IL-6 trans-signalling modulates TLR4-dependent inflammatory responses via STAT1 and STAT3

A thesis submitted for the degree of
Doctor of Philosophy

By

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Abstract

Innate immune responses triggered by the prototypical inflammatory stimulus LPS are mediated by TLR4 and involve the coordinated production of a multitude of inflammatory mediators, especially IL-6 which signals via the shared IL-6 cytokine family receptor subunit gp130. However, the exact role of IL-6, which can elicit either pro- or anti-inflammatory responses, in the pathogenesis of TLR4-driven inflammatory disorders, as well as the identity of signalling pathways activated by IL-6 in a pro-inflammatory state, remain unclear. To define the contribution of gp130 signalling events to TLR4-driven inflammatory responses, we combined genetic and therapeutic approaches based on a series of *gp130*$^{F/F}$ knock-in mutant mice displaying hyperactivated IL-6-dependent Jak/STAT signalling in an experimental model of LPS/TLR4-mediated septic shock. The *gp130*$^{F/F}$ mice were markedly hypersensitive to LPS which was associated with the specific upregulated production of IL-6, but not TNFα. In *gp130*$^{F/F}$ mice, either genetic ablation of *IL-6*, antibody-mediated inhibition of IL-6 receptor signalling, or therapeutic blockade of IL-6 trans-signalling completely protected mice from LPS hypersensitivity. Furthermore, genetic reduction of STAT1 in *gp130*$^{F/F}$:*Stat1*$^{-/-}$ mice and STAT3 activity in *gp130*$^{F/F}$:*Stat3*$^{+/-}$ mice ameliorated LPS hypersensitivity and lowered LPS-induced IL-6 production. Additional genetic approaches demonstrated that the TLR4/Mal (Myd88 dependent) pathway contributed to LPS hypersensitivity and increased IL-6 production in *gp130*$^{F/F}$ mice. Furthermore, macrophages were not the cell type responsible for the LPS hypersensitivity of *gp130*$^{F/F}$ mice. Collectively, these data demonstrate for the first time that IL-6 trans-signalling via STAT1 and STAT3 is a critical modulator of LPS-driven pro-inflammatory responses through cross-talk regulation of the TLR4/Mal signalling pathway, and potentially implicate cross-talk between Jak/STAT and TLR pathways as a broader mechanism that regulates the severity of the host inflammatory response. Moreover, it was found that anti-inflammatory IL-10 signalling was intact in the *gp130*$^{F/F}$ mice.

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university, and to the best of my knowledge and belief, contains no material published or written by another person, except where due reference is made in the text. Where the work in this thesis is part of joint research, the relative contributions of the respective persons are listed in the text.

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Abbreviations

'	minutes
''	seconds
AP1	activation protein 1
Bcl-xl	B-cell lymphoma-extra large
BMMs	bone marrow macrophages
BMP	bone morphogenetic proteins
Btk	brutons tyrosine kinase
CASP	colon ascendens stent peritonitis
cm	centimetre
CO_2	carbon dioxide
CCL5	chemokine (C-C motif) ligand 5
CD	Crohn's disease
CD14	cluster of differentiation 14
CHR	cytokine-binding homology region
CLP	cecal ligation and puncture
DAMPs	danger associated molecular patterns
DCs	dendritic cells
D-Gal	D-galactosamine
DOC	sodium deoxycholate
DSS	dextran sodium sulphate
EF	endotoxin-free
EMSA	electrophoretic mobility shift assay
Erk	extracellular signal regulated kinase
FCS	fetal calf serum
g	gravitational acceleration
GAB1	GRB-associated binding protein
GARG16	glucocorticoid-attenuated response gene 16
GAS	γ-activated sequences
gp130	glycoprotein 130
HEK-293	human embryonic kidney cell line
hrs	hours
i.p.	intraperitoneal
i.v.	intravenously
IBD	inflammatory bowel disease
IFN	interferon
IFNAR	interferon alpha betal receptor alpha chain
IFNGR	IFNγ receptor
IκB	inhibitory subunit κB
IKK	IκB kinase

IL-	interleukin
IP10	interferon-inducible protein 10
IRAK	IL-1 receptor-associated kinase
IRF1	interferon regulatory factor 1
IRF3	interferon response factor 3
IRF9	interferon response factor 9
ISGF3	IFN-stimulated gene factor 3
JAKs	janus tyrosine kinases
JNK	cJun N-terminal kinase
kD	kilodalton
KIR	kinase inhibitory region
LBP	LPS binding protein
LIF	leukaemia inhibitory factor
LPS	lipopolysaccharide
LRR	leucine rich repeat
M	molar
ml	millilitres
MAPKs	mitogen-activated protein kinase
M-CSF	macrophage colony stimulating factor
MEFs	mouse embryonic fibroblasts
MHC	major histocompatibility complex
μl	microlitre
mBSA	methylated bovine serum albumin
mg	milligrams
MyD88	myeloid differentiation factor 88
MyD88s	MyD88 short
NAP1	NAK-associated protein 1
nm	nanometers
NF-κB	nuclear factor-κB
NK	natural killer
O/N	overnight
^{o}C	degrees Celsius
PAMP	pathogen-associated molecular pattern
PBS	Phosphate Buffer Saline
PCR	Genotyping Polymerase Chain Reaction
PI3K	phosphatidylinositol 3-kinases
PIAS	protein inhibitor of activated STAT
PM	peritoneal macrophage
PRRs	pathogen recognition receptors
pY-STAT1	phospho tyrosine STAT1
pY-STAT3	phospho tyrosine STAT3
qPCR	Quantitative Real-Time PCR
RA	rheumatoid arthritis

RAW	mouse leukaemic monocyte macrophage cell line
R	receptor
RANTES	regulated on activation normal T cell expressed and secreted
RPMI	Roswell Park Memorial Institute
RP105	radioprotective 105
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute
RT	room temperature
SES	*Staphylococcus epidermidis* cell-free supernantant
sgp130	soluble gp130
SH2	Src homology 2
SHP2	SH2 domain containing protein-tyrosine phosphatase
sIL-6R	soluble form of the IL-6Rα
Smad	Sma and Mad related proteins
SOCS1	suppressor of cytokine signalling 1
SOS	son of sevenless
STAT	signal tranducers and activators of transcription
STAT3-C	constitutively active STAT3
TAB	TAK1 binding proteins
TEA	triethylamine
TAK1	transforming growth factor-β activated kinase 1
TAK-242	alkyl 6-(N-substituted sulfamoyl) cyclohex-1-ene-1-carboxylate
TGF-β	transforming growth factor β
Th	T helper cell
TIR	Toll/Interleukin-1 receptor
TIRAP	MyD88 adaptor like (Mal)/ TIR-domain containing adaptor protein
TLRs	Toll-like receptors
TNF	tumour necrosis factor
TRAF6	TNF receptor-associated factor-6
TANK	TRAF family member-associated NF-κB activator
TBK1	TANK binding kinase 1
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adaptor protein inducing interferon (IFN) β
Tyk2	tyrosine kinase-2
U-STAT3	Unphosphorylated STAT3
WBC	white blood cell
V	volts
α	alpha
β	beta
δ	delta
κ	kappa
γ	gamma

List of Conference Presentations

Part of the work in this thesis was presented as oral presentations at the following conferences:

2010: Postgraduate Student Symposium Clayton, Australia

IL-6 trans-signalling modulates TLR4 depedent inflammation responses via STAT3. *CJ. Greenhill (presenting author)*

2010: Three Minutes Thesis Competition, Clayton, Australia

Finding the secret ingredient in the cure for Sepsis. *CJ. Greenhill (presenting author)*

2010: Australian Society for Medical Research (ASMR) Student Symposium, Melbourne, Australia

The role of gp130-dependent STAT3 activity on regulating TLR-driven inflammation. *CJ. Greenhill (presenting author)*

2010: Rotary Club presentation, Melbourne, Australia

Report on conference and lab visits overseas. *CJ. Greenhill (presenting author)*

2009: Postgraduate Student Symposium Clayton, Australia

The role of gp130-dependent STAT3 activity on regulating TLR-driven inflammation. *CJ. Greenhill (presenting author)*

2009: Lab visit talk, Edinburgh University, Edinburgh, UK

The role of gp130-dependent STAT1 and STAT3 activity on regulating TLR-driven inflammation. *CJ. Greenhill (presenting author)*

2009: Lab visit talk, Cardiff University, Cardiff, UK

The role of gp130-dependent STAT1 and STAT3 activity on regulating TLR-driven inflammation. *CJ. Greenhill (presenting author)*

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De-regulated gp130 signalling and it's effect on inflammation**.** *CJ. Greenhill, C. Kennedy (presenting authors)*

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The role of gp130-dependent STAT1 and STAT3 activity on regulating TLR-driven inflammation. *CJ. Greenhill (presenting author)*

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2009: International Cytokine Society (ICS) and International Society for Interferon and Cytokine Research (ISICR): "Cellular and Cytokine Interactions in Health and Disease," Lisbon, Portugal
Modulation of TLR4 mediated inflammation by deregulated gp130-STAT signalling. *CJ. Greenhill1 (presenting author), M. Najdovska, P. Hertzog, A. Mansell, BJ. Jenkins*

2009: Southern Health Research Week, Melbourne, Australia
The regulation of TLR4 mediated inflammation by Interleukin 6 signalling. *CJ. Greenhill (presenting author), M. Najdovska, M. Ernst, P. Hertzog, A. Mansell, BJ. Jenkins*

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The regulation of TLR4 mediated inflammation by Interleukin 6 signalling. *CJ. Greenhill (presenting author), M. Najdovska, M. Ernst, P. Hertzog, A. Mansell, BJ. Jenkins*

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The role of gp130 STAT1 and STAT3 activity on regulating TLR-driven inflammation. *CJ. Greenhill (presenting author), M. Najdovska, J. Gould , M. Ernst, P. Hertzog, BJ. Jenkins*

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The potential role of Interleukin 6 and the regulation of STAT1 and STAT3 during inflammation in the TLR4 signalling pathway*. CJ. Greenhill (presenting author), M. Najdovska, J. Gould , M. Ernst , P. Hertzog, BJ. Jenkins*

CHAPTER 1

Literature Review

1.1 Inflammation

Inflammation represents a complex host defence mechanism which comprises of a response from both the innate and adaptive immune systems. The immune response protects multi-cellular organisms from a variety of different pathogens such as viruses, bacteria and fungi, which have the potential to cause infection and disease. Primarily, physical barriers are utilised by the body to protect against pathogens, such as the epithelial layer of the skin to prevent pathogen penetration (reviewed in, Chaplin, 2010). Internally, there are systems at work such as tears, saliva and urine, to expel unwanted pathogens. In addition, cilia in the airways and digestive tract utilise peristalsis to remove pathogens, and gastric acid in the stomach acts as a chemical defence (reviewed in, Chaplin, 2010). Despite these initial protective barriers, pathogens frequently still manage to evade them, consequently triggering an inflammatory response by the immune system. This response involves vasodilatation of the arteries and increased capillary permeability which then allows cells to migrate to the affected site. The recruitment of these cells is controlled by the action of cytokines and chemokines (reviewed in, Chaplin, 2010).

1.1.1 Cytokines and cytokine receptors

Cytokines are a class of small glycosylated regulatory proteins, ranging from approximately 8-40,000 Dalton in mass, that are secreted into the microenvironment to control cellular processes such as differentiation, proliferation, survival, functional maturation and apoptosis (reviewed in, Dinarello, 2000; Pfitzner *et al.,* 2004). Each cytokine has a specific cell surface receptor (R) that triggers intracellular signalling to mediate its effects. The actions of cytokines also depend on their abundance and access to their receptors, often in a cell type dependent manner. Many cytokines also share functions and are considered redundant, as outlined in Table 1.1 (reviewed in, Dinarello, 2000).

Cytokines can be classed based on their structure and the structure of their receptors (reviewed in, Krause, Pestka, 2005). For instance, class I cytokines are made up of alpha (α)-helices ("α-helical cytokines) and they also possess 2 beta (β)-pleated sheets (Rozwarski *et al.,* 1994; Krause, Pestka, 2005) (Fig. 1.1). Examples of the type I cytokines include some of the interleukins (IL-) eg. IL-2, IL-6 and IL-4 (reviewed in, Liongue, Ward, 2007). Class II cytokines consist of two disulfide bridges and the absence of the so-called "WSXWS" motif in the C-terminal part of the extracellular domain (Zdanov, 2004). Class II cytokines include interferon (IFN) α and β, and IL-10 (reviewed in, Theofilopoulos *et al.,* 2005) and are generally involved in minimising damage caused by the immune systems response to insult or injury (reviewed in, Hinck, 2010).

Class I cytokine receptors all possess a 200 amino acid extracellular region which is necessary for ligand-receptor interactions; also referred to as the cytokine-binding homology region (CHR). This CHR resides extracellularly and consists of two tandem fibronectin type III modules, with the N-terminal one containing four conserved cysteines and the C-terminal one harbouring the "WSXWS" (one-letter amino acid code; x is a non-conserved residue) motif (reviewed in, Bazan, 1990; Liongue, Ward, 2007; Hinck, 2010). Class II cytokine receptors are similar to class I in that they possess helical ligand folds, tandem fibronectin type III modules in the extracellular domains of their receptors. Class II cytokine receptors differ because their first and second β-pleated sheet is replaced with an α-helix and they don't possess the class I specific WSxWS box (reviewed in, Bazan, 1990) (Fig. 1.1). In certain class II cytokine receptors the last α-helix has a kink which extends beyond the inner core (Walter, 2002; Langer *et al.,* 2004).

Other classes of cytokines include the tumor necrosis factor (TNF) family, and others such as transforming growth factor β (TGF-β) (reviewed in, Sato, Miyajima, 1994). For example, TNFα exists in two different forms, soluble and transmembrane, the former being a homotrimer of 17 kilodalton (kD) cleaved monomers and the latter a homotrimer of 26kD uncleaved monomers (Tang *et al.,* 1996). Each subunit contains two anti-parallel β pleated sheets (Jones *et al.,* 1989). TGF-β related proteins are secreted as mature peptides which then form homo- or

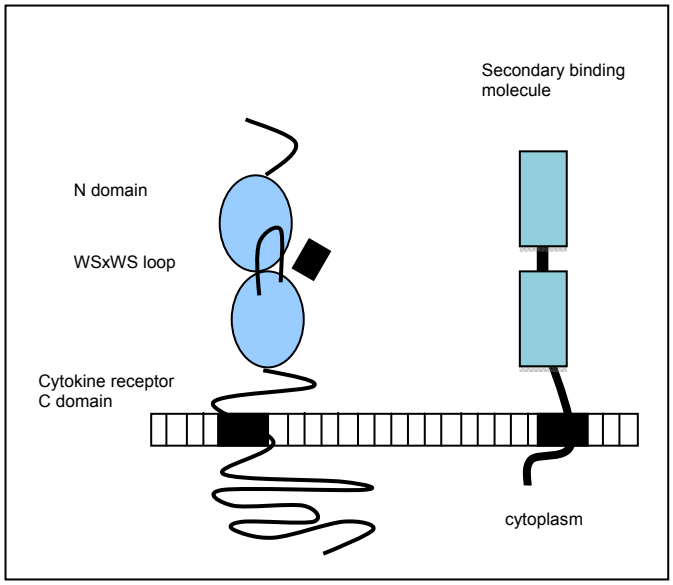

Figure 1.1: Cytokine receptors (Adapted from Ihle, 1995).

Class I and II cytokine receptors utilise two units for interaction with cytokines. The tertiary complex forms a Y shape, where the ligand specific receptor (blue ovals) forms the left arm and the common receptor (green diamonds) forms the right arm with space for the cytokine (black box) to sit in between. Type I cytokine receptors possess a WSxWS motif proximal to the hydrophobic hinge region (Bazan 1990; Hinck, 2010).

hetero-dimers. The secondary structure consists of a large conserved helix and nine β sheets which together delineate two fingers (Herpin *et al.,* 2004).

Cytokines are produced from a variety of different cells, including those of haematopoietic and non-haematopoietic origin, and commonly act in a local manner, only affecting cells in close proximity (reviewed in, Heinrich *et al.,* 1998; Feldmann, 2008). However, cytokines can act on many different target cells and often work in conjunction with other cytokines in an additive manner. Moreover, cytokines can act in a synergistic or antagonistic manner (reviewed in, Heinrich *et al.,* 1998) (Fig. 1.2). For instance, it has been shown that TNFα blocks IL-6 induction of type II acute phase response genes (Baumann *et al.,* 1989; Mackiewicz *et al.,* 1991). Furthermore, IL-1β upregulates the expression of the IL-6 signalling negative regulator, suppressor of cytokine signalling 3 (SOCS3) (Bode *et al.,* 1999; Yang *et al.,* 2004) and prevents IL-6 mediated signal transducer and activator of transcription 3 (STAT3) activation, which is an essential component of the IL-6 signalling pathway (Ahmed, Ivashkiv, 2000; Ahmed *et al.,* 2002). Moreover, it has recently been discovered that IL-1β and TNFα results in mitogen-activated protein kinase (MAPK)-activated protein kinase 2-dependent serine phosphorylation of the glycoprotein 130 (gp130) on serine 782, which consequently results in augmented receptor internalisation and degradation. As a result of decreased gp130 molecules, IL-6-dependent STAT3 activation and gene induction is reduced (Radtke *et al.,* 2010). Conversely, IL-6 production is also stimulated by TNFα and IL-1β (Kohase *et al.,* 1986; Wang *et al.,* 1998) and the former two cytokines stimulate transcription of each other (reviewed in, Papanicolaou *et al.,* 1998) (Fig. 1.2). Furthermore, IL-6 has been shown to antagonise IL-1β and TNFα, however, the specifics of this interaction are unclear (Aderka *et al.,* 1989; Schindler *et al.,* 1990).

Cytokines differ from growth factors as they do not necessarily have a positive effect on proliferation, growth and differentiation, as they can be inhibitory. For example, IL-10 and TGF-β are cytokines which inhibit pro-inflammatory cytokine production during an inflammatory response (Gerard *et al.,* 1993; Dinarello, 2000; Imai, *et al.,* 2000). In addition, cytokines differ from hormones in that they are synthesised rapidly after stimulation and are not stored in glands as pre-formed molecules (reviewed in, Heinrich *et al.,* 1998). The

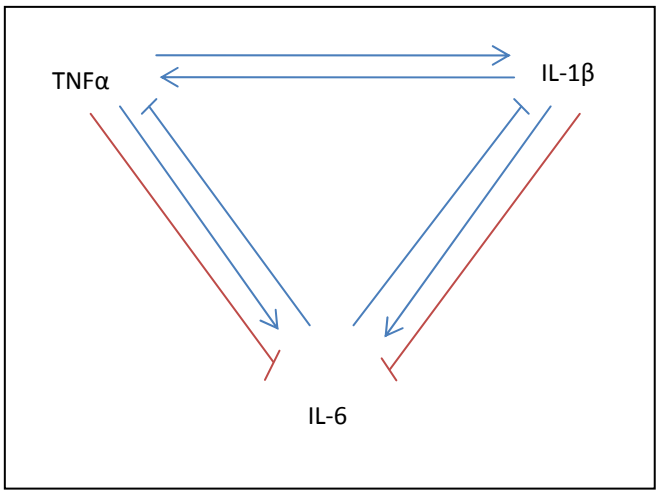

Figure 1.2: Cytokine interactions

Cytokines can act in a synergistic or antagonistic manner. TNFα inhibits and stimulates IL-6, and stimulates IL-1β. IL-6 inhibits TNFα and IL-1β. IL-1β stimulates TNFα and IL-6, however also inhibits IL-6.

synthesis of cytokines is transient, and they can be biologically active in nanomolar (small) concentrations (reviewed in, Heinrich *et al.,* 1998). During inflammation, the production of cytokines is amplified in the circulation and they provide the means by which the adaptive immune response is triggered by the body. Therefore the role of cytokines is vital to the initial innate immune response.

1.1.2 The innate immune response

The innate immune response is non-specific and rapid, and can be activated by, for example, trauma or infection. Other than physical barriers, the innate immune response involves the action of phagocytic cells which do not require prior exposure to the invading organism or 'memory' to be activated (reviewed in, Chaplin, 2010). Phagocytes are a group of cells which include granulocytes and macrophages, both of which engulf and digest pathogens. Granulocytes are also referred to as polymorphonuclear leukocytes and include a variety of white blood cells which are characterised by their granules which form unique staining patterns (reviewed in, Akgul *et al.,* 2001). Included in this group of cells are neutrophils, which create the first line of defence against bacterial and fungal infections (reviewed in, Akgul *et al.,* 2001). Neutrophils are derived in the bone marrow and migrate to infected or inflamed tissues, and upon their retirement undergo spontaneous apoptosis and subsequent removal by macrophages (reviewed in, Akgul *et al.,* 2001). A failure of neutrophils to migrate to the site of infection is associated with a poor outcome during sepsis, however the mechanisms involved are not thoroughly understood (Alves-Filho *et al.,* 2005; Alves-Filho *et al.,* 2008). Other granulocytes involved during the innate immune response are eosinophils, and basophils (Fig. 1.3). Eosinophils possess cytoplasmic granules which contain toxic molecules and enzymes which are effective at combating pathogens such as helminths and other parasites (reviewed in, Chaplin, 2010). They have been shown to be involved in allergic responses, along with mast cells, which release histamines from their granules and produce lipid mediators (reviewed in, Minai-Fleminger, Levi-Schaffer, 2009). Mast cells are not classified as granulocytes, despite being similar to eosinophils (reviewed in, Dranoff, 2004). Another form of granulocyte, the basophil, also releases histamines from their granules (reviewed in, Chaplin, 2010). Other components of the innate immune system include NK cells and complement proteins. NK cells

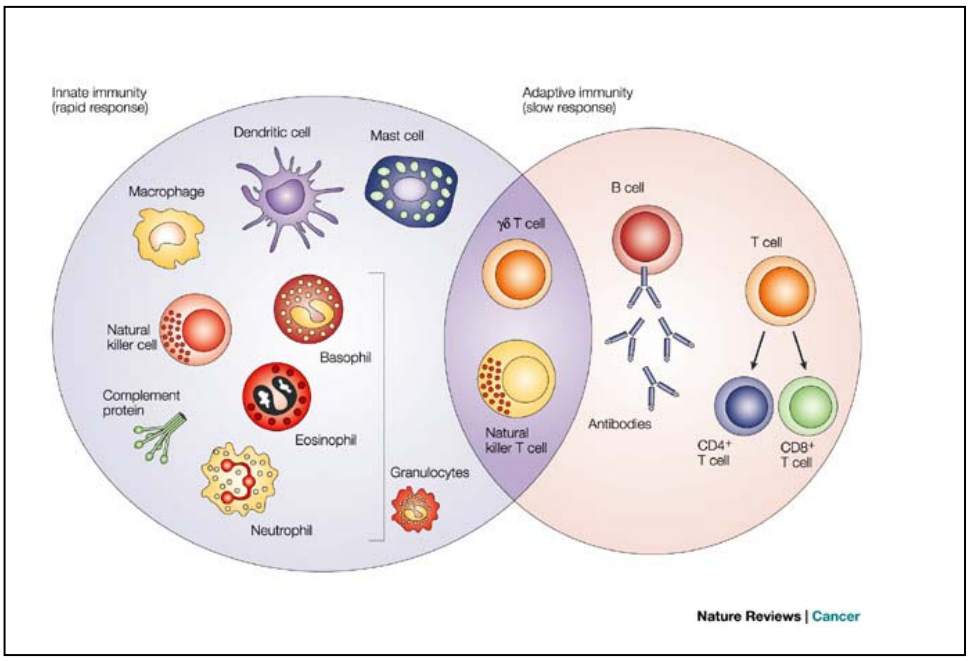

Figure 1.3: Innate and adaptive immune responses (Dranoff, 2004).

