UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE, HEALTH & LIFE SCIENCES

School of Biological Sciences

Effect of a maternal inflammatory challenge during the preimplantation period on offspring development and phenotype

by

Charlotte Lucy Williams

Thesis for the degree of Doctor of Philosophy

September 2009

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

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The preimplantation mammalian embryo is sensitive to its immediate surroundings: alterations to its *in vitro* or *in vivo* environment can affect not only the immediate events of blastocyst formation, but can also give rise to long-term phenotypic consequences during fetal and/or postnatal life. For example, rodent studies have shown that maternal under-nutrition during preimplantation gestation can lead to increased risk of cardiovascular, metabolic and behavioural abnormalities in the adult offspring.

Do other types of maternal challenge similarly impact on the developmental programme with long-lasting consequences? Infection and injury are common in everyday life and normally result in altered homeostasis and generation of an inflammatory response. The aim of my thesis was to study the effects of an inflammatory environment during preimplantation development on the phenotype of the blastocyst, fetus and offspring postnatally. In the first part of the study mouse embryos were cultured *in vitro* in the presence of medium only (control) or increasing concentrations (1-1000 pg/ml) of the inflammatory cytokine, interferon γ (IFN-γ). The second part of the study focused on an *in vivo* model of maternal systemic inflammation where saline (control), 10, 50 or 150 µg/kg lipopolysaccharide (LPS) was administered intraperitoneally (i.p.) to female mice on gestational day 0.5 (GD 0.5).

In vitro culture of mouse embryos with select higher concentrations of IFN-γ resulted in a greater proportion of cavitated embryos (1000 pg/ml) and reduced inner cell mass (ICM) cell number (10 and 1000 pg/ml) without affecting trophectoderm (TE) cell number. *In vivo*, generation of a maternal systemic inflammatory response to LPS administration was confirmed initially. LPS treatment induced sickness behaviour, weight loss and increased the serum concentration of several cytokines, e.g. interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α). Prenatally, the maternal inflammatory challenge resulted in reduced ICM cell number and reduced ICM:TE cell ratio in GD 3.5 blastocysts, but did not affect the number of embryos generated or GD 17 litter size. Furthermore, the GD 17 conceptus was normal in terms of weight of the extra-embryonic tissues and fetal organs. Postnatally, the systemic maternal inflammatory challenge did not alter litter size, birth weight or growth, but did result in altered behaviour, organ/body weight ratios and immune status of adult offspring. In particular, male offspring from 150 µg/kg LPS treated mothers displayed reduced levels of locomotor and exploratory related activity, increased mass of specific fat-pads and increased body mass index (BMI). Furthermore, male offspring from 150 µg/kg LPS treated mothers displayed altered splenic T and B lymphocyte populations with the percentage of B lymphocytes reduced and the percentage of T lymphocytes increased. Both male and female offspring from LPS treated mothers had lower concentrations of

a number of serum cytokines and chemokines, either basally or after directly receiving their own LPS challenge.

My work using a mouse model has shown that maternal inflammation during preimplantation gestation can permanently change the developmental programme, leading to altered adult phenotype, affecting diverse physiological systems. This study implicates maternal immune status during very early gestation as critical in the health of the next generation.

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Declaration of Authorship

I, Charlotte Lucy Williams,

declare that the thesis entitled,

Effect of a maternal inflammatory challenge during the preimplantation period on offspring development and phenotype

and the work presented in the thesis are both my own, and have been generated by me as the result of my own original research. I confirm that:

- this work was done wholly or mainly while in candidature for a research degree at this University;
- where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
- where I have consulted the published work of others, this is always clearly attributed;
- where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
- I have acknowledged all main sources of help;
- where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
- none of this work has been published before submission.

Signed: ……..

Date:……..

Acknowledgements

First I would like to thank The Boss (Prof. Tom Fleming). He has been a great supervisor and has been truly enthusiastic towards me and my research from the very beginning of my PhD. He has always had an open door, a positive attitude and a good sense of humour. I am sure, if you asked him, that he would say the 'L' in Charlotte L. Williams stands for 'last minute'!! I will always be grateful to him for his generosity in both academic and personal situations.

Next I would like to thank all members of Tom's group, both past and present, for kind words or acts that have encouraged and supported me over the last 4 years. In particular I would like to thank Dr Adam Watkins who has been responsible for teaching me most of the experimental and analysis techniques that I have used during my PhD. In addition, he has always been generous and enthusiastic during impromptu discussions and question sessions in the office! Dr Bhav Sheth has been fantastic and I wish to thank her for helpful conversations and teaching me primer design and PCR. But most of all, she has inspired me to be strong and to speak my mind. Thank you also to Dr Judith Eckert for teaching me how to differentially label embryos and image them correctly. I must thank Dr Sally Rosser-Davies for passing on to me a very helpful phrase…..'when eating an elephant, take small bites'!!

I want to say a big thank you to Prof. Sonia Quaratino for all her help with the theory and practice of FACS – a technique that I have discovered I really enjoy. Most importantly I want to thank her for inspiring me to be fearless, ambitious, and strong.

Thank you to Dr Ferdousi Chowdhury for teaching me how to use the Luminex 100 instrument and associated software.

To my fellow PhD students – thank you. To name but a few, Jenny Warner, Philippa Mitchell, Claire Powell, Dan Asby, Shmma Quraishe and Rob Howlin: I am so glad that I've shared the challenges of PhD life with them all.

I must also thank Tiffany, Sally and Yannis (a.k.a. Coffee Club) for many, many wonderful weekend meetings. They are amazing people and have provided balance to my life over the last couple of years.

Doriana Cellura…..da dove iniziare……

Finally, I want to thank my Mum and Dad. Quite simply, I would not be where I am now without their endless help, support and love.

This thesis is dedicated to my Mum and Dad

In order to know where we are going in life, we must know where we have come from.

List of Abbreviations

ACTH	Adrenocorticotrophic hormone
ANOVA	Analysis of variance
AP	Activator protein
APC	Antigen presenting cell
APP	Acute phase protein
APR	Acute phase response
ART	Assisted reproductive technology
ATP	Adenosine triphosphate
BMI	Body mass index
BSA	Bovine serum albumin
CIITA	Class II transactivator
CNS	Central nervous system
CO_2	Carbon dioxide
COX	Cyclooxygenase
CRH	Corticotropin releasing hormone
DC	Dendritic cell
DNA	Deoxyribonucleic acid
Dnmt	DNA methyltransferase
DNP	Dinitrophenyl
DOHaD	Developmental origins of health and disease
FACS	Fluorescence activated cell sorter
FCS	Fetal calf serum
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
GBP	Guanylate-binding protein
GC	Glucocorticoid
G-CSF	Granulocyte colony-stimulating factor
GD	Gestational day
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPI	Glycosylphosphatidylinositol
HCC	Hemofiltrate CC
hCG	Human chorionic gonadotropin
HDAC	Histone deacetylase

HPA	Hypothalamus-pituitary-adrenal
HRBC	Human red blood cell
Hsd11b1	11β-hydroxysteroid dehydrogenase
i.p.	intraperitoneal
i.v.	intravenous
ICM	Inner cell mass
IFN	Interferon
IFNGR	Interferon gamma receptor
Ig	Immunoglobulin
IKK	Inhibitor of κB kinase
IL	Interleukin
IRAK	Interleukin 1 receptor associated kinase
IRF	Interferon regulatory factor
IRS	Interferon responsive sequence
IU	International Units
JAK	Janus kinase
K	Potassium
KC	Keratinocyte-derived cytokine
KSOM	Potassium simplex optimised medium
LBP	LPS binding protein
LMP	Low molecular protein
LOS	Large offspring syndrome
LPS	Lipopolysaccharide
LSD	Least significant difference
mAb	Monoclonal antibody
MAPK	Mitogen activated protein kinase
MCP	Monocyte chemoattractant protein
MD	Myeloid differentiation
MFI	Mean fluorescence intensity
MIP	Macrophage inflammatory protein
mRNA	Messenger ribonucleic acid
MyD88	Myeloid differentiation primary response gene 88
Na	Sodium
NaCl	Sodium chloride
NaOH	Sodium hydroxide

NF-κB	Nuclear factor κB
NK	Natural killer
NO	Nitric oxide
PAF	Platelet activating factor
PAMP	Pathogen associated molecular pattern
PAR	Predictive adaptive response
PBS	Phosphate buffered saline
PBS	Phosphate buffered saline
PE	Phycoerythrin
Ped	Preimplantation embryo development
PEPCK	Phosphoenolpyruvate carboxykinase
PFC	Plaque-forming cell
PG	Prostaglandin
PI	Propidium iodide
PI3 kinase	Phosphatidyl inositol 3-kinase
PKC	Protein kinase C
PMSG	Pregnant mare serum gonadotrophin
PolyI:C	Polyribinosinic-polyribocytidilic acid
PVP	Polyvinylpyrrolidone
RANTES	Regulated upon activation, normal T-cell expressed and secreted
RBC	Red blood cell
RIP	Receptor interacting protein
rm	recombinant mouse
RNA	Ribonucleic acid
rpm	Revolutions per minute
Sal	Saline
SBP	Systolic blood pressure
SEM	Standard error of the mean
SRBC	Sheep red blood cell
SSC	Side scatter
STAT	Signal transducer and activator of transcription
T_{H1}	T helper type 1
TBC	Trophoblast cell
TCR	T cell receptor
TE	Trophectoderm

TIR	Toll-interleukin-1 receptor
TIRAP	TIR domain-containing adaptor protein
TLR	Toll-like receptor
TNBS	Trinitrobenzene sulphonic acid
TNF	Tumour necrosis factor
TRIF	TIR domain-containing adaptor inducing INF-β
uNK	Uterine natural killer
ZGA	Zygotic genome activation

1 Introduction

1.1 Preimplantation mouse embryo development

1.1.1 Morphological aspects of preimplantation development

The process of preimplantation development begins after fertilisation and culminates at gestational day (GD) 4.5 when implantation occurs (Johnson and McConnell, 2004). Thus, the primary morphological aim of preimplantation development is to produce an implantation-competent blastocyst comprising two distinct cell lineages; the extra-embryonic trophectoderm (TE) that will eventually form the placanta; and the embryonic inner cell mass (ICM) that will give rise to the fetus. The single-cell zygote undergoes a total of 7 cleavage divisions to become a blastocyst containing 128-blastomeres (Fig. 1.1) and there is no net growth over this period, so each blastomere becomes progressively smaller with each cleavage division (Johnson and McConnell, 2004).

The first three cleavage divisions produce an embryo composed of 8 spherical, loosely-associated and non-polarised blastomeres (Fig. 1.1c). The process of TE differentiation begins at the late 8-cell stage (approximately 10 hours after cleavage to the 8-cell stage) when the embryo becomes compact (Johnson and McConnell, 2004). At the time of compaction, all 8 blastomeres maximise cell-cell contact by flattening onto one another, and undergo both intercellular adhesion and apico-basal axis polarisation (Fig. 1.1d). The outer apical surface of each blastomere has a stable population of microvilli and is non-adhesive, and the inner basolateral surfaces are adhesive and microvilli-free (Fleming et al., 2004b). Flattening events co-ordinated by the calcium-dependent E-cadherin/catenin system (components of the adherens junction) seem to play a key role in correctly orientating the blastomeres for generation of inner- and outer-cells in subsequent rounds of division (Fleming et al., 2004b; Johnson et al., 1986). When cleavage stage embryos are incubated with anti-E-cadherin antibodies, cellular polarisation still occurs but happens over a more protracted time course and along random axes. In addition, junctional formation and blastocoel formation still take place but ICM is absent or greatly reduced (Johnson et al., 1986).

Cell polarity attained at compaction provides blastomeres with the essential spatial organization to give rise to divergent cell lines at the fourth cell division. Division of polarised 8-cell blastomeres gives rise to the 16-cell morula which is composed of two

distinct cell types (Fig. 1.1e): the outer, polar cells of the TE lineage and the inner, non-polar cells of the ICM (Johnson and McConnell, 2004). The polarised microvillus surface and cortical phenotype is maintained during mitotic cell division, but the cytoplasmic polarity is transiently lost; thus, the cytocotical pole effectively provides the cell with a memory of polarity (Johnson et al., 1988). Cell division is either parallel or perpendicular to the axis of cell polarity: division parallel to the axis of cell polarity is a conservative division because the apical pole is bisected and inherited by both daughter cells; division perpendicular to the axis of cell polarity is a differentiative division since the apical region is not bisected (Johnson and McConnell, 2004). Therefore, one daughter cell is polar because it receives the microvillous apical region, whereas the other daughter cell is non-polar because it receives only the basolateral cell surface. In a fifth cell cycle (16- to 32-cell transition) the outer, polar blastomeres engage in another round of differentiative or conservative division in order to complete the process of lineage segregation within the embryo. If the first allocation (8- to 16-cell transition) of cells to the ICM is relatively small then the embryo can compensate by a relatively large allocation in the second round (16- to 32-cell transition) and *vice versa* (Fleming, 1987; Johnson and McConnell, 2004).

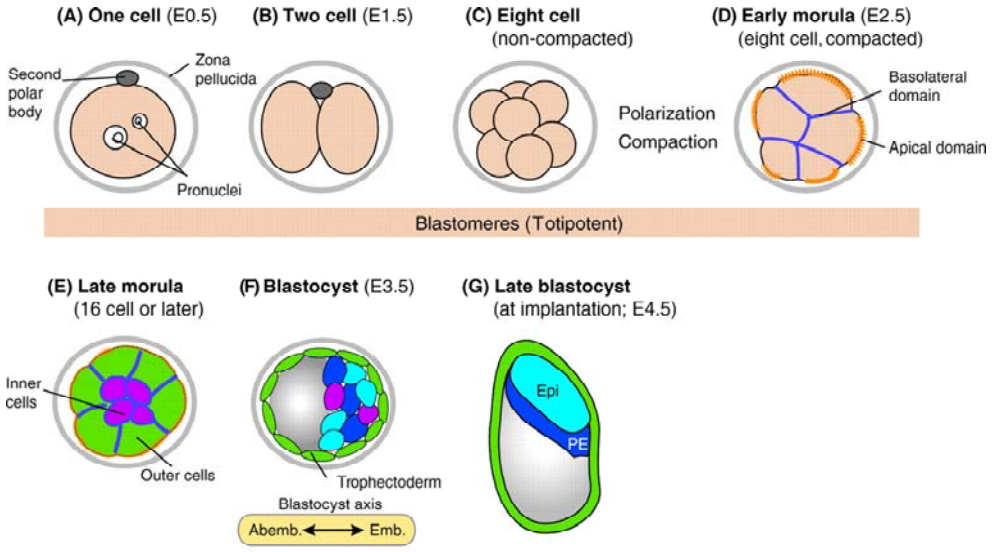


Figure 1.1 Diagram of preimplantation mouse embryo development from the 1-cell to the implantation-competent blastocyst stage (A-G). Epi, epiblast; PE, primitive endoderm; Abemb.-Emb., abembryonic-embryonic axis (Rossant and Tam, 2009).

The embryo described thus far is a morula: a densely clustered ball of 32-cells that does not have an internal cavity. In order for the 32-cell morula to become a blastocyst, it must undergo the process of cavitation. During cavitation the outer,

polarized cells of the 32-cell embryo transport fluid transcellularly into the morula in order to create a fluid filled blastocoel cavity lying between the TE and ICM (Fig. 1.1f). Two major maturational events of the TE are essential for the success of cavitation and maintenance of the blastocoel. Firstly, the tight junctions between the TE cells must be fully formed so that there is a stable permeability seal around the blastocoel to prevent leakage of the blastocoel fluid. Secondly, the Na^+/K^+-ATPase in the basolateral membrane must be fully functioning, as it is this that is responsible for producing the ATP-dependent ion transport required to drive water down its osmotic gradient into the blastocoel. The blastocoel cavity is fully expanded by the 64 cell stage and by the 128-cell stage (GD 4.5) a third cell lineage is apparent (Johnson and McConnell, 2004). This cell lineage is the primitive endoderm (PE; Fig. 1.1g) and forms on the surface of the ICM adjacent to the blastocoel cavity and will form the extraembryonic parietal and visceral endoderm, which will eventually form the yolk sac (Rossant, 2004). At this stage the embryo hatches from its zona pellucida, a glycoprotein-rich acellular coating, and begins the process of uterine attachment and implantation (Johnson and McConnell, 2004).

1.1.2 Zygotic genome activation

Zygotic genome activation (ZGA) is the first major developmental event that occurs following fertilization and is essential for development; 2-cell embryos do not cleave any further when cultured in the presence of the RNA polymerase II inhibitor, α-amanitin (Zeng and Schultz, 2005). During ZGA the developmental programme that is initially directed by maternally inherited components is replaced by the new programme that is coded for by the embryos own genome (Schultz, 2002). ZGA has at least three functions that are essential for developmental progression. The first is to destroy oocyte-derived mRNA transcripts. The second is to replace maternal transcripts with zygotic ones for those common cellular components (e.g. actin) that, if not replaced, will result in developmental arrest due to the inability to execute basic cellular functions. The third is to promote the reprogramming of gene expression that is coupled with generation of novel transcripts that are not expressed in the oocyte (Schultz, 2002). The third function is likely responsible for driving the transformation of the differentiated oocyte into the totipotent blastomeres of the 2-cell embryo.

ZGA has occurred by the 2-cell stage and there is some evidence demonstrating that the 1-cell embryo is, at least partly, transcriptionally active (Aoki et al., 1997; Wang et al., 2004). For example, Aoki et al. found that BrUTP incorporation in the 1-

cell embryo was approximately 20% that of the 2-cell embryo (Aoki et al., 1997). However, intriguingly, a microarray analysis found that the 1-cell embryo contained no α-amanitin-sensitive gene transcripts; which suggested that there was no *de novo* generation of mature mRNA transcripts (Zeng and Schultz, 2005). Upon these findings, the authors suggested that the 1-cell embryo is transcriptionally active, but lacks the ability to produce polyadenylated, translatable mRNA (Zeng and Schultz, 2005). This theory is supported by other lines of evidence suggesting that transcription and translation may be uncoupled processes in the 1-cell embryo. For example, when the plasmid-borne luciferase reporter gene was injected into 1-cell embryos, luciferase mRNA was detected whereas luciferase activity was not detected until after cleavage to the 2-cell stage (Nothias et al., 1996). This group also showed that transcription and translation were temporally tightly coupled events if the luciferase gene was injected at the 2-cell stage (Nothias et al., 1996). This finding thus demonstrated that transcription and translation are uncoupled processes in the 1-cell embryo.

In the 2-cell embryo soon after ZGA, a chromatin-based transcriptionally repressive state becomes apparent, where efficient gene transcription requires the presence of an enhancer (Schultz, 2002). Inducing histone hyperacetylation relieves the repressive state and the requirement for an enhancer (Wiekowski et al., 1993). Such a repressive state could be critical for the precise and controlled regulation of important sequential developmental events. Interestingly, microarray analysis has shown that the types of genes expressed selectively in the 2-cell embryo are those involved with ribosome biogenesis and assembly, protein synthesis, RNA metabolism and transcription (Zeng and Schultz, 2005). This analysis also implicated that the two genes, *c-Myc* and *Hdac1* (Histone deacetylase 1), may be seminal components involved in the expression of crucial developmental genes and production of the transcriptionally repressive state, respectively (Zeng and Schultz, 2005). Indeed, ablation of *c-Myc* function in 2-cell embryos post-ZGA resulted in the arrest of almost all embryos at the 8-cell-morula stage (Paria et al., 1992).

1.1.3 Cell lineage specific transcription factors

Lineage specification of the pluripotent ICM is regulated by restricted expression of key transcription factors, which include Oct4 (also called Pou5f1), Sox2 and Nanog. Oct4 is expressed in the oocyte and all blastomeres of the cleavage stage embryo but by the blastocyst stage, expression is restricted to the ICM (Ovitt and Scholer, 1998). Oct4-

deficient embryos develop to the blastocyst stage but the ICM cells are not pluripotent and are restricted to differentiation along the trophoblast lineage (Nichols et al., 1998). Interestingly, Sox2 acts co-operatively with Oct4 to activate transcription of the gene encoding fibroblast growth factor 4 (FGF4, *Fgf4*) in the ICM and thus contributes to paracrine growth factor signalling in the blastocyst (Nichols et al., 1998). Sox2 is also expressed in all cleavage stage blastomeres and becomes restricted to the ICM at the blastocyst stage (Avilion et al., 2003). Interestingly, Sox2 expression is maintained in the TE and is required for the maintenance of proliferating TE cells, presumably in co-operation with a factor other than Oct4 (Avilion et al., 2003). Oct4 and Sox2 are not the only factors critical for maintaining pluripotency and repressing differentiation. Expression of *Nanog* is first seen in the inner cells of the late morula and is maintained in the ICM at the blastocyst stage (Mitsui et al., 2003). It seems that Nanog function is required for ICM maintenance and repression of PE fate because Nanog-deficient ICM cells differentiate into the extraembryonic endoderm lineage (Mitsui et al., 2003).

Differentiation of the PE and TE depends upon the expression of other sets of transcription factors. Expression of the transcription factor GATA6 is essential for development of the PE from the ICM (Koutsourakis et al., 1999). Interestingly, the *Gata6* gene contains Nanog binding sites in its enhancer region and *GATA6* is upregulated in *Nanog*-deficient cells (Mitsui et al., 2003). Hamazaki et al. have shown that activation of the Grb2/Mek pathway, likely via FGFR signalling, relieves Nanog-mediated repression of *GATA6* expression and thus results in differentiation of PE (Hamazaki et al., 2006). Cdx2 has been found to be essential for TE maintenance because *Cdx2* null mice fail to implant, which is indicative of a major TE defect prior to implantation (Chawengsaksophak et al., 1997). Blastocyst formation initiates in *Cdx2*-null embryos, however Oct4 and Nanog do not become down-regulated in the TE and integrity of the TE is lost (Strumpf et al., 2005). It is clear that lack of Cdx2 does not result in the complete inability to form TE cells in the blastocyst, thus suggesting that there are other factors upstream of *Cdx2*. Recently the transcription factor TEAD4 (TEA domain family transcription factor 4) has been shown to be required for TE formation upstream of *Cdx2*. *Tead4*-null embryos died before implantation, did not form a blastocoel and failed to maintain Cdx2 expression (Nishioka et al., 2008).

Overall, generation of the three cell lineages within the blastocyst seems to depend upon localized expression of both positive and negative factors. Oct4 and Nanog promote pluripotent ICM formation, where Oct4 inhibits TE formation and Nanog

inhibits PE formation. TEAD4 and Cdx2 promote TE formation and inhibit pluripotent ICM formation, while GATA6 promotes PE formation and blocks pluripotent ICM formation.

1.1.4 Epigenetic reprogramming

Different cells and tissues within multicellular organisms acquire different patterns of gene expression during development and epigenetic modifications are thought to be highly important (Morgan et al., 2005). These are covalent modifications of DNA (e.g. DNA methylation) and core histone proteins (e.g. histone acetylation) that regulate gene activity without altering the nucleotide sequence (Godfrey et al., 2007). Therefore, each cell within a multicellular organism has its own epigenetic signature which reflects genotype, developmental history and environmental influences, and ultimately, all of these aspects are reflected in the resulting phenotype (Morgan et al., 2005). DNA methylation is associated with transcriptional silencing (generally, methylated genes are silenced and hypomethylated gene are induced) and heritability of this phenomenon through cell divisions (Bird, 2002). DNA modification takes place post-replication, primarily on cytosine residues (CpG dinucleotides) and often in promoter regions. DNA methyltransferase (Dnmt) enzymes transfer methyl groups on to the C5 position of most of the cytosine residues in the genome (Reik et al., 2001). During normal development, it is a requirement that cells of the embryo undergo major epigenetic 'reprogramming', whereby the original epigenetic marks are removed and different ones are established (Morgan et al., 2005). In, particular, this happens at fertilization when gamete markers are erased and replaced with embryonic markers that are important for early development and pluripotency. The paternal genome that is delivered to the oocyte by the sperm is mostly packaged with protamines rather than histones, which is rapidly reversed upon fertilization. Closely following this histone acquisition is the initiation of paternal genome wide loss of DNA methylation which is complete before DNA replication begins in the paternal pronucleus (Morgan et al., 2005). Exactly, how and why paternal genome demethylation occurs is unknown. However, it is likely that the oocyte cytoplasm contains enzymatic demethylation factors that actively catalyse the process, and it seems that this is important for returning the specialized gamete genomes back to embryonic pluripotency (Fig. 1.2).

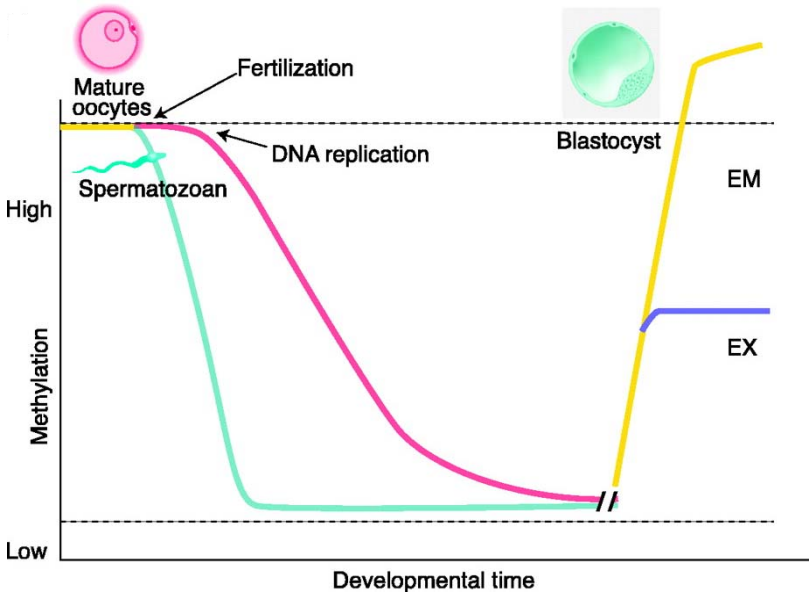


Figure 1.2 Reprogramming of methylation in preimplantation embryos. The paternal genome (green) is demethylated by an active mechanism and the maternal genome (pink) by a passive mechanism. Both are remethylated at implantation to different extents in the embryonic (EM) and the extra-embryonic (EX) lineages (Reik et al., 2001).

Global DNA methylation is lost progressively over the course of the first three cleavage division by a passive mechanism that is likely due to nuclear exclusion of the enzyme (Fig. 1.2), DNA methyltransferase-1 (Dnmt1) (Morgan et al., 2005). At this stage, the only genes that do not become demethylated are the imprinted genes that retain their germ line imprints. It has been postulated that unique histone modifications may guide DNA methylation maintenance of imprinted genes (Morgan et al., 2005). By the blastocyst stage, combinations of active and passive demethylation result in very low levels of methylation on the genome of the TE cells (Fig. 1.2). In contrast, the ICM shows clear signs of extensive *de novo* methylation, which may begin at the late morula stage (Morgan et al., 2005). It is possible that this *de novo* methylation is catalysed by Dnmt3b, as this enzyme is synthesised in cells of the ICM but not the TE. It appears that the epigenetic asymmetry established in the blastocyst is important developmentally, and it is conceivable that a higher degree of epigenetic control is needed in embryonic tissue (i.e. ICM) than in extra-embryonic tissue (i.e. TE). This hypothesis stems from the fact that embryonic tissue derivatives have far greater longevity and undergo far more complex differentiation than extra-embryonic tissues that have limited differentiation potential and life expectancy (Morgan et al., 2005).

1.2 Developmental origins of health and disease

The concept of 'developmental origins of health and disease' (DOHaD) arose from human epidemiological studies relating size at birth to altered risk of adult cardiovascular disease (Barker and Osmond, 1986) and type 2 diabetes (Hales et al., 1991). Many studies have now linked prenatal adversity to the development of a range of adult diseases, which are collectively known as metabolic syndrome: this includes coronary heart disease, stroke, insulin resistance, type 2 diabetes, hyperlipidaemia, hypertension and obesity (Gluckman and Hanson, 2004b). This implies that the risk of developing some chronic noncommunicable diseases in adulthood is influenced not only by an individuals genes and life-style but also by environmental factors acting early in life (Gluckman and Hanson, 2004a). Birth weight can act as an indicator of an adverse prenatal environment and is thus a convenient parameter to use for making associations with postnatal outcomes in the offspring (Gluckman et al., 2007). However, birth weight is an incomprehensive marker because some alterations to the prenatal environment have been shown to result in long term phenotypic changes in the offspring without altering fetal growth; for example, the offspring of women subjected to under-nutrition in early pregnancy during the Netherlands famine did not have reduced birth weight but did have an increased risk of obesity (Painter et al., 2005). It has been proposed that the DOHaD concept is a type of developmental plasticity whereby an organism has the ability to change its structure and function in response to environmental cues (Gluckman et al., 2007). Indeed, developmental plasticity permits the development of a range of phenotypes from a single genotype and is well know to occur in other organisms; for example, in the locust (*Locusta migratoria*), wing shape and metabolism are determined at the larval stage by pheromone signals indicating population density (Applebaum and Heifetz, 1999).

There is now much evidence from experimental animal models to back up the human epidemiological evidence. Such studies have shown that manipulation of the prenatal environment can lead to altered cardiovascular and/or metabolic function in later life (Fowden and Forhead, 2004; Gluckman and Hanson, 2004a). Manipulating maternal nutrition by reducing total nutrient intake in a balanced manner, reducing the protein intake specifically or exposing the mother to a high fat diet all result in similar outcomes e.g. insulin resistance, alterations of the hypothalamic-pituitary-adrenal (HPA) axis, disturbances of endothelial function, hypertension and obesity. For example, in rats, calorie restriction throughout pregnancy lead to hypertension in the

adult offspring (Woodall et al., 1996). Also in a rat model, maternal low protein diet throughout gestation lead to hypertension in offspring at weaning (Langley-Evans et al., 1994). Offspring of rat dams fed a high-fat diet throughout pregnancy displayed hypertension and blunted endothelium-dependent vascular relaxation to acetylcholine (Khan et al., 2003). Administration of glucocorticoids to the mother is also known to produce similar effects; for example, dexamethasone administered to rat dams in the last week of pregnancy resulted in adult offspring that displayed hyperglycaemia and hyperinsulinaemia (Nyirenda et al., 1998).

Two conceptual models have been proposed to explain the DOHaD hypothesis. The 'thrifty genotype' model argued that 'thrifty' genes had been evolutionarily selected for by virtue of the fact that these genes enabled us to cope more successfully with the high energy expenditure and uncertain nutritional intake of our hunter-gather past (NEEL, 1962). However, this explanation was deemed unsatisfactory as it failed too explain either the experimental animal data or patterns of disease seen in some populations (Gluckman and Hanson, 2004a). As an alternative, the 'thrifty phenotype' model was proposed: it stated that the fetus becomes growth retarded in response to adverse conditions *in utero*, and the associated adaptations result in a phenotype better suited to a food deprived postnatal environment (Hales and Barker, 2001). This model also had its limitations as it could readily explain the consequences in individuals born small, but could not explain how programming could continue to operate in individuals born in the upper birth size range, i.e. a well nourished fetus.

The 'predictive adaptive response' (PAR) model has evolved from the 'thrifty phenotype' model in order to avoid the limitations associated with the latter. The PAR model suggests that the adaptive responses made by the fetus need not necessarily be for immediate advantage but may be in expectation of the future post-natal environment (Gluckman and Hanson, 2004b). An important suggestion that is implied by the PAR model is that disease risk is a consequence of the extent of match or mismatch between the environments that are experienced pre-and postnatally; greater risk of disease is associated with greater extent of mismatch (Fig. 1.3).

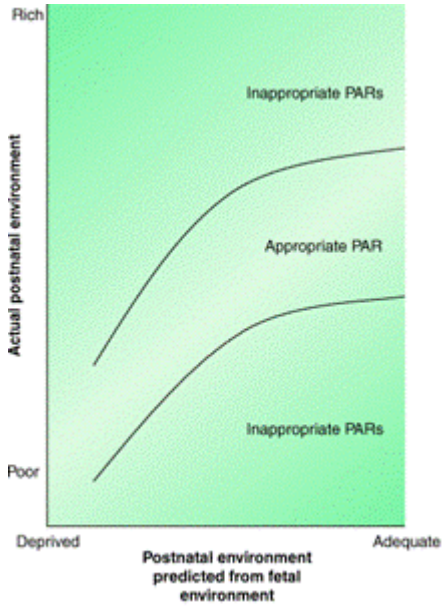


Figure 1.3 The nature of the predictive adaptive response (PAR) is determined by the predicted and actual postnatal environment. The fetus sets a range of homeostatic settings appropriate for postnatal life according to the information it receives in utero. If the actual postnatal environment matches the prenatal prediction, then the PARs are appropriate and disease risk is low; if they do not match, then the disease risk is increased (Gluckman and Hanson, 2004b).

1.3 The effects of altered preimplantation embryo environment

1.3.1 Embryo culture

A number of studies show that pre-implantation embryos are sensitive to their environmental conditions, and that these conditions may influence their developmental programme, not only in the short term, but also in the long term. Initially it was observed that *in vitro* cultured mouse embryos, after transfer into pseudo-pregnant mothers, resulted in reduced fetal growth when compared with their *in vivo* counterparts (Bowman and McLaren, 1970). Further studies of this sort in mice have confirmed these initial findings and have identified culture systems that may act deleteriously on embryo development. It seems that the addition of serum to the culture medium is detrimental to development. Addition of serum to the culture medium resulted in fetuses at gestational day 14 that were 20% smaller than those derived from embryos cultured in the absence of serum or developing *in vivo* (Khosla et al., 2001). Conversely, it has been found that the addition of insulin, albumin or specific amino acids to the culture medium leads to improved rates of implantation and development later in gestation (Kaye and Gardner, 1999; Lane and Gardner, 1997).

Early embryos from cattle and sheep also display sensitivity to their environments. Ovine and bovine embryos that have been cultured *in vitro*, after transfer, often produce young that suffer from a condition known as large offspring syndrome (LOS). LOS is characterized by aberrant fetal and placental development, increased birth weight, myogenesis, dystocia (difficulty in giving birth), dysfunctional perinatal pulmonary activity, organomegally (particularly of the liver and heart) and increased mortality in early post-natal life (Young et al., 1998). The animals that survive have been shown to retain the features of abnormal organ size during later life. The occurrence of LOS is also particularly associated with the addition of sera to the culture medium (Sinclair et al., 1999). Furthermore, adult mice derived from *in vitro* cultured embryos were shown to exhibit behavioural alterations that relate to anxiety and locomotor activity (Ecker et al., 2004; Fernandez-Gonzalez et al., 2004). Watkins et al. recently showed in the mouse that *in vito* culture resulted in increased systolic blood pressure, serum angiotensin-converting enzyme (ACE; catalyses the conversion of Angiotensin I to Angiotensin II; Ang. II is a potent vasoconstrictor and thus acts to increase blood pressure) and hepatic phosphoenolpyruvate carboxykinase (PEPCK; rate limiting enzyme in gluconeogenesis; an increase of which could thus potentially lead to increased blood glucose levels) in adult offspring (Watkins et al., 2007).

1.3.2 Maternal diet

The environmental sensitivity of the embryo can also be demonstrated *in vivo*. The effect of maternal diet is a particularly well studied aspect of *in vivo* model systems and has been shown to impact on pre-implantation phenotype and long term development. In a rat model, maternal low protein diet fed exclusively during the pre-implantation period (control diet was fed for the remainder of gestation and postnatally) resulted in offspring with low birth weight, over-compensatory adolescent growth, onset of adult hypertension and alterations of relative organ size in a gender specific manner. In addition, in the blastocyst, this relatively modest reduction of protein in the maternal diet lead to a reduction of ICM cells in the early blastocyst and a reduction in both TE and ICM cell numbers in the late blastocyst (Kwong et al., 2000). Furthermore, the same maternal treatment was shown to cause an increase in the expression of 11β-hydroxysteroid dehydrodgenase type 1 (*Hsd11b1*; activates glucocorticoids in fetal liver) and PEPCK in fetal liver (Kwong et al., 2007). Taken together, all of these lines of evidence show that the preimplantation embryo is highly sensitive to its environment and that behavioural, cardiovascular and metabolic aspects of postnatal physiology are vulnerable.

1.4 Maternal immune response and its effects on the offspring

Relatively few studies have investigated the effects of maternal infection and inflammation during gestation on the offspring postnatally. There are even fewer studies that have investigated offspring phenotype following maternal immune activation during early gestation. Our knowledge of the effects of maternal infection and inflammation has come from epidemiological studies of human populations and experimental animal models. Furthermore, such studies have tended to focus on individual aspects of offspring physiology or phenotype; e.g. only behaviour or only certain aspects of the immune system. Intriguingly, maternal infection and inflammation during gestation has been shown to lead to alterations in various aspects of postnatal offspring phenotype, including; behaviour (Limosin et al., 2003; Shi et al., 2003), immunity (Reiterova et al., 2006; Yamaguchi et al., 1983), metabolic status (Nilsson et al., 2001) and hypothalamic-pituitary-adrenocortical (HPA) axis functioning (Reul et al., 1994). Thus, it appears that a wide variety of body systems in the offspring are affected by maternal immune activation during gestation.

Key evidence linking maternal infection during gestation to behavioural abnormalities in the offspring has come from human epidemiological research. Such studies found an association between maternal influenza infection during mid-gestation and the development of schizophrenia in genetically susceptible offspring (Brown, 2006; Limosin et al., 2003). Epidemiological evidence also suggests that maternal infection with other pathogens, such as rubella, *Toxoplasma gondii* and Herpes simplex virus type 2, increases the risk of schizophrenia in the offspring (Brown, 2006). This indicated that the maternal immune response, rather than the nature of the pathogen *per se* that was the important risk factor. As such, this raised the possibility that an experimental animal model could be developed. To model maternal infection during mid-late gestation, LPS or the viral mimic PolyI:C (polyriboinosinic-polyribocytidilic acid, a synthetic double-stranded RNA analogue) has been administered to pregnant rodents during mid-late gestation. Indeed, in these animal models, activation of the maternal immune system lead to behavioural, morphological and pharmacological alterations in the offspring relevant to schizophrenia (Fortier et al., 2007; Zuckerman et al., 2003). Furthermore, Smith et al. showed that a single maternal injection of IL-6 on GD 12.5 lead to the same offspring behaviour abnormalities as administration of PolyI:C (Smith et al., 2007). From these animal models it would thus appear that cytokine exposure, resulting from either infection or administration of individual

cytokines, can precipitate schizophrenic-type behavioural abnormalities in the offspring.

Interestingly, it seems that the precise timing of maternal immune activation can result in subtle differences in the behavioural phenotype that is induced in the offspring. Meyer et al. administered PolyI:C to pregnant mice on GD 9 and GD 17 (corresponding to mid- and late-gestation, respectively) and found that some behavioural abnormalities were present in offspring from both treatment times but that other behavioural abnormalities were present in GD 9 offspring but not in GD 17 offspring and vice versa (Meyer et al., 2008; Meyer et al., 2006). No experimental models have investigated schizophrenic behaviour in offspring following very early gestational maternal immune challenge; however, some human epidemiological evidence has shown that this stage may also be at risk. One birth cohort study reported that maternal genital and reproductive infections during periconception increased the risk of schizophrenia in the offspring (Babulas et al., 2006). Another human birth cohort study indicated that the risk of schizophrenia in offspring was increased 7-fold after serologically documented influenza exposure during the first trimester of pregnancy (Brown et al., 2004).

Schizophrenia is most well known for its neuropsychiatric aspect; however, this disease is also associated with an abnormally high prevalence of metabolic syndrome and immune dysfunction (Schorr et al., 2009; Strous and Shoenfeld, 2006). For example, drug-naïve patients with schizophrenia have been shown to have increased intra-abdominal adiposity, impaired glucose tolerance and altered levels of IL-2, IL-6, IL-10 and TNF-α (Spelman et al., 2007; Strous and Shoenfeld, 2006; Thakore et al., 2002). To my knowledge none of the maternal immune activation animal models of schizophrenia have investigated the presence of metabolic or immune abnormalities in the offspring. However, in another study (that did not investigate behavioural parameters) metabolic-type abnormalities were found in rat offspring following maternal LPS administration during mid-gestation. Nilsson et al. challenged rats with 3 repeated doses of LPS (790 µg/kg) during mid-gestation and examined aspects of metabolic functioning in the offspring (Nilsson et al., 2001). Adult male offspring displayed obesity and features of the metabolic syndrome, specifically; increased body weight, increased abdominal fat-pad weights, elevated food intake, increased circulating leptin, insulin resistance and increased serum levels of 17β-estradiol and progesterone. In contrast, adult female offspring in the same study appeared to be

less affected, exhibiting elevated serum testosterone levels, increased basal corticosterone levels and increased weight of the heart and adrenal glands. Furthermore, in another study, pregnant rats were injected with IL-6, TNF-α or dexamethasone (synthetic glucocorticoid) during mid-gestation and the offspring were examined postnatally for a range of physiological and endocrine parameters (Dahlgren et al., 2001). The authors reported an overlapping but non-identical phenotype in the offspring following the different maternal treatments, including; increased body weight in all offspring that was due to increased adiposity, corticosterone response to stress was increased in the IL-6 group, insulin resistance was seen in IL-6 group males, males from the TNF-α group showed decreased locomotor activity whereas the IL-6 females showed increased locomotor activity and plasma testosterone levels were increased in females from the IL-6 and TNF-α groups (Dahlgren et al., 2001). This study indicates that aspects of offspring metabolic and behaviour can be altered by administering individual components of a typical immune response. In another study, pregnant rats received a single challenge with either LPS (at a much lower dose than the Nilsson study; 30 µg/kg) or human red blood cells (HRBC; T cell-dependent foreign antigen) during mid-gestation, and postnatal HPA axis functioning was examined only in the male offspring (Reul et al., 1994). This study reported that adult male offspring from both LPS and HRBC groups displayed increased basal plasma corticosterone levels, whereas after novelty-induced stress, only offspring from the HRBC group showed increased ACTH and corticosterone levels. In addition, decreased levels of mineralocorticoid and glucocorticoid receptors were detected in the hippocampus. These studies thus show that offspring metabolic status can be altered by maternal immune challenge during mid-gestation. To my knowledge no studies have been conducted to investigate the effect of maternal immune activation during early gestation on offspring metabolic status and body composition.

Studies investigating the effect of maternal immune challenge on offspring immune status have tended to focus on the humoral immune system and have reported contradictory results. Immunisation of female mice with sheep red blood cells (SRBCs; T cell-dependent foreign antigen) at any time point from 2 days before fertilisation to GD 16 induced suppression of anti-SRBC (IgM and IgG) plaque-forming cell (PFC) responses in their offspring, which suggested reduced ability to produce antigen-specific antibody (Yamaguchi et al., 1983). However, it should be noted, that this suppressive effect on anti-SRBC PFC response persisted only until ~15 weeks after birth. Interestingly, this group went on to show by adoptive transfer of immune cells,

that it was specifically the maternal $CD4^+$ T cells of SRBC-immunised mice that induced suppression in their offspring (Fujii and Yamaguchi, 1992). Further study by the same group showed that the suppression in the offspring was restricted to the MHC type that was activated during pregnancy and that the suppression in the offspring was a result of the generation of $CD4^+$ suppressor T cells by the offspring (Fujii et al., 1993). Overall, the findings from this set of experiments is much alike the recent findings of Mold et al. (Mold et al., 2008). This study reports that large numbers of maternal cells cross the placenta to reside in fetal lymph nodes (rich in TGF-β which can induce T_{reg} differentiation), inducing the development of $CD4^+CD25^{high}FOXP3^+$ T_{regs} that suppress antimaternal fetal immunity and that persist into early adulthood (Mold et al., 2008). A similar process could thus be the cause of the (transient) anti-SRBC suppression seen in the mouse model above.

In contrast, infection of female mice with the parasite *Toxocara canis* during mid-late gestation (GD 12-18) seemed to be protective for the offspring if they were infected postnatally (Reiterova et al., 2006). When offspring were postnatally infected (6 weeks of age) with the same parasite that their mothers had received (*T. canis*), offspring displayed reduced production of IFN-γ and IL-5, suppressed eosinophilia, increased levels of IgM and IgG1 and reduced larval recovery from the brain and muscles (Reiterova et al., 2006). This finding implied that, in response to postnatal infection, increased levels of protective antibodies were produced in offspring from infected dams, which thus reduced the extent of infection without the requirement of a vigorous immune response, i.e. IFN-γ and IL-5 production and eosinophilia. One important difference between this study and the study that found anti-SRBC suppression in the offspring (discussed above), is that offspring would have received maternally produced anti-*T. canis* IgG antibodies via the placenta and milk. Increased levels of maternally derived antibodies would have been transferred to the offspring of infected dams and thus provided passive protection to the offspring upon reinfection. However, the offspring in this study also displayed increased production of antibody upon reinfection, thus showing that offspring from infected dams had secondary-type antibody response to a primary challenge. This type of response has been found before: Enhanced production of DNA-specific antibodies was found in offspring from dams that had received anti-DNA antibodies two weeks prior to mating (Sasaki et al., 1977). It is thought that during the neonatal period, maternal antibodies induce T-cell dependent idiotypic responses that prime the offspring immune system (Lemke et al., 2009).

1.5 Lipopolysaccharide and Toll-like receptor signalling pathways

Pathogen-associated molecular patterns (PAMPs) are essential components of most micro-organisms; of which lipopolysaccharide (LPS; also known as endotoxin) is an example (Akira et al., 2006). LPS is an abundant and integral constituent of the outer membrane of Gram-negative bacteria (Fig. 1.4a). It is released from the membrane when bacteria divide, die and lyse, and is therefore liberated into host tissues during a Gram-negative bacterial infection (Rietschel et al., 1994). It is widely acknowledged that LPS is of prime importance in host recognition of infection and that it is a highly potent activator of the immune response, even in the absence of other bacterial components (Akira et al., 2006; Van Amersfoort et al., 2003). Intravenous (i.v.) or intraperitoneal (i.p.) injection of LPS triggers an acute systemic inflammatory response that fully mimics the initial phase of a genuine systemic Gram-negative bacterial infection (Beutler, 2004; Dantzer, 2004). Indeed, LPS has been used for decades to model systemic inflammation and sepsis (Beutler, 2004).

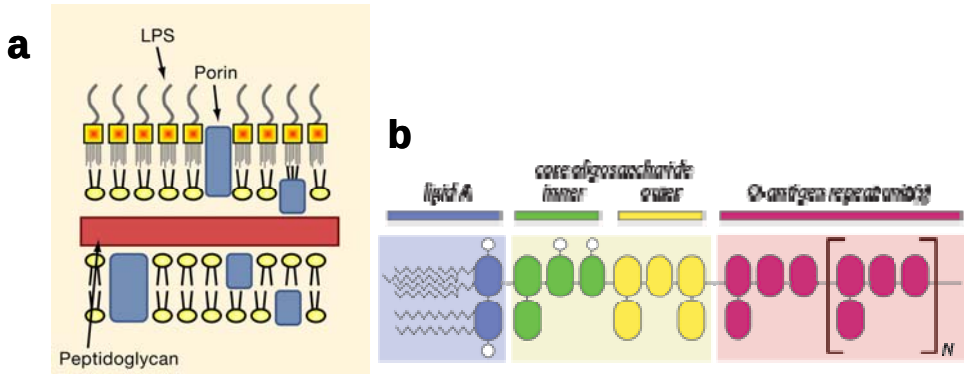


Figure 1.4 (**a**) Schematic representation of a Gram-negative bacterial cell wall showing the position of LPS in the outer membrane; (**b**) general structure of an LPS molecule (Akira et al., 2006).

LPS molecules from all Gram-negative bacteria have the same general structure; that is a polysaccharide attached to a lipid component called lipid A (Fig. 1.4b). The polysaccharide component is made up of a core and a polymer of oligosaccharide molecules (O-antigen). This polysaccharide component is highly variable and specific to each bacterial strain and is bound to lipid A through a core sugar moiety called 2-keto-3-deoxyoctulosonic acid (KDO). Lipid A consists of a phosphorylated glucosamine disaccharide to which long fatty acids are attached (Rietschel et al., 1994). Furthermore, Lipid A is thought to be the component of LPS that is responsible for inducing cytokine expression (Rietschel et al., 1994). Several experiments have demonstrated much variance in the ability of various LPS species to induce the

synthesis of cytokines. For example, LPS molecules from *E.coli* and *Salmonella typhimurium* differentially activate cytokine production in mice deficient in TNF-α and lymphotoxin-α. In these mice, *E.coli* induces the expression of very little IL-1α, IL-1β and IFN-γ, whereas *S. typhimurium* LPS induces the release of high concentrations of these cytokines (Netea et al., 2001). Variation in the three-dimensional structure of lipid A is also observed between different bacterial strains and has been linked to its potency. Lipid A species that have a conical shape (e.g. *E.coli*) have high levels of activity whereas those with a cylindrical shape (*Rhodobacter capsulatum*) are weaker (Schromm et al., 2000).

The innate immune system is able to discriminate between self and invading micro-organisms by expressing receptors for a number of different PAMPs, e.g. LPS, double stranded RNA, peptidoglycans (Vivier and Malissen, 2005). The PAMP-recognising receptors of the innate immune system are known as Toll-like receptors (TLRs); LPS is recognised by TLR4. TLRs are expressed on various immune cells, including antigen presenting cells (APCs; i.e. macrophages and dendritic cells, DCs), neutrophils, B cells, some types of T cells, as well as on non-immune cells such as fibroblasts and epithelial cells (Akira et al., 2006; Poltorak et al., 1998). Recognition of LPS by TLR 4 alone is insufficient for signalling: LPS binding protein (LBP; an acute phase protein present in the blood stream that is produced by the liver), CD14 (a proteinacious co-receptor that is either soluble, or glycosylphosphatidylinositol (GPI)-linked at the cell surface) and myeloid differentiation protein-2 (MD-2; a small protein associated with the extracellular part of TLR4 that is important for LPS recognition and cellular distribution of TLR4) are the three accessory proteins that are also required, which, together with TLR 4, comprise the LPS receptor complex (Fujihara et al., 2003). To begin with LPS must associate with LBP, this complexed LPS then binds to soluble or GPI linked CD14. Next, CD14 facilitates the transfer of LPS to the extracellular portion of the TLR 4/MD-2 receptor complex (Fig. 1.5), which in turn leads to TLR dimerisation and activation of intracellular signalling pathways (Fujihara et al., 2003; Lu et al., 2008b).

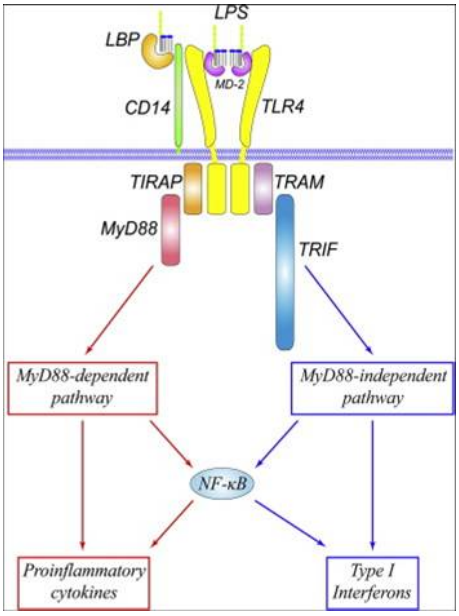


Figure 1.5 Overview of LPS/TLR4 receptor complex and intra cellular signalling pathways (Lu et al., 2008b).

