

Numbers of trophectoderm (TE) and inner cell mass (ICM) cells were determined in blastocyst stage embryos on day 4.5 pc. The zona pellucida of the blastocyst stage embryos were removed by brief incubation with 0.5% pronase (Sigma-Aldrich), before incubation with 10 mM trinitrobenzenesulfonic acid (picric sulfonic acid, Sigma-Aldrich) for 10 min at 4°C, followed by incubation for 10 minute in 0.1 mg/ml anti-dinitrophenyl (Sigma-Aldrich)-BSA at 37°C. The TE cells with bound antibody were subsequently lysed by incubation with complement (C') present in guinea pig serum (IMVS, Adelaide, Australia). Nuclei of lysed TE cells were then labelled by incubation with 10 μg/ml propidium iodide (Sigma-Aldrich) for 5 minute at 37°C in the dark, while ICM and TE cells were further labelled with 6 μg/ml bisbenzimide (Sigma-Aldrich) in ethanol overnight. On the following day, embryos were washed in 100% ethanol, mounted in glycerol, and visualized under the ultraviolet filter of a fluorescence microscope. The number of TE cells (pink) and ICM cells (blue) was determined and expressed as absolute numbers and a ratio.

2.2.5. Mouse blastocyst collection, differential staining and tissue freezing

Naturally mated *Il10* −/− and *Il10* +/+ female mice were sacrificed by cervical dislocation on day 3.5 pc. The uterus was collected and blastocysts were flushed out of both uterine horns with MOPS handling media. The flushed blastocysts were used for cell number analysis by differential staining. Oviduct and uterus tissue were also collected, snap frozen in liquid nitrogen and stored at -80°C.

2.2.6. Assessment of day 17.5 pc pregnancy outcomes

On day 17.5 pc mice were killed by cervical dislocation between 1000 h – 1200 h. The uterus from each female was removed, and the number of viable, dead and resorbing implants was counted. An

implantation site included both viable and non-viable implantations. A pregnant mother was defined as a mother carrying implantation sites (either viable or non-viable). Each viable fetus was dissected from the amniotic sac and umbilical cord; fetal and placental weights were recorded and expressed as absolute numbers and a ratio.

2.3. OFFSPRING ASSESSMENT

2.3.1. Offspring cohort

Plugged females allocated to this cohort continued their pregnancy to term where the number of pups born, length of gestation and birth weight was recorded. All offspring from each female were weaned on day 21 and the littermates of the same sex were housed together. The offspring were weighed on day 1, day 8, and day 21 and on every second week after weaning to 19 weeks of age.

2.3.2. Dual-energy X-ray absorptiometry (DEXA)

All male and female offspring of each mother underwent DEXA scanning at 7, 13 and 19 weeks of age. Each individual mice was anaesthetised (Avertin; Sigma-Aldrich) and was laid on the DEXA equipment to be scanned. The DEXA machine generates a measurement of the bone mineral density (BMD), bone mineral composition (BMC), the total lean mass, total fat mass and overall body tissue mass as well as the percent fat (% fat) of the whole mice or selected region of interest.

2.3.3. Offspring inflammatory response to LPS

At 18 weeks of age, male and female offspring of different mothers from all 4 treatment groups were injected i.p. with 100 μg/kg LPS at 0900h. Two hundred microlitres of blood was collected retro-orbitally at 1230h from anaesthetised mice (Avertin; Sigma-Aldrich) using a glass capillary tube containing approximately 20 μl (1.2 USP units) of heparin (300 USP units per vial of heparin sodium salt diluted in 5 ml PBS; Sigma-Aldrich). Blood was left at 4°C overnight before being centrifuged at 1500xg for 10 min to recover plasma. Plasma samples were stored at -80°C for Luminex multiplex analysis of cytokine content.

2.3.4. Full body post mortem

At 19 weeks, after the final DEXA scan, each offspring was anaesthetised (Avertin; Sigma-Aldrich). Approximately 1 ml of blood was collected by cardiac puncture from anaesthetised mice (Avertin; Sigma-Aldrich) using a 20G needle (BD Biosciences) containing approximately 50 μl (3 USP units) of heparin (300 USP units per vial of heparin sodium salt diluted in 5 ml PBS; Sigma-Aldrich) before the mice was sacrificed by cervical dislocation for a full body post mortem. Blood was left at 4°C overnight before being centrifuged at 1500xg for 10 min to recover plasma. Plasma samples were stored at -80°C for later analysis. The following tissues were the excised and weighed individually; brain, heart, thymus, kidney (left hand and right hand), adrenal gland (left hand and right hand), liver, spleen, uterus, ovaries (left hand and right hand), testicles (left hand and right hand), parametrial fat, epididymal fat, peri renal fat, retroperitoneal fat (including mesenteric fat), epididymis and seminal vesicle glands. The bicep and triceps (left hand and right hand) and the quadriceps and gastrocnemius (left hand and right hand) were taken as representative samples of the muscle tissue. Parametrial and epididymal fat tissues were fixed in 4% paraformaldehyde (Sigma-Aldrich) for further histological analysis.

2.4. QUANTITATIVE REAL TIME – POLYMERASE CHAIN REACTION

2.4.1. RNA Isolation

Whole uterine horns were dissected on day 3.5 pc under RNAse free conditions, the oviducts, ovaries and uterus were removed separately to be snap frozen in liquid nitrogen and stored at -80°C until processing. Total oviduct or uterus RNA extraction was performed by adding 250 μl of Trizol (Invitrogen, Carlsbad, USA) to tubes containing 1.4 mm ceramic beads (Geneworks, Adelaide, Australia). Oviducts or uterus tissue were added and the Precellys (Bertin Technologies, Montigny le Bretonneux, France) was used to homogenise tissue (2 x 15 s). Fifty microlitres of chloroform (Unilab, Ajax Finechem, Tarenpoint, Australia) was then added and samples were gently mixed by hand. Samples were then centrifuged at 12000xg for 15 min at 4°C. Approximately 120 μl of the aqueous RNA phase was removed and placed into a fresh tube. An equal volume of isopropanol (Sigma-Aldrich) was added and 0.5 μl of glycogen (Invitrogen), and the sample was precipitated overnight at -20°C. The following morning, samples were pelleted at 16000xg for 30 min at 4°C. The pellet was then washed twice with ice-cold 75% ethanol and centrifuged at 12000xg for 15 min at 4°C. Excess ethanol was removed and the pellet was air dried for 30-45 min before being resuspended in 20 μl of RNAse free Milli-Q water.

Samples were then treated with DNAse to remove contaminating DNA; using reagents supplied in a TURBO DNA-free kit (Life Technologies, Applied Biosystems, Carlsbad, USA) according to the manufacturer's instructions. Two microlitres of 10x DNAse I buffer and 1 µl of DNAse I were added to each sample and incubated for 30 min at 37oC. Following this, 5 µl of DNAse Inactivating Reagent was incubated with each sample for 2 min. Samples were centrifuged at 12000xg for 2 min at 4oC, and the RNA-containing supernatant was transferred to a clean tube. Extracted RNA was quantified using a NanoDrop Spectrophotometer (Thermo Scientific, Wilmington, USA).

2.4.2. Reverse Transcription, cDNA generation and qRT-PCR

First-strand cDNA was reverse-transcribed from 2 µg RNA using Superscript III (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions, with 200 ng random sequence oligohexamers (Geneworks, Adelaide, Australia) and 500 ng oligo dT_{18} (Proligo, Lismore, Australia) at 52oC for 1 hour, using a GeneAmp PCR System 2700 thermocycler (Applied Biosystems, Foster City, CA). PCR reactions were performed in a final volume of 20 µL, containing 10 µL of SYBR Green, 6 µL of H_2O, 1 µL each of 1 µM forward and reverse primers (see Table 2.1 for list of all primers) and 2 µL of cDNA template or water (negative/non-template control). For each gene of interest, the relative mRNA expression was calculated by comparison with standard curves generated from serial dilutions of pooled placental cDNA, normalised to housekeeper gene, *Actb*. PCR conditions were: 10 minutes at 95oC followed by 40 cycles of 95oC for 15 seconds and 60oC for 45 seconds, using a Rotorgene 6000 (Corbett Life Sciences, Sydney, Australia). After DNA amplification, PCR products were subjected to High Resolution Melt (HRM) analysis.

2.4.3. Oligonucleotide primer design

Oligonucleotide primer pairs were designed using Primer Express version 2 software (Life Technologies, Applied Biosystems). Messenger RNA sequences were downloaded from the Entrez nucleotide database, accessible from the National Centre for Biotechnology Information website (http://www.ncbi.nlm.nih.gov). All primers were purchased from Geneworks (Adelaide, Australia). A list of all sequences for target genes analysed, including product size and Genbank accession number are provided in Table 2.4. Primer specificity was determined by gel electrophoresis (2% agarose gel;

Promega, Madison, USA) to confirm the correct product size and analysis of the dissociation curve and gel electrophoresis was used to exclude the formation of primer dimers or non-specific products.

Table 2.1   PCR primers for RT-PCR analysis

Target mRNA	Primer sequence	Product size (bp)	Genbank
Il6	5' ACAACCACGGCCTTCCCTAC	131	NM_031168
	3' TCCACGATTTCCCAGAGAACA		
Tnfa	5' GTAGCCCACGTCGTAGCAAAC	118	NM_013693
	3' CTGGCACCACTAGTTGGTTGTC		
Il1a	5' CCGACCTCATTTTCTTCTGG	104	NM_010554.4
	3' GTGCACCCGACTTTGTTCTT		
Il1b	5' CCCAAGCAATACCCAAAGAA	133	NM_008361.3
	3' GCTTGTGCTCTGCTTGTGAG		
Ifng	5' GCGTCATTGAATCACACCTG	261	NM_008337.3
	3' TGAGCTCATTGAATGCTTGG		
Il10	5' AGGCGCTGTCATCGATTTCT	103	NM_010548.2
	3' TGGCCTTGTAGACACCTTGGT		
Lif	5' CGCCAATGCTCTCTTCATTTC	113	NM_008501
	3' TCCGATGCTCCACCAACT		
Csf2	5' CCTGGGCATTGTGGTCTACAG	117	NM_009969.4
	3' GGCATGTCATCCAGGAGGTT		
Il12a	5' CTCCTGTGGGAGAAGCAGAC	342	M86672
	3' CAGATAGCCCATCACCCTGT		
Il12b	5' TGACACGCCTGAAGAAGA	366	M86671
	3' AGAGACGCCATTCCACAT		
Actb	5' GTGTGACGTTGACATCCGTAAAG	151	M12481
	3' CTCAGGAGGAGCAATGATCTTGAT		

2.5. ASSAYS

2.5.1. Luminex

Assistance from an experienced laboratory member (Dr David Sharkey, University of Adelaide) was employed in performing the Luminex assay to avoid any operator error. The assay consist of a 96-well plate Milliplex Map Mouse Cytokine/Chemokine Panel kit (Millipore, MA, USA) which tests for 14 specific analytes (GM-CSF, IL-1α, IL-1β, IL-6, IL-10, IP-10, KC, LIF, MCP-1, M-CSF, MIP-1α, MIP-1β, RANTES and TNFα). Preparation of mouse cytokine standard was prepared according to the manufacturer's instruction. All reagents were allowed to warm to room temperature (20 – 25°C) before use in the assay. The filter plate was prewetted by pipetting 200 μl of Wash Buffer into each well of the Microtiter Filter Plate. The plate was sealed and placed in the plate shaker for 10 minutes at room temperature. The Wash Buffer was removed by vacuum and the excess buffer was blotted using absorbent pads or paper towels. Twenty five microlitres of each Standard or Control was added into appropriate wells (Assay Buffer used for the background 0 pg/ml standard) and 25 μl of Assay Buffer was added to the sample wells. Twenty five microlitres of the serum matrix solution was added to the background, standards and control wells before adding 25 μl of sample into the appropriate wells. The Mixed or Premixed Beads were vortex in the Mixing Bottle before adding 25 μl of the beads into each well. The plate was then sealed and covered before incubated with agitation on a plate shaker for 2 hours at room temperature. The fluid was gently removed by vacuum and washed twice with 200 μl/well of Wash Buffer. Twenty five microlitres of Detection Antibodies was added into each well before sealing and covering the plate to be incubated with agitation on the plate shaker for 1 hour at room temperature. 25 μl of Streptavidin-Phycoerythrin was added to each well containing 25 μl Detection Antibodies before sealing and covering the plate to be incubated with agitation on the plate shaker for 30 minutes at room temperature. All contents were then gently removed by vacuum and washed twice with 200 μl/well of Wash Buffer. One hundred fifty microlitres of Sheath Fluid was added to all wells and the beads were resuspended on a plate shaker for 5 minutes before running the plate on the Luminex 200™ System (Luminex Corporation, TX, USA). The Median Fluorescence Intensity data was analysed using a 5-parameter logistic or spline curve-fitting method for calculating cytokine/chemokines concentrations in each sample.

2.6. IMMUNOHISTOCHEMISTRY

2.6.1. Tissue collection, paraffin embedding and sectioning

Epididymal fat tissue from male offspring and parametrial fat tissue from female offspring at 19 weeks were collected and fixed in 4% paraformaldehyde (Sigma-Aldrich) in PBS (pH 7.4) for 24 h at 4°C. Tissue was then washed three times in 1 x PBS over the following two days, and late on the second day, tissue was transferred to a 70% ethanol solution where it remained until tissue processing. Tissue was processed and embedded using the Leica TP1020 Tissue Processor (Leica Microsystems) involving the following dehydration and embedding protocol; 1 h 75% ethanol, 1 h 85% ethanol, 1 h 90% ethanol, 1 h 96% ethanol, 2 x 1 h absolute ethanol (ANALAR), 2 x 1 h 100% Xylene (Ajax Finechem), 2 x 1 h paraffin wax (Ajax Finechem) under vacuum conditions. Tissue was moulded immediately into wax blocks and stored for sectioning on a Leica Rotary Microtome (Leica Microsystems). Sections were cut into 6 µm sections and fixed onto SuperFrost Plus slides (Menzel-Gläser) using a 45°C water bath. Slides were dried overnight at 37°C. Blocks and sections were stored at room temperature prior to staining.

2.6.2. Tissue staining

Epididymal and parametrial fat sections from respective male and female offspring were stained using toluidine blue. Three sections, each 90 µm distance apart, from each offspring were chosen for staining. Tissue sections were dewaxed in Safsolv (Ajax Finechem, Tarren Point, Australia) and dehydrated through graduated dilutions of ethanol for 5 minutes each (2 x 100%, 1 x 90% and 2 x 70% ethanol) and followed by 1 x 5 minutes in MQ water. The sections were stained in 0.1% toluidine blue for 5 minutes and washed three times in MQ water and then rehydrated through a graduated increase in ethanol concentration (the reverse of dehydration protocol) and cleared in two changes of Safsolv (Ajax Finechem) for 5 minutes. The slides were dried and then mounted with coverslips using DPX mountant (Merck, Darmstadt, Germany).

2.6.3. Image analysis and quantification of fat cell size

Stained tissue sections were captured as a digital image using a Nanozoomer 1.0 (Hamamatsu, Shizouka, Japan) at a zoom equivalent to a 40x objective lens. Adipose cell size was quantified utilising the image analysing software, ImageJ (NIH, Maryland, USA) analyse function. The images were

changed to greyscale and the threshold was adjusted to highlight the fat cells for quantification. A total of 600 – 700 adipocytes were counted per slide.

2.7. STATISTICAL ANALYSIS

Advice from a qualified biostatistician was employed to assist in selecting the statistical tests and interaction models for *in vivo* experimental outcomes (Thomas Sullivan, Department of Public Health, University of Adelaide). For data where progeny, fetus or tissues were independent repeated measures of a single treatment (mother), Mixed Models Analysis of Variance was used with the mother as the base subject. The model design compares the groups (4 levels according to two genotypes and two treatments) over time. To allow for differences in growth according to sex, two-way and three-way interactions between group, time and sex were fitted as fixed effects in the model. Litter size was added as a covariate to control for any differences in weight due to litter size. Random effects for mother and progeny within mother were also added to the model so as to control for the dependence in repeated measurements within mice and correlations between mice from the same litter.

Statistical analysis employed for *in vitro* experiments were χ^2-tests for embryo development outcomes compared as percentages while single outcome measures of cell counts were analysed using a one-way ANOVA. All data was presented as the mean ± SEM (standard error of mean) and analysed using SPSS PASW 18 software (SPSS Inc., Chicago, IL).

Chapter 3

Effect of low dose LPS during the pre-implantation period on embryo and fetal development

3.1. INTRODUCTION

As the pre-implantation embryo traverses the female reproductive tract and develops from the zygote to blastocyst stage, it experiences fluctuations in the composition of the surrounding maternal environment, including the availability of nutrients, growth factors and cytokines. The maternal oviduct and uterine environment are critical to the processes of embryonic development and implantation for a successful pregnancy. Growth factors and cytokines mediate signalling between the embryo and the maternal tract to modulate embryo development (Hardy and Spanos, 2002, O'Neill, 2008) and also influence the maternal immune system to induce tolerance and accommodate successful implantation (Orsi, 2008, Robertson et al., 2011). It has now been recognised that the environment existing within the reproductive tract during the pre-implantation period can have a profound effect on embryo development and subsequently affect fetal development and offspring health (Gluckman et al., 2008).

The fetal origins of adult disease (FOAD) or 'fetal programming' hypothesis links perturbations to the uterine environment experienced by the fetus *in utero* with profound consequences in postnatal growth trajectory and adult health (Barker and Clark, 1997). This hypothesis proposes that perturbations to the uterine environment can permanently alter the parameters of metabolic function in the fetus, resulting in an increased risk of progeny developing metabolic disorders including hypertension, type II diabetes and obesity after birth. Evidence supporting this hypothesis has been generated using maternal undernutrition models in mice (Watkins et al., 2008, Fleming et al., 2012) and rats (Kwong et al., 2004), where the result is progeny with a predisposition to adult metabolic disorders characterised by increased adiposity, hypertension, impaired glucose tolerance and altered cholesterol metabolism.

In vitro culture studies in mice have also provided a valuable understanding of the consequences of perturbing the environment surrounding the embryo. The pre-implantation embryo exhibits remarkable plasticity to adapt to the surrounding suboptimal conditions in an attempt to develop to blastocyst stage and even further in order to implant successfully, but this can often lead to adverse effects on further downstream stages of development. *In vitro* perturbations such as extended culture in suboptimal conditions has been shown to result in poor embryo development, altered gene expression such as imprinted gene *H19* and glucose transporter *Slc2a3* and restricted fetal development *in utero* (Lane and Gardner, 1997, Zander-Fox et al., 2010, Lane and Gardner, 2003). After birth, when offspring are exposed to a nutrient-rich diet, the result is accelerated postnatal growth, resulting in obesity, insulin

resistance and increased cardiovascular disease (Sjoblom et al., 2005, Watkins and Fleming, 2009, Watkins et al., 2007).

With the practice of assisted reproductive technologies (ART) being common in Western countries, children are born after *in vitro* fertilisation (IVF) involving hormonal stimulation and culture of pre-implantation embryo in media that do not mimic the normal *in vitro* conditions. The pre-implantation period corresponds with the period of development that occurs *in vitro* in many assisted reproductive techniques, including IVF and ICSI. Embryo culture in both human and animal models demonstrates that this process can result in many developmental changes in offspring generated by these techniques, including low birth weights, increased risks of birth defects and alterations in subsequent postnatal health (Boerjan et al., 2000, Hansen et al., 2005). Therefore, peri-conceptional maternal health would also contribute to ensuring a successful pregnancy and a healthy baby as the hormonal treatments and invasive procedures of ART would be stressful on the maternal environment, even without the additional stress to the embryo of culture *in vitro*.

One important potential stressor on the maternal environment in both natural conception and assisted reproductive technology would be the presence of infection in the reproductive tract. The experiments described in this chapter were devised to investigate the effects on embryo, fetal and placental development from low level maternal systemic inflammation induced by treatment with bacterial product lipopolysaccharide (LPS) during the pre-implantation period. An *in vivo* mouse model of maternal systemic inflammation was established, utilising LPS to elicit a pro-inflammatory cytokine response prior to implantation, specifically on days 2.5 and 3.5 pc. This model was studied in both wildtype *Il10* $^{+/+}$ and *Il10* $^{-/-}$ mice to investigate the effects of maternal deficiency in the anti-inflammatory cytokine IL-10 during LPS treatment. We hypothesised that a pro-inflammatory LPS challenge in the pre-implantation period elicits change in the embryo developmental trajectory that in turn alters fetal growth and postnatal health. Additionally we predicted this would not be due to direct effects of LPS on the developing embryo but rather the consequences of elevated pro-inflammatory cytokines synthesised in the oviduct and/or the uterus. This was addressed by *in vivo* and *in vitro* studies investigating the effects of LPS treatment on the embryo. Fetal and placental development was assessed just prior to the end of gestation on day 17.5 pc.

3.2. EFFECT OF MATERNAL INFLAMMATORY RESPONSE TO LPS DURING THE PRE-IMPLANTATION PERIOD ON *IN VIVO* EMBRYO DEVELOPMENT

There are no studies in the mouse that investigate the effect of low dose LPS given just prior to implantation on blastocyst development. Previous studies investigating the effect of LPS on the pre-implantation embryo development induce maternal inflammatory response on day 0.5 pc, which is at the zygote stage (Deb et al., 2005, Deb et al., 2004, Williams et al., 2011). A 'minimum dose' of 5 μg LPS was found to induce 100% embryonic loss (Deb et al., 2004) and increases DNA damage as embryo development progresses towards implantation (Jaiswal et al., 2009) while a low dose of 0.2 μg LPS was sufficient to reduce cell number in the inner cell mass (ICM) cells (Williams et al., 2011). Cell number is a sign of embryo health as the embryo requires a threshold number of cells for it to develop properly and below a certain number of cells, the result is implantation incompetence or miscarriage due to poor fetal growth (Tam, 1988). The total cell number and the inner cell mass cell and trophectoderm cell ratio (ICM:TE ratio) are well-accepted parameters to evaluate blastocyst development, especially given that the ICM cell population size correlates with implantation potential of the blastocyst (Leppens et al., 1996).

To determine the effect of maternal systemic inflammation during days 2.5 and 3.5 pc on embryo development, wildtype females were treated by i.p. injection of various doses of LPS, ranging from 0.5 μg to 62.5 μg per mouse. The blastocysts were flushed from the uterus and embryo development was assessed according to standard morphological criteria on day 3.5 pc, 4 hours after the second LPS treatment. Females treated with 0.5 μg LPS were observed to retain viable embryos, all at the blastocyst stage of development following LPS injection on both day 2.5 pc and day 3.5 pc in numbers that were comparable to control females (Table 3.1). Females treated with 2.5 μg, 12.5 μg and 62.5 μg LPS were found to have progressively reduced embryo viability. The proportion of mated females from which blastocysts were recovered was not changed at any LPS dose.

The proportion of embryos that were blastocyst stage in females treated with either 2.5 μg or 12.5 μg LPS were not significantly different from the control females, but the females treated with 62.5 μg LPS were found to have fewer embryos at the blastocyst stage (66% reduced vs. control).

The flushed blastocysts were differentially stained to determine the effects of LPS treatment on blastocyst cell number and allocation of the ICM and TE cells. Blastocysts flushed from females treated with 0.5 µg LPS showed no significant difference in either ICM or TE cell numbers to the blastocysts flushed from control PBS treated females (Table 3.2). However, blastocysts from the 2.5, 12.5 and 62.5 µg LPS treatment groups had reduced ICM cell numbers (33% reduced vs. control in the 2.5 µg LPS group, $p < 0.001$; 45% reduced vs. control in the 12.5 µg LPS group, $p < 0.001$; 42% reduced vs. control in the 62.5 µg LPS group, $p < 0.001$) (ANOVA, Sidak post-hoc test, Table 3.2). TE cell numbers were unaffected in all LPS treatment groups (Table 3.2). The total blastocyst cell numbers were unaffected in the 0.5 and 2.5 µg LPS group, but were reduced in the 12.5 and the 62.5 µg LPS group ($p < 0.05$, ANOVA, Sidak post-hoc test, Table 3.2). Alteration in cell numbers caused the ICM:TE cell ratio to be significantly reduced in blastocysts flushed from females treated with 2.5, 12.5 and 62.5 µg LPS groups (33% to 44% reduced vs. control, $p \leq 0.002$, ANOVA, Sidak post-hoc test, Table 3.2).

Table 3.1 **The effect of maternal LPS treatment and dose on incidence of viable embryos and number of blastocyst stage embryos flushed per female.**

Treatment	% female with viable embryos	Number of blastocyst stage embryos per female*
Control	100% (5/5)	6.0 ± 0.6
0.5 µg LPS	100% (5/5)	4.6 ± 0.2
2.5 µg LPS	80% (4/5)	5.0 ± 1.0
12.5 µg LPS	80% (4/5)	5.8 ± 1.6
62.5 µg LPS	80% (4/5)	2.0 ± 1.2 **

* All data expressed as mean ± SEM.

** $p < 0.001$ compared to control (χ^2-test).

 n = 5 females per treatment group.

Table 3.2 **The effect of maternal LPS treatment and dose on cell numbers and allocation to the inner cell mass (ICM) and trophectoderm (TE) in blastocyst stage embryos.**

Treatment	Total cell number*^	TE cells*^	ICM cells*^	ICM:TE*^
Control	53.7 ± 3.0[a]	39.3 ± 2.8[a]	14.4 ± 0.9[a]	0.40 ± 0.03[a]
0.5 µg LPS	49.5 ± 2.4[a]	36.0 ± 2.1[a]	13.4 ± 0.8[a]	0.39 ± 0.03[a]
2.5 µg LPS	46.8 ± 2.2[a]	37.2 ± 2.0[a]	9.6 ± 0.7[b]	0.26 ± 0.02[b]
12.5 µg LPS	43.6 ± 2.0[b]	35.8 ± 1.7[a]	7.8 ± 0.5[b]	0.22 ± 0.01[b]
62.5 µg LPS	42.6 ± 1.6[b]	34.3 ± 1.5[a]	8.3 ± 0.3[b]	0.25 ± 0.01[b]

* All data expressed as mean ± SEM.

^ n = 12-30 blastocyst stage embryos (flushed from 5 females per treatment group)

[a, b] Superscripts represent significant differences within columns at $p < 0.05$, one way ANOVA (Sidak post-hoc test)

3.3. EFFECT OF LPS IN CULTURE MEDIA ON PRE-IMPLANTATION EMBRYO DEVELOPMENT *IN VITRO*

The previous experiment showed that systemic LPS can have a detrimental effect on blastocyst development *in vivo*. To determine whether this was due to a direct effect of LPS on the embryo, embryos were cultured in the presence of LPS at a range of doses. The lowest 25 ng/ml LPS concentration used in the *in vitro* culture was estimated to approximate the lowest 0.5 μg LPS treatment administered in the *in vivo* study assuming an even distribution of LPS through the tissues of a 20 gram mouse. The highest 62.5 μg LPS treatment *in vivo* was calculated to be equivalent to an *in vitro* concentration of 3.125 μg/ml and the highest *in vitro* concentration of 25 μg/ml LPS is equivalent to a lethal dose of 500 μg in an adult mouse.

The embryos were cultured in increasing LPS concentrations from 25 ng/ml to 25 μg/ml and embryo development was assessed daily according to standard morphological criteria. Embryos cultured in LPS concentration of 25 ng/ml, 250 ng/ml and 2.5 μg/ml was observed to have no significant differences in the cleavage, morula and blastocyst stages of development when compared to control embryos cultured in media only (Table 3.3) where embryo development was normal and not affected by the presence of LPS in the media. On the other hand, embryos cultured in 25 μg/ml LPS were observed to be adversely affected in terms of development to the morula stage (15% more inhibited development vs. control, $p < 0.001$, χ^2-test, Table 3.3) and the blastocyst stage (19% more inhibited development vs. control, $p < 0.001$, χ^2-test, Table 3.3) although cleavage was not affected, compared to the control embryos.

The cultured embryos were differentially stained on day 5 of culture (blastocyst stage) to determine the effects of LPS added to the culture media on blastocyst cell number and allocation of the ICM and TE cells. Blastocysts cultured in culture media containing 25 ng/ml, 250 ng/ml or 2.5 μg/ml LPS showed no significant differences in the ICM and TE cell numbers when compared to control blastocysts cultured in culture media only (Table 3.4). However, blastocysts cultured with 25 μg/ml LPS had reduced TE cell numbers (39% reduced vs. control, $p < 0.001$, ANOVA, Sidak post-hoc test, Table 3.4). There was a slight reduction in ICM cell number but it was not significant. The total cell numbers in blastocysts were unaffected in the 25 ng/ml, 250 ng/ml and 2.5 μg/ml LPS treatment groups but were reduced in the 25 μg/ml LPS treatment group (33% reduced vs. control, $p < 0.001$, ANOVA, Sidak post-hoc test, Table

3.4). Alteration in cell numbers caused a significant reduction in the ICM:TE ratio in blastocysts from the 25 μg/ml LPS treatment group (43% increased vs. control, p = 0.26, ANOVA, Sidak post-hoc test, Table 3.4).

Table 3.3   **The effect of LPS concentration in culture media on embryo development *in vitro*.**

Treatment	% cleavage*	% morula*	% blastocyst*
Control	93.7 ± 3.5	90.5 ± 3.1	87.9 ± 1.8
25 ng/ml LPS	92.9 ± 2.7	90.9 ± 2.7	85.9 ± 11.7
250 ng/ml LPS	93.9 ± 2.7	90.9 ± 6.7	84.9 ± 8.6
2.5 μg/ml LPS	92.9 ± 2.7	88.9 ± 2.7	83.8 ± 4.8
25 μg/ml LPS	88.9 ± 6.1	74.8 ± 8.1**	69.7 ± 11.7**

* All data expressed as mean $\pm$ SEM, % morula and % blastocyst are relative to zygote number at onset of culture.