The innate immune response acts as the first line of defence against pathogens. Involved in this response are cells such as granulocytes (basophils, eosinophils and neutrophils), mast cells, macrophages, dendritic cells and natural killer cells. Following this innate immune response comes the slower adaptive immune response which involves B cells and T (CD4+ and CD8+) lymphocytes. Natural killer T cells and γ-delta T cells are cytotoxic lymphocytes which are involved in both the innate and adaptive immune response.

are cytotoxic lymphocytes which have been shown to target tumours (Kiessling *et al.,* 1975). Complement is another component of the innate immune system, which includes plasma, cell surface proteins and regulatory pathways which are involved in elimination of pathogens (reviewed in, Chaplin, 2010).

Another cell type involved in innate immunity is the macrophage, which is derived from a monocyte, predominantly found in bone marrow (where they are produced), blood and spleen. Monocytes then migrate to tissues in response to infection and differentiate into macrophages (or dendritic cells (DCs)) (Fig. 1.3) (reviewed in, Chaplin, 2010), where they release nitric oxide to kill pathogens and multiple inflammatory mediators (or cytokines). The environment in which the macrophages reside influences their release of cytokines, resulting in several different phenotypes. Two of the major categories of macrophage are; pro-inflammatory/antiviral (which release IFN-γ, IL-6, IL-12, and TNFα) and anti-inflammatory (which produce cytokines such as IL-10, the IL-1 receptor antagonist, and TGF-β) (reviewed in, Chaplin, 2010; Geissmann *et al.,* 2010). In addition to this role, tissue macrophages are also phagocytes and play a role during tissue homeostasis. Other phagocytes involved in innate immunity include non-professional cells (the role of which is not primarily phagocytosis) including epithelial cells, endothelial cells and fibroblasts (reviewed in, Takeuchi, Akira, 2010).

Cells involved in innate immunity use specific host receptors and signalling pathways to distinguish between extracellular (non-invasive) and intracellular (invasive) microbes (reviewed in, Takeuchi, Akira, 2010). The receptors involved are termed pathogen recognition receptors (PRRs) and are used to sense conserved motifs presented by microbes. There are five main classes of PRRs, including; membrane bound Toll-like receptors (TLRs), C-type lectin receptors, soluble, cytosolic nucleotide-binding oligomerisation domain (NOD)-like receptors, the PYHIN family (a new family of innate DNA sensors also referred to as AIM2-like receptors (ALRs)) and the retinoic-acid-inducible protein-like helicases (reviewed in, Takeuchi, Akira, 2010, Unterholzner *et al*., 2010). As will be discussed in more detail in section 1.3 below, these PRRs play a central role in coordinating the balanced release of pro- and anti-inflammatory mediators (e.g. cytokines) and the ensuing transition from innate to adaptive immunity, which is critical to the successful resolution of inflammation.

1.1.3 The adaptive immune response

The adaptive immune response is specific to vertebrates, is not activated immediately and results in lifetime-long acquired immunity, or immunological 'memory'. This complex system involves a precise sequence of antigen delivery, inflammatory cytokine release and cellular communication (reviewed in, Geissmann *et al.,* 2008). Essential to this process are monocyte-derived DCs which possess high phagocytic activity when in their immature form, and as they mature acquire the ability to produce cytokines (reviewed in, Geissmann *et al.,* 2010) (Fig. 1.3). In the initiation of adaptive immunity, DCs present antigens from pathogens or infected cells to T cells. This occurs by use of the major histocompatibility complex (MHC) which is attached to peptides (antigens) (reviewed in, Geissmann *et al.,* 2008). T cells are lymphocytes that are classed as CD8+ T cells (cytotoxic T cells), which act to kill cells infected with intracellular microbes, and CD4+ T cells, which regulate cellular immune responses (reviewed in, Chaplin, 2010) (Fig. 1.3). These CD4+ T cells then differentiate into T helper cell 1 (Th1) or Th2 cells, which support cell mediated immune responses or humoral, anti-helminthic and allergic responses, respectively (reviewed in, Chaplin, 2010). Other forms of T cells include T regulatory cells and Th17 cells which collectively play a role in regulating acquired immunity. NK T cells are a type of T cell which express the NK1.1 (CD161) antigen and lack the CD4 and CD8 markers. These cells play an immuno-regulatory role and release large amounts of cytokines such as IL-4, IFNγ and TNFα (reviewed in, Godfrey *et al.,* 2004). Gamma (γ) delta (δ) T cells are another form of cytotoxic cell involved in innate and adaptive immunity (reviewed in, Dranoff, 2004) (Fig. 1.3).

Another class of lymphocytes, B cells, produce antibodies during an immune response (reviewed in, Martensson *et al.,* 2010) (Fig. 1.3). This occurs via the uptake and presentation of antigen on its cell surface, which via interactions with T cells, leads to the induction of different classes of antibody (e.g. IgG, IgM etc.) all which have varying effects. For example, IgE is involved in allergy, IgA is involved in preventing colonisation of mucosal areas (reviewed in, Underdown, Schiff, 1986), IgM is present in the circulation and prevents bacterial and fungal infections, and IgG provides the basis of most antibody based

immunity against pathogens (reviewed in, Woof, Burton, 2004). While the innate and adaptive immune responses exist to protect the host from pathogens, if uncontrolled, they can often be detrimental.

1.1.4 Cytokines and inflammatory disease

If the inflammatory response is not tightly regulated, excessive transcriptional activation of inflammatory mediators such as cytokines and chemokines can result (reviewed in, Pfitzner *et al.,* 2004). Such a scenario can in turn lead to leukocyte-induced tissue destruction and chronic pathologies (reviewed in, Liu, Malik, 2006), including; rheumatoid arthritis (RA), inflammatory bowel disease (IBD) and peritonitis. There are high degrees of cytokines (mRNA and protein) such as IL-6, TNFα and IL-1β in the synovial fluid of RA patients (reviewed in, Buchan, 1988; Feldmann, 1996). Interestingly, TNFα appears to regulate IL-1β and other pro-inflammatory cytokines, leading to the hypothesis that TNFα may be an essential regulator during RA, and hence, could be a potential therapeutic target (reviewed in, Vilcek, Feldmann, 2004). Animal models of collagen-induced arthritis treated with anti-TNFα antibodies have resulted in reduced inflammation, however, no effects were apparent on joint destruction. Conversely, IL-1 blockage (through IL-1 receptor antagonist), results in reduced joint destruction (Joosten *et al.,* 1999). When extended to the clinical setting, however, TNFα treatment has been more effective than targeting IL-1, highlighting the inconsistencies between human and animal models of this disease (reviewed in, Vilcek, Feldmann, 2004; Feldmann, 2008). Phase II/III clinical trials in 1993-1999 of cA2 or 'Remicade®' (a chimeric human-mouse anti-TNFα antibody) in RA indicated the effectiveness of this treatment, however, it was unsure why only one third of patients showed healing of joint damage (reviewed in, Vilcek, Feldmann, 2004). Despite these uncertainties, two other anti-TNFα antibodies are now widely used (Enbrel® and Humira®) to successfully treat RA and other inflammatory disorders, one of these being Crohn's disease (CD) (van Dullemen *et al.,* 1995; Vilcek, Feldmann, 2004). CD is one of the main types of IBD, which is characterised by chronic mucosal inflammation and inflammatory cytokines that are associated with this disease include IL-4, IL-5, IFN-γ, IL-1, IL-6, IL-8 and TNFα (reviewed in, Papadakis, Targan, 2000). Targeting the IL-6

receptor (IL-6R) is also very successful in the treatment for RA and CD, which is discussed in section 1.6.2.

Peritonitis, or inflammation of the peritoneum, has also been associated with high levels of inflammatory cytokines (Enriquez *et al.,* 2002; Klinger *et al.,* 2002). It is a complication often associated with patients on continuous ambulatory peritoneal dialysis; a therapy for kidney failure. Pro-inflammatory cytokines associated with this disease include IL-1α, IL-6, TNFα, IL-12 and IL-4, however, more investigation needs to be done to clarify the mechanisms at play during this condition, as current treatment mainly involves broad spectrum antibiotics (Enriquez *et al.,* 2002; Lai, Leung, 2010).

All these inflammatory diseases highlight the devastating effects an uncontrolled inflammatory response can produce, and the need for further investigation into mechanisms controlling this process. Specifically, the success of the above treatments has not been extended to the treatment of another major inflammatory disease; sepsis.

1.1.5 Sepsis and endotoxic shock

Sepsis is a chronic, systemic inflammatory disease which causes a large strain on public health systems worldwide, with approximately 215,000 deaths and 750,000 cases of sepsis each year in the US alone (reviewed in, Karima *et al.,* 1999). Sepsis kills more people than lung cancer, or breast and bowel cancer combined, costing the US approximately $16 billion annually (Robson, Daniel, 2008). The rate of sepsis has been documented at 8.7 % per year, based on the growth of the US population (Martin *et al.,* 2003). This consequently results in sepsis being the tenth leading cause of death in the US (Hoyert *et al.,* 2001). The incidence of sepsis in Victoria, Australia, was identified as 194 per 100,000 in 2002 (Sundararajan *et al.,* 2005).

Sepsis, is defined as a systemic inflammatory response syndrome (SIRS) resulting from infection, which is a combination of pathological infections and physiological changes (Table 1.2). Sepsis is initially caused by the invasion of tissue that is normally sterile by pathogenic microbes, for example intestinal bacterial overgrowth. Bacteria, viruses and fungi are the main categories of these pathogenic microbes (reviewed in, Nduka, Parrillo, 2009). In 45-60% of cases, sepsis is triggered by Gram-negative bacterial infections and in this scenario is

referred to as endotoxic shock (reviewed in, Karima *et al.,* 1999). Reasons for the increasing incidence of sepsis cases are varied, however, it is predicted that they include the increased use of invasive equipment such as catheters, emergence of antibiotic resistant micro-organisms, increases in occurrence of human immuno-deficiency virus, and increase of the elderly population (Martin *et al.,* 2003). Somewhat ironically, the latter is potentially an unforeseen consequence of improvements in medical care, which have increased the life expectancy in the elderly or patients with disorders, both of whom commonly have a sub-optimal immune response which leads to an increased risk of infection. Patients with sepsis have a more pronounced and prolonged inflammatory response in comparison to normal individuals, which in severe cases can lead to multi-organ dysfunction and death (Martin *et al.,* 2003; Rittirsch *et al.,* 2008). Septic shock is the term used for advanced sepsis when the patient has hypotension and can be life threatening (reviewed in Stearns-Kurosawa *et al.,* 2011).

The molecular pathways leading to sepsis involve complex signalling networks that produce inflammatory cytokines, chemokines, reactive oxygen species, prostaglandins and lipid mediators (reviewed in, Karima *et al.,* 1999). These complex pathways and interactions result in many clinical outcomes, including respiratory distress and failure, renal shutdown, hepatic failure, haematological coagulation, neurological trauma, cardiovascular hypotension and shock (Bohrer *et al.,* 1997; Martin *et al.,* 2003). Studies have shown that hypotension is caused by a translocation of platelets from the circulation to the lungs and a minority to the liver, a condition known as thrombocytopenia (Shibazaki *et al.,* 1996). In addition, leukocytes accumulate in tissues such as the lung, which can lead to leukocyte-induced tissue injury (reviewed in, Karima *et al.,* 1999). Conversely, the associated rapid loss of circulating white blood cells (leukopenia) can also leave a sepsis patient more susceptible to infection (reviewed in, Hinshaw, 1996). During sepsis, endocrinopathy can result in the form of hyperglycemia, insulin resistance or insufficient production of adrenal corticosteroids or vasopressin (Brierre *et al.,* 2004). Furthermore, it has been shown in human sepsis patients, in addition to animal models of sepsis, that the complement system is involved in the pathogenesis of this disease. In the experimental models, the complement system is robustly induced, along with upregulation of C5a receptors in many different organs. The interaction of C5a

with its receptors is thought to be linked to apoptosis of the lymphoid system, a suppression of innate immune functions by blood neutrophils and cardiac dysfunction (Ward & Gao, 2009). There is debate as to whether coagulation is involved in the pathogenesis of sepsis, or whether it is simply a bystander (reviewed in, Stearns-Kurosawa *et al*., 2011). Patients with this disease have clots forming faster than they can be broken down, and they can lodge in the microvascular beds of organs. As a result of increased consumption of platelets and coagulation factors, these levels drop, fibrinogen levels decrease and clotting times are prolonged. This scenario results in increased risk of bleeding in the patients (reviewed in, Stearns-Kurosawa *et al.,* 2011).

In addition to the initial pro-inflammatory cytokine 'storm', sepsis patients often succumb to an immuno-suppressive response that follows as an attempt by the host to control tissue damage (reviewed in, Rittirsch *et al.,* 2008). This involves neutrophils becoming paralysed, resulting in a shut down of vital intracellular signalling pathways and inhibition of the adaptive immune system, including increased apoptosis of lymphocytes and DCs (Solomkin *et al.,* 1981; Hotchkiss, Nicholson, 2006). Damage of vascular endothelial cells has also been shown to be associated with septic shock and is thought to result in multi-organ failure (Mutunga *et al.,* 2001). The immuno-suppressive response also involves the diversion from an initial Th1 to a Th2 response and leaves the individual at risk of nosocomial infections (reviewed in, Rittirsch *et al.,* 2008). Despite this cytokine production, the apoptosis of macrophages and neutrophils remains unchanged or even decreased (Hotchkiss *et al.,* 2002; Hotchkiss *et al.,* 2005; Rittirsch *et al.,* 2008).

Animal models that mimic sepsis and the symptoms described above include three main types; injection of exogenous toxin (eg. lipopolysaccharide (LPS)), modification of the endogenous protective barrier of the animal, such as intestinal leakage (eg. cecal ligation and puncture (CLP), or colon ascendens stent peritonitis (CASP)), and infusion or instillation of exogenous bacteria (reviewed in, Doi *et al.,* 2009). These models all differ in the sepsis related symptoms they trigger, however, are considered a valuable model of this disease in animals. For instance, LPS administration induces systemic inflammation and an increase in pro-inflammatory cytokine production, which mimics the clinical features of sepsis (reviewed in, Wichterman *et al.,* 1980; Remick *et al.,* 2000). The

mechanism of this response will be discussed below in section 1.2. In addition, LPS infusion results in renal injury, increased blood urea nitrogen and neutrophil infiltration (Knotek *et al.,* 2001; Cunningham *et al.,* 2004; Tiwari *et al.,* 2005). Alternatively, CLP surgery involves ligation distal to the ileocecal valve and needle puncture of ligated cecum, which results in leakage of fecal contents into the peritoneum, triggering polymicrobial bacteria release and sepsis (reviewed in, Rittirsch *et al.,* 2008). This procedure usually is accompanied by treatment with fluids and antibiotics (reviewed in, Freise *et al.,* 2001). Mice which are subjected to CLP experience hypotension and an increase in pro-inflammatory cytokine production (reviewed in, Doi *et al.,* 2009). Finally, bacterial infusion models involve the introduction of a single pathogen in a controlled manner, which is similar to the initiation of human sepsis (reviewed in, Doi *et al.,* 2009). In terms of reproducibility of these sepsis models with human disease, CLP most closely mimics human disease symptoms (reviewed in, Rittirsch *et al.,* 2007). However, the multitude of differences between humans and mice (eg. the types of TLRs, age of the effected humans versus age of the experimental mice), and the complex multi-factorial nature of sepsis make it difficult to perfectly represent in an animal model (reviewed in, Rittirsch *et al.,* 2007).

Despite what is known about sepsis, clinical trials testing the efficacy of treatments against this disease have had limited success, and in some instances have unintentionally elevated patient complications and mortality. For instance, despite mouse studies demonstrating that elevated production of the pro-inflammatory cytokine TNFα is a primary agent driving the pathogenesis of (LPS; aka endotoxin)-induced endotoxic shock, neutralising TNFα antibodies have failed to provide effective protection in the human disease and often lead to fatal outcomes (reviewed in, Reinhart, Karzai, 2001; Riedemann *et al.,* 2003). It is likely that problems in the design of these clinical trials and the underlying incorrect assumptions derived from early mouse models of the inflammatory pathways driving the pathogenesis of the disease may have contributed to the failure of these approaches (reviewed in, Nasraway, 2003). In addition, the treatment of patients with sepsis usually occurs at a stage when it is too late to target the mechanisms involved (reviewed in, Rittirsch *et al.,* 2007). Other therapies include the use of IL-1 receptor (IL-1R) antagonists and anti-LPS antibodies, (Kawata *et al.,* 1999; Bartfai *et al.,* 2003; Beutler, 2004), however, no

successful overall outcomes in human disease have been achieved thus far. Therapies attempting to control inappropriate coagulation during sepsis have had some successful outcomes. Targeting activated protein C inhibits cofactors (Va and VIIa), which are involved in the generation of fibrin, resulting in the significant slowing of coagulation (Marlar *et al.,* 1981, reviewed in, Stearns-Kurosawa *et al.,* 2011). Activated C protein therapy has had successful outcomes during a study in the late 1980s with nonhuman primates (Taylor *et al.,* 1987). These studies demonstrated that pretreatment with activated C protein prevented death as a result of gram-negative bacterial *E. coli* challenge (Taylor *et al.,* 1987). This therapy is now being used to treat in patients with severe sepsis. Despite the success of activated C protein therapy during sepsis, there are complications and many factors affect the benefit of treatment.

Therefore, a better understanding of the mechanisms and pathways involved during sepsis and endotoxic shock is urgently required.

1.2 LPS

E. coli is the main Gram-negative bacteria that causes endotoxic shock, affecting approximately 400,000 patients annually in the US (Bohrer *et al.,* 1997). Under normal physiological conditions, *E. coli* is released in very small numbers into the bloodstream and liver, however if released in larger numbers triggered by factors such as surgery, *E. coli* can result in systemic inflammatory responses such as sepsis. LPS is present on the outer membranes of Gram-negative bacteria such as *E. coli*, and is important in maintaining the structure and function of these pathogens, as well as providing a target for the host immune system (reviewed in, Erridge *et al.,* 2002). LPS is comprised of a lipid A moiety (a hydrophilic heteropolysaccharide linked to a hydrophobic lipid portion, which anchors to the outer membrane and is the main determinant of endotoxicity), a core polysaccharide and an O-polysaccharide (Seydel *et al.,* 2000) (Fig. 1.4). Importantly, the conserved lipid A portion of LPS activates host immune cells. The core polysaccharide of LPS is divided into two regions consisting of an inner core, which is adjacent to the lipid A and contains many unusual sugars, and an outer core which is further from the bacterial surface (reviewed in, Erridge *et al.,* 2002). The O chain of LPS differs in length, sugars, sequence, chemical linkage, substitution, and ring forms utilised from one bacterial strain to another (reviewed

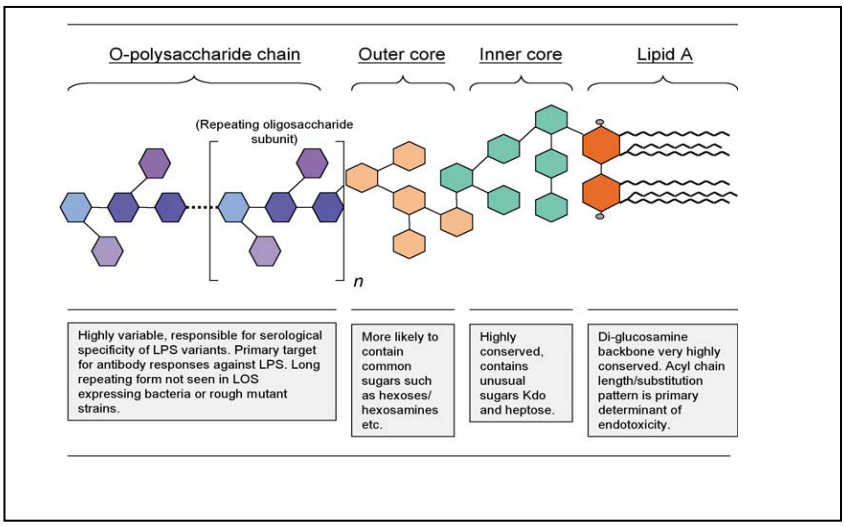

Figure 1.4: LPS structure (Erridge *et al*., 2002).

LPS is comprised of a lipid A moiety (a hydrophilic hetero-polysaccharide linked to a hydrophobic lipid portion, which anchors the molecular to the outer membrane), a core polysaccharide and an O-polysaccharide chain (of varying length).

in, Erridge *et al.,* 2002). LPS is highly toxic and classified as a pathogen-associated molecular pattern (PAMP). Specifically, LPS is the primary PAMP that facilitates host immune recognition of Gram-negative bacteria such as *E. coli* (reviewed in, Takeuchi, Akira, 2001). Consequently, when LPS is administrated to mice systemically, this results in activation of the immune system and release of pro-inflammatory cytokines (reviewed in, Erridge *et al.,* 2002). The best characterised cellular response to LPS is by the macrophage/monocyte lineage, which when stimulated with LPS produce pro-inflammatory cytokines such as TNFα, IL-1β and IL-6 (reviewed in, Holst *et al.,* 1996). Moreover, non-professional cells such as endothelial cells have also been shown to produce inflammatory cytokines in response to LPS (Dauphinee, Karsan, 2006). The mechanism involved in this pro-inflammatory cytokine production involves the use of TLRs.