Upon dimerisation, the intracellular TIR (Toll-interleukin-1 receptor) domain of TLR4 recruits downstream signal transduction adaptor proteins to the complex; these are, MyD88 (myeloid differentiation primary response gene 88), TIRAP (TIR domain-containing adaptor protein), TRIF (TIR domain-containing adaptor inducing IFN-β) and TRAM (TRIF-related adaptor molecule) (Lu et al., 2008b). TLR4 signalling has been broadly divided into two pathways; MyD88-dependent and MyD88-independent (Fig. 1.5). Proinflammatory cytokines (e.g. IL-1, TNF-α, IL-6) are produced via the MyD88-dependent pathway, where TIRAP facilitates the association between MyD88 and TLR4, then MyD88 activates IRAK (IL-1 receptor associated kinase) 4 and 1 which ultimately results in activation of the transcription factors NF-κB (nuclear factor κB) and AP-1 (activator protein 1), via the IKK (IkB kinase) and MAPK (mitogen-activated protein kinase) pathways, respectively. Type I interferons (e.g. IFN-α and IFN-β) are produced via the MyD88-independent pathway, where TRIF activates IKK and MAPK pathways via RIP1 (receptor-interacting protein 1) (Lu et al., 2008b).

1.6 The acute phase response

1.6.1 General aspects and initiation of an acute phase response

The purpose of the acute phase response (APR) is to counteract the immunological stress and restore homeostasis as quickly as possible. This is accomplished in two fundamental ways; firstly, by destruction and removal of the causative organisms

and/or harmful molecules; and secondly, by activating host repair processes (Ceciliani et al., 2002). The APR involves a myriad of changes both local to and distant from the affected site, including; immune, neuroendocrine, CNS, metabolic, hepatic and hematopoietic alterations (Table 1.1).

Affected System	**Nature of Change**
Immune system	• Activation of peripheral innate immune system (e.g. tissue macrophage). • Release of pro-inflammatory molecules (e.g. IL-1, TNF-α, IL-6, IL-8, PGs, histamine). • Recruitment and activation of leukocytes from the blood (e.g. neutrophils). • Increased dilation and permeability of capillary beds, leading to extravasion of fluid and plasma proteins into the tissue (oedema).
Neuroendocrine/HPA system	• Increased CRH release from the hypothalamus. • Increased ACTH release from the anterior pituitary. • Increased glucocorticoid (e.g. cortisol) release from the adrenals.
CNS	• Induction of fever. • Sickness behaviours; e.g. anorexia, adipsia, lethargy, somnolence, anhedonia and decreased social interaction. • Hyperalgesia and allodynia.
Metabolic	• Increased protein catabolism (e.g. breakdown of muscle tissue). • Increased gluconeogenesis (i.e. glucose production from certain amino acids or lactic acid). • Increased adipose tissue lipolysis. • Decreased levels of plasma zinc and iron.
Hepatic	• Increased synthesis of hepatic proteins (the acute phase proteins). • Increased hepatic lipogenesis.
Hematopoietic system	• Increased leukocytosis (white blood cell count). • Increased thrombocytosis (platelet count). • Increased secretion of colony-stimulating factors.

Table 1.1 Summary of the major changes that happen during an acute phase reaction (Ceciliani et al., 2002; Gruys et al., 2005; Konsman et al., 2002).

Activation of the peripheral innate immune system is the first step in the APR. Antigen presenting cells (APCs; i.e. blood macrophages and dendritic cells, DCs) are particularly important because they release a wide range of inflammatory mediators that induce most features of the APR; these include: TNF-α, IL-1β, IL-6, IL-10, IL-12, MIP-1 α/β, MCP-1, RANTES, G-CSF, GM-CSF, platelet activating factor (PAF), prostaglandins, reactive oxygen species (ROS), nitric oxide (NO) and proteases

(Fujihara et al., 2003). Pro-inflammatory cytokines (e.g. TNF-α, IL-1β) act to recruit and activate additional immune help from the circulation. For example, IL-1 and TNF-α act on vascular endothelial cells to induce expression of adhesion molecules that slow down circulating leukocytes and make them adhere to the vascular wall, and chemokines (e.g. IL-8 and MCP-1) chemoattract leukocytes into the affected area (Ceciliani et al., 2002). Once in the affected tissue, leukocytes (e.g. neutrophils) phagocytose micro-organisms and debris, and release toxic free radical metabolites and proteolytic enzymes. Vasoactive molecules such as prostaglandins (PGs), histamine and bradykinin are also released at the site of inflammation. These molecules act in local capillary beds to increase vessel permeability and dilation which results in increased blood flow and, in turn, extravasion of fluid and plasma proteins into the tissue. In addition to their effects on the local vasculature, PGs, histamine and bradykinin also mediate inflammatory pain. In addition, IL-6 is the major mediator for stimulating hepatic secretion of the acute phase proteins (APPs) and TNF-α is important in the catabolism of muscle protein and adipose tissue (Ceciliani et al., 2002; Gruys et al., 2005).

1.6.2 Fever and sickness behaviour

The CNS-related components of the APR, i.e. fever and sickness behaviour, are easily recognised indicators of illness that highlight the abundant cross-talk that must occur between the CNS and peripheral immune system. It is believed that communication between the CNS and peripheral immune system is mediated in two ways: (1) a neural route via afferent neurones that innervate the body site where the infection takes place; and (2) a humoral pathway that involves cytokines and PGs (Konsman et al., 2002). Fever and sickness behaviour are frequently observed after a very short latency following immune stimulation; i.e. more quickly than cytokines could be transcribed, translated and secreted, and furthermore these symptoms frequently abate before the disappearance of blood-borne cytokines (Blatteis et al., 2005; Campisi et al., 2003). For instance, the first cytokines do not appear in the blood following intravenous (i.v.) LPS until at least 30 minutes after its injection, whereas fever is apparent within 10-12 minutes (Blatteis et al., 2005). It seems very likely that the maintenance/latter phases of fever and sickness behaviour are mediated by PGs and cytokines that have been formed in the mean time. Receptors for the pro-inflammatory cytokines are expressed in many regions of the brain: intra-cerebroventricular (icv) administration of IL-1 and IL-6 can induce typical sickness

responses and furthermore, these cytokines are found in the brain after peripheral administration of IL-1 or LPS (Blatteis et al., 2005; Hopkins, 2007; Konsman et al., 2002). Direct transfer of peripherally produced cytokines to the CNS is highly implausible however, because cytokines are relatively large lipophobic molecules that cannot passively cross the blood-brain barrier (Blatteis et al., 2005; Campisi et al., 2003). It is becoming increasingly apparent that induction of other signalling molecules, PGE_2 in particular, is key to the CNS effects (Konsman et al., 2002). It has long been known that administration of cyclo-oxygenase (COX) inhibitors attenuates the febrile and behavioural aspects of sickness (Blatteis et al., 2005). Importantly, Teeling et al. have recently shown that blockade of PG production with the COX inhibitor indomethacin, completely reversed LPS-induced changes in mouse burrowing behaviour without affecting peripheral or CNS cytokine levels (Teeling et al., 2007).

In 1988 Hart put forward the view that sickness behaviour and fever represent a highly organised strategy to fight infection (Hart, 1988). Fever is a transient physiological adjustment to a new homeostatic equilibrium that is characterised by a raised set-point of body temperature (Dantzer, 2004). As such, an individual experiencing fever will feel cold even in thermoneutral environments. Fever is an important aspect of the immune response because the increased body temperature that is achieved acts to stimulate immune cell proliferation and is unfavourable for the growth of many bacterial and viral pathogens. Indeed, hosts that are unable to mount an appropriate fever response have a lower survival rate than those hosts which develop a normal fever (Dantzer, 2004). Metabolic rate is increased quite substantially during fever; therefore, the energy cost of the febrile response is quite high. Increased catabolism of muscle protein and adipose tissue, and gluconeogenesis act to increase glucose availability for these costly energetic processes. Since the metabolic cost of fever is so high, the host must conserve heat and energy. Any unnecessary activity is reduced or stopped completely, hence the lethargy, somnolence, curled-up posture and reduced social interaction that is characteristic of sickness behaviour. The hyperalgesia (extreme sensitivity to pain) and allodynia (exaggerated response to non-painful stimuli, i.e. non-painful stimuli feel painful) that is associated with sickness not only directs recuperative behaviours (e.g. protection of the painful bodily site) to the affected part of the body, but additionally it encourages immobility, and in this way also serves to save energy (Dantzer, 2004). The anorexic component of sickness behaviour seems counter productive in light of the increased energy requirements highlighted above. However, all things considered, finding food and digesting it

require energy expenditure, and doing so would increase the risk of exposure to predation and possible ingestion of further pathogenic organisms. Communication at this level allows reorganisation of host perception and actions, in such a way that is most advantageous to fight infection and recover in optimal time (Dantzer, 2004).

1.6.3 Hypothalamus-piuitay-adrendal (HPA) axis activation

In response to a variety of stressors the hypothalamus releases corticotrophin releasing hormone (CRH) into the hypophyseal portal system, which causes the anterior pituitary gland to secrete adrenocorticotrophic hormone (ACTH) into the general circulation. ACTH then stimulates the adrenal gland to secrete the glucocorticoids (GCs), cortisol, corticosterone and cortisone (mice and rats secrete corticosterone almost exclusively). The HPA axis can be activated by neural afferent stimulation as well as humoral mediators. Neural afferent signals from both the periphery (e.g. pain via peripheral nociceptive pathways) and from higher centres in the CNS (e.g. emotion via the limbic system) can rapidly activate the HPA axis (Hopkins, 2007). Many studies have highlighted hypothalamic IL-1, IL-6 and PGE_2 as potent activators of the HPA axis and much data indicates that humoral IL-6 is an important circulating mediator (Hopkins, 2007; Lenczowski et al., 1997; Sapolsky et al., 2000). The GCs have a range of actions, but are particularly important for altering metabolic and immune functioning. They increase the rate of protein and adipose tissue catabolism, and cause increased plasma glucose concentrations, partly by stimulating hepatic gluconeogenesis. The effects of GCs on the immune system are overwhelmingly immunosuppressive and anti-inflammatory; however, they do have some important permissive actions. Generally, GCs act to inhibit synthesis, release and/or efficacy of a variety of inflammatory mediators. Such inflammatory mediators include; cytokines, chemokines, histamine, PGs, nitric oxide and protease enzymes (Sapolsky et al., 2000). Furthermore, GCs reduce the number of leukocytes at inflammatory foci and in the circulation, and in addition have anti-proliferative and apoptotic actions in these cells. GCs have permissive actions by enhancing the effect of other molecules; for example, they enhance liver APP production by increasing hepatic sensitivity to inflammatory mediators such as IL-6; and they up-regulate the tissue expression of receptors for hormones, cytokines and growth factors (Sapolsky et al., 2000).

1.6.4 Other aspects of the acute phase response

IL-6 is thought to be the major mediator for stimulating hepatic secretion of the acute phase proteins (APPs). The pattern of protein synthesis by the liver is much altered within 4-8 hours of challenge; some proteins are up regulated (positive APP) and some proteins are down regulated (negative APP) (Ceciliani et al., 2002; Gruys et al., 2005). The so called negative APPs tend to be the physiologically expressed proteins that are usually present in the blood (e.g. albumin), whereas the positive APPs have a number of roles that range from initiation and regulation, to resolution of the inflammation. Positive APPs include those from the complement, coagulation and fibrinolytic systems, as well as scavenger proteins, inflammatory response participants and protease inhibitors (Ceciliani et al., 2002). Complement pathways are involved in chemotaxis and opsonisation of pathogenic material; coagulation and fibrinolytic systems help to restore tissue integrity following damage; the scavenger molecule LPS binding protein (LBP) interacts with LPS and transfers it to its cell surface receptors, CD14/TLR-4; protease inhibitors help to prevent excessive tissue damage from pathogen and immune system derived proteases (Ceciliani et al., 2002).

1.7 Rationale

There is now ample evidence, from both *in vitro* and *in vivo* studies, showing that the preimplantation embryo is sensitive to its immediate environment (Fernandez-Gonzalez et al., 2004; Watkins et al., 2008b). Perturbations during preimplantation development have been shown to alter not only the immediate events of blastocyst formation, but also cardiovascular, metabolic and behavioural aspects of offspring phenotype (Fernandez-Gonzalez et al., 2004; Kwong et al., 2000; Watkins et al., 2007; Watkins et al., 2008b). A limited amount of evidence suggests that behaviour and immune function may be altered in offspring from mothers that experienced infection or immune challenge during early gestation. Human epidemiological evidence has indicated that maternal infection during the first trimester increased the risk of offspring developing schizophrenia (Babulas et al., 2006; Brown et al., 2004). In addition, immunisation of female mice with sheep red blood cells (a T cell-dependent antigen) during periconception and early gestation has been shown to reduce the ability of offspring to produce antibody until approximately 15 weeks of age postnatally (Yamaguchi et al., 1983). However, detailed experimental models investigating how maternal inflammation activation during preimplantation gestation affects various aspects of prenatal and postnatal offspring phenotype are severely lacking. Thus, the

aim of my PhD is to investigate the effects of an inflammatory environment during preimplantation development on the phenotype of the blastocyst, fetus and offspring postnatally, with a major focus on an *in vivo* mouse model of maternal systemic infection.

The hypothesis of this thesis is that, in the mouse, maternal systemic inflammation during preimplantation gestation will alter the immediate events of blastocyst formation and will perturb diverse aspects of postnatal offspring phenotype, including; growth, behaviour, systolic blood pressure, body structure and immune status. Thus, the aims of this study are to develop an *in vivo* model of maternal systemic inflammation during preimplantation gestation in the mouse, and examine the resultant phenotype in the blastocyst, fetus and offspring postnatally. First, the maternal immune response to administration of lipopolysaccharide will be confirmed by assessing maternal sickness behaviour, weight loss and serum cytokine profile. Next the immediate effects of maternal LPS-injection will be assessed in the gestational day 3.5 blastocyst by counting the number of cells in the trohectoderm and inner cell mass. The effects on the gestational day 17 fetus will be examined by dissection of the conceptus and its constituent parts. The final part of the study will concentrate on the offspring postnatally. Offspring will be assessed for growth by weekly weight until adulthood. In addition, offspring will undergo a battery of behavioural tests and systolic blood pressure measurements. Prior to cull, a subset of offspring will receive their own direct immune challenge (in the form of an LPS injection) or saline injection. In order to assess immune status of the injected offspring, serum cytokine profiles will be measured using multiplex technology and spleen lymphocyte populations will be examined by flow cytometry. All offspring, regardless of postnatal treatment, will be fully dissected to investigate body structure and the mass of all major organs and fat-pads will be recorded. All postnatal data will be analysed using a multilevel random effects regression model that takes into consideration the hierarchical nature of the dataset (Kwong et al., 2004; Watkins et al., 2008b).

2 Materials and Methods

2.1 Superovulation and *in vitro* procedures

2.1.1 Mice

The MF1 strain mice used throughout this study were bred and housed in the Biomedical Research Facility at the University of Southampton. Prior to experimentation female mice were housed in groups and male studs were housed individually. All animals were maintained under controlled 12 hour light/dark cycles (lights on 0700 hours, lights off 1900 hours), at a temperature of 18-22ºC and were allowed *ad libitum* access to standard rodent chow and tap water.

2.1.2 Superovulation and mating

5-6 week old virgin female MF1 mice were superovulated by intraperitoneal (i.p.) injection of 5 IU pregnant mare serum gonadotropin (PMSG; Intervet; Appendix I) at 1400 hours, then 46-48 hours later with 5 IU human chorionic gonadotropin (hCG; Intervet; Appendix I) and caged individually overnight with MF1 stud males (6 weeks to 6 months of age). The presence of a vaginal plug the following morning was taken as a sign of mating and this was then regarded as gestational day 0.5 (GD 0.5). Plug-positive females were removed from the stud cages and housed in groups until required.

2.1.3 Embryo collection

At GD 1.5 (48 hours post-hCG, two-cell embryo stage) female mice were killed by cervical dislocation (in accordance with Schedule 1 of the 1986 Animal Procedures Act), then their oviducts were dissected out and placed into normal saline solution (0.85% NaCl), pre-warmed to 37ºC. Each oviduct was flushed by inserting a blunt-ended needle (Coppers Needle Work, Birmingham, UK; hypodermic stainless steel needle, 30-gauge, 10mm) a short distance through the aperture of the infundibulum and flushing through pre-warmed (37ºC) H6 medium supplemented with 4 mg/ml bovine serum albumin (H6-BSA; Appendix I). 2-cell embryos with good morphology were selected and washed three times in 20 µl drops of equilibrated (37ºC, 5% CO_2 in air for at least one hour) KSOM medium (Appendix I) covered in mineral oil (Sigma, M8410, embryo culture tested), in culture dishes. The washed embryos were pooled and maintained in equilibrated KSOM at 37ºC, 5% CO_2 in air until required.

2.1.4 Embryo culture with IFN-γ

Lyophilised recombinant mouse IFN-γ ($8.43x10^3$ IU/µg; R&D Systems, 485MI) was reconstituted with PBS (supplemented with 0.1% BSA) to obtain a stock solution of 100 µg/ml. Aliquots of the IFN-γ stock solution were stored at -20ºC until required. For each experiment, IFN-γ stock solution was defrosted and diluted 1:100 with PBS (supplemented with 0.1% BSA), to obtain a 1 µg/ml solution. This solution was then diluted 1:100 with KSOM to obtain a 10 ng/ml solution. Then, serial dilutions of 1:10 (in KSOM) were performed in order to obtain the four experimental IFN-γ concentrations: 1000, 100, 10 and 1 pg/ml. KSOM alone was used as the control. Six to ten, 20 µl drops of control or IFN-γ-containing medium were pipetted onto culture dishes, covered with mineral oil and allowed to equilibrate at 37ºC, 5% CO_2 in air for at least one hour. Pooled embryos were randomly assigned to each of the five groups (control, 1, 10, 100 or 1000 pg/ml IFN-γ) and, in order to minimise dilution of the IFN-γ in the final culture drops, were briefly placed into a 'transfer drop' that was at the same concentration of IFN-γ. For example, embryos that were to be cultured in 1000 pg/ml IFN-γ were placed into a 'transfer drop' at 1000 pg/ml IFN-γ. Embryos were cultured at a density of 10 embryos per 20 µl drop for each IFN-γ concentration, for up to 146.5 hours post-hCG without any change of medium. Embryo development was scored twice daily at 0830 and 1630 hours. Embryos that had developed to the blastocyst stage (Fig. 2.1a) by approximately 117 hours post-hCG were removed from culture. These embryos underwent differential nuclear staining to determine cell number in the trophectoderm (TE) and inner cell mass (ICM) cell lineages.

2.1.5 Differential nuclear staining

The method used was based on that described by Handyside and Hunter (Handyside and Hunter, 1984). The zona pellucidae surrounding the blastocysts were removed by a brief period of incubation (20-30 seconds) in acidic Tyrode's solution (Sigma, T1788, mouse embryo tested; pH 2.3), pre-warmed to 37ºC. Next, blastocysts were transferred to a cavity block containing pre-warmed (37ºC) H6-BSA and allowed to recover for at least 20 minutes. Then, blastocysts were incubated at room temperature in trinitrobenzene sulphonic acid (TNBS; Sigma, P2297; Appendix I) for 10 minutes, followed by three washes in pre-warmed (37ºC) H6-BSA. The embryos were then incubated for a further 10 minutes at room temperature in a 25 µl drop comprised of 10.4 µl goat anti-dinitrophenyl-BSA antibody solution (anti-DNP-BSA; Sigma, D9781 or D9656) and 14.6 µl H6-BSA, followed by three washes in pre-

warmed (37ºC) H6-BSA. Lysis and nuclear staining of the outer TE cells was achieved next by incubating the blastocysts at 37ºC, for 10 minutes, in a 25 µl drop comprised of 23 µl guinea pig complement (Cedarlane, CL4051; Appendix I) and 2 µl propidium iodide (PI; Sigma, P4864; Appendix I). Again, the blastocysts were washed three times in pre-warmed H6-BSA. The blastocysts were fixed in 990 µl of absolute ethanol to which 10 µl of bisBenzimide H 33258 DNA stain (Hoechst 33258, Sigma, B2883; Appendix I) was added. The blastocysts were kept in the ethanol and Hoechst, at 4ºC and in the dark, for a minimum of 90 minutes and a maximum of 1 week. After fixation, the blastocysts were placed into absolute ethanol for 5-8 minutes and then into a small drop of ultra pure glycerol (Amersham). Next, a small volume of glycerol containing the blastocysts was carefully pipetted onto an ethanol-cleaned glass slide, and a cover slip was placed over the drop and pressed down gently.

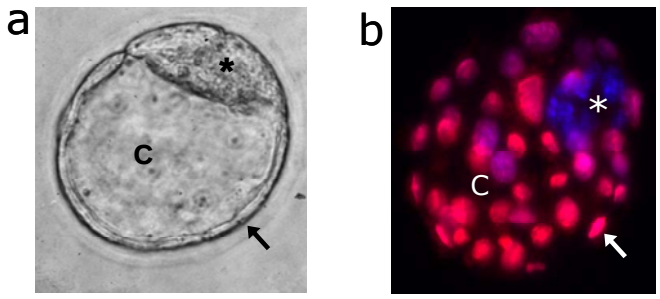


Figure 2.1 Brightfield (**a**) and fluorescent (**b**) blastocyst images. The blastocyst in photograph (**b**) was differentially stained: blue, Hoechst-stained ICM nuclei and red, PI-stained TE nuclei. On both images, * indicates the ICM, arrow indicates the TE and 'C' indicates the blastocoel cavity.

The nuclei of the TE were stained red (PI) and the nuclei of the ICM were stained blue (Hoechst), which meant that the number of cells in each lineage could be differentiated and counted (Fig. 2.1b). The blastocysts were viewed and imaged using a fluorescence microscope (Zeiss Axiovert 200) and MetaMorph software (Universal Imaging Corp., Version 6.2r6). DAPI (blue, Hoechst-stain) and TRITC-CY3 (red, PI-stain) filters were selected and then a Z-series (3-5 µm separations, X20 and X40 magnification) was captured for each blastocyst. A combined blue and red image (Fig. 2.1b) was used for analysis and every Z-section from all blastocyst was examined separately. The manual count application was used to differentially count and record the number of TE and ICM cells in each blastocyst.

2.2 Generation and assessment of maternal inflammation *in vivo*

2.2.1 Natural mating

Details of the mice are as in section 2.1.1. 7-8.5 week old virgin female MF1 mice, weighing between 27 and 33 grams, were naturally mated by housing them individually overnight with MF1 stud males (6 weeks to 6 months of age). Female mice remained with a stud for up to four consecutive nights. Each morning, the females were checked for the presence of a vaginal plug, which was taken as a sign of mating. Plug-negative females remained with the stud male, and plug-positive females were removed from the stud cages, weighed and housed alone.

2.2.2 LPS administration

Naturally mated plug-positive females received a dose of either normal saline (0.85% NaCl; control) or one of three lipopolysaccharide (LPS; *Salmonella enterica enteritidis*, Sigma, L4774) dosages between 1400 and 1600 hours on the day of plug (GD 0.5) when embryos would be at the zygote stage, 13-15 hours post-fertilisation. Dosages of 10, 50 and 150 µg/kg body weight LPS were freshly prepared (lyophilised LPS reconstituted with 0.85% saline; 1 mg/ml) on each occasion and were administered by i.p. injection of 100 µl volumes. The mice were put back into their cages directly following treatment and were housed alone until required.

2.2.3 Assessment of the maternal response to LPS

A group of females (treated as in section 2.2.2) was subjected to a variety of assessments in order to characterise maternal response to the injection of saline (control) or LPS dosages. A subset of these females was assessed for sickness-related behaviours and body weight change. The other subset of these females was sacrificed for collection of blood for analysis of serum cytokine concentrations using a multiplex cytokine assay kit (see section 2.2.3.3).

2.2.3.1 Sickness behaviour and body weight

At 1 hour post-injection, mice underwent an open field test to assess the extent to which locomotor and exploratory-related behaviour had been affected (see section 2.4.2.1). Maternal body weight, and food and water consumption was assessed in addition to the open field test. Mice were weighed at the time of injection and at 24, 48, 72, 96 and 120 hours post-injection (GD 0.5-5.5). Furthermore, mice were

weighed in late gestation, at GD 15.5. The chow and tap water bottle of each mouse were also weighed at the time of injection and at 24, 48, 72, 96 and 120 hours post-injection (GD 0.5-5.5). Mice had access to *ad libitum* chow and tap water throughout all assessments, apart from the 3 minute duration of the open field test.

2.2.3.2 Collection of blood

Female mice from control and LPS treated groups were sacrificed by cervical dislocation at 3, 24, 48 and 72 hours post-injection. Maternal blood samples were taken by cardiac puncture using a needle (BD Microlance 3, 23-gauge) and syringe (BD Plastipak, 1 ml). Briefly, blood was drawn out slowly from the right ventricle by carefully puncturing the right ventricular wall. Care was taken not to damage the atria (which would cause haemorrhage and reduced the volume of blood available for collection) or to suck air into the syringe (which would cause hemolysis) during this process. The blood was immediately transferred to a 1.5 ml tube and placed on ice. The clotted blood samples were then centrifuged at 4ºC, at 9,300 ***g*** for 10 minutes and the resulting serum was aliquoted and stored at -80ºC until analysis.

2.2.3.3 Multiplex cytokine assay

The Bio-Plex Mouse Cytokine 23-Plex Panel kit (Bio-Rad) was used to detect and quantify the following cytokines, simultaneously, in each serum sample: IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 p40 (antagonist), IL-12 p70 (agonist), IL-13, IL-17, eotaxin (CCL11), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), IFN-γ, keratinocyte-derived chemokine (KC; CXCL1), monocyte chemoattractant protein-1 (MCP-1; CCL2), macrophage inflammatory protein-1 α (MIP-1α; CCL3), MIP-1β (CCL4), RANTES (regulated upon activation, normal T-cell expressed and secreted; CCL5) and TNF-α. All buffers, diluents and cytokine standards required to perform the assay were provided in the kit. All samples, standards and blanks were run in duplicate on every plate. The assay was carried out in accordance with the manufacturer's instructions.

In brief, thawed serum samples were diluted 1:4 with mouse sample diluent (12.5 µl serum + 37.5 µl diluent/well). The lyophilized recombinant cytokine standard was reconstituted with standard diluent and a nine-point serial dilution was prepared, where each standard was a 4-fold dilution of the previous tube. All samples and standards were kept on ice until ready for use. Diluted standards and samples (50 µl/well) were added to antibody-conjugated fluorescently-dyed beads in the wells of a

96-well filter plate. The samples and standards were incubated with the beads at room temperature, in the dark, on a microplate shaker (300 rpm), for 1 hour. At the end of the incubation period, the plate was washed three times with wash buffer (100 µl/well/wash). Liquid was sucked out through the filter-bottom of each well by placing the 96-well plate on a vacuum manifold. Next, freshly diluted detection antibody was added to the wells and incubated at room temperature, in the dark, on a microplate shaker (300 rpm) for 30 minutes. The plate was washed a further three times (as above). Freshly diluted streptavidin-phycoerythrin (PE) was then added to the wells and incubated at room temperature, in the dark, on a microplate shaker (300 rpm) for 10 minutes. The plate was washed again (as above). Finally, the beads were resuspended in assay buffer (125 µl/well) and placed on the microplate shaker (300 rpm), in the dark, at room temperature for approximately 10 minutes. The plate was then run on a Luminex 100 (Luminex Corporation) multiplex array instrument.

StarStation (version 2.0; Applied Cytometry Systems, Sheffield, UK) software was used to acquire and analyse all data from the Luminex 100 instrument. The instrument was set to acquire 1000 total events/well. All values were reported minus background, i.e. any background fluorescence detected in the blank sample was subtracted from the value obtained for each of the standards and samples. Outliers were defined as follows: points with negative mean fluorescence intensity (MFI); points with very extreme MFI and concentration. The outliers were removed from the analysis and, where possible, the most appropriate standard curve for each cytokine was established by considering each point in duplicate. The StarStation software then calculated the sample cytokine concentrations (pg/ml) based on these standard curves (Table 2.1), and reported the individual result for each well (as opposed to reporting the mean of the duplicate for each sample).

Cytokine	Expected Concentration Range (pg/ml)	Cytokine	Expected Concentration Range (pg/ml)
IL-1α	0.46 - 30,081	IL-17	0.77 – 50,600
IL-1β	0.26 - 17,208	Eotaxin (CCL11)	0.65 – 42,582
IL-2	0.90 - 58,666	G-CSF	0.60 – 39,312
IL-3	0.64 - 41,713	GM-CSF	0.52 – 33,995
IL-4	0.73 - 47,598	IFN-γ	0.67 – 44,043
IL-5	0.43 - 28,327	KC (CXCL1)	0.45 – 29,709
IL-6	0.35 – 23,116	MCP-1 (CCL2)	0.42 – 27,783
IL-9	0.30 – 19,936	MIP-1α (CCL3)	0.56 – 36,645
IL-10	0.50 – 32,741	MIP-1β (CCL4)	0.54 – 35,161
IL-12 p40	0.27 – 17,713	RANTES (CCL5)	0.44 – 28,968
IL-12 p70	0.22 – 14,330	TNF-α	0.85 – 55,937
IL-13	0.69 – 45,348		

Table 2.1 Expected concentration range (standard 1-9) of each cytokine within the recombinant standard provided in the multiplex kit. The ranges were used to establish standard curves for each of the cytokines.

2.3 Assessment of embryonic and fetal development

2.3.1 Blastocyst collection and analysis of cell number

Uteri were dissected out and placed into normal saline solution that had been pre-warmed to 37ºC. Embryos were then flushed from each uterus by inserting a flushing needle (described in section 2.1.3) a short distance into the cut, anterior end of each uterine horn, and flushing through pre-warmed (37ºC) H6-BSA. The blastocysts were washed three times (as in section 2.1.3) before undergoing differential nuclear staining to determine cell number in the TE and ICM cell lineages (see section 2.1.5).

2.3.2 GD 17 fetal dissection

At GD 17, female mice from each treatment group (as in section 2.2.1 and 2.2.2) were sacrificed by cervical dislocation. The uterus containing the fetuses was dissected out (taking care to remember its orientation), placed into a petri dish containing cold PBS and immediately put onto ice. Next, the wall of each uterine horn was carefully cut open. Each conceptus (i.e. placenta, yolk sac, fetus and amniotic fluid) was 'plucked' from its attachment site, placed on its own in a petri dish containing cold PBS and put onto ice. Each conceptus was blotted to remove excess moisture and was weighed. Each conceptus was then separated into its constituent parts, i.e. placenta, yolk sac and fetus, and each part was weighed individually. After that, using a dissecting microscope (Zeiss Stemi SV6) and fine surgical instruments, each fetus was dissected and fetal brain, liver, left and right kidneys were removed and weighed.

2.4 Assessment of postnatal growth, behaviour, physiology and immune status

A battery of postnatal assessments (Table 2.2) was conducted on the offspring of female mice that had been saline or LPS treated (as in section 2.2.2). Details of each assessment are described in the following sections.

Maternal Treatment	Saline, 10, 50 and 150 µg/kg LPS		
Litter size	Yes	Yes	Yes
Birth weight	Yes	Yes	Yes
Growth	Yes	Yes	Yes
Behaviour	Yes	Yes	Yes
Systolic Blood Pressure	Yes	Yes	Yes
Organ Weight	Yes	Yes	Yes
Offspring Treatment	No treatment	Saline	50 µg/kg LPS
Flow Cytometry of Splenocytes	No	Yes	Yes
Multiplex cytokine assay of serum	No	Yes	Yes

Table 2.2 Summary table of postnatal offspring treatment groups and assessments; those conducted before and after offspring treatment are highlighted.

2.4.1 Birth weight and growth

Female mice from each treatment group were allowed to give birth naturally. Birth took place on GD 19; therefore mothers were checked for birth early in the morning on this day. For each litter, the numbers of live and dead pups were recorded and any dead pups were removed from the cage. The gender of each pup was assessed by examining the external, peri-anal anatomy, and the numbers of males and females were recorded. Each pup was weighed on a digital balance and birth weights were

recorded. The litters were then culled back to six pups per litter. Where possible, three pups of each gender were kept; the ones kept were those that had the median weights for their gender. The pups were then returned to the cage with their mother, and had *ad libitum* access to standard rodent chow and tap water at all times. At 3 weeks of age the pups were weaned; littermates of the same gender were housed together and the mother was culled. The offspring were weighed weekly until 30 weeks of age.

2.4.2 Behaviour

Behaviour was assessed at 4, 5, 6, 8, 11, 14, 17, 20 and 23 weeks of age. At each of these ages mice were tested as follows:

- Day 1 – open field test (afternoon) followed by nesting overnight.
- Day 2 – burrowing (1400-1600 hours) followed by overnight burrowing and consumption of glucose solution.

Mice were housed in their home cages with their littermates when not involved in behavioural tests but underwent all behavioural tests individually. Food and water were available *ad libitum*, except during the glucose solution test when glucose solution was substituted for tap water.

2.4.2.1 Open field

Open field tests (Fig. 2.2c,d) were conducted in the afternoon, in a normally lit room and prior to doing the test, mice were habitualised to the behaviour room for 30 minutes. The open field arena consisted of a white acrylic base enclosed on four sides with transparent, 0.7 cm thick acrylic (dimensions of 27.5 x 27.5 x 20 cm, model ENV-520, Med Associates Inc., Georgia, Vermont). Laser beams crossing the open field arena allowed animal movement to be detected and recorded automatically. Each open field arena was surrounded by an aluminium shield that prevented the mouse under investigation from seeing other rodents. Data was acquired by Activity Monitor software (Version 4.0, Med Associates Inc., Georgia, Vermont) for a 3 minute (180 seconds) period which started as soon as a mouse was detected in the arena (Cunningham et al., 2007). Each mouse was individually placed in the centre of the open field arena and the distance travelled, time spent resting, number of jumps and number of rears was recorded by the computer. After each three minute recording

period, the mouse was returned to its home cage and the open field arena was cleared of any droppings and wiped out with disinfectant solution and paper towels.

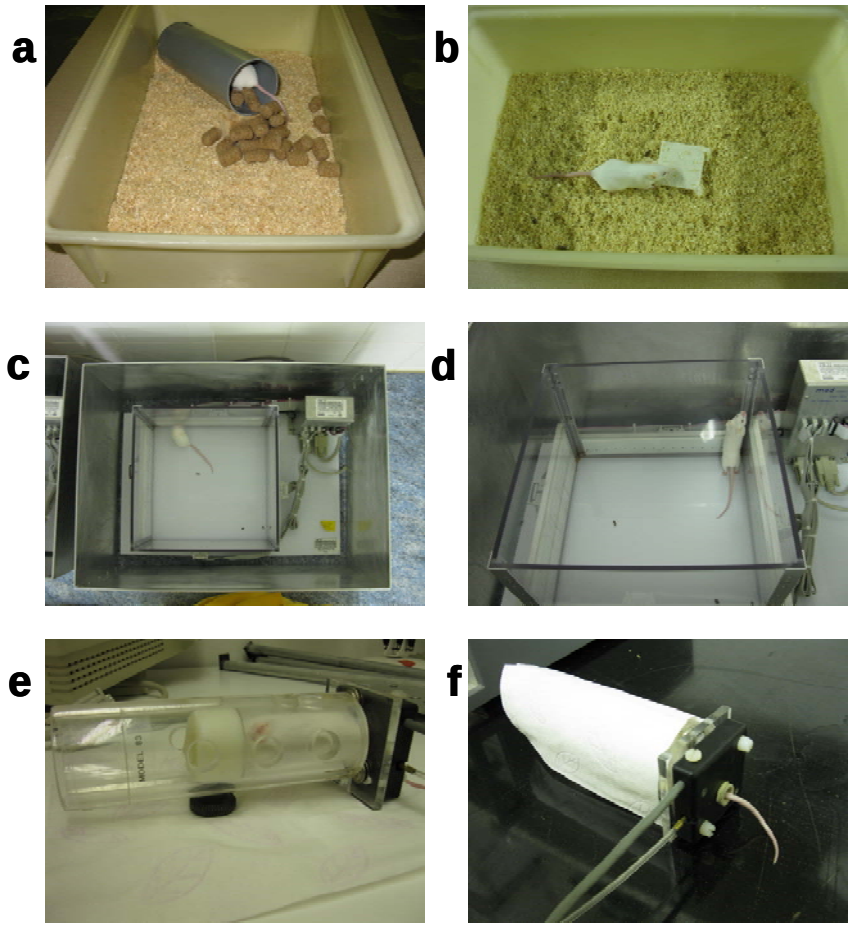


Figure 2.2 Representative images of behavioural and blood pressure assessments. (**a**) Burrowing, (**b**) nesting, (**c**) and (**d**) open field. Blood pressure apparatus, uncovered (**e**) and covered (**f**).

2.4.2.2 Nesting and tap water consumption

Normal tap water consumption was assessed overnight, concurrent with the nesting (see below). Pre-weighed bottles of normal tap water were given at approximately 1600 hours. The bottles were reweighed at 1000 hours the following morning and the amount consumed (for an overnight period of approximately 18 hours) was calculated. At approximately 1600 hours mice were placed individually into cages that contained only sawdust bedding and a pressed cotton square ('Nestlet', Datesand Ltd, Manchester, UK; Fig. 2.2b). The nests were rated the following morning by assigning a 1-5 score based on the following (Deacon, 2006a):

1. Nestlet not noticeably touched (>90% intact).

2. Nestlet partially torn up (50-90% remaining intact).

3. Nestlet mostly shredded, but no identifiable nest site; >50% of the Nestlet is torn up, but <90% of the Nestlet is within a quarter of the cage floor area, i.e. material is not gathered into a nest, but spread around the cage.

4. An identifiable, but flat nest:>90% of the Nestlet is torn up, the material is gathered into a nest within a quarter of the cage floor area, but the nest is flat, with walls less than the mouse body height (when curled up on its side) on >50% of its circumference.

5. A (near) perfect nest: >90% of the Nestlet is torn up, the nest is a crater, with walls larger than the mouse body height (when curled up on its side) on >50% of its circumference.

2.4.2.3 Burrowing

Plastic cylinders (blind ended, 20 cm long, 6.8 cm in diameter, open end supported by a block 3 cm in height) were filled with 190±2 g of standard rodent chow pellets and placed in individual mouse cages (Fig. 2.2a). Mice were placed individually in the burrowing cages at approximately 1400 hours and were left undisturbed until approximately 1600 hours. The chow remaining in the cylinders after two hours was weighed and the amount displaced ('burrowed') was calculated (Deacon, 2006b). For the overnight burrowing, cylinders were returned to the cages immediately (without being topped up with additional chow) after weighing them at 1600 hours. The chow remaining in the cylinders at approximately 1000 hours the following morning was weighed and the amount displaced ('burrowed' during an overnight period of approximately 18 hours) was calculated.

2.4.2.4 Consumption of glucose solution

Glucose solution consumption was assessed overnight, concurrent with the overnight burrowing (section 2.4.2.3 above). A 5% glucose solution (D-(+)-glucose powder ≥99.5%; Sigma, G8270; dissolved 5% w/v in distilled water) in pre-weighed bottles was substituted for the normal tap water at 1600 hours. The bottles were weighed again at approximately 1000 hours the following morning, and the amount consumed (during an overnight period of approximately 18 hours) was calculated.

2.4.3 Systolic blood pressure

Systolic blood pressure (SBP) was measured at 16, 22 and 28 weeks of age by tail cuff plethysmography using an ITC model 229 blood pressure monitor (Linton Instruments, Norfolk, UK). Blood pressure measurements were taken at a room temperature of 27-30ºC which the mice were allowed to acclimatise to for at least 60 minutes prior to readings being taken. In order to take the SBP measurements, mice were restrained in an acrylic tube and their tails were threaded through the tail cuff (Fig. 2.2e,f). The acrylic tube was then covered with a paper towel to provide the mouse with a 'safer' feeling environment (Fig. 2.2f). Each mouse was allowed to acclimatise to the apparatus for approximately 5 minutes prior to readings being taken. Five SBP readings were taken for each mouse at each of the ages studied and the mean value of the three median readings was taken as the SBP of that mouse at that age.

2.4.4 Organ weight and immune status

2.4.4.1 Postnatal treatment groups

Mice were sacrificed between 37 and 42 weeks of age. All animals were weighed the day of cull and, where possible, the mice in each litter were assigned to a postnatal treatment group according to this weight. Two mice (one male and one female) from each litter were assigned to one of three postnatal groups; no treatment (the heaviest male and female), i.p. injection of normal saline (the lightest male and female) or i.p. injection of 50 µg/ml LPS (the median weight male and female; LPS, *Salmonella enterica enteritidis*).

2.4.4.2 Organ collection and multiplex cytokine assay of serum

On the day of cull, littermates from one litter were separated and each mouse was caged individually with *ad libitum* access to tap water but without chow. Injected mice (saline and LPS) were culled 3.5 hours post-injection. All mice were killed by cervical dislocation, blood samples were taken by cardiac puncture and serum was stored (as in section 2.2.3.2). The Bio-Plex Mouse Cytokine 23-Plex Panel kit (Bio-Rad) was used to detect and quantify cytokine concentrations in each serum sample (see section 2.2.3.3). The following organs and fat pads were dissected out, weighed, snap frozen in liquid nitrogen and stored at -80ºC; heart, lungs, liver, left and right kidneys, brain, interscapular fat, retroperitoneal fat, gonadal fat, inguinal fat and mesenteric fat. In addition, the spleen of every mouse was harvested and weighed. Spleens from the

injected mice (saline and LPS) were placed immediately into cold PBS and were kept on wet ice until splenocyte isolation was commenced (no longer than 3 hours).

2.4.4.3 Isolation of splenocytes

Splenocytes were isolated by preparation of single-cell suspensions from spleen and removal of red blood cell (RBC) contamination by hypotonic lysis. A single-cell suspension was prepared by mashing a whole spleen (first cut into 4-5 pieces) through a cell strainer (BD Falcon Cell Strainer, 100 µm pore size, catalogue number 352360) into a 50 ml conical tube, using the plunger from a 1 ml syringe and rinsing regularly with plenty of cold PBS. The resulting cell suspension was centrifuged at 4ºC, at 400 ***g*** for 7 minutes and the supernatant was discarded. Next the cell pellet (dark red in colour due to the presence of RBCs) was re-suspended in 1-5 ml of RBC lysis buffer (ACK buffer; Appendix I) and incubated at room temperature for 10-15 minutes. The tube was then filled with cold PBS, centrifuged at 4ºC, at 400 ***g*** for 7 minutes and the supernatant discarded. The cell pellet (now white in colour indicating that RBCs had been eliminated) was re-suspended in 4 ml of cold PBS supplemented with 1% fetal calf serum (FCS) and 0.02% sodium azide. These freshly isolated splenocytes were then immunostained (section 2.4.4.4) and fixed in preparation for analysis by flow cytometry (section 2.4.4.5).

2.4.4.4 Immunostaining

Freshly isolated splenocytes (section 2.4.4.3) were immunostained using combinations of fluorochrome-conjugated monoclonal antibodies (mAbs) against mouse leukocyte subpopulation markers (Table 2.3).

Four spleens (i.e. the 2 LPS and 2 saline treated animals from one litter) were processed per staining session. In all staining sessions, splenocytes from one mouse were stained with each mAb individually (single-colour tube), with no mAb (cells only, negative control tube) and with two mAbs together (2-colour tube). As such, four tubes (BD Falcon 5 ml round bottom polystyrene test tube, 352032) were prepared (25 µl of cell suspension/tube) for this mouse: cells only (no mAb), CD4, CD8, CD19, and 2-colours (CD4, CD8). For each of the remaining three mice, i.e. three spleens, only two tubes were prepared (also 25 µl of cell suspension/tube): CD19 and 2-colours (CD4, CD8,).

Splenocytes were incubated with antibody in the dark, on ice for 30 minutes. The tubes were then filled with cold PBS (supplemented with 1% FCS and 0.02% sodium azide), centrifuged at 4ºC, at 400 ***g*** for 7 minutes and the supernatant discarded. Next the cells were fixed by adding 1 ml of 4% formaldehyde to each tube and incubating in the dark, on ice for 20 minutes. Finally, a further 3 ml of cold PBS were added to each tube. The splenocytes were maintained in this 1% formaldehyde solution, well sealed, in the dark, at 4°C for up to 1 week before running on the flow cytometer. Splenocytes were centrifuged at 4ºC, at 400 ***g*** for 7 minutes and re-suspended in approximately 400 µl of PBS immediately before being run on the flow cytometer.

Marker of Interest	Fluorochrome	Clone & Isotype	Working Concentration (µg/10^6 cells/100 µl)
CD4	Fluorescein isothiocyanate (FITC)	GK1.5, Rat IgG2b	0.25
CD8	PerCP-Cy5.5	53-6.7, Rat IgG2a	0.25
CD19	Allophycocyanin (APC)	MB19-1, Mouse IgA	0.5

Table 2.3 Summary of the fluorochrome-conjugated mAbs used to immunostain splenocytes for flow cytometry. (All antibodies were purchased from eBioscience and were used at the concentrations recommended by the manufacturer).

2.4.4.5 Flow cytometry

Flow cytometry was performed using a FACSCanto instrument (BD Biosciences) and all data were analysed using FACSDiva software (Version 5.0.3, BD Biosciences). In total, 10,000 or 20,000 events were collected for every tube. Analysis of the collected data was conducted as follows. To start with, singly stained tubes were used to set the appropriate compensation levels. In brief, this was achieved by adjusting the median fluorescence of the positive population so that it was in alignment with the median fluorescence of the negative population. Next, splenocyte populations were identified from a forward scatter (FSC; indicating cell size) vs. side scatter (SSC; indicating cell granularity) plot. These splenocyte populations were gated and analysed further by creating dot plots to examine expression of the fluorescently-labelled cell markers. For tubes labelled with CD19, a SSC vs. APC-CD19 plot was drawn. For tubes labelled with

two colours, a FITC-CD4 vs. PerCP-Cy5.5-CD8 dot plot was created. The percentage of cells in each population and gate of interest was then quantified and displayed.

2.5 Statistics

Throughout the thesis significance was taken to be $P \leq 0.05$ and a trend was considered to exist if a P-value of between 0.051 and 0.1 was observed.

All statistics were carried out using SPSS statistical software (SPSS, version 14). All *in vitro* and *in vivo* blastocyst data (Chapters 3 and 5) and all maternal data (Chapter 4) were analysed with a least significant difference (LSD) one-way analysis of variance (ANOVA) test.

All GD 17 fetal dissection data (Chapter 5) and all postnatal data (Chapter 6) were analysed using a multilevel random effects regression model (Kwong et al., 2004; Watkins et al., 2008b). This method of statistical analysis enabled the complex and hierarchical structure of the data set to be considered accurately. The model not only takes into account between-litter and within-litter variation, but also makes possible the incorporation and evaluation of other measured parameters e.g. litter size.

Pearsons correlation analysis was used were relevant to assess the degree of association between two parameters of interest.

3 Effect of interferon-γ on preimplantation mouse embryo development *in vitro*

3.1 Introduction

The essential role played by cytokines in reproductive and developmental processes is now as well recognised as their immune-modulating properties (Orsi, 2008). Interferon gamma (IFN-γ) is known to have important roles in both the immune and reproductive systems. In the context of the immune system, IFN-γ is a pleiotropic cytokine that is produced predominantly by activated $CD4^+$ T_{H1} lymphocytes, cytotoxic $CD8^+$ T lymphocytes and natural killer (NK) cells. Typically, IFN-γ is involved in antiproliferative and antiviral responses, tumour suppression, macrophage activation, antigen presentation and promoting the differentiation of naïve $CD4^+$ T lymphocytes into T_{H1} lymphocytes (Ramana et al., 2002). The diverse biological actions of IFN-γ in the immune system are mediated mainly by regulation of gene expression within the target cell (Ramana et al., 2002). Classically, IFN-γ signals intracellularly through the Janus kinase/signal transducer and activator of transcription-1 (JAK/STAT1) pathway (Ramana et al., 2002). However, other intracellular signalling pathways, including MAP kinase, PI3-kinase and Src-kinases, are also known to play important roles in regulating the full repertoire of responses brought about by IFN-γ (Gough et al., 2008; Ramana et al., 2002).

In the context of early pregnancy, IFN-γ is arguably most well recognized for its role at the feto-maternal interface during the peri-implantation period. Uterine natural killer (uNK) cell-derived IFN-γ is imperative for normal decidual development and uterine artery remodelling during implantation (Ashkar et al., 2000). In addition, there is some evidence to suggest that IFN-γ plays a functional role in trophoblast cells (TBCs); for example, *in vitro* culture with IFN-γ was found to induce the differentiation of ectoplacental cone cells into cytokeratin-positive TBCs (Athanassakis et al., 2000) and also to enhance TBC phagocytic activity (Albieri et al., 2005). Interestingly, normal TBCs *in vitro* appear to be resistant to the characteristic IFN-γ-induced apoptosis and IFN-γ-induced expression of MHC class II molecules (Hoshida et al., 2007; Murphy et al., 2004).

In comparison, much less is known about the role of IFN-γ during preimplantation development. The uterine fluid of naturally cycling mice has been found to contain

IFN-γ at a concentration of approximately 100 pg/ml at oestrus (Orsi et al., 2007). Furthermore, in an elegant immunohistochemical study mapping the expression of IFN-γ in the normal mouse uterus, IFN-γ was detected in endometrial glands and luminal epithelium, and staining was strongest in estrus-phase uteri (Platt and Hunt, 1998). These experiments demonstrate that IFN-γ is present in the normal, healthy uterus and as such, suggests that the preimplantation embryo is exposed to IFN-γ physiologically. Moreover, the preimplantation mouse embryo has been shown to express the complete IFN-γ receptor (IFNGR1 and IFNGR2 subunits) at all stages from one-cell to the blastocyst (Truchet et al., 2001).

To my knowledge, there are very few studies investigating the effects of *in vitro* culture with IFN-γ on preimplantation development, and all of these studies used very high IFN-γ concentrations. In addition, only one of these studies used recombinant mouse (rm) IFN-γ; limited conclusions can be drawn from the other studies since they cultured mouse embryos in IFN-γ from other species and IFN-γ is known to be highly species specific (Cameo et al., 1999; Hill et al., 1987; Warner et al., 1993). Warner et al. (Warner et al., 1993) used rmIFN-γ in their study and reported a dose-dependent IFN-γ-stimulated increase in preimplantation cleavage rate that was associated with increased cell surface expression of Qa-2 protein. The Qa-2 protein is a MHC class Ib (non-classical) protein that is coded for by the preimplantation embryo development (*Ped*) gene; the presence of which is known to confer an intrinsically fast rate of preimplantation cleavage, i.e. the *Ped* fast phenotype (Wu et al., 1998). The aforementioned Warner study used an inbred, *Ped* fast strain of mice (C57BL/6J); it seems therefore, that exposure to IFN-γ can enhance preimplantation cleavage rate in embryos that are already genetically 'fast' and indeed, the *Ped* gene is known to contain an interferon responsive sequence (IRS) in its promoter (Cai et al., 1996).

Overall, therefore, evidence seems to suggest that the effects of IFN-γ in early pregnancy are not as deleterious as might be expected from what is know of its characteristic actions in the immune system. However, we know very little about how a physiological rmIFN-γ concentration range affects preimplantation mouse embryo development; and furthermore the effect of IFN-γ on embryos that are from an outbred stock of mice, the genetic diversity of which likely better reflects the situation in human populations (Byrne et al., 2007). Furthermore, it is conceivable that altered levels of this cytokine could be found in the uterine fluid, if a female experiences systemic inflammation during the preimplantation period of gestation. As such, the

aim of this study was to examine the direct effect of a physiological rmIFN-γ concentration range on the development of outbred (MF1) preimplantation mouse embryos *in vitro*. Groups of preimplantation embryos were cultured from the 2-cell to the blastocyst stage in 0 (medium only), 1, 10, 100 and 1000 pg/ml rmIFN-γ. The effect of IFN-γ on development was assessed as follows: i) by daily morphological scoring; and ii) by differential nuclear labelling of the two cell lineages (ICM and TE) within the blastocyst.