** $p < 0.001$ compared to control (χ^2-test).

^ n = 99 embryos per treatment group (in 3 replicate culture experiments)

Table 3.4   **The effect of LPS concentration in culture media on blastocyst stage cell numbers, allocation to ICM and TE in blastocyst stage embryos *in vitro*.***

Treatment	Total cell number*^	TE cells*^	ICM cells*^	ICM:TE*^
Control	94.1 ± 3.3[a]	72.4 ± 2.9[a]	21.7 ± 1.8[ab]	0.31 ± 0.03[a]
25 ng/ml LPS	88.3 ± 3.6[a]	65.7 ± 3.0[a]	22.6 ± 1.0[a]	0.36 ± 0.02[ab]
250 ng/ml LPS	86.0 ± 2.9[a]	65.8 ± 3.0[a]	20.3 ± 1.6[ab]	0.34 ± 0.03[ab]
2.5 μg/ml LPS	78.9 ± 4.1[a]	62.0 ± 3.7[a]	16.9 ± 1.0[b]	0.29 ± 0.02[a]
25 μg/ml LPS	63.4 ± 3.1[b]	44.5 ± 2.4[b]	19.0 ± 1.1[ab]	0.44 ± 0.03[b]

* All data expressed as mean ± SEM.

^ n = 23-32 blastocysts per treatment group (in 3 replicate culture experiments)

[a, b] Superscripts represent significant differences within columns at $p < 0.05$, one-way ANOVA (Sidak post-hoc test)

3.4. EFFECT OF LPS AND/OR IL-10 CYTOKINE ADMINISTRATION TO CULTURE MEDIA ON PRE-IMPLANTATION EMBRYO DEVELOPMENT *IN VITRO*

There have been many studies to investigate the effects and function of growth factors and cytokines on embryo development *in vitro* (Hardy and Spanos, 2002). To determine whether IL-10 can influence embryos directly, either on its own or in combination with LPS, additional *in vitro* experiments were performed.

Embryos were cultured in media containing either or both LPS (25 ng/ml) and recombinant mouse IL-10 (rmIL-10; 1 ng/ml) and assessed daily according to standard morphological criteria. Embryos cultured in culture media with LPS or with rmIL-10 cytokine showed no significant differences in progression to the cleavage, morula or blastocyst stages of development when compared to the control embryos cultured in culture media only (Table 3.5). There was a small decrease in the proportion that was at blastocyst stage but this did not reach statistical significance. Similarly, embryos cultured in culture media with both LPS and rmIL-10 cytokine had no significant differences in all three stages of embryo development (Table 3.5).

The cultured embryos were differentially stained on day 5 of culture (blastocyst stage) to assess the effects of LPS and rmIL-10 in the culture media on blastocyst cell number and allocation to the ICM and TE cells. The presence of either LPS or rmIL-10 or the combination of both in the culture media did not have any effect on the number of ICM and TE cells respectively when compared to culture media alone (Table 3.6). The ICM:TE ratio was similarly not affected in any of the three treatment groups.

Table 3.5 The effect of LPS and/or IL-10 cytokine addition to culture media on embryo development *in vitro.*

Treatment	% cleavage*	% morula*	% blastocyst*
Control	92.3 ± 5.5	87.2 ± 4.6	82.1 ± 5.8
25 ng/ml LPS	95.8 ± 0.9	88.1 ± 5.8	81.4 ± 7.6
1 ng/ml rmIL-10	90.7 ± 3.5	87.3 ± 0.6	77.1 ± 9.1
25 ng/ml LPS + 1 ng/ml rmIL-10	94.1 ± 4.3	91.5 ± 2.0	79.7 ± 6.0

* All data expressed as mean $\pm$ SEM, % morula and % blastocyst are relative to zygote number at onset of culture.

There are no significant differences between treatment and control groups (χ^2-test).

^ n = 118 embryos per treatment group (in 3 replicate culture experiments)

Table 3.6 The effect of LPS and/or IL-10 cytokine addition to culture media on cell numbers, allocation to ICM and TE in blastocyst stage embryos *in vitro*.

Treatment	Total cell number*^	TE cells*^	ICM cells*^	ICM:TE*^
Control	86.7 ± 3.6	68.2 ± 2.8	18.4 ± 1.5	0.27 ± 0.02
25 ng/ml LPS	83.7 ± 2.3	66.8 ± 2.1	16.8 ± 0.7	0.26 ± 0.01
1 ng/ml rmIL-10	86.5 ± 2.0	69.8 ± 1.9	16.8 ± 0.7	0.24 ± 0.01
25 ng/ml LPS + 1 ng/ml rmIL-10	84.6 ± 2.6	67.2 ± 2.1	17.4 ± 1.0	0.26 ± 0.01

* All data expressed as mean $\pm$ SEM.

There are no significant differences between treatment and control groups (one-way ANOVA).

^ n = 18-25 blastocyst stage embryos (in 3 replicate culture experiments)

3.5. EFFECT OF ADMINISTRATION OF LPS DURING THE PRE-IMPLANTATION PERIOD ON FETAL AND PLACENTAL DEVELOPMENT

No studies have investigated the effect of maternal systemic inflammation prior to implantation. Previous studies carried out in our laboratory showed that administering LPS to pregnant females on day 17 pc will induce preterm labour (Robertson et al., 2006). Studies in interleukin-10 knockout (*Il10 -/-*) mice have shown that while the IL-10 cytokine is not essential for normal pregnancy outcome (White et al., 2004), IL-10 does play a role in protecting pregnancy from the adverse effects of an inflammatory challenge with LPS in mid or late gestation (Robertson et al., 2006, Robertson et al., 2007). Our studies have also shown that the IL-10 cytokine regulates inflammatory cytokine synthesis to protect against LPS-induced abortion and fetal growth restriction in mice (Robertson et al., 2007).

To examine the effect of maternal LPS treatment and IL-10 deficiency on fetal and placental development, an initial dose response experiment was performed by a technical officer (Camilla Dorian, University of Adelaide) to determine the appropriate LPS dose for this model, where doses of 0.1 μg, 0.3 μg and 0.5 μg LPS or PBS were injected in both *Il10 +/+* and *Il10 -/-* females mated to males of the same genotype on days 2.5 and 3.5 pc. The females were examined on day 17.5 pc, where the total implantation sites and resorbing implantation sites were counted, and the weights of every individual fetus and placenta were measured. The fetal:placental ratio is a measure of placental efficiency where a higher ratio equals to a more efficient placenta; thus larger placentas are more inefficient than smaller placenta at transporting nutrients to the fetus. Pregnancy viability was assessed by observing the proportion of plugged females following mating that progressed to viable pregnancies in late gestation.

While pregnancy viability was not affected by all three LPS doses in the *Il10 +/+* females, pregnancy viability was severely reduced by 63% the 0.3 μg LPS dose in the *Il10 -/-* females when mated to *Il10 -/-* males while no viable pregnancy was observed in the 0.5 μg LPS treatment group. Similar increased susceptibility to LPS was observed in the fetal weights, where in *Il10 +/+* females were reduced by 10% in the 0.5 μg treatment group while fetal weights of *Il10 -/-* females fetal weight was reduced by 22% in the 0.3 μg treatment. Thus the combination of both maternal and fetal IL-10 deficiency was found to severely increase the adverse effects of LPS treatment on fetal growth. Other parameters including resorption rate, placental weight and number of total implantation sites were not affected by any of the three LPS doses in either *Il10 +/+* or *Il10 -/-* genotypes.

For subsequent experiments, a 0.5 µg LPS dose was chosen based on the initial dose response experiment. Since in subsequent experiments the females from both genotypes would be mated to *Il10* $^{+/+}$ males, it was assumed that the resulting heterozygous (*Il10* $^{+/-}$) fetuses from the *Il10* $^{+/+}$ x *Il10* $^{-/-}$ matings would be better able to tolerate the 0.5 µg LPS dose and progress to a viable pregnancy as differences in fetal growth in *Il10* $^{+/+}$ mice were only observed in the 0.5 µg LPS dose. The *Il10* $^{+/+}$ x *Il10* $^{-/-}$ pairing was rationalised to be a better model, as it would allow us to address the effects of LPS on the pre-implantation embryo in an IL-10 deficient maternal environment, without confounding effects of fetal IL-10 deficiency.

To examine the effect of maternal LPS treatment and IL-10 deficiency on fetal and placental development, *Il10* $^{+/+}$ and *Il10* $^{-/-}$ female mice were mated with *Il10* $^{+/+}$ males and treated with the chosen 0.5 µg LPS dose (i.p.) on both days 2.5 and 3.5 pc.

Pregnancy viability in the PBS-treated control *Il10* $^{-/-}$ females was found to be reduced by 28% compared to the control *Il10* $^{+/+}$ females (Figure 3.1A). Following LPS treatment on days 2.5 and 3.5 pc, pregnancy viability was observed to be reduced by 11% in the *Il10* $^{+/+}$ females and 28% in the *Il10* $^{-/-}$ females compared to the respective PBS-treated controls. The reduction in pregnancy viability in *Il10* $^{+/+}$ females treated with LPS, did not reach significance compared to the *Il10* $^{+/+}$ controls. In contrast, the reduction in pregnancy viability observed in the *Il10* $^{-/-}$ females was significant relative to the *Il10* $^{-/-}$ controls ($p < 0.05$, χ^2-test, Figure 3.1A). Maternal IL-10 deficiency was found to significantly reduce pregnancy viability by 44% in the LPS-treated *Il10* $^{-/-}$ females compared to the LPS-treated *Il10* $^{+/+}$ females ($p < 0.01$, Figure 3.1A). There was no difference in the number of total implantation sites, due to either maternal LPS treatment or maternal IL-10 deficiency (Figure 3.1B). The percentage of viable implantation sites per mated female was not significantly affected by maternal LPS treatment or maternal genotype (Figure 3.1C). The percentage of implantation sites undergoing resorption was not significantly affected by maternal LPS treatment or maternal genotype (Figure 3.1D).

The weight of fetuses in *Il10* $^{+/+}$ and *Il10* $^{-/-}$ females was observed to be reduced after maternal LPS treatment (9% reduced vs. control *Il10* $^{+/+}$, $p = 0.003$; 29% reduced vs. control *Il10* $^{-/-}$, $p < 0.001$) (ANOVA, Sidak post-hoc test, Figure 3.1E). Thus, the impact of LPS on fetal growth was greater in the *Il10* $^{-/-}$ females (16% reduced vs. LPS-treated *Il10* $^{+/+}$ fetuses, $p < 0.001$, Figure 3.1E). This was despite

the fact that in PBS-treated controls, fetal weights were elevated with maternal IL-10 deficiency, with fetuses from the *Il10* -/- females being heavier than the *Il10* +/+ fetuses (8% increased, p = 0.025, Figure 3.1E). The mean placental weight from *Il10* +/+ females was increased after maternal LPS treatment (8% increased vs. PBS-treated control *Il10* +/+, p = 0.015, Figure 3.1F) while there was no effect of maternal LPS treatment on the placental weight in *Il10* -/- females. The fetal:placental ratio was found to be significantly reduced after LPS treatment in both *Il10* +/+ mice (16% reduced vs. PBS-treated control *Il10* +/+, p < 0.001, Figure 3.1G) and *Il10* -/- mice (30% reduced vs. PBS-treated control *Il10* -/-, p < 0.001, Figure 3.1G). There was no effect of maternal IL-10 deficiency on the extent of change in fetal:placental ratio in LPS-treated compared to PBS-treated control mice.
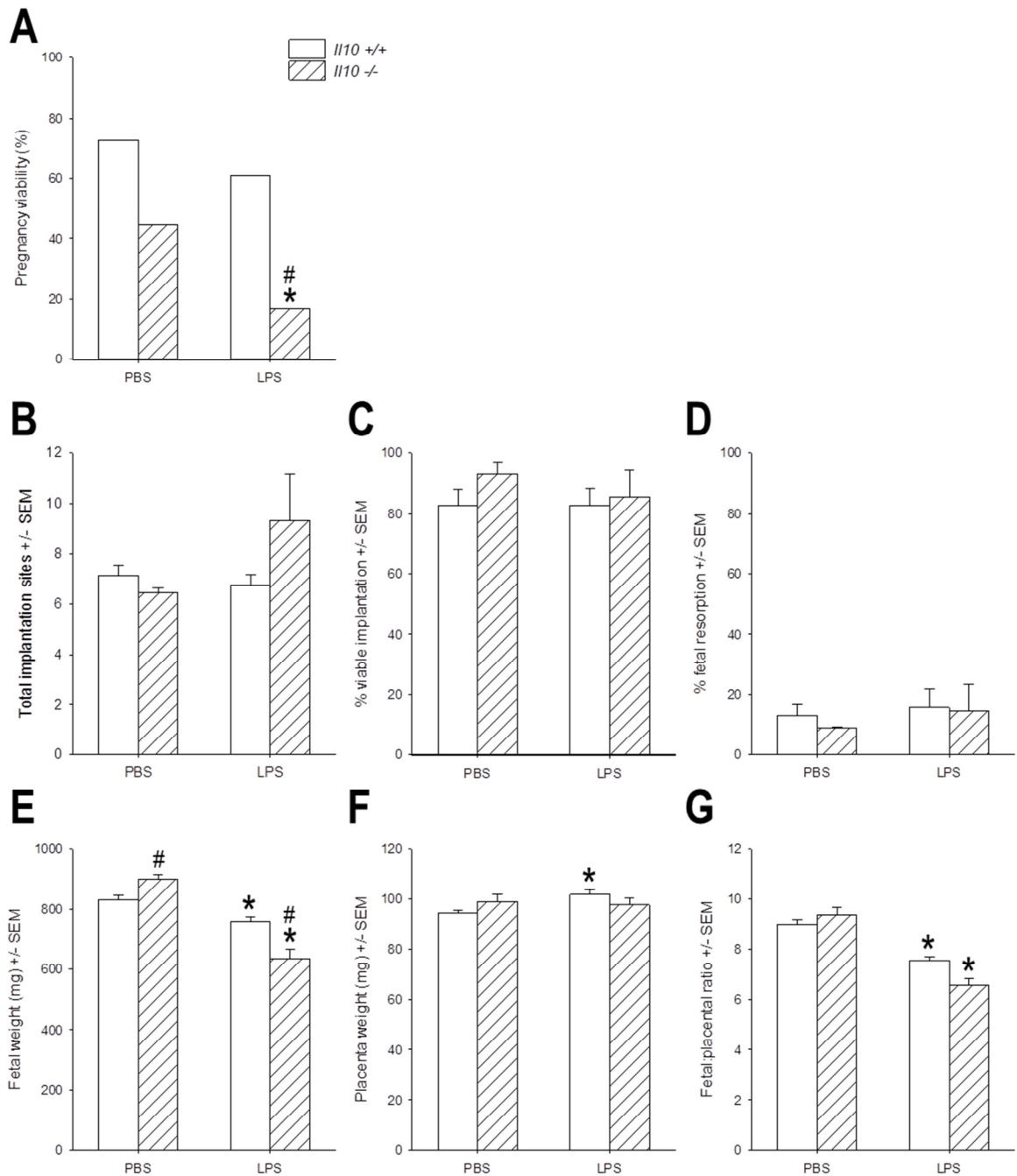
Figure 3.1   The effect of low dose LPS on fetal and placental development in *Il10* ⁺ᐟ⁺ and *Il10* ⁻ᐟ⁻ females at day 17.5 pc.

Il10 ⁺ᐟ⁺ (open bars) and *Il10* ⁻ᐟ⁻ (shaded bars) females were mated to *Il10* ⁺ᐟ⁺ males and injected with 0.5 µg/mouse LPS or PBS on days 2.5 and 3.5 pc (A) the percentage of plugged females progressing to viable pregnancies, (B) the mean ± SEM number of total implantation sites per mated mouse, (C) the percentage of viable implantation sites, (D) the percentage of implantation sites undergoing resorption, (E) the mean ± SEM fetal weight of each implantation sites per group, (F) the mean ± SEM placenta weight of each implantation site per group, and (G) the mean ± SEM fetal:placental ratio of each implantation site per group. n = 16 - 22 females per group; Data was compared by χ2-test (A) and one way ANOVA (B-G), *p < 0.05 compared with PBS group within the same genotype, #p < 0.05 compared with *Il10* ⁺ᐟ⁺ group of the same treatment.

3.6. DISCUSSION

This study was undertaken to investigate the significance of low level maternal systemic inflammation during the pre-implantation period on development of the embryo, fetus and placenta. As hypothesised, a pro-inflammatory LPS challenge in the pre-implantation period elicited changes in the embryo developmental trajectory that in turn altered fetal growth. The experiments in this chapter establish a model whereby maternal systemic inflammation induced by LPS during the pre-implantation period influence the fetus in later gestation. As LPS was shown to not directly impact development of the embryo at low and moderate doses, this model appears to reflect indirect effects of LPS mediated via the maternal tract. This interpretation is consistent with previous studies which have shown that LPS-induced intra-uterine fetal death induced by LPS administration in mid-gestation is principally due to maternal effects and not fetal sensitivity to LPS (Kohmura et al., 2000).

The LPS dose for the maternal systemic inflammation model was determined in a preliminary dose response experiment, although it was not directly comparable to the subsequent experiments as the $Il10$ $^{-/-}$ females were mated to males of the same genotype. Since both the mother and the fetus were IL-10 deficient, the extent of the difference reported due to maternal LPS treatment that can be attributed to IL-10 deficiency $in\ utero$ is unclear. Thus, the $Il10$ $^{-/-}$ females were mated to $Il10$ $^{+/+}$ males to specifically address the effect of maternal IL-10 deficiency on the pre-implantation embryo in this model. The low LPS dose used in this model mimics a minor inflammatory infection where the pre-implantation embryo can tolerate the altered maternal environment and progress to implant. In the $in\ vivo$ study, the female mice treated with the 0.5 μg LPS did not show any visible physical signs of distress or sickness such as fever, ruffled coat, shivering or reduced mobility. In contrast, when the LPS doses were increased up to 25-fold, the mice showed reduced mobility, shivering and hunching. Embryo development was similarly affected as the LPS dose increased, where the number of embryos developing into the blastocyst stage was reduced due to arrested development. This suggests a mechanism whereby at low dose, the embryo senses and adapts to the altered maternal environment. While most cytokine receptors are expressed on both TE and ICM cells, LIFR is reported to be expressed solely on ICM cells (Nichols et al., 1996). This difference in receptor expression could possibly contribute to the prominent reduction in ICM cells observed in this study, however since LIF was not down-regulated, but was up-regulated and only in $Il10$ $^{-/-}$ mice, this does not fully explain the differential effect.

The significance of the *in vitro* experiments in this chapter was crucial to establishing the maternal systemic inflammation model and to provide a model in which the contribution of cytokines in the mechanism of LPS can be investigated. The *in vitro* culture experiments support the utility of the model as minimal direct effects of LPS were observed in development when embryos were cultured in the presence of LPS, at concentrations similar to the doses utilised *in vivo*.

Previous studies have linked maternal infection of both viral and bacterial origins during pregnancy with psychiatric disorders including autism in later development of the offspring (Atladóttir et al., 2010, Ciaranello and Ciaranello, 1995). A recent study published during the course of this work was done in a similar manner to our model where maternal systemic inflammation was induced by increasing doses of LPS, but on the day of zygote formation (day 0.5 pc) (Williams et al., 2011). Similar findings of reduced ICM cell numbers were observed after maternal LPS treatment although the proportion of embryos at blastocyst stage was not affected by maternal LPS. This can be explained by the different timing of LPS treatment as gene expression and susceptibility to various environment insults is known to be different throughout the different stages of embryo development (Guo et al., 2010). The response to maternal systemic inflammation may differ in the zygote as opposed to the blastocyst as embryonic genome activation in the mouse begins at the late 2-cell stage (Schultz, 2002, Wang et al., 2004a). *In vitro* studies have shown differences in response to various compounds including ammonium and amino acids at different developmental stages (Zander et al., 2006, Lane and Gardner, 1997).

The experiment involving the culture of embryos together in the presence of LPS and/or rmIL-10 cytokine showed that IL-10 has no direct effect on embryo development. This finding validates the criteria of our maternal systemic inflammation model. The IL-10 cytokine is expressed throughout pregnancy by epithelial cells and leukocytes in the endometrium and placenta (Lin et al., 1993, Moore et al., 1993). It is not known whether the IL-10 receptor (IL-10R) is expressed on the mouse pre-implantation embryo. However there is evidence of the IL-10 cytokine being secreted by human pre-implantation embryo from IVF studies (Ozornek et al., 1995, Domínguez et al., 2008). Studies in the *Il10* $^{-/-}$ mice have shown that maternal IL-10 deficiency does not affect fertility, however it does affect fetal and postnatal growth trajectory (White et al., 2004). This current study, particularly the data from the PBS-treated control *Il10* $^{-/-}$ mice supports the earlier findings where maternal IL-10 deficiency resulted in increased fetal weights of pups from *Il10* $^{-/-}$ females (White et al., 2004).

Although the effect of maternal LPS treatment on the proportion of mated mice progressing to viable pregnancies was consistent with the preliminary LPS dose response experiment, it is unclear if the non-viable pregnancies were a result of poor embryo development or implantation failure in the altered maternal tract. As no resorption sites were observed on day 17.5 pc, late gestation fetal loss was not the cause, and loss at an earlier time point can be inferred. This observation relates to an 'all or none' phenomenon. The 'all or none' invokes the concept that embryonic exposure to insults before organogenesis results in either no adverse embryonic outcome or in embryonic death (Adam, 2012). Many studies have looked at various perturbations during pregnancy and found factors including as mutagen and radiation exposure to induce either pregnancy loss (full resorption) or normal pregnancy with increased risk of congenital aberrations (Rutledge et al., 1992, Nagao et al., 1991, Kim et al., 2001b). Some maternal effects were also found to act in an 'all or none' fashion, where perturbation during early pregnancy either results in successful pregnancy or complete pregnancy failure (Erlebacher et al., 2004, Schelbach et al., 2013).

Although fetal resorption and the number of viable implants were not affected by LPS treatment or maternal IL-10 deficiency, fetal weights were reduced regardless of maternal IL-10 genotype, indicating that early pregnancy LPS treatment had an adverse effect regardless of the presence of maternal IL-10. However, a protective effect of maternal anti-inflammatory IL-10 in modulating the effects of LPS treatment was evident as the impact was greater in the $Il10^{-/-}$ fetuses. This is consistent with previous studies of maternal LPS treatment in mid (day 9.5 pc) and late (day 17 pc) gestation (Robertson et al., 2007, Robertson et al., 2006), where effects were more pronounced in the absence of IL-10.

The shift in fetal weights resulted in a reduction in the fetal:placental weight ratio, an index of placental efficiency. Since placenta weight was not affected by maternal LPS or IL-10 deficiency, this suggests that the placentas may be functionally altered by LPS regardless of $Il10$ genotype. This indicates that LPS has more impact on fetal development as opposed to placental development. This is consistent with the finding that LPS treatment differentially affected the ICM cells, since the fetal is largely derived from the ICM compartment. Similar effects on both ICM and fetal growth are seen in other studies where cytokines or nutrition are perturbed in the peri-conception period (Sjöblom et al., 1999, Kwong et al., 2000).

Previous studies have shown that the embryo is sensitive to environmental perturbations during the pre-implantation period mediated by maternal diet and *in vitro* culture (Kwong et al., 2000, Sjoblom et al., 2005). Studies in fetal growth restriction models have demonstrated that the changes in placental morphology resulted in reduced fetal growth (Sjoblom et al., 2005, Roberts et al., 2001a). The changes we reported in the fetal and placental development are likely to be influenced by perturbations in the maternal environment caused by LPS. One possibility is that these are mediated by altered cytokine signalling between the embryo-maternal interface, and thus contributes to embryo development, implantation competence and subsequent fetal development.

Chapter 4

Cytokine environment during the pre-implantation period

4.1. INTRODUCTION

The previous chapter explains how a mouse model was established to study maternal systemic inflammation utilising LPS during the pre-implantation period. The effect of perturbation in the maternal environment during this critical period was investigated in relation to the physiological outcomes of pregnancy in a natural mating context. It was shown that systemic inflammation due to administration of bacterial LPS, a TLR4 ligand, during the pre-implantation period can alter subsequent fetal development. Although the previous chapter describes experiments showing that maternal systemic inflammation can influence pregnancy outcome, the mechanism of how LPS acts on the pre-implantation embryo to alter this developmental outcome is not clear.

During the pre-implantation period, the embryo develops in the absence of direct cell contact with the maternal reproductive tract before implantation occurs. As the embryo traverses through the tract, its growth and development is influenced by the fluctuations of the physiochemical composition of the surrounding maternal environment, including cytokines and growth factors which are secreted by the oviduct and uterine epithelium. Embryos express cytokine receptors and several cytokines have different effects on the embryo, including increasing the rate of blastocyst formation (GM-CSF, IGF-II and others) or the rate of apoptosis (TNFα) (Hardy and Spanos, 2002, Schultz and Heyner, 1993).

It is possible that the anti-inflammatory IL-10 cytokine can play a role in modulating the adverse effects of maternal systemic inflammation with LPS. The IL-10 cytokine was originally thought to be secreted by type 2 helper cells (T$_H$2 cells) and inhibits cytokine production by T$_H$1 cells and by activated macrophages (Fiorentino et al., 1991). However the recent literature reports that IL-10 is produced by a wide variety of cells, including T$_H$1, T$_H$2 and T$_H$17 cells, T$_{reg}$ cells, CD8$^+$ T cells and B cells as well as non-hematopoietic cells including epithelial cells (Kubo and Motomura, 2012). In the female reproductive tract, IL-10 is expressed by epithelial cells lining the uterus and oviduct (Sallinen et al., 2000, Orsi et al., 2007). In the previous chapter, we reported that the absence of maternal IL-10 increases the adverse effect of LPS on pregnancy rates and fetal growth. This is consistent with the knowledge of IL-10 being the central regulator of the response to LPS, acting to limit the LPS-induced expression of pro-inflammatory cytokines, such as TNFα, IL-1 and IFNγ (Howard et al., 1993, Gerard et al., 1993, de Waal Malefyt et al., 1991, Fiorentino et al., 1991). Since embryos do not directly respond to

IL-10 (previous chapter), this observation indicates that IL-10 regulated pathways in maternal cells, such as cytokine production, are responsible for the effect on development of embryos.

The previous chapter describes the effects of maternal systemic perturbation with LPS on fetal and placental development. However, the experimental strategy used in chapter 3 does not exclude the possibility that any changes in fetal and placental development are due to an altered uterine receptivity to embryo implantation, as opposed to altered blastocyst development after maternal perturbation with LPS. By utilisation of qPCR and embryo transfer in this chapter, we aim to understand the mechanism by which maternal systemic perturbation with LPS influences embryo development to result in restricted fetal growth. We expect the qPCR experiments to be informative as to which cytokines are produced in the reproductive tract after activation by TLR4 ligand, as would occur in the event of a local or bacterial systemic infection.

The embryo transfer experiment described in this chapter was designed to overcome the confounding issue of any impact of LPS on maternal tract receptivity and to identify to what extent maternal LPS treatment influences pregnancy and implantation success through effects mediated on the developing embryo. The experiments utilised embryos transferred at the blastocyst stage. Transferred embryos were conceived and underwent three rounds of blastomere cleavage to the 8-cell stage in a normal environment prior to LPS administration at days 2.5 and 3.5 pc. All blastocysts were then transferred to a normal environment in untreated recipients. Thus embryo development from the compacted morula to the blastocyst stage occurred in an altered maternal environment, but uterine receptivity in recipients is not perturbed.

An additional experiment utilising etanercept, a soluble TNFα antagonist in combination with LPS was performed to further dissect if TNFα is one of the main cytokines that mediates the action of LPS. Etanercept (trade name Enbrel®) is a recombinant fusion protein that consists of the soluble TNF receptor (p75) linked to the Fc portion of the human IgG1 (TNFR:Fc) and has high affinity for binding TNFα (Moreland et al., 1997). Etanercept is commercially available as one of the TNFα inhibitors used to treat inflammatory diseases such as rheumatoid arthritis and psoriasis (Sills et al., 2001, Moreland et al., 1997).

By comparison with the findings from the maternal systemic inflammation experiments described in Chapter 3, the experiments outlined in this chapter attempt to dissect apart the mechanism of how LPS impacts on the pre-implantation embryo via alteration of the cytokines produced by the maternal tract.

4.2. EFFECT OF LPS ADMINISTRATION DURING THE PRE-IMPLANTATION PERIOD ON CYTOKINE ENVIRONMENT IN THE REPRODUCTIVE TRACT

The changes we have reported in fetal and placental development are likely to be mediated by maternal perturbation caused by LPS during the pre-implantation period. Pre-implantation embryo development, attachment and implantation, uterine tissue remodelling required for implantation and modulation of the maternal immune response to pregnancy are all factors which can contribute to optimising fetal growth and pregnancy outcome. Each of these alone or in combination could be altered by the activation of inflammatory cytokine expression in the maternal reproductive tract.

To determine a possible mechanism for reduced pregnancy rates and fetal growth described in section 3.5, cytokine expression in the reproductive tract after maternal LPS treatment was analysed. A total of 12 *Il10* $^{+/+}$ and *12 Il10* $^{-/-}$ females (6 per treatment group) were administered (i.p.) 12.5 μg LPS or PBS on days 2.5 and 3.5 pc. Four hours after the injection on day 3.5 pc, the oviduct and uterus were collected and flushed to confirm the presence of embryos. QPCR was performed on mRNA extracted from the oviduct and uterus to examine the levels of cytokine expression following maternal LPS treatment.

The presence of viable embryos was confirmed in the reproductive tract to ensure all females in the experiment were pregnant, as embryo fertilisation does not occur with every mating. The higher dose of 12.5 μg LPS was administered instead of the 0.5 μg LPS dose utilised in earlier experiments. This was chosen with the understanding that tissue responses to LPS occur in an escalating dose-response manner (Berg et al., 1995, Anton et al., 2012). The 12.5 μg LPS dose would more likely reveal a detectable activation of LPS-induced cytokine expression in these tissues.