1.3 TLRs

TLRs are evolutionary-conserved pathogen recognition receptors which recognise specific microbial components of invading pathogens (reviewed in, O'Neill, Bowie, 2007). The Toll gene was originally discovered in *Drosophila* and was shown to be the code for a group of proteins which play a role in embryonic dorso-ventral patterning (Hashimoto *et al.,* 1988). They were, however, also found to be necessary for immune responses against the fungus *Aspergillus fumigatus* (Lemaitre *et al.,* 1996). TLRs are characterised by an extracellular PAMP-recognition leucine rich repeat (LRR) motif, as well as a cytoplasmic Toll/Interleukin-1 receptor (TIR) domain which has structural similarity to the IL-1R family and facilitates association with specific intracellular adaptor proteins (Fig. 1.5) (reviewed in, Watters *et al.,* 2007). There are 13 members of the mammalian TLR family, 10 (TLR1-10) in humans and 12 in mice (TLR1-9 and TLR11-13), each with the ability to recognise a unique set of PAMPs (see Table 1.3 for details) (Rock *et al.,* 1998; Takeuchi *et al.,* 1999; Du *et al.,* 2000; Hemmi *et al.,* 2000; Chuang, Ulevitch, 2001; Zhang *et al.,* 2004). TLR12 and 13 are relatively uncharacterised; however recent studies suggest that along with TLR11, they play a role in host immune surveillance in the central nervous system (Mishra *et al.,* 2008). This diverse spread of specificities of TLRs is due to the insertions at positions 10 and 15 of LRRs and also by the differential use of accessory proteins

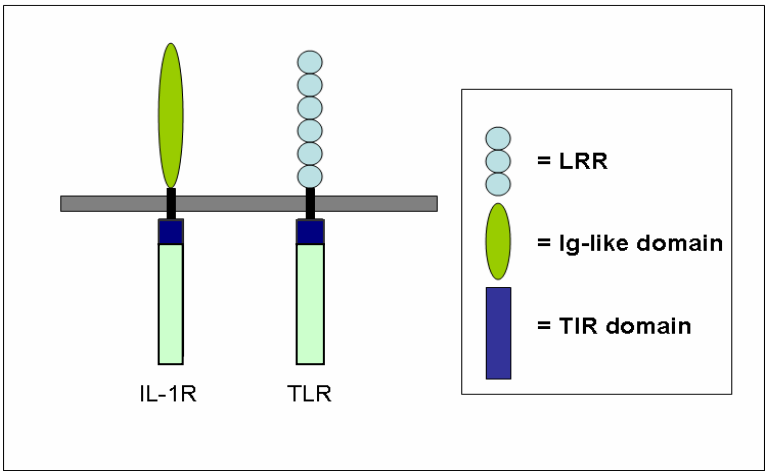

Figure 1.5: TLR structure (Adapted from Bilak et al., 2003).
The TLR and IL-1R receptor families possess the common cytoplasmic TIR domain. Immunoglobulin-like modules in the IL-1R receptor family distinguish it from the TLR family, which have LRRs as the extracellular domain.

(Fig. 1.6) (Bell *et al.,* 2003; Watters *et al.,* 2007). This diversity of adaptor usage also widens the repertoire of TLR responses, so that the immune response can potentially recognise all microbes (reviewed in, Beutler, 2004).

TLR4 was the first member of the TLR family to be characterised and is critical in the recognition of LPS (Mollen *et al.,* 2006). The discovery of TLR4 was due to the experiments in C3H/HeJ mice, which have an impaired ability to respond to LPS due to a mutation in the TLR4 gene which leads to a $Pro^{712}His$ substitution in the cytoplasmic signalling domain of TLR4 (Poltorak *et al.,* 1998). These mice are highly susceptible to Gram-negative infection, and in addition to TLR4-deficient mice, are hypo-responsive to LPS (Poltorak *et al.,* 1998; Hoshino *et al.,* 1999). In addition to this research, Medzhitov *et al*., (1997) also discovered the human homologue of Toll activated immune pathways.

LPS-induced TLR4 responses are dependent upon the binding of LPS binding protein (LBP) to LPS. LBP resides in the sera and is produced in the liver as an acute-phase protein which then binds the lipid A component of LPS and brings it into contact with cluster of differentiation 14 (CD14) on macrophages/monocyte host cells (Goldblum *et al.,* 1994; Hailman *et al.,* 1994; Akira, Hoshino, 2003). LBP is thought to be involved in an immune response against small amounts of LPS early during an infection, a theory confirmed by the lack of response of $LBP^{-/-}$ mice to LPS (Schumann *et al.,* 1990; Jack *et al.,* 1997). However, these mice were highly susceptible to infection caused by *Salmonella typhimurium*, as a result of inadequate phagocytosis and killing of the micro-organisms (Jack *et al.,* 1997). Furthermore, mice treated with anti-LBP antibodies were highly susceptible to small amounts (250 CFU/mouse) of *Klebsiella pneumoniae*, a Gram-negative bacteria (Le Roy *et al.,* 2001).

CD14 is a glycosylphosphatidylinositol (GPI)-anchored protein which is also found in the blood in its soluble form (sCD14), and increases in concentration during systemic response to infection (Hailman *et al.,* 1996). sCD14 is utilised by cells which do not express membrane bound CD14, such as endothelial cells, which can then respond to low levels of LPS via sCD14 (Kitchens *et al.,* 2001). Although CD14 is an acute phase protein, sCD14 has also been shown to suppress cellular responses to LPS (Kitchens *et al.,* 2001; Bas *et al.,* 2004). Specifically, once LPS has been recognised by monocytes, human sCD14 can limit the amount of LPS that remains bound to these cells, hence, reducing cytokine responses

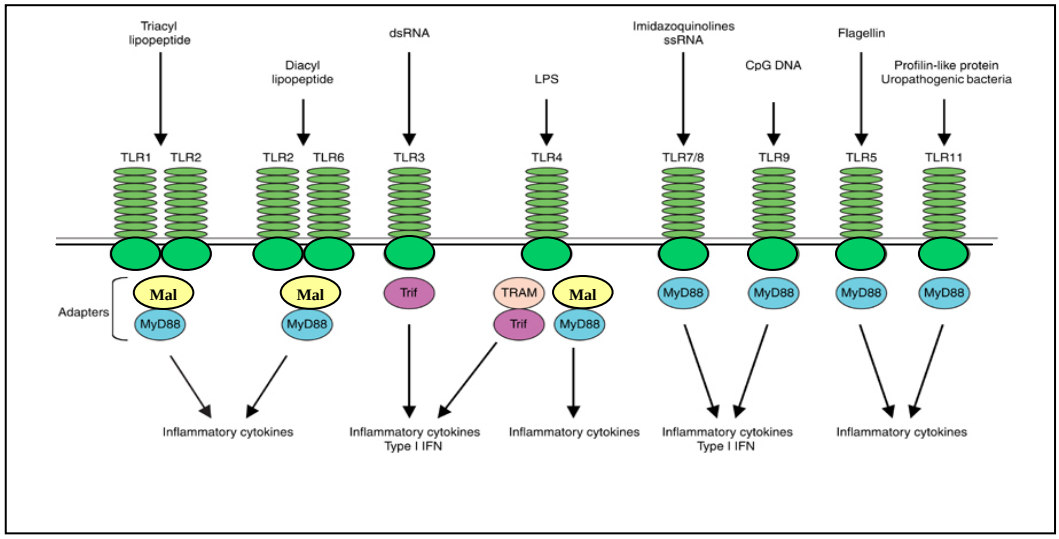

Figure 1.6: TLR mediated immune responses (Adapted from Kawai, Akira, 2006).

The TLRs utilise various adaptors in their signalling pathways to diversify their responses. TLR2 signals via Mal and MyD88 when in a hetero-dimer with TLR1 or TLR6. TLR4 signals via TRIF and TRAM as the MyD88 independent arm of the pathway, and Mal and MyD88 as the MyD88 dependent arm. TLR5, 7/8 and 11 all utilise MyD88 alone.

(Kitchens *et al.,* 2001). In addition to this regulatory role of sCD14 to control LPS reactions within the bloodstream, cellular CD14 is also essential for TLR4 signalling as evidenced by the fact that *CD14*$^{-/-}$ mice are resistant to LPS (Haziot *et al.,* 1996). Furthermore, anti-CD14 antibodies administered to rabbits resulted in a 50-fold increased *Shigella* invasion in the intestinal mucosa when compared with controls (Haziot *et al.,* 1996; Wenneras, 2000), suggesting the importance of LBP and CD14 during the control of infection and in the recognition of LPS (Heumann, 2001).

In addition to CD14 and LBP, MD2 is another extracellular accessory molecule necessary for TLR4 responses to LPS (Medvedev *et al.,* 2007). Initial studies indicated that TLR4 and MD2 are physically associated together, and also that stimulating stable transfectants (expressing TLR4 alone or with MD2) with LPS only resulted in downstream nuclear factor-κB (NF-κB) activity in those with TLR4 and MD2 (Shimazu *et al.,* 1999). Further evidence that MD2 is necessary for LPS signalling was provided by a study demonstrating that transfection of cells (CD14 transfected Chinese hamster ovary-KI fibroblasts containing an MD2 mutant) with wild-type MD2 restored their response (e.g. activation of MAPKs or secretion of IL-6) to bacterial endotoxin (Schromm *et al.,* 2001).

Collectively, the resulting LPS/LBP/CD14/MD2 complex that is formed from all these molecules then has the ability to bind to TLR4 and initiate the early myeloid differentiation factor 88 (MyD88)-dependent pathway and the later MyD88-independent pathway (Fig. 1.7) (reviewed in, Palsson-McDermott, O'Neill, 2004).

1.3.1 TLR intracellular signalling

There are two main pathways involved in TLR signalling to activate NF-κB, the MyD88-dependent and -independent pathways. The MyD88-dependent pathway utilises the TIR domain containing adaptors MyD88 and MyD88 adaptor like (Mal, also known as TIR-domain containing adaptor protein (TIRAP)), whereas the MyD88-independent pathway utilises TIR-domain-containing adaptor protein inducing interferon (IFN)β (TRIF, also known as TICAM-1) and TRIF-related adaptor molecule (TRAM, also known as TICAM-2) (Kawai *et al.,* 2001; Kenny, O'Neill, 2008) (Fig. 1.7). These adaptors initiate TLR signalling via their association through TIR-TIR interactions which are created as a result of TLR

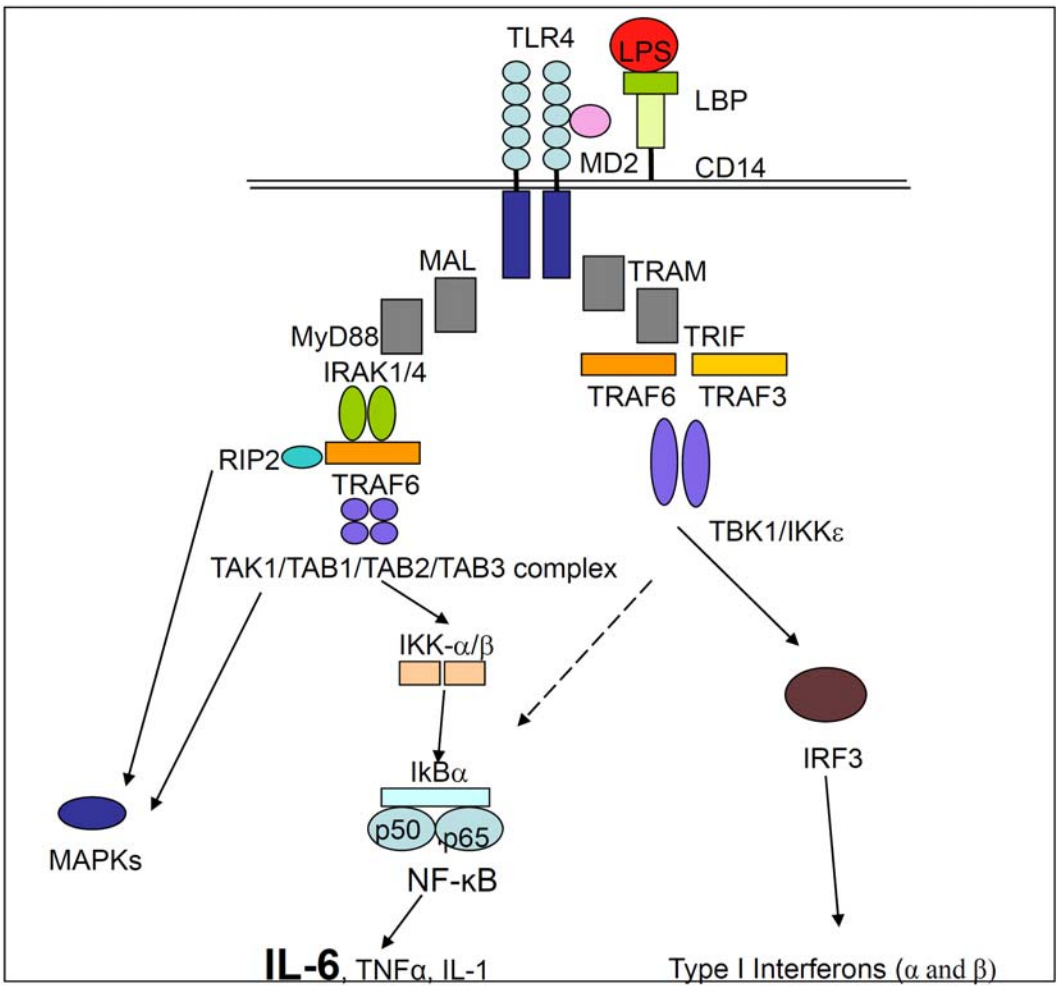

Figure 1.7: TLR4 pathway (Adapted from Takeuchi, Akira, 2010).

LBP, which is present in the host's bloodstream, is the first point of contact by the immune system to LPS. The association between LPS and LBP induces the formation of a high affinity complex which also comprises of CD14 and MD2 accessory receptors, which present LPS to TLR4. The TLR4 receptor then homodimerises and triggers the signalling cascades essential for the production of pro- inflammatory cytokines, IFNs and activation of the MAPK pathway.

dimerisation (reviewed in, Kenny, O'Neill, 2008). TLR adaptors are conserved among many different species, with 26 adaptors being present in the sea urchin (Sodergren *et al.,* 2006; O'Neill, Bowie, 2007).

Overall, the most widely utilised adaptor molecule in used by TLRs is MyD88 (Fig. 1.6), which could account for the redundancy of the TLR pathways. However, the differential use of these various adaptors also diversifies the responses of individual TLRs (Fig. 1.6) (reviewed in, Kawai, Akira, 2005). TLR4 utilises all these adaptor molecules and signals via the MyD88-dependent and -independent pathways (reviewed in, Kawai, Akira, 2005) (Fig. 1.6,1.7). TLR2 signals via the MyD88-dependent pathway, and utilises Mal as an adaptor (reviewed in, Kawai, Akira, 2005) (Fig. 1.6). TLR5, 7 and 9 only use the MyD88-dependent pathway, but do not utilise Mal, whereas TLR3 signals via the MyD88-independent pathway alone, which utilises TRIF (Kobayashi *et al.,* 2006) (Fig. 1.6). Due to the involvement of TLR4 in the recognition of LPS/Gram-negative bacteria which is the cause of the majority of sepsis cases, this review will be focusing primarily on the TLR4 signalling pathway and the relevance of these adaptors accordingly.

1.3.2 The TLR4 signalling pathway components

MyD88 is a 296 amino acid protein which consists of the TIR domain and the amino-terminal death domain (Kawai, Akira, 2005). The generation of MyD88-deficient mice (*MyD88*$^{-/-}$) has demonstrated the critical role MyD88 plays in the induction of pro-inflammatory cytokines during TLR4 signalling (Kawai *et al.,* 1999). For instance, *MyD88*$^{-/-}$ mice were resistant to LPS-induced shock after administration of a high dose of LPS, whereas wild-type mice died within 96 hours (hrs) of LPS administration (Kawai *et al.,* 1999) and were protected from developing sepsis in a polymicrobial septic peritonitis disease model (Weighardt *et al.,* 2002). In addition, the production of pro-inflammatory cytokines such as TNFα, IL-1β and IL-6 was abolished in *MyD88*$^{-/-}$ macrophages stimulated with LPS, further supporting the role of MyD88 as vital to the inflammatory response (Kawai *et al.,* 1999). However, delayed activation of NF-κB and MAPKs in response to LPS in *MyD88*$^{-/-}$ macrophages and the production of IFN-inducible genes such as interferon-inducible protein 10 (IP-10) and glucocorticoid-attenuated response gene 16 (GARG16), suggested that a component of the TLR4

pathway exists that does not involve MyD88 (Kawai *et al.,* 2001). This pathway was later referred to as the MyD88-independent pathway (see above), and thus accounted for previous observations that LPS induces the expression of type 1 interferons (Hamilton *et al.,* 1996).

The discovery of Mal was the first indication that different TLRs recruit a variety of adaptor molecules (Horng *et al.,* 2001). Mal is similar to MyD88 as it contains a TIR domain, however, the N terminal domain of Mal is 75 amino acids smaller and lacks a death domain (Fitzgerald *et al.,* 2001). Mal acts as a bridging adaptor, and brings MyD88 into physical contact with TLR4. The importance of Mal in the TLR4 signalling pathway has been confirmed by the generation of Mal-deficient mice (*Mal*$^{-/-}$). These mice have normal responses to TLR5, 7, and 9 agonists, however they were completely resistant to LPS-induced shock (Horng *et al.,* 2002; Yamamoto a *et al.,* 2002). In addition, macrophages from these *Mal*$^{-/-}$ mice failed to produce cytokines such as IL-6, TNFα and IL-1β in response to LPS (Yamamoto a *et al.,* 2002). Despite these observations, *Mal*$^{-/-}$ mice were still able to induce late phase NF-κB, MAPK and interferon response factor 3 (IRF3) activation. Therefore, to eliminate the possibility that these adaptors may function in a redundant manner, the MyD88 and Mal double knock-out mouse was generated (Yamamoto a *et al.,* 2002). These mice still induced IRF3 activation and induction of genes such as IP10 and GARG16, which was similar to wild-type mice in response to LPS, further suggesting that additional adaptors are involved in the MyD88-independent pathway of TLR4 signalling (Yamamoto a *et al.,* 2002).

The adaptors involved in the MyD88-independent pathway include TRIF and TRAM (Yamamoto b *et al.,* 2002; Fitzgerald *et al.,* 2003). TRIF is 712 amino acids in size and is expressed in a variety of human tissues (Yamamoto a *et al.,* 2003). In TRIF-deficient mice (*TRIF*$^{-/-}$), activation of IRF3, IFNβ and pro-inflammatory cytokine production is impaired in response to TLR4 stimulation (Yamamoto a *et al.,* 2003). Mice deficient in both TRIF and MyD88 are unable to activate NF-κB in response to LPS, indicating that TRIF is responsible for MyD88-independent signalling (Yamamoto a *et al.,* 2003; Hirotani *et al.,* 2005).

TRAM is 235 amino acids in size and activates IRF3 and NF-κB to induce interferon type 1 production (Fitzgerald *et al.,* 2003; Yamamoto b *et al.,* 2003).

TRAM is upstream of TRIF in this pathway and brings it physically into contact with TLR4, facilitating its activation (Oshiumi *et al.,* 2003). Mice deficient in TRAM have reduced cytokine production in response to LPS, and MyD88-independent IFN-inducible gene production is abolished, illustrating the critical role for TRAM in this pathway (Yamamoto a *et al.,* 2003).

1.3.3 The TLR4/MyD88-dependent pathway

The TLR4/MyD88-dependent pathway mentioned above results in the activation of NF-κB (reviewed in, Kaisho, Akira, 2006). TLR4 homodimerisation and tyrosine phosphorylation is necessary for its signalling capacity, as is the recruitment of MyD88 to TLR4 via Mal (Medvedev *et al.,* 2007; Watters *et al.,* 2007; Kenny, O'Neill, 2008). This function of Mal is necessary as TLR4 and MyD88 are both electropositive, resulting in the inability of these molecules to bind each other (Dunne *et al.,* 2003; O'Neill, Bowie, 2007). In order to function, Mal has been shown to be cleaved by caspase 1, as suppressing this process resulted in an inhibition of TLR4 signalling (Dunne *et al.,* 2003; O'Neill, Bowie, 2007; Watters *et al.,* 2007). It is thought that this cleavage results in a conformational change which exposes residues necessary for the interaction of Mal and other signalling molecules. Another requirement for the activation of Mal is tyrosine phosphorylation by Brutons tyrosine kinase (Btk) (reviewed in, Watters *et al.,* 2007). Once activated, this TLR4/Mal/MyD88 complex then recruits the serine/threonine kinases IL-1 receptor-associated kinase (IRAK)1 and IRAK4 via their N-terminal death domain (reviewed in, Watters *et al.,* 2007), and following a series of events (Figure 1.7) ultimately leads to activation and nuclear translocation of NF-κB and the induction of many inflammatory cytokine genes, such as IL-6, TNFα and IL-1β (reviewed in, Chen, 2005; Watters *et al.,* 2007; Akira *et al.,* 2006).

Prior to stimulation, NF-κB exists in the cytoplasm as a heterodimer, which can consist of a combination of p50/NF-κB1, p52/NF-κB2, RelA (p65), c-Rel and RelB. The most characterised heterodimer combination of these Rel and NF-κB proteins is p50/RelA(p65) and p50/c-Rel, which is sequestered in an inactive form in the cytoplasm and has been termed the ‘canonical NF-κB pathway’ which leads

to the IκBα degradation mentioned above (reviewed in, Pomerantz, Baltimore, 2002). However, the 'alternate pathway' consists of p52/RelB proteins initiating target gene transcription (reviewed in, Brown *et al.,* 2008). These two pathways have differing roles depending on the cell context and stimulus, and can control identical or altered gene sets (reviewed in, Beinke, Ley, 2004).

It should also be noted that another pathway implicated in the activation of NF-κB is phosphatidylinositol 3-kinases (PI3K)/Akt, which has also been shown to play an important role in response to LPS through the serine/kinase RIP2 (Dauphinee, Karsan, 2006). TAK1 also activates the serine/threonine kinases mitogen-activated protein (MAPKs), including cJun N-terminal kinase (JNK) and p38. Furthermore, it has been shown that MAP3K TPL2 is required for the activation of extracellular signal regulated kinase (Erk) (Banerjee *et al*., 2006). These pathways are involved in cell differentiation, survival and apoptosis as well as regulating pro-inflammatory cytokine production, however the mechanisms of this activation are not fully understood (Dauphinee, Karsan, 2006).