3.2 Methods

3.2.1 Superovulation and embryo collection

Female MF1 mice were superovulated and mated with MF1 males (section 2.1.2). Embryos were flushed from oviducts at the 2-cell stage (GD 1.5, ~48 hours post-hCG) and temporarily maintained in equilibrated KSOM at 37ºC, 5% CO_2 in air (section 2.1.3).

3.2.2 Embryo culture with IFN-γ

Embryos were cultured from 2-cell to blastocyst stage (up to 146.5 hours post-hCG) in KSOM only (control) or increasing concentrations of IFN-γ (8.43×10^3 IU/µg); 1, 10, 100 and 1000 pg/ml, without any changes of medium (section 2.1.4). The developmental stage of each embryo was assessed twice daily at 0830 and 1630 hours. An embryo was described as compact when no individual cell outlines could be discerned clearly. An embryo that had a visible cavity of any size was described as cavitated.

3.2.3 Differential nuclear staining

Embryos that had developed to the blastocyst stage by approximately 117 hours post-hCG were removed from culture and differentially stained (section 2.1.5). After mounting, the embryos were viewed under a fluorescence microscope and the numbers of TE and ICM nuclei were counted.

3.2.4 Statistics

All statistics (section 2.5) were carried out using SPSS statistical software (SPSS, version 14). Developmental stage data and blastocyst cell number data were analysed with a LSD one-way ANOVA test. Pearsons correlation analysis was used to assess the degree of association between increasing IFN-γ concentrations and blastocyst cell

number and the degree of association between percentage of embryos cavitated and percentage of embryos arrested. Developmental stage data were additionally converted to Z-scores (using SPSS), which were analysed using a LSD one-way ANOVA. The Z-score transformation used the entire developmental stage dataset to standardise the developmental stage to the same scale, with a mean of 0 and a standard deviation of 1. This transformation allowed for analysis between treatment groups by comparing the developmental stage curve for each treatment as a whole, hence avoiding the use of multiple comparisons across time.

3.3 Results

3.3.1 Developmental rate of embryos cultured *in vitro* with increasing concentrations of IFN-γ

This set of experiments shows that embryo culture with increasing concentrations of IFN-γ (1, 10, 100 and 1000 pg/ml) has no effect on development before cavitation. Fig. 3.1a-c illustrates the similarity between treatment groups at every time point, up to the 4-cell (Fig. 3.1a), 8-cell (Fig. 3.1b) and compact (Fig. 3.1c) stages of development. However, embryo culture with increasing concentrations of IFN-γ did appear to have an effect on cavitation, as shown in Fig. 3.1d. Of all treatment groups, control embryos (Fig. 3.1d, solid black line) have the slowest cavitation rate and the lowest percentage of cavitated embryos at 138.5 hours post-hCG (~60% maximum cavitated). Embryos cultured in 1 and 100 pg/ml IFN-γ (Fig. 3.1d, black dashed and blue lines, respectively) are similar to one another, and have a higher percentage of embryos cavitated, with approximately 9% more cavitated embryos than control at 138.5 hours post-hCG (not significant). Embryos cultured in 10 and 1000 pg/ml IFN-γ (Fig. 3.1d, green and red lines, respectively) are also similar to one another, and have the highest percentage of embryos cavitated out of all treatment groups, with approximately 17% more cavitated embryos than control at 138.5 hours post-hCG (not significant).

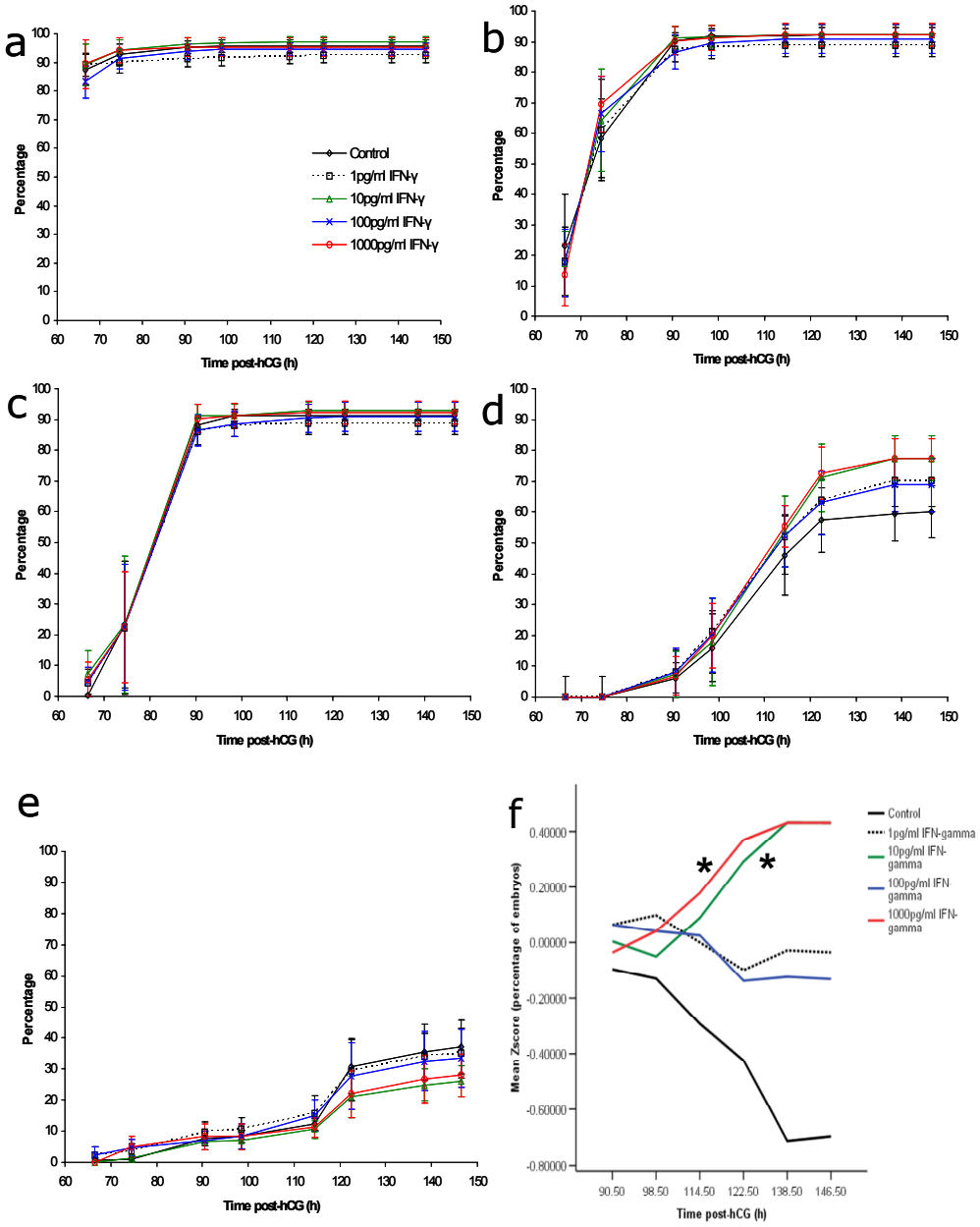


Figure 3.1 Developmental stage data for preimplantation embryos cultured *in vitro* with KSOM and increasing concentrations of IFN-γ (1, 10, 100 and 1000 pg/ml). IFN-γ had no effect at any concentration, at any time point on embryo development (**a**) to the 4-cell stage and beyond, (**b**) the 8-cell stage and beyond, (**c**) compaction and beyond, (**d**) on the percentage of embryos that cavitated or (**e**) on the percentage of arrested embryos. Z-scores analysis showed that the overall percentage of cavitated embryos was increased (P≤0.05) in 10 and 1000 pg/ml IFN- γ treated groups when compared with control (**f**). Data in (**a**) to (**e**) are mean±SEM; *denotes that the entire curve is significantly different (P≤0.05) from the control group. n=918 embryos in total; n=180-190 embryos per treatment group; experimental n=4.

Assessment of overall embryo development using Z-score analysis supported the suggestion that embryo culture with increasing concentrations of IFN-γ (1, 10, 100 and

1000 pg/ml) has no effect on developmental rate up to the 4-cell, 8-cell and compact stages of development (plots not shown). Interestingly however, the Z-score analysis showed that embryos cultured in the presence of 10 and 1000 pg/ml IFN-γ, overall, have a significantly (P≤0.05) higher percentage of cavitated embryos than control (Fig. 3.1f).

	Pearson Correlation for cavitated embryos (%) vs. arrested embryos (%)	
Time post-hCG (h)	*r*-value	P-value
90.5	-0.767	<0.01
98.5	-0.739	<0.01
114.5	-0.609	<0.01
122.5	-0.889	<0.01
138.5	-0.724	<0.01
146.5	-0.726	<0.01

Table 3.1 Correlation analysis assessed the relationship between the percentage of cavitated embryos and the percentage of arrested embryos at each time point post-hCG (h) after cavitation had become evident (i.e. from 90.5 h post-hCG). Correlation coefficients (*r*-value) showed that as the % of cavitated embryos increased the % of arrested embryos decreased (P≤0.01) at every time point examined.

There appeared to be an increased percentage of cavitated embryos in the treatment groups where there was a decreased percentage of arrested embryos. This suggested that survival was improved in the treatment groups that exhibited a high percentage of cavitated embryos concomitantly with a low percentage of arrested embryos. For example, at 122 hours post-hCG, the percentage of embryos cavitated was 73% (high) and 58% (low) for the 1000 pg/ml rmIFN-γ and control treated groups respectively, whereas a reciprocal pattern was seen for the percentage embryos arrested, which was 22% (low) and 31% (high) respectively, for the same groups. Indeed, correlation analysis revealed a strong and significant (P≤0.01) negative relationship between the percentage of cavitated embryos and the percentage of arrested embryos at each time point post-hCG (Table 3.1). Therefore, the percentage of cavitated embryos increased as the percentage of arrested embryos decreased, which suggested improved survival.

3.3.2 Blastocyst cell number and ICM:TE ratio of embryos cultured *in vitro* with increasing concentrations of IFN-γ

Analysis of blastocyst cell number revealed a lineage-specific alteration in embryos that had been cultured in the presence of particular concentrations of IFN-γ (Fig. 3.2a).

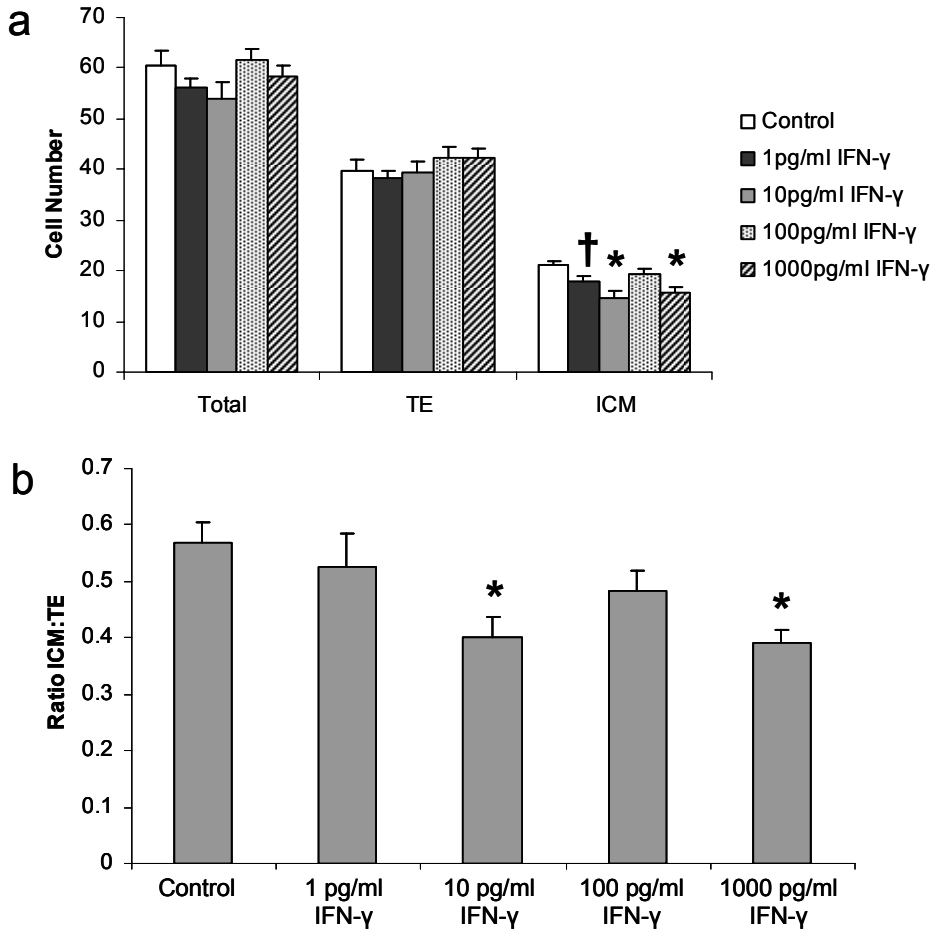


Figure 3.2 Blastocyst cell number (**a**) and ICM:TE cell ratios (**b**) of embryos cultured *in vitro* with KSOM and increasing concentrations of IFN-γ (1, 10, 100 and 1000 pg/ml). Embryo culture in the presence of 10 and 1000 pg/ml IFN-γ reduces ICM:TE cell ratio (**b**), which appears to be attributable to reduced cell number in the ICM lineage specifically (**a**). Values are mean±SEM; *P≤0.05; † P≤0.1. n=176 embryos in total; n=27-49 embryos per treatment group; experimental n=2.

Across all treatment groups, the mean total blastocyst cell number ranged from 54 to 62 cells (Fig. 3.2a). Blastocysts from the 10 pg/ml IFN-γ treatment group had the lowest mean total cell number, while blastocysts from the 100 pg/ml IFN-γ treatment group had the highest mean total cell number. However, no statistical differences in total cell number were found to exist. In addition, no statistical differences in TE cell

number were found to exist. Across treatment groups the mean TE cell number ranged from 38 to 42 cells (Fig. 3.2a). Blastocysts from the 1 pg/ml IFN-γ treatment group had the lowest mean TE cell number, while blastocysts from the 1000 pg/ml IFN-γ treatment group had the highest mean TE cell number. Statistically significant differences were however found to exist specifically within the ICM cell lineage (Fig. 3.2a). Blastocysts from the 10 and 1000 pg/ml IFN-γ treatment groups contained fewer (P≤0.05) ICM cells (mean of 15 and 16 ICM cells, respectively) than control blastocysts (mean of 21 ICM cells). Blastocysts from the 1 pg/ml IFN-γ treatment group also contained fewer (P≤0.1) ICM cells (mean of 18 ICM cells). Essentially, there appeared to be a concentration dependent decrease in ICM cell number as IFN-γ concentration increased. Indeed, a negative correlation (r = -0.165, P≤0.05; Table 3.2) was found to exist between ICM cell number and increasing concentrations of IFN-γ. No such correlation was found to exist when total and TE cell number were analysed (Table 3.2).

	Pearson Correlation	
	r-value	P-value
Total cells	0.007	0.929
TE cells	0.097	0.202
ICM cells	-0.165	0.029*
ICM:TE ratio	-0.207	0.006*

Table 3.2 Correlation analysis assessed the relationship between increasing IFN-γ concentrations and blastocyst cell number. Correlation coefficients (*r*-value) showed that ICM cell number and ICM:TE ratio decreased as concentration of IFN-γ increased. No such correlation was found to exist when total and TE cell number were considered. *P≤0.05.

Unsurprisingly, the reduced ICM number had the effect of reducing ICM:TE cell ratio in blastocysts from the 10 and 1000 pg/ml IFN-γ treatment groups when compared with control. Control blastocysts were found to have an ICM:TE ratio of 0.57, whereas blastocysts from the 10 and 1000 pg/ml IFN-γ treatment groups had a reduced (P≤0.05) ICM:TE ratio (ICM:TE ratio of 0.40 and 0.39, respectively). Moreover, a negative correlation (r = -0.207, P≤0.05; Table 3.2) was also found to exist between ICM:TE ratio and increasing concentrations of IFN-γ.

3.4 Discussion

In this part of the study mouse preimplantation embryos were cultured from the 2-cell to the blastocyst stage in medium only or increasing concentrations of rmIFN-γ within a physiological range; the effects of which were assessed by twice-daily scoring of developmental stage and by quantification of cell number in the ICM and TE of blastocysts. To summarise, in this chapter I have shown that; i) select concentrations of rmIFN-γ (1000 pg/ml) result in an overall greater percentage of cavitated embryos; and ii) essentially, there is a dose dependent reduction in ICM cell number as rmIFN-γ concentrations increase. The results obtained for the 100 pg/ml rmIFN-γ treatment group are curious because they are similar to the control group for every parameter examined, and therefore do not fit in with the dose-dependent pattern that was observed with the other treatment groups.

Development of embryos from all treatment groups was similar until the compact stage and, in general, proceeded with the slight developmental delay that is well known to occur in *in vitro* cultured embryos when compared with embryo development *in vivo* (Sharkey, 1998; Watkins et al., 2007). Cavitation was affected to the greatest extent since *in vivo*, cavitation would have occurred by 96 hours post-hCG in most embryos, whereas only 15-20% of embryos had cavitated by this time in this study. In addition, the final percentage of embryos cavitating in the control group is low, at only 60%. Embryos could have been compromised for the following technical reasons: embryos were taken out of the incubator for a short time twice-daily for scoring purposes, during which time detrimental alterations in temperature or medium-pH could have occurred; also, embryos were cultured for the duration of the experiment (~4 days) in the same medium, without any changes or additions to the media, therefore there may have been a progressive lack of nutrients and/or build-up of toxic metabolites. All treatment groups were exposed to the same routine, therefore it is interesting that rmIFN-γ seemed to improve the maximum percentage of cavitated embryos (discussed below).

It is clear from this study that preimplantation mouse embryo development does not require an exogenous source of rmIFN-γ, since the control group were cultured in KSOM-medium free of IFN-γ. Other lines of evidence confirm this: it seems that IFN-γ is not absolutely essential for development, since IFN-γ and IFN-γ-receptor knockout mice are fertile and produce viable young (Dalton et al., 1993; Huang et al., 1993; Lu et al., 1998). It should be noted however, that the rate of fetal resorbtion in IFN-γ

knockout mice is very high and that surviving implantation sites are extremely abnormal (Ashkar and Croy, 1999; Ashkar et al., 2000). This suggests therefore, that IFN-γ is not essential for preimplantation development but becomes so for healthy implantation. Despite IFN-γ appearing not to be essential for preimplantation development, it is clear that the preimplantation embryo is exposed to IFN-γ. Orsi et al. (Orsi et al., 2007) found IFN-γ to be at a concentration of approximately 100 pg/ml (~0.1 IU/ml; WHO standard units=1000 IU/μg IFN-γ) in mouse uterine fluid at estrus. In addition, Ashkar et al. (Ashkar and Croy, 1999) found a similar amount of IFN-γ, ~0.1 IU/uterus (~100 pg/uterus), in homogenates of cycling mouse uterus, and up to 10 IU/implantation site during mid-pregnancy.

The Jak/STAT1 pathway mediates many of the classical immune functions of IFN-γ, of which most would be deleterious to the preimplantation embryo, e.g. its anti-proliferative and pro-apoptotic actions. Indeed, STAT1 knockout mice produce viable young, which suggests that Jak/STAT1 signalling is not essential for gestational development (Durbin et al., 1996). Interestingly, Truchet et al. (Truchet et al., 2004) reported the presence of constitutively phosphorylated (activated) STAT1 in the nucleus throughout mouse preimplantation development; in spite of this however, very low or undetectable levels of mRNA were detected for the IFN-γ primary response gene, interferon regulatory factor 1 (IRF-1). This finding suggested the presence of a transcriptional inhibition mechanism which, according to some recent evidence in mice, could involve histone deacetylase 1 (HDAC1). In one study, RNAi-mediated reduction of HDAC1 in preimplantation mouse embryos resulted in histone hyperacetylation, elevated expression of a subset of genes and a developmental delay (Ma and Schultz, 2008). In another study, treatment of mouse trophoblast cells with a HDAC-inhibitor alleviated the repression of IFN-γ -inducible gene expression of genes, including; IRF1, class II transactivator (CIITA), low molecular protein (LMP), guanylate-binding protein (GBP) (Choi et al., 2009). Taken together this evidence suggests that any response made by the preimplantation mouse embryo to IFN-γ must occur via a STAT1-independent pathway. As noted in the introduction, other intracellular signalling pathways, including MAP kinase, PI3-kinase and Src-kinases, are known to play important roles in regulating the full repertoire of IFN-γ-mediated responses (Gough et al., 2008; Ramana et al., 2002). Several signal transduction pathways are known to be involved in preimplantation embryo development and include some of the STAT1-independent pathways that are activated by IFN-γ, e.g. MAP kinase and PI3-kinase (Zhang et al., 2007).

Interestingly, the PI3-kinase/Akt pathway is known to be important mediator of anti-apoptotic survival signals in the preimplantation embryo (Zhang et al., 2007). In the present study, a strong negative correlation was found to exist between the percentage of embryos cavitated and the percentage of embryos arrested, which was suggestive of improved survival. Therefore, it seemed that 10 and 1000 pg/ml rmIFN-γ treated groups may have had improved survival, because a lower percentage of arrested embryos were seen in these treatment groups that had the greatest percentage of cavitated embryos and *vice versa*. However, it was impossible to ascertain whether the embryos were cavitated or not upon arrest.

Significantly reduced ICM cell numbers and ICM:TE ratios were seen in the 10 and 1000 pg/ml rmIFN-γ treated groups that also displayed the highest percentage of embryos cavitated and the lowest percentage of embryos arrested. Taken together these data suggest that select, higher physiological concentrations of rmIFN-γ (10 and 1000 pg/ml) have a positive effect on the percentage of embryos cavitated, which is associated with improved survival and reduction in ICM and ICM:TE ratio. Reduced ICM cell number could have occurred for the following reasons; reduced proliferation, increased differentiation or increased apoptosis. Specific assays for each of these eventualities were not conducted therefore definitive conclusions could not be made. However, no change in total cell number was observed therefore increased ICM apoptosis is unlikely. As such, the reduced ICM:TE ratio is more likely to be due to reduced proliferation of the ICM specifically, or increased differentiation towards the TE cell lineage. A very interesting recent study conducted by Lu et al. (Lu et al., 2008a) found that activation of the Ras-MAPK/ERK1/2 pathway is highly important for Cdx2 expression and TE differentiation from *in vitro* cultured embryonic stem cells, and that it is asymmetrically expressed at the apical membrane of the 8-cell embryo. In addition activation of PKC isoforms in the preimplantation embryo has been shown to contribute to the regulation of cavitation (Eckert et al., 2004a; Eckert et al., 2004b). It is possible, therefore, that IFN-γ-mediated activation of the PKC and Ras-MAPK/ERK1/2 pathways induced precocious and/or increased TE cell differentiation, which lead to reduced cell number in the ICM compartment.

To conclude, in the present study select higher doses of rmIFN-γ (10 and 1000 pg/ml) were found to increase the maximum percentage of cavitated embryos and to reduce the ICM:TE ratio in the blastocyst, which was specifically due to a reduction in ICM cell number. It is possible that these effects were due to IFN-γ-mediated

modulation of signalling pathways (e.g. Ras-MAPK/ERK1/2, PI3-K/Akt, PKC) that are important for preimplantation development. An effect on survival potentially being mediated by PI3-K/Akt; and TE differentiation mediated by PKC and Ras-MAPK/ERK1/2 is possible. The results obtained for the 100 pg/ml rmIFN-γ treatment group are curious because they are similar to the control group for every parameter examined, and therefore do not fit in with the dose-dependent pattern that was observed with the other treatment groups. It is interesting that this 100 pg/ml rmIFN-γ is the same as the IFN-γ concentration found in mouse uterine fluid at oestrus and in oestrus mouse uterus homogenate (Ashkar and Croy, 1999; Orsi et al., 2007). Taken together, this leads me to suggest that there may be an 'optimal' IFN-γ concentration provided by the maternal reproductive tract. Furthermore, it should be born in mind any IFN-γ signalling that occurs in the preimplantation embryo is subject to interaction and cross-regulation by the many different factors present within the complex milieu of the uterine environment.

4 The maternal response to lipopolysaccharide administration

4.1 Introduction

Lipopolysaccharide (LPS) is an abundant and integral constituent of the outer membrane of Gram-negative bacteria. It is released from the membrane when bacteria divide, die and lyse, and is therefore liberated into host tissues during a Gram-negative bacterial infection (Rietschel et al., 1994). It is widely acknowledged that LPS is of prime importance in host recognition of infection and that it is a highly potent activator of the immune response, even in the absence of other bacterial components (Akira et al., 2006; Van Amersfoort et al., 2003). Intravenous (i.v.) or intraperitoneal (i.p.) injection of LPS triggers an acute systemic inflammatory response that fully mimics the initial phase of a genuine systemic Gram-negative bacterial infection (Beutler, 2004; Dantzer, 2004). Indeed, LPS has been used for decades to model systemic inflammation and sepsis (Beutler, 2004).

Very minor inflammatory responses that occur within a particular tissue often remain localised and do not evoke a systemic response. In contrast, more severe tissue inflammation and inflammation directly within the circulation (as with i.v. and i.p. injection of LPS), evoke an acute systemic inflammatory reaction known as the acute phase reaction (APR). LPS is recognised by Toll-like receptor (TLR) 4, which is expressed predominantly by cells of the innate immune system (Fujihara et al., 2003; Poltorak et al., 1998). Of these, antigen presenting cells (APCs; i.e. macrophages and dendritic cells, DCs) are particularly important because they release a wide range of inflammatory mediators that induce most features of the APR; these include: TNF-α, IL-1β, IL-6, IL-10, IL-12, MIP-1 α/β, MCP-1, RANTES, G-CSF, GM-CSF, platelet activating factor (PAF), prostaglandins, reactive oxygen species (ROS), nitric oxide (NO) and proteases (Fujihara et al., 2003). The APR lasts 1-2 days and encompasses a wide range of responses that reflect the host's concerted effort to neutralise the pathogenic stimulus and subsequently restore homeostasis (Dantzer, 2004). Major features of the APR include; production of hepatic acute phase proteins (APPs), increased adrenocorticotropic hormone (ACTH) and glucocorticoid (GC) release, catabolism of muscle and adipose tissue, leukocytosis, fever, display of sickness

behaviour (e.g. lethargy, somnolence, anhedonia, impaired cognitive function, anorexia and adipsia) and reduced body weight (Ceciliani et al., 2002; Dantzer, 2004).

The features of an APR provide focus for the quantitative assessment of immune activation, and many are relatively straight forward to assess, e.g. weight loss. Many studies involving the systemic administration of LPS have measured circulating cytokine concentrations, body weight, food and water consumption and locomotor/exploratory activity (frequently by open field test). Teeling et al. (Teeling et al., 2007) conducted an open field test 2 hours after i.p. administration of 1, 10 and 100 µg/kg LPS (*Salmonella abortus* equi) to mice and reported reduced levels of activity. Also in this study, circulating concentrations of the major proinflammatory cytokines were found to be elevated compared with control three hours after i.p. injection of 100 µg/kg LPS. In another study, Ogimoto et al. (Ogimoto et al., 2006) injected mice i.p. with 50 µg/kg LPS (*Escherichia coli* serotype 055:B5) and examined its effects on food and water consumption, and body weight. When compared with vehicle injected controls at 20 hours post-injection, LPS injected mice exhibited reductions in all of the aforementioned parameters: food consumption was 63% of control, water consumption was 58% of control and a loss of ~1.4 g of body weight was observed.

Here in this part of the study I wanted to assess the extent of maternal immune activation following LPS administration on the first day of gestation (GD 0.5). Thus, female mice were injected i.p with 0 (saline only), 10, 50 or 150 µg/kg LPS (*Salmonella enterica enteritidis*) on GD 0.5, and subsequently underwent the following assessments: open field test at 1 hour post-injection to examine locomotor and exploratory activity; measurement of food and water consumption every 24 hours for the first 5 days (GD 0.5-5.5) to assess the extent of anorexia and adipsia; measurement of body weight every 24 hours for the first 5 days (GD 0.5-5.5) and also once in late gestation (GD 15.5) to determine any longer lasting effects on maternal weight gain. In addition, serum samples were collected every 24 hours for the first 3 days (GD 0.5-3.5) to track maternal serum cytokine concentrations using multiplex technology.

4.2 Methods

4.2.1 Natural mating and LPS administration

Female MF1 mice (controlled for age and weight) were naturally mated with MF1 males (section 2.2.1). Successfully mated females (vaginal-plug positive) received a dose of either normal saline (0.85% Sodium chloride, control) or one of three LPS (*Salmonella enterica enteritidis*, Sigma) doses between 1400 and 1600 hours on the day of plug (GD 0.5). LPS doses of 10, 50 and 150 µg/kg body weight were administered by i.p. injection of 100 µl volumes (section 2.2.2).

4.2.2 Assessment of the maternal response to LPS

These females were subjected to a variety of assessments in order to characterise maternal response to the injection of saline (control) or LPS dosages. One subset of females was assessed for sickness-related behaviours and body weight change. The other subset of females was instead sacrificed to allow the collection of blood for analysis of serum cytokine concentrations using a multiplex cytokine assay kit (section 2.2.3.3).

4.2.2.1 Sickness behaviour and body weight

At 1 hour post-injection mice underwent an open field test (section 2.2.3.1) as a quantitative measure of sickness behaviour. Each mouse was individually placed in the centre of the open field arena and the distance travelled, time spent resting, number of jumps and number of rears was recorded. Maternal body weight, and food and water consumption was also assessed. Mice were weighed at the time of injection and at 24, 48, 72, 96 and 120 hours post-injection (GD 0.5-5.5). In addition, the mice were weighed in late gestation, at 360 hours post-injection (GD 15.5). The chow and tap water bottle of each mouse were also weighed at the time of injection and at 24, 48, 72, 96 and 120 hours post-injection (GD 0.5-5.5). Mice had access *ad libitum* to chow and tap water throughout all assessments, apart from the 3 minute duration of the open field test.

4.2.2.2 Collection of blood and multiplex cytokine assay

Female mice from control and LPS treated groups were sacrificed by cervical dislocation at 3, 24, 48 and 72 hours post-injection. Maternal blood samples were taken by cardiac puncture. The clotted blood samples were centrifuged at 4ºC, at 9,300 ***g*** for 10 minutes and the resulting serum was stored in aliquots at -80ºC until

analysis (section 2.2.3.2). The Bio-Plex Mouse Cytokine 23-Plex Panel kit (Bio-Rad) was used to detect and quantify 23 cytokines simultaneously, in each serum sample. The multiplex assay plates were read on a Luminex 100 instrument and results were analysed using StarStation software (section 2.2.3.3).

4.2.3 Statistics

All statistics (section 2.5) were carried out using SPSS statistical software (SPSS, version 14). All data were analysed with a LSD one-way ANOVA. Pearsons correlation analysis was used to assess the degree of association between increasing LPS dosages and relevant maternal parameters of interest.

4.3 Results

4.3.1 Sickness behaviour: maternal open field

A noticeable reduction in open field activity was evident in LPS treated mice at 1 hour post-injection (Fig. 4.1). Compared with saline-injected controls, LPS treated mice displayed a 28% ($P \leq 0.1$; 10 and 150 µg/kg) or 36% ($P \leq 0.05$; 50 µg/kg) reduction in distance travelled (Fig. 4.1a). Control mice rested for 52% of the duration of the open field test, whereas 10 µg/kg LPS treated mice rested for 59% of the time and 50 and 150 µg/kg LPS treated mice for 63% and 61% of time respectively ($P \leq 0.05$; Fig. 4.1b). Mice treated with 10 µg/kg LPS reared 31% less than control mice, while 50 and 150 µg/kg LPS treated mice reared 35% and 37.5% less respectively, than control mice ($P \leq 0.1$; Fig. 4.1c). Of all parameters tested in the open field, LPS administration affected jumping activity to the greatest extent (Fig. 4.1d). LPS treated mice jumped 90-95% less ($P \leq 0.05$) than saline-injected control mice.

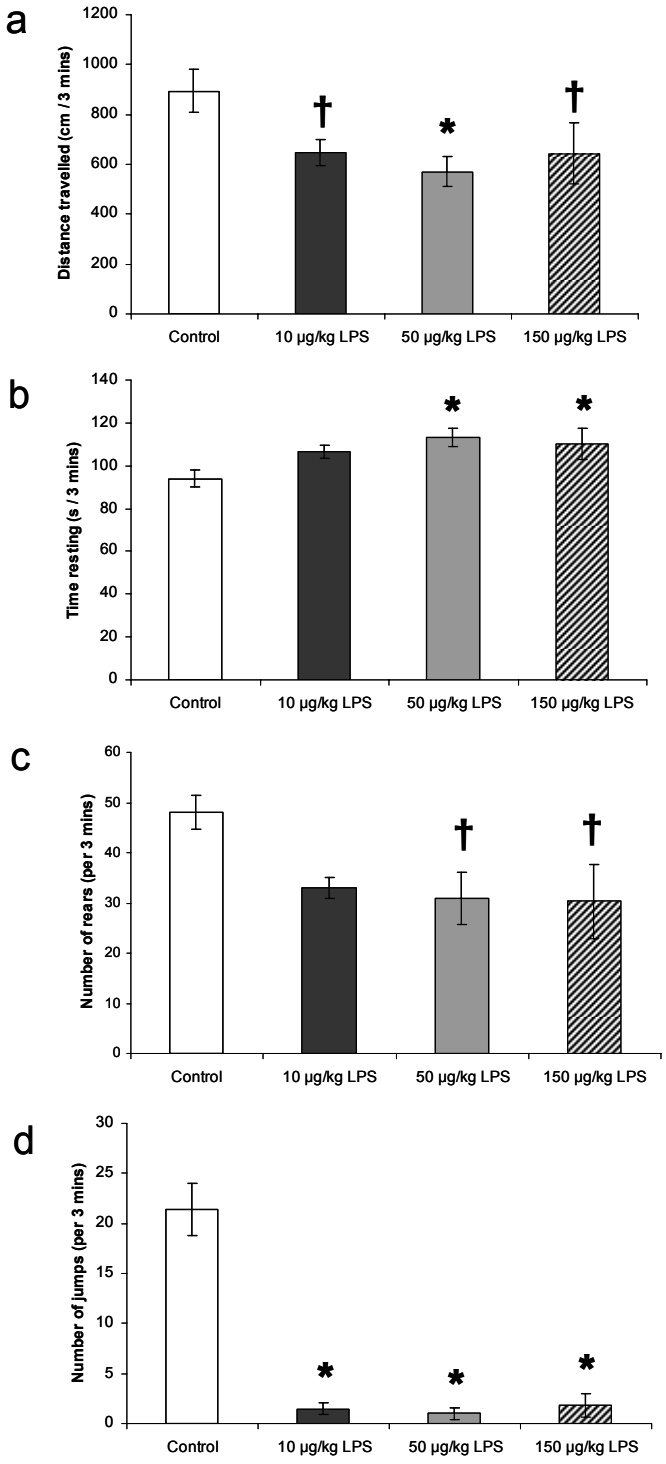


Figure 4.1 Assessment of maternal sickness behaviour: A noticeable reduction in open field activity was evident in LPS treated mice at 1 hour post-injection. Compared with saline-injected controls, LPS treated mice; travelled a shorter distance (**a**), spent more time resting (**b**), reared less (**c**) and jumped less (**d**). Values are mean±SEM; *P≤0.05; † P≤0.1. n=5-7 per treatment group.

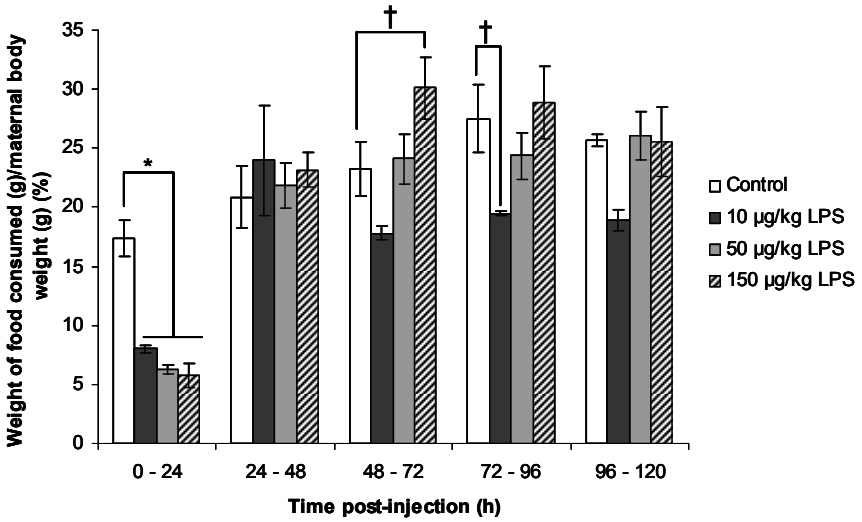
4.3.2 Sickness behaviour: maternal food consumption

In the first 24 hours post-injection (GD 0.5–1.5), LPS treated mice had significantly ($P \leq 0.05$) reduced food consumption when compared with saline-injected controls (Fig. 4.2a). Thus, between 0 and 24 hours post-injection, saline-injected control mice consumed 1.7-fold more food than 10 µg/kg LPS treated mice ($P \leq 0.05$) and, 2.5- and 2.8-fold more than 50 and 150 µg/kg LPS treated mice, respectively ($P \leq 0.05$; Fig. 4.2a). Females entering the experiment were controlled for body weight (all females weighed between 27 g and 33 g) but nevertheless, in order to ensure that small variations in body weight were not influencing food consumption, the amount consumed in each 24 hour period post-injection was expressed in relation to pre-injection maternal body weight (Fig. 4.2b). Here, LPS treated mice had significantly ($P \leq 0.05$) reduced food consumption compared with saline-injected controls between 0 and 24 hours post-injection (Fig. 4.2b). Food consumption in all treatment groups (including saline-injected controls) increased between 24 and 48 hours post-injection (GD 1.5–2.5) compared with the previous 24 hours (Fig. 4.2a,b). Between 24 and 48 hours post-injection food consumed by control mice increased 1.2-fold, and food consumed by 10, 50 and 150 µg/kg LPS treated mice increased 3-fold, 3.5-fold and 4-fold, respectively. Food consumption between 24 and 48 hours was similar for all treatment groups (21-24% of pre-injection body weight). Patterns of food consumption were also similar between treatment groups from 48 to 120 hours post-injection (GD 2.5-5.5), except for a trend ($P \leq 0.1$) in 150 µg/kg LPS treated mice consuming more at 48-72 hours post-injection and 10 µg/kg LPS treated mice less at 72-96 hours post-injection compared with controls (Fig. 4.2a,b). Cumulative food consumption is shown in Fig. 4.2c. Here, consumption by control mice was increased compared to all LPS treated groups equally at 24 and 48 hours post-injection.

a

	Weight of food (g) consumed at the following times post-injection:				
	0 -24 h	24 – 48 h	48 – 72 h	72 – 96 h	96 – 120 h
Control	4.7 ± 0.38	6.2 ± 0.77	6.9 ± 0.77	8.1 ± 0.15	7.1 ± 0.79
10 µg/kg LPS	2.7 ± 0.25*	7.4 ± 1.57	5.6 ± 0.00	5.8 ± 0.15†	5.9 ± 0.38
50 µg/kg LPS	1.9 ± 0.08*	6.4 ± 0.59	7.1 ± 0.54	7.1 ± 0.44	7.7 ± 0.59
150 µg/kg LPS	1.7 ± 0.29*	6.6 ± 0.40	8.6 ± 0.81	8.3 ± 0.91	7.4 ± 0.92

b



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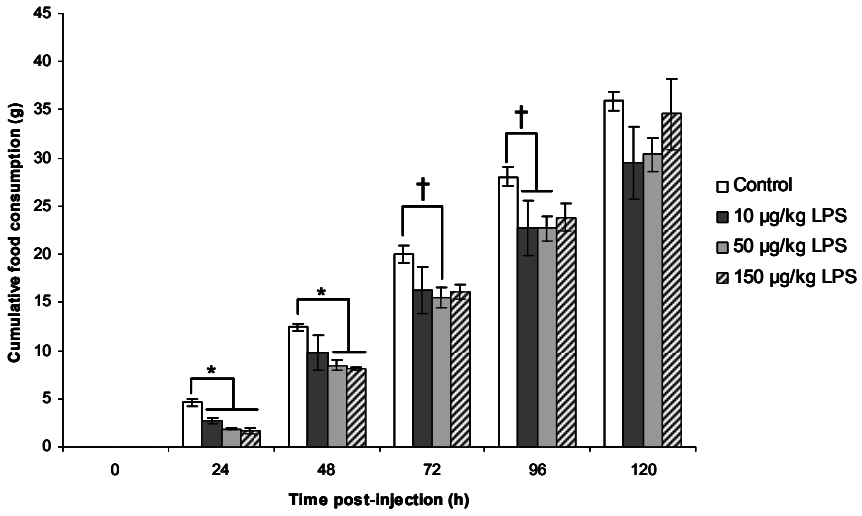


Figure 4.2 Maternal food consumption following i.p. injection of saline or LPS doses: (**a**) raw weight of food consumed during each 24 hour period post-injection, (**b**) food consumption normalised to pre-injection body weight for each 24 hour period post-injection (%), (**c**) cumulative weight of food consumed at each 24 hour time point post-injection. Values are mean±SEM; *P≤0.05; † P≤0.1. n=5-7 per treatment group.

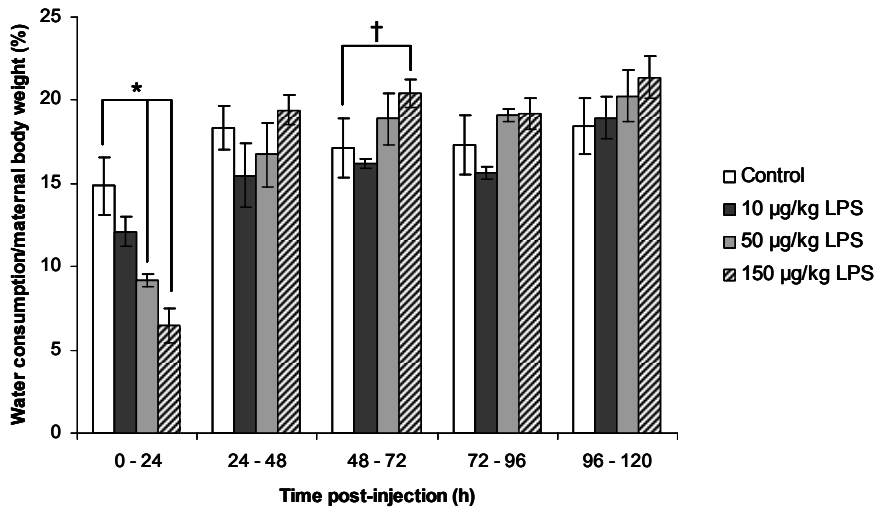
4.3.3 Sickness behaviour: maternal water consumption

Maternal water consumption is shown in Fig. 4.3. Raw volume of water consumed was significantly reduced ($P \leq 0.05$) in 50 and 150 µg/kg LPS treated mice versus controls at 24 hours post-injection (Fig. 4.3a). Thereafter, raw water volume consumed was similar between groups. Water consumption/maternal body weight (%) was similarly reduced in 50 and 150 µg/kg LPS treated mice versus controls at 24 hours post-injection before stabilising across groups thereafter (Fig. 4.3b). Cumulative water consumption was also reduced at 24 hours post-injection in the 50 and 150 µg/kg LPS treated mice versus control (Fig. 4.3c).

a

	Volume of water (ml) consumed at the following times post-injection:				
	0 -24 h	24 – 48 h	48 – 72 h	72 – 96 h	96 – 120 h
Control	4.4 ± 0.56	5.0 ± 0.58	5.1 ± 0.56	5.1 ± 0.55	5.5 ± 0.53
10 µg/kg LPS	3.7 ± 0.26	4.7 ± 0.52	4.8 ± 0.09	4.8 ± 0.21	5.8 ± 0.28
50 µg/kg LPS	2.7 ± 0.06*	4.9 ± 0.49	5.9 ± 0.18	5.9 ± 0.27	6.0 ± 0.41
150 µg/kg LPS	1.9 ± 0.30*	5.5 ± 0.24	5.8 ± 0.23†	5.5 ± 0.23	6.1 ± 0.32

b



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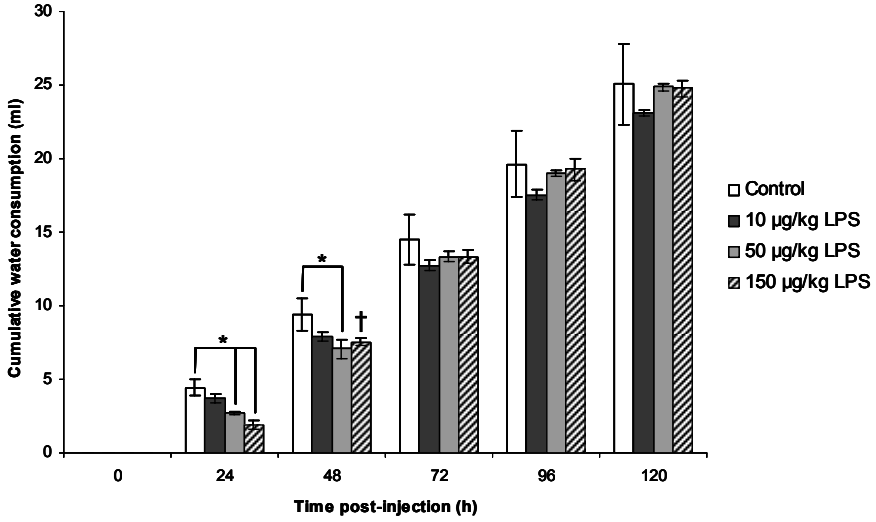


Figure 4.3 Maternal water consumption following i.p. injection of saline or LPS doses: (**a**) raw volume of water consumed during each 24 hour period post-injection, (**b**) water consumption normalised to pre-injection body weight for each 24 hour period post-injection (%), (**c**) cumulative volume of water consumed at each 24 hour time point post-injection. Values are mean±SEM; *P≤0.05; † P≤0.1. n=5-7 per treatment group.

4.3.4 Maternal body weight

Maternal body weight following treatment is shown in Fig. 4.4. These were similar between groups up to 120 hours post-injection except for a significant reduction in 150 µg/kg LPS treated mice at 24 and 48 hours post-injection (P≤0.05) and a trend reduction at 72, 96 and 120 hours post-injection (P≤0.1). By late gestation (360 hours post-treatment, GD 15.5), mice from 10 and 50 µg/kg LPS treatment were heavier than saline-injected control mice (P≤0.1; Fig. 4.4).

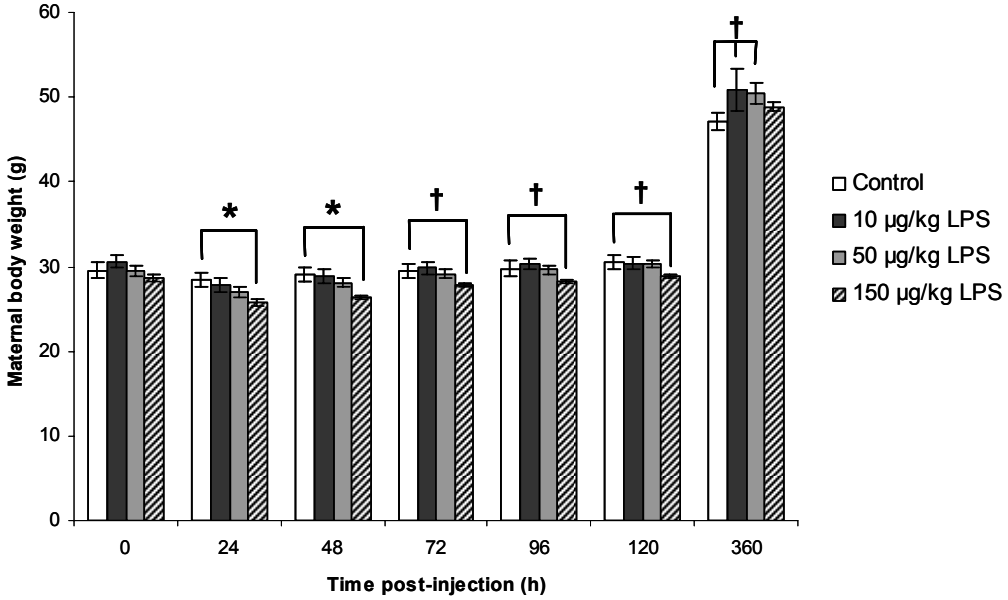


Figure 4.4 Maternal body weight following i.p. injection of saline or LPS doses. Reduction in maternal body weight was evident in LPS treated mice at 24 hours post-injection (GD 1.5), significantly so (P<0.05) in 150 µg/kg LPS treated mice. Regain of maternal body weight had begun by 48 hours post-injection (GD 2.5) and was slowest in 150 µg/kg LPS treated mice. By late gestation (360 hours post-injection, GD 15.5), maternal body weight was increased (P<0.1) in 10 and 50 µg/kg LPS treated mice when compared with saline-injected controls. Values are mean±SEM; *P≤0.05; † P<0.1. n=5-7 per treatment group.

Post-injection maternal body weight was normalised to pre-injection maternal body weight by expressing the former as a percentage of the latter (Fig. 4.5). This showed that all groups initially lost weight over ~96 hours before regaining their post-injection body weight. This weight loss was enhanced in all LPS treated groups. Thus, at 24 and 48 hours post-injection, control mice had a significantly greater body weight ratio compared to all LPS treated groups (P≤0.05).

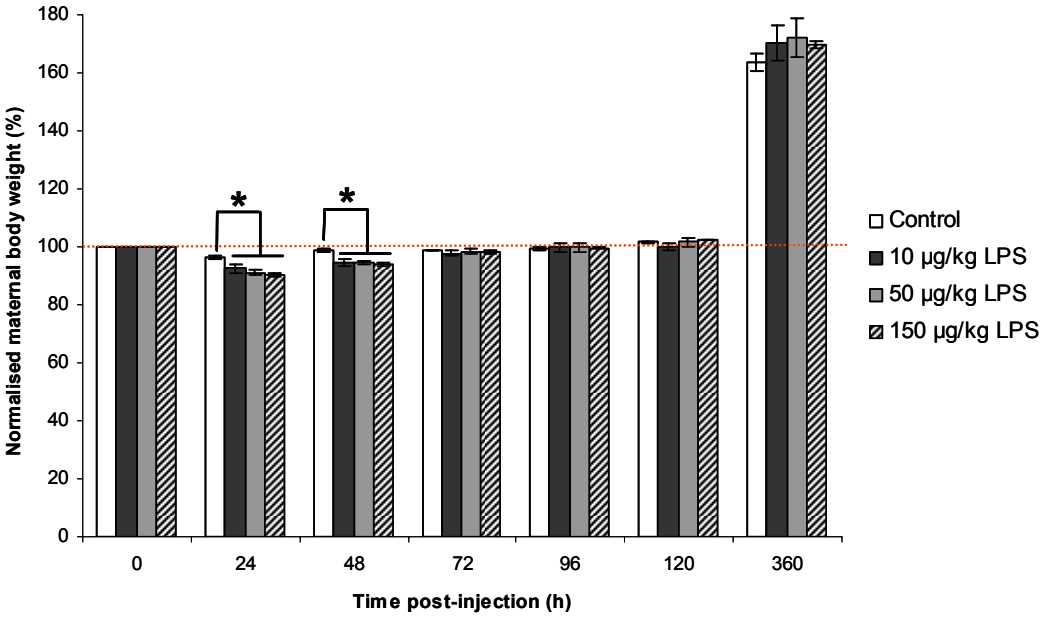


Figure 4.5 Normalised maternal body weight following i.p. injection of saline or LPS doses (%). Maternal body weight was reduced (P<0.05) in all LPS treated groups at 24 and 48 hours post- injection (GD 1.5 and 2.5) when compared with saline-injected controls. Regain of body weight was evident from 48 hours (GD2.5) post-injection and mice from all treatment groups regained their pre-injection body weight by 96 hours (GD4.5) post-injection. Dashed orange line indicates pre-injection body weight. Values are mean±SEM; *P≤0.05. n=5-7 per treatment group.