4.2.1. Cytokine gene expression in day 3.5 pc oviduct tissue

To determine the effect of maternal systemic inflammation induced by LPS on cytokine gene expression during the pre-implantation period, qPCR was performed on mRNA extracted from oviduct tissues collected from control and LPS-treated *Il10* $^{+/+}$ and *Il10* $^{-/-}$ females, 4 hours after the second 12.5 µg LPS i.p. injection on day 3.5 pc. Of ten cytokine genes that were analysed, three were found to be up-regulated after LPS treatment compared to the control *Il10* $^{+/+}$ oviduct tissues, including *Tnfa* (5.9-fold increased, $p < 0.001$, Figure 4.1B), *Il1b* (3.3-fold increased, $p = 0.025$, Figure 4.1D), and *Il10* (4.0-fold increased, $p = 0.004$, Figure 4.1F) (vs. PBS-treated *Il10* $^{+/+}$ oviduct tissues, ANOVA).

In *Il10* $^{-/-}$ mice, nine out of ten cytokines were found to be differentially expressed in the oviduct after maternal LPS treatment, including *Il6* (6.9-fold increased, $p = 0.01$, Figure 4.1A), *Tnfa* (9.5-fold increased, $p = 0.002$, Figure 4.1B), *Il1a* (2.6-fold increased, $p = 0.015$, Figure 4.1C), *Il1b* (3.5-fold increased, $p = 0.001$, Figure 4.1D), *Ifng* (4.4-fold increased, $p = 0.002$, Figure 4.1E), *Il10* (2.0-fold increased, $p < 0.001$, Figure 4.1F) (see below), *Lif* (2.2-fold increased, $p = 0.004$, Figure 4.1G) *Csf2* (2.3-fold increased, $p = 0.028$, Figure 4,1H) and *Il12b* (10.9-fold increased, $p < 0.001$, Figure 4.1J) (vs. PBS-treated *Il10* $^{-/-}$ oviduct tissues, ANOVA).

Il10 gene expression was detected in the *Il10* $^{-/-}$ mice in the qPCR experiments, as the *Il10* gene in *Il10* $^{-/-}$ mice is not excised but is disrupted by the insertion of a neomycin cassette, resulting in a non-functional *Il10* gene (Kuhn et al., 1993). The genomic status of our *Il10* $^{-/-}$ breeding colony was confirmed using three specific PCR primers for the IL-10 mutation as previously reported (Gazzinelli et al., 1996). Therefore, the mRNA expression of *Il10* observed in the *Il10* $^{-/-}$ tissues is expected as the *Il10* primers used in this experiment detect the sequence upstream of the inserted neo cassette.

Expression of *Il10* was found to be up-regulated by 4.6-fold in control PBS-treated *Il10* $^{-/-}$ oviduct tissues compared with control PBS-treated *Il10* $^{+/+}$ oviduct tissues ($p = 0.004$, Figure 4.1F, ANOVA). in contrast, expression of six cytokines were found to be further up-regulated in LPS-treated *Il10* $^{-/-}$ oviduct tissues compared with LPS-treated *Il10* $^{+/+}$ oviduct tissues including *Il6* (2.8-fold increased, $p = 0.013$, Figure 4.1A), *Il1a* (4.6-fold increased, $p = 0.02$, Figure 4.1C), *Il10* (2.3-fold increased, $p < 0.001$, Figure 4.1F), *Csf2* (5.8-fold increased, $p = 0.001$, Figure 4.1H), *Il12a* (16.9-fold increased, $p = 0.032$, Figure 4.1I) and *Il12b* (79.5-fold increased, $p < 0.001$, Figure 4.1J). However, the expression of *Tnfa* was found to be

down-regulated by 0.5-fold in LPS-treated $Il10^{-/-}$ oviduct tissues compared with LPS-treated $Il10^{+/+}$ oviduct tissues (p = 0.001, Figure 4.1B).

Figure 4.1 Cytokine gene expression profile in day 3.5 pc oviduct tissue following maternal LPS treatment during the pre-implantation period.

mRNA was quantified by qPCR from oviduct tissues collected from *Il10* [+/+] (open bars) and *Il10* [-/-] (shaded bars) females treated with either 12.5 μg/mouse LPS or PBS (n = 6 per group) and normalised to expression of reference gene *β-actin*. Transcripts analysed were (A) *Il6*, (B) *Tnfa*, (C) *Il1a*, (D) *Il1b*, (E) *Ifng*, (F) *Il10*, (G) *Lif*, (H) *Csf2*, (I) *Il12a* and (J) *Il12b*. Data is presented as mean ± SEM and the effects of treatment and genotype were compared using ANOVA; cytokine expression in oviduct tissues from PBS-treated *Il10* [+/+] females was normalised to 1. * $p < 0.05$ compared to PBS group within the same genotype, # $p < 0.05$ compared to LPS-treated *Il10* [+/+] females, † $p < 0.05$ compared to PBS-treated *Il10* [+/+] females.
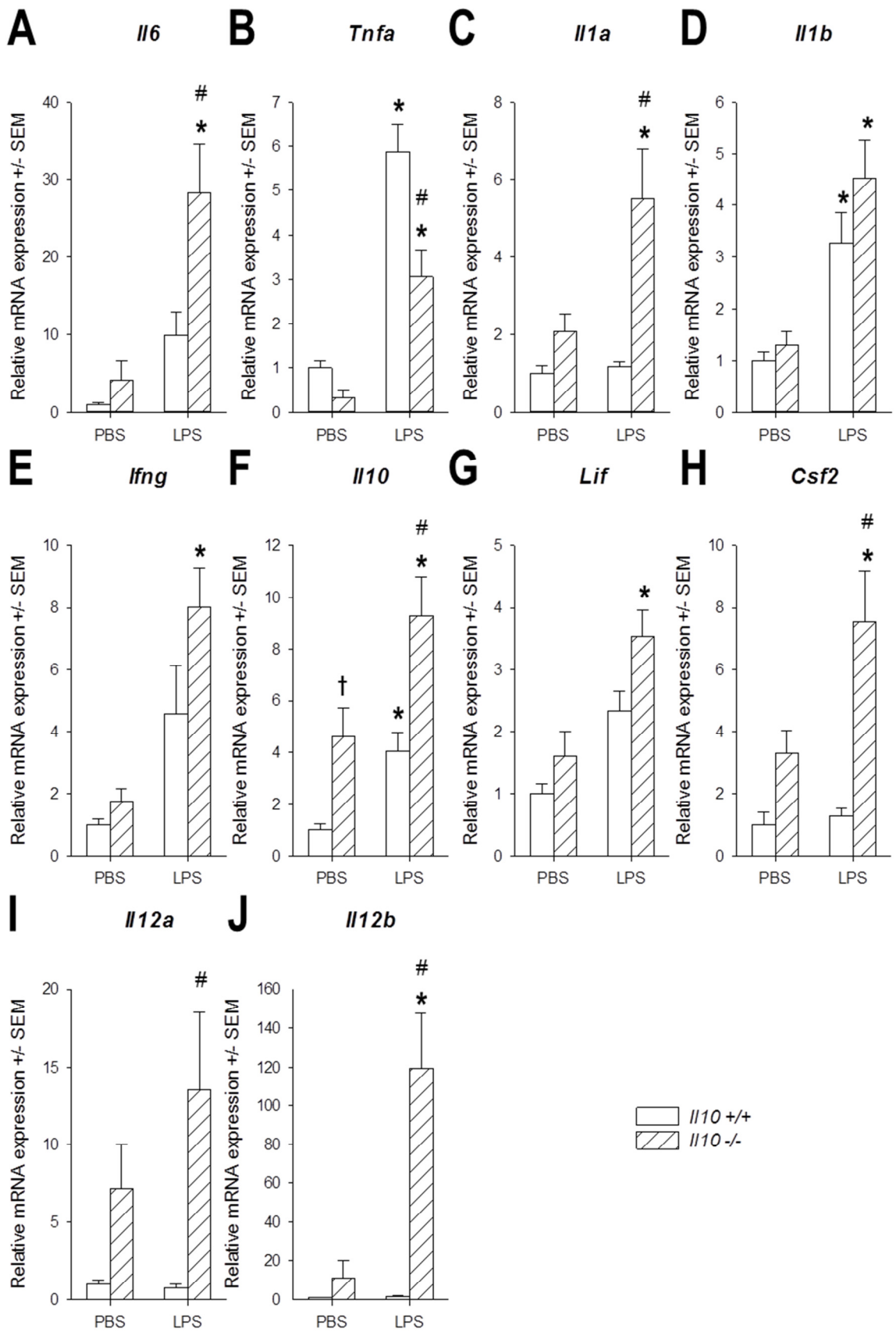
4.2.2. Cytokine gene expression in day 3.5 pc uterus tissue

To determine the effect of maternal systemic inflammation induced by LPS on cytokine gene expression during the pre-implantation period, qPCR was performed on mRNA extracted from uterus tissues collected from control and LPS-treated *Il10* $^{+/+}$ and *Il10* $^{-/-}$ females, 4 hours after the second LPS treatment by i.p. injection on day 3.5 pc. Following maternal LPS treatment, none of the ten cytokine genes analysed were found to be differentially expressed in the uterus when compared to the control *Il10* $^{+/+}$ uterus. There was a trend to increased *Tnfa* and *Ifng*, but these did not reach statistical significance in part due to between sample variations in expression levels.

In *Il10* $^{-/-}$ mice, three cytokines were found to be up-regulated in the *Il10* $^{-/-}$ uterus following maternal LPS treatment, including *Tnfa* (14.0-fold increased, $p < 0.001$, Figure 4.2B), *Ifng* (4.4-fold increased, $p < 0.001$, Figure 4.2E) and *Il12b* (16.6-fold increased, $p < 0.001$, Figure 4.2 J) (vs. control *Il10* $^{-/-}$ uterus, ANOVA).

The identical three cytokines were also found to be differentially expressed due to maternal IL-10 deficiency following LPS treatment, including *Tnfa* (2.4-fold increased, $p = 0.001$, Figure 4.2B), *Ifng* (1.5-fold increased, $p = 0.001$, Figure 4.2E) and *Il12b* (59.9-fold increased, $p < 0.001$, Figure 4.2J) when compared to LPS-treated *Il10* $^{+/+}$ uterus, ANOVA).

Figure 4.2   Cytokine gene expression profile in day 3.5 pc uterus tissue following maternal LPS treatment during the pre-implantation period.

mRNA was quantified by qPCR from uterus tissues collected from *Il10* [+/+] (open bars) and *Il10* [-/-] (shaded bars) females treated with either 12.5 μg/mouse LPS or PBS (n = 6 per group) and normalised to expression of reference gene *β-actin*. Transcripts analysed were (A) *Il6*, (B) *Tnfa*, (C) *Il1a*, (D) *Il1b*, (E) *Ifng*, (F) *Il10*, (G) *Lif*, (H) *Csf2*, (I) *Il12a* and (J) *Il12b*. Data is presented as mean ± SEM and the effects of treatment and genotype were compared using ANOVA; cytokine expression in uterus tissues from PBS-treated *Il10* [+/+] females was normalised to 1. * $p < 0.05$ compared to PBS group within the same genotype, [#] $p < 0.05$ compared to LPS-treated *Il10* [+/+] females.
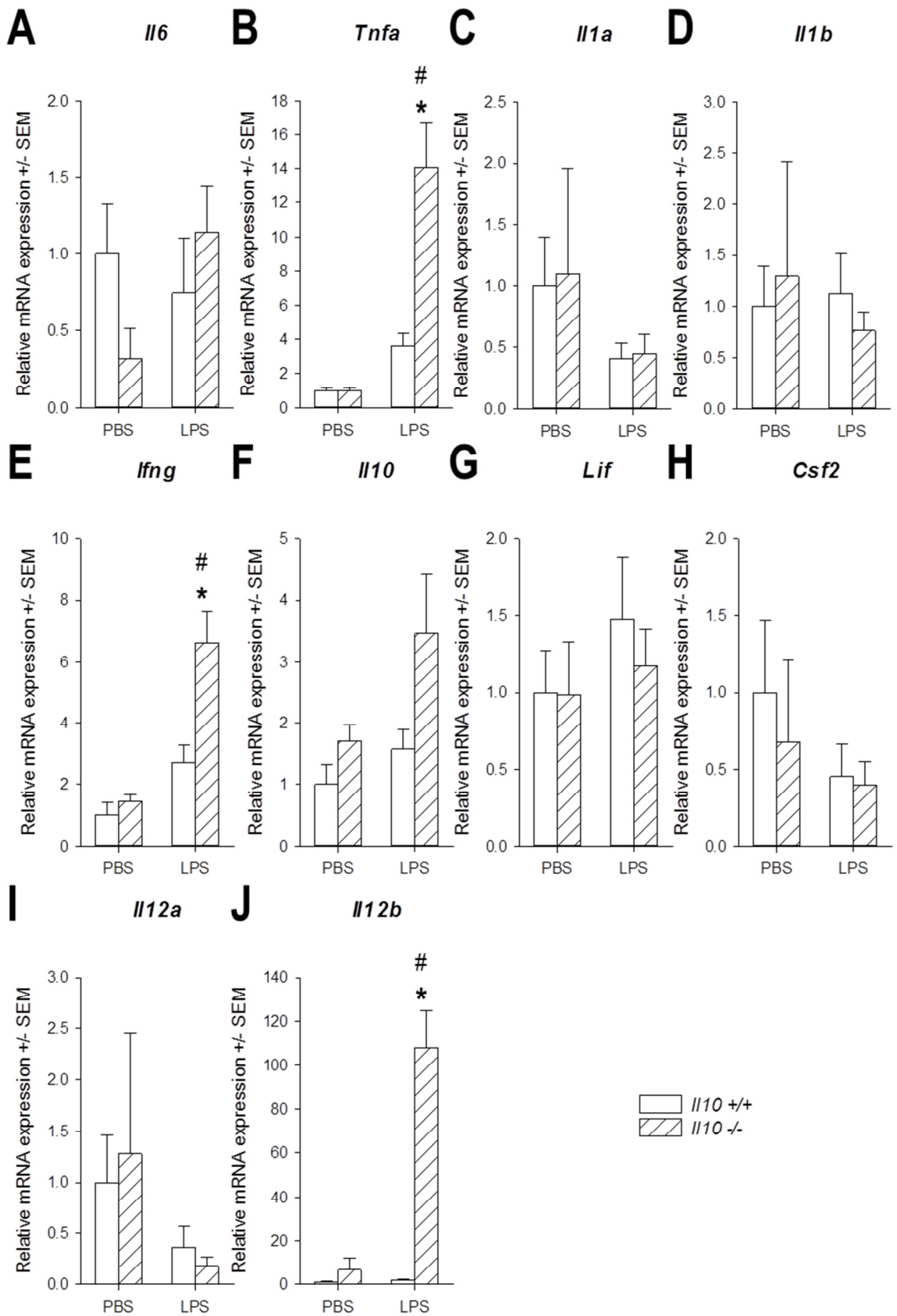
4.3. EFFECT OF TNFα ANTAGONIST ETANERCEPT (Enbrel®) ON FETAL AND PLACENTAL DEVELOPMENT FOLLOWING LPS ADMINISTRATION DURING THE PRE-IMPLANTATION PERIOD

The previous experiments indicates that maternal systemic inflammation induced by LPS during the pre-implantation period up-regulates cytokine expression in the oviduct and uterus, including the pro-inflammatory TNFα, IFNγ and IL-12 p40. The activation of LPS-induced cytokine genes indicates that these cytokines may play a role in mediating the effects of LPS on the embryo and may provide a mechanism by which the embryo senses the altered maternal environment.

Previous studies in mice have shown that LPS-induced inflammation results in an increase in pro-inflammatory cytokine production including TNFα, and that this cytokine is associated with pregnancy loss and restricted fetal growth (Challis et al., 2009, Renaud et al., 2011). Administration of recombinant TNFα recapitulates the effect of LPS in increasing the rate of fetal resorption and pregnancy failure (Chaouat et al., 1990). Other studies have revealed adverse programming effects of TNFα on pre-implantation embryo development, where inhibition of cell proliferation and induction of cell death in the ICM of rat blastocysts was observed (Pampfer et al., 1994a, Pampfer et al., 1997b). It has been suggested that anti-TNF therapies may be useful in treatment of women with infertility and recurrent miscarriages (Clark, 2010) and previous studies in our laboratory have shown that treatment to inhibit TNFα synthesis using the TNFα antagonist, etanercept (Enbrel®) can protect $Il10^{-/-}$ mice from LPS-induced abortion on day 9.5 pc (Robertson et al., 2007).

To examine whether etanercept is able to protect the pre-implantation embryo from the adverse effects of maternal systemic inflammation with LPS, etanercept was administered in conjunction with LPS treatment on days 2.5 and 3.5 pc (i.p.) in $Il10^{+/+}$ females mated to $Il10^{+/+}$ males. The etanercept dose chosen for this experiment was 100 μg per mouse and the females were administered etanercept or PBS at 0900h on days 2.5 and 3.5 pc, prior to LPS administration at 1100h. This dose was chosen as it was shown previously to be effective in protecting from the effects of LPS. This experiment resulted in four treatment groups (2x2 factorial design); control (PBS) treatment, LPS treatment, etanercept treatment and LPS + etanercept treatment. The females were examined on day 17.5 pc, where the total implantation sites and resorbing implantation sites were counted, and the weights of every fetus and placenta were measured.

Pregnancy viability in the LPS treatment group was found to be reduced by 26% when compared to control females (p = 0.021, χ^2-test, Figure 4.3A), similar to previous findings reported in Figure 3.1A. Treatment with 100 μg etanercept alone did not cause any significant differences or adverse effect on the proportion of mated females progressing to viable pregnancy. On the other hand, treatment of etanercept before and after LPS treatment did not improve outcomes compared to LPS alone, and was found to cause a more substantial reduction in pregnancy viability (by 50%) when compared to the control group (p < 0.001, χ^2-test, Figure 4.3A).

There was no significant difference in the number of total implantation sites in the LPS-treated and etanercept-treated groups when compared to the control group (Figure 4.3B). However, females treated with LPS and etanercept was found to displayed more implantation sites compared to the control group (p = 0.002, ANOVA, Sidak post-hoc test, Figure 4.3B). The increased number of implantation sites in the LPS + etanercept-treated group was also significantly different compared to the LPS-treated and the etanercept-treated group (p = 0.029 vs. LPS group, p = 0.002 vs. etanercept group) (Figure 4.3B). The percentage of viable implantation sites per mated female was not significantly affected by either LPS or etanercept treatment during the pre-implantation period (Figure 4.3C). Similarly, the percentage of implantation sites undergoing resorption was not significantly affected in any of the three treatment groups (Figure 4.3D).

The weight of fetuses in the LPS-treated group was reduced when compared to the control group (18% reduced vs. control in the LPS treatment, p < 0.001) (mixed model ANOVA, Figure 4.3E). Fetal weight was not affected in the etanercept-treated and the LPS + etanercept-treated group when compared to the control group. Fetal weights in the LPS + etanercept group was not different to the PBS control or the LPS + etanercept treatment groups, indicating that the etanercept may have a modest effect on fetal growth, but was not able to fully protect fetal growth from adverse effects of maternal LPS treatment.

There was no significant difference in the mean placental weights in all 3 treatment group compared to the control group (Figure 4.3F). Similarly, there was no significant differences in the fetal:placental ratio in all 3 treatment groups compared to the control group (Figure 4.3G).
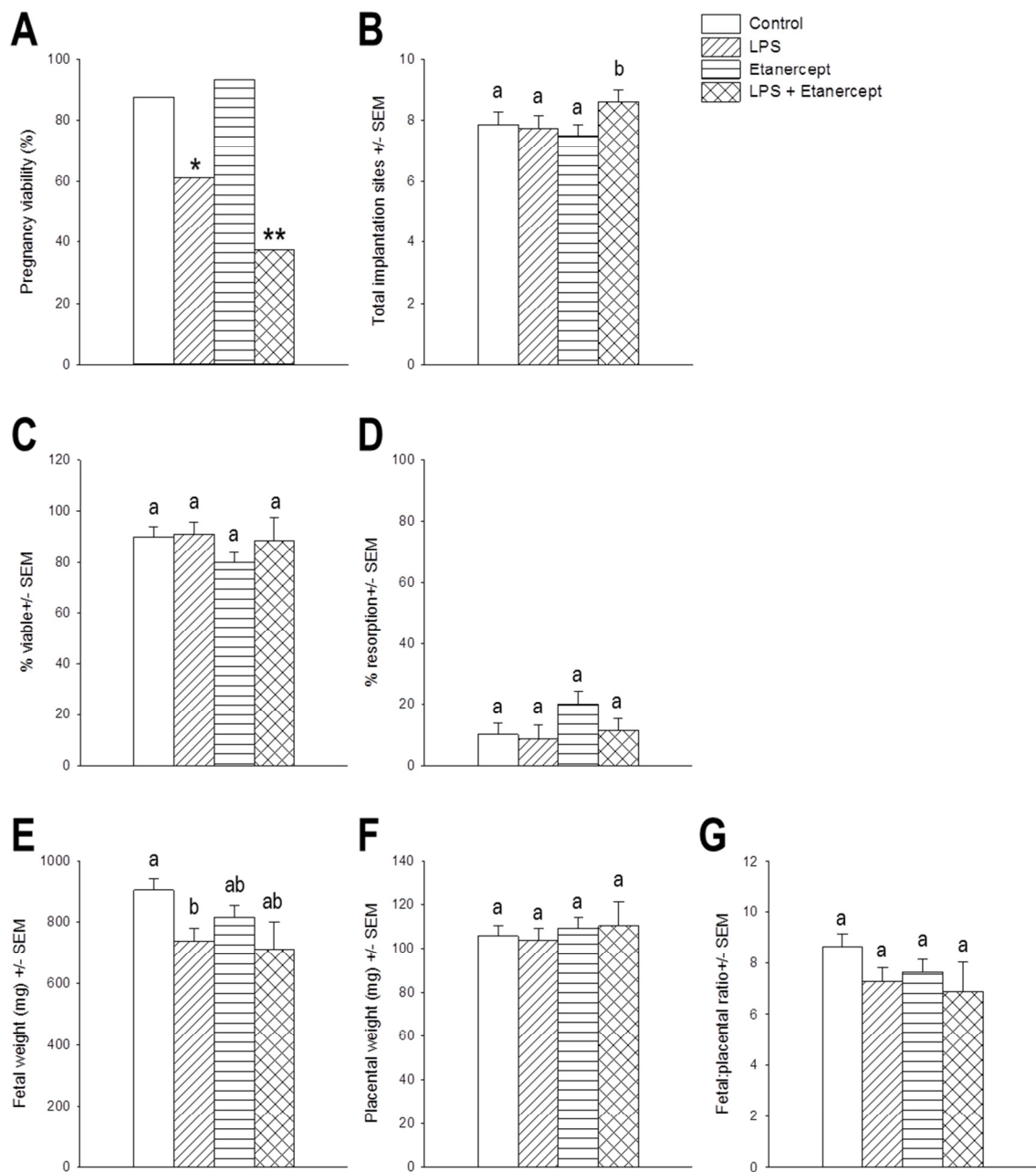
Figure 4.3   The effect of maternal treatment with LPS and etanercept (Enbrel) during the pre-implantation period on day 17.5 pc fetal and placental development.

All *Il10* [+/+] control (PBS) females (open bars), LPS-treated females (diagonal lined bars), etanercept-treated females (horizontal lined bars) and LPS + etanercept-treated females (cross lined bars) were mated with *Il10* [+/+] males and were treated with LPS (0.5 µg) and/or etanercept (100 µg/mouse) on days 2.5 and 3.5 pc. (A) the percentage of plugged females progressing to viable pregnancies, (B) the mean ± SEM number of total implantation sites per mated female, (C) the percentage of viable implantation sites, (D) the percentage of total implantation sites undergoing resorption, (E) the mean ± SEM fetal weight, (F) the mean ± SEM placenta weight, and (G) the mean ± SEM fetal:placental ratio. n = 16 mated females per treatment group; Data was compared by χ^2-test (A), one way ANOVA (B) and mixed model ANOVA including litter size as a covariate (C-G), *p < 0.05 and **p < 0.01 when compared to control group and [a,b] superscripts represents significant differences at p < 0.05 (Sidak post-hoc test).

4.4. EFFECT OF LPS ADMINISTRATION DURING THE PRE-IMPLANTATION PERIOD ON FETAL AND PLACENTAL DEVELOPMENT FOLLOWING EMBRYO TRANSFER

To determine the relative contribution of maternal systemic inflammation with LPS on embryonic development as opposed to uterine receptivity to implantation, embryo transfer experiments were carried out at the blastocyst stage of development. Due to low success rates when embryo transfer was performed in *Il10* $^{+/+}$ females, Swiss female recipients were used as an alternative method. *Il10* $^{+/+}$ females were mated to *Il10* $^{+/+}$ males and were treated (i.p.) with either 0.5 μg LPS or PBS on days 2.5 and 3.5 pc. Embryos were flushed 4 hours after the second LPS injection on day 3.5 pc and transferred immediately into the uterus of pseudopregnant Swiss females (day 2.5 pc) mated with vasectomised *Il10* $^{+/+}$ males with 6-8 blastocysts per recipient.

Implantation rates and fetal survival were unaffected by donor LPS treatment following embryo transfer when assessed on day 17.5 (Table 4.1). However when viable implantation sites were expressed as a percentage of total transferred embryos, fetal survival was found to be reduced by 28% in the LPS-treated donor group ($p = 0.007$, t-test, Table 4.1).

Embryos transferred from donor females treated with LPS resulted in lower fetal weight on day 17.5 pc compared to control donor embryos (21% reduced $p < 0.001$, t-test, Figure 4.4A). In contrast, placental weight was found to be increased in the recipient females that received embryos from LPS-treated donors compared to control embryos (13% increased, $p = 0.003$, Figure 4.4B). The shift in fetal and placenta weights resulted in a reduction in fetal:placental ratio in the LPS-treated donor embryos when compared to control donor embryos (31% decrease, $p < 0.001$, Figure 4.4C). These changes observed with donor LPS treatment are comparable in nature and scale to the findings reported in section 3.5 of the previous chapter.

Table 4.1   Effect of donor LPS treatment on pregnancy parameters on day 17.5 pc following embryo transfer

	Control (n = 6)	0.5 µg LPS (n = 6)
Total embryos transferred	44	40
Embryos implanted in pregnant recipients	42	31
% implantation/embryo transferred	95.8 ± 4.2	80.6 ± 9.5
% viable implantation /embryo transferred	82.3 ± 4.2	54.2 ± 7.3 **
% viable implantation sites/total implantation sites	86.5 ± 4.8	70.6 ± 8.6

Data are expressed as mean ± SEM.

The number of embryo recipient is shown in parentheses; donor to recipient transfer ratio is 1:1.

The number of embryos transferred was 6 - 7 per recipient.

** $p < 0.05$ compared to control (independent t-test)
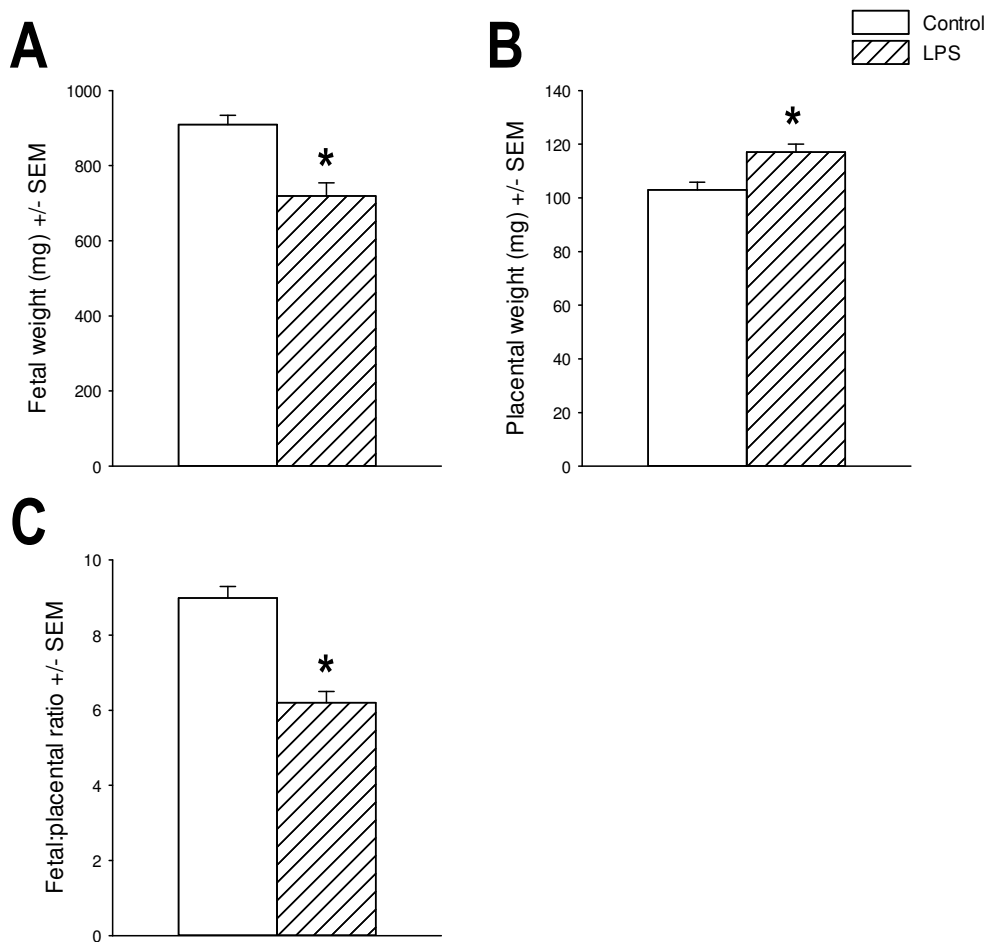
Figure 4.4   **The effect of LPS treatment in donor females during the pre-implantation period on fetal and placental development at day 17.5 pc following embryo transfer.**

Il10 [+/+] control (PBS) donors (open bars) and LPS-treated donors (shaded bars) were mated with vasectomised *Il10* [+/+] males, treated on days 2.5 and 3.5 pc before embryo transfer into day 2.5 pc Swiss recipients. (A) the mean ± SEM fetal weight, (B) the mean ± SEM placenta weight, and (C) the mean ± SEM fetal:placental ratio. n = 6 recipients per group; Data was compared by independent t-test, *p < 0.05 when compared to control group.