1.3.4 The TLR4/MyD88-independent pathway

As mentioned above, the TLR4/MyD88-independent pathway involves the integral adaptors TRIF and TRAM. The activation of this pathway (unlike the MyD88-dependent pathway, which is initiated at the cell surface) occurs in the endosomal compartment after TLR4 internatlisation (Kagan *et al*., 2010). TRAM is a bridging adaptor (like Mal), in that it is required to couple TLR4 and TRIF together (reviewed in, Kenny, O'Neill, 2008). TRAM requires regulation by serine phosphorylation via PKCε following LPS stimulation, which is thought to cause TRAM to dissociate from the membrane and induce downstream signalling (McGettrick *et al.,* 2006). Following the TRAM and TRIF interaction, TRIF then binds TRAF3 and TRAF6 via its N-terminal TRAF binding motifs (Sato *et al.,* 2003; Takeuchi, Akira, 2010). This interaction then promotes the activation of NF-κB (reviewed in, Takeuchi, Akira, 2010) (Fig. 1.7). In parallel, TRAF family member-associated NF-κB activator (TANK), binding kinase 1 (TBK1) and IKKε (IKKi) then bind to TRIF via TRAF3. Furthermore, TRAF3 and NAK-associated protein 1 (NAP1) mediate the activation of TBK1 by bridging the gap between this molecule and TRIF (Hacker *et al.,* 2006; Oganesyan *et al.,* 2006). This key

event results in activation of IRF3 which then binds to IFN sensitive response elements in the promoter regions of pro-inflammatory type I IFN genes (i.e. α and β) to promote their transcription (reviewed in, Palsson-McDermott, O'Neill, 2004) (Fig. 1.7). Mice deficient in IRF3 fail to produce IFNβ in response to LPS, illustrating the critical role for this regulatory factor during this arm of the TLR4 pathway (Sakaguchi *et al.,* 2003). These mice are also resistant to LPS-induced endotoxic shock (Sakaguchi *et al.,* 2003).

1.3.5 Type I IFNs

Type I IFNs are pleiotropic cytokines which include 15 members, all of which have similar structure and biological activities, are induced in response to virus, and share receptor subunits (reviewed in, Isaacs, Lindenmann, 1957; Weissmann, Weber, 1986; Pestka *et al.,* 1987; Hertzog *et al.,* 1994; Stark *et al.,* 1998). Type I IFNs are involved in the modulation of inflammation and anti-viral responses by the host, including apoptotic cell death, activation of adaptive immunity, activation of natural killer cells and haematopoietic stem cell proliferation (reviewed in, Takaoka, Yanai, 2006; Takeuchi, Akira, 2010). These processes occur through IFN signalling via the interferon receptors (IFNAR), which consist of two subunits; IFNAR1 and IFNAR2 (Novick *et al.,* 1994) (Fig. 1.8). The intracellular portion of the receptors is associated with the members of the janus tyrosine kinase (Jak) family which includes Jak1, Jak2, Jak3 and tyrosine kinase-2 (Tyk2) (reviewed in, Yeh, Pellegrini, 1999). These Jaks are then tyrosine phosphorylated upon ligand binding and then dock and tyrosine phosphorylate STAT1 and STAT2 (reviewed in, Darnell *et al.,* 1994; Bonjardim *et al.,* 2009). These activated STATs then dissociate from the receptor and form a complex with interferon response factor 9 (IRF9), called IFN-stimulated gene factor 3 (ISGF3), which translocates to the nucleus to activate target genes via IFN-stimulated response elements (Haque, Williams, 1994; Bluyssen *et al.,* 1996; van Boxel-Dezaire *et al.,* 2006) (Fig. 1.8). Examples of such genes include IFN-inducible protein 10 (IP10), GARG16 and immune-responsive gene 1 (reviewed in, Akira, Hoshino, 2003). In addition to STAT1 and STAT2, STAT3 and STAT5 are activated by type I IFNs (Yang *et al.,* 1998). IFNα stimulation can lead to the

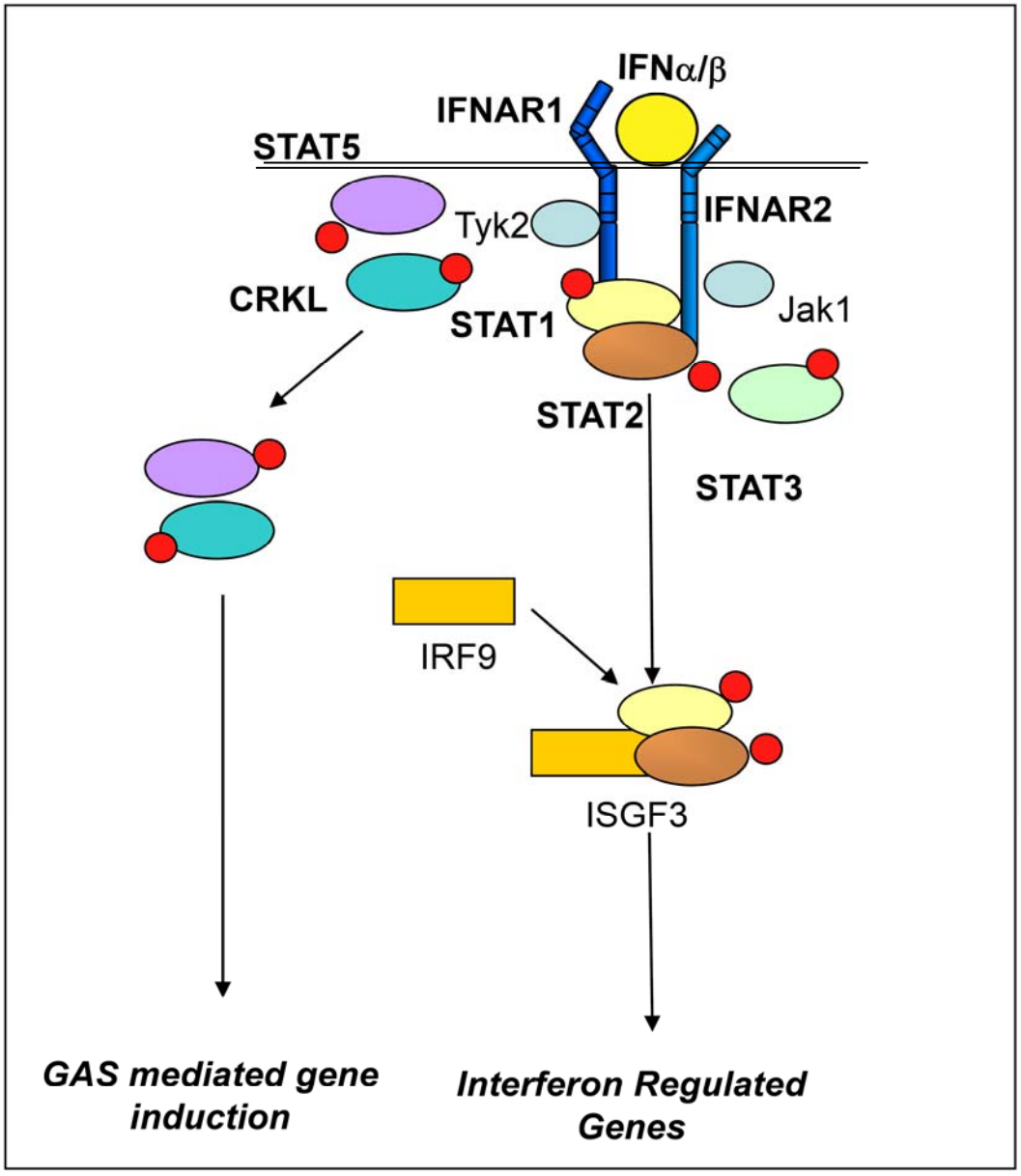

Figure 1.8: IFNAR pathway (Adapted from Takaoka, Yanai, 2006).
Type I IFNs signal via the IFNAR1/2 receptor complex. The intracellular portion of the receptors are associated with Jaks, which are phosphorylated (red dots) upon signal activation and then dock and activate STAT1/2, which complex with IRF9. This complex (ISGF3) then translocates to the nucleus to activate target genes via IFN-stimulated response elements (ISRE). STAT5 dimerises with CRKL and translocates to the nucleus to activate GAS mediated genes, and STAT3 is involved in the activation of IFN regulated genes.

formation of STAT1 homodimers that bind to γ-activated sequences (GAS) promoter elements (Tassiulas *et al.,* 2004; Caraglia *et al.,* 2005; Gimeno *et al.,* 2005). STAT5 is thought to associate with Tyk2, which recruits and phosphorlyates v-crk sarcoma virus CT10 nocogene homologue (avian)-like and STAT5, leading to their dimerisation and translocation into the nucleus to activate GAS-mediated gene expression (reviewed in, Takaoka, Yanai, 2006). STAT3 forms homodimers and heterodimers with STAT1 during type I IFN signalling and has been shown to support the ISGF3 induction of anti-viral genes and is also thought to be involved in the activation of PI3K (Yang *et al.,* 2001; Ho, Ivashkiv, 2006). Studies of the IFNAR1 knock-out mice indicate that they are resistant to LPS (Mahieu *et al.,* 2006), however they are susceptible to viral infections (Muller *et al.,* 1994) and IFNβ knock-out mice are also resistant to lethal endotoxemia (Karaghiosoff *et al.,* 2003).

1.3.6 Negative regulators of TLRs

In order to control the potential exaggerated expression of TLR mediated pro-inflammatory cytokine production, which can lead to destructive effects such as septic shock, negative regulators are utilised (reviewed in, Lang, Mansell, 2007). These factors may also be responsible for LPS tolerance, which results in a down regulation of this inflammatory pathway, however the mechanics of this process are not well understood (reviewed in, Biswas, Lopez-Collazo, 2009).

One of these regulators is SOCS1, which was identified as the first member of this class of proteins (Yoshimura *et al.,* 1995). Along with the other 7 members of this family (SOCS2-7 and CIS), these proteins are induced rapidly in response to cytokines and other inflammatory mediators such as LPS (reviewed in, Starr, Hilton, 1998; Yasukawa *et al.,* 2000; Yoshimura *et al.,* 2007). SOCS proteins interfere with signalling by targeting the receptor complex to the proteasome for degradation via the ubiquitin pathway (Zhang *et al.,* 1999). Alternatively, SOCS proteins bind to and inhibit Jak catalytic activity via their Src homology 2 (SH2) domain and kinase inhibitory region (KIR), respectively (Endo *et al.,* 1997; Krebs, Hilton, 2001; Yoshimura *et al.,* 2003) (Fig. 1.9). As an example of the specificity of SOCS proteins, SOCS1 down-modulates IFN/STAT1 signalling by binding to the activation loop of Jaks on the receptor via their KIR and SH2 domains, thus inhibiting tyrosine phosphorylation and consequential STAT1 activation

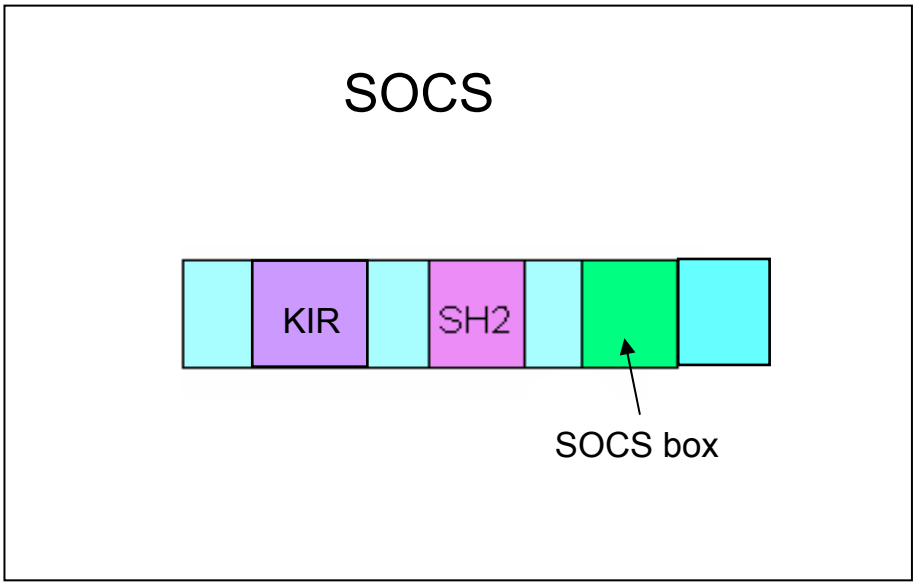

Figure 1.9: SOCS structure (Adapted from Alexander, 2002)

SOCS molecules consist of a SH2 domain which is involved in tyrosine binding, a KIR domain which is necessary for Jak phosphorylation, and a SOCS box which binds to the ubiquitin transferase system, which results in receptor/cytokine complex proteasomal degradation.

(Yasukawa *et al.,* 1999). This inhibition is thought to be specific to IFNAR1, as co-immunoprecipitation experiments have demonstrated the association of IFNAR1 and SOCS1 (Fenner *et al.,* 2006). To illustrate the importance of this receptor subunit in SOCS1 negative regulation, *SOCS1*$^{-/-}$ mice, which are hypersensitive to LPS, survive longer when crossed onto an IFNAR1 (not IFNAR2) knock-out background (Fenner *et al.,* 2006). In addition to its ubiquitin-transferase activity via the SOCS box (Zhang *et al.,* 1999) (Fig. 1.9), SOCS1 is proposed to interact with p65 and result in proteasomal degradation, leading to down-regulation of NF-κB (reviewed in, Kobayashi *et al.,* 2006). Moreover, Mansell *et al.* (2006) have identified SOCS1 as interacting with Mal and causing the ubiquitin-proteasome-dependent degradation which is seen to occur within 15-30 minutes of activation by TLR2 or TLR4. It is thought that this process involves phosphorylation of Mal by Btk following TLR stimulation, and then the binding of Mal to the SH2 region of SOCS1 (Mansell *et al.,* 2006).

In addition to SOCS1, TLRs are regulated through many intracellular mechanisms. For example, MyD88 short lacks the intermediate linker sequence which is evident in the full-length MyD88 molecule and can inhibit LPS activation of NF-κB in monocytes by preventing IRAK4 activation (Janssens *et al.,* 2002). Other intracellular negative regulators include sterile α- and armadillo-motif-containing protein, a protein of 690 amino acids which has been shown to be a negative regulator of TRIF (Mink *et al.,* 2001; Couillault *et al.,* 2004). In addition IRAK-M has been shown to impair IRAK-1 association with TRAF6 (Kobayashi *et al.,* 2002). Furthermore, Toll-interacting protein has been shown to sequester the activity of IRAK-1, resulting in decreased NF-κB activation, and A20 cleaves the polyubiquitin chain in TRAF6 which interferes with NF-κB translocation to the nucleus (Boone *et al.,* 2004).

Regulators of TLR signalling can also occur in the extracellular region of the cell. For example, 'soluble decoy proteins' (reviewed in, Lang, Mansell, 2007) such as sTLR4, which is an isoform of the TLR4 gene, has been shown to inhibit NF-κB activation (Iwami *et al.,* 2000), most likely by preventing the interaction between TLR4 and CD14/MD2 (Hyakushima *et al.,* 2004).

Transmembrane regulators are another form of negative regulation of TLRs, which interfere with TLR ligand binding or sequester TLR adaptors. One such

protein is called radioprotective 105 (RP105), a homologue of TLR4 which lacks a signalling domain. RP105 has been shown in a human embryonic kidney cell line (HEK-293) to interact with TLR4/MD2 complexes following LPS stimulation and inhibit LPS binding (Divanovic *et al.,* 2005).

As well as playing a positive role in LPS signalling (described in section 1.6.1), PI3K has also been assigned a negative regulatory role, as inhibition of the PI3K-Akt pathway results in upregulation of pro-inflammatory cytokine production in endothelial cells and monocytes (Schabbauer *et al.,* 2004). PI3K has also been shown to associate with TRIF after LPS stimulation and inhibit NF-κB activity, but not affect the IRF3 part of the pathway (Aksoy *et al.,* 2005).

1.4 Anti-inflammatory cytokines

In addition to previously mentioned negative regulators of TLR signalling, anti-inflammatory cytokines are also up-regulated in the body during an inflammatory response to control the exaggerated production of pro-inflammatory cytokines (reviewed in, Opal, DePalo, 2000).

1.4.1 IL-10

A key anti-inflammatory cytokine which is triggered by the TLR4 pathway is IL-10, an 18kD glycoprotein produced by Th2 cells, B cells, monocytes, macrophages, and keratinocytes (Fiorentino *et al.,* 1989; Moore *et al.,* 2001). IL-10 signals by binding to its receptor complex comprising of IL-10R1 and IL-10R2, which induces Jak1 and Tyk2 activation. This activation results in STAT3 tyrosine phosphorylation, homodimerisation, and translocation to the nucleus (Kotenko *et al.,* 1997; O'Farrell *et al.,* 1998; Riley *et al.,* 1999; Moore *et al.,* 2001) (Fig. 1.10). These dimers then bind to IL-10 inducible genes and cause gene expression, for example SOCS3 and B cell lymphoma 3 (Cassatella *et al.,* 1999; Kuwata *et al.,* 2003). STAT3 has been shown to bind to the IL-10 promoter, and can up-regulate its expression in humans (Benkhart *et al.,* 2000).

In addition, research has suggested that SOCS3 is essential for IL-10 anti-inflammatory signalling. For instance, the ability of IL-10 to inhibit TNFα and nitric oxide production is reduced in peritoneal macrophages isolated from *SOCS3*$^{+/-}$ mice (Berlato *et al.,* 2002). Interestingly however, SOCS3 is also thought to be a potential negative regulator of IL-10 anti-inflammatory functions.

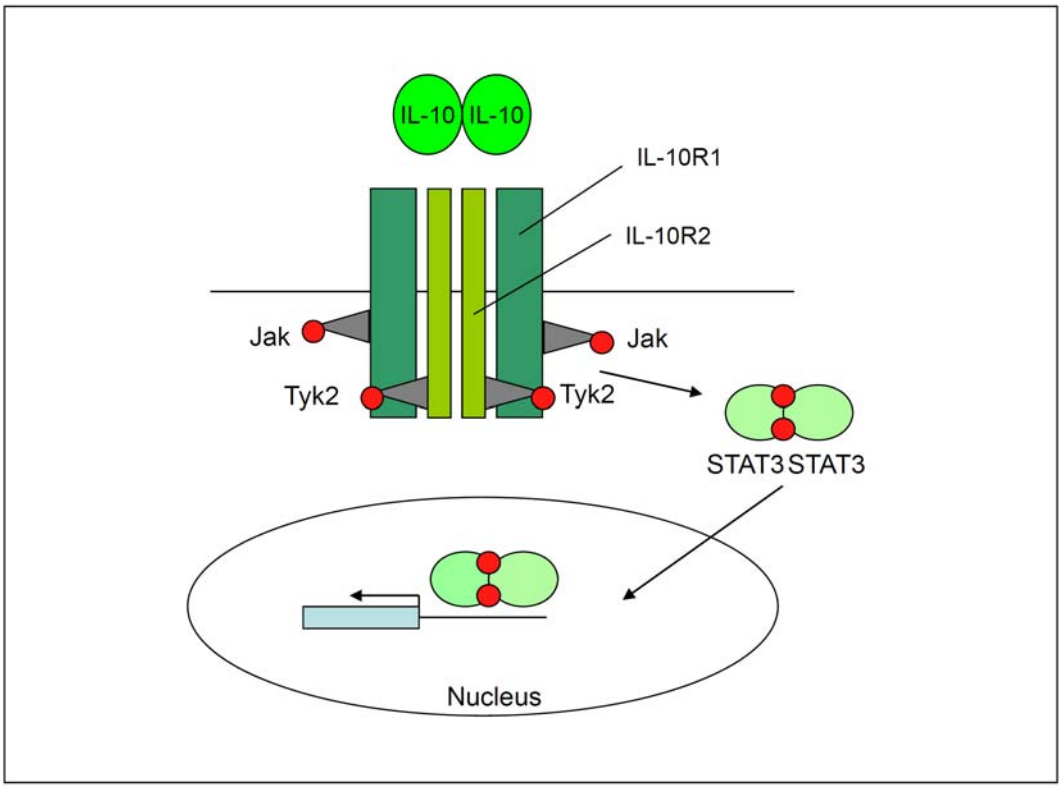

Figure 1.10: IL-10 signalling pathway (Adapted from Kotenko *et al*., 1997). The IL-10 homodimer binds to the IL-10R1 and IL-10R2 chain complex to initiate signal transduction involving Jak-STAT activation. The IL-10R2 chain is essential for the intracellular receptor complex to be active and to initiate signalling.

This negative regulatory function was assumed based on its role as a repressor of other pathways and the fact that it is up-regulated by many intracellular pathogens for survival (Stoiber *et al.,* 2001; Imai *et al.,* 2003; Qasimi *et al.,* 2006). Previously, it has shown that increased SOCS3 expression in macrophages decreases STAT3 tyrosine phosphorylation and potential activity by IL-10 (Berlato *et al.,* 2002). Moreover, SOCS3 inhibited STAT3 transcription induced through gp130 to a higher degree than STAT3 transcription induced through the IL-10 receptor (Yasukawa *et al.,* 2003). By contrast, SOCS3-deficient peritoneal macrophages (generated by the Cre-loxP system) were shown to have an intact suppression of LPS-induced TNFα production when treated with IL-10 (Yasukawa *et al.,* 2003). These discrepancies are thought to be explained by time-dependent mechanisms; that IL-10 is SOCS3-dependent early on and then switches to a SOCS3-independent mechanism for inhibition of TNFα (Qasimi *et al.,* 2006). However, further research into this area is necessary.

IL-10 has been shown to exert its anti-inflammatory role by inhibition of the production of pro-inflammatory cytokines such as TNFα, IL-1β and IL-6 by activated monocytes/macrophages (de Waal Malefyt *et al.,* 1991; Fiorentino *et al.,* 1991). Studies in human monocytes have indicated that after LPS stimulation, IL-10 stabilises IκBα, thus preventing NF-κB activation and nuclear translocation, and therefore resulting in a reduction in TNFα release (Shames *et al.,* 1998). In accordance with these results, Schottelius *et al.,* (1999) showed that IL-10 inhibits nuclear translocation of NF-κB by blocking IκBα degradation in response to TNFα stimulation in THP-1 cells (a human monocytic cell line) (Schottelius *et al.,* 1999).

Evidence of IL-10 anti-inflammatory activity is clear from IL-10-deficient mice, which develop chronic entercolitis, similar to inflammatory bowel disease (Kuhn *et al.,* 1993). Additional evidence of the anti-inflammatory role of IL-10 comes from its ability to prevent experimental colitis in rats when IL-10 gene transfer was undertaken (Barbara *et al.,* 2000). In addition, when administered with corticosteroids, IL-10 has shown to prevent rodent chronic granulomatous inflammation (Herfarth *et al.,* 1998). Furthermore, IL-10 treatment protected mice from a lethal injection of endotoxemia (Howard *et al.,* 1993) and inflammatory

bowel disease patients have shown clinical improvements when treated with IL-10 (Schreiber *et al.,* 1995).