4.3.5 Maternal serum cytokines

I will first characterise the general features of the cytokine profiles observed before focusing on LPS-induced changes in the profiles. The profile of each cytokine over the course of this experiment proved to be quite different between treatment groups (Fig. 4.6). Little change was seen in the serum profile of most cytokines from saline-injected control mice during the course of the experiment, however some cytokines were seen to increase between GD 0.5 and GD 3.5 (3 and 72 hours post-injection; Fig. 4.6). In control mice the following cytokines were increased at GD 3.5 (72 hours post-injection) compared with GD 0.5 (3 hours post-injection); IL-3 (2.2-fold; P=0.081), IL-4 (2.2-fold; P=0.008), IL-12-p40 (2.4-fold; P=0.045), IL-12-p70 (2.4-fold; P=0.076), GM-CSF (1.5-fold; P=0.004), RANTES (2.4-fold; P=0.058), TNF-α (4-fold; P=0.076). A common pattern emerged for the LPS treated groups. Firstly, at 3 and 24 hours post-injection, serum cytokine concentrations from the 10 and 50 µg/kg LPS treated groups were similar to each other, whereas the 150 µg/kg LPS treated group tended to be higher. Secondly, by 48 hours post-injection, serum cytokine concentrations from

all LPS treated groups tended to be similar to one another (Fig. 4.6). In many cases this pattern appeared to be a consequence of 150 μg/kg LPS treatment inducing higher serum cytokine concentrations than 10 and 50 μg/kg LPS treatment initially, and subsequently cytokine concentrations falling more slowly in 150 μg/kg LPS treated mice than in 10 and 50 μg/kg LPS treated mice. Thirdly, this resulted in all LPS treated groups only achieving equivalent serum cytokine concentrations to each other by 48 hours post-injection (Fig. 4.6). Serum concentrations of many cytokines from LPS treated mice reached a peak at 3 hours post-injection, e.g. IL-2, IL-3 and RANTES. Serum concentrations of IL-1α and IL-9 from LPS treated mice reached a peak later, at 24 hours post-injection. It was the case with almost all of the cytokines, that treatment with 150 μg/kg LPS resulted in the highest serum cytokine concentrations at 3 and 24 hours post-injection (Fig. 4.6).

Analysis of the effect of LPS treatment on cytokine profile (versus controls) also showed diverse effects. Thus, in many cases the cytokine concentration was between 2- and 5-fold higher in serum from 150 μg/kg LPS than in serum from saline-injected control mice; for example, at 3 hours post-injection, IL-3 was increased 3-fold ($P=0.002$), IL-4 was increased 2-fold ($P=0.002$) and Eotaxin was increased 5-fold ($P=0.02$; Fig. 4.6). Also at 3 hours post-injection, in serum from 150 μg/kg LPS treated mice, a rather more striking increase was seen compared with control in; IL-12-p40 (48-fold; $P=0.001$), MCP-1 (58-fold; $P=0.004$), and RANTES (52-fold; $P=0.044$). Interestingly, at 48 hours post-injection, serum concentrations of IL-12-p70 and TNF-α from LPS treated mice were at their highest and LPS treated groups were similar to one another (Fig. 4.6). As stated above, by 48 hours post-injection serum cytokine concentrations from all LPS treated groups tended to be similar to one another. For some cytokines this meant that their concentrations were also similar to control (e.g. IL-4, GM-CSF), but for other cytokines their concentrations were several-fold increased or decreased compared with control. Concentrations of IL-2, IL-10, and IL-13 were all decreased compared with control at 48 hours post-injection. Of these cytokines, IL-2 exhibited the most extreme reduction since 10, 50 and 150 μg/kg LPS treatment groups were less than control by 8-, 34- and 21-fold (all $P\leq0.001$), respectively (Fig. 4.6). In contrast, the concentrations of the following cytokines were increased 2-fold to 4-fold compared with control at 48 hours post-injection ($P\leq0.1$ and $P\leq0.05$); IL-6, IL-12-p70, RANTES and TNF-α (Fig. 4.6).

NB: The multiplex assay was able to detect and quantify the full concentration range of every cytokine at every time point examined, except for G-CSF, KC and IL-6 at 3 hours post injection (discussed below; marked by '**!**' in Fig. 4.6). The data presented here for G-CSF, KC and IL-6 at 3 hours post injection are inaccurate and under-representative of actual concentrations, particularly for the LPS treated groups. In these cases, 40-100% of readings could not be converted to concentrations because the values obtained were above the upper limit of detection of the assay; for example, no G-CSF concentrations were calculated for the 10 µg/kg LPS treatment group at 3 hours post-injection because all samples had readings above the upper limit of detection.

Figure 4.6 (overleaf) Maternal serum cytokine concentrations (pg/ml) at 3, 24, 48 and 72 hours post-injection (GD 0.5-3.5). Graphs for each of the 23 cytokines are displayed in name order; first the interleukins in numerical order and then all others in alphabetical order. Values mean ±SEM; n=5-7 per treatment group.

* P≤0.05 compared with saline-injected control group

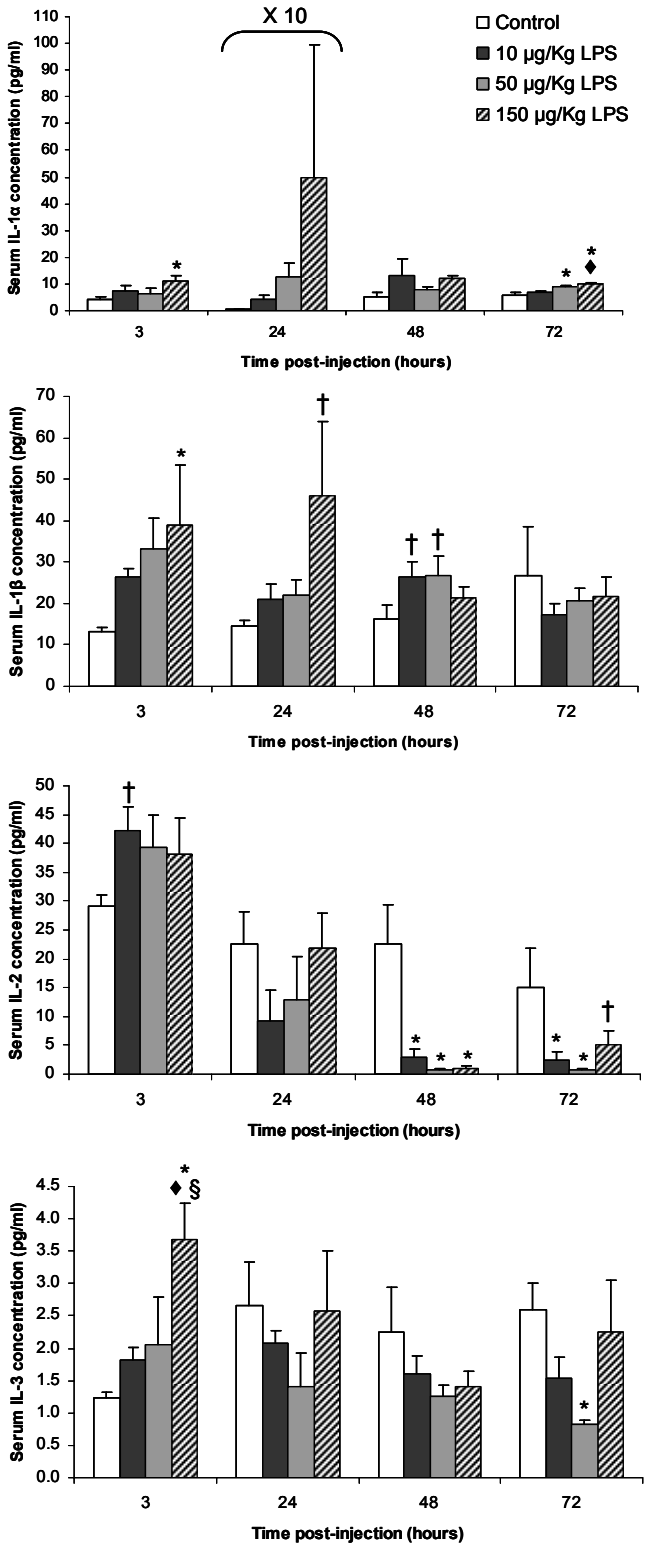
♦ P≤0.05 compared with 10 µg/kg LPS group

§ P≤0.05 compared with 50 µg/kg LPS group

† P≤0.1 compared with saline-injected control group

NB: At certain time points, cytokine concentrations were very high; as such, values at these time points were divided by 10 or 30 to allow all bars to be displayed on the same graph, on the same scale. A horizontal bracket with 'X 10' or 'X 30' indicates that the values for those bars should be multiplied by 10 or 30 to obtain the actual concentration.

! - indicates technical problems with quantification of cytokine concentrations



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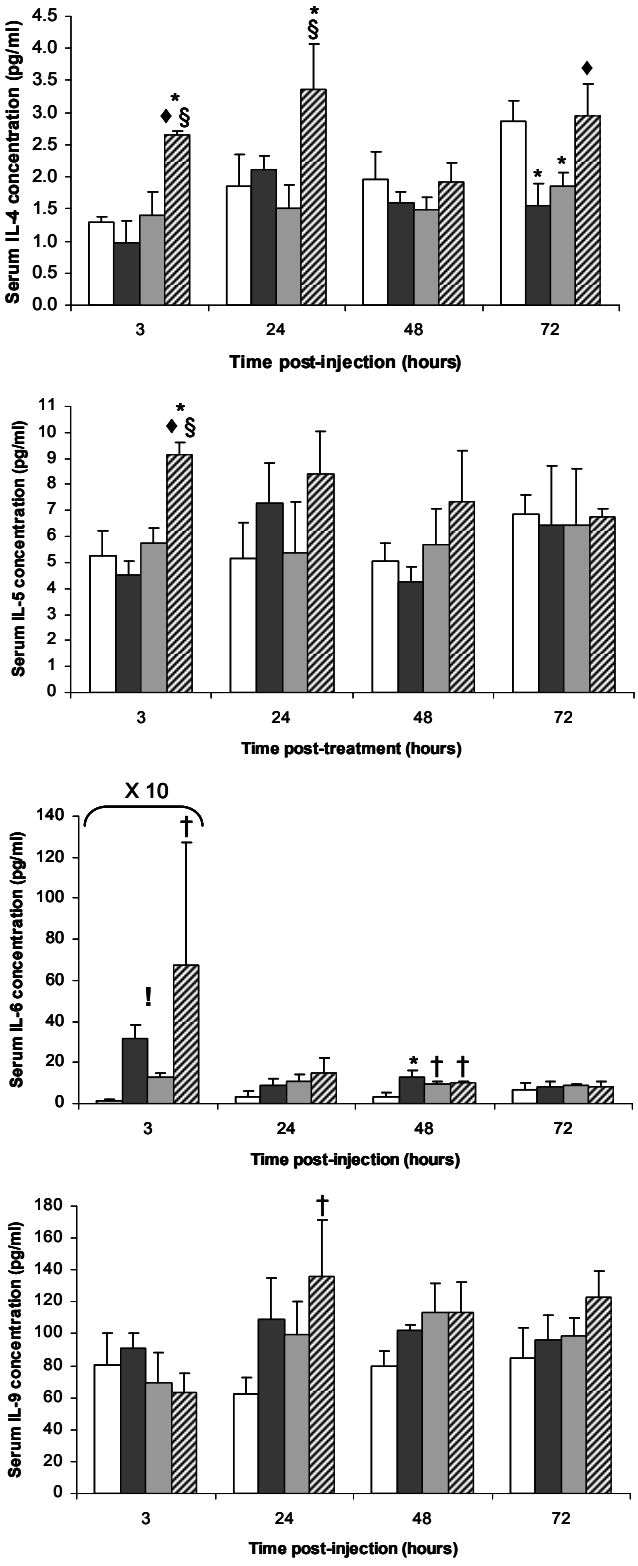

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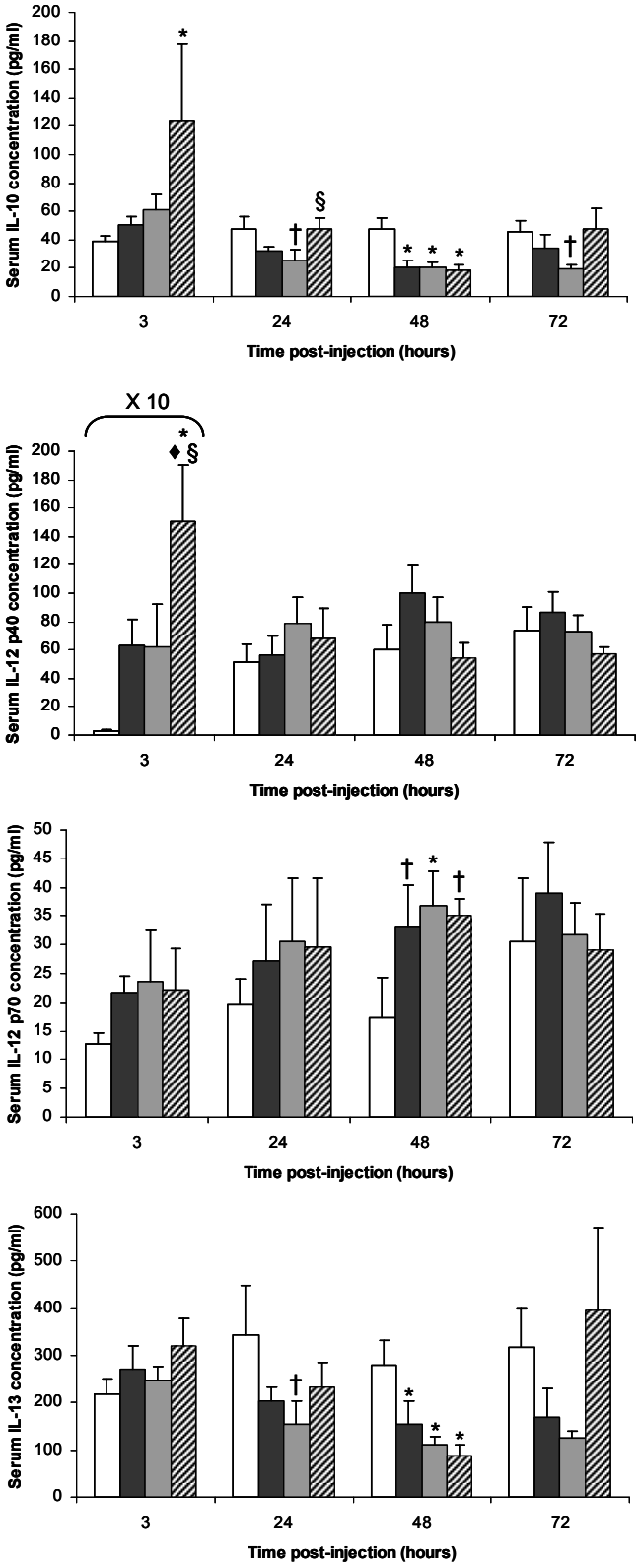

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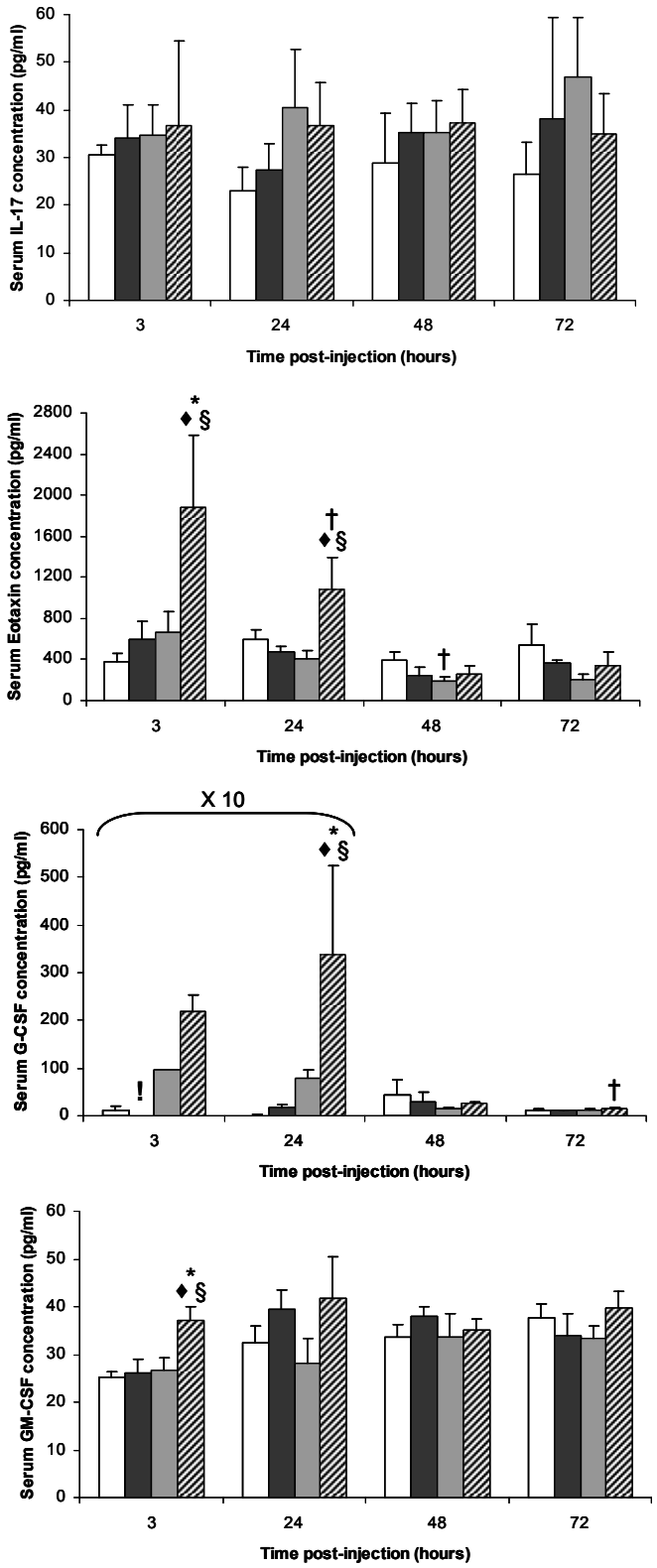

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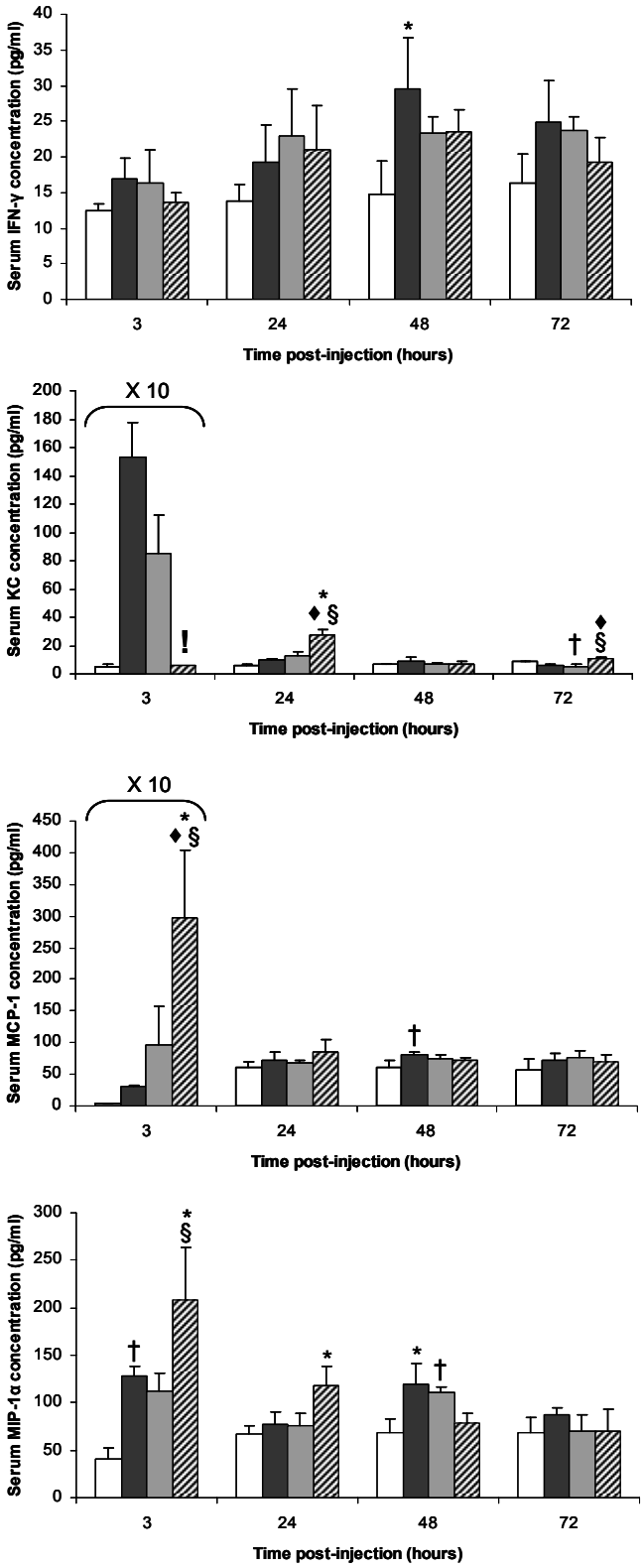

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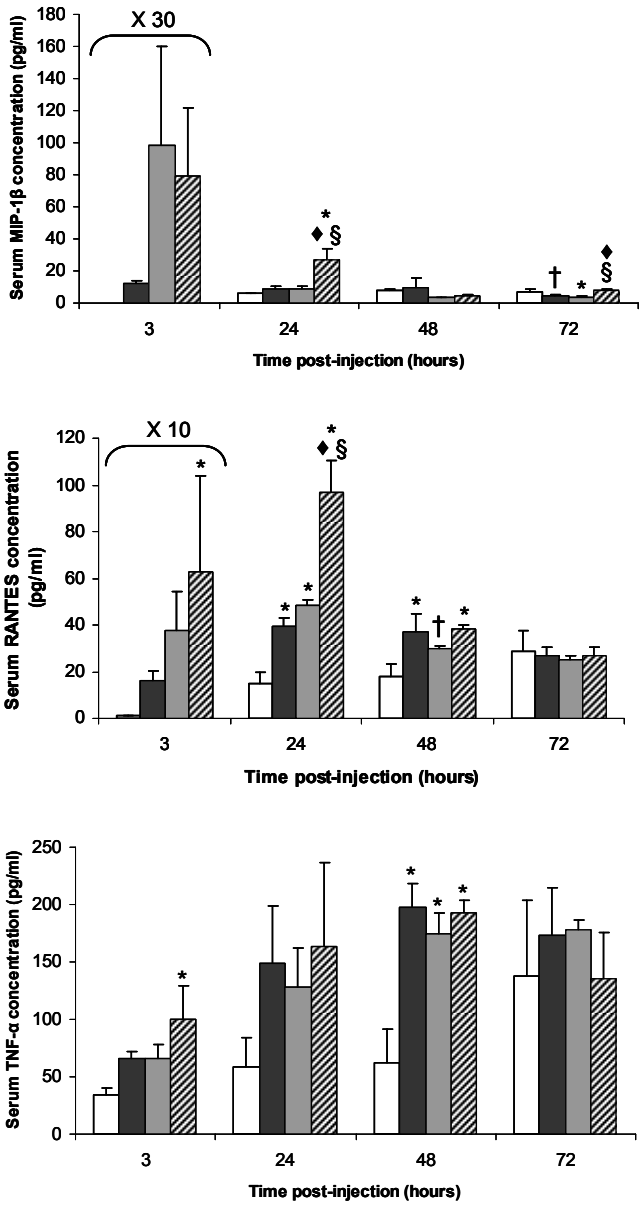


Fig. 4.6 (*end*)

4.4 Discussion

In this part of the study I examined the maternal response to i.p. LPS administration on the first day of gestation (GD 0.5). This, to my knowledge, is the first time that the maternal response to LPS has been studied at such an early stage of gestation. The response to systemic administration of LPS has been well studied in non-pregnant murine models and is known to result in an APR, of which characteristic features include; sickness behaviour, reduced body weight and increased circulating

concentrations of proinflammatory cytokines. My findings here indicate that the maternal APR during very early gestation is comparable to that typically observed in non-pregnant mice following i.p. injection of LPS within a similar dosage range.

As expected, open field activity was found to be reduced at 1 hour post-injection in all LPS treated groups compared with control. LPS treated mice travelled a shorter distance and spent more time resting. Interestingly, jumping in the open field was reduced by 90-95% in the LPS treated mice and was the most severely affected open field parameter assessed in this study. It is perhaps not surprising that jumping was more severely affected given the physically strenuous action involved. All LPS doses reduced open field activity at 1 hour post-injection to a broadly similar level; for example, compared with control, mice treated with 10 µg/kg LPS reared ~31% less and mice treated with 50 and 150 µg/kg LPS reared only ~35% less. Teeling et al. (Teeling et al., 2007) conducted an open field test 2 hours after i.p. administration of LPS (*Salmonella abortus* equi) to mice and reported ~36% less rears in mice treated with 10 µg/kg LPS and ~56% less rears in mice treated with 100 µg/kg LPS. Teeling et al. clearly observed a more pronounced dose-dependent reduction in rearing than that seen in the present study. Open field testing in the present study was conducted at 1 hour post-injection whereas the open field testing in the Teeling study was conducted at 2 hours post-injection, and likely contributes to the difference in results.

The Teeling study also reported increased plasma concentrations of the major proinflammatory cytokines (IL-1β, IL-12-p70, IL-6, IFN-γ, KC, IL-10, TNF-α) three hours after i.p. injection of 100 µg/kg LPS (using a Mesoscale Discovery multiplex kit). In the present study, IL-1β, IL-10 and TNF-α were all found to be increased at 3 hours after injection with LPS, particularly so in the 150 µg/kg LPS group. Although, IL-12-p70 concentrations were increased in LPS treated mice at 3 hours, this was not statistically significant and interestingly, IFN-γ concentrations were not increased. Accurate comparisons cannot be made for circulating G-CSF, KC and IL-6 concentrations due to aforementioned problems with quantification of the very high MFI values obtained. Indeed, it has previously been reported in a study using multiplex cytokine technology (TNF-α, IL-6, MCP-1) that IL-6 concentrations could not be quantified in serum following i.p. LPS injection (1000 µg/kg, *E.coli* K-235), because the MFI measured was much greater than that ascribed to the highest standard (Ao et al., 2005). However, it should be noted that the samples with unquantifiable MFI

values could be rerun at a higher dilution in order to bring them into the range of the standard curve.

Ogimoto et al. (Ogimoto et al., 2006) injected mice i.p. with 50 μg/kg LPS (*Escherichia coli* serotype 055:B5) and examined its effects on food and water consumption, and body weight. When compared with vehicle injected controls at 20 hours post-injection, LPS injected mice exhibited reductions in all of the aforementioned parameters: food consumption was 63% of control, water consumption was 58% of control and a loss of ~1.4 g of body weight was observed (Ogimoto et al., 2006). As expected, the present study showed that LPS administration resulted in reduced food and water consumption and also reduced body weight at 24 hours post-injection. Furthermore, the reductions were quantitatively similar for the 50 μg/kg LPS treated group in the present study as they were for 50 μg/kg LPS treated mice in the Ogimoto study: the corresponding results in the present study were as follows; food consumption was ~38% of control, water consumption was ~61% of control and a loss of ~1.4 g of body weight was observed.

There did not appear to be any clear pattern relating food/water consumption to serum cytokine concentrations. This may at first appear surprising since the brain is known to express receptors for proinflammatory cytokines and furthermore, that both the administration of such cytokines peripherally, and directly into the brain, results in the same sickness behaviours as LPS administration, including anorexia and adipsia. However, in light of some recent studies, the disengagement seen here between food/water consumption and serum cytokine concentrations may not be so unexpected. Pre-treatment of mice with the non-selective cyclooxygenase (COX) inhibitor indomethacin, has been shown to reverse LPS-induced reduction in burrowing activity and to attenuate LPS-induced anorexic actions, without altering the peripheral production of proinflammatory cytokines (Naoi et al., 2006; Teeling et al., 2007). Consequently, it seems that prostaglandins can affect behaviour independently of peripheral cytokines. However, PG levels were not assessed in the current study therefore this association could not be confirmed here.

The concentration of many cytokines is known to change in the maternal circulation as pregnancy progresses, with many beginning to increase substantially at mid-gestation and reaching high levels by late pregnancy (Orsi et al., 2006). For example, Orsi et al. highlighted the following cytokines as increasing markedly in late

pregnancy; TNF-α, IL-1β, IL-2, IL-6, IL-10, IL-12p40, IL-12p70 and IL-18 (Orsi et al., 2006). Interestingly, in the present study, several serum cytokines in control mice were found to increase through preimplantation gestation (i.e. GD 3.5 compared with GD 0.5), these included; IL-3 (2.2-fold; P=0.081), IL-4 (2.2-fold; P=0.008), IL-12-p40 (2.4-fold; P=0.045), IL-12-p70 (2.4-fold; P=0.076), GM-CSF (1.5-fold; P=0.004), RANTES (2.4-fold; P=0.058), TNF-α (4-fold; P=0.076). Thus, the present study echoes the findings in the Orsi study (mentioned above).

In 1993 the Th1/Th2 paradigm of pregnancy was proposed, which essentially stated that successful pregnancy was a Th2 phenomena (Wegmann et al., 1993). Since then however, it has been acknowledged that, despite being a helpful stepping stone in our understanding of the immunology of pregnancy, it is a gross over simplification, as discussed extensively in a review by Gérard Chaouat (Chaouat, 2007). In the present study, the types of cytokines that were seen to increase physiologically during the preimplantation period included, chemokines (RANTES), hematopoietic growth factors (GM-CSF), proinflammatory (IL-12-p40, IL-12-p70, TNF-α) and T helper type 2 (T_{H2}; IL-3, IL-4) cytokines. Thus, the data presented in this chapter further support the idea that the T_{H1}/T_{H2} paradigm is an over simplification of events. Murine endometrial leukocyte infiltration and maternal hyporesponsiveness to paternal MHC antigens is mediated by seminal fluid and involves local alterations in a number of chemokines and cytokines including GM-CSF and RANTES (Johansson et al., 2004; Robertson et al., 1997). Thus, it is possible that the uterus is a source of the systemic GM-CSF and RANTES. TNF-α is reported to be present in the preimplantation uterus and is suggested to have an important role to play in trophoblast invasion of the uterus due to it inducing trophoblastic expression of matrix metalloprotease 9 (MMP9) (Cohen et al., 2006). It is thus possible that the systemic TNF-α could also be from a uterine source. In addition, the uterus could be a source of the IL-12 found in the serum: a moderate expression of IL-12 family members has been found to be necessary for normal uterine receptivity to implantation, whereby it is thought that IL-12 induces IFN production which in turn activates uNK cells to direct appropriate spiral artery remodelling (Ledee-Bataille et al., 2005). Furthermore, it has been shown that during the third trimester of normal human pregnancy peripheral blood monocytes are primed to produce IL-12 (Sacks et al., 2003). The increase in the T_{H2} type cytokines, IL-3 and IL-4, could be mediated by progesterone-induced blocking factor (PIBF). Progesterone is essential for the maintenance of pregnancy and induces the release of

PIBF from lymphocytes during pregnancy, where PIBF acts to skew the T_{H1}/T_{H2} balance in favour of T_{H2} cytokine production (Szekeres-Bartho and Wegmann, 1996).

Patterns of maternal serum cytokine concentrations differed between treatment groups in the days following LPS injection. Injection of LPS, particularly the highest dose (150 μg/kg), induced acute (3 and 24 hours post-injection) increases in maternal serum concentrations of a broad range of cytokines, including; chemokines (e.g. Eotaxin, MCP-1, MIP-1α, MIP-1β, RANTES), hematopoietic growth factors (e.g. G-CSF, GM-CSF), proinflammatory (e.g. IL-1β, TNF-α), T_{H1} (e.g. IL-2) and T_{H2} (e.g. IL-3, IL-4, IL-5, IL-10) type cytokines. Later, at 48 hours post-LPS, concentrations of IL-2, IL-10 and IL-13 were all decreased compared with control, whereas IL-6, IL-12p70, RANTES and TNF-α were all increased compared with control. Interestingly, those that were decreased at 48 hours are predominantly anti-inflammatory T_{H2} type (i.e. IL-10 and IL-13) and those that were increased are predominantly proinflammatory (i.e. IL-6, IL-12p70 and TNF-α). This pattern of serum cytokines could be due in part to cross regulation of cytokine production. For example, IL-10 is known to have potent immunosuppressive effects on macrophages, suppressing the production of proinflammatory mediators (e.g. IL-6, IL-12, TNF-α). Since the IL-10 concentration was reduced at 48 hours, it is possible that there was reduced macrophage suppression which thus created a permissive environment for the release of proinflammatory cytokines from macrophages. Exactly how fluctuations in maternal serum cytokines influence intrauterine cytokine composition is unclear. Indeed, large discrepancies were found between the intrauterine and serum compartments for some cytokines in the mouse during estrus (Orsi et al., 2007). It seems likely therefore, that the compartmentalisation of the uterine lumen 'protects' it from being directly influenced by fluctuations in serum cytokine concentrations. However, it is quite possible that serum cytokine concentrations have an indirect effect on uterine luminal composition and could thus potentially alter preimplantation embryo development. The composition of the luminal fluid is impossible to ascertain in the present study because uterine fluid was not collected, but it should be noted that LPS treatment did affect blastocyst cell lineage allocation (as discussed in Chapter 5).

In this chapter, I have demonstrated activation of the maternal immune response resulting from i.p. injection of 10, 50 and 150 μg/kg LPS. Furthermore, this maternal immune response during very early gestation is comparable to that typically observed in non-pregnant mice following i.p. injection of LPS within a similar dosage range.

5 Effect of maternally administered LPS during the preimplantation period on development of the blastocyst and fetus

5.1 Introduction

The preimplantation mammalian embryo is well known to be sensitive to alterations in its surrounding environment (Watkins et al., 2008a). Evidence from both *in vitro* and *in vivo* studies indicate that altered preimplantation embryo environment can affect not only the immediate events of blastocyst formation, but can also give rise to long-term phenotypic consequences during fetal and/or postnatal life (Fleming et al., 2004a; Watkins et al., 2008a). Furthermore, certain lines of evidence suggest that blastocyst cell number is indicative of implantation potential (Richter et al., 2001), fetal development (Lane and Gardner, 1997) and postnatal phenotype (Kwong et al., 2000; Watkins et al., 2007). Implantation potential of human blastocysts following assisted reproductive technology (ART) was found to be improved if blastocysts had larger ICMs (presumably comprising more cells) (Richter et al., 2001). Lane and Gardner (Lane and Gardner, 1997) found a significant positive correlation between fetal development and blastocyst and ICM cell number, whereas interestingly, there was no relationship with TE cell number. Kwong et al. (Kwong et al., 2000) showed, in a rat model, that maternal low protein diet during preimplantation development led to reduced ICM and TE cell number in the mid-late blastocyst and also to altered postnatal phenotype, including; reduced birth weight, increased growth, increased systolic blood pressure and altered organ/body weight ratios for the liver and kidneys. In another study, *in vitro* culture of mouse embryos was found to reduce TE and ICM cell number in the blastocyst and subsequent to embryo transfer, resulted in increased offspring systolic blood pressure postnatally (Watkins et al., 2007).

Maternal infection and inflammation specifically during early gestation has been associated with increased rates of abortion in human and animal studies. A human birth cohort study found that bacterial vaginosis was associated with increased risk of miscarriage in the first trimester but did not affect conception rates in women undergoing *in vitro* fertilisation (Ralph et al., 1999). Furthermore, in an animal study, female mice had a 54% abortion rate following maternal infection with the parasite *Toxocara canis* on the first day of gestation (Reiterova et al., 2003). In support of this

finding, Deb et al. (Deb et al., 2004) administered a range of LPS (*Salmonella Minnesota* Re-595) doses i.p. to pregnant female mice on the first day of gestation and found that 250 µg/kg LPS caused total embryonic loss and that 50, 100 and 200 µg/kg LPS resulted in 52%, 56% and 86% embryonic loss, respectively. Very little else is known about the effects of maternal immune activation during very early gestation on pre-and post-implantation embryo development. Thus, in this part of the study I sought to characterise the phenotype of blastocysts and fetuses from mothers that received an immune challenge during preimplantation development. Pregnant female mice were injected i.p with 0 (saline only), 10, 50 or 150 µg/kg LPS (*Salmonella enterica enteritidis*) on GD 0.5. To assess blastocyst phenotype, uteri of females from each treatment group were flushed at GD3.5, whereupon blastocysts were differentially labelled to allow quantification of cell number in the TE and ICM lineages. Post-implantation fetal development was assessed in each treatment group by GD17 fetal dissection where placenta, yolk sac, fetus and a selection of fetal organs were weighed.

5.2 Methods

5.2.1 Natural mating and LPS administration

Female MF1 mice (controlled for age and weight) were naturally mated with MF1 males (section 2.2.1). Successfully mated females (vaginal-plug positive) received a dose of either normal saline (0.85% Sodium chloride, control) or one of three LPS (*Salmonella enterica enteritidis*, Sigma) doses between 1400 and 1600 hours on the day of plug (GD 0.5). LPS doses of 10, 50 and 150 µg/kg body weight were administered by i.p. injection of 100 µl volumes (section 2.2.2).

5.2.2 Embryo collection and differential nuclear staining

Female mice from each treatment group were sacrificed at GD 3.5 whereupon embryos were flushed from uteri and temporarily maintained in equilibrated KSOM. Blastocysts were washed three times before undergoing differential nuclear staining. After mounting, differentially stained blastocysts were viewed under a fluorescence microscope and the numbers of TE and ICM nuclei were counted (section 2.3.1).

5.2.3 GD 17 fetal dissection

At GD 17, female mice from each treatment group were sacrificed. The uterus containing GD 17 fetuses was dissected out and placed in cold PBS on ice. Each

conceptus was weighed and separated into its constituent parts, i.e. placenta, yolk sac, fetus, whereupon each part was weighed. A selection of organs (brain, liver, left and right kidneys) from each fetus was then rapidly dissected out and weighed (section 2.3.2).

5.2.4 Statistics

All statistics (section 2.5) were carried out using SPSS statistical software (SPSS, version 14). All blastocyst data were analysed with a LSD one-way ANOVA test. All GD 17 fetal dissection data were analysed using a multilevel random effects regression model (Kwong et al., 2004; Watkins et al., 2008b).

5.3 Results

5.3.1 Embryo number

The total number of embryos recovered from GD 3.5 uteri did not differ between treatment groups (Table 5.1). Furthermore, the proportion of embryos that were blastocysts did not differ between treatment groups, either in terms of raw number of blastocysts recovered or when normalised as a percentage of the total number of embryos flushed (Table 5.1, bottom row).

	Maternal treatment group (µg/kg LPS)			
	Control	10	50	150
Total number of embryos ±SEM	13.9 ± 1.2	15.3 ± 1.0	12.1 ± 1.3	15.0 ± 0.6
Number of blastocysts ±SEM (% of total)	8.6 ± 1.6 (64.5)	13.3 ± 0.9 (87.0)	8.7 ± 2.3 (65.0)	9.3 ± 3.3 (62.5)

Table 5.1 Embryos flushed from control and LPS-injected mothers at GD 3.5. There were no differences between treatment groups in total number of embryos flushed, number of embryos that were blastocysts or the percentage of embryos that were blastocysts (number of blastocysts as % of total flushed). n=4-8 mothers per treatment group.

5.3.2 Cell number and ICM:TE ratio of blastocysts from control and LPS treated mothers

Blastocysts from the 10 µg/kg LPS treatment group had 10.2% more cells in total than control blastocysts (P=0.003; Fig. 5.1a). In contrast, blastocysts from the 50 and

150 µg/kg LPS treatment groups had fewer cells in total than control, reduced by 11.4% (P=0.025) and 19.5% (P=0.001), respectively (Fig. 5.1a). The increased total cell number in the 10 µg/kg LPS treatment group appeared to be due to an increase in TE cell number specifically, as blastocysts from the 10 µg/kg LPS treatment group had 21.2% more TE cells than control (P<0.001; Fig. 5.1a). TE cell number in blastocysts from the 50 and 150 µg/kg LPS treatment groups was not different from control (Fig. 5.1a). Strikingly, ICM mass cell number was reduced in all LPS treatment groups compared with control; the 10 µg/kg LPS group was reduced by 14.3% (P=0.002) and the 50 and 150 µg/kg LPS groups were both reduced by 33.3% (P<0.001; Fig. 5.1a).

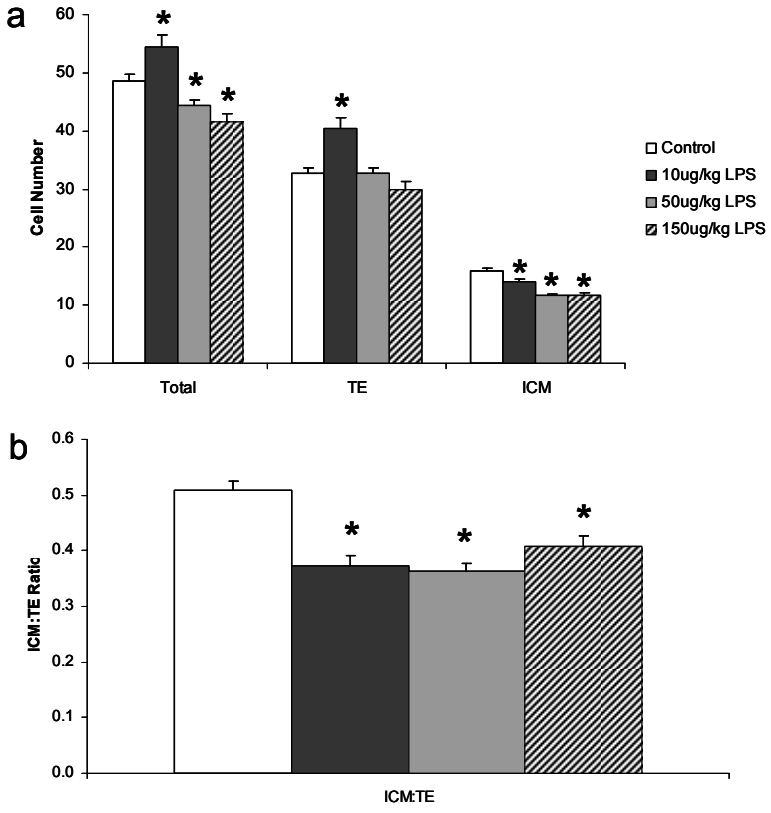


Figure 5.1 (**a**) Cell number and (**b**) ICM:TE cell ratio of blastocysts from control and LPS treated mothers. Values are mean±SEM; *P≤0.05. n= 33-50 blastocysts per treatment group; n=4-8 mothers per treatment group.

The alterations in cell number resulted in reduced ICM:TE cell ratios in all LPS treatment groups compared with control; the 10 µg/kg LPS treatment group ratio was reduced by 37.8%, the 50 µg/kg LPS treatment group ratio was reduced by 41.7% and the 150 µg/kg LPS treatment group ratio was reduced by 24.4% (all groups P<0.001; Fig. 5.1b). The reduced ICM:TE cell ratio in the 50 and 150 µg/kg LPS treatment

groups seemed to be caused by a reduction in ICM cell number specifically. However, in the 10 μg/kg LPS treatment group, the magnitude of the increase in TE cell number was greater than the reduction in ICM cell number, thus the increase in TE cell number is likely to have contributed most to the reduced ICM:TE ratio in this group (Fig. 5.1b).

5.3.3 GD 17 fetal dissection

No differences in mean total fetus number, or mean fetus number in the left or right uterine horn (Table 5.2) were observed between treatment groups.

	Maternal treatment group (μg/kg LPS)			
	Control	10	50	150
Total number of fetuses ±SEM	13.0 ± 0.0	14.0 ± 1.5	14.7 ± 0.9	13.0 ± 0.0
Number of fetuses in left horn ±SEM	6.0 ± 0.6	7.3 ± 0.9	7.0 ± 0.6	6.0 ± 1.0
Number of fetuses in right horn ±SEM	7.0 ± 0.6	6.7 ± 0.7	7.7 ± 1.2	7.0 ± 1.0

Table 5.2 GD 17 litter size; mean total number of fetuses and mean number of fetuses in each uterine horn. No differences in total fetus number, or fetus number in the left or right uterine horn were observed between treatment groups. n=2-3 mothers per treatment group.

Raw weight of the placenta, yolk sac (both Fig. 5.2a), conceptus and fetus (both Fig. 5.2b) did not differ between treatment groups. In addition, no differences between treatments were found for fetal liver, brain, left and right kidneys, either when analysed as raw organ weight (data not shown) or when normalised to fetal body weight (Fig. 5.2c,d).

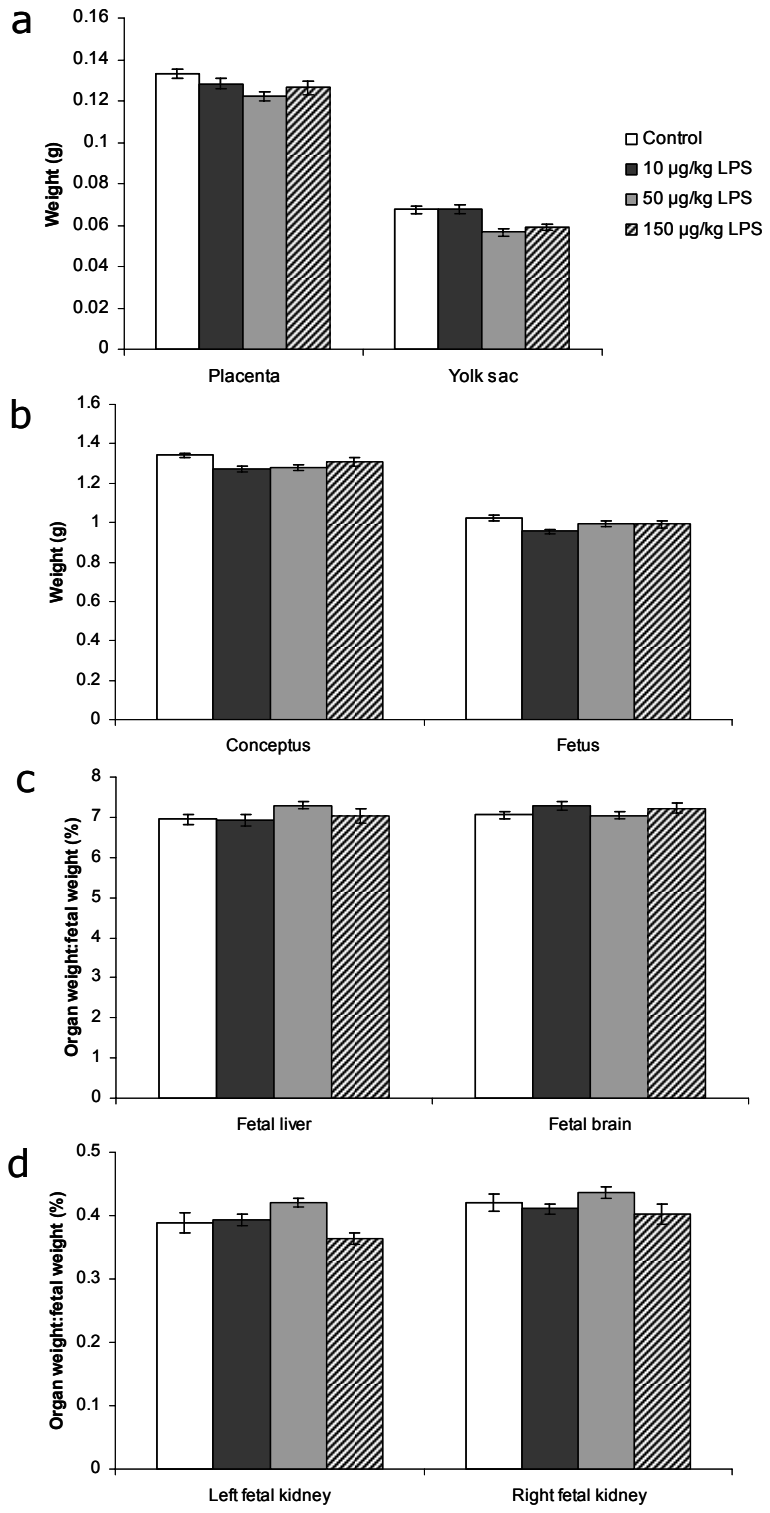


Figure 5.2 (**a**) Weight of GD 17 placenta and yolk sac; (**b**) conceptus and fetus weight at GD 17. Normalised GD 17 fetal organ weight (%), (**c**) fetal liver and fetal brain; (**d**) left and right fetal kidneys. Values are mean±SEM; n= 12-17 fetuses per treatment group; n=2-3 mothers per treatment group.

5.4 Discussion

In this chapter I have shown that administration of 10, 50 and 150 µg/kg LPS to pregnant mice on GD 0.5 resulted in significantly reduced ICM:TE cell ratio in the GD 3.5 blastocyst, but did not affect the number of embryos generated, the GD 17 litter size or GD 17 fetal phenotype, in terms of litter size, weight of conceptus, fetus, placenta, yolk sac, fetal liver, fetal brain or fetal kidneys.

Maternal infection and inflammation specifically during early gestation has been associated with increased rates of abortion in human and animal studies (Deb et al., 2004; Ralph et al., 1999; Reiterova et al., 2003). The study conducted by Deb et al. (Deb et al., 2004) was relevant to the present study because this group administered a similar range of LPS doses i.p. to pregnant female mice on the first day of gestation. They found that 250 µg/kg LPS caused total embryonic loss and that 50, 100 and 200 µg/kg LPS resulted in 52%, 56% and 86% embryonic loss, respectively. In contrast, I found no effect of maternal LPS administered on GD 0.5 on embryonic loss, since numbers of embryos at GD 3.5 were similar to fetal litter size at GD 17. This disparity is in spite of both studies using equivalent LPS doses, administered i.p. at the same time point of gestation and in a mouse model. Additionally, in this study I have confirmed that an inflammatory response is induced in mothers following LPS administration (Chapter 4), therefore the lack of an effect on embryonic loss cannot be attributed to a weak/absent maternal immune response. The major differences between the studies are in the type of LPS and the strain of mouse used: the Deb study used Park strain mice and *Salmonella Minnesota* Re-595 LPS, whereas MF1 mice and *Salmonella enterica enteritidis* LPS were used in the present study.