4.5. EFFECTS OF MATERNAL LPS TREATMENT DURING PRE-IMPLANTATION PERIOD ON FETAL AND PLACENTAL DEVELOPMENT IN NATURAL MATING AND EMBRYO TRANSFER

Utilising the data obtained from the experiment carried out in chapter 3, the effects of embryo transfer on fetal and placental development could be assessed. The two groups are comparable as the time of gestation in natural mating groups (day 17.5 pc) was equivalent to the embryo transfer groups (day 17.5 pc in recipient females). However, as the recipient females were of a different genotype, the effects of embryo transfer on fetal and placental development could not be fully assessed as maternal effects may be different based on the maternal genetic background. Alternatively, the effects of maternal LPS treatment on fetal and placental development in both natural mating and embryo transfer conditions are comparable.

Similar trends were observed in the fetal and placental development in both natural mating and embryo transfer groups. A reduction in fetal weights following embryo exposure to maternal LPS treatment was observed in both groups compared to their respective control groups (9% and 21% reduction respectively in natural mating and embryo transfer, $p < 0.05$, Table 4.2, independent t-test) while increased placental weight following maternal LPS treatment was observed in both groups compared to their respective control groups (9% and 14% increment respectively in natural mating and embryo transfer, $p < 0.05$). Similar changes in fetal:placental weight ratios were also observed in both groups following maternal LPS treatment (17% and 31% reduction vs. respective control group, $p < 0.05$).

Table 4.2 **Effect maternal LPS treatment on fetal and placental development in natural mating and embryo transfer**

	Natural mating Control #	Natural mating 0.5 μg LPS #	Embryo transfer Control ^	Embryo transfer 0.5 μg LPS ^
Fetal weight (mg)	832 ± 15	759 ± 14 *	909 ± 25	719 ± 35 *
Placental weight (mg)	94 ± 1	102 ± 2 *	103 ± 3	117 ± 3 *
Fetal:placental weight ratio	9.0 ± 0.2	7.5 ± 0.1 *	9.0 ± 0.3	6.2 ± 0.3 *
Mean litter size	6.8 ± 0.4	6.7 ± 0.5	6.0 ± 0.5	4.0 ± 0.4

Data are expressed as mean ± SEM.

n = 15 and 11 *Il10* $^{+/+}$ females for PBS control and LPS treatment group.

^ n = 6 recipient females per group. Donor to recipient transfer ratio is 1:1.

* $p < 0.05$ compared to respective control groups (independent t-test).

4.6.   DISCUSSION

This study was undertaken to investigate the mechanism by which LPS acts during the pre-implantation period to alter late gestation outcomes in the maternal systemic inflammation model. The experiments presented in this chapter show that (1) the effects of systemic LPS are largely effected at the level of the pre-implantation embryo as opposed to maternal tract receptivity, and (2) the effects on the embryo are likely to be mediated by elevated production of inflammatory cytokines in the oviduct and the uterus. The changes in cytokine expression levels after maternal LPS treatment included elevated production of factors known to have adverse effect on embryo survival and programming and these were evident in both the oviduct and uterus so could exert effects at both the cleavage and blastocyst stages of development.

The experiments described in Chapter 3 demonstrated that maternal systemic inflammation elicited by LPS lead to a dramatic decrease in pregnancy rates and fetal growth. The experiments in this chapter show the changes in implantation success and fetal growth is likely to be secondary to LPS acting indirectly on the pre-implantation embryo via the maternal tract. As the embryo has no direct contact with the maternal tract during this period, cytokines provide a signalling mechanism whereby the embryo can sense and respond to the changes occurring during the peri-conceptional through to the pre-implantation period, as embryos express cytokine receptors from conception to implantation (Hardy and Spanos, 2002). Cytokines are implicated in critical reproductive processes including modulating embryo development, endometrial receptivity and implantation competence (Kaye and Harvey, 1995, Stewart et al., 1992). The balance of cytokines levels in the maternal environment appears critical to ensuring competent implantation and successful pregnancy, as an imbalance of pro-inflammatory and anti-inflammatory cytokines can lead to implantation failure or embryo degradation (Winger et al., 2011, Pampfer et al., 1994a, Pampfer et al., 1997b).

It was a limitation of this study that the oviduct and uterine tissues were both evaluated at the same time point, on day 3.5 pc. At this time, the blastocyst stage embryo is located in the uterus, having transitioned from the oviduct approximately 24 h earlier. However as maternal LPS treatment was given on both days 2.5 and 3.5 pc, the cytokine gene expression profile in the oviduct tissue was assumed to be indicative of the oviductal environment the embryo is exposed at morula stage on day 2.5 pc. The shift in cytokine mRNA levels induced by LPS in the maternal tract indicates that the embryo is likely to

be exposed to pro-inflammatory TNFα which is known to be detrimental to embryo development and viability (Wuu et al., 1999). *In vivo* studies in rodents have shown that mouse blastocysts express the TNFα receptor and exposure to TNFα results in increased cell death, particularly in the ICM cells that later form the fetal tissue (Pampfer et al., 1994b). Maternal diabetes, which is known to induce high levels of TNFα in the reproductive tract, revealed that the adverse effects of TNFα resulted in programming effects on the pre-implantation embryo and those effects were carried over when blastocysts from diabetic rats were flushed and cultured in vitro for 48 hours (Pampfer et al., 1994c, Pampfer et al., 1997a, Pampfer et al., 1997b). LPS-induced fetal resorption is known to be associated with intrauterine production of TNFα (Gendron et al., 1990). This was found to be mediated by LPS binding to Toll-like receptor 4 (TLR4) that is expressed in both human and mouse reproductive tract epithelial cells (Soboll et al., 2006, Allhorn et al., 2008). TLR4 binds LPS and stimulates up-regulated expression and secretion of pro-inflammatory cytokines including TNFα, IL-1α and IL-6 (Soboll et al., 2006). This is consistent with various systemic inflammation models in rodents during different time points throughout gestation where administration of LPS induced TNFα and other cytokines in the maternal serum and reproductive tissues (Hodyl et al., 2007, Robertson et al., 2007, Robertson et al., 2006, Williams et al., 2011).

Although the cytokine gene expression in the PBS-treated *Il10* [+/+] and *Il10* [-/-] mice were not significantly different, it appears that with larger n values, differences in *Tnfa*, *Csf2* and *Il12a* may become apparent. As *Il10* [-/-] mice are reported to have a disposition to chronic enterocolitis (Berg et al., 1996), their normal cytokine profile could be altered by compensating for deficiency in IL-10 in order to dampen a pro-inflammatory profile. However additional experiments would be needed to evaluate this. It was not possible to evaluate whether changes in cytokine gene expression were accompanied by changes in protein levels. Initial exploratory experiments revealed that it is extremely difficult to recover the very small volumes of fluid (estimated to be 2-3 μl) from mouse oviduct.

Despite the fact that TNFα is one of the major pro-inflammatory cytokines induced by maternal LPS treatment and its well-known adverse effects on embryos, the use of a soluble TNFα receptor etanercept, did not protect the embryo from maternal LPS treatment. Previous studies utilising etanercept have shown that similar doses of etanercept were able to protect the fetus from the harmful effects of LPS during mid-gestation and was able to attenuate TNFα and several other pro-inflammatory

cytokines downstream of TNFα such as IL-6, IL-1α and IL-12p40 (IL-12b) (Robertson et al., 2007). Renaud et al. demonstrated that etanercept decreases LPS-induced fetal death and this was correlated to alterations in the uteroplacental perfusion (Renaud et al., 2011). A possible explanation could be that the local concentration of etanercept reaching pro-inflammatory cytokines surrounding the embryo was insufficient to neutralise the elevated TNFα and protect the embryo. It is also possible that etanercept was able to inhibit the adverse effects of TNFα, but other pro-inflammatory cytokines were still harmful. The embryo expresses receptors for IFNγ and its development is profoundly inhibited by this cytokine (Truchet et al., 2001), so even if TNFα were neutralised it is likely the elevated IFNγ induced by LPS in both $Il10^{+/+}$ and $Il10^{-/-}$ mice would have adverse effects.

Taken together, the data from experiments here and in Chapter 3 allow us to surmise that cytokines may be a major mechanism of action for LPS effects on the embryo in our model. However, the possibility that altered uterine receptivity caused by maternal systemic inflammation may contribute to the effects reported in chapter 3 required investigation. Thus, in an attempt to further dissect the action of LPS in early pregnancy, embryo transfer was utilised so that implantation and ongoing fetal development could be achieved independently of any effects of LPS on the maternal environment at implantation.

Using this experimental approach, implantation rates were similar regardless of donor LPS treatment, although there was evidence of reduced post-implantation survival in LPS-exposed embryos. Importantly, the programming effect of LPS on fetal and placental development was clearly maintained after embryo transfer. The changes seen in fetal-placental development from LPS-treated donor embryos were similar to the changes seen in the embryos from LPS-treated females in chapter 3. This indicates that the effect of maternal LPS is carried over with the pre-implantation embryo after transfer and suggests that any effects of altered cytokine expression in embryos were exerted before embryo recovery from donors. As the recipient mothers were of a different genetic background, it is possible that there is an effect of uterine genotype on fetal and placental development. However, a similar scale of change in altered fetal and placental development due to LPS was observed in Swiss as in the C57Bl/6 mothers, suggesting that the effect of LPS on embryo programming manifests independently of uterine genotype. Incidentally, this also does not allow us to completely rule out an effect of LPS on maternal receptivity. As implantation rates were not significantly affected by donor LPS treatment, it is possible

that the reduction in pregnancy rates observed in the maternal systemic inflammation model in chapter 3 may be in part due to altered maternal tract receptivity caused by LPS treatment. It would be interesting in future experiments to examine effects of LPS on endometrial determinants of implantation success such as induction of immune tolerance and associated leukocyte changes.

The extent to which the embryo transfer experiment is useful for understanding the effect of LPS on the embryo is confounded by the major effects of the transfer process which independently affects fetal and placental development. When the data were analysed according to maternal environment (recipients or natural mating), substantial effects of embryo transfer alone were evident. At the time of embryo transfer, the pregnant recipients were designated equivalent to day 2.5 pc and were killed 15 days later at day 17.5 pc. Since embryos were transferred as blastocysts, they may have implanted earlier than day 4.5 pc, resulting in a slightly advanced fetal and placental development compared to the pups from the natural mating experiments. Although the pups were larger following embryo transfer in the control group, fetal development was still restricted after donor LPS treatment. The larger difference between the control and LPS groups in the embryo transfer experiment compared to the natural mating groups could be due to a combination of maternal LPS treatment and embryo transfer effects.

In addition, the embryos utilised in the embryo transfer in the experiment were developed in natural conditions, devoid of any ovarian hyper-stimulation with gonadotrophins, which is standard practice for embryo transfer experiments. A stimulated oviductal environment has been demonstrated to be detrimental towards the developmental competence of the pre-implantation embryo (Van der Auwera et al., 1999). Furthermore, the immediate transfer of blastocysts as was conducted in our protocol reduces stress on the embryo resulting from the procedure, compared with protocols involving embryo culture *in vitro*.

There is the possibility that mediators apart from cytokines could be altered after maternal LPS exposure. These agents could contribute to the adverse effects of LPS on the embryo and this was not evaluated in our experiments. One potential mediator could be nitric oxide (NO), which was found to be elevated in the decidual tissue and uterus when LPS-induced embryonic resorption occurs in mice (Ogando et al., 2003). Another possibility is reactive oxygen species (ROS) which are shown to be involved in LPS-induced intrauterine growth restriction and skeletal retardation (Xu et al., 2006b).

In summary, the findings we reported in this chapter reveal that cytokines are likely to be involved in mediating the adverse effects of maternal systemic inflammation caused by LPS. The results from this chapter provide an indication that the effects of maternal LPS treatment were carried over or 'programmed' in the pre-implantation embryo for subsequent development and support the conclusion that programming effects of maternal LPS treatment is largely due to effects on the embryo and not maternal tract receptivity to implantation. However, we cannot exclude the possibility that altered uterine receptivity may be a contributing factor in our model. To more fully distinguish the effects of the altered maternal tract from the intrinsic effects on the embryo, additional embryo transfer strategies could be utilised. Transfer of blastocysts from control and LPS-treated donors into control and LPS-treated recipients would give some indication of the effects of LPS on uterine receptivity to embryo implantation and placental development. However, assessment of uterine receptivity may prove to be difficult due to the direct effects of embryo transfer on development. An altered protocol would be required as the embryo transfer described in this chapter was performed on day 2.5 pc recipients, while the LPS treatments are administered on both days 2.5 and 3.5 pc. Wiebold et al have shown that day 2.5 pc recipients have higher pregnancy and embryo survival rates compared to day 3.5 pc recipients (Wiebold and Anderson, 1986). Assessment of the importance of *Il10* status may also be difficult as the background strain for the *Il10* $^{+/+}$ and *Il10* $^{-/-}$ mice is C57Bl/6, which are known to be even more sensitive to embryo manipulation than other strains. Consistent with this, our preliminary experiments in C57Bl/6 recipients showed very poor implantation rates that would be incompatible with a successful pregnancy outcome.

Chapter 5

Postnatal development following cytokine environment perturbation during the pre-implantation period

5.1.   INTRODUCTION

The previous chapters describe the impact of maternal LPS treatment during the pre-implantation period on embryo and fetal development and the importance of maternal IL-10 in protecting the resulting fetus from the adverse effects of LPS. The mechanism of action of LPS on the pre-implantation embryo was examined in chapter 4. These experiments provide evidence for the importance of events occurring during the pre-implantation period, which is highly sensitive to perturbations that can result in implantation failure or altered outcomes for the pregnancy.

As mentioned in chapter 3, the fetal origins of adult disease hypothesis links adverse intrauterine environment with offspring phenotype in adult life (Barker and Clark, 1997). Many studies have focused on insults during the post-implantation period, during mid or late gestation. However, in the last decade there have been more perturbation studies targeting the early pregnancy period, as it has been recognised that the maternal environment during the peri-conceptional period, where the embryo forms and implants, is critical for pregnancy success. Even minor changes occurring during this time can influence the embryo to result in long term consequences for the fetus and neonate.

Several studies have shown that perturbations during early pregnancy can alter pregnancy outcomes and/or act to 'program' development in the resulting offspring. The most studied insults to date are maternal diet and embryo culture. Maternal undernutrition during the pre-implantation period has been shown to alter postnatal phenotype and to increase susceptibility to cardiovascular and metabolic diseases in rodents (Kwong et al., 2000, Watkins et al., 2008). Studies in rats revealed that maternal obesity at conception increases the risk of obesity in adult male offspring (Shankar et al., 2008).

Due to the wide use of ART techniques in Western countries, a lot of research has been done to investigate the long term health effects in children derived from ART. It is widely known that ART techniques, including IVF and ICSI are associated with increased risks of birth and congenital heart defects in ART children (D'Souza et al., 1997, Hansen et al., 2005, Tararbit et al., 2012, Savage et al., 2011). A study in mice has shown that embryo culture increases systolic blood pressure in adult female offspring (Watkins et al., 2007). Sjoblom et al. found that embryo culture in normal media results in accelerated postnatal growth and increased body mass and adiposity in the adult male offspring

compared to the *in vivo* counterparts, while addition of GM-CSF into the media alleviates many effects of culture on postnatal growth (Sjoblom et al., 2005).

However, few studies have looked at the long term effects of infection or inflammation during the pre-implantation period. One study has shown that maternal LPS treatment at the zygote stage resulted in sex-specific changes in the adult progeny, including increased fat pad:body weight ratio and increased body mass index (BMI) in the male progeny (Williams et al., 2011).

The experiments outlined in this chapter were designed to examine the effects of maternal LPS treatment during the pre-implantation period on growth trajectory, body morphometry and immune response to LPS in resultant progeny.

In many studies evaluating postnatal growth trajectory, mice are killed throughout the experiment for full body composition in order to measure growth at different time points. However, this method requires a large number of mice and results in a lower number of analysed progeny at the final time point of the experiment. Dual energy X-ray absorptiometry (DEXA) scanning, which was originally developed to examine bone mineral density, has been used to efficiently measure lean tissue and fat volume in humans and rodents. Over the duration of our postnatal study DEXA analysis was utilised, where the progeny were anaesthetised and scanned at 7, 13 and 19 weeks of age. This method eliminated the need to sacrifice the mice at earlier time points of the experiment, and allowed multiple *in vivo* measurements of bone and tissue composition to be collected at various time points for every individual offspring.

An additional experiment was carried out to investigate the effects of maternal LPS treatment during the pre-implantation period on the immune response of the adult progeny to LPS challenge. LPS is known to elicit an inflammatory response, including in gestational tissues where increased expression of TNFα, IL-6 and IFNγ in the serum and amniotic fluid is seen 4 hours following LPS administration (Robertson et al., 2007, Robertson et al., 2006, Oskvig et al., 2012). Several previous studies have shown that the LPS response can be attenuated by previous LPS exposure (Hodyl et al., 2007, Shanks et al., 1995, Shanks et al., 2000, Urakubo et al., 2001). Williams et al. has shown that maternal LPS treatment during

the zygote stage results in a blunted immune response to LPS challenge in offspring. Several serum cytokines including IL-1α, IL-1β and IL-10 were reduced in the progeny derived from LPS-treated females when compared to the control progeny (Williams et al., 2011). Therefore, the adult progeny from our model were treated with LPS to investigate whether exposure to systemic LPS as a pre-implantation embryo alters the later life response to LPS.

To compare the 4 groups (2 genotypes and 2 treatments) in the postnatal weight and DEXA data over time, a linear mixed effects model was fitted to the data where group, time and interaction between group and time were fitted as fixed effects. Differences in growth according to sex were analysed in a 3-way interaction between group, sex and time. Litter size was included as a covariate to control for differences in weight due to litter size. To statistically analyse each offspring as a repeated measurement for each female (mother), random effects for female and offspring within female were also added to the model to control for the dependence in repeated measurements within offspring and correlations between offspring from the same litter. A similar approach was utilised to analyse the full body composition. For Luminex data, the time variable was omitted as data was collected at one time point only.

5.2. EFFECT OF MATERNAL SYSTEMIC INFLAMMATION DURING PRE-IMPLANTATION PERIOD ON POSTNATAL GROWTH AND DEVELOPMENT

In chapter 3, maternal LPS treatment during the pre-implantation period was found to restrict fetal development in $Il10^{+/+}$ and $Il10^{-/-}$ females at day 17.5 pc. To determine the extent to which maternal systemic inflammation by LPS during the pre-implantation period affects postnatal outcomes for those fetuses which survive pregnancy, a third cohort of $Il10^{+/+}$ and $Il10^{-/-}$ females mated to $Il10^{+/+}$ males were allowed to give birth and the growth of their progeny was assessed. A total of 52 litters from 52 mothers were generated ($Il10^{+/+}$ PBS = 13; $Il10^{+/+}$ LPS = 15; $Il10^{-/-}$ PBS = 14; $Il10^{-/-}$ LPS = 10), resulting in 296 mice born.

Pregnancy viability (the proportion of mated females progressing to viable pregnancy) in the PBS-treated $Il10^{-/-}$ females was reduced by 12% compared to control $Il10^{+/+}$ females (Figure 5.1A). Following LPS treatment on days 2.5 and 3.5 pc, pregnancy viability was reduced by 12% in the $Il10^{+/+}$ females and 27% in the $Il10^{-/-}$ females compared to the respective PBS-treated controls. Similar to the results reported in chapter 3, the reduction in pregnancy viability in the $Il10^{+/+}$ females treated with LPS did not reach significance while the reduction in pregnancy viability observed in the $Il10^{-/-}$ females was significant relative to the $Il10^{-/-}$ controls ($p < 0.05$, χ^2-test, Figure 5.1A). Maternal IL-10 deficiency and LPS treatment together were found to significantly reduce pregnancy viability by 27% compared to the PBS-treated $Il10^{+/+}$ ($p < 0.05$, Figure 5.1A).

Gestational length showed no significant difference between the four treatment groups with a mean gestational time of 19.5 ± 0.2 days for each group (Figure 5.1B). At the time of birth, there was no significant difference in litter size due to maternal LPS treatment in either $Il10^{+/+}$ or $Il10^{-/-}$ females compared to the respective PBS controls (Figure 5.1C). However, litter size did vary due to maternal IL-10 deficiency where PBS-treated $Il10^{-/-}$ females had fewer pups (5.8 ± 0.3 pups) when compared to PBS-treated $Il10^{+/+}$ females (7.2 ± 0.3 pups) ($p = 0.019$, ANOVA, Figure 5.1C).

Newborn progeny derived after LPS treatment from both $Il10^{+/+}$ and $Il10^{-/-}$ females displayed no difference in birth weight compared to progeny of PBS-treated controls (1.32 ± 0.02g for $Il10^{+/+}$ and 1.37 ± 0.02g for $Il10^{-/-}$) (mixed model ANOVA, Figure 5.1D). Similarly, no significant interaction was

observed between progeny weight at day 8 with maternal LPS treatment (3.62 ± 0.2g for PBS-treated *Il10* [+/+] and 3.73 ± 0.2g for PBS-treated *Il10* [-/-], Figure 5.1E).
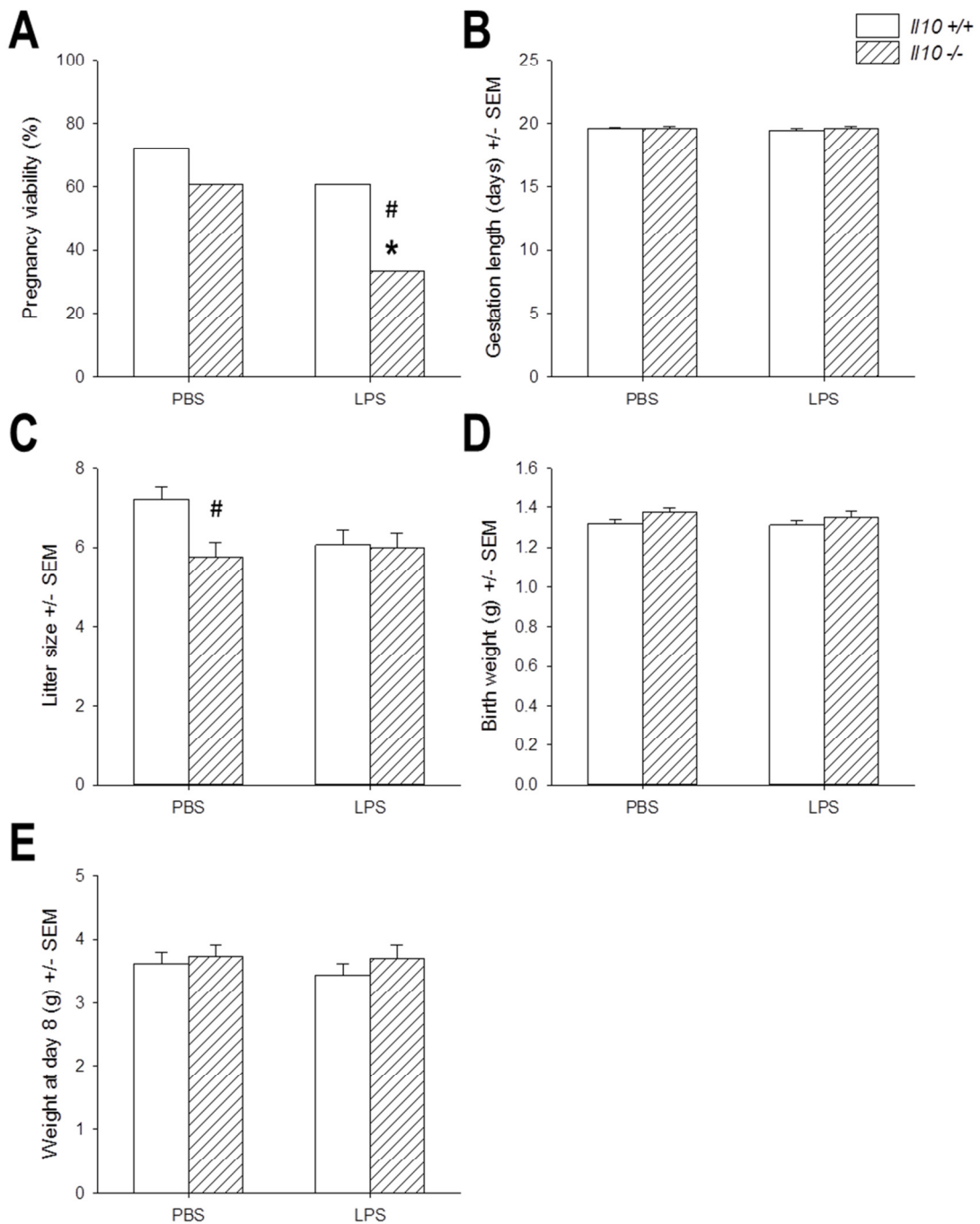
Figure 5.1   Effect of low dose LPS at days 2.5 and 3.5 pc on pregnancy parameters at term.

All *Il10* ^{+/+} females (open bars) and *Il10* ^{-/-} females (shaded bars) were mated with *Il10* ^{+/+} males and were treated with 0.5 μg LPS or PBS on days 2.5 and 3.5 pc. (A) the percentage of plugged females progressing to viable pregnancies, (B) the mean ± SEM number of gestation days, (C) the mean ± SEM number of pups in each litter per mated female, (D) the mean ± SEM birth weight of each progeny per treatment group, and (E) the mean ± SEM weight at day 8 of each progeny per treatment group. n = 19 - 30 mated females per group; Data was compared by χ^2-test (A), one way ANOVA (B and C) and mixed model ANOVA (D and E), *p < 0.05 when compared to control group and # p < 0.05 when compared with *Il10* ^{+/+} group of the same treatment. Data for 24 hours and day 8 are combined from both male and female progeny.

Pups were weaned at three weeks of age and housed with littermates of the same sex, then weighed at 2 week intervals until 19 weeks of age. When analysing the male and female ratio within each litter, the number of male pups born within a litter were generally higher in all 4 groups. No significant differences were found in ratio of male to female progeny between treatment groups (Table 5.1).

Table 5.1   **Effect of maternal LPS treatment during the pre-implantation period on progeny sex ratio.**

Treatment	n	Male/females pups in total	Fraction male pups per litter*^	Fraction female pups per litter*^	Male:female ratio*^
Il10 +/+ PBS	13	45/38	0.52 ± 0.05	0.44 ± 0.06	1.47 ± 0.20
Il10 +/+ LPS	15	51/29	0.67 ± 0.05	0.41 ± 0.05	1.89 ± 0.39
Il10 -/- PBS	14	45/31	0.62 ± 0.05	0.35 ± 0.04	1.95 ± 0.44
Il10 -/- LPS	10	36/21	0.64 ± 0.07	0.36 ± 0.07	1.33 ± 0.19

n = number of mothers per treatment group.

*All data expressed as mean ± SEM. No significant differences between control and treatment groups (ANOVA).

^Sex was determined when pups were weaned at 3 weeks.

Progeny weight at 3 weeks of age onwards was analysed according to sex and the data from male and female progeny are reported separately as the mixed model analysis revealed an interaction between sex and maternal LPS treatment on postnatal growth. Comparison of the *Il10* +/+ females revealed that male progeny derived from LPS-treated mothers displayed a 12% reduction ($p < 0.001$) in weight at 5 weeks of age when compared to male progeny of PBS-treated mothers (Figure 5.2A). This reduction in male progeny weight was also observed at 7 weeks of age (8% reduced, $p = 0.002$), 9 weeks of age (8% reduced, $p = 0.001$), 11 weeks of age (6% reduced, $p = 0.009$) and 13 weeks of age (5% reduced, $p = 0.015$) when compared to male progeny of PBS-treated *Il10* +/+ mothers (Figure 5.2A, 5.2B, mixed model ANOVA).

Likewise, the effect of LPS treatment in the *Il10* -/- females also resulted in a significant reduction in male progeny weight at 7 weeks of age (6% reduced, $p = 0.03$), 9 weeks of age (7% reduced, $p = 0.007$), 11 weeks of age (5% reduced, $p = 0.019$), 13 weeks of age (4% reduced, $p = 0.044$) and 15 weeks of age (5% reduced, $p = 0.017$) when compared to male progeny of PBS-treated *Il10* -/- mothers (Figure 5.2A, 5.2C).

Analysis of progeny weight from both *Il10* ⁺/⁺ and *Il10* ⁻/⁻ PBS-treated mothers revealed that male progeny derived from *Il10* ⁻/⁻ mothers are generally heavier than those of *Il10* ⁺/⁺ mothers. A significant increase in progeny weight was observed from week 9 until the end of the experiment at 19 weeks (6% increased at week 9, $p = 0.01$), (7% increased at week 11, $p = 0.002$), (7% increased at week 13, $p = 0.003$), (8% increased at week 15, $p = 0.002$), (10% increased at week 17, $p < 0.001$) and (7% increased at week 19, $p < 0.001$) when compared to progeny from PBS-treated *Il10* ⁺/⁺ mothers (Figure 5.2A).

Maternal IL-10 deficiency resulted in a similar increase in weight in the resulting male progeny after maternal LPS treatment. Male progeny derived from LPS-treated *Il10* ⁻/⁻ mothers were observed to be heavier when compared to the male progeny from LPS-treated *Il10* ⁺/⁺ mothers (7% increased at week 7, $p = 0.018$), (8% increased at week 9, $p = 0.004$), (8% increased at week 11, $p = 0.002$), (8% increased at week 13, $p = 0.001$), (7% increased at week 15, $p = 0.006$), (9% increased at week 17, $p < 0.001$) and (7% increased at week 19, $p < 0.001$) (Figure 5.2A).