More recently it has suggested IL-10 may also have immuno-stimulatory properties. Specifically, IL-10 augments the ability of preactivated human-purified CD8+ T cells to drive proliferation via IL-2 (Groux *et al.,* 1998). When pre-incubating CD4+ lymphocytes with IL-10, their ability to produce cytokines post-activation is enhanced (Lelievre *et al.,* 1998). Furthermore, IL-10 potentiates the *in vitro* growth and differentiation of activated human B lymphocytes (Rousset *et al.,* 1992). To confirm this pro-inflammatory role, IL-10 administrated intravenously (i.v.) into human subjects 1hr after LPS i.v. injection resulted in enhanced IFNγ release, in addition to increasing LPS-mediated cytotoxic T cell lymphocyte and NK activation (Lauw *et al.,* 2000). Therefore, the treatment of patients with inflammatory diseases with IL-10 may have some disadvantages and further understanding of the role of this cytokine and its signalling molecules needs to be investigated. Collectively, the research above suggests cell type differences may affect how IL-10 behaves, hence a more thorough understanding of these mechanisms is needed.

1.4.2 TGF-β1

TGF-β1, which belongs to the TGF-β super-family that includes 20 highly related cytokines known as the bone morphogenetic proteins (BMP) (reviewed in, Rider, Mulloy, 2010), plays a role in maintaining homeostasis, immunosuppression, extracellular matrix production, as well as other activities such as cell proliferation, differentiation and tissue repair (reviewed in, Massague, 1990; Roberts, Sporn, 1993; Alexandrow, Moses, 1995; Massague, 1998). TGF-β signalling involves a set of transmembrane receptor serine/threonine kinases and the activation of cytoplasmic signal transducer proteins Sma and Mad related proteins (Smad)2 and Smad3, which form complexes with Smad4 and translocate to the nucleus to transcriptionally-induce target genes (reviewed in, Massague, 1998) (Fig. 1.11). Smad7 negatively regulates this signalling pathway via competition for phosphorylation by the activated receptor complex (Nakao *et al.,* 1997) (Fig. 1.11).

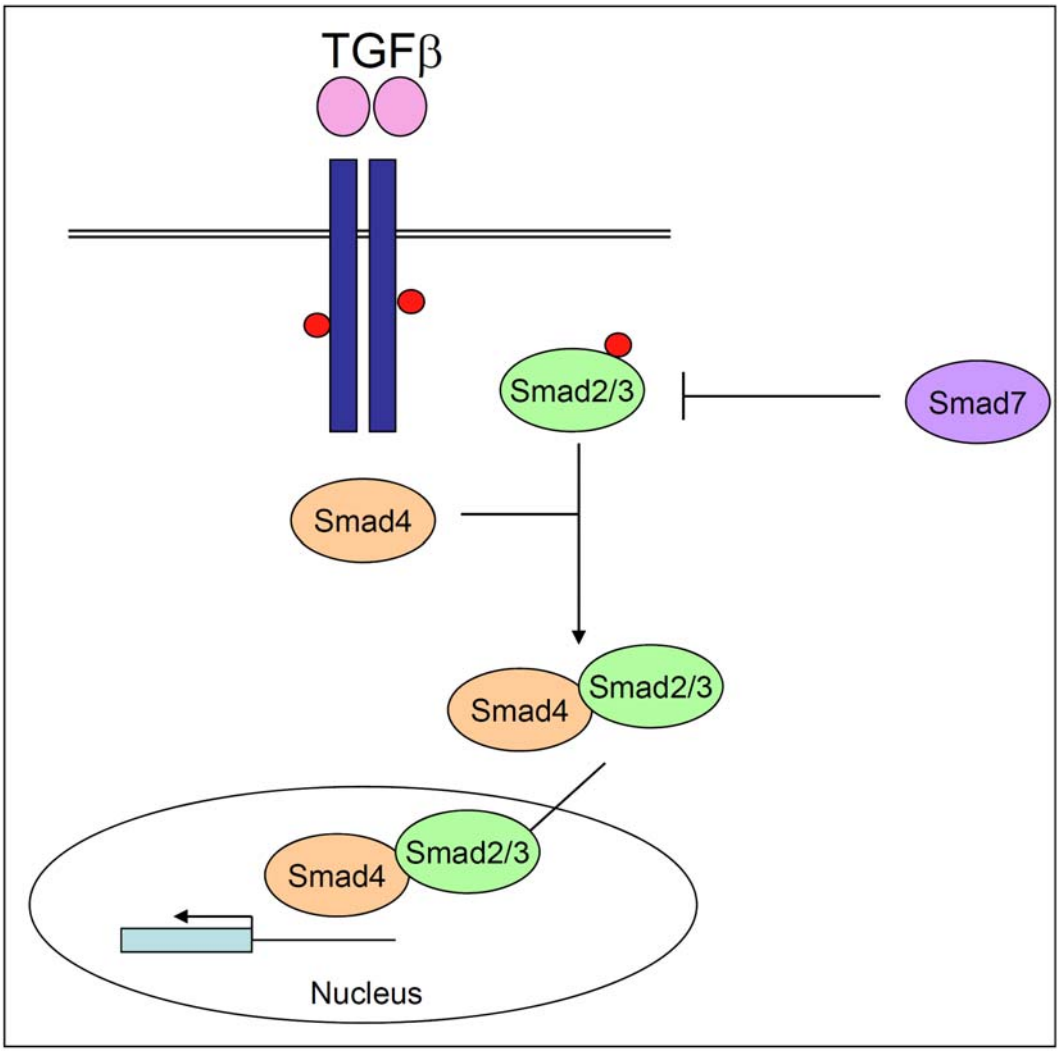

Figure 1.11: TGFβ signalling (Adapted from Massague, 1998).

TGFβ signalling involves a set of trans-membrane receptor serine/threonine kinases which activate Smad2 and Smad3. These Smads then form heteromeric complexes with Smad4 and translocate to the nucleus to target specific gene activation. Smad7 negatively regulates this signalling pathway via competition for phosphorylation by the activated receptor complex.

Deficiency of TGF-β1 in mice results in uncontrolled inflammation, with infiltration of inflammatory cells into vital organs and up-regulation of inflammatory cytokines such as TNFα and IL-1β, and death occurs within 3-4 weeks (Kulkarni *et al.,* 1993; McCartney-Francis *et al.,* 2004). Although the identity of a pathogen that is causing the initiation of the rampant inflammatory response in these mice is unknown, inflammation may be triggered by endogenous stimuli (McCartney-Francis *et al.,* 2004). Moreover, these mice were hypersensitive to LPS, with 50% of *Tgf-βI*$^{-/-}$ mice failing to survive 12hrs after LPS challenge, and 90% by 24hrs (Kulkarni *et al.,* 1993; McCartney-Francis *et al.,* 2004). These mice had increased NF-κB activation and TLR4 expression, the latter being detected 3 days after birth. As this TLR4 up-regulation was initiated so early, it may have predisposed these mice to aberrant activation of the TLR4 pathway by normal gut flora (McCartney-Francis *et al.,* 2004). Furthermore, a transgenic mouse model was developed which lacked TGF-β signalling in the intestine due to expression of a dominant negative form of TGF-β type II receptor under the control of an intestinal promoter (Hahm *et al.,* 2001). These mice acquired spontaneous colitis and had increased susceptibility to dextran sodium sulphate (DSS)-induced IBD. Moreover, inhibiting the over-expression of Smad7 in the T cells and mucosa of IBD patients restores the ability of TGF-β1 to signal, thus down-regulating this inflammatory response (Monteleone *et al.,* 2001). SOCS3 has also been shown to inhibit TGF-β1/Smad3 signalling in macrophages (Liu *et al.,* 2008). In addition, TGF-β1 can deactivate macrophages by reducing their release of hydrogen peroxide (Tsunawaki *et al.,* 1988) and nitric oxide (Ding *et al.,* 1990). It also reduces the cytotoxic activity of macrophages (Nelson *et al.,* 1991) and their production of TNFα and IL-1β (Espevik *et al.,* 1987; Bogdan *et al.,* 1992). Moreover, when administered to rats, TGF-β1 arrested LPS-induced hypotension and decreased mortality (Perrella *et al.,* 1996).

Although the exact mechanism by which TGF-β1 modulates TLR4 responses is still unknown, TGF-β can induce MyD88 ubiquitination and degradation by the proteasome, thus inhibiting TLR4 mediated signalling (reviewed in, Watters *et al.,* 2007). Additional research has shown that TGF-β1 can also down-regulate NF-κB by post-translational modifications of the IκBα protein in epithelial cells (Arsura *et al.,* 1997). Furthermore, it has been shown

that TGF-β1 can inhibit LPS-induced activity of JNK in mouse macrophages (Imai *et al.,* 2000), as well as down-regulate activation protein 1 and CD14 receptor expression (Imai *et al.,* 2000).

Despite the overwhelming anti-inflammatory role TGF-β1 plays during the inflammatory response, there has been evidence of it behaving in a pro-inflammatory manner. For instance, transgenic mice which express TGF-β1 in the liver are more susceptible to endotoxemia (Vodovotz *et al.,* 1998), and transgenic mice with hepatocyte-specific TGF-β1 over-expression have increased inflammatory cytokine expression and mortality rates following LPS administration when compared to wild-type controls (Garcia-Lazaro *et al.,* 2005).

Although TGF-β1 is induced in patients with sepsis (Marie *et al.,* 1996), its clinical use in the treatment of sepsis has had limited success (reviewed in, Rittirsch *et al.,* 2008).

1.5 Implications of TLR pathway components during human sepsis

TLR signalling components themselves have been implicated in the excessive pro-inflammatory mediator production during human sepsis. For example, during disease, danger associated molecular patterns (DAMPs) from the invading micro-organisms activate the immune response and consequential up-regulation of TLR expression (reviewed in, Mollen *et al.,* 2006; Uematsu, Akira, 2007; Nduka, Parrillo, 2009) leading to an exaggerated inflammatory response and the above-mentioned tissue damage. The degree of NF-κB activation has been associated with disease severity in patients with septic shock (Arcaroli *et al.,* 2006; Nduka & Parrillo, 2009). Furthermore, it has recently been discovered that polymorphisms in the human Mal allele were associated with the pathogenesis of sepsis (Ferwerda *et al.,* 2009). Polymorphisms in TLR4 have also been associated with sepsis and Gram-negative infections in humans (Feterowski *et al.,* 2003). Previous research has shown the potential to target TLR signalling components during sepsis. For example; curcumin, auranofin, cinnamaldehyde and acrolein all prevent TLR homodimerisation (Youn *et al.,* 2006; Lee *et al.,* 2008; Youn *et al.,* 2008). Molecules that inhibit the binding of MyD88 to TLR are also emerging which have shown to inhibit LPS-mediated TLR4 signalling (Lee *et al.,* 2007), as well as cell penetrating peptides that attach to BB loop sequences on TLR4 to

inhibit LPS signalling (Toshchakov & Vogel, 2007). TLR antagonists are currently undergoing clinical trials for the treatment of sepsis, however certain studies in humans have not shown benefits, possibly due to the increase in sepsis cases stemming from Gram-positive or fungal causes (reviewed in, Rittirsch *et al.,* 2008). Ideally, a partial agonist for TLR4 could be used during Gram-negative sepsis, rather than blocking the entire signalling pathway, so that the remaining signalling would provide protective immunity towards infection (reviewed in, O'Neill *et al.,* 2009). For instance, TAK-242 (alkyl 6-(N-substituted sulfamoyl) cyclohex-1-ene-1-carboxylate), a small-molecule inhibitor of protein-protein interactions has been shown to selectively inhibit TLR4-mediated TNFα, IL-6 and IL-12 production possibly by targeting MD2 (Ii *et al.,* 2006), indicating its potential as a therapy for inflammatory disease. However, successful treatment has been limited by differences in individual's genetics and responses to infection (reviewed in, Cooke, Hill, 2001) and further research into this area is required.

The components of TLR pathways, in addition to the pro-inflammatory cytokines they produce (mentioned above), have devastating consequences in the case of inflammatory diseases such as septic shock. Understanding more thoroughly the molecular pathways involved in this process and the actions of the production of cytokines downstream of TLRs is of primary importance. Due to the limited success with treatments targeting TNFα during septic shock, this places emphasis on the need to further research into other pro-inflammatory cytokines and their impacts during inflammatory disease. One such cytokine is IL-6, as it is a major marker used to identify sepsis in patients (Hack *et al.,* 1989).

1.6 The pro-inflammatory cytokine; IL-6

IL-6 is a prototypical inflammatory cytokine that is produced in response to all TLRs, especially TLR4. IL-6 is a monomeric cytokine that is approximately 20kD in size and is produced by various cells including T cells, B cells, monocytes/macrophages, fibroblasts and endothelial cells (reviewed in, Heinrich *et al.,* 1998). IL-6 was initially discovered as a molecule that initiated B cell differentiation into antibody-producing plasma cells, and in addition it was shown to mediate proliferation and differentiation signals of T cells (Hirano *et al.,* 1986; Suda *et al.,* 1988). IL-6 has a wide spectrum of biological functions such as promoting acute phase protein synthesis in the liver and notably has been shown

to play a role in immune regulation, haematopoiesis and oncogenesis (Muraguchi *et al.,* 1981; Hirano *et al.,* 1985; Ikebuchi *et al.,* 1987; Kawano *et al.,* 1988; Lotz *et al.,* 1988; Hirano *et al.,* 1997; Ito, R. *et al.,* 1997; Smith *et al.,* 2001).

IL-6 belongs to a family of cytokines which includes, IL-11, IL-27, ciliary neurotrophic factor, cardiotrophin, leukaemia inhibitory factor (LIF) and oncostatin M (reviewed in, Heinrich *et al.,* 1998) (Fig. 1.12). These cytokines all feature a similar tertiary structure which is made up of a conserved four α-helical bundle, as in section 1.1.1. In addition, the IL-6 family members all signal via the gp130 receptor subunit, which largely accounts for their similar and redundant physiological responses, the most widely studied of which is IL-6 (reviewed in, Heinrich *et al.,* 1998).

1.6.1 IL-6 signalling

The key receptor molecule utilised during IL-6 signalling is gp130, a ubiquitously-expressed 130kD type 1 cytokine receptor that consists of an extracellular region, a cytoplasmic domain and a single transmembrane segment (reviewed in, Heinrich *et al.,* 1998; Muller-Newen, 2003) (Fig. 1.13). The extracellular domain of gp130 consists of the cytokine-binding homology region which comprises of five fibronectin type III domains, and the N-terminal immunoglobulin-like domain. The CHR is made up of two of the fibronectin type III domains and contains the four conserved cysteines and the WSXWS motif (see section 1.1.1) (Bazan, 1990; Kishimoto *et al.,* 1995; Chow *et al.,* 2002) (Fig. 1.13). These cytokines signal via receptor complexes containing gp130 homodimers or heterodimers. For instance, IL-6 and IL-11 signal via a gp130 homodimer, whereas LIF signals via the LIF receptor/gp130 heterodimer complex (reviewed in, Heinrich *et al.,* 1998) (Fig. 1.12). IL-6/gp130 signalling is initiated upon binding of IL-6 to its specific IL-6 receptor α-subunit (IL-6Rα) on target cells with low affinity. IL-6Rα is comprised of a transmembrane domain, a signal peptide, an extra-cellular region and a short cytoplasmic tail (Varghese *et al.,* 2002) (Fig. 1.13). The association of the membrane-bound gp130 signal transducing β subunit and IL-6R/IL-6 constitutes a high affinity hexameric receptor complex (Varghese *et al.,* 2002) consisting of duplicate gp130, IL-6 and

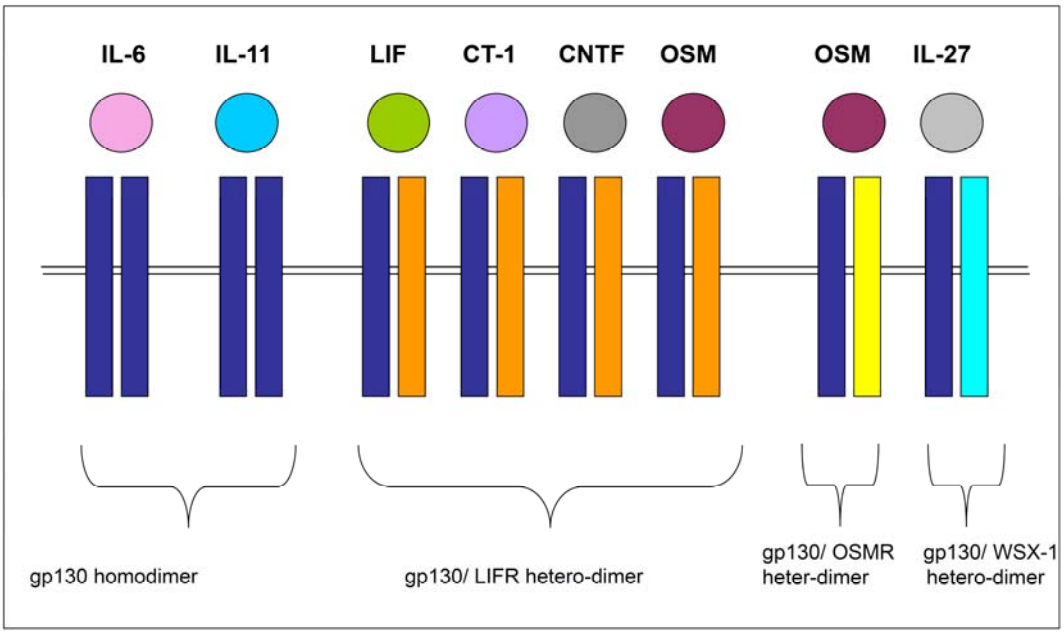

Figure 1.12: IL-6 family of cytokines (Adapted from Heinrich *et al*., 1998). The IL-6 family of cytokines all signal via the gp130 receptor subunit. This can be as a homodimer, in the case of IL-6 and IL-11, or as a heterodimer. LIF, CT-1, CNTF and OSM all signal via the gp130 and LIFR subunits. OSM utilises gp130 in combination with OSM receptor, and IL-27 signals via gp130 and WSX-1 receptor complex.

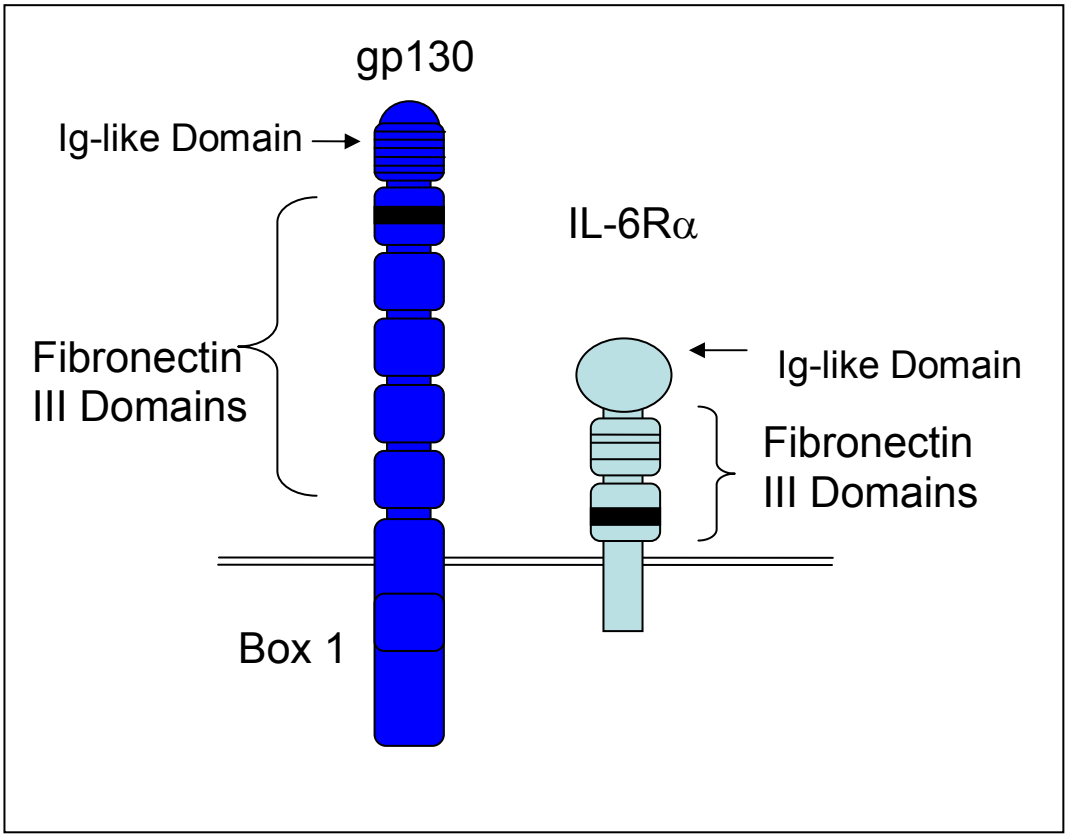

Figure 1.13: gp130 and IL-6 structures (Kishimoto *et al*., 1995).

The IL-6Rα consists of an Ig-like domain which recognises IL-6 and a cytokine homology binding region (CHR) which is composed of 2 fibronectin modules, which contain conserved cysteine residues (black lines) and a WSXWS motif (black box) and a short intracellular tail. The gp130 receptor also contains a CHR which comprises of 5 fibronectin domains, an Ig-like domain and a intra-cellular Box-1 motif which binds to Jaks.

IL-6Rα molecules, and this structure was confirmed from crystallisation studies (Boulanger *et al.*, 2003).

Due to gp130 lacking any intrinsic tyrosine activity, gp130 utilises its cytoplasmic 'Box-1' proline-rich motif to bind to Jaks, which are constitutively associated with gp130 (Tanner *et al.,* 1995; Muller-Newen, 2003) (Fig. 1.13). Binding to gp130 activates Jak1 and Jak2, which then dimerise and trans-phosphorylate each other and consequently tyrosine phosphorylate the receptor cytoplasmic domain to create docking sites for SH2 domain-containing signalling proteins (reviewed in, Hirano *et al.,* 1997; Heinrich *et al.,* 1998). In particular, these include STAT1 and STAT3 which bind to the 2 or 4 C-terminal tyrosine residues (Y), respectively (Fig. 1.14). Hence, IL-6 predominantly activates STAT3 and to a lesser extent STAT1 (reviewed in, Stahl *et al.,* 1995; Gerhartz *et al.,* 1996; Kuropatwinski *et al.,* 1997; Heinrich *et al.,* 1998; Heinrich *et al.,* 2003). The STATs are then tyrosine phosphorylated (Fig. 1.14) by the Jaks, and STATs then form heterodimers or homodimers in the cytoplasm and translocate to the nucleus to induce target gene expression (reviewed in, Heinrich *et al.,* 1998; Bild *et al.,* 2002; Heinrich *et al.,* 2003) (Fig. 1.14). STAT3 induces anti-apoptotic and pro-proliferative genes, whereas STAT1 target genes are pro-apoptotic and anti-proliferative (reviewed in, Regis *et al.,* 2008). STAT3 target genes include STAT3, SOCS3, B-cell lymphoma-extra large (Bcl-xl), B-cell lymphoma 2 and vascular endothelial growth factor, whereas STAT1 target genes include STAT1, SOCS1, the anti-viral gene OASA, IP10 and IFN regulatory factor 1 (IRF1) (reviewed in, Regis *et al.,* 2008). Serine phosphorylation of STAT3 and STAT1 is thought to occur in the nucleus and enhances the transcriptional potency and DNA binding capacity of these dimers (Wen *et al.,* 1995; Heinrich *et al.,* 2003).