In the present study, reduced ICM:TE ratio in the 50 and 150 µg/kg LPS treatment groups was due to a reduced ICM cell number specifically, whereas in the 10 µg/kg LPS treatment group the reduced ICM:TE ratio seemed to be a consequence of both increased TE cell numbers and reduced ICM cell numbers. The magnitude of the increase in TE cell number was greater than the reduction in ICM cell number, thus the increase in TE cell number is likely to have contributed most to the reduced ICM:TE ratio in the 10 µg/kg LPS group. This result suggests that proliferation of TE cells was enhanced specifically in the 10 µg/kg LPS treatment group, since TE cell number was unaffected in the 50 and 150 µg/kg LPS treatment groups. Although not statistically significant, the percentage of embryos at the blastocyst stage of development was higher in the 10 µg/kg LPS treatment group (87%) than in the other treatment groups

(62.5-65%). Therefore, I suspect that the TE cells in embryos from the 10 µg/kg LPS treatment group had a greater propensity for proliferation, differentiation and maturation, which lead to increased TE cell number and promoted cavitation, i.e. blastocyst formation.

In contrast to the effects on TE cell number, the present study revealed that ICM cell number was reduced in all LPS treatment groups (vs. control). Similarly, a study in the rat reported a 15% reduction in ICM cell number in early blastocysts from females fed a low protein diet during the preimplantation stage of gestation (Kwong et al., 2000). Furthermore, blastocysts from streptozotocin-induced diabetic rats were found to have reduced cell number which was specifically due to reduced ICM cell number (Pampfer et al., 1997). It is important to note however, that the mechanisms resulting in reduced blastocyst cell number were different in the two studies highlighted above. Blastocyst cell number in the low protein maternal diet model was reportedly reduced due to slowed cellular proliferation rather than apoptosis (Kwong et al., 2000). In contrast, the reduced blastocyst cell number in the maternal diabetes model was due to high levels of cell death within the ICM (Pampfer et al., 1997). In the present study assays for cell death and/or cell proliferation were not conducted, therefore it is not possible to conclude whether the reduced ICM cell number seen in blastocysts from LPS treated mothers was due to increased cell death, reduced proliferation or a combination of the two.

The placenta develops from the TE cell lineage and the entire fetus is derived from the ICM cell lineage, therefore reductions in blastocyst cell number deplete the pool of cells available for subsequent stages of development. In this study administration of 10, 50 and 150 µg/kg LPS to pregnant female mice on GD 0.5 did not alter GD 17 fetal litter size, weight of conceptus, fetus, placenta, yolk sac, fetal liver, fetal brain or fetal kidneys, despite significant decreases in ICM cell number in blastocysts from all LPS groups and increased TE cell number in the 10 µg/kg LPS group. It is interesting to note that the rat low protein maternal diet model also showed no reduction in litter size and no changes in the size of GD 10 deciduum or visceral yolk sac despite reduced blastocyst cell number (Kwong et al., 2000). Experimental reduction of preimplantation embryo cell number resulted in decreased post-implantation viability and caused a delay in the timing of gastrulation and morphogenesis (Hishinuma et al., 1996; Power and Tam, 1993; Tam, 1988). However, interestingly, these studies revealed a remarkable restorative capacity of the fetus, whereby increased

proliferation compensated for earlier losses in blastocyst cell number so that fetal size was comparable to control by approximately GD 10-12 (Hishinuma et al., 1996; Power and Tam, 1993; Tam, 1988). Thus, in the present study, a possible explanation for altered blastocyst cell number without alterations in fetal phenotype could due to a compensatory fetal growth phase prior to GD 17 when the dissections took place. Examination of an earlier post-implantation time point potentially could reveal an effect of maternal LPS treatment.

Overall, the effects of 10-150 µg/kg LPS administered to mothers i.p. on GD 0.5 were subtle; embryo number and implantation capacity were not affected and the altered blastocyst cell number appeared to have been restored by late gestation, since GD 17 fetal phenotype was found to be normal. Other studies have indicated that treatments which result in altered blastocyst cell number can also result in altered postnatal physiology (Kwong et al., 2000; Watkins et al., 2007), thus the altered blastocyst cell number found in the present study may herald alterations in postnatal offspring phenotype. The postnatal consequences of maternal immune activation during very early preimplantation gestation are the subject of the next chapter.

6 Effect of maternally administered LPS during preimplantation development on postnatal growth, behaviour and physiology

6.1 Introduction

Maternal infection and inflammation during gestation can lead to alterations in various aspects of postnatal offspring phenotype, including; behaviour (Limosin et al., 2003; Shi et al., 2003), immunity (Reiterova et al., 2006; Yamaguchi et al., 1983), metabolic status (Nilsson et al., 2001) and hypothalamic-pituitary-adrenocortical (HPA) axis functioning (Reul et al., 1994).

Key evidence linking maternal infection during gestation to behavioural abnormalities in the offspring has come from human epidemiological research carried out over the last 20 years. These studies have indicated that maternal influenza infection during mid-gestation is a significant risk factor for development of schizophrenia in genetically susceptible offspring (Brown, 2006; Limosin et al., 2003). To model maternal infection during mid-late gestation, LPS or the viral mimic PolyI:C (polyriboinosinic-polyribocytidilic acid, a synthetic double-stranded RNA analogue) has been administered to pregnant rodents. In these animal models, activation of the maternal immune system during mid-late gestation lead to behavioural, morphological and pharmacological alterations in the offspring relevant to schizophrenia (Fortier et al., 2007; Zuckerman et al., 2003). These data thus imply that the maternal immune response, rather than a particular pathogen, is the important link between prenatal maternal infection and postnatal behavioural abnormalities.

Studies investigating the effect of maternal immune challenge on offspring immune status have tended to focus on the humoral immune system and have reported contradictory results. Immunisation of female mice with sheep red blood cells (SRBCs; T cell-dependent foreign antigen) at any time point from 2 days before fertilisation to GD 16 induced suppression of anti-SRBC (IgM and IgG) plaque-forming cell (PFC) responses in their offspring, which suggested reduced ability to produce antigen-specific antibody (Yamaguchi et al., 1983). However, it should be noted, that this suppressive effect on anti-SRBC PFC response persisted only until ~15 weeks after birth. In contrast, infection of female mice with the parasite *Toxocara canis* during mid-late gestation (GD 12-18) seemed to be protective for the offspring if they were

infected postnatally (Reiterova et al., 2006). When offspring were postnatally infected (6 weeks of age) with the same parasite that their mothers had received (*T. canis*), offspring displayed reduced production of IFN-γ and IL-5, suppressed eosinophilia, increased levels of IgM and IgG1 and reduced larval recovery from the brain and muscles (Reiterova et al., 2006). The latter study implied that, in response to postnatal infection, increased levels of protective antibodies were produced in offspring from infected dams, which thus reduced the extent of infection without the requirement of a vigorous immune response, i.e. IFN-γ and IL-5 production and eosinophilia. Hodyl et al. (Hodyl et al., 2007) administered 3 repeated doses of LPS (200 μg/kg) to rat dams during mid-late gestation (GD 16, 18 and 20) and reported severely blunted plasma cytokine (IL-1β and TNF-α) production in 19 day old offspring upon offspring challenge with LPS. In contrast, the cytokine response of adult offspring in the Hodyl study was no different to control, but adult female offspring from LPS treated mothers had fewer leukocytes, which the authors attributed to an accelerated rate of immunosenescence.

Alterations in offspring metabolic status and HPA axis functioning have been reported following maternal immune activation during gestation. Nilsson et al. (Nilsson et al., 2001) challenged rats with 3 repeated doses of LPS (790 μg/kg) during mid-gestation and examined aspects of metabolic functioning in the offspring. Adult male offspring displayed obesity and features of the metabolic syndrome, specifically; increased body weight, increased abdominal fat-pad weights, elevated food intake, increased circulating leptin, insulin resistance and increased serum levels of 17β-estradiol and progesterone. In contrast, adult female offspring in the same study appeared to be less affected, exhibiting elevated serum testosterone levels, increased basal corticosterone levels and increased weight of the heart and adrenal glands. In another study, pregnant rats received a single challenge with either LPS (30 μg/kg) or human red blood cells (HRBC) during mid-gestation, and postnatal HPA axis functioning was examined only in the male offspring (Reul et al., 1994). This study reported that adult male offspring from both LPS and HRBC groups displayed increased basal plasma corticosterone levels, whereas after novelty-induced stress, only offspring from the HRBC group showed increased ACTH and corticosterone levels. In addition, decreased levels of mineralocorticoid and glucocorticoid receptors were detected in the hippocampus.

In the majority of the studies discussed above, maternal immune challenge occurred at a post-implantation time point, most frequently during mid- late gestation. Very limited knowledge is available about offspring phenotype following maternal immune challenge during early gestation. In humans, one birth cohort study reported that maternal genital and reproductive infections during periconception increased the risk of schizophrenia in the offspring (Babulas et al., 2006). Another human birth cohort study indicated that the risk of schizophrenia in offspring was increased 7-fold after serologically documented influenza exposure during the first trimester of pregnancy (Brown et al., 2004). In relation to offspring immune status, as mentioned above, immunisation of female mice with SRBC at any time point from 2 days before fertilisation to GD 16 induced suppression of anti-SRBC (IgM and IgG) PFC responses in their offspring (Yamaguchi et al., 1983).

The postnatal consequences of other types of challenge sustained during preimplantation development have been studied, and have established preimplantation development as being a sensitive stage during which a number of postnatal phenotypes may be induced from one genotype. The postnatal consequences of *in vitro* culture have been found to include; altered locomotor and anxiety-related behaviour (Ecker et al., 2004; Fernandez-Gonzalez et al., 2004), altered organ/body weight ratios (Fernandez-Gonzalez et al., 2004) and increased systolic blood pressure (Watkins et al., 2007). *In vivo*, feeding of a low protein maternal diet exclusively during preimplantation development lead to altered birth weight and growth, increased blood pressure and altered locomotor and anxiety-related behaviour in the offspring postnatally (Kwong et al., 2000; Watkins et al., 2008b). Such findings show that preimplantation embryo environment can affect the structure and function of many different body systems.

Here, in this part of the study, I sought to characterise the postnatal phenotype of offspring from mothers that received an immune challenge during preimplantation development. Pregnant female mice were injected i.p with 0 (saline only), 10, 50 or 150 µg/kg LPS (*Salmonella enterica enteritidis*) on GD 0.5 and were allowed to give birth naturally. Offspring were assessed for growth, behaviour and systolic blood pressure at several time points throughout life, and were fully dissected at the time of cull in order to assess organ/body weight ratios. In addition, a subset of offspring was injected with saline or 50 µg/kg LPS prior to sacrifice in order to assess immune status in terms of serum cytokine concentrations and spleen lymphocyte populations.

6.2 Methods

6.2.1 Natural mating and LPS administration

Female MF1 mice (controlled for age and weight) were naturally mated with MF1 males (section 2.2.1). Successfully mated females (vaginal-plug positive) received a dose of either normal saline (0.85% Sodium chloride, control) or one of three LPS (*Salmonella enterica enteritidis*, Sigma) doses between 1400 and 1600 hours on the day of plug (GD 0.5). LPS doses of 10, 50 and 150 µg/kg body weight were administered by i.p. injection of 100 µl volumes (section 2.2.2).

6.2.2 Birth weight and growth

Female mice from each treatment group were allowed to give birth naturally, which took place on GD 19. Litter size, gender and birth weight was recorded. Litters were culled back to six pups per litter; the three median-weight pups of each gender were kept. Offspring were weaned at three weeks of age and were housed with littermates of the same gender thereafter. Offspring were weighed weekly until 30 weeks of age. Mice had *ad libitum* access to chow and tap water throughout (section 2.4.1).

6.2.3 Behaviour

Behaviour (section 2.4.2) was assessed at 4, 5, 6, 8, 11, 14, 17, 20 and 23 weeks of age. At each of these ages mice were tested as follows:

- Day 1 – open field test (afternoon) followed by nesting overnight (sections 2.4.2.1 and 2.4.2.2).
- Day 2 – burrowing (1400-1600 hours) followed by overnight burrowing and consumption of 5% glucose solution (sections 2.4.2.3 and 2.4.2.4).

6.2.4 Systolic blood pressure

Systolic blood pressure (SBP) was measured at 16, 22 and 28 weeks of age by tail cuff plethysmography. Each mouse was allowed to acclimatise to the apparatus for approximately 5 minutes prior to readings being taken. Five SBP readings were taken for each mouse at each of the ages studied and the mean value of the three median readings was taken as the SBP of that mouse at that age (section 2.4.3).

6.2.5 Organ weight and immune status

Mice were sacrificed between 37 and 42 weeks of age. Prior to sacrifice, mice from each litter received either; no treatment, i.p. injection of normal saline (0.85%) or i.p. injection of 50 µg/ml LPS. Two mice (one male and one female) from each litter were assigned to each of these three postnatal treatment groups (section 2.4.4.1). Injected mice (saline and LPS) were culled 3.5 hours post-injection. All mice were killed by cervical dislocation, blood was taken by cardiac puncture and serum was stored at -80ºC. The Bio-Plex Mouse Cytokine 23-Plex Panel kit (Bio-Rad) was used to detect and quantify cytokine concentrations in each serum sample (section 2.2.3.3). Organs and fat pads were dissected out, weighed, snap frozen in liquid nitrogen and stored at -80ºC (section 2.4.4.2). Splenocytes were isolated from the spleens of the injected mice (saline and LPS) by preparation of single-cell suspensions (section 2.4.4.3). Freshly isolated splenocytes were immunostained using combinations of fluorochrome-conjugated monoclonal antibodies (mAbs) against mouse leukocyte subpopulation markers (section 2.4.4.4). Flow cytometry was performed using a FACSCanto instrument and all data were analysed using FACSDiva software (section 2.4.4.5).

6.2.6 Statistics and calculations

All statistics (section 2.5) were carried out using SPSS statistical software (SPSS, version 14). Pre-treatment maternal body weight data was analysed with a LSD one-way ANOVA test. All postnatal data were analysed using a multilevel random effects regression model which takes into account the hierarchical nature of the data set with between-litter and within-litter variation (Kwong et al., 2004; Watkins et al., 2008b). Thus, differences identified between treatment groups are independent of maternal origin of litter and gestational litter size (i.e. all pups, before standardisation of litter size), unless otherwise stated. Body mass index (BMI) was calculated as follows: [Body weight in grams/(body length in centimetres)^2] x 100.

6.3 Results

All results in this section were based on replicates of 6 mothers (i.e. 6 litters) per treatment group. Results for litter size, offspring gender ratio and birth weight (section 6.3.1) were based on non-standardised offspring numbers (i.e. all pups born). Results for all offspring experiments i.e. offspring growth, behaviour, systolic blood pressure and tissue weights (sections 6.3.2-6.3.5) were based on standardised

offspring numbers. Results for offspring immune status were based on selected offspring from standardised litters (section 6.3.6).

6.3.1 Gestational parameters, litter size and birth weight

Female mice were controlled for age and weight, and were randomly allocated to treatment groups however, females in the 10 and 50 µg/kg LPS groups were heavier than control (P=0.029 and P=0.026, respectively; Table 6.1). There was no difference in the length of gestation. Compared with control, litter size was 1.3-fold larger in the 10 µg/kg LPS group (P=0.008) and there was a trend towards larger litter size (1.2-fold, P=0.070) in the 50 µg/kg LPS group (Table 6.1). In addition, the number of male offspring born was 1.4-fold larger in 10 µg/kg LPS group (vs. control; P=0.028; Table 6.1). Offspring gender ratio (male:female) and birth weight was not significantly different between treatment groups, except for a trend towards reduced birth weight in male offspring (vs. control; P=0.093; Table 6.1).

	Maternal treatment (µg/kg LPS)			
	saline	10	50	150
Maternal pretreatment body weight (g)	28.6 (±0.4)	30.3* (±0.3)	30.3* (±0.7)	28.9 (±0.6)
Gestation length (days)	19	19	19	19
Litter size	10.8 (±0.6)	13.7* (±0.6)	12.7† (±0.3)	11.3 (±1.0)
Number of male pups	5.5 (±0.6)	7.7* (±0.7)	6.2 (±0.5)	5.8 (±0.7)
Number of female pups	5.3 (±0.4)	6.0 (±0.5)	6.5 (±0.5)	5.5 (±0.6)
Male:Female ratio	1.1 (±0.17)	1.4 (±0.21)	1.0 (±0.16)	1.1 (±0.18)
Birth weight (g)	1.62 (±0.02)	1.57 (±0.01)	1.57 (±0.01)	1.64 (±0.02)
Male birth weight (g)	1.65 (±0.03)	1.57† (±0.02)	1.59 (±0.02)	1.66 (±0.03)
Female birth weight (g)	1.58 (±0.03)	1.56 (±0.02)	1.54 (±0.02)	1.61 (±0.02)

Table 6.1 Maternal pre-treatment body weight and effect of early gestational maternal inflammation on gestation length, litter size, male:female ratio and birth weight. Values are mean(±SEM); *P≤0.05; † P≤0.1 (vs. control). n=6 mothers per treatment group; n=65-82 offspring per treatment group (32-39 females and 33-46 males).

6.3.2 Offspring growth

No differences in mean weekly body weight were evident between treatment groups for either female or male offspring up to 30 weeks of age (Fig. 6.1a,b).

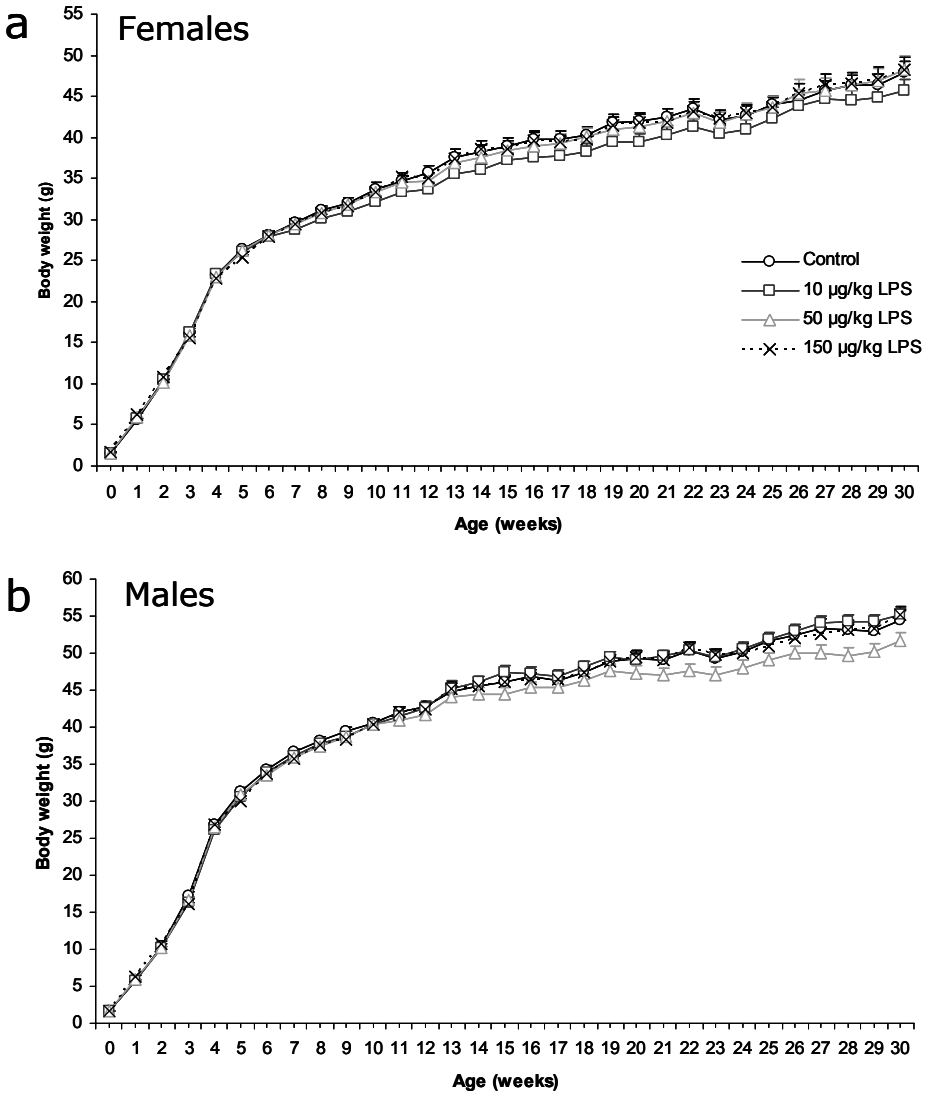


Figure 6.1 Growth of female (**a**) and male (**b**) offspring from birth to 30 weeks of age. Growth is not different between treatments at any time point. Values are mean±SEM. n=6 mothers per treatment group; n= 18 females and 18 males per treatment group.

6.3.3 Behaviour

For each individual mouse, behavioural tests were repeated 9 times between 4 and 23 weeks of age. In general, open field activity (section 6.3.3.1) and nesting activity (section 6.3.3.2) declined upon repeated exposure, whereas burrowing activity

(section 6.3.3.3) seemed to improve with repetition. Consumption of 5% glucose solution (section 6.3.3.4) appeared to decrease in male offspring with repetition, but did not vary much between time points for female offspring.

6.3.3.1 Open field

When examined on a weekly basis, minimal differences were found between treatment groups in open field activity, for either female (Fig. 6.2) or male (Fig. 6.3) offspring. However, differences were detected at select time points and are detailed as follows. Compared with control, 150 µg/kg LPS females reared less at week 8 (reduced by 61.7%, P=0.001; Fig. 6.2d) and 150 µg/kg LPS males reared less at weeks 4 (reduced by 20.6%, P=0.029), 6 (reduced by 53.2%, P=0.045), 11 (reduced by 51.7%, P=0.013), 14 (reduced by 41.2%, P=0.033) and 20 (reduced by 58.2%, P=0.011; Fig. 6.3d). In addition, 150 µg/kg LPS males jumped less than control at weeks 6 (80.3% less, P=0.031) and 17 (91.2% less. P=0.034; Fig. 6.3c), and rested for longer at week 6 (12.9% longer, P=0.040; Fig. 6.3b).

No significant differences between treatments were found for female offspring in distance travelled or number of jumps (Fig. 6.2a,c). However, a trend was detected for 50 µg/kg LPS females to spend longer resting at week 6 (vs. control, 9% longer, P=0.097; Fig. 6.2b). Additionally, compared with control, trends towards reduced rearing activity were detected in 10 µg/kg LPS females at week 8 (reduced by 44.4%, P=0.065) and in 150 µg/kg LPS females at week 11 (reduced by 49.4%, P=0.072; Fig. 6.2d). Compared with control, a trend towards reduced distance travelled was seen in 150 µg/kg LPS males at week 8 (47.4% reduction, P=0.072) and in 50 and 150 µg/kg LPS males at week 11 (reduced by 24.6%, P=0.091 and 30.8%, P=0.069, respectively; Fig. 6.3a). A trend was also detected for 150 µg/kg LPS males to rest longer than control at weeks 8 and 11 (10.5% and 8.3% longer, respectively, P≤0.1; Fig. 6.3b). Trends towards reduced jumping were seen in 150 µg/kg LPS males (vs. control) at weeks 8 (75.5% less. P=0.086), 11 (70.7% less. P=0.051), and 20 (70.8% less. P=0.064; Fig. 6.3c). In addition, there was a trend towards 10 µg/kg LPS males jumping less than control at week 20 (64.6% less, P=0.065; Fig. 6.3c). A trend was also detected for 150 µg/kg LPS males to rear less at week 8 (vs. control, reduced by 48%, P=0.064). All differences and trends were independent of maternal origin and gestational litter size.

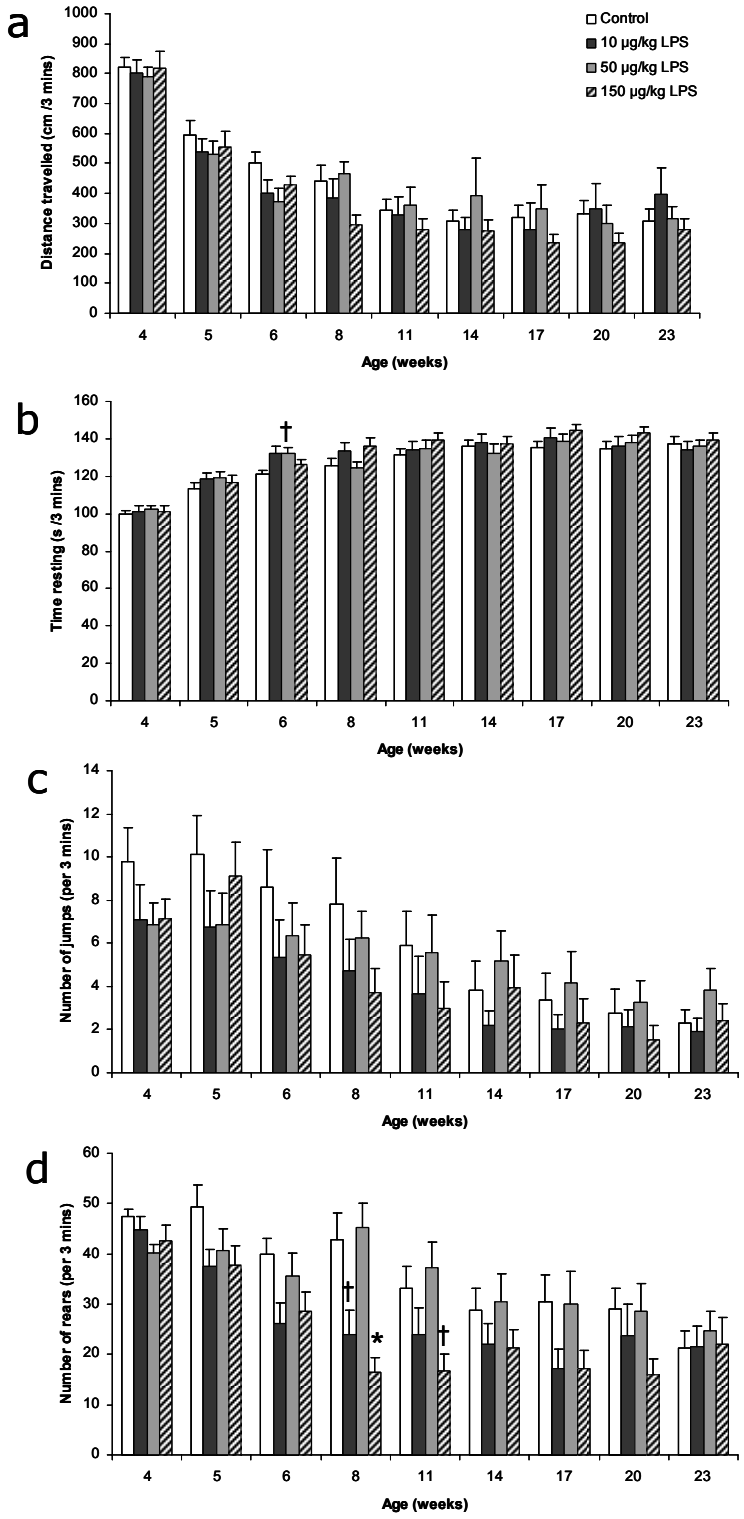


Figure 6.2 Open field assessment of locomotor and exploratory behaviour in female offspring: (**a**) distance travelled, (**b**) time resting, (**c**) number or jumps and (**d**) number of rears. Values are mean±SEM; *P≤0.05; † P≤0.1; independent of maternal origin and litter size. n=6 mothers per treatment group; n= 18 females per treatment group.

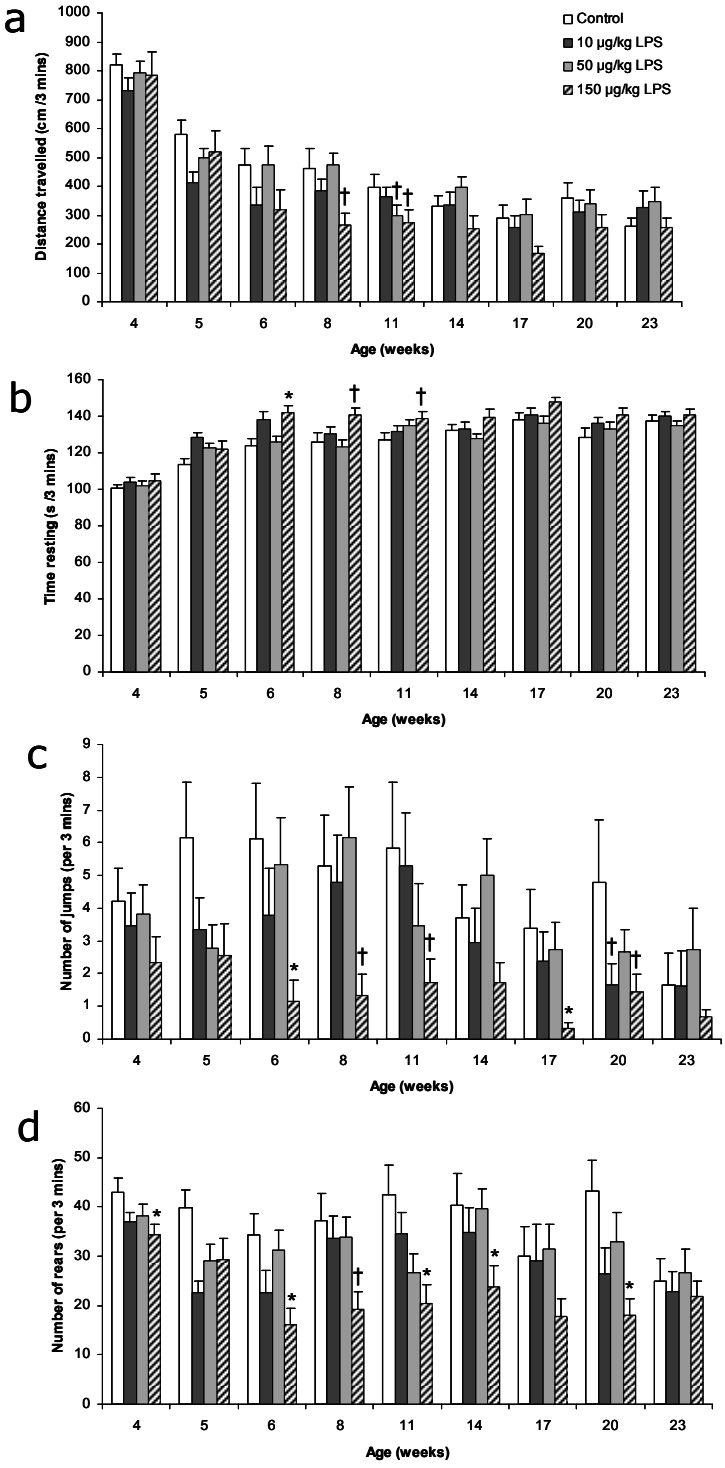


Figure 6.3 Open field assessment of locomotor and exploratory behaviour in male offspring: (**a**) distance travelled, (**b**) time resting, (**c**) number or jumps and (**d**) number of rears. Reduction in open field activity was evident in male offspring from the 150 µg/kg LPS group. Values are mean±SEM; *P≤0.05; † P≤0.1; independent of maternal origin and litter size. n=6 mothers per treatment group; n= 18 males per treatment group.

Life-time open field activity of female and male offspring was integrated by averaging all weekly data for each open field parameter (Fig. 6.4). Male 150 µg/kg LPS offspring displayed reduced life-mean number of jumps and rears (reduced by 67.4%, P=0.027 and 40.1%, P=0.015, respectively; Fig. 6.4c,d) and a trend towards increased life-mean time resting (increased by 7.5%, P=0.059), but no differences in life-mean distance travelled (Fig. 6.4a). Female 150 µg/kg LPS offspring displayed a trend towards reduced life-mean number of rears (reduced by 32.1%, P=0.064; Fig. 6.4d). No differences existed between treatment groups for females when life-mean distance travelled, time resting or number of jumps were examined (Fig. 6.4a,b,c). All differences and trends in life-time mean data were independent of maternal origin and gestational litter size.

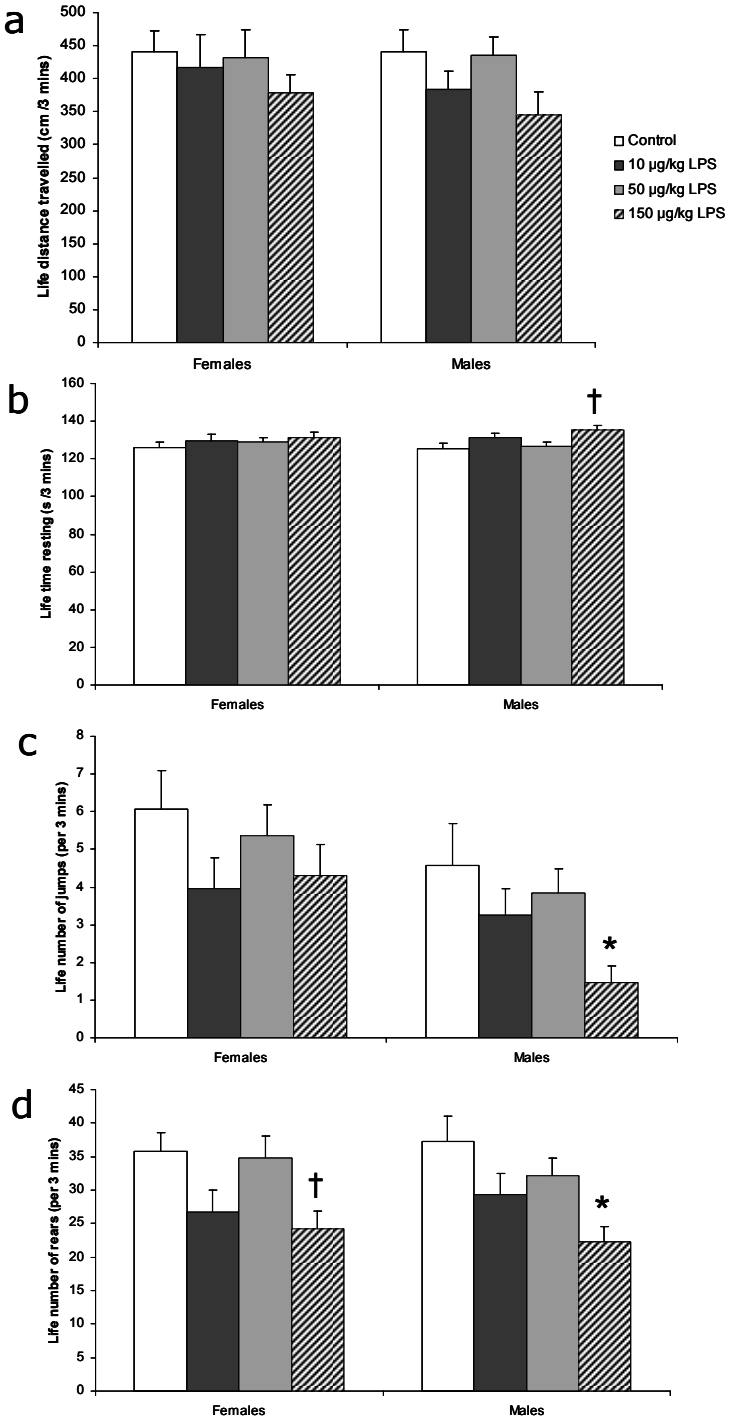


Figure 6.4 Life-time open field activity of individual female and male offspring was integrated by averaging all weekly data for each open field parameter: (**a**) distance travelled, (**b**) time resting, (**c**) number or jumps and (**d**) number of rears. Male offspring from the 150 µg/kg LPS group displayed reduced life-time open field activity. Values are mean±SEM; *P≤0.05; † P≤0.1; independent of maternal origin and litter size. n=6 mothers per treatment group; n= 18 females and 18 males per treatment group.

6.3.3.2 Nesting

At week 14, 50 and 150 µg/kg LPS males had significantly increased nest building activity (increased by 9.1% and 11.1%, respectively, P≤0.01, versus control; Fig. 6.5b). A trend towards increased nest building activity was seen in 150 µg/kg LPS males at week 6 (increased by 6.1%, P=0.087, *versus* control; Fig. 6.5b) and by 10 µg/kg LPS males at week 14 (increased by 4.8%, P=0.059, versus control; Fig. 6.5b). In contrast, at week 23, 10 µg/kg LPS males exhibited a trend towards reduced nest building activity (reduced 12.8%, P=0.089, versus control; Fig. 6.5b). No differences in female nesting behaviour were seen between treatments at any time point. However, at week 8, 150 µg/kg LPS females exhibited a trend towards reduced nest building activity (reduced 15.9%, P=0.092, *versus* control; Fig. 6.5a). No differences were observed between treatment groups for life-time mean nesting, for either females or males (Fig. 6.5c). All data were independent of maternal origin and gestational litter size.

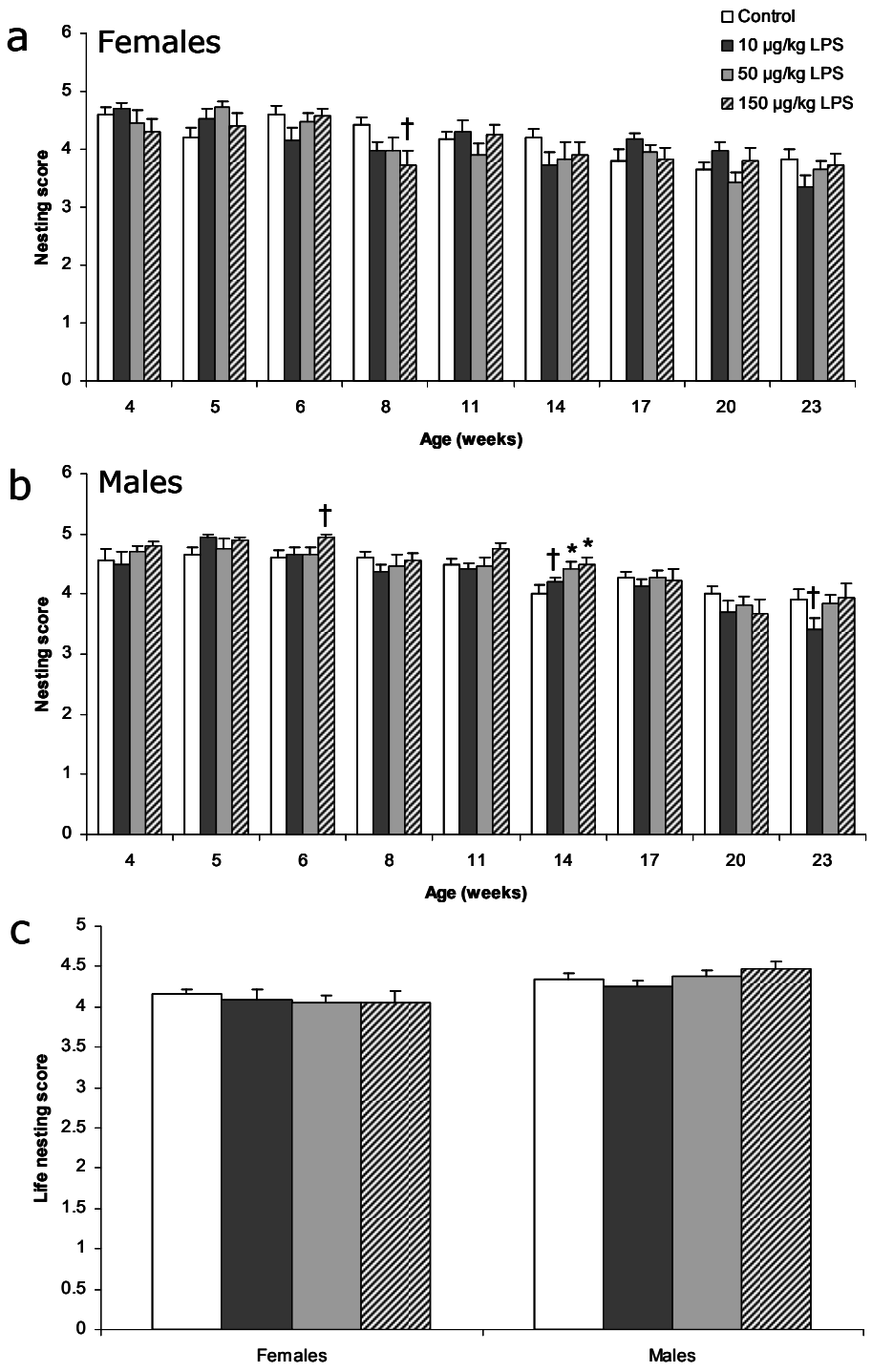


Figure 6.5 Nesting scores of (**a**) female and (**b**) male offspring across time; and (**c**) life-time nesting scores. Overall, little difference in nesting was seen between groups. Values are mean±SEM; *P≤0.05; † P≤0.1; independent of maternal origin. n=6 mothers per treatment group; n= 18 females and 18 males per treatment group.

6.3.3.3 Burrowing

No differences were seen between treatment groups for burrowing between 1400 and 1600 hours for either male or female offspring (Fig. 6.6a,b), although a trend was detected at week 23 for 50 µg/kg LPS females to burrow less than control (reduced by 7.7%, P=0.087; Fig. 6.6a). In addition, there were no significant differences between treatments for life-time mean burrowing between 1400 and 1600 hours for female or male offspring (Fig. 6.6c). These data were independent of maternal origin and gestational litter size. The only difference seen between treatment groups for burrowing overnight was at week 23, where 50 µg/kg LPS females burrowed less than control (reduced by 6.5%, P=0.046; Fig. 6.7a). These data were independent of maternal origin, whereas gestational litter size was found to have a positive influence (+4.6 g per value increase in litter size, P=0.032). There were no differences between treatment groups for life-time mean burrowing overnight, for either female or male offspring (Fig. 6.7a,b,c).

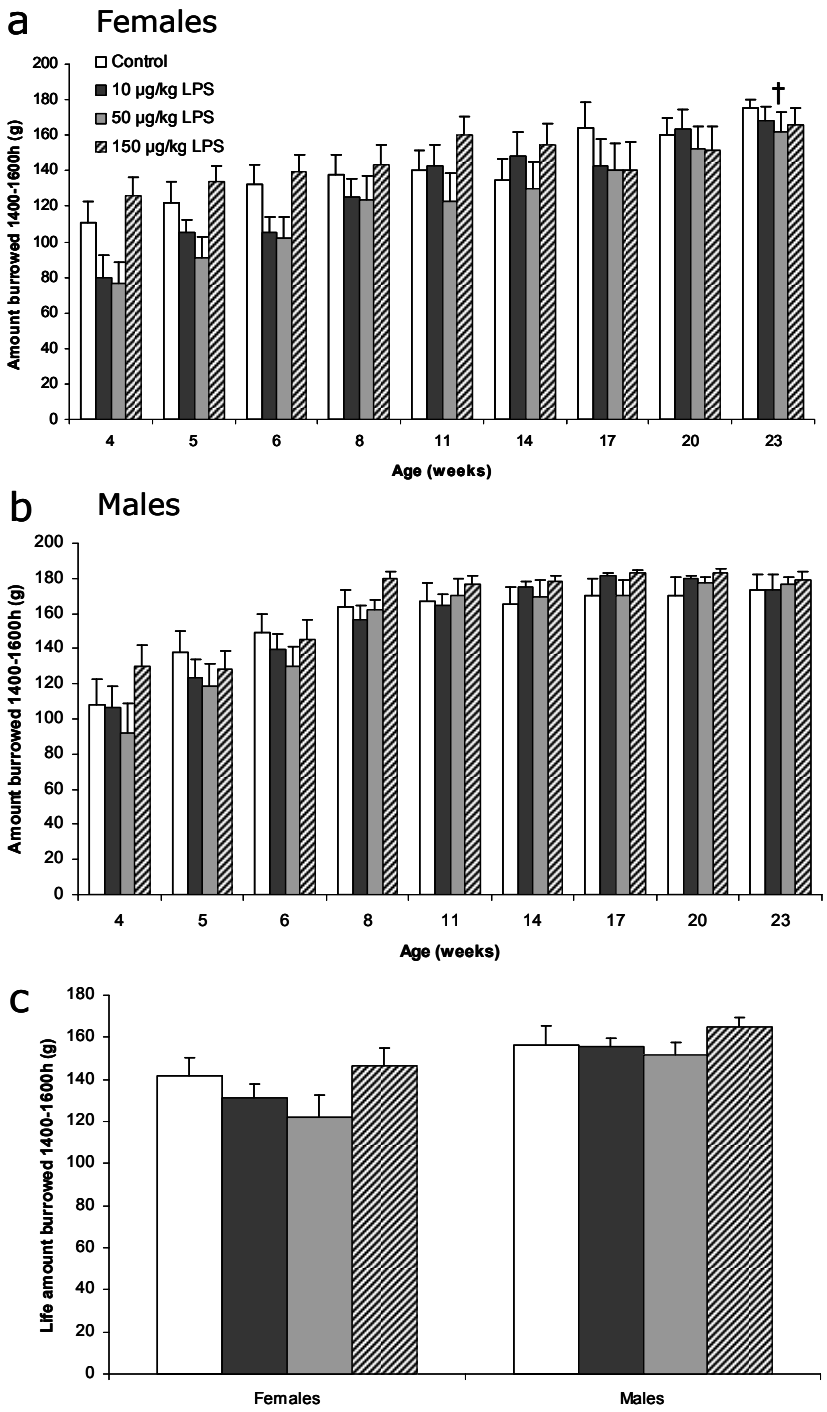


Figure 6.6 Amount burrowed from 1400-1600h for (**a**) female and (**b**) male offspring across time; and (**c**) life-time amount burrowed from 1400-1600h. Little difference was seen between groups. Values are mean±SEM; † P≤0.1; independent of maternal origin. n=6 mothers per treatment group; n= 18 females and 18 males per treatment group.

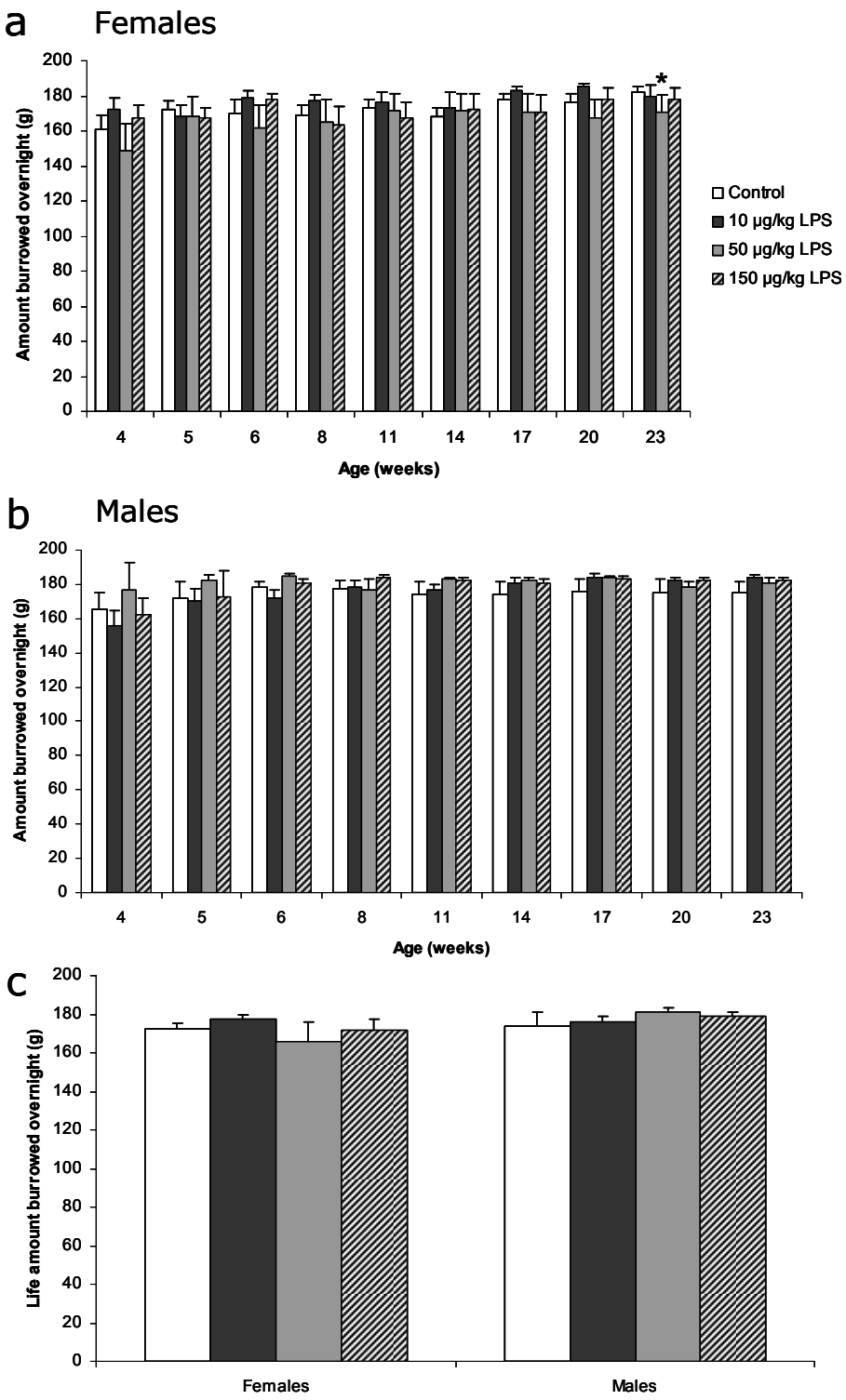


Figure 6.7 Amount burrowed overnight for (**a**) female and (**b**) male offspring across time; and (**c**) life-time amount burrowed overnight. Little difference was seen between groups. Values are mean±SEM; *P≤0.05; independent of maternal origin. n=6 mothers per treatment group; n= 18 females and 18 males per treatment group.

6.3.3.4 Consumption of glucose solution and tap water

When examined on an individual weekly basis, 150 µg/kg LPS females consumed more 5% glucose solution than control at week 5 (increased by 36.4%, P=0.038). At weeks 6 and 8, a trend was detected for 150 µg/kg LPS females to consume more 5% glucose solution than control (increased by 41.2%, P≤0.1; Fig. 6.8a). Furthermore, when compared with control, there was a trend towards 150 µg/kg LPS females having increased life-time mean consumption of 5% glucose solution (increased by 36%, P=0.094; Fig. 6.8c). Male offspring displayed no differences between treatments at any time point, or when life-time mean consumption of 5% glucose solution was analysed (Fig. 6.8b). Tap water consumption was not different between treatments for females or males, at any time point (data not shown).