In contrast, postnatal growth of female progeny from *Il10* ⁺/⁺ and *Il10* ⁻/⁻ mothers was less affected compared to their male littermates. Female progeny from LPS-treated *Il10* ⁺/⁺ mothers were significantly lighter in weight from week 5 to week 9 when compared to progeny of PBS-treated *Il10* ⁺/⁺ mothers (12% reduced at week 5, $p = 0.005$), (9% reduced at week 7, $p = 0.011$) and (8% reduced at week 9, $p = 0.015$) (Figure 5.3A, 5.3B, mixed model ANOVA).

No significant effect of LPS was observed in growth trajectory of the female progeny of *Il10* ⁻/⁻ females (Figure 5.3A, 5.3C). Regardless of IL-10 genotype, female progeny from *Il10* ⁺/⁺ and *Il10* ⁻/⁻ mothers displayed similar body weights throughout their postnatal development and the combination of maternal LPS treatment and IL-10 deficiency did not alter the development of female progeny.
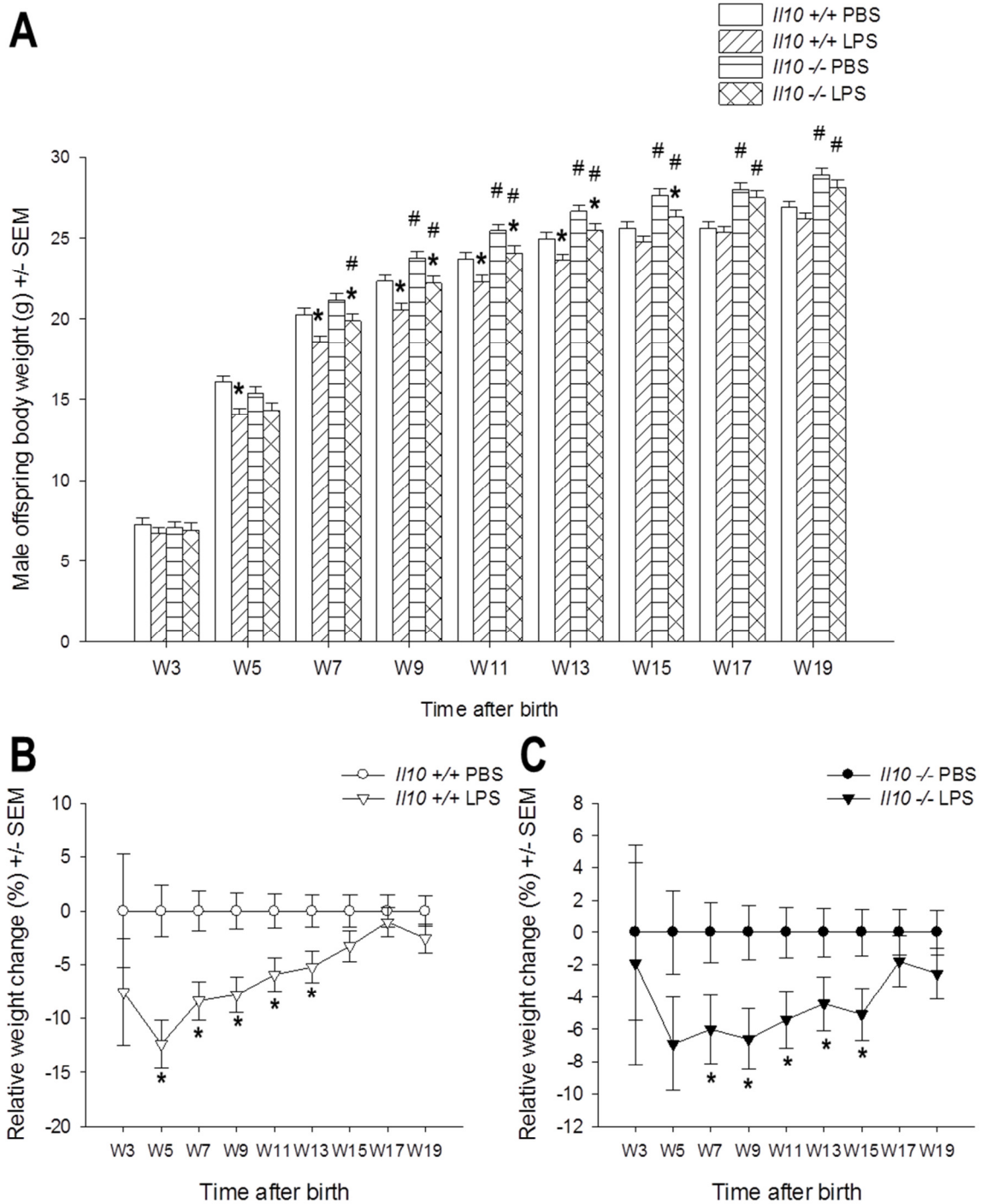
Figure 5.2   Growth trajectory of male progeny of *Il10 +/+* and *Il10 -/-* mothers from birth till 19 weeks.

Body weights (g) of male progeny of PBS-treated *Il10 +/+* (open bars/open circles), LPS-treated *Il10 +/+* (diagonal lined bars/open triangles), PBS-treated *Il10 -/-* (horizontal lined bars/closed circles) and LPS-treated *Il10 -/-* (cross lined bars/closed triangles) females mated to *Il10 +/+* males and treated on days 2.5 and 3.5 pc. n = 45 and 51 male progeny from PBS-treated and LPS-treated *Il10 +/+* mothers and 45 and 36 male progeny from PBS-treated and LPS-treated *Il10 -/-* mothers. Data was analysed by mixed model ANOVA, $*p < 0.05$ when compared to control group of the same genotype, $\#p < 0.05$ when compared to *Il10 +/+* genotype with the same treatment. Data are expressed as the estimated marginal mean ± SEM in (A) and percent relative change in weight in (B-C).
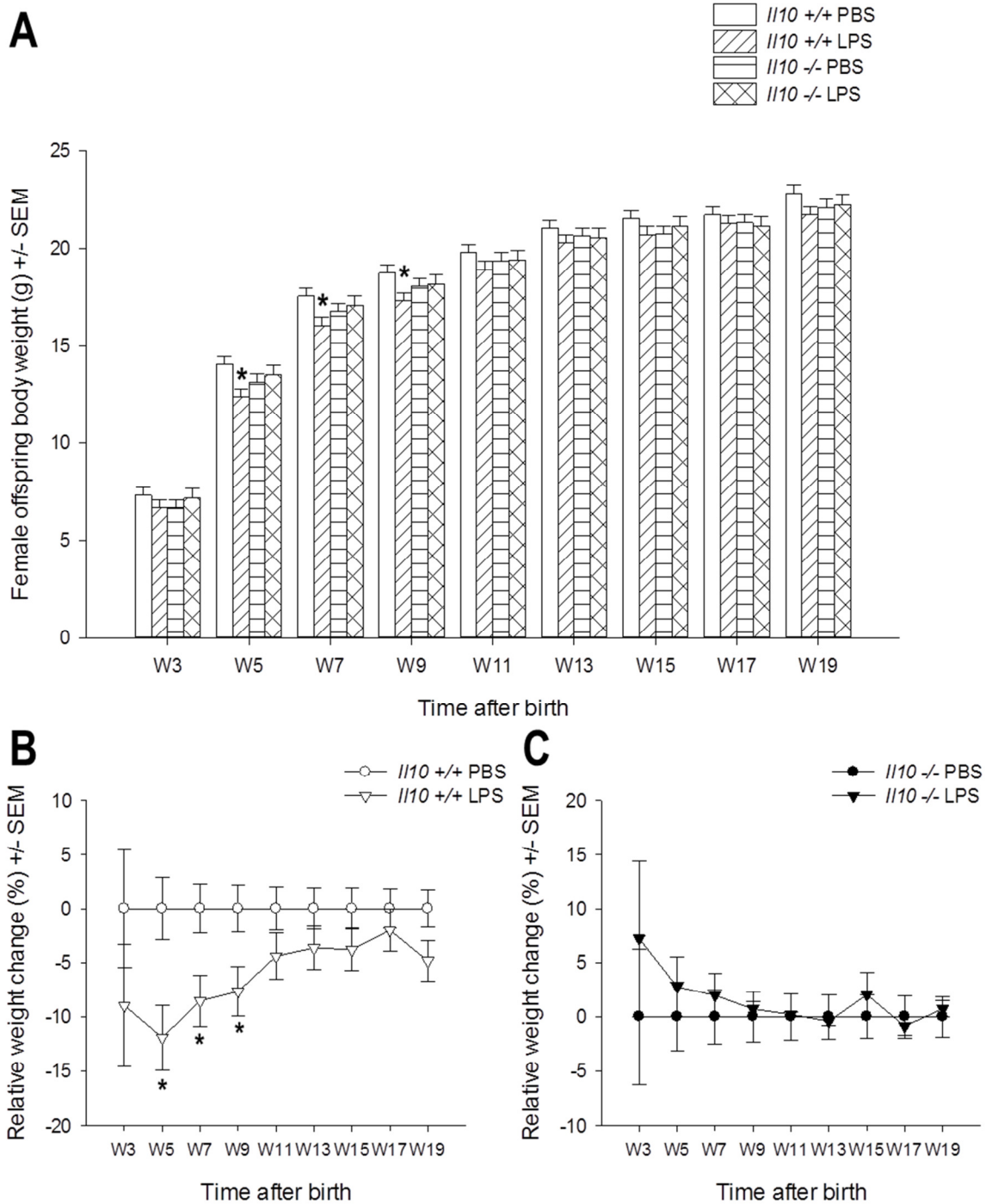
Figure 5.3 Growth trajectory of female progeny of *Il10 +/+* and *Il10 -/-* mothers from birth till 19 weeks.

Body weights (g) of female progeny of PBS-treated *Il10 +/+* (open bars/open circles), LPS-treated *Il10 +/+* (diagonal lined bars/open triangles), PBS-treated *Il10 -/-* (horizontal lined bars/closed circles) and LPS-treated *Il10 -/-* (cross lined bars/closed triangles) females mated to *Il10 +/+* males and treated on days 2.5 and 3.5 pc. n = 38 and 29 female progeny from PBS-treated and LPS-treated *Il10 +/+* mothers and 31 and 21 female progeny from PBS-treated and LPS-treated *Il10 -/-* mothers. Data was analysed by mixed model ANOVA, *$p < 0.05$ when compared to control group of the same genotype. Data are expressed as the estimated marginal mean ± SEM in (A) and percent relative change in weight in (B-C).

5.3. EFFECT OF MATERNAL LPS TREATMENT DURING PRE-IMPLANTATION ON BONE AND BODY COMPOSITION OF PROGENY.

To investigate the effects of maternal LPS treatment and maternal IL-10 deficiency on postnatal bone and body composition in the resulting progeny, DEXA analysis was performed throughout the experiment, at 6 week intervals at the 7, 13 and 19 week time points. At each time point, the mice were fasted for 4 hours before being anesthetised with Avertin and subjected to DEXA scanning. The image acquisition time on the DEXA machine is less than 5 minutes. The parameters measured directly by DEXA are the bone mineral density (BMD) and the %fat of the trunk tissue. From those results, the bone, fat and lean mass is computed by the DEXA software. The output is as shown in Figure 5.4. The DEXA results were normalised to individual body weight and displayed according to sex although there was no interaction between group, sex and time point.
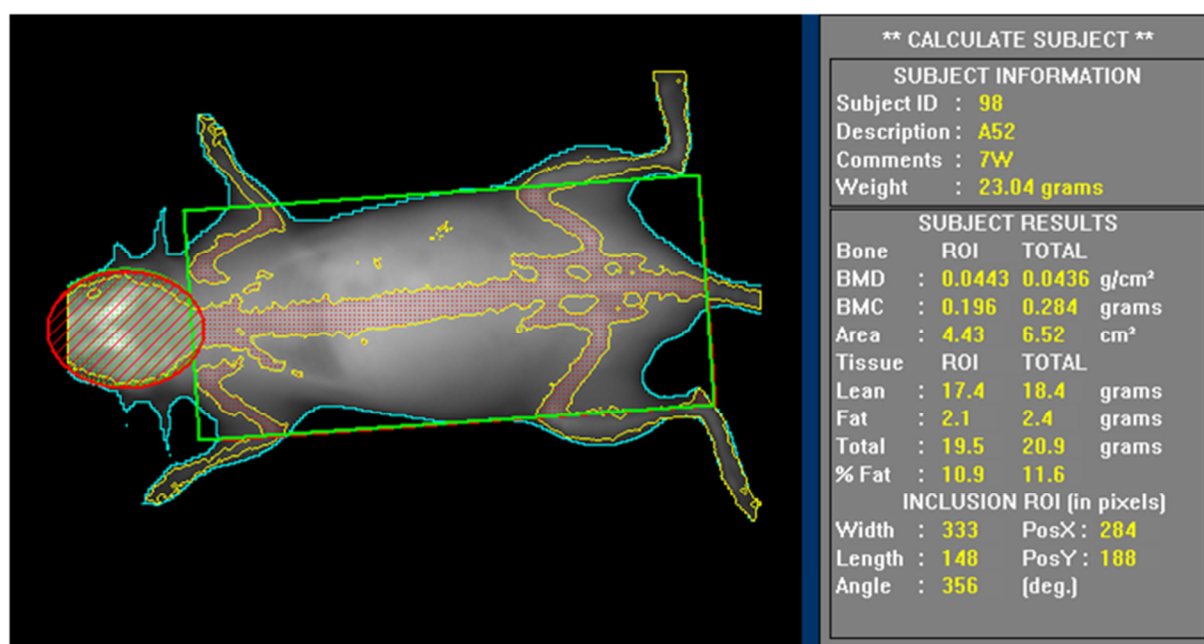
Figure 5.4   Example of DEXA analysis output of an individual mouse.

Each male and female offspring derived from *Il10* +/+ and *Il10* -/- females treated with PBS or 0.5 μg LPS on days 2.5 and 3.5 pc were fasted for 4 hours prior to being anaesthetised and subjected to DEXA analysis at the ages of 7, 13 and 19 weeks. The green box represents the region of interest (ROI) while the red circle is the exclusion area to avoid accounting for the brain tissue in the analysis.

5.3.1. Bone composition

Maternal IL-10 genotype and sex were found to be significant determinants of the bone mineral density (BMD), where only the male progeny was affected and maternal LPS treatment did not alter the BMD of the progeny. Male progeny derived from PBS-treated *Il10 $^{-/-}$* mothers displayed increases in BMD when compared to the progeny derived from PBS-treated *Il10 $^{+/+}$* mothers. An increment of 4% and 6% respectively in BMD was observed at week 13 (p = 0.026) and at week 19 (p = 0.001) (Figure 5.5A, mixed model ANOVA).

No effect of maternal LPS treatment or IL-10 deficiency was observed in the BMD of the female progeny of *Il10 $^{+/+}$* and *Il10 $^{-/-}$* mothers at any time point (Figure 5.5B).

Maternal IL-10 deficiency was found to be a significant determinant of the bone mineral content (BMC), followed by maternal LPS treatment in the male progeny but only at 19 weeks of age. Male progeny derived from PBS-treated *Il10 $^{-/-}$* mothers displayed a significant increase of 17% in BMC when compared to the male progeny derived from PBS-treated *Il10 $^{+/+}$* mothers (p < 0.001, Figure 5.5C). Maternal LPS treatment in *Il10 $^{+/+}$* mothers resulted in a 9% increase in BMC of the resulting male progeny when compared to their control counterparts (p = 0.022) while the combination of both maternal IL-10 deficiency and LPS treatment resulted in a further increase of 8% in male progeny from LPS-treated *Il10 $^{-/-}$* mothers when compared to male progeny from LPS-treated *Il10 $^{+/+}$* mothers (p = 0.036, Figure 5.5C).

No effect of maternal LPS treatment or IL-10 deficiency was observed in the BMC of the female progeny at any time point. However, female progeny from LPS-treated *Il10 $^{-/-}$* mothers displayed an increase of 11% in BMC at 19 weeks of age when compared to female progeny from PBS-treated *Il10 $^{-/-}$* mothers (p = 0.035, Figure 5.5D).
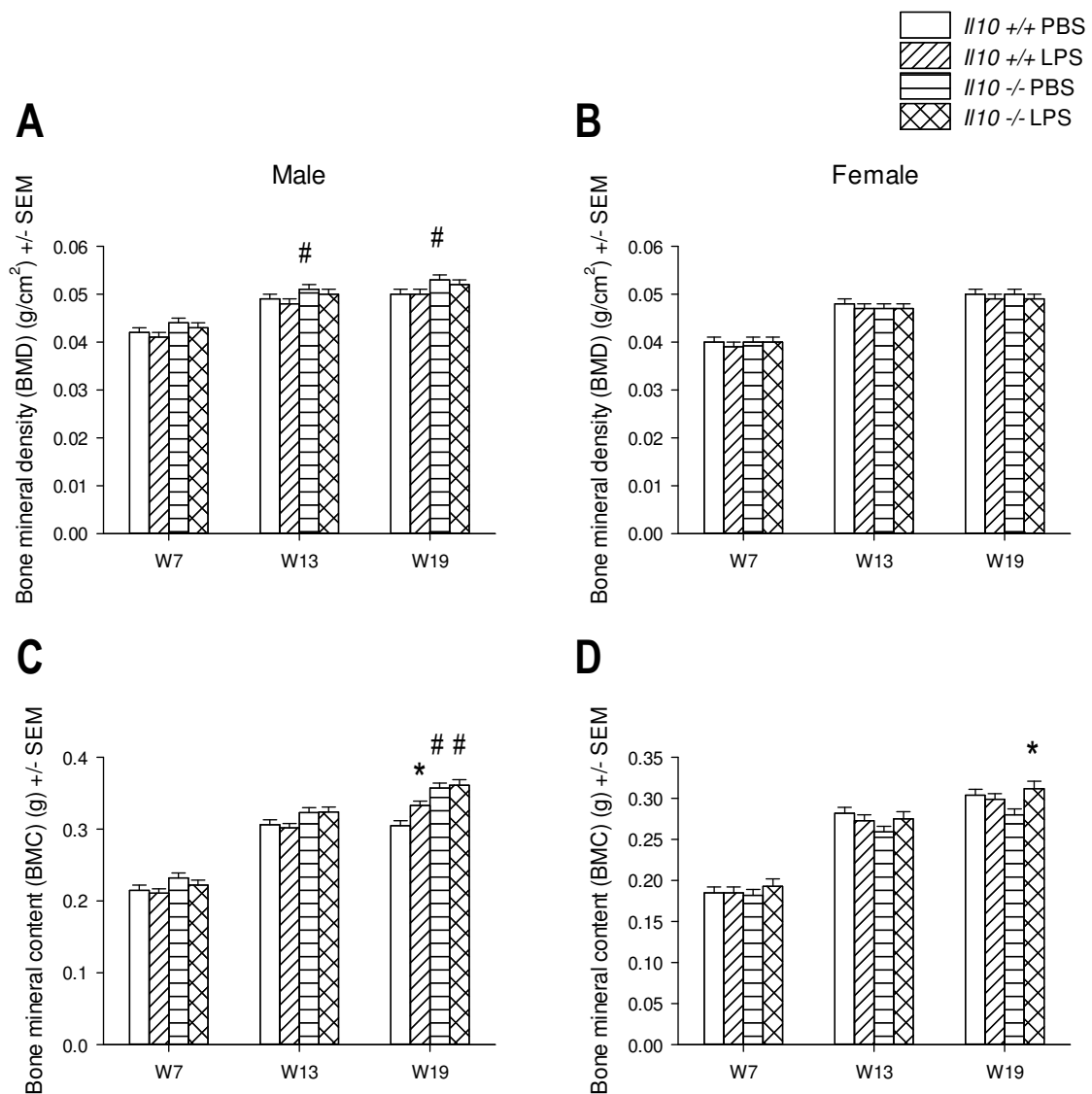
Figure 5.5   Bone composition of adult male and female progeny of *Il10 +/+* and *Il10 -/-* mothers at week 19.

(A, B) Bone mineral density (BMD) and (C, D) bone mineral content (BMC) of *Il10 +/+* and *Il10 +/-* male (left column) and female (right column) progeny at 7, 13 and 19 weeks of age. The progeny were derived from PBS-treated *Il10 +/+* (open bars/open circles), LPS-treated *Il10 +/+* (diagonal lined bars/open triangles), PBS-treated *Il10 -/-* (horizontal lined bars/closed circles) and LPS-treated *Il10 -/-* (cross lined bars/closed triangles) females mated to *Il10 +/+* males and treated on days 2.5 and 3.5 pc. n = 45 and 51 male progeny and 38 and 29 female progeny from PBS-treated and LPS-treated *Il10 +/+* mothers; 45 and 36 male progeny and 31 and 21 female progeny from PBS-treated and LPS-treated *Il10 -/-* mothers. All data are expressed as relative estimated marginal means ± SEM, corrected to individual body weight. Data was analysed by mixed model ANOVA, *p < 0.05 when compared to control group of the same genotype, #p < 0.05 when compared to *Il10 +/+* genotype with the same treatment.

5.3.2. Body composition

Analysis of body composition during postnatal development of male and female progeny derived from PBS and LPS-treated *Il10* +/+ and *Il10* -/- females utilising DEXA revealed that sex and maternal IL-10 genotype are significant determinants of lean tissue mass. An increase of 8% and 8% respectively in lean tissue mass was observed in male progeny derived from PBS-treated *Il10* -/- mothers at week 13 (p = 0.009) and week 19 (p = 0.003) when compared to male progeny derived from PBS-treated *Il10* +/+ mothers (Figure 5.6A, mixed model ANOVA). Similarly, increases of 8% and 9% respectively were observed in male progeny derived from LPS-treated *Il10* -/- mothers at week 13 (p = 0.025) and week 19 (p = 0.003) when compared to male progeny derived from LPS-treated *Il10* +/+ mothers (Figure 5.6A).

No effects of maternal LPS treatment or IL-10 deficiency on lean tissue mass were observed in the female progeny derived from either *Il10* +/+ or *Il10* -/- mothers at any time point (Figure 5.6B).

Sex was also found to be a significant determinant of fat tissue mass, where only male progeny were affected by maternal LPS treatment. Male progeny derived from LPS-treated *Il10* +/+ mothers displayed a reduction of 19% in fat tissue mass at 7 weeks of age when compared to male progeny derived from PBS-treated *Il10* +/+ mothers (p = 0.033, Figure 5.6C). In contrast, male progeny derived from LPS-treated *Il10* -/- mothers displayed a reduction of 16% in fat tissue mass at 13 weeks of age when compared to male progeny derived from PBS-treated *Il10* -/- mothers (p = 0.039, Figure 5.6C).

No effects of maternal LPS treatment or IL-10 deficiency on fat tissue mass were observed in the female progeny derived from either *Il10* +/+ or *Il10* -/- mothers at any time point (Figure 5.6D).

Consistent with the postnatal trajectory result observed in section 5.2, male progeny derived from *Il10* -/- mothers displayed a significant increase in total tissue mass compared to male progeny derived from *Il10* +/+ mothers. Increments of 9% and 7% respectively were observed in male progeny derived from PBS-treated *Il10* -/- mothers at week 13 (p = 0.008) and week 19 (p = 0.033) when compared to male progeny derived from PBS-treated *Il10* +/+ mothers (Figure 5.6E). Similarly, the combination of maternal LPS treatment and IL-10 deficiency resulted in increases of 8% and 8% respectively in total tissue mass

in male progeny derived from LPS-treated *Il10* $^{-/-}$ mothers at week 13 (p = 0.048) and week 19 (p = 0.011) when compared to male progeny derived from LPS-treated *Il10* $^{+/+}$ mothers (Figure 5.6E).

No effects of maternal LPS treatment or IL-10 deficiency on total tissue mass were observed in the female progeny derived from either *Il10* $^{+/+}$ or *Il10* $^{-/-}$ mothers at any time point (Figure 5.6F).

The percentage of body fat measured by DEXA was found to be affected by maternal LPS treatment only in the male progeny derived from *Il10* $^{+/+}$ mothers. Progeny derived from LPS-treated *Il10* $^{+/+}$ mothers displayed a significant reduction of 16% in body fat at 19 weeks of age when compared to male progeny derived from PBS-treated *Il10* $^{+/+}$ mothers (p = 0.033, Figure 5.6G). No significant effect of maternal LPS on percentage of body fat was observed in the male progeny derived from *Il10* $^{-/-}$ mothers.

In contrast, the percentage of body fat in female progeny was found to be affected by maternal LPS treatment only in the female progeny derived from *Il10* $^{-/-}$ mothers. Progeny derived from LPS-treated *Il10* $^{-/-}$ mothers displayed a significant reduction of 18% in body fat at 13 weeks of age when compared to female progeny derived from PBS-treated *Il10* $^{-/-}$ mothers (p = 0.006, Figure 5.6H). No significant effect on percentage of body fat was observed in the female progeny derived from *Il10* $^{+/+}$ mothers.

Figure 5.6   Body composition of adult male and female progeny of *Il10* ^{+/+} and *Il10* ^{-/-} at week 19.

DEXA analysis of the (A, B) lean tissue mass (g), (C, D) fat tissue mass (g), (E, F) total tissue mass (g) and (G, H) percent body fat (%) of *Il10* ^{+/+} and *Il10* ^{-/-} male (left column) and female (right column) progeny at 7, 13 and 19 weeks of age. The progeny were derived from PBS-treated *Il10* ^{+/+} (open bars/open circles), LPS-treated *Il10* ^{+/+} (diagonal lined bars/open triangles), PBS-treated *Il10* ^{-/-} (horizontal lined bars/closed circles) and LPS-treated *Il10* ^{-/-} (cross lined bars/closed triangles) females mated to *Il10* ^{+/+} males and treated on days 2.5 and 3.5 pc. n = 45 and 51 male progeny and 38 and 29 female progeny from PBS-treated and LPS-treated *Il10* ^{+/+} mothers; 45 and 36 male progeny and 31 and 21 female progeny from PBS-treated and LPS-treated *Il10* ^{-/-} mothers. Data are expressed as relative estimated marginal means ± SEM, corrected to individual body weight (A-F) and percent body fat (G-H). Data was analysed by mixed model ANOVA, *p < 0.05 when compared to control group of the same genotype, #p < 0.05 when compared to *Il10* ^{+/+} genotype with the same treatment.
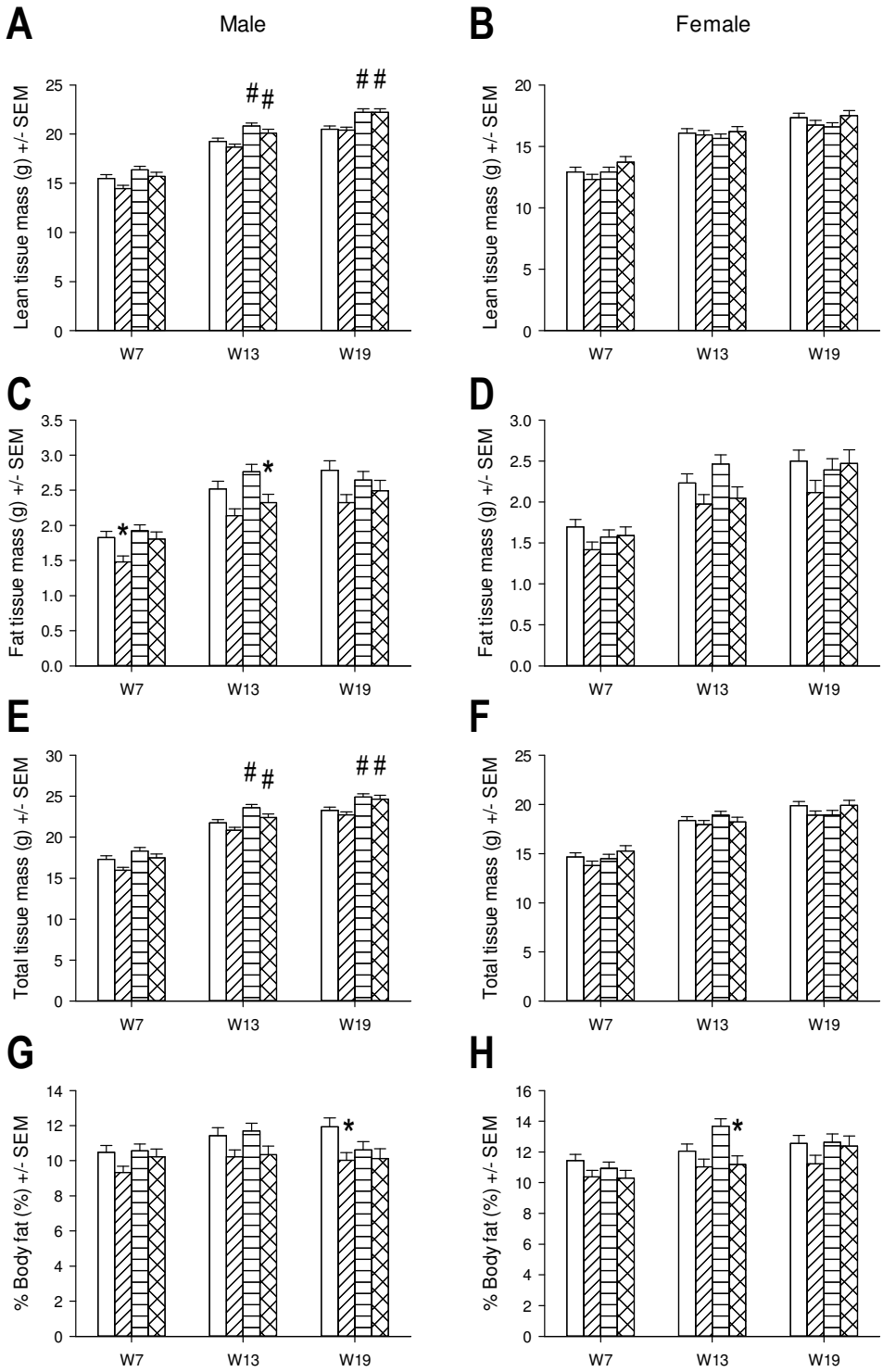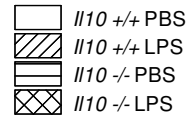
5.4. EFFECT OF MATERNAL SYSTEMIC INFLAMMATION DURING PRE-IMPLANTATION PERIOD ON BODY COMPOSITION OF PROGENY

The previous section demonstrated the effects of maternal LPS treatment and maternal IL-10 deficiency on the bone and body composition measured via non-invasive DEXA throughout the 19 week life course. The result showed that maternal LPS treatment affects the overall fat deposition, mainly in the male progeny. In a second set of analyses, progeny derived from both $Il10^{+/+}$ and $Il10^{-/-}$ mothers treated with PBS or 0.5 µg LPS were sacrificed following the final DEXA scan (19 weeks of age) and full body post mortems were carried out to determine the effect of maternal LPS treatment and maternal IL-10 deficiency on the anatomical development of the offspring. Full body composition was performed on a total of 258 progeny, 152 males and 106 females from 52 litters. Tissue weights were assessed as both absolute weights and relative weight (organ weight to lean body weight) to correct for differences in body weight and body fat of every individual mouse. The mesenteric fat was collected together with retroperitoneal fat. However smaller fat pads (the interscapular and inguinal fat pads) were not measured, since it was not practical to achieve this in a reasonable time frame for all autopsies.