The membrane proximal pY_{757} residue in gp130 also acts as a binding site for the SH2 domain containing protein-tyrosine phosphatase (SHP2) (Stahl *et al.,* 1995). SHP2 is tyrosine phosphorylated by the Jaks (Schaper *et al.,* 1998), following which it binds the growth factor receptor bound protein 2 (GRB) 2 (Fukada *et al.,* 1996) and GRB-associated binding protein (GAB1) adaptor molecules (Takahashi-Tezuka *et al.,* 1998) which ultimately facilitate the activation of the RAS/Erk/MAPK pathway (Bennett *et al.,* 1994; Li *et al.,* 1994; Takahashi-Tezuka *et al.,* 1998; Heinrich *et al.,* 2003) (Fig. 1.15). The PI3K/AKT

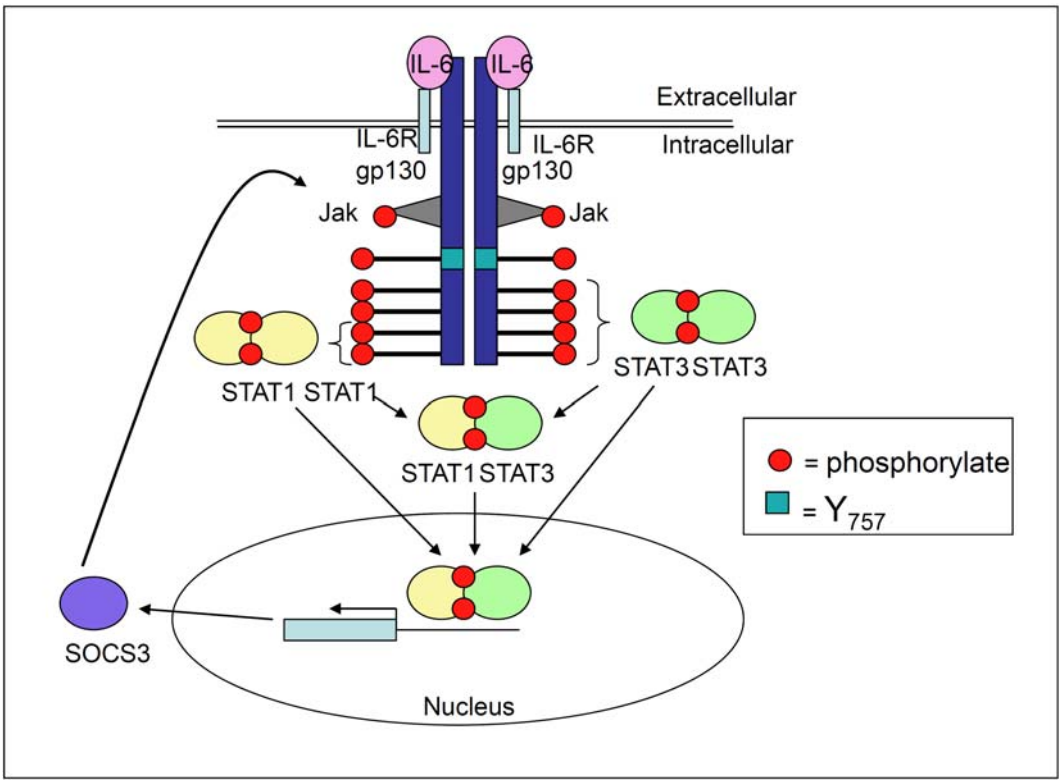

Figure 1.14: IL-6/STAT signalling cascades (Adapted from Heinrich *et al*., 1998).

IL-6 coupled with the IL-6Rα promotes gp130 homodimerisation which results in the trans-phosphorylation of cytoplasmic Jaks. The cytoplasmic tyrosine residues then become phosphorylated and 4 of these residues create docking sites for STAT3 and 2 of them, STAT1. The STATs are then phosphorylated and form hetero- or homo-dimers, translocate to the nucleus, and bind specific gene promoter sequences to induce gene transcription.

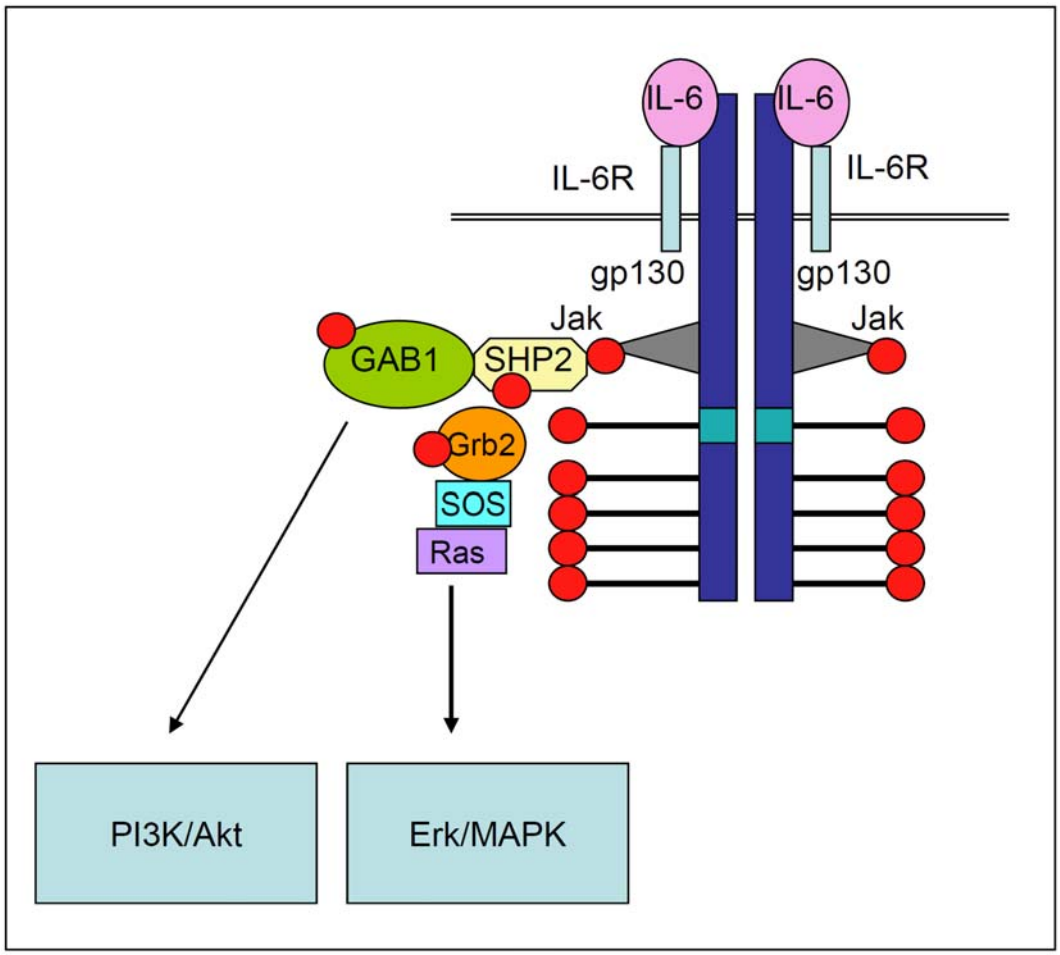

Figure 1.15: Erk/MAPK and PI3/Akt pathways (Adapted from Takahashi-tezuka *et al*., 1998).

SHP2 binds to the Y757 region of the gp130 cytoplasmic domain and associates with GAB1. This then activates the PI3K/Akt pathway which is predominately involved in promoting cell proliferation and survival by anti-apoptosis actions. The Erk/MAPK is activated by the interaction of SHP2 and GRB2, SOCS and RAS, which is involved in promoting cell proliferation and survival and pro-proliferative actions.

pathway is also activated by IL-6, and is likely to involve GAB1 interacting with PI3K (Takahashi-Tezuka *et al.,* 1998; Heinrich *et al.,* 2003) (Fig. 1.15).

1.6.2 IL-6 and inflammatory disorders, including endotoxic shock

A pro-inflammatory role for IL-6 has been associated with many inflammatory diseases, including sepsis (Hack *et al.,* 1989), IBD/colitis (Atreya *et al.,* 2000) and RA (Hirano *et al.,* 1988). For instance, IL-6 is significantly increased above normal levels of healthy individuals in the serum of patients with sepsis (Hack *et al.,* 1989). IL-6 mRNA levels were higher in IBD patient gut biopsies than in controls (Stevens *et al.,* 1992) and IL-6 is elevated in sera and intestinal tissues of CD patients (Gross *et al.,* 1992). In accordance with the research indicating IL-6 is pro-inflammatory, targeting IL-6 signalling in humans with humanised anti-IL-6R antibody has been beneficial for many inflammatory diseases such as RA, Castleman's disease and possibly CD (Wendling *et al.,* 1993; Nishimoto *et al.,* 2000; Ito *et al.,* 2004; Nishimoto, Kishimoto, 2004).

The pro-inflammatory role for IL-6 has also been supported by several animal models for these inflammatory conditions. For example, IL-6-deficient mice *(IL-6$^{-/-}$)* are resistant to experimentally-induced colitis and arthritis (Alonzi *et al.,* 1998; Gay *et al.,* 2006; Nowell *et al.,* 2009). In mice, experimentally-induced RA and IBD are attenuated by abrogating IL-6 signalling with a neutralising antibody against the IL-6R (Takagi *et al.,* 1998; Atreya *et al.,* 2000).

Despite the above studies indicating a pro-inflammatory role for IL-6, research has also indicated that IL-6 plays an anti-inflammatory role. For instance, levels of IL-1 receptor antagonist (which blocks IL-1α and IL-1β) and TNF antagonists (soluble TNF receptor, which blocks TNFα) in the plasma were increased in cancer patients receiving recombinant IL-6 administration (Tilg *et al.,* 1994). In addition, *IL-6$^{-/-}$* mice suffer an increased mortality rate in response to endotoxin, further supporting its role as anti-inflammatory (Xing *et al.,* 1998).

A non-essential role during LPS-induced inflammation has also been assigned to IL-6, since *IL-6$^{-/-}$* mice display similar acute phase responses to wild-type mice following LPS administration (Fattori *et al.,* 1994).

The diverse portfolio and often opposing roles of IL-6 during the inflammatory response might be explained, at least in part, by its ability to initiate 2 modes of signalling: "classical" signalling via the interaction of IL-6 with its

membrane-bound IL-6Rα subunit (reviewed in, Heinrich *et al.,* 2003), and "trans-signalling" via a naturally-occurring soluble (s) IL-6Rα (reviewed in, Jones b *et al.,* 2005).

1.6.3 IL-6 trans-signalling

In addition to classical IL-6 signalling, trans-signalling is a mechanism utilised by IL-6 to increase its repertoire of cellular targets, largely due to the restricted expression of membrane-bound IL-6Rα primarily on hepatocytes, monocytes, macrophages and some lymphocytes (reviewed in, Taga, Kishimoto, 1997; Jones b *et al.,* 2005; Rose-John *et al.,* 2006). Trans-signalling functions via a naturally occurring soluble form of the IL-6Rα (sIL-6R) which binds to IL-6 and then signals via gp130 as per conventional signalling described above in section 1.6.1 (reviewed in, Jones, Rose-John, 2002) (Fig. 1.16). There are two mechanisms that control the production of sIL-6R; alternatively spliced mRNA and proteolytic cleavage of a membrane anchored protein at a site that is close to the cell surface (Lust *et al.,* 1992; Rose-John, Heinrich, 1994; Althoff *et al.,* 2000; Mullberg *et al.,* 2000; Althoff *et al.,* 2001). This cleavage is thought to be undertaken by ADAM metallopeptidase domain 10 (ADAM10) and ADAM17 (Matthews *et al.,* 2003; Chalaris *et al.,* 2007). Many cell types such as embryonic stem cells (reviewed in, Rose-John, 2002; Humphrey *et al.,* 2004), various neural cells (Marz *et al.,* 1998; Marz *et al.,* 1999), early haematopoietic progenitor cells (Peters *et al.,* 1997; Peters *et al.,* 1998), T cells (Atreya *et al.,* 2000), smooth muscle cells (Klouche *et al.,* 1999) and endothelial cells (Romano *et al.,* 1997) are only responsive to IL-6 when the sIL-6R is present.

A negative regulator of this trans-signalling process is soluble gp130 (sgp130), which has been shown to exclusively target IL-6 responses driven by the sIL-6R and does not inhibit responses via classical IL-6R signalling (Narazaki *et al.,* 1993; Murakami-Mori *et al.,* 1996; Montero-Julian *et al.,* 1997; Jones b *et al.,* 2005) (Fig. 1.16). The molecular basis for this selective inhibition is that IL-6 alone doesn't bind to gp130 in its membrane bound or soluble form, rather it associates with IL-6R first (Jostock *et al.,* 2001; Rose-John, Neurath, 2004) (Fig. 1.16). Sgp130 has been detected in the circulation and is generated by mRNA splicing, which produces different splice variants, each of which are thought to have different functions (Tanaka *et al.,* 2000). There are at least 3 forms of sgp130

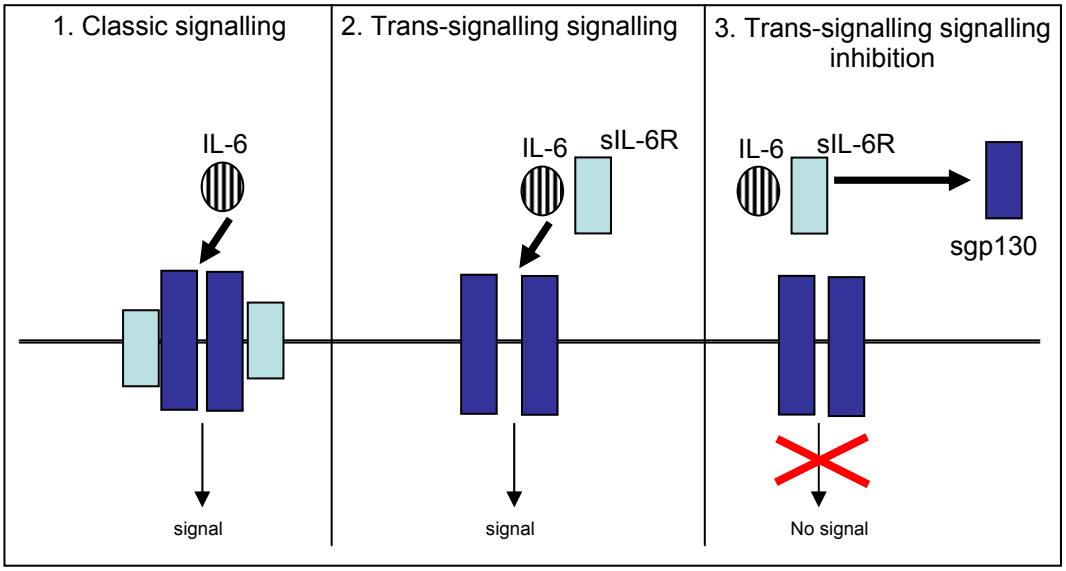

Figure 1.16: IL-6 trans-signalling (Adapted from Rose-John *et al*, 2003). Trans-signalling functions via a naturally-occurring soluble form of the IL-6R (sIL-6R) which binds to IL-6 and then signals via gp130 (2) as per classical signalling (1). A negative regulator of this trans-signalling process is soluble gp130, which has been shown to exclusively target IL-6 responses (3) driven by the sIL-6R and does not inhibit responses via membrane bound (classical) IL-6R signalling.

that have been identified, weighing 50, 90 and 110kD respectively (Narazaki *et al.,* 1993; Zhang *et al.,* 1998). Their roles during disease are unknown, however, but they are thought to regulate IL-6 signalling (Narazaki *et al.,* 1993). For example, one of these variants, a 50kD protein labelled gp130-RAPS (gp130 of the rheumatoid arthritis antigenic peptide-bearing soluble form) with a unique amino acid sequence, Asn-Ile-Ala-Ser-Phe in its COOH-terminus, is thought to be an auto-antigen in RA, which has an inhibitory effect on IL-6, and auto-antibodies to it play a role in the progression of RA (Tanaka *et al.,* 2000).

During inflammation, IL-6 trans-signalling has been shown to orchestrate leukocyte recruitment, activation and apoptotic clearance (Jones a, 2005). In addition, IL-6 trans-signalling has been linked to the induction of the fever response which involves increased L-selectin-induced leukocyte adhesion to tissue sections (Chen *et al.,* 2004). Specifically, human peripheral blood leukocytes incubated in the presence of sgp130 prevented thermal activation of L-selectin-dependent adhesion (Chen *et al.,* 2004). Interestingly, it has been shown that there is an increased amount of sIL-6R in the synovial fluid of rheumatoid and juvenile arthritis patients, which correlates with increased disease incidence (Kotake *et al.,* 1996; Desgeorges *et al.,* 1997). This process is thought to be directed via STAT3, as shown by Nowell, Williams (2009) using the *gp130*$^{F/F}$ mouse model (see below in section 1.8 for a detailed description).

IL-6 trans-signalling has also been implicated in the pathogenesis of experimentally-induced inflammation models, such as peritonitis and colitis (Atreya *et al.,* 2000; Hurst *et al.,* 2001). For instance, treatment with sgp130 suppressed colitis activity in animal models of chronic intestinal inflammation associated with induced apoptosis of T cells of the lamina propria in the colons of mice (Atreya *et al.,* 2000). In the case of peritonitis, it is thought that sIL-6R plays a role in controlling the pattern of leukocyte recruitment during disease (Atreya *et al.,* 2000; Hurst *et al.,* 2001). Specifically, sIL-6R released from the neutrophil infiltrate (in the peritoneal cavity) regulates CXC and CC chemokine expression, which suppresses neutrophil recruitment and attracts mononuclear leukocytes which then results in the clearance of infection (Hurst *et al.,* 2001).

A consistent theme about trans-signalling is its proposed involvement in promoting the pathogenesis of inflammatory disease, which may therefore explain the paradox of IL-6 having both pro- and anti- inflammatory effects; this

alternative signalling pathway could potentially be responsible for the pro-inflammatory actions of IL-6. However, further research on the role of IL-6 trans-signalling during septic shock is required.

1.6.4 Negative regulators of IL-6 signalling

Based on the obvious disease associations with deregulated IL-6 production and signalling, it is perhaps not surprising that the tight regulation of IL-6 signalling is important in order to maintain homeostasis and prevent disease. Indeed, deregulated activation of gp130-dependent STAT3 activation has been linked with IBD (Alonzi *et al.,* 1998; Suzuki *et al.,* 2001) and various cancers (Bromberg, J. F. *et al.,* 1999; Buettner *et al.,* 2002; Yu, Jove, 2004) including those of haematopoietic and epithelial origin (Grandis *et al.,* 1998; Catlett-Falcone *et al.,* 1999), liver (Li *et al.,* 2006; Ogata *et al.,* 2006), prostate (Lou *et al.,* 2000; Gao *et al.,* 2005; Abdulghani *et al.,* 2008), cervical (Chen, C. L. *et al.,* 2007) and colon (Corvinus *et al.,* 2005), illustrating the importance of negative regulation of these pathways. To date, three main classes of negative regulators have been identified; SOCS, protein inhibitor of activated STAT (PIAS) proteins and tyrosine phosphatases (reviewed in, O'Shea, Watford, 2004).

SOCS3 behaves in a similar manner to SOCS1 utilising the ubiquitin-transferase system mentioned in section 1.3.6. SOCS3 is a STAT3 target gene and is predominantly induced by IL-6 family cytokines, following which it binds to gp130 at position Y_{757} (in mice, 759 in humans) in competition with SHP2 and prevents the phosphorylation of the receptor and STATs (reviewed in, Ilangumaran *et al.,* 2004) (Nicholson *et al.,* 2000) (Fig. 1.14). SOCS3 knock-out mice are embryonic lethal and die at mid-gestation due to placental insufficiency (Roberts *et al.,* 2001), therefore, the use of SOCS3 conditional knock-outs in a range of cells and organs including macrophages and liver have been created to study its role in response to cytokines (Croker *et al.,* 2003). As predicted, these SOCS3 conditional knock-outs display prolonged STAT3 activation, illustrating the importance of this regulatory molecule to down-modulate IL-6 signalling (Croker *et al.,* 2003). In addition, the deletion of the *Socs3* gene in haematopoietic and endothelial cells illustrated the critical role for SOCS3 in the homeostatic regulation of the inflammatory response (Croker *et al.,* 2003; Howard *et al.,* 2004). Interestingly, it has been shown that SOCS3 inhibits the activation of TRAF6 and

TAK1 following IL-1 stimulation in HEK293 cells (Frobose *et al.,* 2006), hence it could potentially have a negative regulatory effect on TLR signalling. In addition, ectopic expression of SOCS3 in macrophages has been shown to inhibit LPS mediated TNFα expression (Qasimi *et al.,* 2006).

The PIAS family of proteins consist of 4 members; PIAS1, PIAS3, PIASx and PIASγ, some of which are alternatively spliced to create isoforms with different functions (reviewed in, O'Shea, Watford, 2004) (Fig. 1.17). PIAS proteins inhibit specific STAT proteins by different modes. For instance, PIAS1 prevents STAT1-dependent signalling by blocking the DNA-binding activity of STAT1 (Liu *et al.,* 2004). These functions were discovered by the observation that PIAS-deficient mice have augmented antiviral and antimicrobial responses and therefore, a reduction in susceptibility to these pathogens (Liu *et al.,* 2004). These mice were also hypersensitive to LPS and an increase in specific IFN gene induction, however IL-6 gene induction was unaffected (Liu *et al.,* 2004). These studies illustrate that PIAS is a regulator of IFN signalling. Furthermore, PIAS3 inhibits STAT3 and STAT5 signalling by inhibiting STAT DNA binding in the nucleus (Chung *et al.,* 1997; O'Shea, Watford, 2004). This interaction was discovered by isolating PIAS3 (which bound to STAT3) and found that it specifically bound to and affected transcriptional activity of STAT3 and not STAT1 (Chung *et al.,* 1997). In contrast to PIAS1 and PIAS3, PIASy and PIASx inhibit their STATs (STAT1 and STAT4, respectively) by mechanisms other than by preventing DNA association (Liu *et al.,* 2001; Arora *et al.,* 2003). For example, PIASx can regulate chromatin structure by recruitment of histone deacetylases (reviewed in, O'Shea, Watford, 2004).