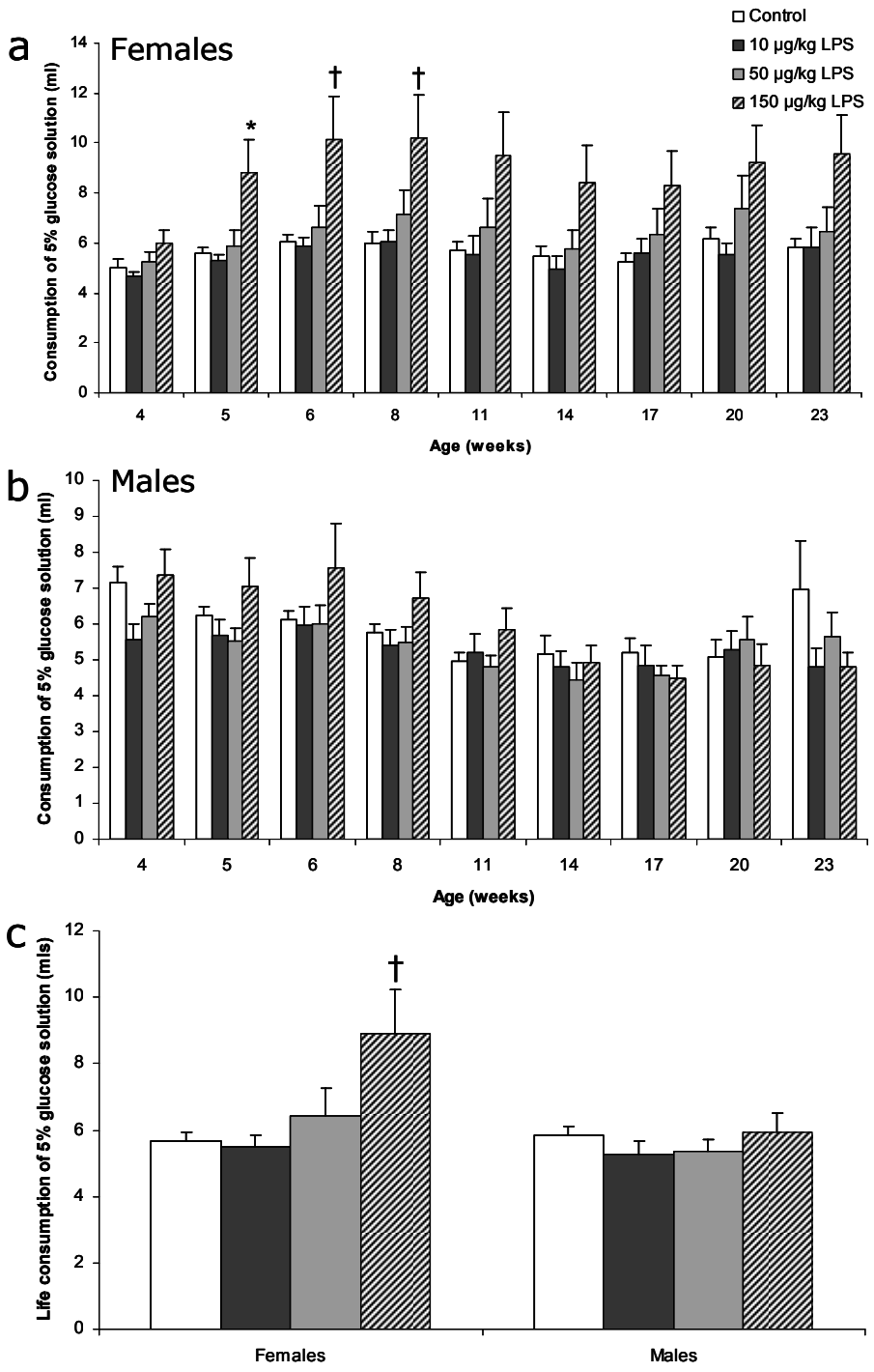


Figure 6.8 Consumption of 5% glucose solution for (**a**) female and (**b**) male offspring across time; and (**c**) life-time consumption of 5% glucose solution. Female offspring from the 150 µg/kg LPS group tended to consume more glucose solution compared with control. Values are mean±SEM; *P≤0.05; † P≤0.1; independent of maternal origin and litter size. n=6 mothers per treatment group; n= 18 females and 18 males per treatment group.

6.3.4 Systolic blood pressure

When compared with control, 10 μg/kg LPS males displayed increased systolic blood pressure (SBP) at weeks 16 and 28 (increased by 4.5%, P=0.035 and 7%, P=0.040, respectively; Fig. 6.9b). Furthermore, life-time mean SBP was significantly increased in 10 μg/kg LPS males (increased by 3.9%, P=0.029; *versus* control; Fig. 6.9c). There were no differences in SBP or life-time mean SBP between treatments for female offspring (Fig. 6.9a). All data were independent of maternal origin and gestational litter size.

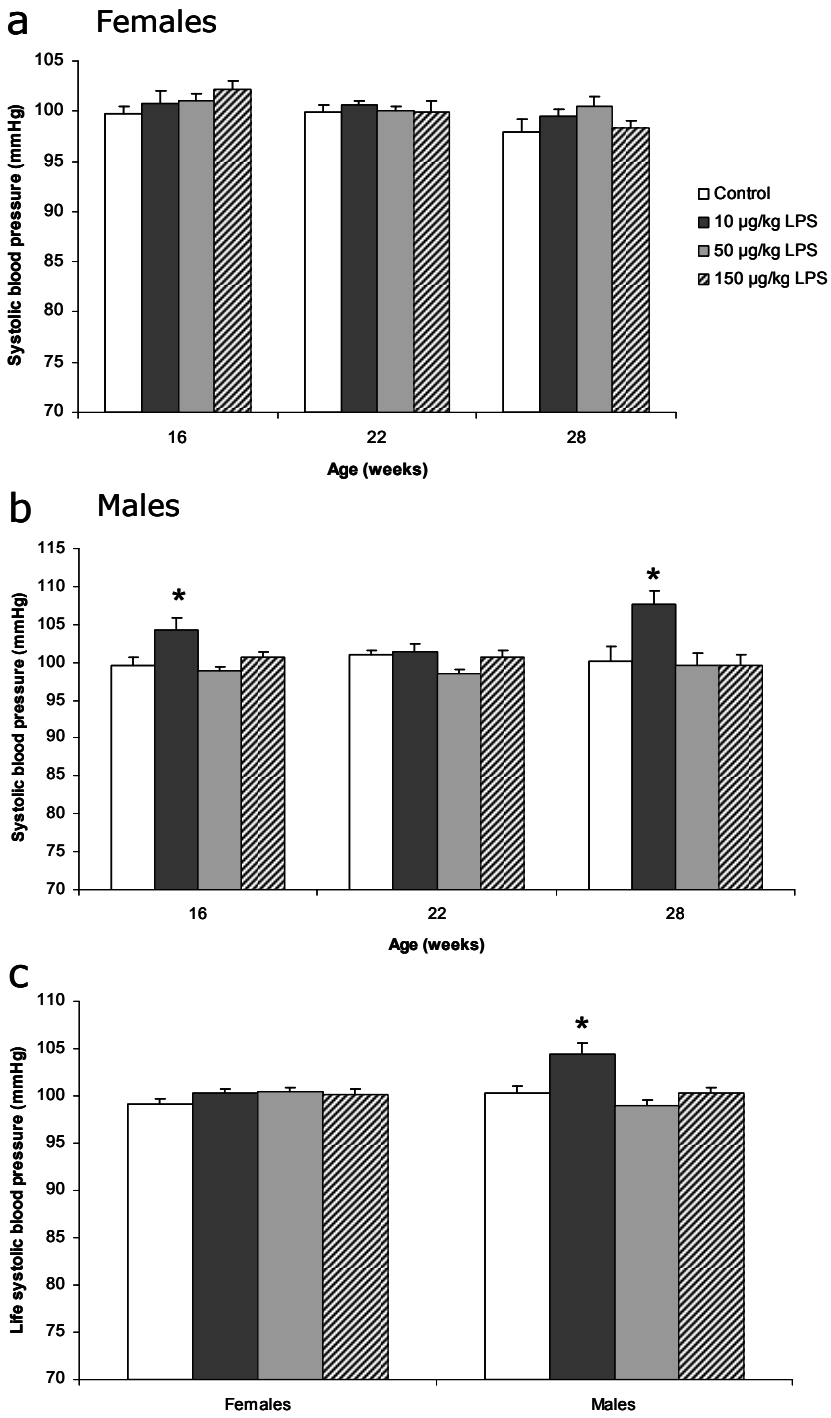


Figure 6.9 Systolic blood pressure (SBP) of (**a**) female and (**b**) male offspring across time; and (**c**) life-time SBP. Overall, male offspring from the 10 µg/kg LPS group had higher SBP than control. Values are mean±SEM; *P≤0.05; independent of maternal origin and litter size. n=6 mothers per treatment group; n= 18 females and 18 males per treatment group.

6.3.5 Organ and fat-pad weights

All organ and fat-pad weights were normalised by expression as a percentage of body weight. Organ/body weight ratios in male offspring appeared to have been affected to a greater extent than in female offspring. Compared with control, 150 µg/kg LPS males had increased retro-peritoneal fat/body weight ratio (increased by 28.5%, P=0.013) and inguinal fat pad/body weight ratios (increased by 27.8%, P=0.031; Fig. 6.12c,d), and displayed a trend towards increased inter-scapular fat/body weight ratios (increased by 21.1%, P=0.089; Fig. 6.12c). BMI was significantly increased in 150 µg/kg LPS males compared with control (increased by 6.9%, P=0.033; Fig. 6.13c), although total fat/body weight ratio and carcass/body weight ratio (Fig. 6.13a,b) did not differ between treatments. In addition, when compared with control, 150 µg/kg LPS males tended to have decreased lung/body weight ratios (decreased by 9.8%, P=0.098; Fig. 6.12a), and 50 µg/kg LPS males tended to have reduced liver/body weight ratios (reduced by 9.5%, P=0.052; Fig. 6.12b). In female offspring no differences between treatments were seen for any organ or fat pad, except liver (Fig. 6.10). Females from 50 µg/kg LPS treatment had significantly reduced liver/body weight ratio compared with control (reduced by 11.6%, P=0.033; Fig. 6.10b), whereas 10 and 150 µg/kg LPS females displayed a trend level reduction (both of the latter treatments reduced by 9.3%, P=0.057; Fig. 6.10b). All data were independent of maternal origin and gestational litter size.

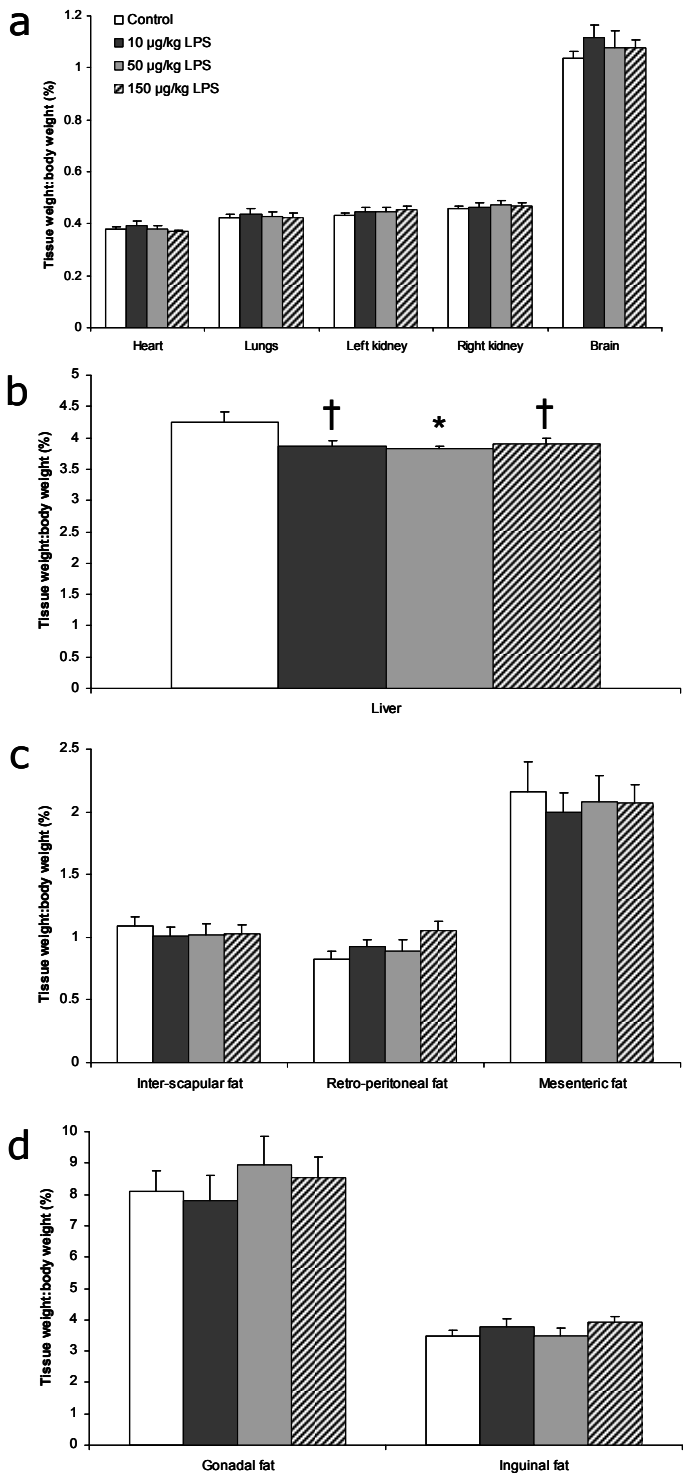


Figure 6.10 Normalised organ and fat-pad weights of female offspring (%): (**a**) major organs; (**b**) liver; (**c**) and (**d**) fat-pads. Female offspring from LPS treated groups had proportionally reduced liver mass compared with control. Values are mean±SEM; *P≤0.05; † P≤0.1; independent of maternal origin and litter size. n=6 mothers per treatment group; n= 18 females per treatment group.

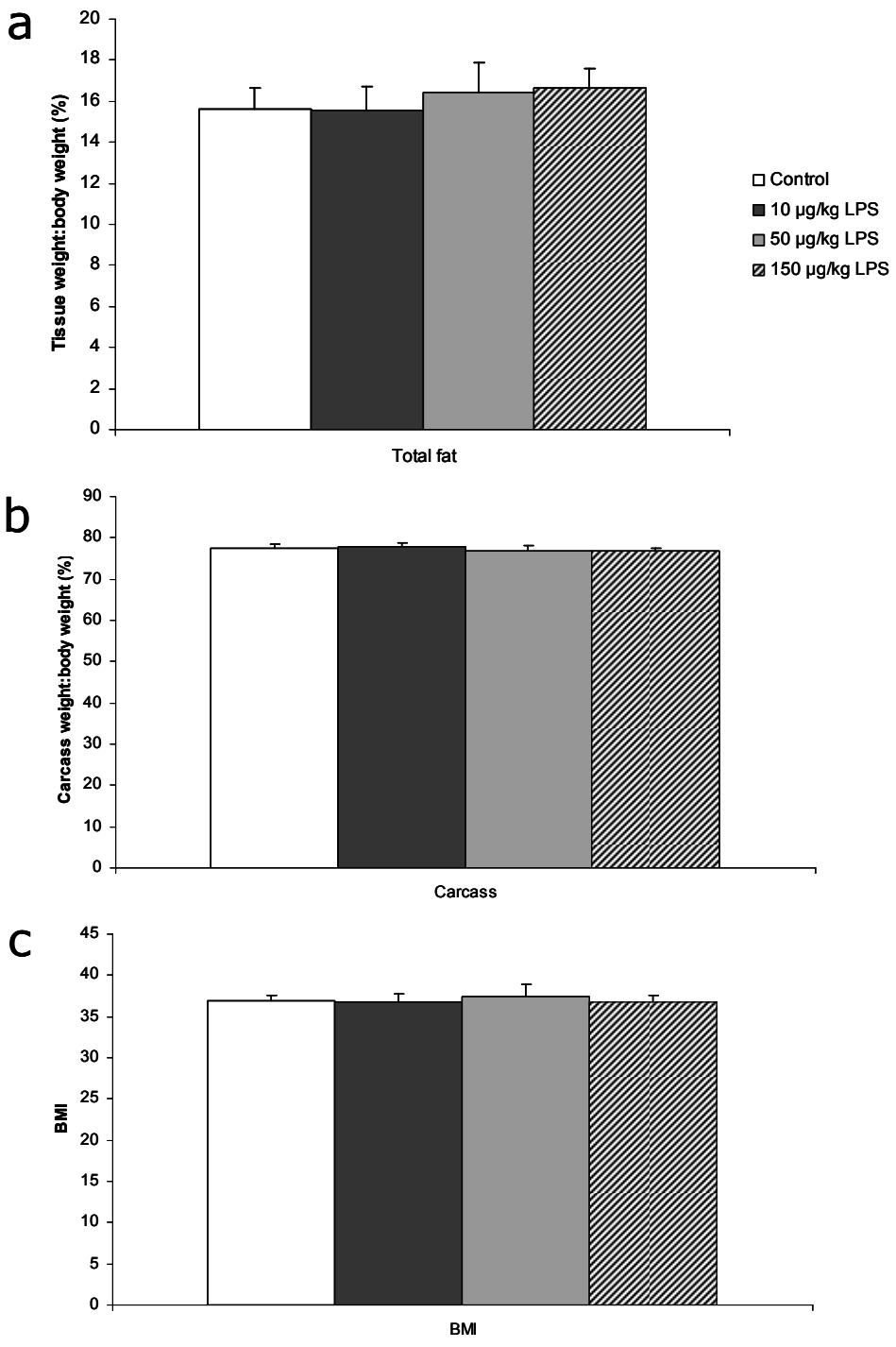


Figure 6.11 (**a**) Normalised total fat mass (%), (**b**) normalised carcass weight (%) and (**c**) BMI for female offspring. Values are mean±SEM. n=6 mothers per treatment group; n= 18 females per treatment group.

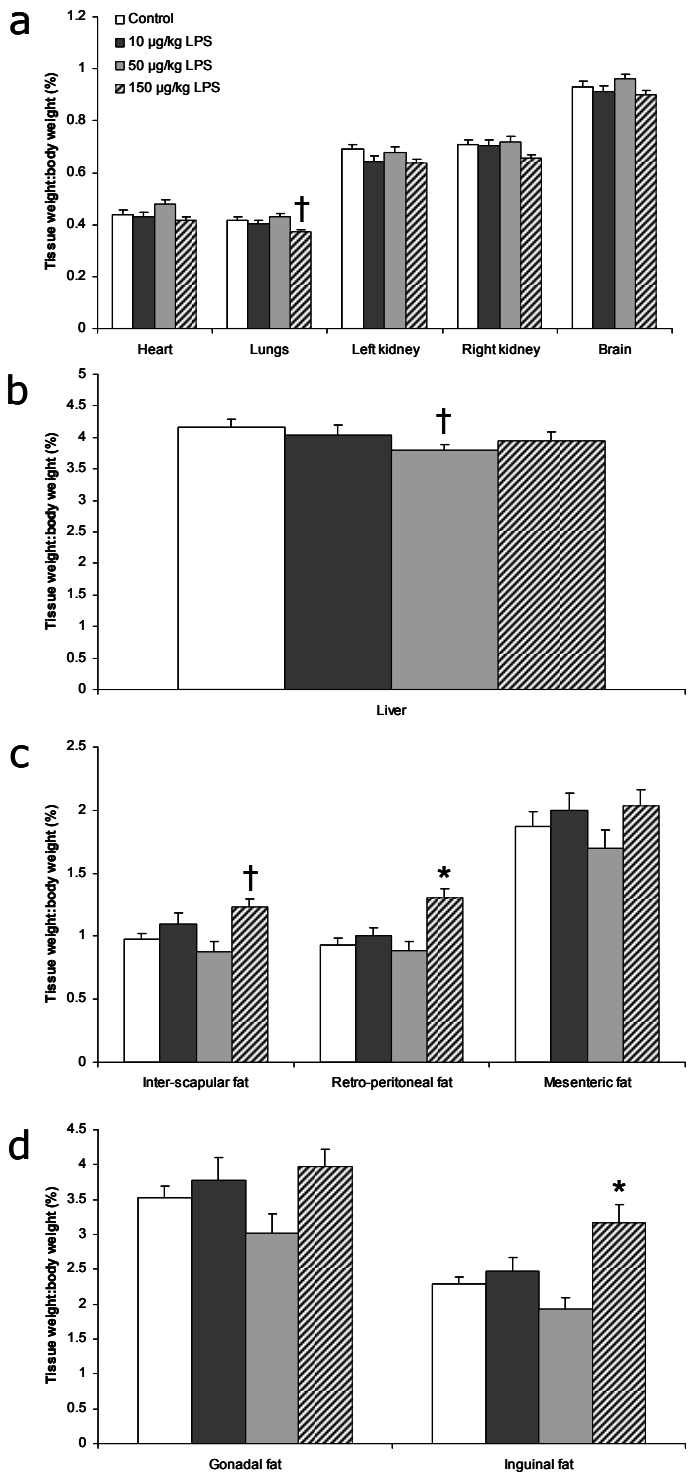


Figure 6.12 Normalised organ and fat-pad weights of male offspring (%): (**a**) major organs; (**b**) liver; (**c**) and (**d**) fat-pads. Compared with control, male offspring from the 150 µg/kg LPS group tended to have proportionally reduced organ mass and increased fat-pad mass. Values are mean±SEM; *P≤0.05; † P≤0.1; independent of maternal origin and litter size. n=6 mothers per treatment group; n= 18 males per treatment group.

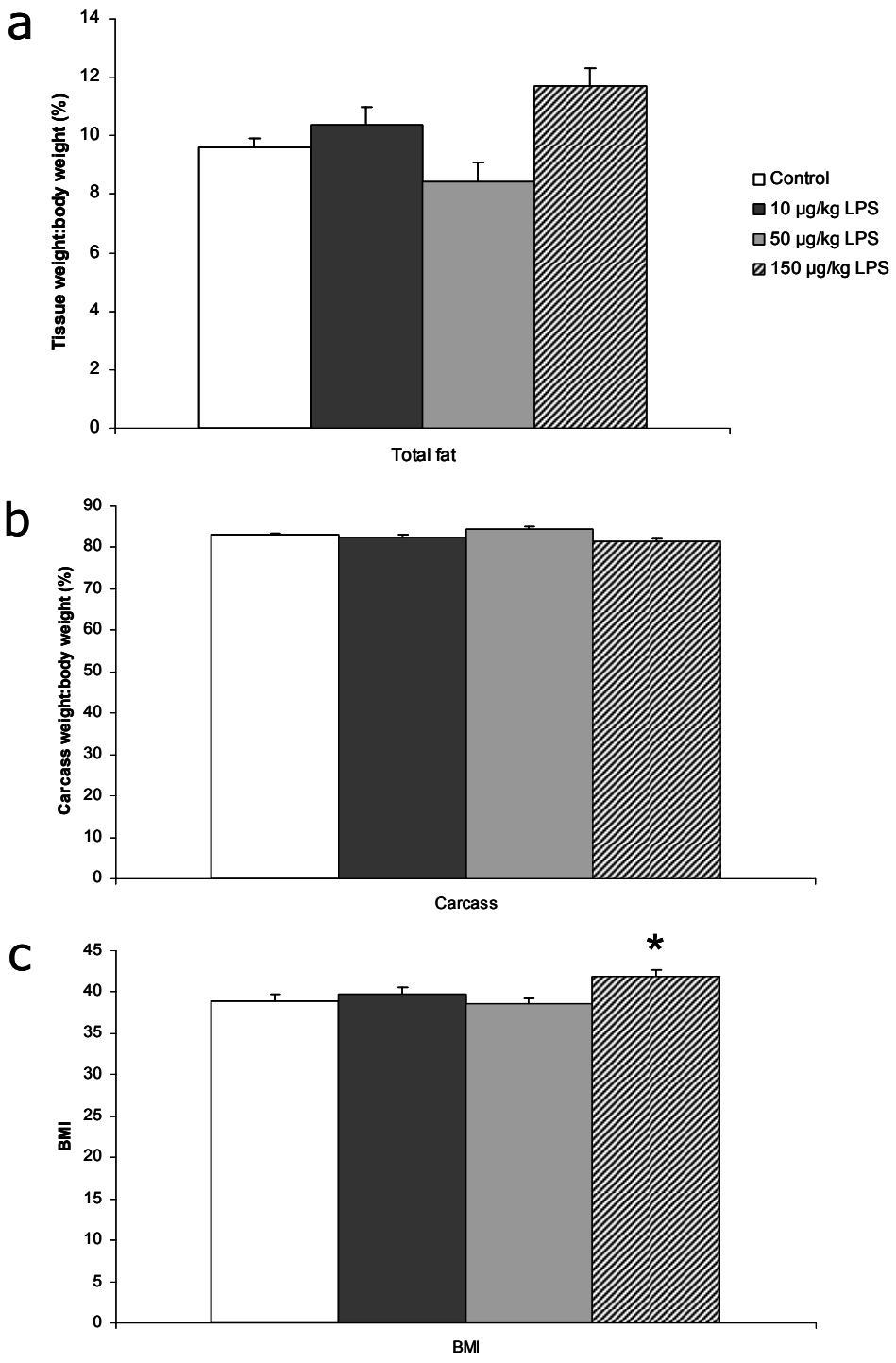


Figure 6.13 (**a**) Normalised total fat mass (%), (**b**) normalised carcass weight (%) and (**c**) BMI for male offspring. Male offspring from the 150 µg/kg LPS group had increased BMI compared with control (P≤0.05). Values are mean±SEM;*P≤0.05; independent of maternal origin and litter size. n=6 mothers per treatment group; n= 18 males per treatment group.

6.3.6 Immune status

In this part of the study, serum cytokine concentrations and splenocyte populations were quantified in LPS-injected (immune challenged) and saline-injected (non-immune challenged control) adult offspring from mothers that received saline, 10, 50 or 150 µg/kg LPS on GD 0.5. After postnatal challenge (i.e. saline or 50 µg/kg LPS), offspring fell into eight different treatment groups, saline-injected offspring from saline, 10, 50 or 150 µg/kg LPS mothers and LPS-injected offspring from saline, 10, 50 or 150 µg/kg LPS mothers, and were designated as follows: Sal:Sal, 10 LPS:Sal, 50 LPS:Sal, 150 LPS:Sal, Sal:LPS, 10 LPS:LPS, 50 LPS:LPS, 150 LPS:LPS (Table 6.2).

Maternal treatment group	Offspring treatment group	Combined treatment group
Sal	Sal	Sal:Sal
10 LPS	Sal	10 LPS:Sal
50 LPS	Sal	50 LPS:Sal
150 LPS	Sal	150 LPS:Sal
Sal	LPS	Sal:LPS
10 LPS	LPS	10 LPS:LPS
50 LPS	LPS	50 LPS:LPS
150 LPS	LPS	150 LPS:LPS

Table 6.2 Summary of treatment groups following postnatal offspring challenge.

A number of comparisons were made when analysing the data.

1. The effect of maternal treatment on saline-injected offspring was examined by comparing the saline-injected offspring from different maternal treatment groups (Sal:Sal, 10 LPS:Sal, 50 LPS:Sal, 150 LPS:Sal).

2. The effect of maternal treatment on LPS-injected offspring was examined by comparing the LPS-injected offspring from different maternal treatment groups (Sal:LPS, 10 LPS:LPS, 50 LPS:LPS, 150 LPS:LPS).

3. The effect of LPS-injection within a maternal treatment group was examined by comparing saline-injected and LPS-injected offspring from the same maternal treatment group (e.g. Sal:Sal vs. Sal:LPS, 10 LPS:Sal vs. 10 LPS:LPS, 50 LPS:Sal vs. 50 LPS:LPS, 150 LPS:Sal vs. 150 LPS:LPS).

6.3.6.1 Offspring serum cytokines

Figures 6.14 and 6.15 show the serum cytokine concentrations obtained for female and male offspring, respectively.

Figure 6.14 (overleaf) Serum cytokine concentrations (pg/ml) of female offspring 3.5 hours post-injection of saline (open bars; offspring = Sal) or 50 µg/kg LPS (dark bars; offspring = LPS). Maternal treatments; Sal = saline, 10 LPS = 10 µg/kg LPS, 50 LPS = 50 µg/kg LPS, 150 LPS = 150 µg/kg LPS.

Graphs for each of the 23 cytokines are displayed in name order; first the interleukins in numerical order and then all remaining cytokines in alphabetical order.

Values mean±SEM; * P≤0.05; † P≤0.1 when comparing the effect of maternal treatments on offspring. Bars have an open star ☆ or open diamond ◊ (P≤0.05), or an open triangle △ (P≤0.1), when comparing the effect of offspring treatment within the same maternal treatment. Differences are independent of maternal origin and litter size unless otherwise stated; n=6 mothers per maternal treatment group; n=4-6 females per combined treatment group.

NB: The data presented here for IL-6, G-CSF and KC are inaccurate and should be interpreted with caution. Some readings could not be converted to concentrations because the values obtained were above the upper limit of detection of the assay.

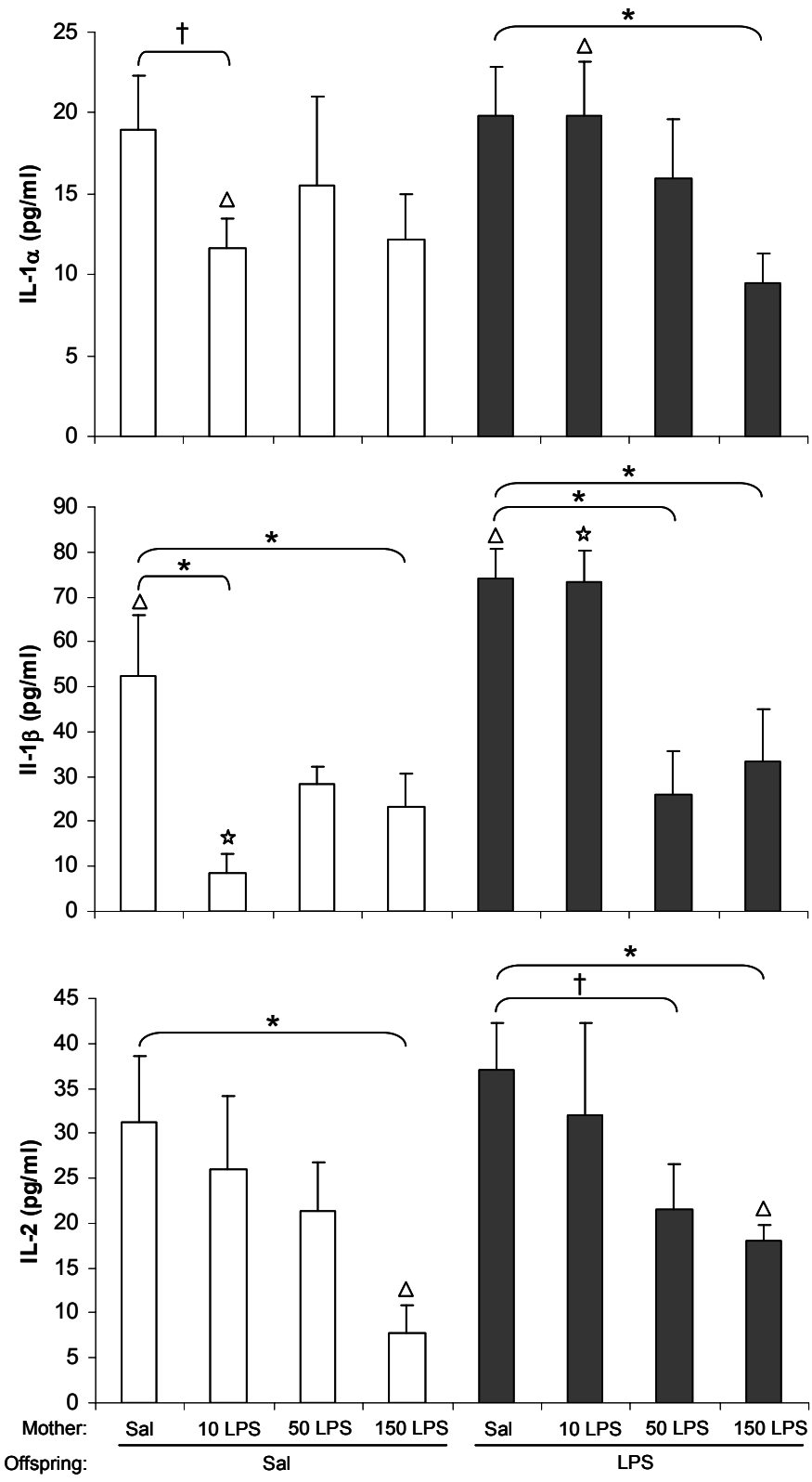
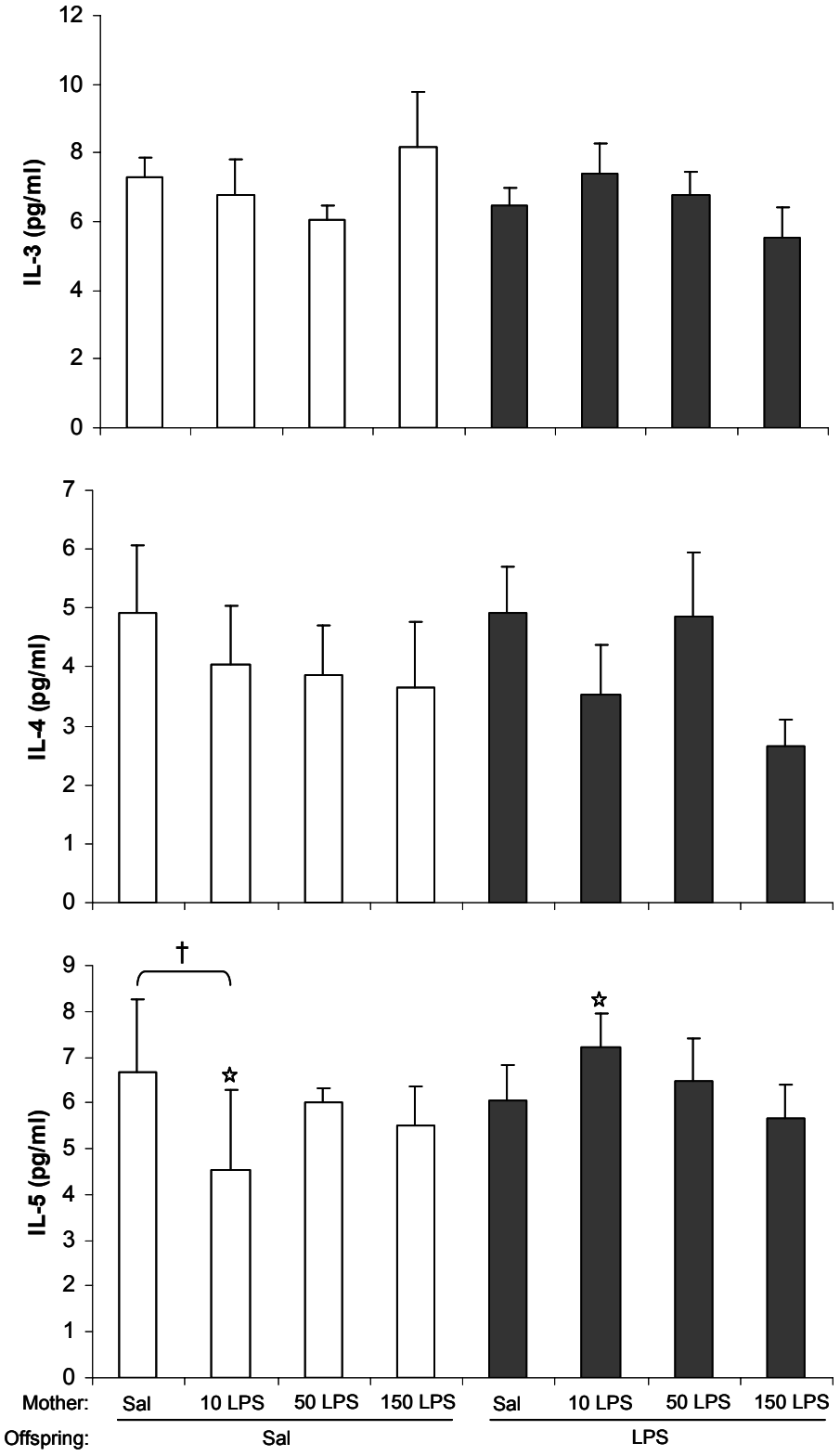


Fig. 6.14 females (*Continued*)



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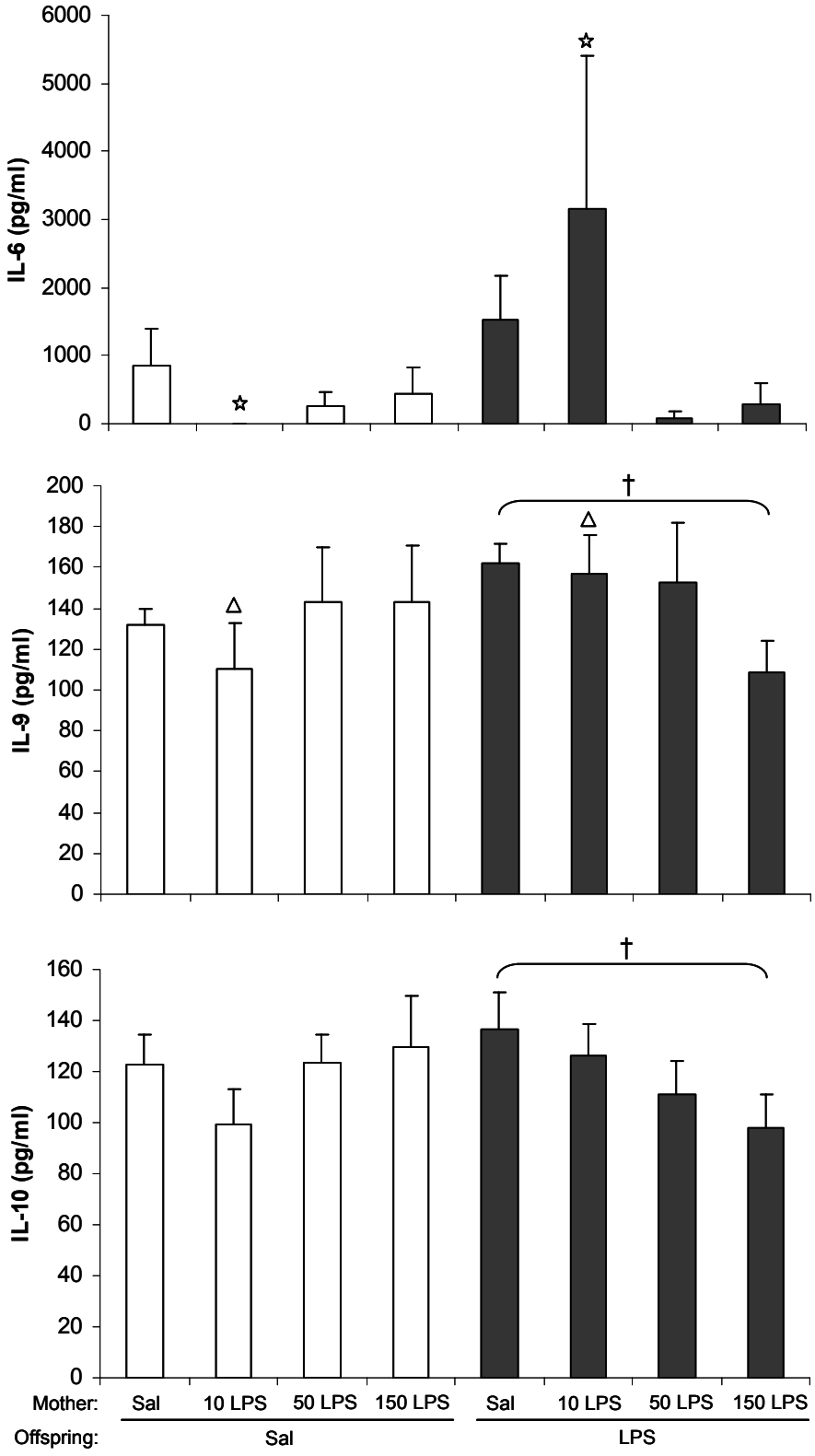

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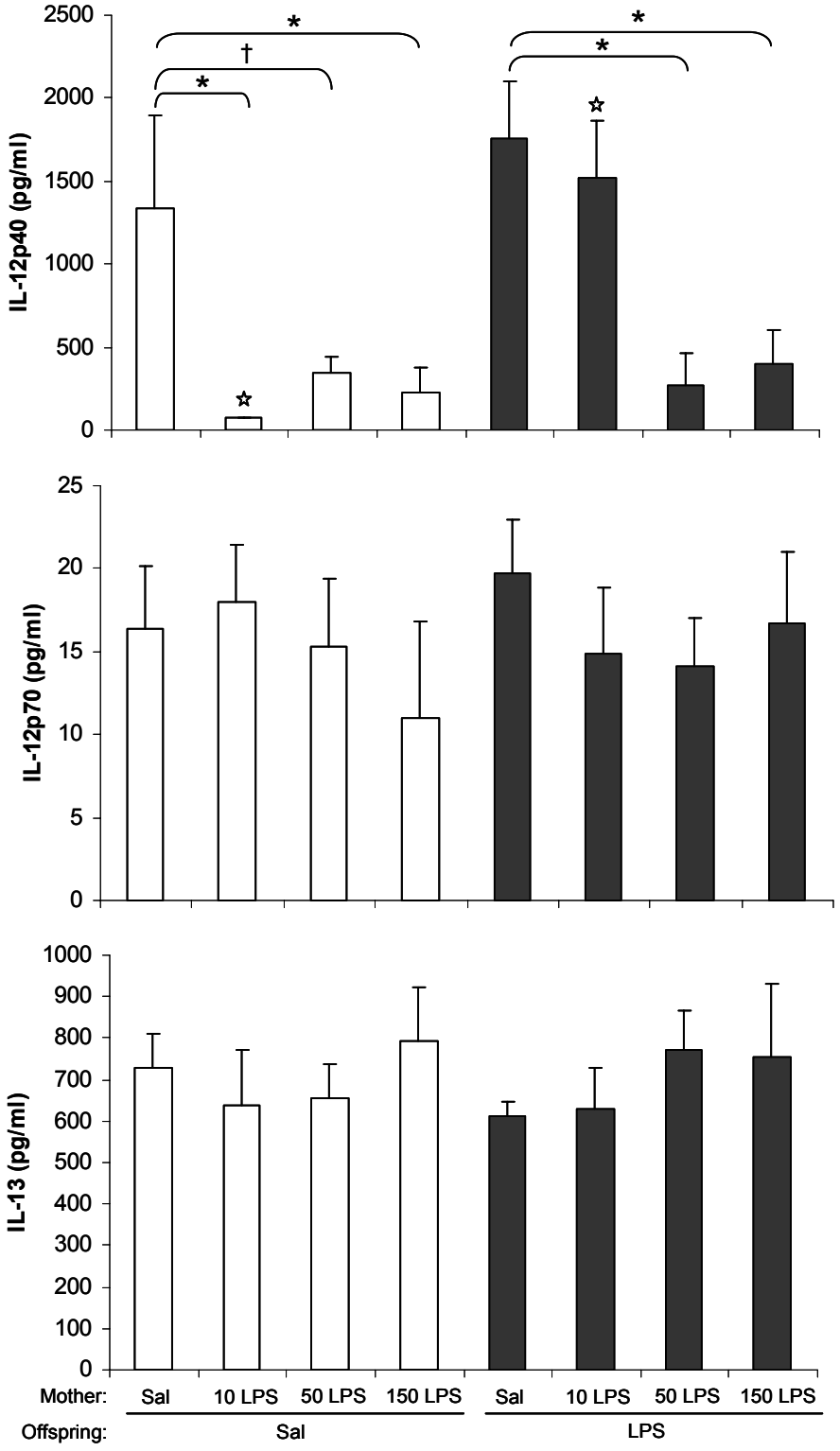

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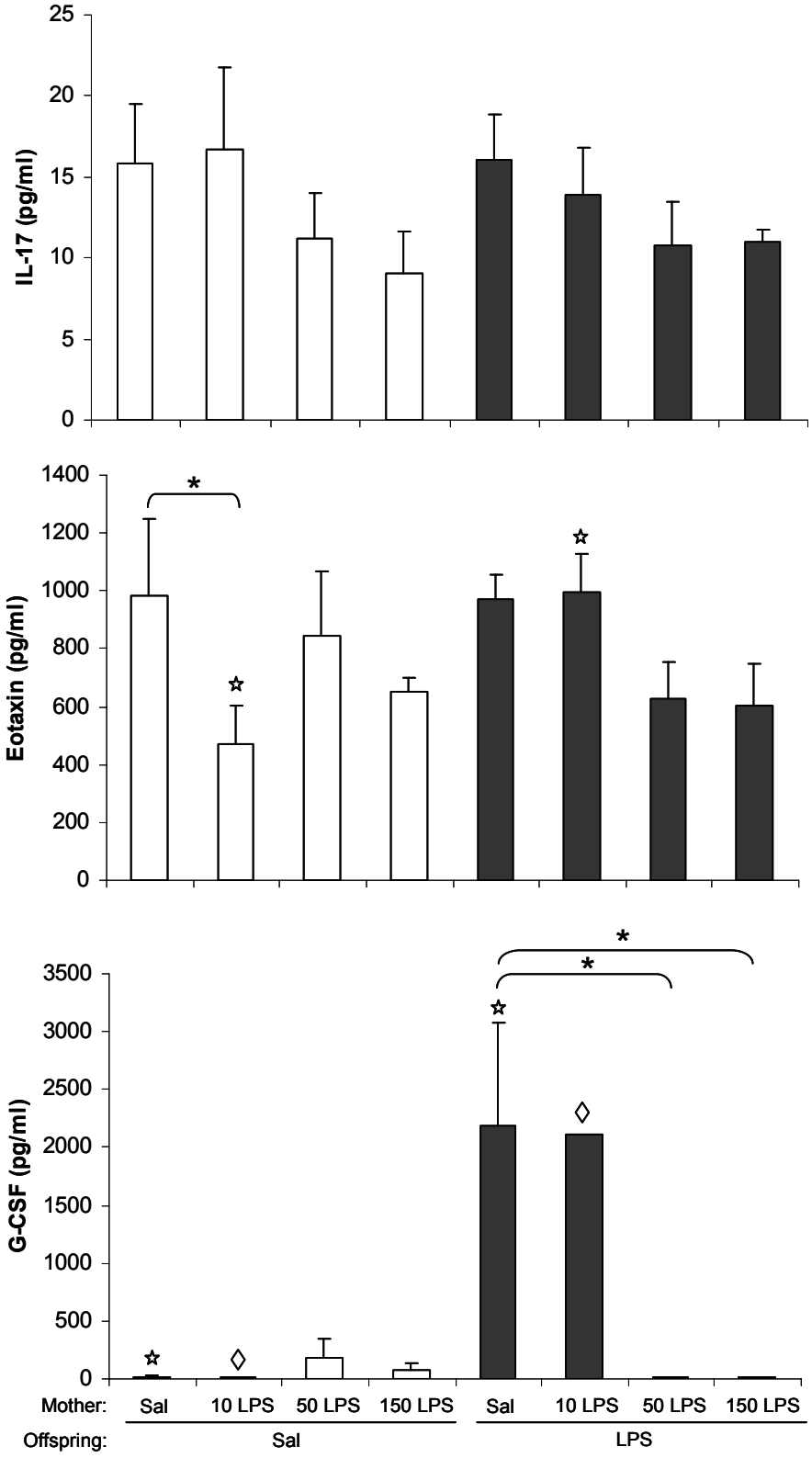

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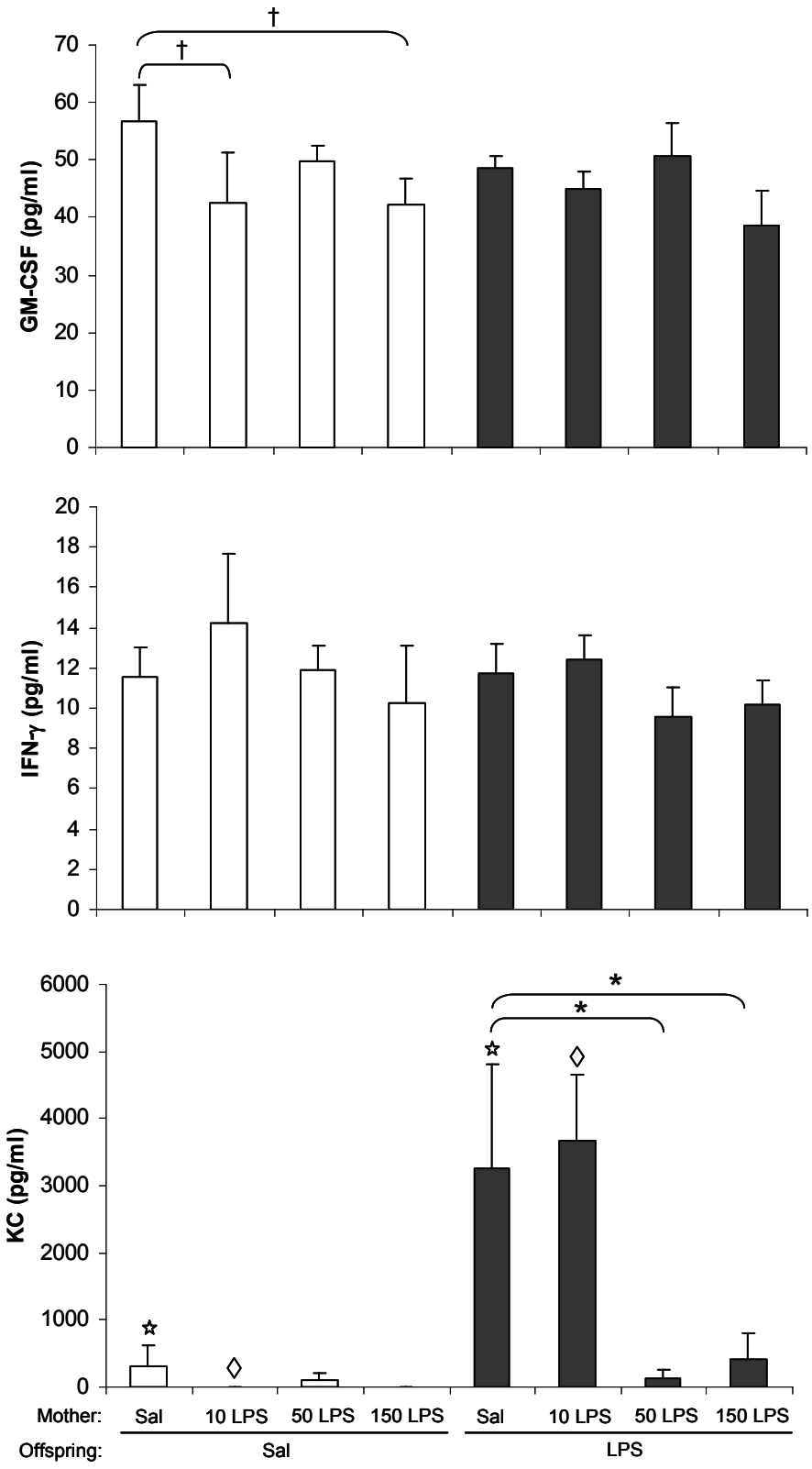