Maternal LPS treatment in the $Il10^{+/+}$ mothers did not result in any changes in the absolute mass of any organ with the exception of a 18% reduction in the absolute mass in thymus tissue of the male progeny ($p = 0.002$, Table 5.2, mixed model ANOVA).

In the male progeny of $Il10^{-/-}$ mothers, maternal LPS treatment did not result in any change in absolute mass of any organ. However, the combination of maternal LPS treatment and IL-10 deficiency resulted in an increase in the absolute mass of the quadriceps (13% increase, $p = 0.048$) and testes (21% increase, $p = 0.002$) (compared to male progeny of LPS-treated $Il10^{+/+}$ mothers, Table 5.2). A significant increase of 37% was observed in the total central fat weight ($p = 0.002$), which consists of epididymal fat (41% increased, $p = 0.008$), retroperitoneal fat (29% increase, $p = 0.001$) and peri-renal fat (59% increase, $p = 0.008$) of male progeny of LPS-treated $Il10^{-/-}$ mothers when compared to males of LPS-treated $Il10^{+/+}$ mothers (Table 5.2). The shift in central fat mass resulted in a significant reduction in muscle to fat ratio by 18% in the male progeny of LPS-treated $Il10^{-/-}$ mothers when compared to males of LPS-treated $Il10^{+/+}$ mothers ($p = 0.031$).

Following correction of the organ weight to lean body weight revealed that the changes observed in the absolute mass were still maintained. The relative mass of the thymus was reduced by 14% in the males from LPS-treated *Il10* $^{+/+}$ mothers compared to control *Il10* $^{+/+}$ mothers (p = 0.018, Table 5.3). Similarly, no significant changes in the relative mass of any organ were reported in male progeny of *Il10* $^{-/-}$ mothers.

The effect of IL-10 genotype and maternal LPS treatment resulted in an increase of 31% and 13% in the relative weight in the spleen and testes of males from LPS-treated *Il10* $^{-/-}$ mothers compared to males from LPS-treated *Il10* $^{+/+}$ mothers (p = 0.034 and p = 0.011). The relative mass of total central fat was also similarly increased by 27% when comparing between male progeny derived from LPS-treated *Il10* $^{+/+}$ and *Il10* $^{-/-}$ mothers (p = 0.013), which was a result of increases in epididymal fat (35% increased, p = 0.008), retroperitoneal fat (22% increase, p = 0.003) and peri-renal fat (55% increase, p = 0.01) (Table 5.3).

Table 5.2 Effect of maternal LPS treatment during pre-implantation period and *Il10* genotype on body composition (absolute weight) of male adult progeny.

Absolute weight	*Il10* +/+ PBS (n = 35)	*Il10* +/+ LPS (n = 47)	*Il10* -/- PBS (n = 39)	*Il10* -/- LPS (n = 31)
Total body weight (g)	26.88 ± 0.38	26.19 ± 0.36	28.89 ± 0.40	28.15 ± 0.44
Lean body weight (g)	26.35 ± 0.52	25.64 ± 0.46	27.48 ± 0.50	27.55 ± 0.65
Muscle:fat ratio	0.59 ± 0.02	0.65 ± 0.02	0.56 ± 0.02	0.53 ± 0.03 #
Total Central Fat (mg)	863 ± 44	737 ± 40	961 ± 43	1010 ± 57 #
Epididymal Fat (mg)	324 ± 20	266 ± 18	350 ± 19	374 ± 26 #
Retroperitoneal Fat (mg)	436 ± 18	391 ± 16	501 ± 17	506 ± 23 #
Peri-renal Fat (mg)	105 ± 9	81 ± 8	123 ± 9	129 ± 12 #
Combined Muscle (mg)	496 ± 14	465 ± 12	525 ± 13	519 ± 18
Gastrocnemius (mg)	149 ± 4	140 ± 4	155 ± 4	155 ± 6
Quadriceps (mg)	192 ± 5	178 ± 5	203 ± 5	202 ± 7 #
Biceps (mg)	32 ± 2	29 ± 2	35 ± 2	32 ± 3
Triceps (mg)	123 ± 3	118 ± 3	129 ± 3	130 ± 4
Brain (mg)	428 ± 5	414 ± 5	422 ± 5	426 ± 7
Heart (mg)	141 ± 3	135 ± 3	146 ± 3	147 ± 4
Lungs (mg)	184 ± 5	180 ± 4	180 ± 6	180 ± 6
Thymus (mg)	55 ± 2	45 ± 2 *	51 ± 2	53 ± 2
Kidneys (L + R) (mg)	181 ± 4	170 ± 4	180 ± 4	180 ± 5
Adrenals (L + R) (mg)	2 ± 0	3 ± 0	2 ± 0	3 ± 0
Liver (mg)	1374 ± 38	1271 ± 33	1425 ± 36	1397 ± 47
Spleen (mg)	69 ± 6	66 ± 5	84 ± 6	94 ± 8 #
Seminal Vesicle (mg)	270 ± 9	254 ± 8	256 ± 9	247 ± 11
Testes (L + R) (mg)	91 ± 3	81 ± 3	98 ± 3	98 ± 4 #
Epididymis (L + R) (mg)	101 ± 2	93 ± 2 *	99 ± 2	100 ± 2

All data presented as estimated marginal means ± SEM.

The number of male progeny analysed is shown in parentheses.

The effect of maternal LPS treatment and *Il10* genotype was compared by Mixed Model Linear Repeated Measures ANOVA.

* $p < 0.05$ when comparing with PBS treatment of the same genotype

# $p < 0.05$ when comparing with *Il10* +/+ genotype of the same treatment.

Table 5.3   **Effect of maternal LPS treatment during pre-implantation period and *Il10* genotype on body composition (relative weight) of male adult progeny.**

Relative weight	*Il10* +/+ PBS (n = 35)	*Il10* +/+ LPS (n = 47)	*Il10* -/- PBS (n = 39)	*Il10* -/- LPS (n = 31)
Total Central Fat (%)	3.27 ± 0.15	2.87 ± 0.13	3.50 ± 0.14	3.61 ± 0.19 #
Epididymal Fat (%)	1.23 ± 0.07	1.03 ± 0.06	1.28 ± 0.06	1.39 ± 0.08 #
Retroperitoneal Fat (%)	1.66 ± 0.06	1.52 ± 0.06	1.83 ± 0.06	1.86 ± 0.07 #
Peri-renal Fat (%)	0.40 ± 0.03	0.31 ± 0.03	0.45 ± 0.03	0.48 ± 0.04 #
Combined Muscle (%)	1.89 ± 0.03	1.82 ± 0.02	1.91 ± 0.03	1.88 ± 0.04
Gastrocnemius (%)	0.57 ± 0.01	0.55 ± 0.01	0.57 ± 0.01	0.57 ± 0.01
Quadriceps (%)	0.73 ± 0.01	0.70 ± 0.01	0.74 ± 0.01	0.75 ± 0.02
Biceps (%)	0.12 ± 0.01	0.11 ± 0.00	0.13 ± 0.01	0.12 ± 0.01
Triceps (%)	0.47 ± 0.01	0.46 ± 0.01	0.47 ± 0.01	0.47 ± 0.01
Brain (%)	1.63 ± 0.03	1.62 ± 0.03	1.54 ± 0.03	1.58 ± 0.04
Heart (%)	0.54 ± 0.01	0.53 ± 0.01	0.53 ± 0.01	0.54 ± 0.01
Lungs (%)	0.70 ± 0.03	0.73 ± 0.02	0.66 ± 0.03	0.66 ± 0.03
Thymus (%)	0.21 ± 0.01	0.18 ± 0.01 *	0.18 ± 0.01	0.20 ± 0.01
Kidneys (L + R) (%)	0.69 ± 0.01	0.66 ± 0.01	0.66 ± 0.01	0.66 ± 0.02
Adrenals (L + R) (%)	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
Liver (%)	5.21 ± 0.12	4.96 ± 0.11	5.19 ± 0.11	5.18 ± 0.15
Spleen (%)	0.26 ± 0.02	0.26 ± 0.02	0.30 ± 0.02	0.34 ± 0.02
Seminal Vesicle (%)	1.02 ± 0.04	1.00 ± 0.03	0.93 ± 0.03	0.92 ± 0.04
Testes (L + R) (%)	0.35 ± 0.01	0.32 ± 0.01	0.36 ± 0.01	0.36 ± 0.01 #
Epididymis (L + R) (%)	0.39 ± 0.01	0.37 ± 0.01	0.36 ± 0.01	0.37 ± 0.01

All data presented as estimated marginal means ± SEM, relative weight = organ weight/lean body weight x 100.

The number of male progeny analysed is shown in parentheses.

The effect of maternal LPS treatment and *Il10* genotype was compared by Mixed Model Linear Repeated Measures ANOVA.

* $p < 0.05$ when comparing with PBS treatment of the same genotype

# $p < 0.05$ when comparing with *Il10* +/+ genotype of the same treatment.

In female progeny derived from PBS and LPS-treated *Il10* ⁺/⁺ females, maternal LPS treatment resulted in a reduction of 6% in the brain (p = 0.03) and 11% in the gastrocnemius (p = 0.03) when comparing the absolute organ mass in female progeny derived from PBS and LPS-treated *Il10* ⁺/⁺ females (Table 5.4, mixed model ANOVA).

In contrast, no effect of maternal LPS treatment and IL-10 deficiency was observed in the female progeny of LPS-treated *Il10* ⁻/⁻ mothers. However, the absolute lean body weight of female progeny from control *Il10* ⁻/⁻ mothers was found to be significantly lower by 7% when compared to the lean body weight of female progeny from control *Il10* ⁺/⁺ mothers (p = 0.041) although there was no significant difference observed in total body weight or central fat. The gastrocnemius and quadriceps of female progeny from control *Il10* ⁻/⁻ mothers was found to be lighter by 13% and 12% when compared to female progeny from control *Il10* ⁺/⁺ mothers (p = 0.022 and p = 0.029). A similar reduction in absolute organ weight was also observed in the lungs (11% reduced, p = 0.034) and liver (14% reduced, p =0.033) (compared to female progeny of control *Il10* ⁺/⁺ mothers, Table 5.4).

The differences observed in absolute organ mass were not maintained when the organ weights were corrected to lean body weights, when no effect of maternal LPS treatment and *Il10* genotype was observed (Table 5.5).

Table 5.4   Effect of maternal LPS treatment during pre-implantation period and *Il10* genotype on body composition (absolute weight) of female adult progeny.

Absolute weight	*Il10* +/+ PBS (n = 35)	*Il10* +/+ LPS (n = 26)	*Il10* -/- PBS (n = 25)	*Il10* -/- LPS (n = 20)
Total body weight (g)	22.82 ± 0.40	21.71 ± 0.42	22.07 ± 0.42	22.23 ± 0.50
Lean body weight (g)	22.21 ± 0.33	20.78 ± 0.40	20.65 ± 0.43 #	21.74 ± 0.50
Muscle:fat ratio	0.51 ± 0.02	0.56 ± 0.03	0.49 ± 0.03	0.52 ± 0.04
Total Central Fat (mg)	761 ± 34	651 ± 40	748 ± 43	738 ± 51
Parametrial Fat (mg)	257 ± 18	218 ± 22	268 ± 23	255 ± 27
Retroperitoneal Fat (mg)	409 ± 14	359 ± 16	381 ± 17	392 ± 20
Peri-renal Fat (mg)	101 ± 9	75 ± 10	100 ± 11	91 ± 13
Combined Muscle (mg)	392 ± 10	355 ± 11	354 ± 12	381 ± 14
Gastrocnemius (mg)	120 ± 3	107 ± 3 *	105 ± 4 #	114 ± 4
Quadriceps (mg)	154 ± 4	137 ± 5	135 ± 5 #	150 ± 6
Biceps (mg)	26 ± 1	21 ± 1	27 ± 1	24 ±1
Triceps (mg)	94 ± 2	89 ± 2	88 ± 2	92 ± 3
Brain (mg)	430 ± 6	404 ± 7 *	411 ± 7	430 ±8
Heart (mg)	123 ± 3	116 ± 4	112 ± 4	114 ± 5
Lungs (mg)	182 ± 4	170 ± 6	162 ± 5 #	166 ± 6
Thymus (mg)	63 ± 3	55 ± 3	57 ± 4	66 ± 4
Kidneys (L + R) (mg)	140 ± 3	132 ± 4	131 ± 4	134 ± 5
Adrenals (L + R) (mg)	3 ± 0	4 ± 0	4 ± 0	4 ± 0
Liver (mg)	1198 ± 34	1132 ± 40	1035 ± 43 #	1212 ± 50
Spleen (mg)	75 ± 5	69 ± 6	83 ± 7	74 ± 8
Uterus (mg)	64 ± 4	67 ± 6	76 ± 6	79 ± 7
Ovary (L + R) (mg)	8 ± 0	7 ± 0	7 ± 1	8 ± 1

All data presented as estimated marginal means ± SEM.

The number of female progeny analysed is shown in parentheses.

The effect of maternal LPS treatment and *Il10* genotype was compared by Mixed Model Linear Repeated Measures ANOVA.

* $p < 0.05$ when comparing with PBS treatment of the same genotype

# $p < 0.05$ when comparing with *Il10* +/+ genotype of the same treatment.

Table 5.5 **Effect of maternal LPS treatment during pre-implantation period and *Il10* genotype on body composition (relative weight) of female adult progeny.**

Relative weight	*Il10* +/+ PBS (n = 35)	*Il10* +/+ LPS (n = 26)	*Il10* -/- PBS (n = 25)	*Il10* -/- LPS (n = 20)
Total Central Fat (%)	3.40 ± 0.15	3.13 ± 0.18	3.63 ± 0.19	3.40 ± 0.23
Parametrial Fat (%)	1.15 ± 0.09	1.06 ± 0.10	1.30 ± 0.11	1.18 ± 0.13
Retroperitoneal Fat (%)	1.89 ± 0.05	1.77 ± 0.06	1.85 ± 0.06	1.81 ± 0.07
Peri-renal Fat (%)	0.42 ± 0.03	0.36 ± 0.04	0.48 ± 0.04	0.42 ± 0.05
Combined Muscle (%)	1.78 ± 0.03	1.70 ± 0.03	1.71 ± 0.03	1.75 ± 0.04
Gastrocnemius (%)	0.54 ± 0.01	0.51 ± 0.01	0.51 ± 0.01	0.52 ± 0.01
Quadriceps (%)	0.69 ± 0.01	0.66 ± 0.02	0.65 ± 0.02	0.69 ± 0.02
Biceps (%)	0.12 ± 0.01	0.10 ± 0.01	0.12 ± 0.01	0.11 ± 0.01
Triceps (%)	0.43 ± 0.01	0.43 ± 0.01	0.43 ± 0.01	0.43 ± 0.01
Brain (%)	1.95 ± 0.02	1.95 ± 0.03	2.00 ± 0.03	1.98 ± 0.03
Heart (%)	0.55 ± 0.01	0.56 ± 0.01	0.54 ± 0.02	0.53 ± 0.02
Lungs (%)	0.83 ± 0.02	0.82 ± 0.03	0.79 ± 0.03	0.77 ± 0.04
Thymus (%)	0.28 ± 0.01	0.27 ± 0.02	0.28 ± 0.02	0.31 ± 0.02
Kidneys (L + R) (%)	0.62 ± 0.01	0.63 ± 0.01	0.63 ± 0.01	0.62 ± 0.02
Adrenals (L + R) (%)	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
Liver (%)	5.38 ± 0.13	5.43 ± 0.16	5.01 ± 0.17	5.58 ± 0.19
Spleen (%)	0.34 ± 0.03	0.33 ± 0.03	0.39 ± 0.03	0.35 ± 0.04
Uterus (%)	0.29 ± 0.02	0.32 ± 0.03	0.37 ± 0.03	0.36 ± 0.03
Ovary (L + R) (%)	0.03 ± 0.00	0.03 ± 0.00	0.04 ± 0.00	0.04 ± 0.00

All data presented as estimated marginal means ± SEM, relative weight = organ weight/lean body weight x 100.

The number of female progeny analysed is shown in parentheses.

The effect of maternal LPS treatment and *Il10* genotype was compared by Mixed Model Linear Repeated Measures ANOVA.

* $p < 0.05$ when comparing with PBS treatment of the same genotype

# $p < 0.05$ when comparing with *Il10* +/+ genotype of the same treatment.

5.5. VALIDATION OF THE ACCURACY AND PRECISION OF THE DEXA APPROACH IN BODY FAT MEASUREMENT

It is clear in the previous sections that the fat data obtained by DEXA did not closely relate to the weight data obtained from the full body post mortems. In particular, on the male progeny of *Il10* $^{-/-}$ mothers treated with LPS (see Figure 5.6C and Table 5.2). To investigate the relationships between DEXA body fat data acquired from the male and female progeny from *Il10* $^{+/+}$ and *Il10* $^{-/-}$ mothers, the 19 week DEXA body fat data was correlated with the excised fat tissue measurements acquired during the and full body post mortems. Total central fat (g) tissue excised was compared to DEXA fat (g) while % body fat from excised data was calculated from total central fat/body weight x 100.

The relationship between excised fat tissue and DEXA fat tissue was found to be moderate but significantly correlated (0.54) ($p < 0.001$, Table 5.6 & Figure 5.7A, B). When the data was analysed separately according to maternal treatment group, similar correlations (0.41 – 0.62) were observed between excised fat tissue and fat tissue measured by DEXA. This correlation was found to be significant for all treatment groups ($p < 0.001$). Excised fat tissue from LPS-treated *Il10* $^{+/+}$ progeny were the best correlated to DEXA values while control *Il10* $^{-/-}$ progeny were less correlated to DEXA (Figure 5.7A and 5.7B)

The relationship between excised % body fat and DEXA measured % body fat was also found to be moderate but positively correlated (0.45) ($p < 0.001$, Table 5.6 & Figure 5.7C, D). A similar relationship was observed in the correlation between excised % body fat and DEXA measured % body fat when the groups were analysed separately (0.39 - 0.49) and the correlations were significant in all 4 groups ($p \leq 0.001$, Table 5.6 & Figure 5.7C, D).

Total mass measured by DEXA was found to strongly correlate to the actual weight of the mice (0.91 – 0.97) and the correlations was significant in all four groups ($p < 0.001$, Table 5.6 & Figure 5.7E, F). Although overall, both data are moderately well correlated, DEXA measurements seem to overestimate the post mortem measurement. .

Table 5.6   Correlations and differences between DEXA versus excised data in progeny from *Il10* +/+ and *Il10* -/- mothers.

Group	Fat tissue (g)		% body fat (%)		Total mass (g)	
	Correlation	Significance	Correlation	Significance	Correlation	Significance
All groups	0.540	<0.001 **	0.453	<0.001 **	0.957	<0.001 **
***Il10* +/+ PBS**	0.550	<0.001 **	0.402	0.001 **	0.971	<0.001 **
***Il10* +/+ LPS**	0.623	<0.001 **	0.493	<0.001 **	0.973	<0.001 **
***Il10* -/- PBS**	0.414	<0.001 **	0.398	0.001 **	0.967	<0.001 **
***Il10* -/- LPS**	0.595	<0.001 **	0.482	<0.001 **	0.916	<0.001 **

Fat tissue = correlation between excised fat vs. DEXA measured fat

% body fat = correlation between excised fat/body weight X100 vs. DEXA measured % body fat

Total mass = correlation between actual body weight vs. DEXA measured total mass
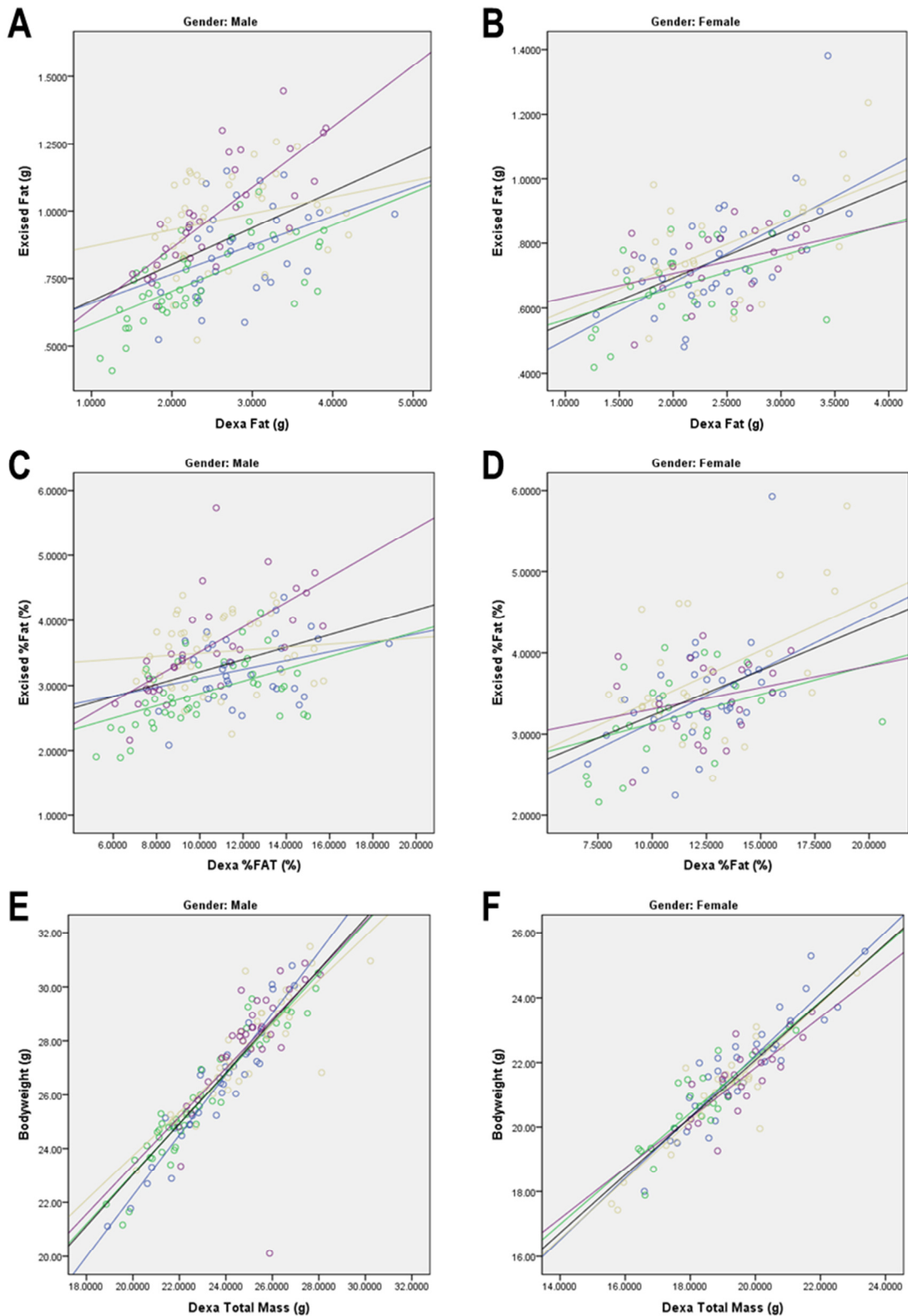
Figure 5.7   The relation between excised and DEXA measured fat tissue.

Correlation between excised and DEXA acquired (A, B) fat tissue (g), (C, D) % body fat (%) and (E, F) body weight (g) from male and female progeny from all 4 groups (black line), PBS-treated *Il10* [+/+] (blue), LPS-treated *Il10* [+/+] (green), PBS-treated *Il10* [-/-] (yellow) and LPS-treated *Il10* [-/-] (purple) mothers.

5.6. EFFECT OF MATERNAL SYSTEMIC INFLAMMATION DURING PRE-IMPLANTATION PERIOD ON FAT CELL SIZE OF MALE AND FEMALE PROGENY OF *Il10* $^{+/+}$ AND *Il10* $^{-/-}$ MOTHERS

To investigate the effects of maternal LPS treatment on the fat cell size in male and female progeny of *Il10* $^{+/+}$ and *Il10* $^{-/-}$ mothers, sections of epididymal and parametrial fat tissue from male and female progeny were stained with toluidine blue and the fat cell size was measured using ImageJ software. One male and one female progeny of each litter were randomly selected for fat cell size quantification, unless the litter consisted of single-sex progeny.

No significant effect of maternal LPS treatment was observed on the fat cell size in either male or female progeny (mixed model ANOVA, Figure 5.8). Similarly, no significant difference in fat cell size was observed when comparing progeny of *Il10* $^{+/+}$ and *Il10* $^{-/-}$ mothers.

Figure 5.8   Effect of maternal systemic inflammation during pre-implantation period on fat cell size of male and female progeny of *Il10* ^{+/+} and *Il10* ^{-/-} mothers.

Histology sections of epididymal fat tissue (A, C, E, G) and parametrial fat tissue (B, D, F, H) from male and female progeny of *Il10* ^{+/+} and *Il10* ^{-/-} mothers. The progeny were derived from PBS-treated *Il10* ^{+/+} (open bars), LPS-treated *Il10* ^{+/+} (diagonal lined bars), PBS-treated *Il10* ^{-/-} (horizontal lined bars) and LPS-treated *Il10* ^{-/-} (cross lined bars) females mated to Il10 +/+ males and treated on days 2.5 and 3.5 pc (I, J). n = 13 and 14 male progeny and 13 and 11 female progeny from PBS-treated and LPS-treated *Il10* ^{+/+} mothers; 12 and 9 male progeny and 11 and 9 female progeny from PBS-treated and LPS-treated *Il10* ^{-/-} mothers. Data are expressed as relative estimated marginal means ± SEM and analysed by mixed model ANOVA.
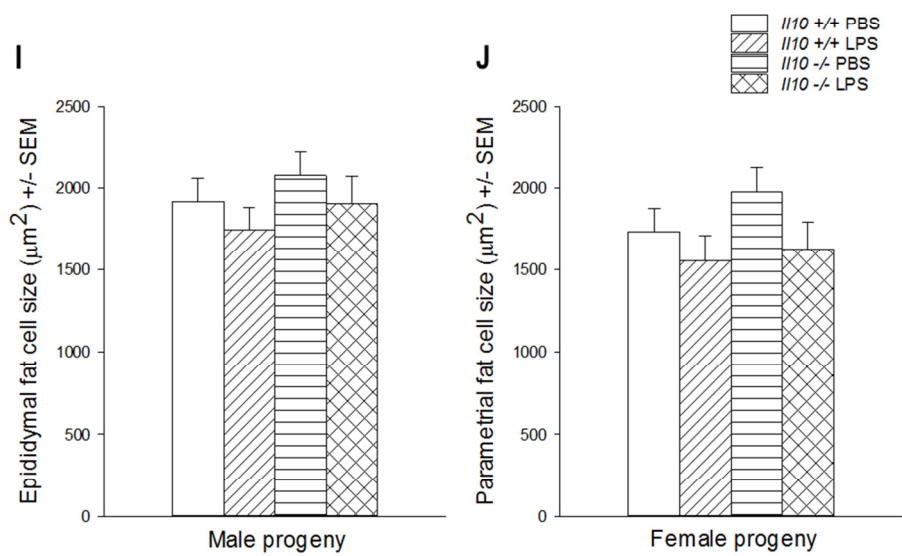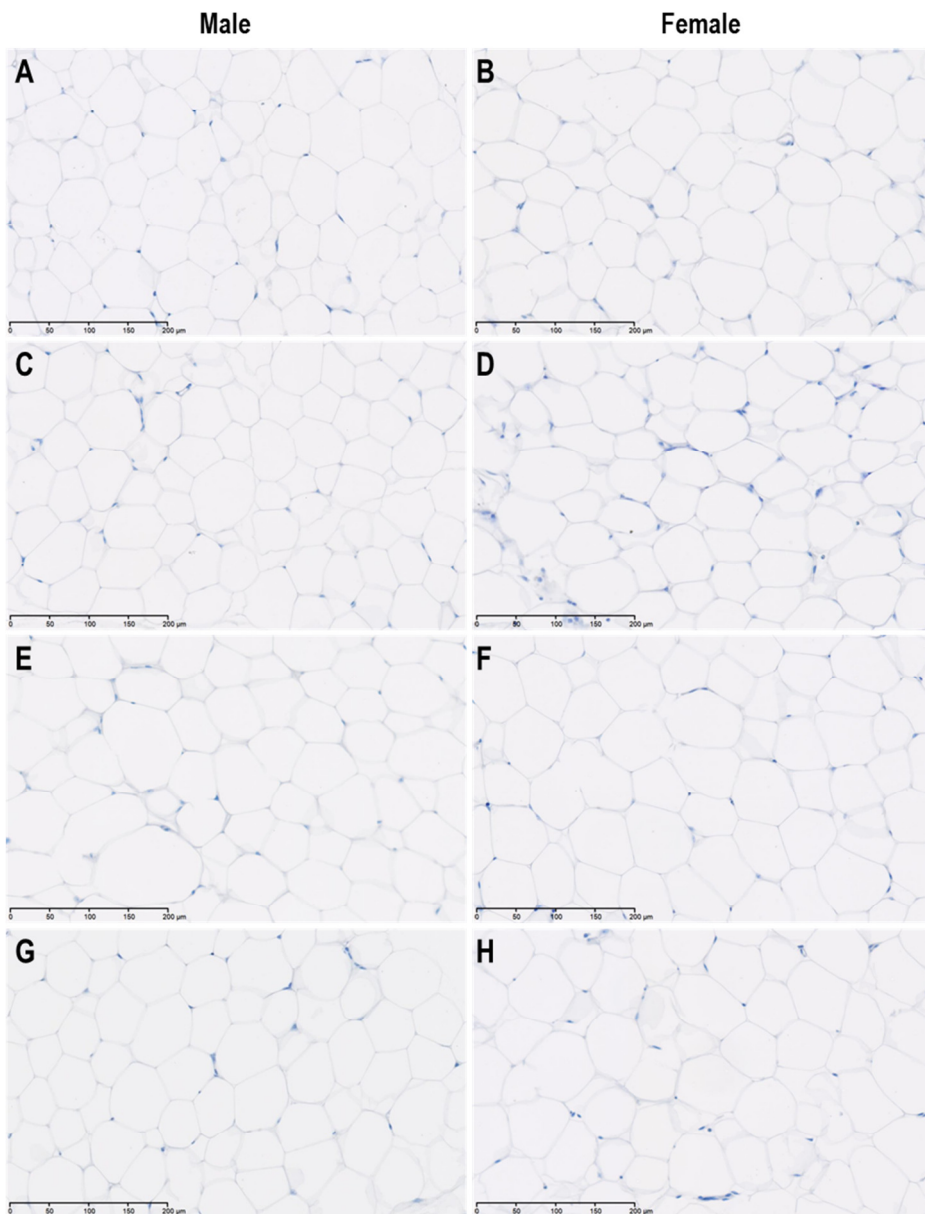
5.7. EFFECT OF MATERNAL SYSTEMIC INFLAMMATION DURING PRE-IMPLANTATION PERIOD ON PROGENY IMMUNE RESPONSE TO LPS CHALLENGE

To determine whether maternal LPS treatment during the pre-implantation period has any effect on progeny immune response to LPS, a number of male and female progeny from PBS and LPS-treated *Il10* $^{+/+}$ and *Il10* $^{-/-}$ mothers were challenged with 100 μg/kg LPS at 18 weeks of age and serum was collected 3.5 hours post-LPS injection. Analysis of the serum cytokine concentrations was performed using a Luminex assay where a selection of 14 cytokines were detected by the multiplex assay including GM-CSF, IL-1α, IL-1β, IL-6, KC, LIF, MCP-1, M-CSF, MIP-1α, MIP-1β, RANTES, IP-10, IL-10 and TNFα. A total of 29 male and 23 females were analysed in this experiment. The serum cytokine concentrations of progeny derived from PBS-treated *Il10* $^{+/+}$ and *Il10* $^{-/-}$ mothers acted as controls.