The final group of negative regulators are cytosolic and membrane bound tyrosine phosphatases which inhibit Jak activity; the SHP family (reviewed in, O'Shea, Watford, 2004). There are two members of the SHP family, SHP1 and SHP2, which are both comprised of N-terminal, SH2 domains and a C terminal protein-tyrosine phosphatase domain (reviewed in, Wormald, Hilton, 2004) (Fig. 1.17). Both SHP family members bind to phosphotyrosine residues of various cytokine receptors via their SH2 domains. For instance, SHP1 can associate with Jak2 in an SH2 domain-independent manner (Jiao *et al.,* 1996). SHP1 is expressed mainly in cells of the haematopoietic system, and mice deficient in SHP1 or with

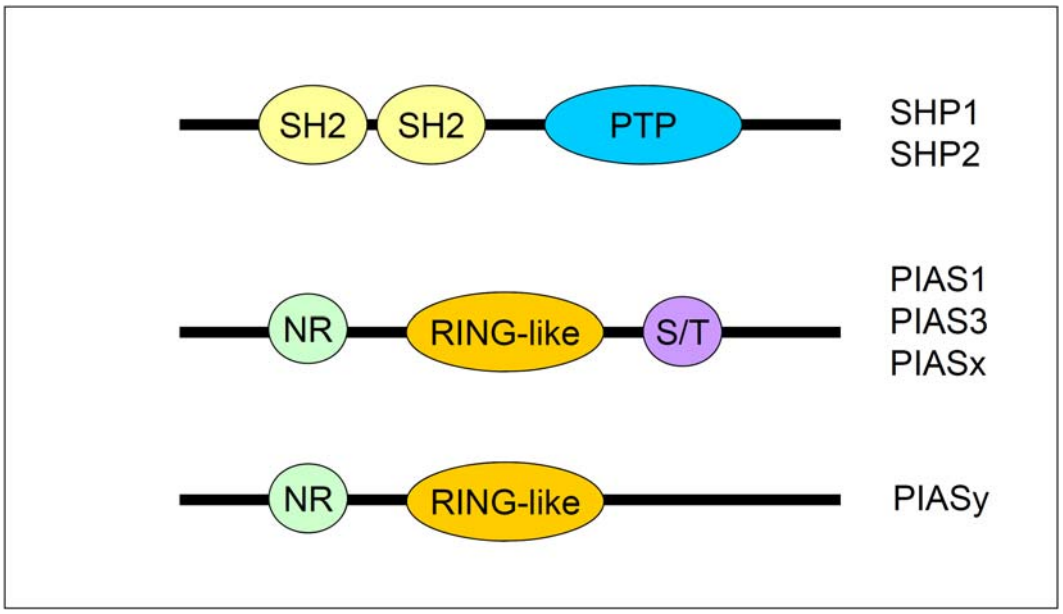

Figure 1.17: Domain structures of negative regulators of cytokine signalling (Adapted from Wormwald *et al*., 2004).

SHP1 and SHP2 are comprised of N-terminal and SH2 domains and a C terminal protein-tyrosine phosphatise domain (PTP*)*. PIASs are comprised of L*XX*LL nuclear receptor interaction motif (NR) and RING-like zinc binding domains. PIAS1, PIAS3, and PIASx contain a C-terminal serine/threonine-rich region that is not present in PIASy.

reduced SHP1 die as a result of severe inflammation (reviewed in, Neel *et al.,* 2003; Salmond, Alexander, 2006).

In contrast, SHP2 is a positive regulator of certain signalling such as Erk/MAPK and PI3K/AKT pathways (reviewed in, Neel *et al.,* 2003). Mice with a deletion in the exon 3 of the *Shp2* gene are embryonic lethal, as SHP2 has been shown to be necessary for efficient gastrulation and cardiac development (reviewed in, Neel *et al.,* 2003). Furthermore, SHP2 negatively regulates cytokine signalling, such as via the gp130 receptor (Symes *et al.,* 1997; De Souza *et al.,* 2002). SHP2 exerts this negative regulation by binding to Y_{757} in a similar manner to SOCS3, albeit with less efficiency (De Souza *et al.,* 2002). SHP2 also negatively regulates the IL-6 pathway by binding to Jaks and preventing their tyrosine phosphorylation, consequently inhibiting STAT activity (Yin *et al.,* 1997).

1.6.5 STATs

As described above, STAT proteins are vital factors involved in cytokine signalling, and comprise of a family of 7 latent transcription factors. These family members mediate intracellular signalling involved in development, cell growth, proliferation and apoptosis (reviewed in, Battle, Frank, 2002; Stephanou, Latchman, 2005). They are activated by intrinsic receptor tyrosine kinases, or by Jaks as discussed above (Ehret *et al.,* 2001). Over the last decade *in vitro* studies have shown that the same STAT molecule can be activated by multiple growth factors and cytokines, and specific cytokines can activate more than one STAT protein (Ehret *et al.,* 2001). For example, STAT1 is predominantly utilised by the pro-inflammatory type II IFN IFNγ, which signals via IFNγ receptor I and II (IFNGRI/ IFNGR2), as well as by IL-6 and type I IFNs. STAT3, however, is activated by IL-6, IL-10, IFNγ, type I IFNs, epidermal growth factor and platelet derived growth factor to name a few (Decker *et al.,* 1991; Lew *et al.,* 1991; Akira *et al.,* 1994; Schindler, Darnell, 1995). These STAT proteins were identified as critical mediators of virtually all cytokine-driven cellular responses (reviewed in, Bromberg & Darnell, 2000).

All STATs are initially latent before becoming activated/phosphorylated and translocate to the nucleus to activate certain DNA promoter sequences to induce gene expression (reviewed in, Heinrich *et al.,* 1998), including that of themselves as has been shown for STAT1 and STAT3 (Yang, *et al.,* 1998). The

translocation of STAT3 into the nucleus requires its binding to an importin-α-importin-β dimer to facilitate passage through the nuclear pore complex (reviewed in, Reich, 2009). The activation of STATs is transient, lasting only minutes to hrs before decreasing to basal levels (reviewed in, Bromberg & Darnell, 2000). After inducing gene transcription, STATs are de-phosphorylated and inactivated by nuclear tyrosine phosphatases, following which they return to the cytoplasm for successive activation (Haspel *et al.,* 1996). It has also been shown that STATs can dimerise in the absence of phosphorylation, and may possibly be capable of regulating pathways of signal transduction (Braunstein *et al.,* 2003). For instance, it has been shown that un-phosphoryalted STAT1 and STAT3 can act as transcription factors by binding to factors such as NF-κB (Ganster *et al.,* 2001; Yang, *et al.,* 2007).

STATs are comprised of an oligomerisation domain at the N terminus, a coiled-coil domain (4-helix bundle), the DNA binding domain (β-barrel), a linker domain (connector domain), the SH2 domain and the transactivation domain (Becker *et al.,* 1998; Chen, *et al.,* 1998; Heinrich *et al.,* 2003) (Fig. 1.18). The SH2 domain is responsible for the binding of STATs to phospho-tyrosine residues on other molecules as well as themselves. Indeed, the dimerisation of STATs is essential for DNA binding, which occurs at the DNA binding domain of the molecule, located centrally (amino acids 300-480) (Shuai *et al.,* 1994; Heinrich *et al.,* 1998). The transactivation domain of STATs at the C-terminus is partially regulated by serine phosphorylation, as mentioned previously, which is thought to be required for full transcriptional activity of STATs (reviewed in, Heinrich *et al.,* 1998). When this serine residue was mutated to an alanine on STAT1, transcriptional activity was significantly reduced by approximately 80% (Wen *et al.,* 1995). In addition, this serine to alanine mutation in STAT3 resulted in a substantial reduction in luciferase target gene expression driven by STAT3-binding sites, also indicating that phospho-serine is needed for maximal transcriptional activation (Wen *et al.,* 1995).

1.6.6 STAT1 and inflammation

STAT1 is primarily activated in response to IFNs, and to a lesser extent by IL-6 and its related family members (Deberry *et al.,* 1997; Heinrich *et al.,* 2003).

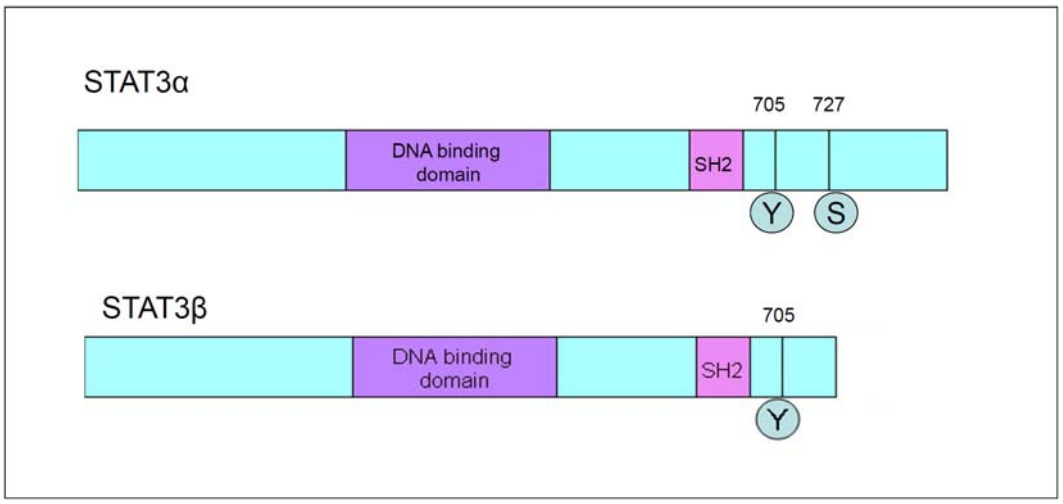

Figure 1.18: STAT3 isoforms (Adapted from Yoo *et al*., 2002).

The SH2 region of STAT3 binds to tyrosine residues, and DNA binding domain to promoter sequences. STAT3β lacks the serine 727 residue which is needed for serine phosphorylation and full transcriptional activity. The tyrosine 705 residue is present in both isoforms.

The importance of STAT1 for IFN biological function has been shown by *Stat1* knock-out mice, which are highly susceptible to viral disease due to defects in IFN-mediated STAT1 signalling (Durbin *et al.,* 1996). Furthermore, STAT1-deficiency in mice protects against LPS-driven endotoxic shock (Karaghiosoff *et al.,* 2003). Conversely, hyperactivation of STAT1 in *Socs1*$^{-/-}$ mice results in hypersensitivity to IFNγ, which in turn leads to increased resistance to viral infection (reviewed in, Yoshimura *et al.,* 2007). However, these mice are also hypersensitive to LPS and spontaneously develop systemic inflammation, which causes multi-organ failure and early death at 2-3 weeks of age (Naka *et al.,* 1998; Kinjyo *et al.,* 2002). Direct evidence for the role of excessive IFNγ signalling through STAT1 activation in driving these pathologies was provided by either the administration of anti-IFNγ antibodies to these mice or by crossing them onto an *Ifnγ*$^{-/-}$ background, which improves their survival (Alexander, 1999). A likely explanation for the hyper-inflammatory phenotype of these mice, at least in response to LPS, was the observation that macrophages from *Socs1*$^{-/-}$ mice produce elevated pro-inflammatory cytokines in response to LPS (Kinjyo *et al.,* 2002). Despite these observations revealing that STAT1 is pro-inflammatory, the role that STAT1 plays in transducing biological responses driven by the IL-6 family cytokines remains poorly understood.

1.6.7 STAT3 and inflammation

STAT3 is expressed in most cell types including lymphocytes, endothelial cells, mast cells, macrophages, neutrophils and dendritic cells (reviewed in, Heinrich *et al.,* 1998). STAT3 was originally discovered as an acute phase response factor, and has been shown to have a role in proliferation and anti-apoptotic functions and is a known oncogene (Alonzi *et al.,* 2001; Reich, 2009). This was confirmed by the use of a constitutively active STAT3 molecule (STAT3-C) which was created through cysteines inserted in the SH2 domain (reviewed in, Bromberg *et al.,* 1999). Consequently, this molecule spontaneously dimerises, accumulates in the nucleus and drives gene transcription. STAT3-C was shown to transform fibroblasts following transfection, and when these cells were injected into nude mice, they formed tumours (reviewed in, Bromberg *et al.,* 1999). STAT-C transformed cells also have increased amounts of *Bcl-*X_L mRNA,

supporting the role of STAT3 as anti-apoptotic (reviewed in, Bromberg *et al.,* 1999).

When it comes to inflammation, however, the role of STAT3 is controversial, as it has been shown to have anti- and pro-inflammatory roles, which has prompted the need for further research into this area. While genetic ablation of *Stat3* in mice leading to embryonic lethality demonstrates the essential role that STAT3 plays during embryogenesis (Takeda *et al.,* 1997), *Stat3* conditional knock-outs were necessary to assess the function of STAT3 in specific cell types and the immune system. Kano and colleagues have created a conditional knock out of STAT3 in endothelial cells, by using the Cre-*loxP* recombination system, which results in increased susceptibility to LPS-induced endotoxic shock in mice (Kano *et al.,* 2003). Furthermore, septic peritonitis is exacerbated by CLP in mice with conditional deletion of *Stat3* in macrophages and neutrophils (Matsukawa *et al.,* 2003). Moreover, macrophage-specific deletion of *Stat3* in mice spontaneously leads to chronic enterocolitis (Takeda *et al.,* 1999). In addition, the specific deletion of *Stat3* in haematopoietic progenitors (Welte *et al.,* 2003) in mice spontaneously leads to chronic intestinal inflammation similar to that observed in IL-10-deficient mice (Kuhn *et al.,* 1993). Collectively, these studies suggest an anti-inflammatory role for STAT3, which is due largely to its activation by the anti-inflammatory IL-10, discussed in section 1.4.1.

Persistent STAT3 tyrosine phosphorylation has been implicated in the pathogenesis of many human inflammatory diseases such as human ulcerative colitis (Suzuki *et al.,* 2001) Crohn's disease (Atreya *et al.,* 2000) and RA (Shouda *et al.,* 2001), suggesting that STAT3 can also be pro-inflammatory. Several experimentally-induced inflammatory mouse models also support these observations. Mice subjected to experimentally-induced colitis had a higher level of activated STAT3 in their colons and this correlated with disease severity (Suzuki *et al.,* 2001). In IL-6-deficient mice, this STAT activation was reduced, as was the degree of colitis, confirming the role of IL-6-induced STAT3 activation in this disease model. Furthermore, targeting IL-6 trans-signalling in mice counteracts the STAT3 control of experimental inflammatory arthritis (Nowell *et al.,* 2009). Moreover, when STAT3 phosphorylation is inhibited *in vivo* by 'stattic' (an inhibitor of STAT3 phosphorylation) in an experimental model of

sepsis, systemic inflammation is prevented, accompanied by an increase in survival (Pena *et al.,* 2010), indicating STAT3 is pro-inflammatory.

While these studies highlight a key pro-inflammatory role for STAT3 (activated via gp130-acting cytokines) in specific disease settings, as mentioned above, STAT3 has also been assigned an anti-inflammatory role which is invariably associated with IL-10 signalling. In this regard, IL-6 signals via STAT1 and STAT3 heterodimers and homodimers, whereas IL-10 predominantly signals via a STAT3 homodimer (Kotenko *et al.,* 1997; Moore *et al.,* 2001). Considering the pro-inflammatory nature of STAT1, the relative balance of STAT1 (pro-inflammatory) and STAT3 (anti- or pro-inflammatory) activation by IL-6, which may depend on the cellular context (Haan *et al.,* 2005), is therefore likely to impact on the overall outcome of its role during inflammation. To illustrate the interchangeable nature of these signalling molecules, a study which genetically ablated *Stat3* in mouse embryonic fibroblasts (MEFs) illustrated that IL-6 was able to mediate an IFN-type response as indicated by up-regulation of MHC, increased anti-viral activity and augmented IFN-specific target genes mediated by STAT1 (Costa-Pereira *et al.,* 2002). As well as IL-6 being able to switch to an IFN response (mediated by STAT1), IFN signalling can also switch to an IL-6 type response (mediated by STAT3). MEFs and bone marrow macrophages (BMMs) deficient for STAT1, when stimulated with IFN, were able to induce a STAT3-type response, suggested by increased proliferation and augmented STAT3 gene induction (Qing, Stark, 2004). These studies demonstrate how cytokines (such as IL-6 and IFNγ) can utilise the same signalling molecules, yet exhibit opposing inflammatory outcomes. In addition, the kinetics of STAT3 activation can also influence whether its effects are pro- or anti-inflammatory. For instance, when STAT3 is up-regulated by deleting SOCS3 in macrophages, the IL-6 response becomes anti-inflammatory (Yasukawa *et al.,* 2003) because STAT3 activation is sustained comparable to that seen following IL-10 stimulation (Yasukawa *et al.,* 2003).

Another possible explanation for the opposing roles of STAT3 during inflammation may be provided by the fact that STAT3 has two isoforms, STAT3α (full-length) and STAT3β (truncated) (Schaefer *et al.,* 1995; Caldenhoven *et al.,* 1996). STAT3β is generated by an alternative splicing event leading to 7 distinct residues replacing the C-terminal 55 amino acids (Yoo *et al.,* 2002) (Fig. 1.18).

These STAT3α and STAT3β isoforms can form homodimers or heterodimers with each other or with STAT1 in transfected cells (Schaefer *et al.,* 1995; Caldenhoven *et al.,* 1996). The homodimers bind identical DNA sites, however STAT3β dimers are slightly more stable and therefore demonstrate greater overall DNA binding capacity than STAT3α (Park *et al.,* 2000). In contrast to the conditional *Stat3* deletion models in which both isoforms of *Stat3* are deleted, mice engineered to lack *Stat3β* only are hypersensitive to endotoxin-induced inflammation in the liver and have reduced recovery from endotoxic shock, indicating STAT3β has anti-inflammatory properties (Yoo *et al.,* 2002). Conversely, *Stat3α* knock-out mice die within 24hrs of birth, indicating its role in post-natal function (Yoo *et al.,* 2002). Therefore, the relative amount of STAT3α and STAT3β present in specific cell types may impact on the overall STAT3-associated inflammatory outcome (Maritano *et al.,* 2004).

More recently, another molecular explanation for the opposing pro- and anti-inflammatory roles of STAT3 has been provided. Whilst STAT3 transcriptional activity has classically been associated with tyrosine phosphorylation, emerging evidence has suggested that non-tyrosine phosphorylated STAT3 may also behave as a transcription factor after IL-6 stimulation (Yang *et al.,* 2007). Indeed, a recent study by Yang *et al.* (2007) has revealed that following STAT3 activation in response to gp130-acting cytokines, non-tyrosine-phosphorylated STAT3 co-operates with NF-κB to bind to the promoter of pro-inflammatory genes such as IL-8, RANTES, IL-6, MET and MRAS to induce their transcription. This implies that the levels of un-phosphorylated STAT3 are important in determining the cellular response during inflammation.

Overall, studies on the role of STAT3 during inflammation are conflicting, prompting the need for further investigation. A possible avenue of research that could help explain these inconsistencies is cross-talk between STAT3 and other pathways.

1.7 Cross-talk between Jak/STAT and NF-κB pathways

As mentioned above, it has been shown that unphosphorylated STAT3 (U-STAT3) can interact with NF-κB components to initiate pro-inflammatory gene

transcription of genes with κB elements (Yang *et al.,* 2007). This interaction has also been previously shown by Yoshida (*et al.,* 2004) where they reported that U-STAT3 forms a complex with the p65 subunit of phosphorylated NF-κB on the κB sequence in the human IL-8 promoter. This complex formed in response to IL-1β and is dependant upon signal transduction through the carboxyl terminus of TRAF6 (Yoshida *et al.,* 2004). Moreover, it was shown that following stimulation with IL-1β and IL-6, pY-STAT3 and phophorylated p65 can form a complex and STAT3 interacts with nonconsensus sequences at the 3' boundary of NF-κB elements of the serum amyloid A gene (Hagihara *et al.,* 2005).

A more specific link between STATs and TLR4-initiated NF-κB signalling pathways has also been established. Previous research has observed that STAT1 serine phosphorylation is induced by TLR2 and 4 signalling in macrophages within 30 minutes of stimulation (Rhee *et al.,* 2003), suggesting the involvement of an unknown yet direct mechanism distinct from the IFN/Jak pathway that causes this phosphorylation. While this indicates possible cross-talk between STAT1 and TLR signalling during the inflammatory response, this area of research is poorly developed.

With respect to STAT3, LPS has been shown to induce STAT3 phosphorylation and SOCS3 expression in the liver and hypothalamus in a MyD88-dependant manner (Yamawaki *et al.,* 2010). Furthermore, blocking STAT3 activity inhibits LPS-mediated IL-1β and IL-6 production in RAW264.7 (mouse leukaemic monocyte macrophage cell line) cells (Samavati *et al.,* 2009), yet another example of the potential cross-talk between STAT3 and the TLR4/NF-κB pathway. Moreover, the systemic inhibition of Jak kinases decreased LPS-mediated acute lung injury (Severgnini *et al.,* 2005), systemic cytokine production and mortality. Furthermore, global over-expression of SOCS3 *in vivo,* either via gene delivery (Fang *et al.,* 2005) or intracellular protein delivery (Jo *et al.,* 2005), protects mice against LPS challenge. In addition, STAT3 has been shown to modulate the p65RelA/p50NF-κB pathway because when STAT3 phosphorylation is inhibited in peritoneal macrophages from mice (transfected with the NF-κB gene reporter), the LPS-mediated induction of NF-κB activation is reduced (Pena *et al.,* 2010). It is also noteworthy that in THP1 (human acute monocytic leukemia cell line) cells pretreated with an inhibitor of STAT3

phosphorylation, decreased levels of p65, p50, p52, Rel-B and c-Rel in nuclear extracts following LPS stimulation were observed (Pena *et al.,* 2010).

The ability of STAT1 and NF-κB to interact has been shown from studies that indicate that when IFNγ and TNFα work in synergy to initiate inflammation, they require STAT1α and NF-κB to co-operate (Ohmori *et al.,* 1997). Although no direct interaction between STAT1 and NF-κB was observed, the authors suggested a weak interaction *in vivo* was possible. Other studies have indicated that STAT1 and NF-κB operate in a cell specific and complex manner in the regulation of the hiNOS gene (Ganster *et al.,* 2001). Specifically, it was shown that in STAT1 mutant fibroblasts, over-expression of NF-κB activates hiNOS promoter-reporter expression, indicating that STAT1 inhibits NF-κB function in these cells. (Ganster *et al.,* 2001). It is thought that STAT1 and NF-κB possibly bind in a protein-protein-DNA complex (Ganster *et al.,* 2001). Consequently, the levels of STAT1 could be important in the regulation of gene expression levels during inflammation.