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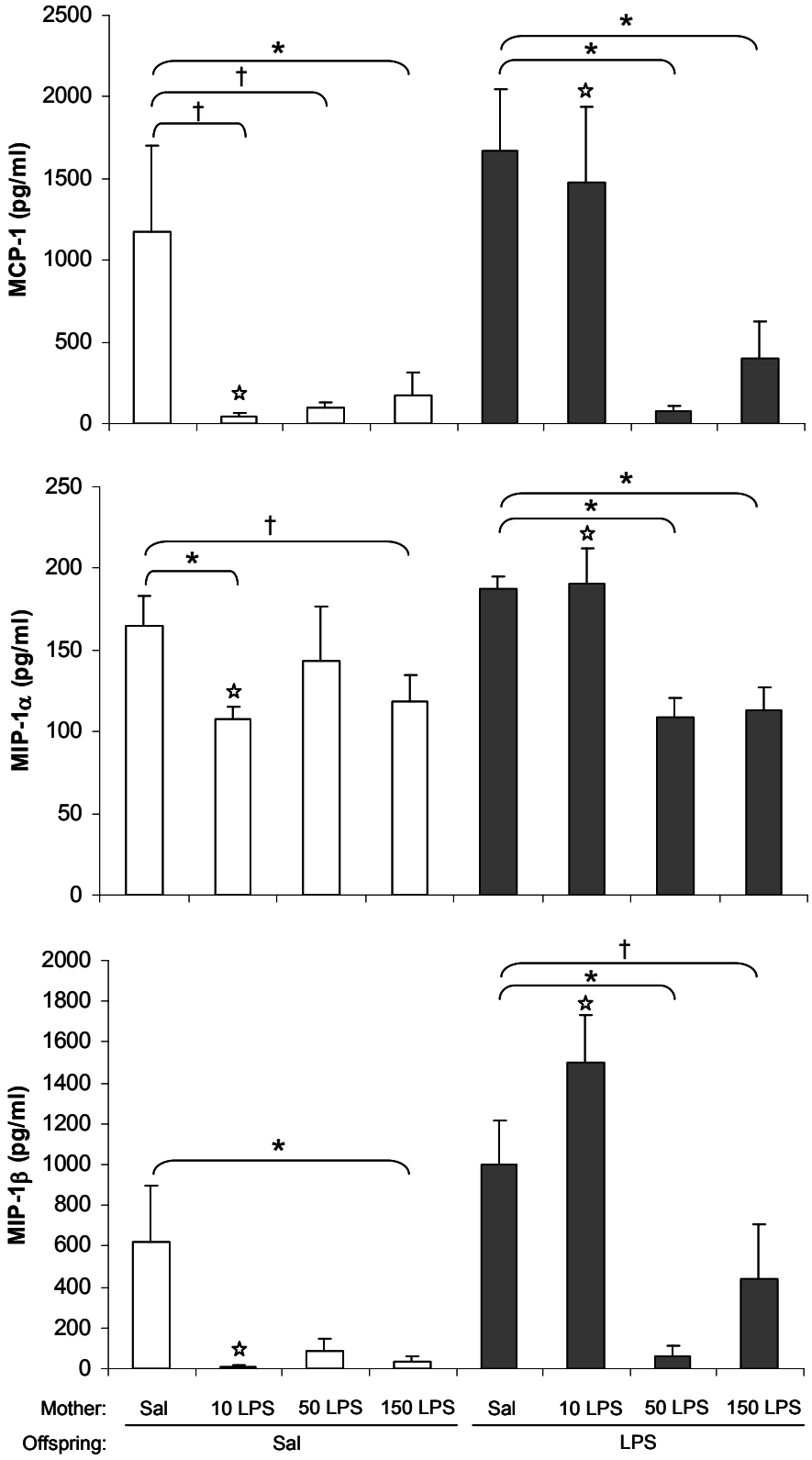

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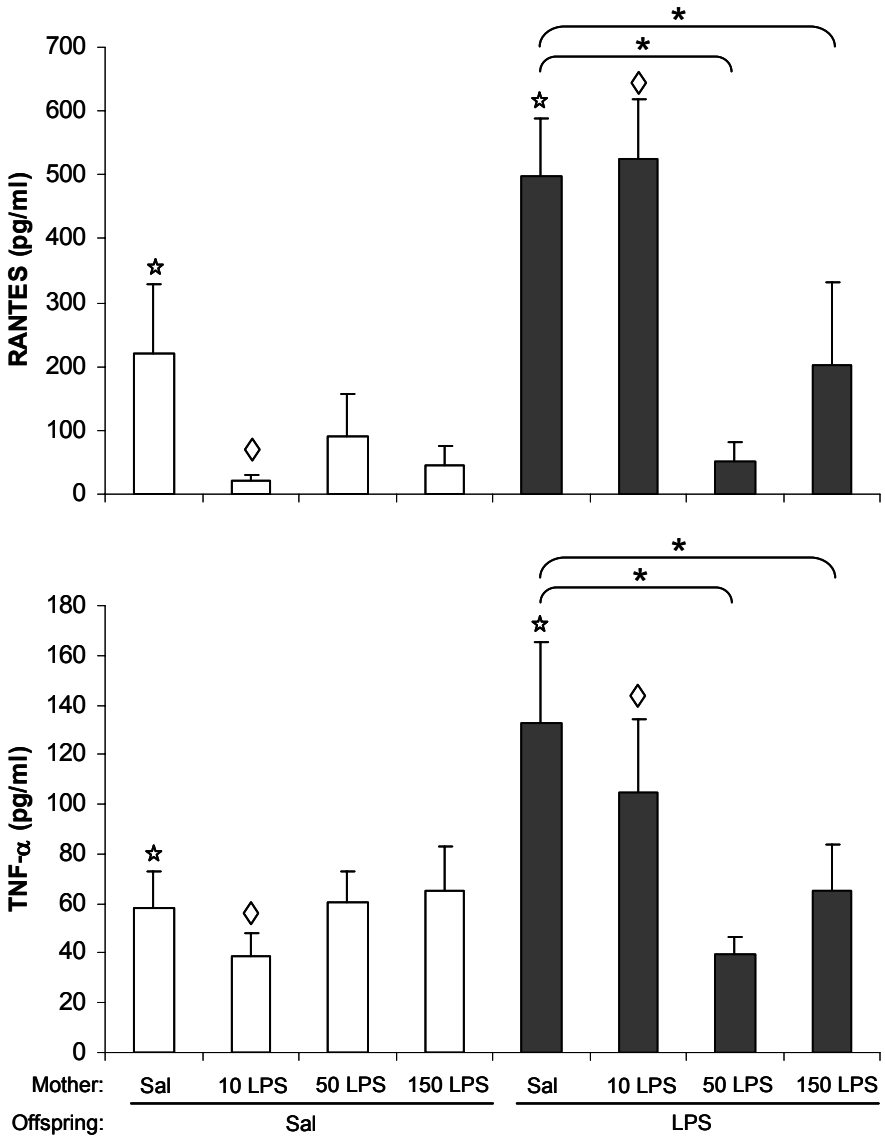


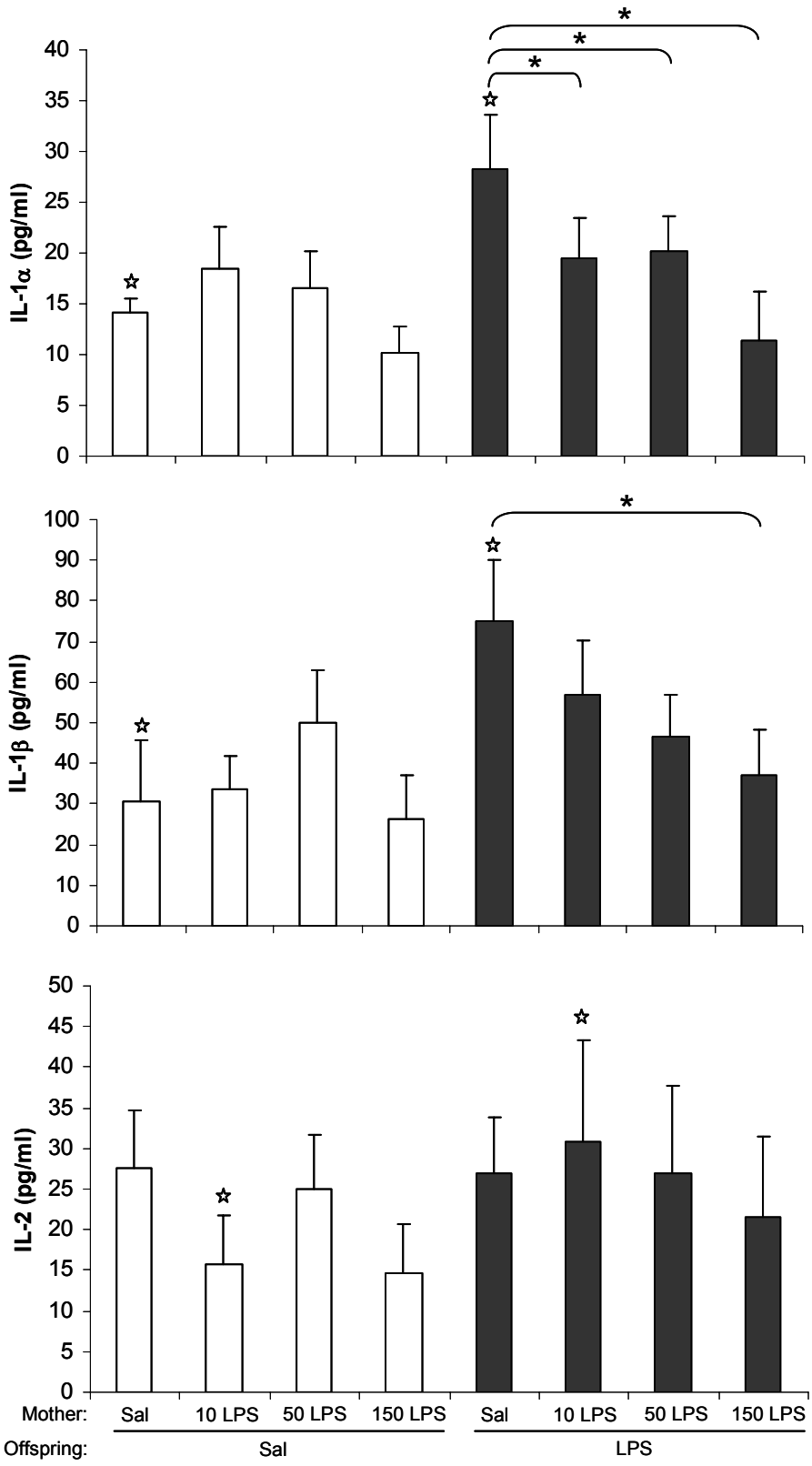
Fig. 6.14 females (*end*)

Figure 6.15 (overleaf) Serum cytokine concentrations (pg/ml) of male offspring 3.5 hours post-injection of saline (open bars; offspring = Sal) or 50 µg/kg LPS (dark bars; offspring = LPS). Maternal treatments; Sal = saline, 10 LPS = 10 µg/kg LPS, 50 LPS = 50 µg/kg LPS, 150 LPS = 150 µg/kg LPS.

Graphs for each of the 23 cytokines are displayed in name order; first the interleukins in numerical order and then all remaining cytokines in alphabetical order.

Values mean±SEM; * P≤0.05; † P≤0.1 when comparing the effect of maternal treatments on offspring. Bars have an open star ☆ or open diamond ◊ (P≤0.05), or an open triangle △ (P≤0.1), when comparing the effect of offspring treatment within the same maternal treatment. Differences are independent of maternal origin and litter size unless otherwise stated; n=6 mothers per maternal treatment group; n=5-6 males per combined treatment group.

NB: The data presented here for IL-6, G-CSF and KC are inaccurate and should be interpreted with caution. Some readings could not be converted to concentrations because the values obtained were above the upper limit of detection of the assay.



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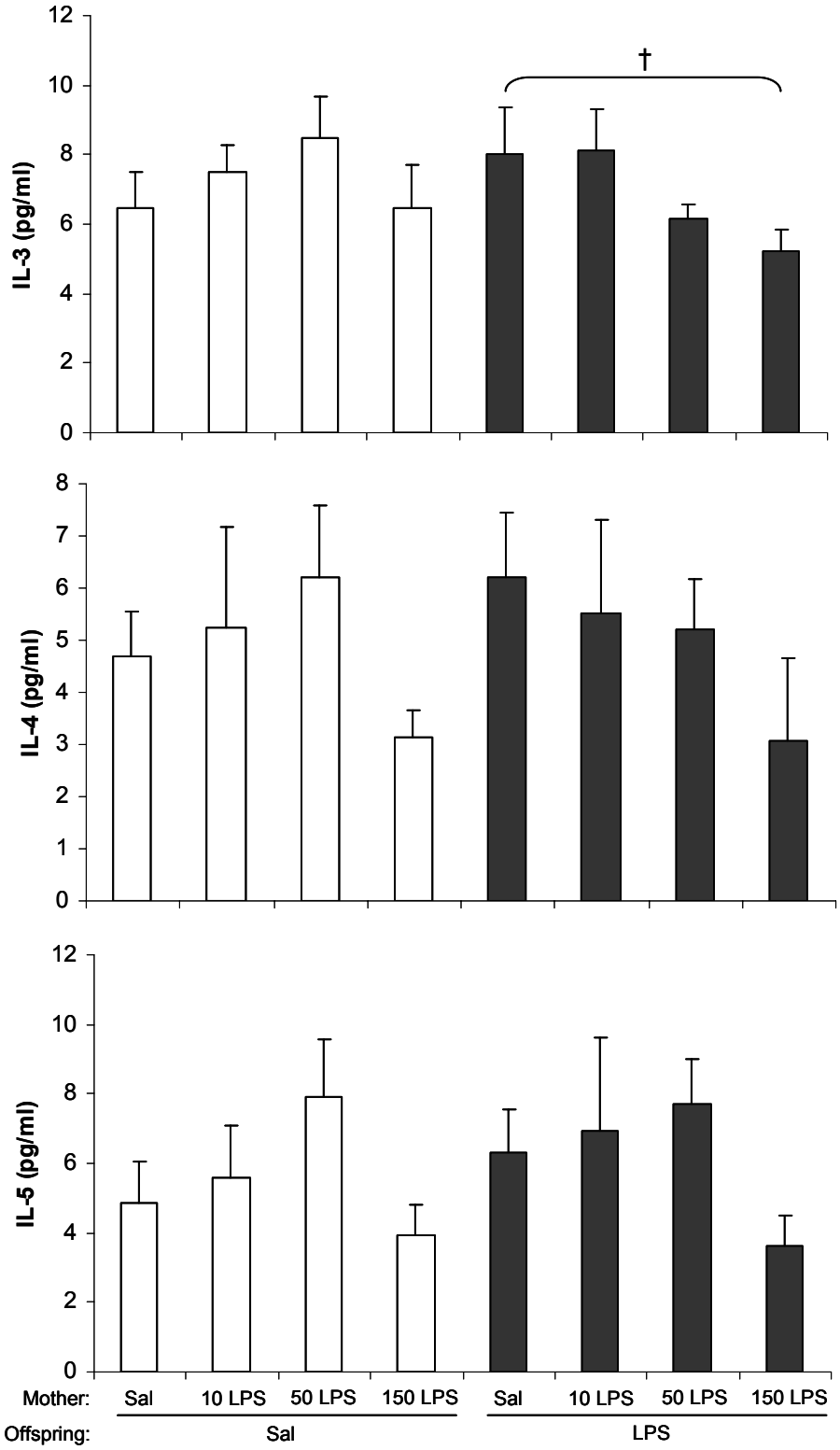

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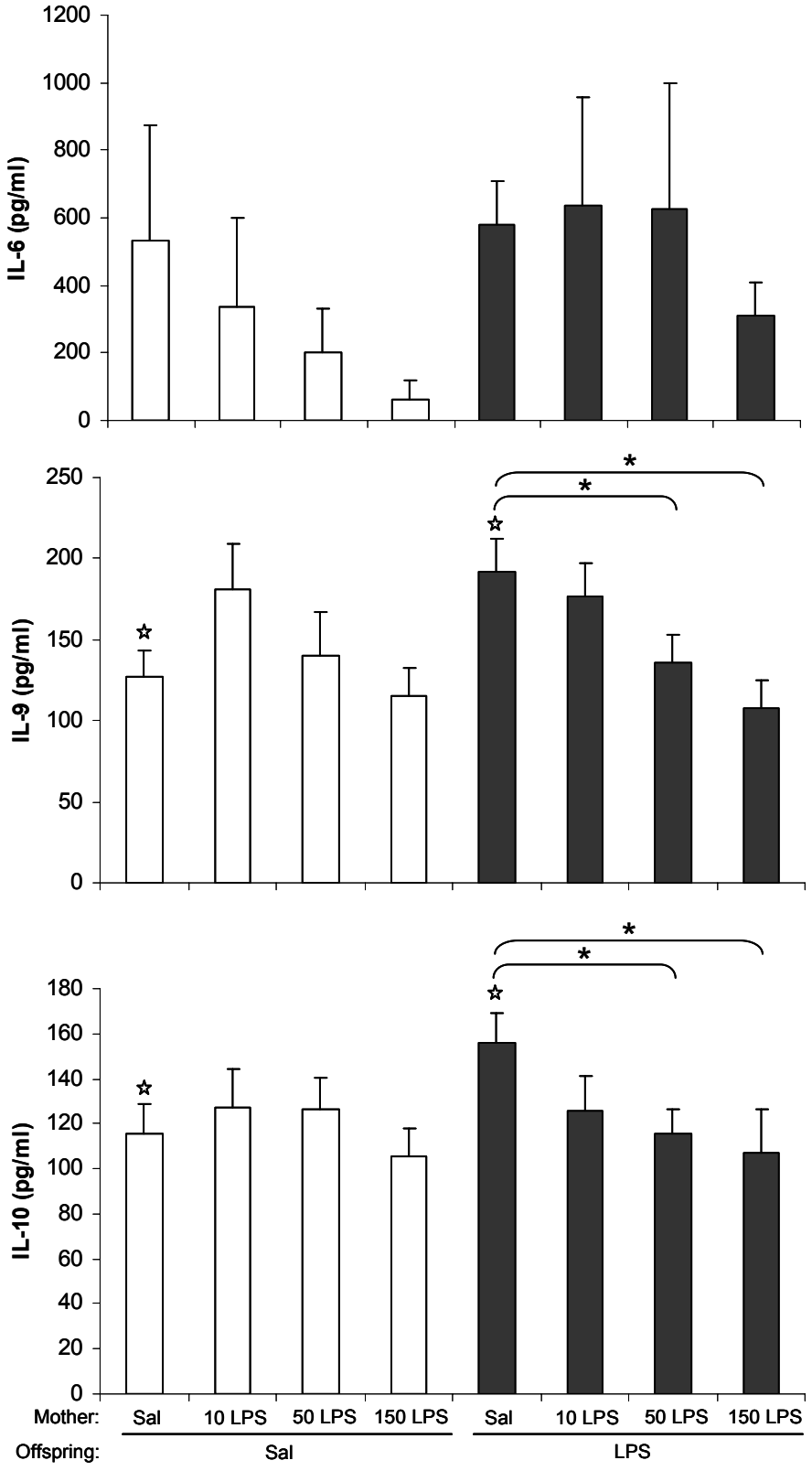

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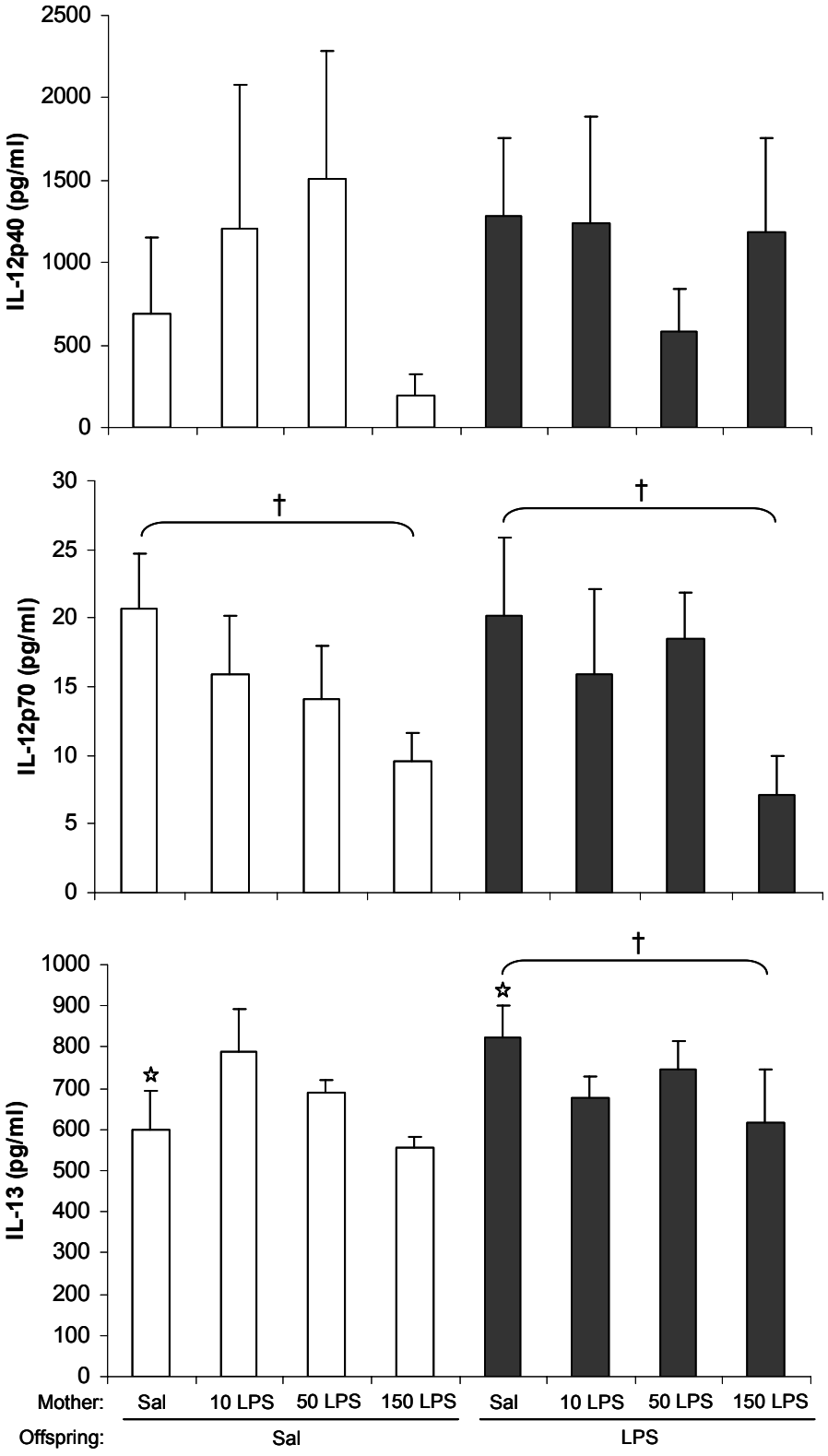

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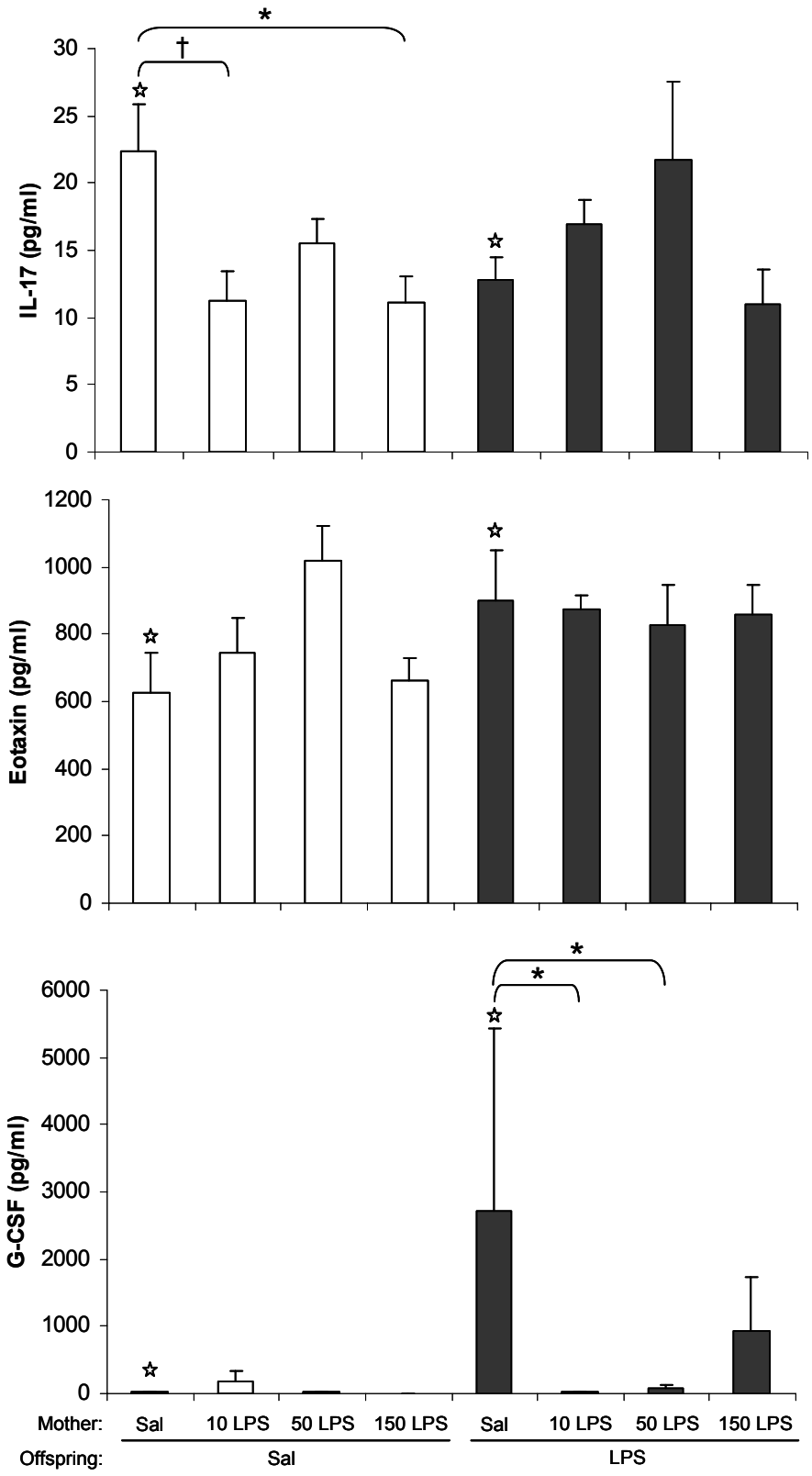

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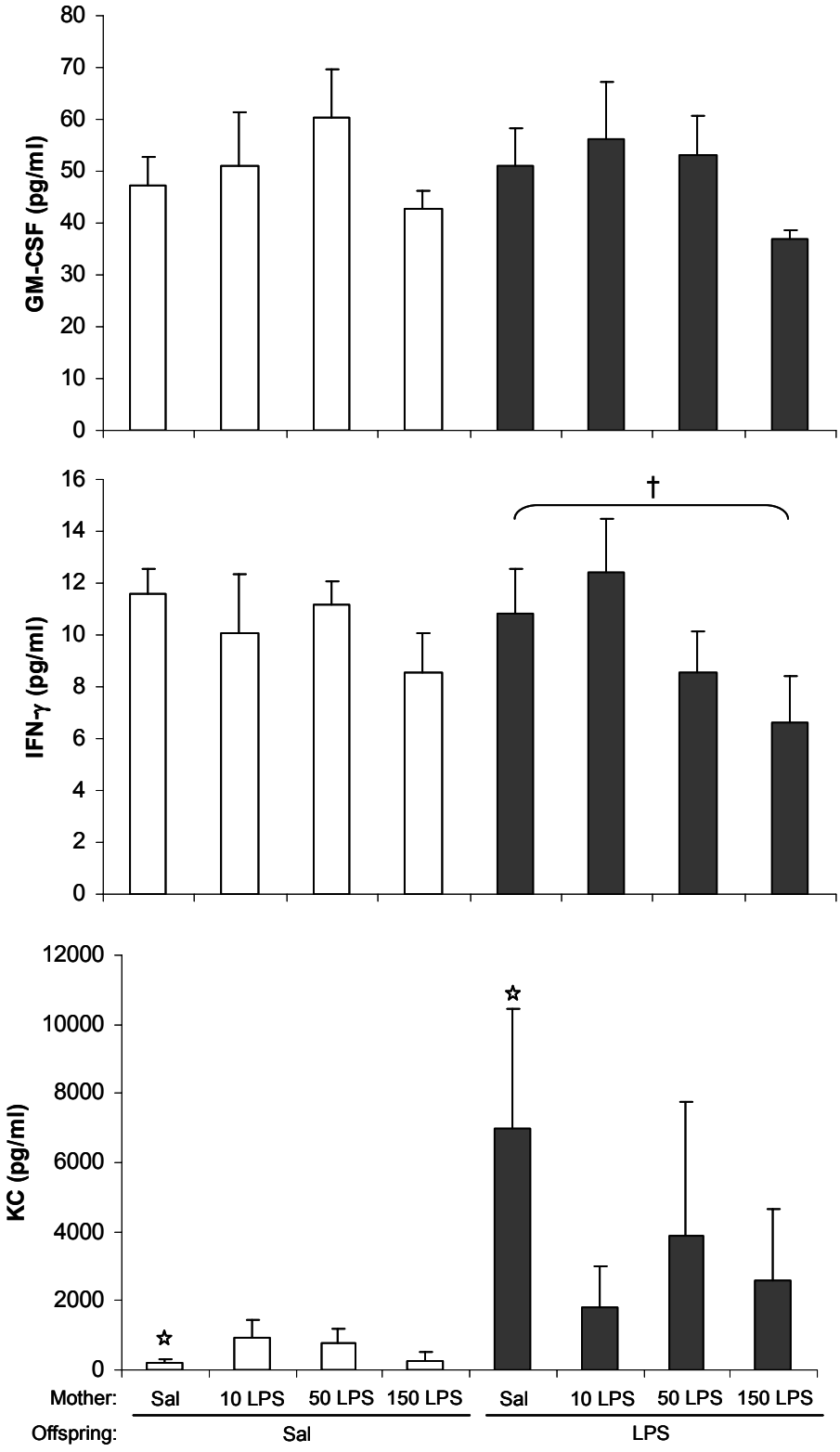

Fig. 6.15 males (*Continued*)

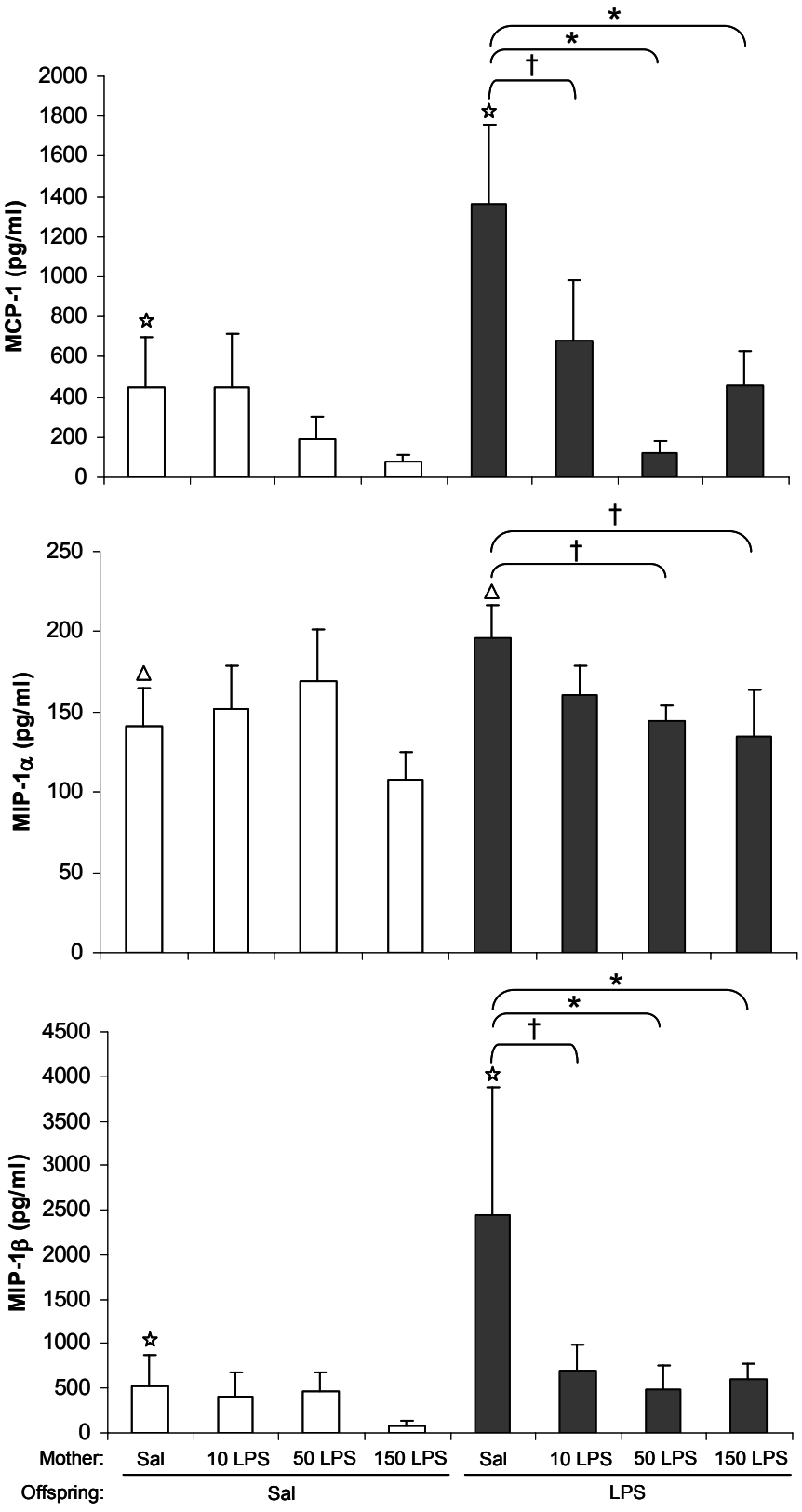

Fig. 6.15 males (*Continued*)

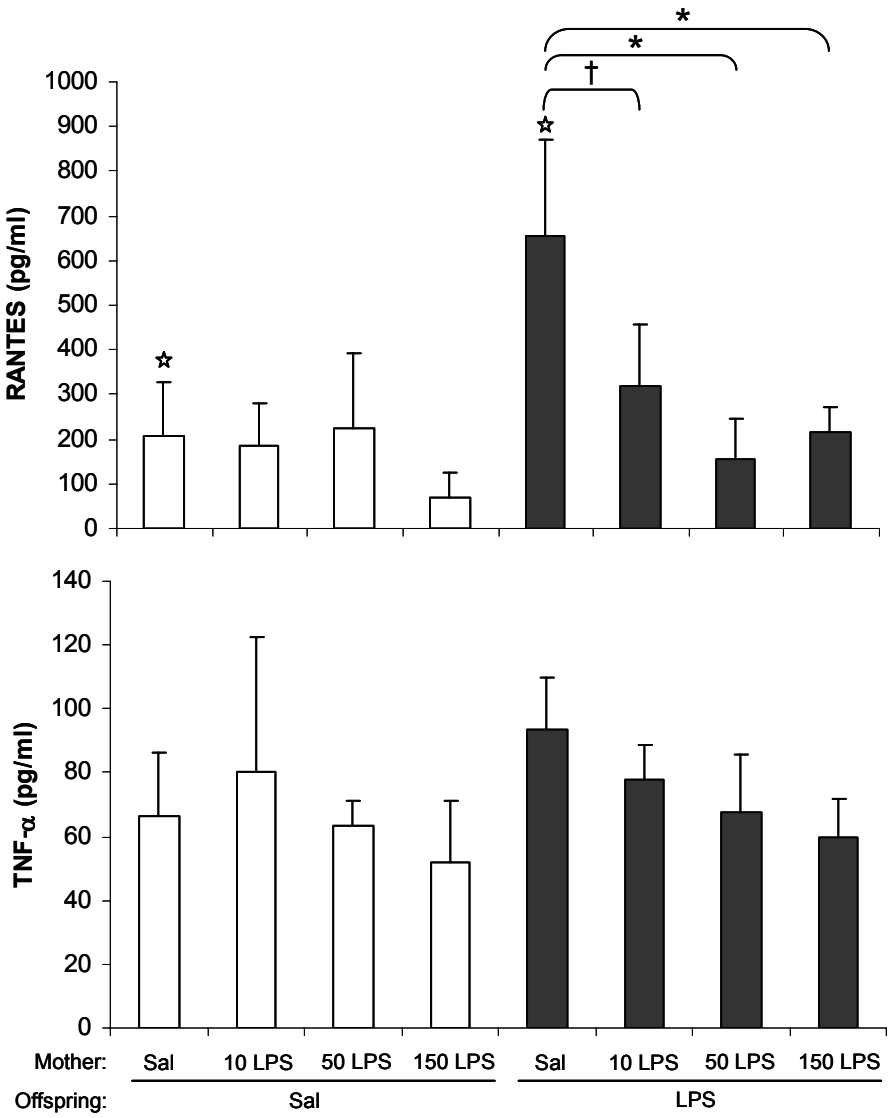

Fig. 6.15 males (*end*)

NB: The multiplex assay was able to detect and quantify the full concentration range of every cytokine at every time point examined, except for IL-6, G-CSF and KC. Thus the data presented here for IL-6, G-CSF and KC should be interpreted with caution. Many readings could not be converted to concentrations because the values obtained were above the upper limit of detection of the assay.

Next, I describe the cytokine data (Figs. 6.14 and 6.15, presented above), in a way that reflects the various comparisons made during analysis (see section 6.3.6).

Comparison 1: The effect of maternal treatment on saline-injected offspring.

Basal (saline-injected) serum concentrations of many cytokines were lower in offspring from maternal LPS treatment groups (i.e. 10 LPS:Sal, 50 LPS:Sal and 150 LPS:Sal) when compared with control (Sal:Sal). Interestingly, this pattern was more prominent in females than in males. A summary of the significant and trend level results for comparison 1 are shown below in Table 6.3.

Table 6.3 Summary table of the significant and trend level results for comparison 1. The effect of maternal treatment on saline-injected offspring.

Treatment Group (vs. Sal:Sal)	Cytokine	Change (% decrease vs. Sal:Sal)	P-value
MALES			
10 LPS:Sal	IL-17	49.6	0.058
150 LPS:Sal	IL-12p70	53.6	0.072
	IL-17	50.4	0.008
FEMALES			
10 LPS:Sal	IL-1α	38.4	0.098
	IL-1β	83.7	0.003
	IL-5	32.8	0.089
	IL-12p40	94.5	0.022
	Eotaxin	52.6	0.041
	GM-CSF	25.3	0.079
	MCP-1	95.9	0.061
	MIP-1α	34.5	0.021
50 LPS:Sal	IL-12p40	74.6	0.086
	MCP-1	92.1	0.079
150 LPS:Sal	IL-12p70	53.6	0.072
	IL-17	50.4	0.008
	IL-1β	55.3	0.033
	IL-2	75.3	0.015
	IL-12p40	83.1	0.019
	GM-CSF	25.4	0.064
	MCP-1	85.4	0.038
	MIP-1α	27.6	0.062
	MIP-1β	93.9	0.048

Comparison 2: The effect of maternal treatment on LPS-injected offspring.

After challenge with 50 µg/ml LPS, offspring from LPS-treated mothers (i.e. 10 LPS:LPS, 50 LPS:LPS and 150 LPS:LPS) generally had lower serum cytokine concentrations than offspring from control mothers (Sal:LPS). Essentially, serum cytokine concentrations were lowest in offspring from mothers that received the highest doses of LPS (i.e. Sal:LPS vs 50 LPS:LPS and Sal:LPS vs 150 LPS:LPS). This pattern was evident in both male and female offspring. Table 6.4 (below) summarises the significant and trend level results for comparison 2.

Table 6.4 Summary table of the significant and trend level results for comparison 2. The effect of maternal treatment on LPS-injected offspring (table continued on next page).

Treatment Group (vs. Sal:LPS)	Cytokine	Change (% decrease vs. Sal:LPS)	P-value
MALES			
10 LPS:LPS	IL-1α	30.9	0.030
	G-CSF	99.3	0.042
	MCP-1	49.7	0.091
	MIP-1β	71.5	0.057
	RANTES	51.2	0.085
50 LPS:LPS	IL-1α	28.7	0.048
	IL-9	29.0	0.033
	IL-10	26.2	0.038
	G-CSF	97.4	0.023
	MCP-1	90.9	0.003
	MIP-1α	26.6	0.053
	MIP-1β	80.5	0.032
	RANTES	76.3	0.013
150 LPS:LPS	IL-1α	59.6	0.003
	IL-1β	50.8	0.043
	IL-3	35.0	0.064
	IL-9	43.8	0.007
	IL-10	31.3	0.022
	IL-12p70	64.9	0.063
	IL-13	25.4	0.078
	IFN-γ	38.9	0.071
	MCP-1	66.2	0.016
	MIP-1α	31.3	0.090
	MIP-1β	75.4	0.047
	RANTES	67.2	0.026

Treatment Group (vs. Sal:LPS)	Cytokine	Change (% decrease vs. Sal:LPS)	P-value
FEMALES			
50 LPS:LPS	IL-1β	65.0	0.001
	IL-2	42.0	0.052
	IL-12p40	84.9	0.005
	G-CSF	99.4	<0.001
	KC	96.1	0.001
	MCP-1	95.6	0.004
	MIP-1α	42.1	0.002
	MIP-1β	93.8	0.006
	RANTES	89.6	0.001
	TNF-α	70.0	0.003
150 LPS:LPS	IL-1α	52.0	0.027
	IL-1β	55.0	0.004
	IL-2	51.2	0.043
	IL-9	32.9	0.067
	IL-10	28.2	0.061
	IL-12p40	77.4	0.005
	G-CSF	99.5	<0.001
	KC	87.6	0.004
	MCP-1	76.1	0.010
	MIP-1α	39.6	0.003
	MIP-1β	56.0	0.054
	RANTES	59.1	0.018
	TNF-α	51.0	0.027

Comparison 3: The effect of offspring LPS challenge within a maternal treatment group.

In offspring from control mothers, the serum concentration of many cytokines increased following LPS-injection i.e. Sal:Sal vs. Sal:LPS. In addition, the serum concentration of many cytokines increased following LPS-injection in offspring from mothers treated with 10 μg/kg LPS (i.e. 10 LPS:Sal vs. 10 LPS:LPS). However, the serum concentration of very few cytokines increased following LPS-injection in offspring from mothers treated with 50 and 150 μg/kg LPS. Table 6.5 (below) summarises the significant and trend level results for comparison 3.

Table 6.5 Summary table of significant and trend level results for comparison 3. The effect of offspring LPS challenge within a maternal treatment group.

Treatment Group	Cytokine	Change (% increase)	P-value
MALES			
Sal:Sal vs Sal:LPS	IL-1α	50.0	0.001
	IL-1β	59.2	0.004
	IL-9	33.4	0.003
	IL-10	25.8	0.028
	IL-13	27.5	0.017
	IL-17	42.9 (decrease)*	0.016
	Eotaxin	30.5	0.011
	G-CSF	99.3	0.017
	KC	97.3	0.021
	MCP-1	67.2	0.015
	MIP-1β	78.5	0.014
	RANTES	68.3	0.018
10 LPS:Sal vs 10 LPS:LPS	IL-2	48.7	0.016
FEMALES			
Sal:Sal vs Sal:LPS	IL-1β	29.3	0.088
	G-CSF	99.0	<0.001
	KC	90.3	0.004
	RANTES	55.7	0.019
	TNF-α	55.9	0.015
10 LPS:Sal vs 10 LPS:LPS	IL-1α	40.9	0.089
	IL-1β	88.4	<0.001
	IL-5	37.5	0.030
	IL-6	99.9	0.046
	IL-9	29.5	0.066
	IL-12p40	95.2	0.007
	Eotaxin	53.1	0.029
	G-CSF	99.4	0.001
	KC	99.7	<0.001
	MCP-1	96.7	0.007
	MIP-1α	43.6	0.003
	MIP-1β	99.2	<0.001
	RANTES	96.0	<0.001
	TNF-α	63.4	0.036
150 LPS:Sal vs 150 LPS:LPS	IL-2	57.5	0.052

To summarise, the serum concentration of many cytokines was lower in saline-injected offspring from LPS injected mothers (vs. saline-injected offspring from saline-injected mothers; comparison 1). Following LPS challenge, the serum concentration of many cytokines increased in offspring from control and 10 μg/kg LPS treated mothers, but did not increase in offspring from 50 and 150 μg/kg LPS treated mothers (comparison 3). Often this meant that the serum concentration of many cytokines following LPS challenge was not different when comparing offspring from 10 μg/kg LPS mothers to control, but remained lower in offspring from 50 and 150 μg/kg LPS treated mothers (comparison 2).

General pattern of altered and unaltered cytokines

During analysis it became clear that, broadly, individual offspring serum cytokines were either affected or unaffected by maternal LPS-treatment (Table 6.6).

- **Affected** (Table 6.6): Serum cytokines concentrations were significantly or trend level affected by maternal LPS treatment in saline-and/or LPS-treated offspring (vs offspring from saline treated control mothers).
- **Unaffected** (Table 6.6): Serum cytokines concentrations were unaffected by maternal LPS treatment, whether LPS-injected or saline-injected.

Offspring gender	Cytokines affected by maternal LPS-treatment		Cytokines unaffected by maternal LPS-treatment	
Males	IL-1α	IL-17	IL-2	KC
	IL-1β	G-CSF	IL-4	TNF-α
	IL-3	IFN-γ	IL-5	
	IL-9	MCP-1	IL-6	
	IL-10	MIP-1α	IL-12p40	
	IL-12p70	MIP-1β	Eotaxin	
	IL-13	RANTES	GM-CSF	
Females	IL-1α	G-CSF	IL-3	
	IL-1β	GM-CSF	IL-4	
	IL-2	KC	IL-6	
	IL-5	MCP-1	IL-12p70	
	IL-9	MIP-1α	IL-13	
	IL-10	MIP-1β	IL-17	
	IL-12p40	RANTES	IFN-γ	
	Eotaxin	TNF-α		

Table 6.6 Serum cytokine concentrations in offspring (saline- and/or LPS-treated) were either affected or unaffected by maternal LPS treatment. Criteria for inclusion in a particular category were based on the presence of a statistically significant or trend level result for one or more treatment group when compared with control, for both LPS- and saline-injected offspring.

6.3.6.2 Offspring splenocytes

Splenocyte populations were identified from forward scatter (FSC) vs. side scatter (SSC) plots. From the FSC vs. SSC plot of every mouse examined, two populations of splenocytes were evident (referred to here as population A and population B). Population A represented 10-20% of total events and consisted of small cells with a low degree of granularity (Fig. 6.16, top panel). In contrast, population B represented 35-45% of total events and consisted of larger cells with a greater degree of granularity (Fig. 6.16, bottom panel). Splenocyte populations A and B were analysed for expression of CD19 (B lymphocytes), CD4 (T helper lymphocytes) and CD8 (cytotoxic T lymphocytes; Fig. 6.16).

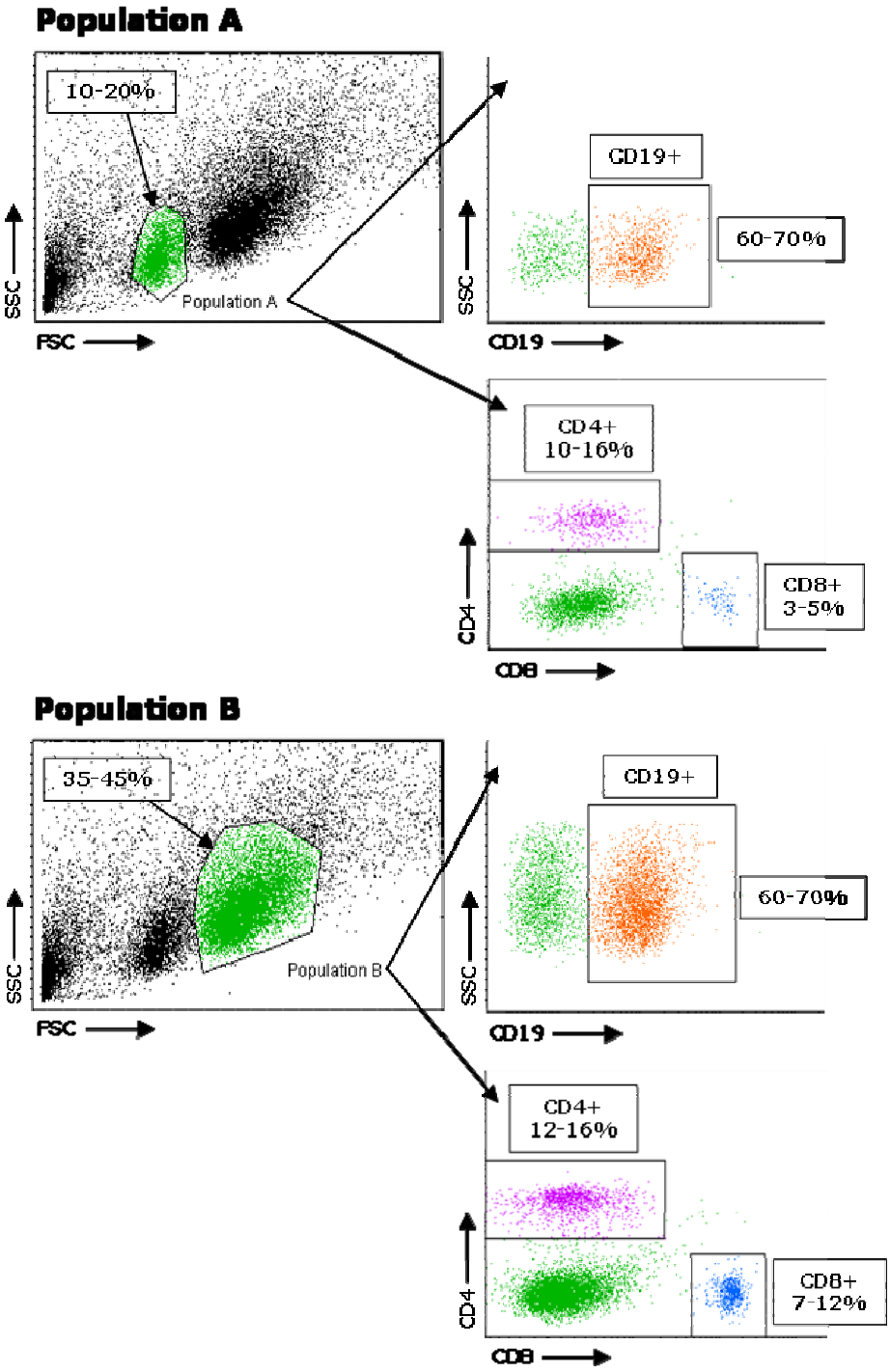

Figure 6.16 Flow cytometry plots showing splenocyte populations and gating strategy (this example is representative of all mice in this study). The forward scatter vs. side scatter (FSC vs. SSC) plot revealed the presence of 2 main cell populations; population A, consisting of small cells with a low degree of granularity, and population B, consisting of larger cells with a greater degree of granularity. The percentage of cells expressing, CD19 (B lymphocytes), CD4 (T helper lymphocytes) and CD8 (cytotoxic T lymphocytes) were then examined in both of these cell populations separately.

CD19$^+$, males: Saline-injected male offspring from 150 µg/kg LPS mothers had 5.7% fewer CD19$^+$ cells in population B than saline-injected male offspring from control mothers (P=0.042; Fig. 6.17b). In addition, saline-injected male offspring from 10 µg/kg LPS mothers displayed a trend towards fewer CD19$^+$ cells in population B than saline-injected male offspring from control mothers (3.8% less; P=0.083; Fig. 6.17b). Interestingly, LPS-injected male offspring from 150 µg/kg LPS mothers also displayed a trend towards fewer CD19$^+$ cells in population B than LPS-injected male offspring from control mothers (5% less; P=0.077; Fig. 6.17b). LPS-injected male offspring from 10 µg/kg LPS mothers displayed a trend towards more CD19$^+$ cells in population B than saline-injected male offspring from the same maternal treatment group (5.2% more; P=0.057; Fig. 6.17b). There were no differences between treatments in the percentage of population A CD19$^+$ cells in males, although LPS-injected offspring from 50 µg/kg LPS mothers displayed a trend towards fewer CD19$^+$ cells than LPS-injected male offspring from control mothers (6.8% less; P=0.091; Fig. 6.17a).

CD4$^+$, males: Saline-injected male offspring from 150 µg/kg LPS mothers had 4.2% more CD4$^+$ cells in population A than saline-injected male offspring from control mothers (P=0.031; Fig. 6.18a). Interestingly, LPS-injected male offspring from 150 µg/kg LPS mothers also displayed a trend towards more CD4$^+$ cells in population A than LPS-injected male offspring from control mothers (3.5% more; P=0.062; Fig. 6.18a). There were no differences between treatments in the percentage of population B CD4$^+$ cells in males, although LPS-injected offspring from 10 and 150 µg/kg LPS mothers displayed a trend towards more CD4$^+$ cells than LPS-injected male offspring from control mothers (2%, P=0.078 and 4.1%, P=0.057 more respectively; Fig. 6.18b).