In male progeny of *Il10* $^{+/+}$ mothers, maternal LPS treatment during the pre-implantation period resulted in a significant decrease in serum IL-10 (55% decreased, p = 0.016, Figure 5.9M) when compared to the male progeny of control *Il10* $^{+/+}$ mothers (mixed model ANOVA). Maternal IL-10 deficiency was found to result in a significant increase in both IL-6 (168% increased, p = 0.006, Figure 5.9D) and KC (283% increased, p < 0.001, Figure 5.9E) in the male progeny of control *Il10* $^{-/-}$ mothers when compared to the male progeny of control *Il10* $^{+/+}$ mothers. The combination of maternal LPS treatment and IL-10 deficiency resulted in a significant increase of 130% in serum KC in the male progeny of LPS-treated *Il10* $^{-/-}$ mothers, compared with the male progeny of *Il10* $^{+/+}$ mothers (p = 0.034, Figure 5.9E). As expected, low serum IL-10 concentration was displayed in the male progeny of control *Il10* $^{-/-}$ (86% reduced, p < 0.001, Figure 5.9M) although IL-10 was clearly present as the mothers were mated to *Il10* $^{+/+}$ males and thus progeny are *Il10* heterozygotes. The combination of maternal LPS treatment and IL-10 deficiency resulted in no significant differences in cytokines in the male progeny of *Il10* $^{-/-}$ mothers, compared with the male progeny of *Il10* $^{-/-}$ mothers with the exception of IL-6 and LIF, which was reduced by 53% and 32% respectively (p = 0.023, Figure 5.9D; p = 0.01, Figure 5.9F).

There was no effect of maternal LPS treatment observed in the serum cytokine concentrations of female progeny of *Il10* $^{+/+}$ mothers in response to LPS challenge, however the combination of maternal LPS treatment and IL-10 deficiency was found to increase serum IL-6 by 81% in the female progeny from LPS-treated *Il10* $^{-/-}$ mothers when compared to the female progeny of control *Il10* $^{-/-}$ mothers (p = 0.001, Figure 5.10D). The combination of maternal LPS treatment and IL-10 deficiency resulted in a significant

increase of 65% in serum TNFα in the female progeny of LPS-treated *Il10* $^{-/-}$ mothers, compared with the female progeny of *Il10* $^{+/+}$ mothers (p = 0.024, Figure 5.10N). Similar to the male progeny result, serum IL-10 concentration was reduced by 88% in the female progeny of control *Il10* $^{-/-}$ mothers when compared to the female progeny of control *Il10* $^{+/+}$ mothers (p < 0.001, Figure 5.10M). A similar finding to the male progeny data was observed where there is a trend towards a lower concentration of serum IL-10 in the female progeny derived from LPS-treated *Il10* $^{+/+}$ mothers compared to progeny derived from PBS-treated *Il10* $^{+/+}$ mothers (p = 0.138 Figure 5.10M).

When all progeny data was combined, an effect of maternal LPS treatment was significant in the concentrations of serum IL-10 in the progeny derived from *Il10* $^{+/+}$ mothers (48% decreased, p = 0.001, Figure 5.11M). Maternal IL-10 deficiency was found to result in a significant increase in IL-6 (127% increased, p = 0.009, Figure 5.11D), KC (168% increased, p = 0.001, Figure 5.11E), LIF (96% increased, p = 0.021, Figure 5.11F), TNFα (78% increased, p = 0.12, Figure 5.11N) and a significant decrease in IL-10 (86% decreased, p < 0.001, Figure 5.11M) in the progeny of control *Il10* $^{-/-}$ mothers when compared to the progeny of control *Il10* $^{+/+}$ mothers. The combination of maternal LPS treatment and IL-10 deficiency resulted in a significant increase of in KC (104% increased, p = 0.023, Figure 5.11E), MCP-1 (34% increased, p = 0.019, Figure 5.11G), TNFα (100% increased, p = 0.001, Figure 5.11N) and a significant decrease in IL-10 (68% decreased, p = 0.049, Figure 5.11M) in the progeny of LPS-treated *Il10* $^{-/-}$ mothers, compared with the progeny of LPS-treated *Il10* $^{+/+}$ mothers.
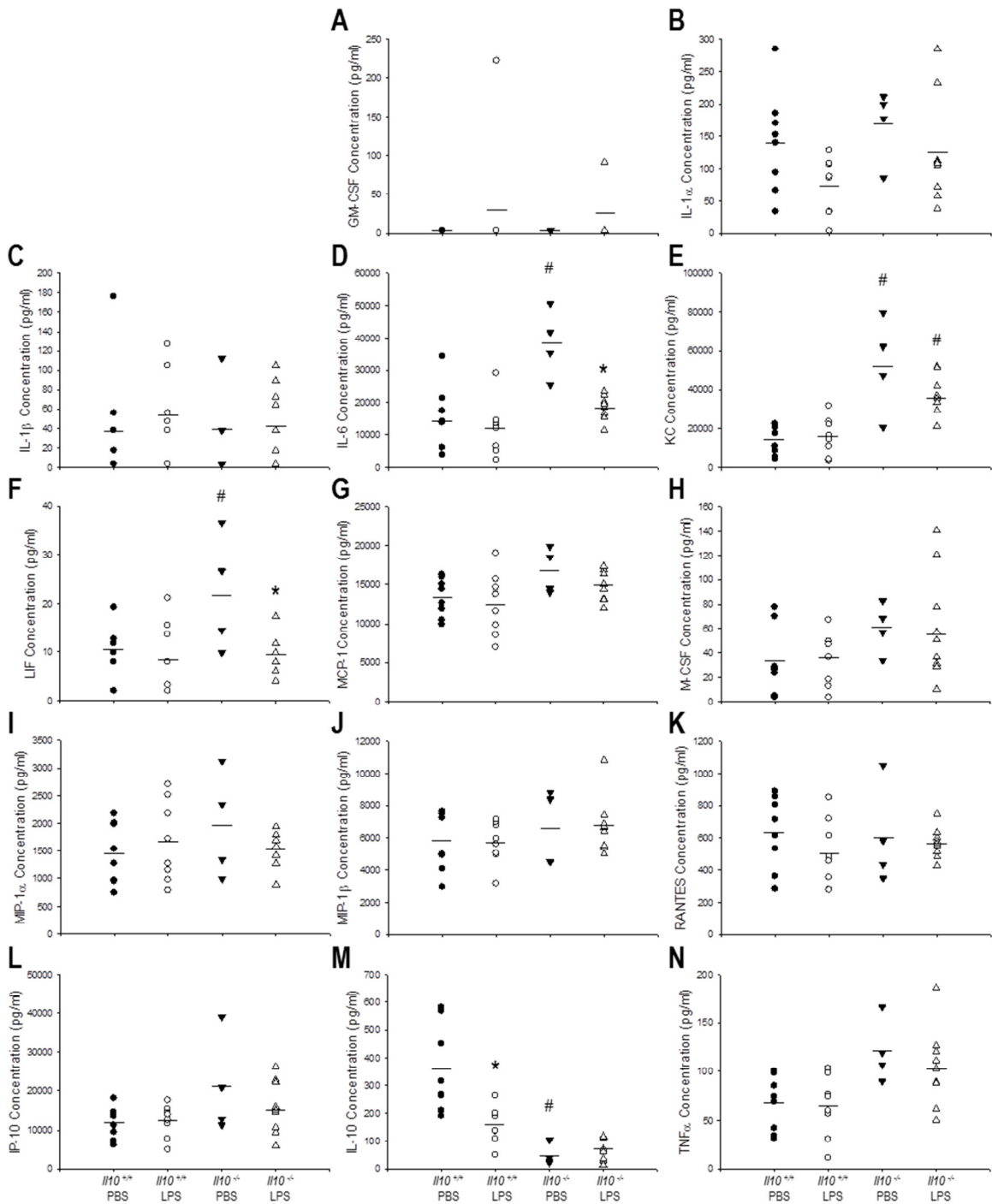
Figure 5.9   Serum cytokine concentrations (pg/ml) of adult male progeny from *Il10* +/+ and *Il10* -/- females 3.5 hours after LPS challenge.

Male progeny derived from PBS-treated *Il10* +/+ females (closed circles), LPS-treated *Il10* +/+ females (open circles), PBS-treated *Il10* -/- females (closed triangles) and LPS-treated *Il10* -/- females (open triangles) were challenged with 100 µg/kg LPS at 18 weeks of age and serum was collected 3.5 hours later. n = 8 and 8 male progeny from PBS-treated and LPS-treated *Il10* +/+ mothers and 4 and 9 male progeny from PBS-treated and LPS-treated *Il10* -/- mothers. All data was presented as individual points with the estimated marginal means and analysed by mixed model ANOVA, *$p < 0.05$ when compared to control group of the same genotype, #$p < 0.05$ when compared to *Il10* +/+ genotype with the same treatment.
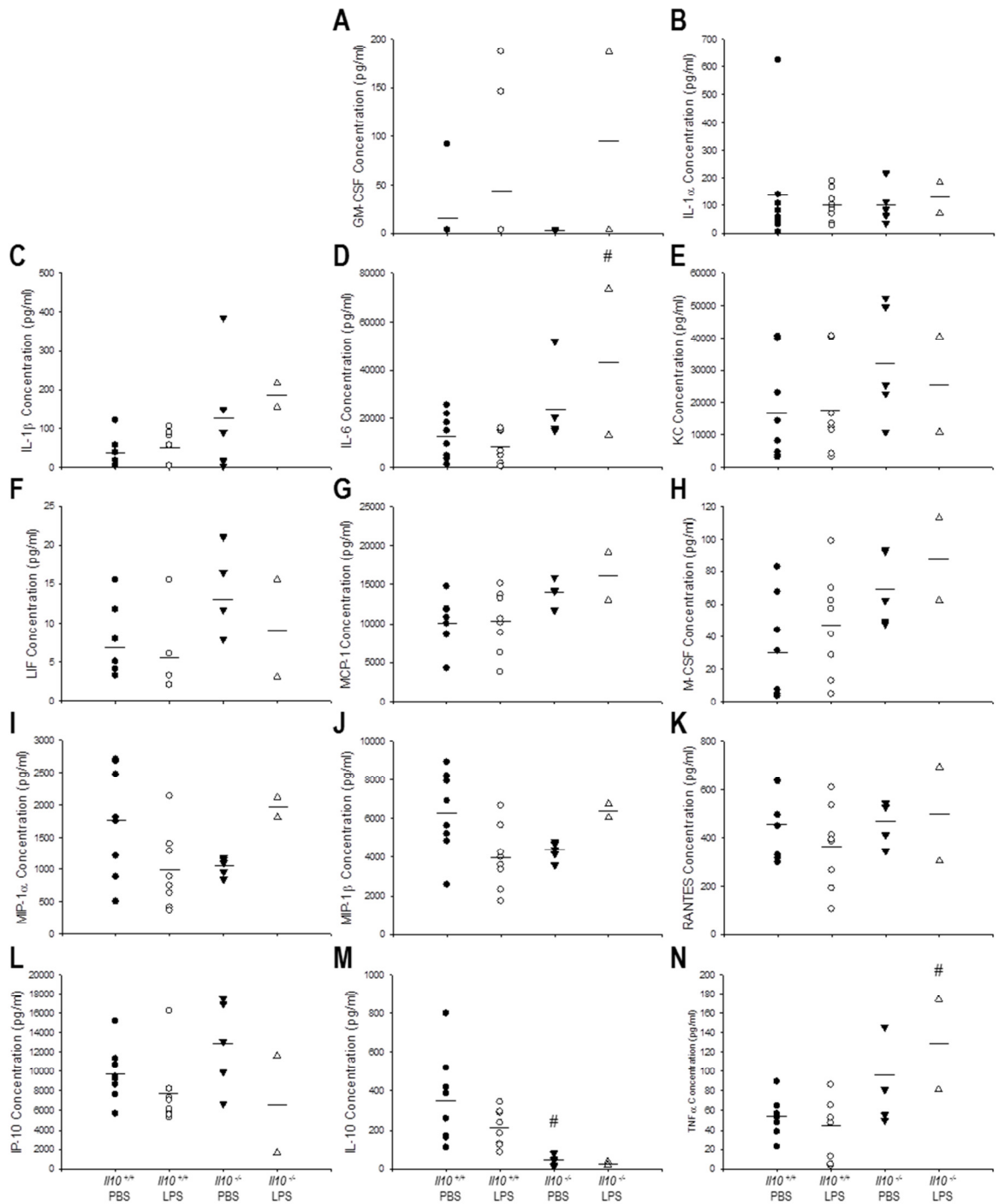
Figure 5.10 Serum cytokine concentrations (pg/ml) of adult female progeny from *Il10* $^{+/+}$ and *Il10* $^{-/-}$ females 3.5 hours after LPS challenge.

Female progeny derived from PBS-treated *Il10* $^{+/+}$ females (closed circles), LPS-treated *Il10* $^{+/+}$ females (open circles), PBS-treated *Il10* $^{-/-}$ females (closed triangles) and LPS-treated *Il10* $^{-/-}$ females (open triangles) were challenged with 100 µg/kg LPS at 18 weeks of age and serum was collected 3.5 hours later. n = 8 and 8 female progeny from PBS-treated and LPS-treated *Il10* $^{+/+}$ mothers and 5 and 2 female progeny from PBS-treated and LPS-treated *Il10* $^{-/-}$ mothers. All data was presented as individual points with the estimated marginal means and analysed by mixed model ANOVA, *p < 0.05 when compared to control group of the same genotype, #p < 0.05 when compared to *Il10* $^{+/+}$ genotype with the same treatment.
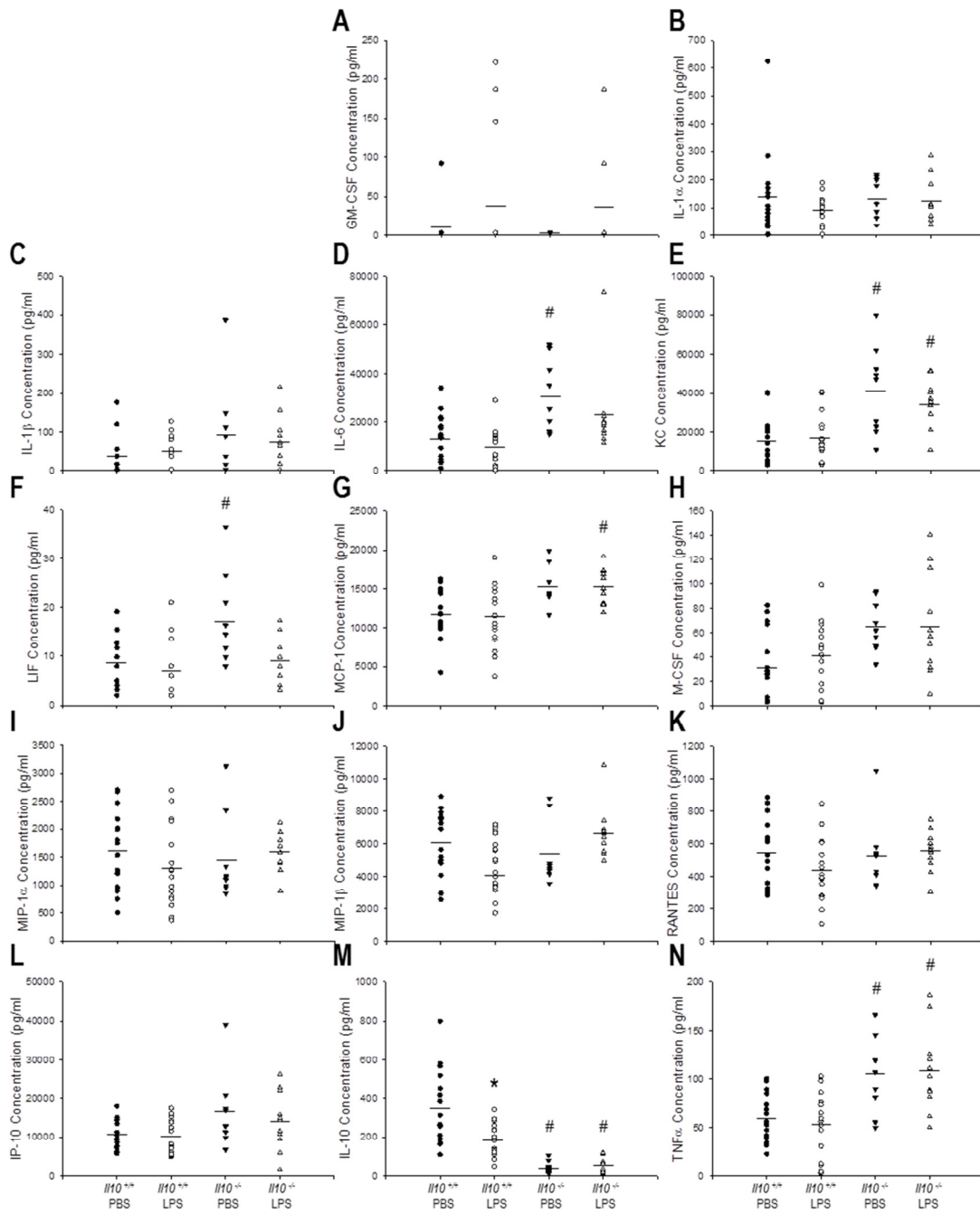
Figure 5.11 Serum cytokine concentrations (pg/ml) of adult male and female progeny from _Il10_ [+/+] and _Il10_ [-/-] females 3.5 hrs after LPS challenge.

All progeny derived from PBS-treated _Il10_ [+/+] females (closed circles), LPS-treated _Il10_ [+/+] females (open circles), PBS-treated _Il10_ [-/-] females (closed triangles) and LPS-treated _Il10_ [-/-] females (open triangles) were challenged with 100 μg/kg LPS at 18 weeks of age and serum was collected 3.5 hours later. n = 16 and 16 progeny from PBS-treated and LPS-treated _Il10_ [+/+] mothers and 9 and 11 progeny from PBS-treated and LPS-treated _Il10_ [-/-] mothers. All data was presented as individual points with the estimated marginal means and analysed by mixed model ANOVA, *p < 0.05 when compared to control group of the same genotype, #p < 0.05 when compared to _Il10_ [+/+] genotype with the same treatment.

5.8. DISCUSSION

This study was undertaken to investigate possible long term effects of maternal LPS treatment during the pre-implantation period on the development of the resulting offspring. As described in the previous two chapters, maternal LPS treatment during the pre-implantation period resulted in restricted fetal development and this was likely to be mediated by actions on embryos of inflammatory cytokine production induced by LPS in the oviduct and the uterus.

The experiments presented in this chapter demonstrate that the effects of systemic LPS experienced by the pre-implantation embryo alter postnatal growth trajectory, particularly in the male progeny. When analysing the postnatal development of male and female progeny of $Il10^{+/+}$ and $Il10^{-/-}$ mothers, sex was found to be the most significant determinant of growth trajectory. Regardless of maternal IL-10 genotype, the male offspring from mothers treated with LPS during the pre-implantation period experienced slower growth post-weaning, although this was not maintained beyond 13 weeks. Post mortem body morphometry, and non-invasive DEXA measurements of these progeny, indicated that the changes in postnatal development were largely due to changes in the accumulation of fat tissue in the male progeny. Most notably, male offspring of $Il10^{-/-}$ mothers were programmed to have elevated fat mass when mothers were exposed to LPS in the pre-implantation period. No such effect of LPS was seen in $Il10^{+/+}$ mothers treated with LPS, indeed DEXA analysis suggested male offspring were less fat after LPS treatment in early life.

During maternal systemic inflammation with LPS, the pre-implantation embryo is exposed to pro-inflammatory cytokines in the maternal reproductive tract. The body morphometry at autopsy revealed that while the adult male progeny from LPS-treated $Il10^{+/+}$ and $Il10^{-/-}$ mothers did not display changes in fat mass compared to their PBS-treated control counterparts, the adult male progeny from LPS-treated $Il10^{-/-}$ mothers displayed an increase in fat mass compared to adult male progeny from LPS-treated $Il10^{+/+}$ mothers. This indicates that maternal IL-10 may play a role in protecting the embryo from the adverse effects of LPS exposure during the pre-implantation period in programming altered phenotype in offspring.

The sex-specific observations in our model were similar to those observed in nutritional models of fetal growth restriction (Desai et al., 2005, Kind et al., 2005) where at post mortem the accumulation of

abdominal fat in male progeny from LPS-treated mothers. Studies displaying fetal growth restriction showed that the progeny may be predisposed to other metabolic diseases related to the fetal origins of adult disease hypothesis (Barker et al., 1989) and babies displaying fetal growth restriction *in utero* combined with accelerated growth after birth were found to have a predisposition to metabolic disorders such as obesity and diabetes in later life (reviewed in (Symonds, 2007)). The experiments described in this chapter are the first to demonstrate long term effects on the development of progeny due to early perturbation in the maternal cytokine environment by LPS and its interaction with the lack of maternal IL-10 during pregnancy.

Body composition measurements and fat distribution are important to understand the mechanisms involved in metabolic regulation. Abdominal obesity has been shown to be strongly associated with metabolic disorders such as diabetes and cardiovascular diseases such as hypertension in humans and mice (Ritchie and Connell, 2007, Chen et al., 2012). However, the effect of LPS treatment during the pre-implantation period on the metabolic parameters such as glucose, insulin, leptin and adiponectin of these progeny was not measured in this model and would be of interest to measure in future experiments. Obesity has also been linked to inflammation, where high levels of circulating TNFα and IL-6 were observed in overweight and obese adults (de Heredia et al., 2012, Xu et al., 2003).

Sex-specific differences in the progeny are often observed in developmental origins of health and diseases (DOHaD)-related studies (Kwong et al., 2000, Watkins et al., 2007, Watkins et al., 2008). However, the underlying mechanisms are not fully understood. As previous studies have shown that the male progeny are often more susceptible than females and display greater effects of programming compared to the female progeny (Ozaki et al., 2001, Sjoblom et al., 2005). As the first round of cleavage occur faster in male embryos (Mittwoch, 1993), it may be that male embryos are more likely to respond to the surrounding environmental influences and are therefore more susceptible to maternal perturbations during early pregnancy. A study by Hong et al. has shown that females are protected from obesity by ovarian hormones. Ovariectomised female mice were found to gain more body weight compared to non-ovariectomised females and weight gain was similar to that seen in male mice (Hong et al., 2009). This may explain why female progeny are less affected by maternal LPS treatment in the current study.

Regardless of maternal IL-10 status, maternal LPS treatment during the pre-implantation period plays a role in the developmental outcomes of the progeny. Significant changes to the physical structure and the immune response of the progeny were similar to other studies evaluating prenatal LPS exposure, which has been shown to either enhance or de-sensitise the progeny's response to LPS. Xu et al. have shown that a pre-treatment of low dose LPS 24 hours prior to a high dose LPS treatment was able to attenuate LPS-induced fetal death during late gestation, but a pre-treatment 4 hours prior enhances the sensitivity to LPS (Xu et al., 2007). Williams et al. reported a pronounced blunted response in various cytokines observed in LPS-treated progeny derived from MF1 mice following previous maternal LPS treatment during the zygote stage (Williams et al., 2011). A study by Shanks et al. showed that exposure of neonatal rats to low dose LPS helps to protect them from adjuvant-induced arthritis in later life (Shanks et al., 2000). In the current study, we found only scant evidence of any change in cytokine response after early life exposure to LPS, and then only in the *Il10* $^{-/-}$ mice. As the progeny of *Il10* $^{-/-}$ mothers are heterozygous for IL-10 cytokine instead of homozygous due to the fathers being *Il10* $^{+/+}$ mice, this may explain the low response of the progeny to the LPS challenge at 19 weeks compared to the progeny of *Il10* $^{+/+}$ mothers.

The effects of maternal LPS treatment was very clear during the pre-implantation and fetal stage, but less so during postnatal development of the progeny, it is possible that the effects of maternal LPS treatment in the progeny were less apparent than expected due to the low dosage of LPS chosen for our model. As mentioned in Chapter 4, responses to LPS treatment occur in a dose escalating manner and a more significant effect could be observed when LPS dosage is increased.

The maternal IL-10 status had less impact on the progeny outcome compared to the other determinants in this experiment. Compared to the previous two chapters, maternal IL-10 deficiency does not seem to substantially alter postnatal developmental outcomes. The only effect was evidence of increased fat in males when maternal IL-10 id deficient. This may be due to capacity in offspring of compensatory pathways that modulate adverse effects of early life programming.

Although the DEXA readings have proven to be time, cost and labour-effective in the process of following the development of each individual offspring, the extent to which DEXA was useful in understanding the effects of maternal LPS treatment and IL-10 deficiency was confounded by the fact

that the fat depots in the mice could not be discriminated between visceral and subcutaneous fat depots, in contrast to fat depots measured during the post mortems. It might be possible to work out the specific area in the mice and evaluate the effects of maternal LPS treatment on each fat depot as shown in a method designed for this purpose recently shown by Chen et al. (Chen et al., 2012).

Comparison between DEXA and post mortem data revealed different findings in the two methods used to measure fat tissue. Various studies have been done in mice to validate DEXA data against post mortem data and most have found DEXA to be a useful tool when measuring bone mineral density and bone mineral content (Nagy et al., 2001, Provyn et al., 2008). While some studies support the use and accuracy of DEXA as a non-invasive method to measure fat tissue (Provyn et al., 2008, Chen et al., 2012), correlation of our data from the two methods revealed that the DEXA software tends to overestimate both the fat tissue mass and percentage of body fat values compared to autopsy analysis by dissection of tissue. This inaccuracy of body fat measurement has been noted in two other studies where the DEXA software either underestimates or overestimates the percentage of body fat in the mice (Brommage, 2003, Nagy and Clair, 2000). Our current DEXA data in the lean and total tissue support the findings in the postnatal trajectory and post mortem data. However, as our DEXA data for fat tissue correlates only moderately to the post mortem data, the traditional approach of dissection was viewed as be more reliable in our experiments in measuring body fat. Future experiments could include excising subcutaneous fat depots to compare between DEXA and post mortem findings, as it is possible that having not included the additional subcutaneous depots contributed to the discrepancy between the two analyses.

In addition, another contributing factor that may also play a part in this model is the IL-10 status of the progeny derived from $Il10^{-/-}$ females. As the progeny are heterozygous for IL-10, the presence of IL-10 cytokine inherited from the father in the progeny might be the reason that their postnatal development was less affected by maternal IL-10 deficiency than in previous studies (White et al., 2004). While the aim of this model was to understand the effects of maternal LPS treatment and maternal IL-10 deficiency during early pregnancy, it would be interesting in future experiments to repeat this experiment with homozygous $Il10^{-/-}$ progeny from $Il10^{-/-}$ X $Il10^{-/-}$ matings.