CD8$^+$, males: Saline-injected male offspring from 50 µg/kg LPS mothers had 3.4% more CD8$^+$ cells in population B than saline-injected male offspring from control mothers (P=0.008; Fig. 6.18d). LPS-injected male offspring from 50 µg/kg LPS mothers displayed a trend towards more CD8$^+$ cells in population A than LPS-injected male offspring from control mothers (0.8% more; P=0.078; Fig. 6.18c).

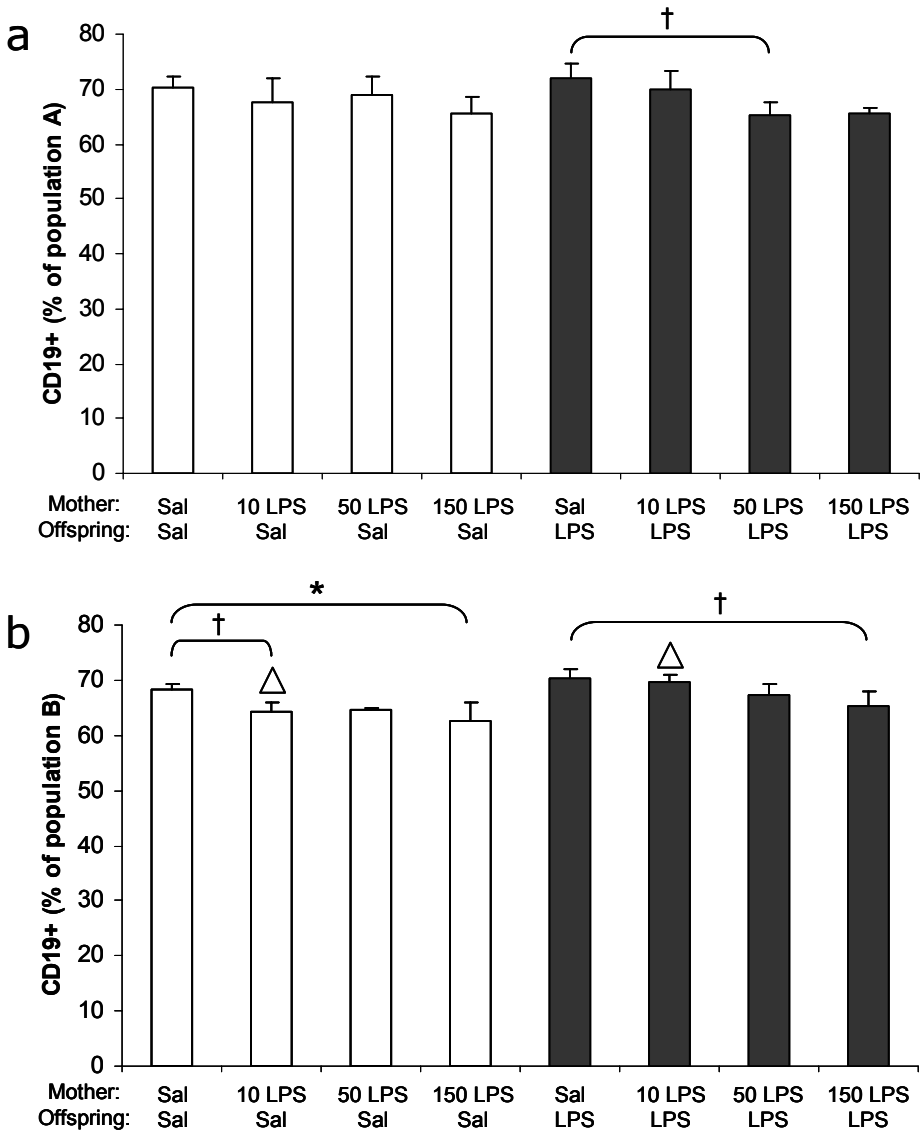

Figure 6.17 Percentage of B lymphocytes ($CD19^+$) in spleens of male offspring from each treatment group; (**a**) $CD19^+$ cells in population A (%), (**b**) $CD19^+$ cells in population B (%). Values are mean±SEM; * P≤0.05; † P≤0.1 when comparing the effect of maternal treatments on offspring. Bars have an open triangle (P≤0.1) when comparing the effect of offspring treatment within the same maternal treatment. Differences are independent of maternal origin and litter size. n=5-6 mothers per treatment group; n= 5-6 male offspring per treatment group.

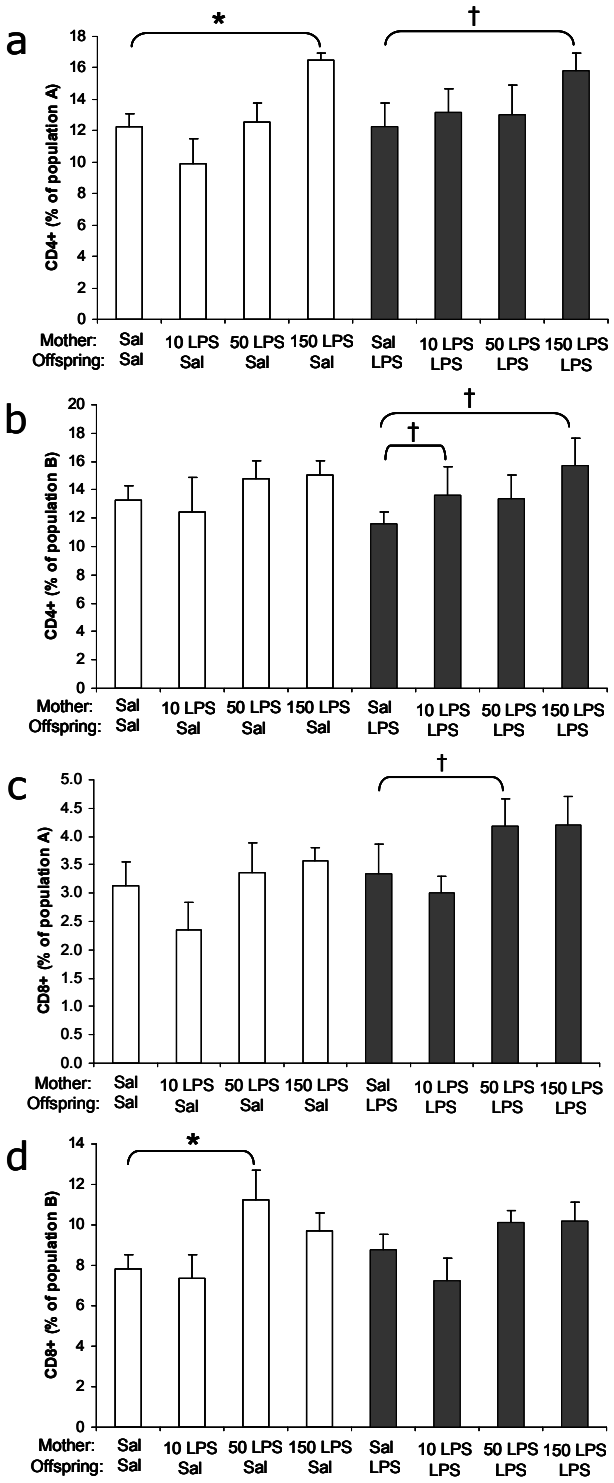

Figure 6.18 Percentage of T helper ($CD4^+$) and cytotoxic T ($CD8^+$) lymphocytes in spleens of male offspring; (**a**) $CD4^+$ in population A, (**b**) $CD4^+$ in population B, (**c**) $CD8^+$ in population A, (**d**) $CD8^+$ in population B. Values are mean±SEM. * $P \leq 0.05$; † $P \leq 0.1$ when comparing the effect of maternal treatments on offspring. Differences are independent of maternal origin and litter size. n=5-6 mothers per treatment group; n= 5-6 male offspring per treatment group.

LPS-injected female offspring from 150 µg/kg LPS mothers had fewer $CD8^+$ cells in population B than saline-injected female offspring from the same maternal treatment group (3.3% less; P=0.040; Fig. 6.20d). There were no differences between treatment groups for female offspring in the percentage of population A $CD8^+$ cells (Fig. 6.20c). Furthermore, there were no differences between treatment groups for female offspring in the percentage of $CD19^+$ or $CD4^+$ cells in either population A or B (Fig. 6.19a,b and Fig. 6.20a,b, respectively).

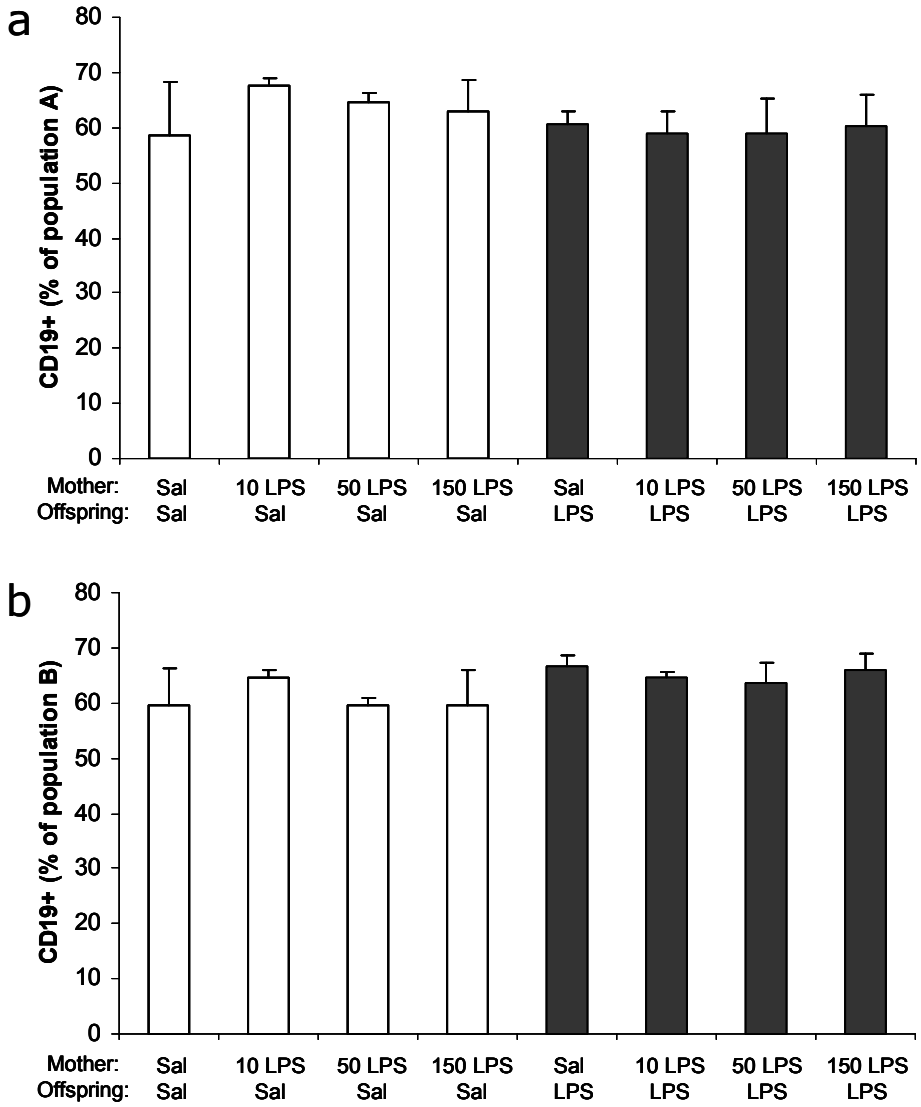

Figure 6.19 Percentage of B lymphocytes ($CD19^+$) in spleens of female offspring from each treatment group; (**a**) CD19+ cells in population A (%), (**b**) $CD19^+$ cells in population B (%). Values are mean±SEM; n=4-6 mothers per treatment group; n= 4-6 female offspring per treatment group.

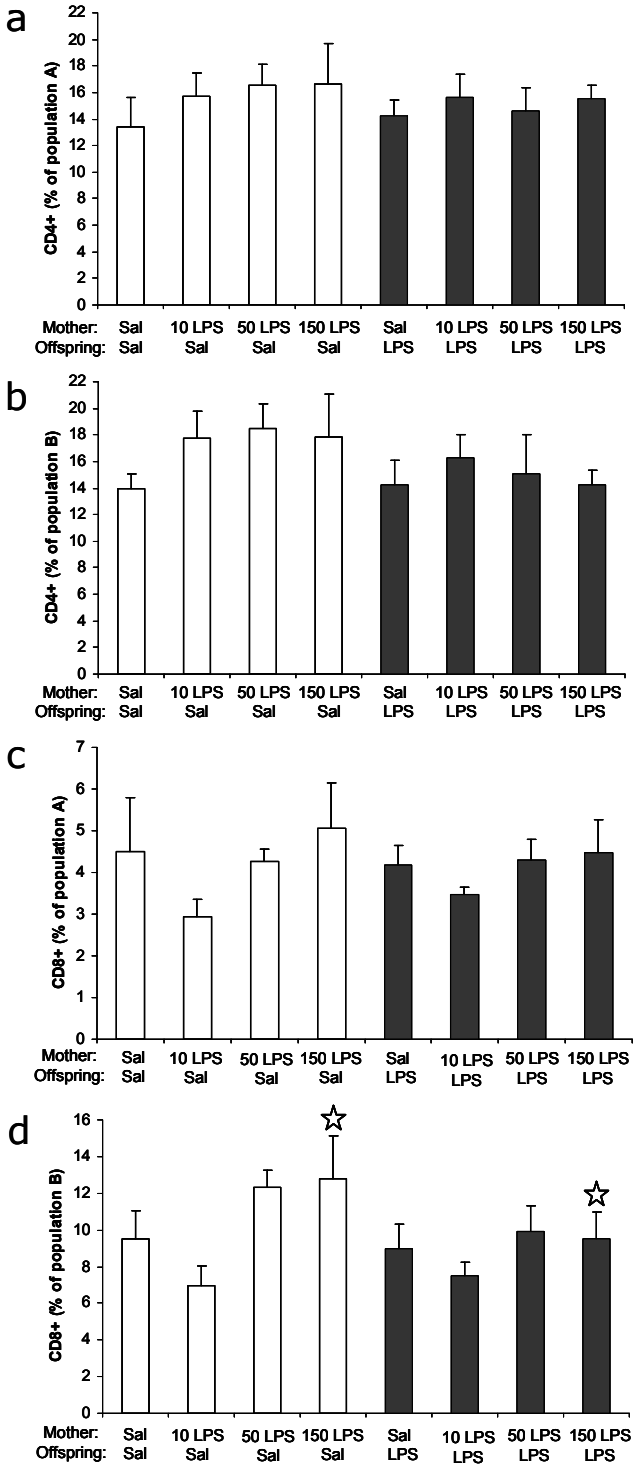

Figure 6.20 Percentage of T helper ($CD4^+$) and cytotoxic ($CD8^+$) T lymphocytes in spleens of female offspring; (**a**) $CD4^+$ in population A, (**b**) $CD4^+$ in population B, (**c**) $CD8^+$ in population A, (**d**) $CD8^+$ in population B. Values are mean±SEM. Bars have an open star ($P \leq 0.05$) when comparing the effect of offspring treatment within the same maternal treatment. Differences are independent of maternal origin and litter size. n=4-6 mothers per treatment group; n= 4-6 female offspring per treatment group.

6.4 Discussion

The evidence presented in this chapter showed for the first time that administration of 10, 50 and 150 µg/kg LPS to pregnant mice on GD 0.5 did not alter birth weight but did result in altered postnatal offspring phenotype, affecting behaviour, systolic blood pressure (SBP), tissue/body weight ratios, body mass index (BMI), the serum concentration of many cytokines and lymphocyte populations in the spleen (Table 6.7).

Offspring phenotype	Males (µg/kg LPS)			Females (µg/kg LPS)		
	10	50	150	10	50	150
Locomotor and exploratory behaviour	-	-	↓	-	-	-
SBP	↑	-	-	-	-	-
Liver/body weight	-	-	-	-	↓	-
Retro-peritoneal fat/body weight	-	-	↑	-	-	-
Inguinal fat/body weight	-	-	↑	-	-	-
BMI	-	-	↑	-	-	-
Basal serum cytokines (saline-injected)	↓	↓	↓↓	↓	↓	↓↓
Immune challenged serum cytokines (LPS-injected)	↓	↓	↓↓	↓	↓	↓↓
Spleen B lymphocyes	-	-	↓	-	-	-
Spleen T helper lymphocytes (CD4+)	-	-	↑	-	-	-
Spleen cytotoxic T lymphocytes (CD8+)	-	↑	-	-	-	-

Table 6.7 Summary of the phenotypic alterations seen in offspring from immune challenged mothers (vs. offspring from control mothers).

In particular, male offspring from 150 µg/kg LPS treated mothers displayed reduced locomotor and exploratory-related behaviour, increased fat-pad mass (retro-peritoneal and inguinal fat/body weight ratios), increased BMI, reduced serum cytokine concentrations, reduced percentage of splenic B lymphocytes ($CD19^+$) and increased percentage of splenic T helper lymphocytes ($CD4^+$). Male offspring from 10 and 50 µg/kg LPS treated mothers were much less affected. The 50 µg/kg LPS males displayed reduced serum cytokine concentrations and increased percentage of splenic

cytotoxic T lymphocytes ($CD8^+$). The 10 µg/kg LPS males displayed reduced serum cytokine concentrations and, unexpectedly, increased SBP. Overall, female offspring were less affected than their male counterparts: the only major alteration in phenotype was reduced serum cytokine concentrations and this was observed in female offspring from all LPS treatment mothers. However, interestingly, liver/body weight ratio was significantly reduced in 50 µg/kg LPS females and to trend level in 10 and 150 µg/kg LPS females.

In the present study, birth weight was not altered by any of the maternally administered LPS doses. This finding supports the results of the previous chapter (Chapter 5), where no differences in GD 17 conceptus or fetus weight were found following the same maternal LPS treatments as used in this chapter. Birth weight can act as an indicator of an adverse prenatal environment and is thus a convenient parameter to use for making associations with postnatal outcomes in the offspring (Gluckman et al., 2007). However, birth weight is an incomprehensive marker because some alterations to the prenatal environment have been shown to result in long term phenotypic changes in the offspring without altering fetal growth; for example, the offspring of women subjected to under-nutrition in early pregnancy during the Netherlands famine did not have reduced birth weight but did have an increased risk of obesity (Painter et al., 2005). Thus, in the present study birth weight did not act as an indicator of alterations in other aspects of offspring phenotype, e.g. behavioural changes.

Despite its unsuitability as a marker, birth weight has proven to be invaluable in epidemiological studies for identifying links between adverse prenatal environments and postnatal outcomes, identifying not only an association with cardiovascular and metabolic abnormalities but also those pertaining to behaviour and mental health (Schlotz and Phillips, 2009). Low birth weight has been associated with a range of behavioural and metal health outcomes in offspring, including; low IQ, behavioural problems in children and adolescents (e.g. hyperactivity, inattention), antisocial personality disorders, stress reactivity and schizophrenia (Schlotz and Phillips, 2009). Schizophrenia is particularly interesting in the context of the present study because of the increasingly documented link between maternal immune activation and the occurrence of this brain disorder in the resultant offspring (Meyer et al., 2008). Indeed, an important mouse study demonstrated that offspring from mothers treated with IL-6, but not IL-1β, IFN-γ or TNF-α, during mid-gestation displayed the same

postnatal behavioural phenotype as mid-gestational PolyI:C administration (Smith et al., 2007). Human birth cohort studies have implicated the early stages of gestation as a vulnerable period. One such study reported that maternal genital and reproductive infections during periconception increased the risk of schizophrenia in the offspring (Babulas et al., 2006). Another of these studies indicated that the risk of schizophrenia in offspring was increased 7-fold after serologically documented influenza exposure during the first trimester of pregnancy (Brown et al., 2004). The evidence presented in this chapter shows that preimplantation stage maternal immune activation in the mouse can result in altered behaviour in the adult offspring. This finding supports the concept that prenatal environment impacts upon behavioural phenotype and provides evidence that maternal inflammation can induce offspring behavioural abnormalities by influencing the preimplantation embryo, well before the appearance of identifiable brain structures.

This finding joins a selection of other studies in mice where behavioural abnormalities have been documented in offspring following a preimplantation stage challenge, e.g. *in vitro* embryo culture and low protein maternal diet *in vivo* (Ecker et al., 2004; Fernandez-Gonzalez et al., 2004; Watkins et al., 2008b). Alterations in locomotor, exploratory and anxiety-related behaviours are a consistent feature of all of the studies; however, gender-specific differences are apparent and the direction of change in such behavioural activities also differs. In the present study, only male offspring from 150 µg/kg LPS treated mothers exhibited reduced locomotor and exploratory-related behaviour. Interestingly, the *in vitro* culture studies also showed effects in only male offspring, but reported increased locomotor activity and decreased anxiety (Ecker et al., 2004; Fernandez-Gonzalez et al., 2004). Furthermore, increased locomotor and exploratory behaviour was also demonstrated following preimplantation low protein maternal diet *in vivo*, but only female offspring were affected (Watkins et al., 2008b). Taken together, these findings suggest that, in the mouse, locomotor, exploratory and anxiety-related offspring behaviours are particularly sensitive to preimplantation embryo environment, and that the nature of the preimplantation challenge may influence the direction in which these behaviours are altered and the gender of the offspring in which they occur.

In this chapter, gender and treatment group-specific alterations were also found in offspring BMI and adiposity. As was seen with the behavioural alterations, only male offspring from 150 µg/kg LPS treated mothers were affected. Males from 150 µg/kg

LPS mothers had significantly increased BMI, despite there being no significant difference in raw body mass. However, to explain this discrepancy, it should be noted that males from 150 µg/kg LPS mothers did weigh ~2 g more than control males at cull, whereas there was absolutely no difference in body length (mean body lengths were within ~2 mm of each other). As such, it seems the small difference in body weight was more influential when the relationship between body weight and body length ('height') was considered in the BMI calculation. The increased BMI in males from 150 µg/kg LPS mothers appeared to be due to increased adiposity, specifically of the retro-peritoneal and inguinal fat-pads. Also in the males from 150 µg/kg LPS mothers, body weight ratios of all organs examined seemed to be reduced, however this was not statistically significant for any organ except for a trend seen in the lung/body weight ratio. In female offspring, there was no effect of maternal LPS treatment on the weight of any tissue except the liver, where liver/body weight ratio was reduced significantly in 50 µg/kg LPS females and to trend level in 10 and 150 µg/kg LPS females. Disproportionate body structure in male offspring, affecting particularly adipose tissue, has been observed in other studies. Culture of preimplantation mouse embryos *in vitro* increased the body weight of only male offspring (Sjoblom et al., 2005). Similar to the present study, these male offspring displayed central adiposity (including increased retro-peritoneal fat) and no change was seen in the mass of any major organ except the brain, where brain/lean body weight ratio was decreased (Sjoblom et al., 2005). Disproportionate body structure has also been observed in male offspring following preimplantation low protein maternal diet in the rat (Kwong et al., 2000). In the rat preimplantation low protein diet study male offspring kidney/body weight ratios were increased whereas liver/body weight ratios were decreased, however no information was presented regarding adipose tissue (Kwong et al., 2000).

Interestingly, a disproportionate offspring body structure similar to that seen in the present study (where LPS was administered in early gestation) was observed in a rat study where mothers were challenged with LPS (790 µg/kg) during mid-gestation (Nilsson et al., 2001). In this rat study, male offspring displayed increased abdominal adiposity (specifically of retro-peritoneal and epididymal fat-pads) but there were no differences in the mass of muscle or other major organs (however, no data was presented for kidney or liver). Importantly, a wide range of physiological and endocrine parameters were investigated in this study, which allowed the authors to demonstrate a phenotype whereby male offspring displayed not only central obesity,

but also other features relevant to the metabolic syndrome, including; insulin resistance, increased plasma leptin concentrations, hyperphagia, increased 17β-estradiol and increased progesterone (Nilsson et al., 2001). Nilsson et al (Nilsson et al., 2001) also found in male offspring increased hippocampal glucocorticoid receptor (GR) protein and reduced glucocorticoid (corticosterone) response to stress, but no change in hippocampal or hypothalamic leptin receptor protein and no change in blood pressure. Furthermore, in another study, pregnant rats were injected with IL-6, TNF-α or dexamethasone (synthetic glucocorticoid) during mid-gestation and the offspring were examined postnatally for a range of physiological and endocrine parameters (Dahlgren et al., 2001). The authors reported an overlapping but non-identical phenotype in the offspring following the different maternal treatments, including; increased body weight in all offspring that was due to increased adiposity, corticosterone response to stress was increased in the IL-6 group, insulin resistance was seen in IL-6 group males, males from the TNF-α group showed decreased locomotor activity whereas the IL-6 females showed increased locomotor activity and plasma testosterone levels were increased in females from the IL-6 and TNF-α groups (Dahlgren et al., 2001). Despite a larger LPS dose being administered at a later gestational time point in the rat study, there are certain similarities with the results presented here in this chapter, namely the increase in male offspring adiposity without any alteration in blood pressure (males from 150 μg/kg LPS mothers). In the present study no endocrine parameters or receptor expression experiments were conducted, therefore to see if further similarities exist, it would be interesting to conduct such investigations in the early gestational inflammation model used in the present study.

The elevated systolic blood pressure (SBP) displayed by only male offspring from 10 μg/kg LPS treated mothers was somewhat unexpected because; i) elevated SBP was not seen in offspring from any other treatment group, and ii) all other phenotypic alterations have been seen in the highest LPS group (e.g. behaviour, increased adiposity and BMI). Interestingly, GD 3.5 blastocysts from 10 μg/kg LPS treated mothers displayed an unexpected increase in TE cell number (Chapter 5) and moreover, both total litter size and the number of male pups was increased in the 10 μg/kg LPS group. These observations suggest that maternal treatment with 10 μg/kg LPS may increase TE cell number in blastocysts, which may enhance their ability to implant and thus result in increased litter size. In addition, there may be a preferential effect on male embryos because a specific increase in the number of neonatal male offspring was observed. Indeed, it has been found that male embryos cleave faster

than female embryos (Mittwoch, 1993); therefore male embryos could be more responsive to the apparent effects of the 10 μg/kg LPS maternal treatment. I suggest that the elevated SBP seen only in the male offspring from 10 μg/kg LPS treated mothers is associated with the alterations discussed above; perhaps acting in a 'classical DOHaD' manner, whereby a suboptimal in utero environment, resulting from decreased availability of maternal resources due to increased litter size, induces offspring cardiovascular abnormalities. However, it is not possible to confirm this hypothesis from the results obtained in the present study.

In the present study, serum cytokine concentrations were the only parameter examined where both males and females from all LPS treatment groups were affected to some extent. The major finding was that serum cytokine responses were blunted in response to LPS-injection in offspring from LPS treated mothers, where the offspring from the highest treatment groups (50 and 150 μg/kg) were most affected. Interestingly, the basal (saline-injected) level of a larger number of the cytokines appeared to be blunted in female offspring than in their male counterparts; for example MCP-1 concentration was blunted in both saline- and LPS-injected female offspring whereas the same cytokine was blunted only in LPS-injected male offspring.

A pattern appeared to exist in the type of cytokine that was 'blunted vs. unaffected' in offspring from LPS treated mothers: the classical pro-inflammatory and antigen presenting cell (APC; e.g. monocyte, macrophage and dendritic cell) produced cytokines seemed to be more affected than the T helper cell type (T_H) cytokines. For example, IL-1β is a typical pro-inflammatory APC produced cytokine that was blunted and IL-4 is typical T_H cytokine that was unaffected in female and male offspring from LPS treated mothers (see table of main cellular sources and actions of cytokines in Appendix II). It could be that this pattern of affected cytokines (i.e. 'the APC-produced are affected but the T_H -produced are unaffected' idea) is seen because of the timing of the serum collection and/or the nature of the challenge used in this study. It is possible that the kinetics of the immune response are affected by maternal LPS-treatment, thus conducting a time course assay rather than looking at a single time point would be informative. Furthermore, LPS is known to stimulate the production of pro-inflammatory cytokines, thus if a T_H inducing challenge was given, more effect may have been seen in the T_H cytokines e.g. IL-4. However, if it were the nature of the immune challenge received by the offspring postnatally that was causing this pattern, then changes in the basal (saline-injected) cytokine concentrations would not

have been seen. Interestingly, upon immune-challenge offspring from 10 μg/kg LPS treated mothers often displayed cytokine concentrations equivalent to immune-challenged offspring from control mothers, whereas serum cytokine concentrations of offspring from 50 and 150 μg/kg LPS treated mothers remained lower. Thus, for many cytokines offspring from 10 μg/kg LPS treated mothers were able to mount a serum response comparable to offspring from control mothers, whereas offspring from higher, 50 and 150 μg/kg LPS treated mothers did not have this capacity.

Monocytes and APCs are cells of the innate immune system that express the LPS receptor, Toll-like receptor-4 (TLR4), and are the main cell type responsible for producing large amounts of several cytokines in response to TLR4-LPS stimulation (Benoit et al., 2008). The findings in this chapter thus suggest that the APCs of offspring from immune challenged mothers have a reduced capacity to produce or secrete certain cytokines. It is intriguing that in the offspring from 10 μg/kg LPS treated mothers administration of immune challenge (i.e. LPS injection) elicited a response that was comparable to control, even when the same cytokine was at a much lower concentration than control in non-immune challenged state (i.e. saline injection). Based on this observation, it seems likely that the effect is at the level of transcription or translation for many of the cytokines because upon LPS stimulation, the dampening effect was readily reversed and cytokine levels comparable to control were achieved within the same time frame. This was the case in the offspring from 10 μg/kg LPS mothers whereas the blunting was pronounced even after LPS stimulation in offspring from 50 and 150 μg/kg LPS mothers, suggesting that more permanent changes were present. Interestingly, post-transcriptional control is very important in determining the level of cytokine production in immune cells (Anderson, 2008). Post-transcriptional control has several important features that are potentially relevant to the findings in the findings in this chapter; i) cytokines can be translated from stored mRNA molecules more rapidly than if a new transcripts had to be synthesised; ii) 'metabolic economy' because energy spent in transcribing the mRNA is not wasted because it can be stored and translated at a later time if the correct stimulus is applied; iii) sensors of microbial infection such as TLRs are linked to intracellular signalling pathways that regulate the translation and decay of mRNA (Anderson, 2008).

How could these immune changes fit with the DOHaD hypothesis? The immune system is very expensive in terms of energy (Lochmiller and Deerenberg, 2000; Newsholme and Newsholme, 1989); moreover, an immune response is extra expense

in energetic terms and the sickness behaviour that arises from such a response could put an organism at risk, for example in terms of predation (Dantzer, 2004; Jin et al., 1995). Thus, if the developing fetus 'senses' an inflammatory external environment *in utero* via the mother, it may predict being born into a pathogen rich environment. Thus, to avoid excess energetic expense and risk associated with sickness behaviour, the offspring may make adaptations to its immune system so that it operates at a lower level basally and reacts minimally and only when really necessary e.g. during heavy infection rather than to less serious infections. This may appear to be a great risk for the offspring, however despite the blunted immune status, during the course of my experimentation at no time did I notice any overt sign of extra burden of illness or disease, especially considering the animals were kept well into adult life and were not housed in specific pathogen free conditions (SPF). It would be interesting to investigate the immune status of these offspring more fully, in terms of antibody levels and components of the complement system for example, and also to see what effect a different type of immune challenge would have, e.g. a T cell-dependent antigen such as SRBCs or ovalbumin.

The spleen was examined for lymphocyte composition (T and B cells) because it is easily accessible, is the largest single secondary lymphoid organ of the body and contains up to 25% of the body's mature lymphocytes (Fu and Chaplin, 1999). The disregulated cytokine production may have contributed to the disregulated lymphoid homeostasis seen in the spleen of offspring from mothers treated with LPS. However, unlike with the cytokines, sex-specific and treatment group specific differences were found in the splenic lymphocyte populations. As was seen with many of the other phenotypes discussed in this chapter, only male offspring were affected and it was mainly those from the highest LPS treatment groups (50 and 150 μg/kg LPS). Interestingly, the affected offspring in the present study displayed increased T cells, where $CD4^+$ were more affected than $CD8^+$, and decreased B cells.

This pattern of disregulated lymphoid homeostasis is reminiscent of that seen in a mouse model of X-linked severe combined immunodeficiency (XSCID) where the common receptor γ chain (γ_c; the shared component of the receptors for IL-2, IL-4, IL-7, IL-9, IL-15) is deficient (Nakajima et al., 1997). These mice display diminished numbers of bone marrow and spleen B cells, and $CD4^+$ T cell accumulation in the spleen (with no effect on $CD8^+$ T cells). The $CD4^+$ T cells have abnormally high rates of proliferation and apoptosis, but the rate of proliferation is greater than the rate of

apoptosis thus the cells increase in number (Nakajima et al., 1997). Furthermore, these $CD4^+$ T cells have an activated phenotype that was shown to be T cell receptor (TCR) dependent (which could be specific for either self or environmental antigen). Indeed, >80% of such mice develop inflammatory bowel disease which the authors propose is consistent with the possibility of self-reactive T cells (Nakajima et al., 1997). Thus, the disregulated lymphoid homeostasis seen in male offspring from 150 µg/kg LPS treated mothers could be a result of altered signalling via the γ_c due to the disregulated cytokine concentrations seen in these animals. This aspect warrants further investigation in the current model because if this hypothesis is accurate, maternal immune activation during early gestation could predispose to auto-immune disease, e.g. inflammatory bowel disease, as noted above.

In conclusion, the results presented in this chapter demonstrate that the preimplantation embryo is sensitive to maternal inflammation, resulting in long-term alterations to the structure and function of diverse body systems within the offspring, including, body fat and BMI, behaviour and immune status. These findings cannot be directly applied to the human population; however, mouse models such as these allow experimentation that would be unethical to conduct in the human and provides insight into the areas that warrant further study in the human.

7 General discussion

The preimplantation mammalian embryo is sensitive to its immediate surroundings, and alterations to its *in vitro* or *in vivo* environment can affect not only the immediate events of blastocyst formation, but can also give rise to long-term phenotypic consequences during fetal and/or postnatal life. For example, rodent studies have shown that maternal under-nutrition during preimplantation gestation can lead to increased risk of cardiovascular, metabolic and behavioural abnormalities in the adult offspring (Kwong et al., 2000; Watkins et al., 2008b). To date, a low protein diet is the only preimplantation stage maternal challenge for which short- and long-term offspring phenotype has been investigated. Thus, do other types of maternal challenge similarly impact on the developmental programme with long-lasting consequences? Infection and injury are common in everyday life and normally result in altered homeostasis and generation of an inflammatory response. The aim of my PhD was to study the effects of an inflammatory environment during preimplantation development on the phenotype of the blastocyst, fetus and offspring postnatally, with a major focus on an *in vivo* mouse model of maternal systemic inflammation.

In the initial phase of the project (Chapter 3), MF1 mouse embryos were cultured in the presence of IFN-γ in order to model, *in vitro*, cytokine changes that may occur in the uterine fluid, *in vivo*, during an inflammatory response. IFN-γ is known to be produced during an inflammatory response and also that it is present in uterine fluid, but little is known about its effects on preimplantation embryo development. The results show that select higher doses of rmIFN-γ (10 and 1000 pg/ml) increase the maximum percentage of cavitated embryos and reduce the ICM:TE ratio in the blastocyst, the latter due specifically to a reduction in ICM cell number. Furthermore, a strong negative correlation was found to exist between the percentage of embryos cavitated and the percentage of embryos arrested, which suggested improved survival. Other studies have shown that IFN-γ STAT1-dependent signalling does not occur in the preimplantation embryo, thus it is a possibility that IFN-γ is acting via STAT1-independent signalling pathways. Some of the STAT1-independent signal transduction pathways that are activated by IFN-γ are known to be involved in preimplantation embryo development e.g. MAP kinase and PI3-kinase (Zhang et al., 2007). Thus, it is possible that the findings presented in Chapter 3 were due to IFN-γ-mediated modulation of signalling pathways that are important for preimplantation development; for example, an effect on survival could have been mediated via PI3-K/Akt signalling

and TE differentiation could have been mediated via PKC and Ras-MAPK/ERK1/2 signalling.

The reason for the reduced blastocyst ICM number was not investigated in the present study, but the degree of apoptosis could be investigated by TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling) or Annexin V (detects the phospholipid, phosphatidylserine, that translocates from the internal to the external layer of the plasma membrane during early apoptosis) labelling and proliferation could be examined by BrdU assay. In addition, the timing of the exposure to IFN-γ could be important in determining the outcome in the blastocyst. In this study, IFN-γ was present in the culture media continually from the 2-cell to the blastocyst stage. It would be interesting to examine the effect of IFN-γ over more discrete stages of preimplantation development, e.g. from 2-cells to 8-cells stage or from compaction to blastocyst stage. This would give better insight into periods of embryo sensitivity to this cytokine. Certain lines of evidence suggest that blastocyst cell number is indicative of implantation potential (Richter et al., 2001), fetal development (Lane and Gardner, 1997) and postnatal phenotype (Kwong et al., 2000; Watkins et al., 2007). As such, the reduced ICM:TE ratio caused by exposure to IFN-γ suggests that implantation potential and postnatal physiology could be affect if an embryo was exposed to similar concentrations of IFN-γ *in vivo*. Transfer of embryos cultured in IFN-γ into pseudopregnant females would enable implantation potential and postnatal offspring phenotype to be assessed.

The remainder of this thesis (Chapters 4, 5 and 6) concerns the *in vivo* model of maternal systemic infection during preimplantation gestation and the pre- and postnatal consequences to the offspring. To model maternal systemic inflammation *in vivo*, saline (control) 10, 50 or 150 μg/kg lipopolysaccharide (LPS) was administered intraperitoneally (i.p.) to female mice on gestational day 0.5 (GD 0.5). The maternal response to LPS administration was investigated in Chapter 4. The findings confirmed the induction of an inflammatory response by these doses of LPS and indicated that the maternal APR during very early gestation is comparable to that typically observed in non-pregnant mice following i.p. injection of LPS within a similar dosage range. Interestingly, locomotor activity at 1 hour post-injection, food consumption over the first 24 hours post-injection and normalised body weight at 24 hours post-injection were reduced by an equivalent amount in all maternal LPS treatment groups, whereas many of the serum cytokines examined were significantly increased only in the

mothers that received the highest LPS dose, i.e. 150 μg/kg. The latter observation thus placed maternal cytokines, rather than acute under-nutrition (due to the LPS-induced anorectic effect), as potential key mediators of LPS-dose dependent phenotypic changes seen in later experiments. However, when studying the effects of maternal challenge on offspring development, it is not the status of the mother *per se* that is important, but rather how changes in the maternal compartment alter the uterine environment. Indeed, a key study highlighted that there is strict compartmentalisation of many cytokines in the uterine fluid compared with serum in the mouse during estrus, for example, the concentration of G-CSF in uterine fluid was 173-fold greater than in serum (Orsi et al., 2007). As such, it will be extremely important to determine the cytokine composition of the maternal uterine fluid following systemic administration of LPS. In doing this, insight would be gained into the composition of the preimplantation embryo's immediate surroundings and would thus help to identify more precisely potential mechanisms involved in offspring phenotype induction.

The effects of maternal inflammation during early gestation on prenatal development are presented in Chapter 5. The main finding in this part of the project was that all LPS doses resulted in significantly reduced ICM cell number and ICM:TE cell ratio in the GD 3.5 blastocyst. No experiments were performed in the present study to determine the cause of the reduction in ICM cell number; therefore it would be interesting to do Annexin V labelling or TUNEL in order to assess the extent of apoptosis in these blastocysts. Reduction in ICM cell number has potentially far reaching consequences because all fetal tissues derive from the ICM and reductions in this lineage deplete the available pool of stem cells, potentially resulting in delayed and/or reduced growth and development (Hishinuma et al., 1996; Power and Tam, 1993; Tam, 1988). However, it is likely that increased post-implantation cell proliferation was able to compensate for the earlier low blastocyst ICM cell number (Hishinuma et al., 1996; Power and Tam, 1993; Tam, 1988) because no gross effects were found in GD 17 fetal phenotype, in terms of litter size, weight of conceptus, fetus, placenta, yolk sac, fetal liver, fetal brain or fetal kidneys. Other studies have indicated that treatments which result in altered blastocyst cell number can also result in altered postnatal physiology (Kwong et al., 2000; Watkins et al., 2007). This was also true in the present study, as altered phenotype was evident in the offspring postnatally.

The evidence presented in the final results chapter (Chapter 6) showed, for the first time, that maternal inflammation during early gestation can alter diverse aspects of postnatal offspring phenotype. Offspring from mothers treated with 150 µg/kg LPS were particularly affected, and in most cases male offspring were affected to a greater extent than females. The major findings were as follows: male offspring from 150 µg/kg LPS treated mothers displayed reduced locomotor and exploratory-related behaviour, increased fat-pad mass (retro-peritoneal and inguinal fat/body weight ratios), increased BMI, reduced serum cytokine concentrations, reduced percentage of splenic B lymphocytes and increased percentage of splenic T helper lymphocytes ($CD4^+$). For female offspring the only major finding was reduced serum cytokine concentrations in offspring from all LPS treated mothers. The increased mass of certain fat-pads hints that there may be metabolic alteration in these animals. Thus, it would be interesting to investigate serum levels of leptin, insulin, glucose and corticosterone, and to assess glucocorticoid receptor expression in the hippocampus and hypothalamus. The alterations in offspring immune status are a particularly novel and interesting finding, not only because the same animals also have behavioural and body structure abnormalities, but also because the offspring immune system has received very little attention in the DOHaD literature. The immune disregulation seen in this study warrants further study as the reasons for the changes seen here are not clear at present.

In several examples of DOHaD-related programming of offspring phenotype, epigenetic mechanisms have been shown to be the molecular basis underpinning physiological plasticity (Lillycrop et al., 2008; Lillycrop et al., 2007). It will be interesting to evaluate whether the altered offspring phenotype discovered in my work can be explained by epigenetic processes. Other elements that should be investigated (from the findings in Chapter 6) include concentrations of other cytokines (e.g. IFN-α and β, TGF-β), antibody levels and the complement system. In addition, FACS analysis of T and B lymphocytes in blood, bone marrow, thymus and lymph nodes could be analysed to assess if the same patterns are observed in these tissues as have been seen in the spleen. This analysis could be extended by trying to assess more precisely the different lymphocyte subtypes, e.g. are the increased $CD4^+$ T cells mainly $CD25^{high}$ $FOXP3^+$ regulatory T cells (T_{regs})? Furthermore, innate immune cells (monocytes, macrophages, dendritic cells, natural killer cells) should be assessed further, especially when considering the reduced levels of cytokines seen in this study. In addition, the various cell types could be isolated and studied *ex vivo* in order to delineate the

cytokine producing abilities of each cell type separately. By doing this it may be possible to pinpoint more precisely the source of the immune disregulation. In addition, it would be interesting to assess the immune response to different challenges (e.g. PolyI:C as a viral mimic and SRBCs or ovalbumin as a T-cell-dependent stimulus) and at different ages (perhaps neonatally and at adolescence). Finally, only one time point was examined in the present study, thus some of the observed cytokine differences may be due to altered kinetics. The kinetics of the immune response could be examined by performing a time course assay post-immune challenge.

8 References

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Appendix I

Pregnant mare serum gonadotropin (PMSG; Intervet)

1 vial contained 5000 IU PMSG in freeze-dried form.

This was reconstituted with 20 ml of sterile 0.85% Sodium Chloride (NaCl) solution (saline) to give 250 IU/ml. Aliquots of 400 µl were prepared and stored at -20°C.

When required, one 400 µl aliquot of PMSG was defrosted and diluted with 1.6 ml of sterile 0.85% saline (to yield 50 IU/ml). For superovulation, 100 µl (5 IU) of this PMSG solution was injected intraperitoneally.

Human chorionic gonadotropin (hCG; Intervet)

1 vial contained 1500 IU hCG in freeze-dried form

This was reconstituted with 3 ml of sterile 0.85% Sodium Chloride (NaCl) solution (saline) to give 500 IU/ml. Aliquots of 200 µl were prepared and stored at -20°C.

When required, one 200 µl aliquot of hCG was defrosted and diluted with 1.8 ml of sterile 0.85% saline (to yield 50 IU/ml). For superovulation, 100 µl (5 IU) of this hCG solution was injected intraperitoneally.

H6-BSA

Stock B (stored at 4-8°C for up to 2 weeks)

Deionized water, sterile	10 ml
Sodium hydrogen carbonate ($NaHCO_3$)	0.2106 g

Stock E (stored at 4-8°C for up to 3 months)

Deionized water, sterile	50 ml
Hepes	2.9785 g

Stock F (stored at 4-8°C for up to 3 months)

Sodium chloride (NaCl)	4.720 g
Potassium chloride (KCl)	0.110 g
Sodium dihydrogen orthophosphate ($NaH_2PO4.2H_2O$)	0.060 g
Magnesium chloride ($MgCl_2$)	0.100 g
D-glucose	1.000 g

DL-lactic acid	3.4 ml
Deionized water, sterile	Made-up to 100 ml

Stock G (stored at 4-8°C for up to 2 weeks)

Deionized water, sterile	10 ml
Pyruvic acid	0.030 g
Penicillin	0.060 g
Streptomycin	0.050 g

Stock H (stored at 4-8°C for up to 3 months)

Deionized water, sterile	10 ml
Calcium chloride dihydrate ($CaCl_2.2H_2O$)	0.260 g

20% Sodium Chloride

Deionized water, sterile	10 ml
Sodium chloride (NaCl)	2.0 g

To prepare 100 ml of H6-BSA:

Deionized water, sterile	78 ml
Stock B	1.6 ml
Stock E	8.4 ml
Stock F	10 ml
Stock G	1.0 ml
Stock H	1.0 ml
20% Sodium chloride (NaCl)	0.6 ml
BSA (Sigma, embryo culture tested, A3311)	0.4 g

pH adjusted to 7.4; osmolarity adjusted to 270-280 mOsm; sterile filtered (0.22 µm filter); aliquots stored at 4°C.

KSOM culture medium

2x stock KSOM

Sodium chloride (NaCl)	1.110 g

Potassium chloride (KCl)	0.0373 g
Potassium dihydrogen orthophosphate (KH_2PO_4)	0.0095 g
Magnesium sulphate 7-hydrate ($MgSO_4.7H_2O$)	0.0099 g
Lactic acid	0.362 ml
Sodium pyruvate	0.0044 g
Glucose	0.0072 g
Sodium bicarbonate ($NaHCO_3$)	0.420 g
Penicillin	0.01256 g
Streptomycin	1 ml
EDTA	4 ml
Tissue culture grade water	Made-up to 100 ml

5 ml aliquots stored at -80°C for up to 2 months.

1x KSOM (10 ml)

The following was added to 5 ml of thawed 2x KSOM stock:

Calcium chloride ($CaCl_2$; 100x stock 25 mg/ml)	100 µl
Non-essential amino acids (100x stock; 10 mM)	50 µl
Essential amino acids (50x stock)	100 µl
L-glutamine (200 mM)	50 µl
Deionized water, sterile	4.75 ml
BSA	0.040 g

Sterile filtered (0.2 µm filter); osmolarity adjusted to 255±5 mOsm with 20% NaCl; aliquots stored at 4°C for up to 1 week.

Trinitrobenzene Sulphonic Acid (TNBS)

TNBS is also known as Picrylsulfonic acid.

Diluted 1:10 in PBS with 0.1% polyvinylpyrrolidone (PVP).

pH adjusted to 7.4 with Sodium Hydroxide (NaOH); stored in the dark at 4ºC.

Guinea pig complement

Reconstituted in 1 ml of ice-cold water.

Diluted 1:10 in cold H6-BSA; stored at -80°C.

Propidium Iodide

1 mg/ml in distilled water; aliquots stored in the dark at 4°C.

Hoechst 33258 (bisBenzimide H 33258)

2.5 mg/ml in distilled water; aliquots stored in the dark at -20°C.

ACK buffer for lysis of red blood cells

Deionized water	1 L
Ammonium Chloride (NH_4Cl)	8.29 g
Potassium Hydrogen Carbonate ($KHCO_3$)	1 g
Sodium-EDTA (Na_2-EDTA)	37.2 mg

pH adjusted to 7.2-7.4; sterile filtered (0.2 µm filter); stored at 4°C.

Appendix II

Cytokine	Main sources	Main Activities
IL-1α	Monocytes, macrophages, DCs, T and B cells, NK cells, some non-immune cells	Proinflammatory, induction of fever and APR, induces IL-2 release, B cell maturation and proliferation
IL-1β	As for IL-1α	As for IL-1α
IL-2	T cells	Stimulates growth and differentiation of T cells, B cells and NK cells
IL-3	T cells, mast cells, eosinophils	Hematopoietic growth factor for myeloid lineage cells
IL-4	T cells (Th2), mast cells, bone marrow stromal cells	Promotes growth and development of T and B cells, immunoglobulin class switch to IgG1 and IgE.
IL-5	T cells (Th2), mast cells, eosinophils	Hematopoietic growth factor specifically for eosinophils
IL-6	Macrophages, monocytes, T cells and B cells, some non-immune cells	Proinflammatory, induces APR, B cell differentiation and antibody production, hematopoiesis.
IL-9	T cells (Th2)	Proliferation of T cells and erythroid precursors
IL-10	T cells (CD4+ Th2 and CD8+), B cells, macrophages and monocytes, monocytes	Stimulates proliferation of B cells and mast cells, contributes to IgA synthesis and secretion by B cells, antagonises generation of Th1 cells
IL-12 p40	Monocytes, macrophages, DCs, neutrophils, non-immune cells	p40 homodimer antagonises the actions of IL-12 p70
IL-12 p70	Monocytes, macrophages, DCs, B cells	Induces differentiation of Th1 T cells, induces the production of IFN-γ from T cells and NK cells
IL-13	T cells (Th2), mast cells and NK cells	Anti-inflammatory, upregulates IgE production by B cells
IL-17	T cells	Induces the production of cytokines and hematopoietic molecules from non-immune cells

Continued…

Cytokine	Main sources	Main Activities
Eotaxin (CCL11)	TNF-activated monocytes, IFN-γ stimulated endothelial cells	Chemoattracts eosinophils
G-CSF	Macrophages, bone marrow stromal cells, non-immune cells	Hematopoietic factor for growth and differentiation of neutrophils
GM-CSF	Monocytes, macrophages, T cells, non-immune cells	Hematopoietic factor for growth and differentiation of granulocytic and monocytic cells
IFN-γ	T cells (Th1 CD4+ and CD8+), NK cells	Upregulates MHC expression, affects activation, growth and differentiation of T cells, B cells and macrophages
KC (CXCL1)	Monocytes, macrophages, DCs, non-immune cells	Chemoattracts and activates neutrophils and basohils
MCP-1 (CCL2)	Activated immune and non-immune cells	Chemoattracts and activate monocytes, DCs, basophils, NK cells and T cells
MIP-1α (CCL3)	Activated immune and non-immune cells	Chemoattracts and activates CD8+ T cells, NK cells, monocytes, macrophages, DCs, B cells, eosinophils
MIP-1β (CCL4)	Activated immune and non-immune cells	Chemoattracts and activates CD4+ T cells, NK cells, monocytes, macrophages, DCs, B cells, eosinophils
RANTES (CCL5)	Activated immune and non-immune cells	Chemoattracts monocytes, memory T cells, basophils and eosinophils
TNF-α	Monocytes, macrophages, T cells, B cells, non-immune cells	Proinflammatory, growth and differentiation of a wide variety of cells, cytotoxic for many types of transformed cells