LPS challenge in the adult offspring revealed that the progeny derived from LPS-treated *Il10 +/+* mothers had an altered cytokine response. Combination of maternal LPS treatment and IL-10 deficiency resulted in reduced serum concentration of LIF cytokine in the male progeny. Our findings were similar to other studies which showed that either a pre-implantation or prenatal exposure to LPS results in an altered response to postnatal exposure to LPS (Shanks et al., 2000, Williams et al., 2011, Xu et al., 2007). Male progeny from LPS-treated was found to have reduced serum IL-10 concentrations. Due to the low offspring number that was challenge with LPS, this warrants further investigation in future experiments to confirm whether pre-implantation maternal LPS exposure will result in either a hypo-responsive or hyper-responsive response towards postnatal LPS exposure. While a hypo-responsive cytokine response to LPS would result in a more tolerant phenotype in the offspring against future infectious insults, a reduced IL-10 response could take longer to eliminate the infection instead.

In summary, the experiments in this chapter provide an indication of altered postnatal development in the resulting progeny due to maternal LPS treatment, and an interaction with maternal IL-10 deficiency. However, since the effects of low dose maternal LPS treatment during the pre-implantation period were less evident during postnatal development and adulthood, any further conclusions made from these experiments are somewhat limited. There are some obvious experiments that could shed more light on this question, such as increasing the LPS dosage or extending the length of the postnatal experiment, as mentioned above. Assessment of the progeny phenotype, metabolic status and fertility may be informative on other subtle phenotype changes in this model. Also, looking at the F2 generation of our maternal systemic inflammation model would be of interest to see if the effect of maternal LPS treatment in the parent could extend than in the *in utero* period to an effect on the F2 progeny as well.

Chapter 6

General discussion and conclusion

6.1. DISCUSSION AND CONCLUSION

It is widely acknowledged that the maternal environment surrounding the embryo during the pre-implantation period is critical for the establishment and maintenance of pregnancy. Recently through *in vivo* and *in vitro* studies, it has been demonstrated in mice and humans that the cytokine milieu of the maternal tract is an important factor in mediating signalling to the embryo from the maternal tract to aid development and implantation during the pre-implantation period. Environmental factors such as inflammation, diet and stress has been shown to alter cytokine expression systemically (Lopez, Madan et al., 2009, Diz-Chaves et al., 2013). To date, only a few studies have investigated the effects of maternal systemic inflammation in early pregnancy, and these suggest that the results are reduced cell number and impaired implantation. It is becoming more evident that perturbations in the pre-implantation uterine environment or manipulations to pre-implantation embryos have long term consequences, often giving rise to adverse developmental changes in subsequent offspring. However, no previous study has determined the extent to which embryo exposure to maternal systemic inflammation prior to implantation effects fetal and neonatal development and the health status of offspring.

The studies described in this thesis are the first to describe the impact of low level maternal systemic inflammation with bacterial LPS during the pre-implantation period on outcomes for the embryo, fetus and adult development. The experiments reported herein provide compelling evidence of the supporting role of cytokines in the maternal reproductive tract in mediating maternal-embryo communication, and suggest that the balance in expression of these cytokines can influence embryo development and optimal pregnancy outcome. In addition, these experiments reveal the importance of the anti-inflammatory IL-10 cytokine as it appears to play a protective role against adverse outcomes in the development of the adult offspring.

In Chapter 3, a detailed characterisation was undertaken to identify the effects of maternal systemic inflammation during the pre-implantation period on the development of the embryo, fetus and placenta. Maternal systemic inflammation was induced by LPS prior to implantation, on days 2.5 and 3.5 pc, in order to mimic a minor inflammatory infection which, the pre-implantation embryo can tolerate and survive to implant. Previous studies have shown that circulating levels of pro-inflammatory cytokines increase within 1-3 hours of LPS challenge and decline to baseline levels within 6 hours (Berg et al.,

1995, Robertson et al., 2007). The two doses of LPS challenge were chosen to mimic the normal pathology of an inflammatory infection, where a sustained exposure to LPS would be expected. Our *in vivo* results mirrored those of similar studies where LPS reduces viability and ICM number in the blastocyst (Deb et al., 2004, Deb et al., 2005, Williams et al., 2011) and was increasingly effective in a dose dependent manner, with significant effects at doses of 12.5 µg LPS and higher. In order to elucidate the possible mechanism by which systemic inflammation acts on the embryo prior to implantation, *in vitro* experiments were carried out to investigate whether LPS acts directly on the embryo. *In vitro* culture of embryos with various doses of LPS had minimal direct effects on embryo development, with effects only at the highest dose of 25 µg/ml LPS. Similarly, *in vitro* culture of embryos with both LPS and IL-10 cytokine also did not alter embryo development. These studies validated the criteria for our maternal systemic inflammation model, where the contribution of cytokines in the mechanism of LPS could be investigated. We then went on to show that indeed cytokines expression in the oviduct is altered by LPS administration. As the pre-implantation embryo is known to express cytokine receptors (Robertson et al., 1994, Sharkey et al., 1995), our findings demonstrate the component molecular players are in place for plasticity of the pre-implantation embryo to utilise cytokines to sense and adapt to an altered maternal environment.

Our studies went further to show that while low level inflammation induced by 0.5 µg LPS did not affect blastocyst development, this dose was sufficient to alter fetal and placental development. The fetal growth restriction seen in our model was similar to that observed in previous studies where maternal systemic inflammation was induced by LPS in mid and late gestation (Robertson et al., 2007, Robertson et al., 2006). In addition, our findings also showed that maternal IL-10 deficiency increased the adverse effect of LPS on fetal weight. The significant reduction in fetal:placental weight ratio suggested a possible reduction in functional competence of the placentae to provide for the fetus as a result of maternal systemic inflammation. The *Il10 $^{-/-}$* mothers in our model were mated to *Il10 $^{+/+}$* fathers, which results in heterozygous conceptus developing in an IL-10 deficient environment. Therefore our finding suggests a possible programming effect on the pre-implantation embryo following maternal LPS treatment, and indicates that IL-10 is a regulator of this communication pathway.

Our primary interest was the mechanism by which inflammation alters the maternal environment to results in changes to developing fetuses and subsequent progeny. As the embryo has no direct contact

with the maternal tract during the pre-implantation period, and cytokines are known provide a signalling mechanism for maternal-embryo communication (Hardy and Spanos, 2002), this raised the question of the role of cytokines in mediating the altered developmental trajectory.

The work presented in Chapter 4 demonstrated that cytokines are likely to be the major mechanism of action for LPS on the embryo as maternal systemic inflammation results in elevated levels of pro-inflammatory cytokines such as TNFα and IFNγ in day 3.5 pc oviduct and uterus tissues from LPS-treated mothers, regardless of the maternal IL-10 genotype. The absence of maternal IL-10 cytokine resulted in higher levels of pro-inflammatory cytokine expression in the maternal tract compared to wildtype mice. Maternal IL-10 deficiency results in delayed clearance of pro-inflammatory cytokines from the maternal tract (Berg et al., 1995) and this in turn, would increase the time of exposure of the embryos to these harmful factors. The activation of LPS-induced cytokine expression indicated that these cytokines may play a role in mediating effects of LPS on the embryo, as an imbalance of pro-inflammatory and anti-inflammatory cytokines has previously been shown to lead to implantation failure or embryo degradation (Chaouat et al., 1990, Pampfer et al., 1994a, Pampfer et al., 1997b).

The use of a soluble TNFα receptor, etanercept, in an attempt to protect the embryo from adverse effects of LPS was unsuccessful despite the fact that TNFα is a major adverse cytokine induced by LPS. Our finding differs from previous studies which reported that etanercept was able to protect the fetus from LPS-induced fetal death during mid-gestation and attenuate TNFα and other pro-inflammatory cytokine expression (Robertson et al., 2007, Renaud et al., 2011). It is possible that the concentration of etanercept used to protect the fetus during mid-gestation was insufficient to neutralise the elevated TNFα surrounding the embryo in the tract, or if etanercept was able to attenuate TNFα, the embryo was still exposed to other pro-inflammatory cytokines such as IFNγ. There are also additional adverse cytokines such as TRAIL which can also have pro-apoptotic effects in embryos (Riley et al., 2004), which we did not measure in the current study. This may reflect the fact that the embryo is more vulnerable during the pre-implantation period as compared to post-implantation, when the maternal-fetal interface may be able to provide some protection against perturbation in the maternal environment.

One of the observations in our early perturbation model was the presence of the 'all or none' phenomenon, a concept by which embryonic exposures to insults that occur before organogenesis result in either no overt adverse embryonic outcome, or alternatively in embryonic death (Adam, 2012). The observation of maternal perturbation during early pregnancy resulting in either a successful pregnancy or pregnancy loss in our model is similar to other early perturbation studies (Erlebacher et al., 2004, Schelbach et al., 2013). This finding again highlights the vulnerability of the pre-implantation embryo to perturbations and the adaptability of those embryos that implanted successfully. It may be that the reproductive system has biological mechanisms to ensure that pregnancy can only progress if the fetus is able to withstand the insult, in order to maximise the outcome of each reproductive investment.

Since we could not exclude the possibility of an altered maternal receptivity in our model, embryo transfer was utilised to determine whether the LPS effect on the embryo and fetal development was evident in an unchallenged maternal environment. However, due to the limitations of using the C57Bl/6 genetic background as a recipient strain, an alternative and more effective recipient strain (Swiss) was utilised. The use of embryo transfer in this experiment revealed that the effects of LPS on fetal and placental development were clearly maintained even when the pseudopregnant recipient mouse was not exposed to LPS. It could not be excluded that these results were in part confounded by the difference in the recipient genetic background. Although implantation rates did not vary, the percentage of viable implantations was significantly reduced. A possibility to overcome this confounding issue would be to use F1 hybrid of the C57Bl/6 strain in order to increase the success of embryo transfer compared to C57Bl/6. Reciprocal embryo transfers between control and LPS-treated donor and recipients would also give more indication of the effects of LPS on maternal uterine receptivity. Same gestational day donor to recipient embryo transfers would be also more beneficial in future experiments to solve these issues.

Chapter 5 aimed to determine some of the effects of maternal systemic inflammation on the pre-implantation period on the postnatal development of the resulting progeny. Prior to the completion of gestation, there was a significant reduction in the fetal and placental parameters in the LPS-treated group regardless of maternal IL-10 genotype. However, there was no significant difference in birth weight of the pups following birth. After birth, male progeny derived from LPS-treated mothers showed

growth restriction post weaning but this was not sustained through to the end of the 19 week experiment. The sex-specific findings in our model were similar to previous rodent studies in nutritional models of fetal growth restriction which show accumulation in both body weight and fat tissue of the male progeny derived from affected mothers (Desai et al., 2005, Kind et al., 2005). While we did not find significant differences in the size of the fat cells, post-mortem analysis revealed that the combination of maternal LPS treatment and IL-10 deficiency prior to implantation resulted in a significant accumulation of the total central fat tissue in the male progeny compared to wildtype LPS-treated groups. This finding suggests a protective role of maternal IL-10 cytokine in the event of early maternal perturbation, and highlights the likely importance of this cytokines in protection for peri-conceptional inflammatory perturbation.

We went on further to show that the progeny derived from LPS-treated $Il10^{+/+}$ mothers had an altered cytokine response when challenged with LPS during adulthood. The combination of maternal LPS treatment and IL-10 deficiency was found to blunt serum concentration of LIF cytokine in the male progeny. Our findings were similar to other studies which showed that either a pre-implantation or prenatal exposure to LPS results in an altered response to postnatal exposure to LPS (Shanks et al., 2000, Williams et al., 2011, Xu et al., 2007). While it was not significantly different in the female progeny, we observed a significantly lower concentration of serum IL-10 in the male progeny derived from LPS-treated $Il10^{+/+}$ mothers compared to their control counterparts not exposed to LPS in early life. Indeed, this observation was significant when the concentration of serum IL-10 in overall progeny was analysed. This observation suggests a blunted IL-10 response as a result of maternal LPS treatment and this would be of interest to pursue further, possibly with a higher LPS dose. A possible mechanism behind this blunted response could be due to alterations in the TLR4 sensing and/or signalling pathway of the progeny in response to LPS. This would be of interest to investigate in future experiments. It might be beneficial for the offspring if the pre-implantation exposure to LPS would result in a more tolerant phenotype in the offspring postnatally. However the reverse could result in severe consequences or be potentially fatal in an event of a life-threatening infection. Clearly, further studies are required to understand the cause and effect relationships between peri-conception exposures and adult immune capability.

Barker and colleagues proposed the fetal origins of adult disease hypothesis that adverse intrauterine conditions at various stages of gestation alters the trajectory of growth, resulting in fetal growth restriction and predisposing to other metabolic diseases in adult life (Barker and Clark, 1997, Barker et al., 1989). An extension of this hypothesis leads to the thrifty phenotype hypothesis which proposes the epidemiological associations between poor fetal and infant growth and the subsequent development of type 2 diabetes and the metabolic syndrome. This programming can result from the effects of poor nutrition in early life, which produces permanent changes in glucose-insulin metabolism (Hales and Barker, 1992). Many studies have demonstrated that altering the uterine environment or the embryonic environment during early pregnancy can induce phenotype changes that have adverse consequences in the health outcomes of subsequent offspring. Although many studies have depicted changes in growth or metabolic parameters of offspring, little is known about the underlying mechanisms to fetal programming. One of the proposed mechanisms relates to altered placental development due to changes in epigenetic modification of the embryo (Cooney et al., 2002, Fortier et al., 2008). In recent years, other proposed mechanisms such as mitochondria and reactive oxygen species (ROS) have also been reported. Mitochondrial abnormalities have been proposed as it has been shown that cellular mitochondrial content is determined very early in development, with these numbers being fixed and perpetuated throughout the developed organism (McConnell and Petrie, 2004, Taylor et al., 2005). As mitochondrial DNA is solely inherited from the mother in humans and rodents, several studies have shown that factors such as maternal diabetes, alcohol consumption and repeated ovarian stimulation can result in mitochondrial dysfunction and growth retardation in the offspring (Wang et al., 2009, Xu et al., 2005, Chao et al., 2005). Repeated ovarian stimulation has also been shown to induce oxidative stress, and ROS has been linked to growth retardation and can result in long term impairment of the cardiovascular system (Franco Mdo et al., 2002, Karowicz-Bilinska et al., 2002, Raab et al., 2009, Xu et al., 2006b).

Sex-specific differences are often observed in developmental programming studies (Kwong et al., 2000, Watkins et al., 2007, Watkins et al., 2008). However, the underlying mechanisms for this interaction with programming are not fully understood as some environmental factors seem to differentially affect one sex over the other. Several studies have shown that the male progeny are more susceptible than females in displaying effects of developmental programming (Ozaki et al., 2001, Sjoblom et al., 2005). This has been suggested to be due to the speed at which male embryos develop, with male embryos

are more likely to respond and adapt to the changes in its environment caused by perturbations during early pregnancy (Mittwoch, 1993).

Although the experiments in this thesis, along with other works that study maternal inflammation, do not address the mechanisms behind the altered fetal development and fat mass accumulation in the progeny, epigenetic changes in the pre-implantation embryo leading to placental insufficiency is a possible cause. As the cells in the pre-implantation embryo has the potential to form all the cells in the adult body, any changes experienced by these cells can have downstream effects on many tissues or organs. Studies of mouse somatic cell nuclear transfer embryos suggest the process of DNA methylation remodelling targets specific genomic regions to ensure the appropriate methylation patterns are established for continuing development (Chan et al., 2012). Certain sequences have been identified as being protected from DNA methylation remodelling, including repetitive transposon-derived sequences and imprinted genes (Hackett and Surani, 2013). Imprinted genes, which are functionally mono-allelic in a parental-origin specific manner and subject to multiple layers of epigenetic control of expression, have been hypothesised to be particularly vulnerable to environmental perturbation (Jirtle and Skinner, 2007). Overexpression of IL-15 was shown to cause chromosomal instability and DNA methylation in other cell lineages (El-Omar et al., 2000) and this suggests the possibility for the same to occur in the embryo. The importance of imprinted genes in the developmental control of growth and placentation likely explains the high incidence of their alteration in manipulations resulting in changes to offspring growth and development (Radford et al., 2012). However, Radford et al. has also reported that their unique mechanism in epigenetic control of expression does not render them more or less susceptible or protected than bi-allelically expressed genes from perturbation in expression induced by maternal undernutrition in either F1 or F2 generation (Radford et al., 2012). Analysis of the expression of imprinted genes in the pre-implantation embryos including *H19* and *IGF2R* that governs fetal and placental development could provide a better understanding behind the programming mechanism.

At this stage, it is not clear how an altered cytokine environment could impact the epigenome in the embryo. One possibility is that cell stress induced by insufficient embryotrophic cytokines, or elevated embryotoxic cytokines, could alter the epigenetic machinery or influence epigenetic marks at particular loci (McConnell and Petrie, 2004, Manikkam et al., 2012, Bertoldo et al., 2014).

The molecular studies described in Chapter 4 showed increased gene expression in pro-inflammatory cytokines in the oviduct and the uterus following maternal systemic inflammation. Assessing embryo gene expression of cytokines and other stress response genes such as heat shock proteins and apoptotic genes would provide a greater insight into the response of the embryo to perturbed environments, and how this gives rise to embryos of reduced developmental competence resulting in poorer placental development and/or altered fetal development. Incidentally, TLR4 was found to mediate intrauterine growth restriction following systemic Campylobacter rectus infection in mice (Arce et al., 2012), which indicate TLR4 as a potential target to investigate in future experiments. Although we did not find any significant differences in fat cell size in our male progeny, another approach would be to measure fat cell size in the other central fat tissues to investigate cell size differences. To further investigate the effects of maternal systemic inflammation on the adult progeny, metabolic parameters such as glucose, insulin, leptin and adiponectin could be measured to investigate whether the phenotype change in progeny are associated with susceptibility to metabolic disorders. Another aspect of interest would be to investigate if the effects of maternal systemic inflammation in the F1 progeny would extend to an effect on the F2 progeny as well.

Future directions from this study could involve using alternative knockout mouse models to investigate the effects of maternal systemic inflammation on the early embryo. As LPS is known to act through the TLR4 pathway (Chow et al., 1999), utilising mice with null mutation in TLR4 gene (*Tlr4* $^{-/-}$) or in the TLR4 adaptor protein, Myd88 (*Myd88* $^{-/-}$) could aid in understanding the mechanism by which LPS acts on the pre-implantation embryo. From a different perspective, mouse models with existing systemic inflammation such as the human TNFα transgenic mouse (Keffer et al., 1991) which is used to study rheumatoid arthritis (RA) would be of interest to investigate the effects of constant inflammation on early pregnancy. Another model to consider may be an obese mouse model as maternal obesity has been shown to be associated with increased inflammatory response (Ramsay et al., 2002). The use of these mouse models does not require manipulation and could relate better to understanding the mechanism of maternal perturbation in humans, as maternal obesity, asthma and autoimmune diseases such as RA and multiple sclerosis (MS) are an issue for women of reproductive age.
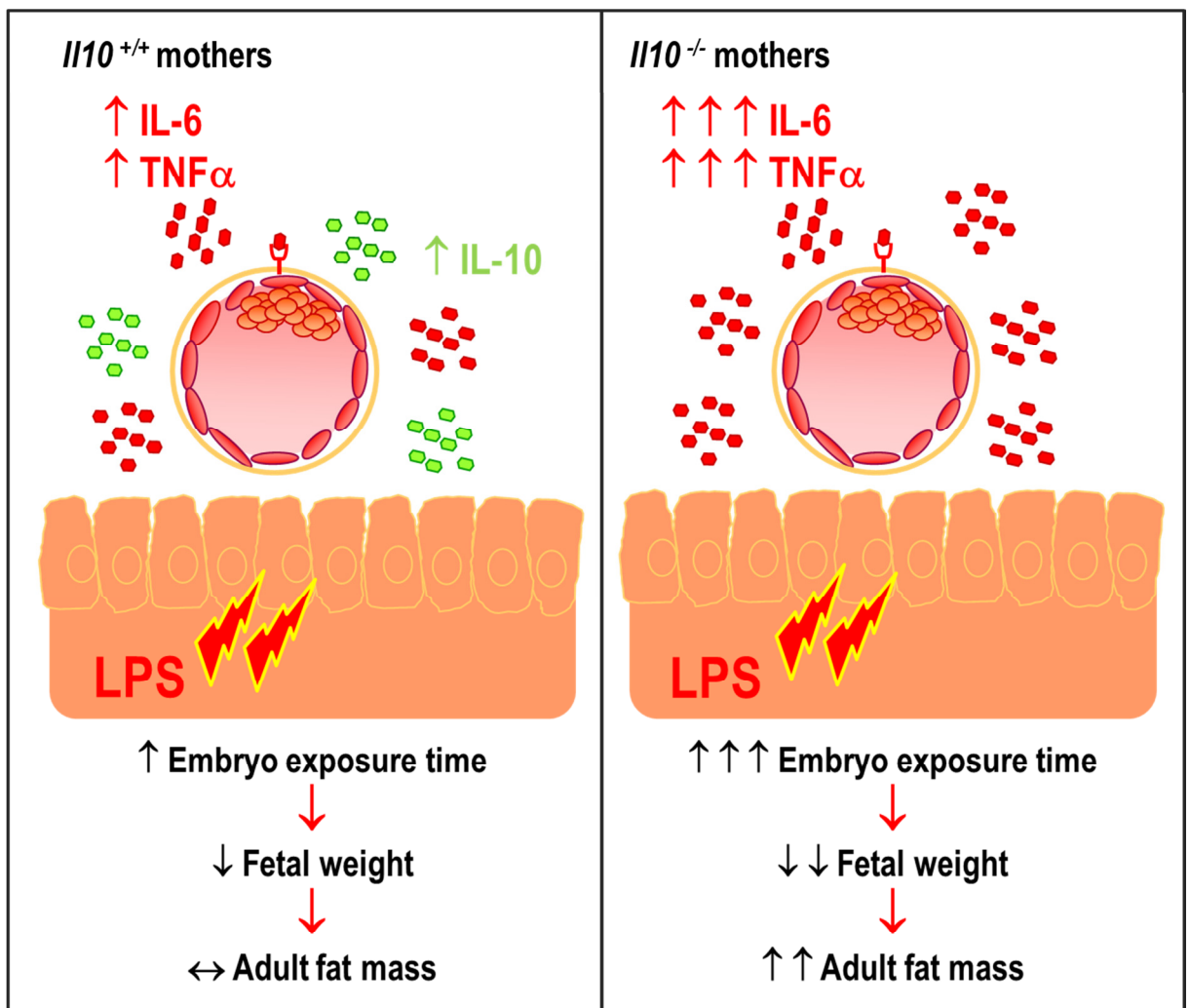
Figure 6.1   Schematic illustration of mechanism of maternal systemic inflammation during the pre-implantation period.

This illustration depicts the consequences of maternal systemic inflammation with bacterial LPS on days 2.5 and 3.5 pc. Studies described in this thesis show that cytokines mediate the inflammatory response to the pre-implantation embryo via cytokine receptors express on the embryos. The absence of maternal anti-inflammatory IL-10 cytokine in the embryo environment delayed clearance of the pro-inflammatory cytokines, prolonging embryo exposure to the adverse environment which alters fetal development. Thrifty phenotype was evident as fat mass accumulation was found in adult progeny from LPS-treated IL-10 deficient mothers compared to their IL-10 sufficient counterparts.

A similar approach would be test oviductal fluid from women with existing systemic inflammation for inflammatory cytokine expression and possibly follow a cohort of these women through pregnancy and early childhood development. These inflammation models could include women with sexually transmitted infections (STI), such as Chlamydia, gonorrhoea and trichomoniasis, which have been shown to have high levels of IL-1β and lactoferrin in the vaginal fluid (Spear et al., 2011) or women with periodontal diseases. Women with periodontal diseases have been associated with higher preterm risk

and low birth weight (Lopez et al., 2002, Shirmohammadi et al., 2009, Tucker, 2006) and it was shown in rats that periodontitis results in both local and systemic concentrations of pro-inflammatory cytokines where high IL-6 and TNFα concentrations in the uterus (Bain et al., 2009). Other models related to common occurrences of infection in women could include gastrointestinal diseases. The human gut microbiome is intensely populated with microbial cells (> 100 trillion) (Abel, 2006) and the intestinal epithelium is plays an important part in separating the sterile host from harmful pathogens (Schulenburg et al., 2004). Perturbations to the intestinal epithelium cells are related to important genetic risk factors of inflammatory bowel disease (IBD) and can originate intestinal inflammation (Cader and Kaser, 2013). Pro-inflammatory IFNγ and TNFα has been shown to disrupt in epithelial barrier function by altering lipid composition in membrane microdomains of tight junction (Li et al., 2008).

Other future directions could also include investigating perturbation during the pre-implantation period through non-infectious inflammatory models, by using damage-associated molecular pattern molecules (DAMPs) such as HMGB1, heat shock proteins (HSPs) and hyaluronan. This would be beneficial towards understanding the role of cytokines during this crucial time as well since DAMPs have also been shown to act through the TLR pathways and induce expression of pro-inflammatory cytokines in inflammatory diseases and preterm labour (Foell et al., 2007, Romero et al., 2011).

In summary, we identified for the first time that cytokines play a role as mediators of programming the embryo during the pre-implantation maternal systemic inflammation. The inflammatory milieu of the maternal tract not only alters blastocyst, fetal and placental development but also leads to permanent sex-specific changes in the growth trajectory, adiposity and innate immune response in the adult offspring. We reported the importance of maternal IL-10 cytokine in protecting the embryo from adverse effects of maternal systemic inflammation. These findings in our model emphasise the role of cytokines in the mechanism of embryo sensing and responding to adapt to its environmental conditions. However, it is important to acknowledge that although correlations with altered cytokines are shown, ultimately we did not discount the possibility of other soluble maternal tract factors in mediating effects of LPS treatment on embryos. This substantiates the need for further analysis of the underlying molecular mechanisms.

Additionally, it is important to investigate the applicability of these concepts in humans. The studies described in this thesis could be extrapolated to humans as systemic inflammation caused by infection is unpredictable when women are trying to conceive naturally or through ART techniques. These studies are valid considering cytokine gene polymorphism is a natural phenomenon, through which individuals in outbred populations have varying cytokine production. Women who have IL-10 gene polymorphisms may naturally produce insufficient IL-10 cytokine, which might then increase the risk of programming the embryo in an event of a systemic inflammation prior to implantation. IL-10 polymorphism has been studied in both men and women and there was an association between IL-10 -2849 SNP and fertility, as these women were twice as likely to have no offspring in their marriage (van Dunne et al., 2006). Association of IL-10 -1082 SNP and recurrent miscarriages have also been shown (Medica et al., 2009), suggesting that maternal IL-10 is important for the maintenance of a successful pregnancy. In this regard it is important for us to understand the role of cytokines in mediating interactions between the female reproductive tract and the embryo as this may have consequences for the long-term health outcomes of subsequent progeny. Such studies may eventually provide new opportunities for therapeutic intervention in pregnancies for women with chronic inflammation or utilising ART techniques. For example, if the findings in this study can be replicated, it would seem sensible to advise women to avoid conception during inflammatory illness, and or to seek treatments for STIs or local reproductive tract infection before attempting to conceive.

Chapter 7

Appendices

Table 7.1   Composition of G1.2 and G2.2 embryo culture media

Component (mM)	G1.2	G2.2
NaCl	85.16	85.16
KCl	5.5	5.5
NaH2PO4.2H2O	0.5	0.5
$CaCl_2.2H_2O$	1.8	1.8
$MgSO_4.7H_2O$	1.0	1.0
$NaHCO_3$	25.0	25.0
Na pyruvate	0.32	0.1
Na lactate (L-isoform)	10.5	5.87
Glucose	0.5	3.15
Glutamine	1.0	1.0
Taurine	0.1	0.0
Non-essential amino acids	All	All
Essential amino acids	None	All
EDTA	0.01	0.0
Human serum albumin	5.0 g/l	0.01 g/l

All reagents purchased from Sigma-Aldrich. For essential and non-essential amino acids, see Table 7.3. Adapted from (Gardner and Lane, 1997)

Table 7.2   Formulation of MOPS buffer for embryo handling

Component (g/L)	MOPS
NaCl	5.552
KCl	0.41
$NaH_2PO_4\text{-}H_2O$	0.035
$MgSO_4\text{-}7H_2O$	0.246
Na Lactate (L)	1.17
Glucose	0.09
Penicillin (optional)	0.06
$NaHCO_3$	0.21
Phenol Red	0.001
Pyruvic Acid	0.035
$CaCl_2\text{-}H_2O$	0.246
Glutamax solution	10 ml
Taurine	0.0125
Non-Essential amino acids	10 ml
MOPS	4.197
BSA	0.000004

Glutamax solution (Invitrogen). All other reagents purchased from Sigma-Aldrich. Non-Essential amino acids solution, see Table 7.3. Adapted from (Gardner et al., 2004).

Table 7.3   Concentration of amino acids used in G1, G2 and MOPS medium

Non-essential amino acids	Concentration (mM)	Essential amino acids	Concentration (mM)
Alanine	0.1	Arginine	0.6
Asparagine	0.1	Cystine	0.1
Aspartate	0.1	Histidine	0.2
Glutamate	0.1	Isoleucine	0.4
Glycine	0.1	Leucine	0.4
Proline	0.1	Lysine	0.4
Serine	0.1	Methionine	0.1
		Phenylalanine	0.2
		Threonine	0.4
		Tryptophan	0.05
		Tyrosine	0.2
		Valine	0.4
		Glutamine	1.0

All amino acids purchased from Sigma-Aldrich. Adapted from (Gardner and Lane, 1997).

Chapter 8

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