Collectively, these studies indicate a potential cross-talk between STATs and the TLR4-driven NF-κB signalling pathway, however, the exact mechanisms involved are unclear. This cross-talk could underlie the augmented pro-inflammatory cytokine production evident during septic shock. Therefore, to investigate whether deregulated STAT activation augments TLR4-driven inflammatory responses, the mice described below were used.

1.8 Genetic manipulation of IL-6 signalling pathways; the *gp130*$^{F/F}$ mouse model

A drawback of existing mouse models, particularly those which are deficient in STAT1 or STAT3, is often the effects/phenotypes which can be attributed to loss of signalling by cytokines other than IL-6, which also utilises these STAT factors (Suzuki *et al.,* 2001). For example, increased inflammatory cytokine production (Kano *et al.,* 2003; Matsukawa *et al.,* 2003) in *Stat3*-deficient cells were due to the inability of IL-10 to signal. Accordingly, this makes it difficult to identify how IL-6 can influence specific biological functions, such as pro- and anti-inflammatory responses, *in vivo*. In addition, these models don't

address the clinical relevance of disease as in most inflammatory disease states STAT3 is increased (as discussed previously).

To overcome these obstacles and identify the physiological requirement of individual signalling molecules activated via gp130 for biological responses elicited by the IL-6 family of cytokines, *gp130*$^{F/F}$ mice were generated. These mice are homozygous for a phenylalanine knock-in substitution of the cytoplasmic Y_{757} in gp130 which, as a consequence of abolishing binding of both SHP2 and SOCS3, simultaneously mediates impaired SHP2/MAPK activation and excessive STAT1/3 signalling and target gene expression (Tebbutt *et al.,* 2002). Interestingly, these mice spontaneously develop multi-organ inflammation (e.g. gastritis, peritonitis) driven by hyperactivation of STAT3, since this pathology is alleviated in *gp130*$^{F/F}$:*Stat3*$^{+/-}$ mice which have normalised STAT3 activation levels (Jenkins a *et al.,* 2005). Furthermore, STAT1 is involved in this inflammatory phenotype, although to a lesser extent (Ernst et al., 2008). Thus, the *gp130*$^{F/F}$ mouse provides a unique biological tool to determine whether imbalanced activation of specific 'endogenous' signalling pathways (e.g STAT1, STAT3) downstream of gp130-acting cytokines (e.g. IL-6) augments TLR-mediated inflammatory responses. To date minimal work has been carried out on the *gp130*$^{F/F}$ mouse in terms of endotoxic shock, thus presenting the opportunity to utilise this model in the hope of further understanding the mechanisms behind sepsis. To further assess the contribution of STATs, cytokines and TLR signalling pathways towards the inflammatory phenotype displayed by the *gp130*$^{F/F}$ mice, the following compound mutant mice were generated: *gp130*$^{F/F}$:*Stat1*$^{-/-}$, *gp130*$^{F/F}$:*Stat3*$^{+/-}$, *gp130*$^{F/F}$:*IL-6*$^{-/-}$, *gp130*$^{F/F}$:*Ifnar2*$^{-/-}$ mice, *gp130*$^{F/F}$:*Mal*$^{-/-}$ mice and mice with Mal knocked out of the macrophages; *gp130*$^{F/F}$:*LySMMal*$^{-/-}$ (Tebbutt *et al.,* 2002; Jenkins a *et al.,* 2005; Fenner *et al.,* 2006; Jenkins *et al.,* 2007; Ernst *et al.,* 2008; Greenhill *et al.,* 2010) (described in detail in section 2.2.1).

1. 9 Hypothesis and aims of this project

1.9.1 Introduction to my project

Endotoxic shock is a chronic inflammatory disease which is triggered by LPS via the TLR4 signalling cascade. This pathway induces a host of pro-inflammatory mediators such as IL-6, which signals through the gp130 receptor

and STAT1 and STAT3. Interestingly, IL-6 can be both pro- and anti-inflammatory, depending on the cellular context. STAT3 also has pro- and anti-inflammatory roles and current conditional knock-out studies are insufficient at explaining these conflicting results. Therefore, I wish to use the *gp130*$^{F/F}$ mouse model (with elevated STAT1 and STAT3 activation), as well as its compound mutants, to elucidate the role of IL-6 and STATs in the context of inflammation.

1.9.2 Hypothesis

Based on the inflammatory phenotype observed in *gp130*$^{F/F}$ mice, the importance of STAT activation on this condition, and the fact that TLRs are the key drivers of this response, I hypothesised that STAT over-activation may directly or indirectly augment TLR signalling. I also hypothesize that anti-inflammatory signalling pathways in the *gp130*$^{F/F}$ mice may be disrupted due to the mutation in these mice during LPS-induced endotoxic shock.

1.9.3 Overall Aims

The overall aim (Aim 1) of this project is to assess whether or not deregulated STAT activation in the *gp130*$^{F/F}$ mice augments TLR-driven inflammatory responses; specifically TLR4 in response to LPS-induced endotoxic shock. In addition, I aim to assess which pro-inflammatory cytokine/s are associated with this inflammatory phenotype, and which components of the TLR4 pathway are involved. Furthermore, I aim (Aim 2) to assess whether the signalling capabilities of a key anti-inflammatory cytokine IL-10 was impaired in the *gp130*$^{F/F}$ mice following LPS-induced endotoxic shock.

1.9.4 Aim 1

Therefore the first aim of this PhD project was to investigate whether deregulated STAT activation augments TLR4 driven inflammatory responses using the following mice treated with 4mg/kg of re-purified LPS. These mice were monitored for survival over 72hrs, and serum and spleen from LPS-treated mice were collected for analysis of pro-inflammatory cytokine production. The livers were also collected for Western blot analysis of STAT activation. This series of mice allows us to assess whether genetically reducing the activity of one STAT

(while the other STAT remains hyper-activated) impacts on the inflammatory responses seen in the *gp130*$^{F/F}$ mutant mouse models.

Gp130 mutant mouse models

1. The following gp130-STAT signalling mutant mice will be used:

i) *gp130*$^{+/+}$ displaying normal STAT1/3 activity

ii) *gp130*$^{F/F}$ displaying higher STAT1/3 activity

iii) *gp130*$^{F/F}$:*Stat3*$^{+/-}$ displaying higher STAT1 with relatively normal STAT3 activity

iv) *gp130*$^{F/F}$:*Stat1*$^{-/-}$ displaying no STAT1 with higher STAT3 activity

2. To identify whether IL-6 is involved in controlling the deregulated gp130 signalling pathways that underlie the inflammatory pathologies observed in response to LPS in the *gp130*$^{F/F}$ mice, the following mutant mice will be used:

(i) *gp130*$^{F/F}$:*IL-6*$^{-/-}$ unable to signal via IL-6

In addition a series of therapeutic approaches targeting IL-6 will be employed, namely:

(i) *gp130*$^{F/F}$ receiving therapeutic intervention targeting IL-6 signalling (2B10; anti-IL-6R antibody)

(ii) *gp130*$^{F/F}$ receiving therapeutic intervention targeting IL-6 trans-signalling (sgp130Fc)

(iii) *gp130*$^{+/+}$ receiving therapeutic intervention targeting IL-6 trans-signalling (sgp130Fc) (to test the general applicability of using sgp130Fc as a treatment for endotoxic shock)

3. To determine specifically which TLR4 pathway(s) are affected by the deregulated gp130 signalling in the *gp130*$^{F/F}$ mice consequently resulting in LPS hypersensitivity and spontaneous multi-organ inflammation, the following mice will be generated:

(i) *gp130*$^{F/F}$:*Mal*$^{-/-}$ unable to signal via the MyD88-dependant pathway

(ii) *gp130*$^{F/F}$:*Ifnar2*$^{-/-}$ unable to signal via the MyD88-independant pathway

In addition a therapeutic approach targeting type I IFN signalling will be employed, namely:

(i) *gp130*$^{F/F}$ receiving therapeutic intervention targeting the MyD88-independent pathway (Mar-1 antibody)

4. To examine the role of macrophages in the LPS hypersensitivity of the *gp130*$^{F/F}$ mice*, gp130*$^{F/F}$:*LysM*-Cre*/Mal* mice will be generated and treated with LPS.

1.9.5 Aim 2

While SOCS3 is a key regulator of IL-6/STAT3 signalling, it is somewhat controversial whether or not SOCS3 also down-modulates the anti-inflammatory actions of IL-10 as discussed previously (Berlato *et al.,* 2002). Since SOCS3 has increased basal expression in the *gp130*$^{F/F}$ mice (Jenkins a *et al.,* 2005), this may impair the ability of IL-10 to signal, consequently resulting in the inflammatory phenotype observed in these animals. Therefore, the second aim of this project was to determine whether SOCS3, a STAT3 target gene, may impair anti-inflammatory IL-10 responses in *gp130*$^{F/F}$ mice. This was done by stimulating macrophages from *gp130*$^{F/F}$ mice with LPS with or without IL-10 and assessing the inflammatory readouts.

Table 1.1: Cytokines involved during inflammation

Cytokine	**Cell source**	**Targets**	**Actions**	**Inflammatory status**
Interleukin **(IL)-6**	-macrophage/monocyte -dendritic cell -endothelial cells -T helper 2 cell -fibroblasts -Bcells Keratinocytes	-liver -B cell -T cells -T helper 17 cells	-synthesis and release of acute phase proteins -proliferation -immune regulation - patho-	pro- and anti-inflammatory

	-mesangium cells -tumour cells		physiological role in inflammation, immune regulation, haematopoiesis and oncogenesis	
Tumour necrosis factor **(TNF)α**	-macrophage -T cells -B cells -natural killers cells -smooth muscle cells	-endothelium cell -all immune cells eg: neutrophils, lymphocytes	-induction and maintenance of the inflammatory immune response -inducer of apoptosis -inducer of cell survival -endotoxin-induced tumor necrosis -activator of fever/shock	pro-inflammatory
IL-1β	-macrophage/monocyte -endothelial cells -lymphocytes -B cells	-lymphocytes -endothelial cell -hepatocytes -lymphocytes (T and B cells) -fibroblasts -neutrophils -NK cells	-synthesis and release of acute phase response proteins -activator of fever -inductor of inflammatory response	pro-inflammatory
Interferon	-macrophages	-dendritic cells	-anti-viral	pro-

(IFN)β	-dendritic cells -T cells	-T cells -B cells	-anti-bacterial -activating natural killer cells	inflammatory
chemokine (C-C motif) ligand 5 (**CCL5)/** Regulated on Activation Normal T Cell Expressed and Secreted **(RANTES)**	-T cells -platelets	-eosinophils -monocytes -T cells (CD4+) -basophils -leukocytes	-recruits leukocytes to inflammatory sites -degranulation of basophils -respiratory burst in eosinophils -activation of T cells	pro-inflammatory
IL-10	-macrophage/monocyte -T helper 2 cell -B cells	-macrophage -dendritic cell	-inhibitor of IL-12 production -inhibitor of pro-inflammatory cytokine synthesis	anti-inflammatory
Transforming growth factor (TGF)-β1	-most cell types eg: -B cells -myeloid cells	-regulatory T cells -T cells -T helper 17 cells -natural killer cells -B cells -myeloid cells -lymphoid cells	-regulation of cell growth -tissue repair -regulator of cell proliferation -regulator of immunity -immunosuppressive -enhance the formation of	anti-inflammatory

			extracellular matrix	

Adapted from; (Carswell *et al.,* 1975; Degliantoni *et al.,* 1985; Durum *et al.,* 1985; Cuturi *et al.,* 1987; Hack *et al.,* 1989; Jones, E. Y. *et al.,* 1989; Kishimoto, 1989; Schall *et al.,* 1990; Kameyoshi *et al.,* 1992; Kuna *et al.,* 1992; Rot *et al.,* 1992; Alam *et al.,* 1993; Bacon *et al.,* 1995; Lawrence, 1996; Hirano *et al.,* 1997; Heinrich *et al.,* 1998; Song *et al.,* 2000; Yoo *et al.,* 2002; Gaur, Aggarwal, 2003; Bonniaud *et al.,* 2005; Naiki *et al.,* 2005; Takaoka, Yanai, 2006; Karampetsou *et al.,* 2010; Takeuchi, Akira, 2010; Yang, L. *et al.,* 2010)

Table 1.2: Criteria for the systemic inflammatory response syndrome (SIRS)

Criterion	**Value**
Temperature	>38◦C or <36◦C
Heart Rate	>90 beats per minute
Respiratory rate	>20 or PaCO2 <32 mm Hg
White blood cell count	>12 K or <4 K mm−3, or >10% bands

-For a diagnosis of SIRS to be made, two of the four criteria need to be present (Bone *et al*., 1992, reviewed in Stearns-Kurosawa *et al.,* 2011)

Table 1.3: TLRs and their agonists

TLR	TLR agonist	Location
TLR2 dimerises with TLR1&6	Recognises the most extensive range of microbial components, bacterial lipoproteins from Gram-positive bacteria, mycoplasma, fungi and viruses - TLR1/2 complex recognises triacylated lipopeptides -TLR2/6 complex recognises diacylated lipoproteins	Plasma membrane
TLR3	Responds to the viral double stranded RNA in the endolysosome and is involved in the recognition of polyinosinic polycytidyllic acid (poly I:C)	Endoplasmic
TLR4	Gram-negative bacterial LPS, heat shock proteins, extracellular domain A in fibronectin, and several viral proteins, and the H5NI avian influenza virus	Plasma membrane
TLR5	Recognises flagellin from flagellated bacteria	Plasma membrane
TLR7 (human TLR8)	TLR7 and human TLR7/8 recognise single stranded RNAs from RNA viruses	Endoplasmic

	and bacteria	
TLR9	TLR9 responses to unmethylated DNA with CpG motifs produced from bacteria and viruses	Endoplasmic
TLR10 co-localises with TLR2	Triacylated lipopeptides and a wide variety of other microbial-derived agonists	Phagosome
TLR11 (mice only)	TLR11 responds to T.gondii to induce DCs Possibly involved in host surveillance of the central nervous system	Plasma membrane

Adapted from; (Okamura *et al.,* 2001; Takeda, 2005; Gondokaryono *et al.,* 2007; Watters *et al.,* 2007; Imai *et al.,* 2008; Mishra *et al.,* 2008; Takeuchi, Akira, 2010, Guan et al., 2010)

CHAPTER 2

Materials & Methods

2.1 LPS preparation

2.1.1 Purification of LPS

Lyophilized LPS powder from *Escherichia coli* (K-235) (Sigma-Aldrich, Saint Louis, MO, USA) was water-phenol extracted to ensure it was pure from contaminants that may stimulate TLRs other than TLR4. This was done by resuspending a 12.5milligrams (mg)/ millilitre (ml) LPS solution in endotoxin-free (EF) distilled water containing 0.2% triethylamine (TEA). To 500 microlitres (µl) of this LPS solution, 25µl of 10% sodium deoxycholate (DOC) (Sigma) and 500µl of water-saturated phenol (Sigma) was added, then vortexed intermittently for 5 minutes (') at room temperature (RT), then for a further 5' on ice. The mixture was then centrifuged at 4 degrees Celsius (^{o}C) for 2' at 14,000 revolutions per minute (rpm)/ 15500 g (gravitational acceleration) and the top aqueous layer transferred to a clean eppendorf tube (Eppendorf, Hamburg, Germany). Then 425µl of 0.2% TEA plus 22µl of 10% DOC were added to the phenol phase of the original tube, and the solution was vortexed, incubated and centrifuged as described above. The aqueous phase was then combined with the one previously collected and 500µl of water-saturated phenol was added to the aqueous phase and the solution was vortexed, incubated and centrifuged again. The aqueous phase was then mixed with 2.2ml of 100% ethanol plus 30µl of 3 molar (M) sodium acetate (Sigma) (in EF water, pH=5.2). The solution was vortexed and placed at -20^{o}C for 1hr and then centrifuged at 4 ^{o}C for 10' at 15,500 g to pellet the LPS. The supernatant was discarded and the pellet was washed in -20^{o}C 100% ethanol and centrifued at 4^{o}C for 2' at 15,500 g. The supernatant was discarded and the LPS pellets were left to air dry. Each pellet was then resuspended in 500µl of 0.2% TEA to give a final LPS concentration of 12.5mg/ml. Aliquots of repurified LPS were stored at -20^{o}C. To confirm the absence of contamination in the LPS, macrophages deficient in TLR4 could have been stimulated and their inflammatory response examined.

2.1.2 LPS bioassay

To quantify the purified LPS, a bioassay was performed. RAW-elam cells (with an NF-κB reporter gene) were plated at 5 x 10^4 cells per well in a 96-well plate. The next day cells were stimulated with 25, 10, 5, 2.5, 1, 0.5, 0.25 and 0.1ng/ml of repurified LPS as well as an earlier batch of quantified repurified LPS (for comparison) for 5-6hrs. Cell media was discarded and 50µl of lysis buffer was added to each well and left to sit for 10' at RT. Lysis buffer was then transferred to a new well and 50µl of Luciferase Substrate Solution (Luciferase Assay System, Promega, Madison, WI, USA) was added to each well for luciferase readings on a 96-well plate (Becton Dickinson, Franklin Lakes, NJ, USA). The previously quantified LPS was used to create a standard curve to then calculate the repurified LPS concentration.

2.2 Animal work

2.2.1 Mouse generation and housing

The generation of *gp130*$^{F/F}$ and compound *gp130*$^{F/F}$ mutant mice heterozygous for *Stat3* (*gp130*$^{F/F}$:*Stat3*$^{+/-}$), homozygous null for *IL-6* (*gp130*$^{F/F}$*:IL-6*$^{-/-}$) and homozygous null for *Stat1* (*gp130*$^{F/F}$:*Stat1*$^{-/-}$) has been previously described (Tebbutt *et al.,* 2002; Jenkins a *et al.,* 2005; Jenkins *et al.,* 2007; Ernst *et al.,* 2008). Mice homozygous null for the *Ifnar2* gene have previously been generated (Fenner *et al.,* 2006). Mice homozygous null for the *Mal* gene (*Mal*$^{-/-}$) were generated from mice in which exons 2 and 3 of the *Mal* gene were flanked with LoxP sites, and then crossed with EIIA-Cre mice to generate null mice (Dunne, A., O'Neill, L., Mansell, A. and Hertzog, P. manuscript in preparation). These mice were then crossed with *gp130*$^{F/F}$ mice to generate the compound mutant *gp130*$^{F/F}$*:Ifnar2*$^{-/-}$ and *gp130*$^{F/F}$*:Mal*$^{-/-}$ mice. Mice homozygous null for the Mal gene in macrophages and neutrophils (*LysM*-Cre/*Mal)* were generated from mice using LysMCre targeting, (protocol described in (Clausen *et al.,* 1999)). These mice were then crossed with *gp130*$^{F/F}$ mice to generate *gp130*$^{F/F}$*:LysM-*

Cre/*Mal* mice. All experiments were performed following Animal Ethics approval from the Monash Medical Centre "A" Committee, and included *gp130*$^{+/+}$ (wild-type) littermate controls that were genetically matched on a mixed 129Sv x C57BL/6 background. All mice were maintained under specific pathogen-free conditions and were age-matched for each experiment.

2.2.2 Genotyping Polymerase Chain Reaction (PCR)

PCR was used to amplify a specific region of genomic DNA to determine the genotype of mice. DNA preparation was done on tails for all mouse colonies (except *gp130*$^{F/F}$:*Ifnar2*$^{-/-}$) and genotyping for *gp130, Stat1, Stat3, IL-6*, *LysMCre* and *Mal* was performed by Technical Assistant Eva Vidacs. Briefly, DNA was prepared from mouse tails using tail buffer (Appendix II) and proteinase K (Roche, Mannheim, Germany) to digest overnight (O/N) at 55^{o}C. The following day samples were mixed with 5M NaCl, incubated at RT, centrifuged and the aqueous phase collected into a new tube, mixed with isopropanol and centrifuged to pellet the DNA. DNA was washed in ethanol, incubated at 37^{o}C for 3hrs, resuspended in MilliQ (Millipore, Bedford, MA, USA) water and left to dissolve O/N at 37^{o}C. DNA then was purified using a UNIFILTER ® 800 (Whatman, USA) plate under vacuum using PBB Binding Buffer (Qiagen, Germany) and PE wash buffer (Qiagen) and then eluted using 0.25% TE buffer (Appendix II).

Genotyping for *Ifnar2* was done on *gp130*$^{F/F}$*:Ifnar2*$^{-/-}$ and control *gp130*$^{F/F}$ mouse DNA prepared separately from above. This involved digesting mouse tails as described above, following which, 1µl of RNAse A (10mg/ml) (Roche) was added and digested at 37^{o}C for 1hr. An equal volume of phenol:chloroform (1:1) (Invitrogen, San Diego, CA, USA) (600µl) was then added to digested tail solution, mixed and centrifuged at 15,500 g for 8'. This process was repeated twice (the last step only adding chloroform). DNA was then eluted with 1ml 100% ethanol, washed in 70% ethanol, centrifuged at 15,500 g for 5' and resuspended in 1/3 dilution of T.E buffer (in water) O/N at 37^{o}C. Initially, DNA quantification and purity was determined using a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA), analysing the absorbance at 260/280 nanometres (nm) and 260/230nm.

For PCR screening, reactions were setup to a final concentration containing: 100ng of genomic DNA, 5µl PCR Buffer (Appendix II), 1.25µl of 10mM dNTPs (Appendix II), 200ng forward and reverse oligo primers (sequences in Appendix III), 2.5µl DMSO and MilliQ water up to 50µl per reaction. PCR was performed on either a Gene Amp PCR system 2400 (Applied Biosciences, Foster City, CA, USA) or a gradient PCR Fast Thermal Cycler (Bio-Rad). Reactions were performed using the following conditions/steps:

Initial denaturation	94^{o}C for 5’	
Further denaturation	94^{o}C for 30’	35 cycles
Annealing	58^{o}C for 45’	
Extension	72^{o}C for 2’	
Final extension	72^{o}C for 5’	

Agarose gels were made using 1 x TAE buffer, agarose (1.5%) (Appendix I) and 2.5µl of 10mg/ml ethidium bromide (25µg/ml) (Appendix II) (to visualise bands). The samples of DNA had loading dye (Appendix II) added to them and were loaded into wells of a wide mini Sub^{TM} agarose gel frame. The gel was run at 90-100 volts (V) for approximately 40’ in 1 x TAE buffer. The DNA bands were then visualised under UV light and photographed. This procedure was identical for all PCR reactions, regardless of the method of DNA purification.

2.2.3 Irradiation and bone marrow reconstitution of mice

Mice were irradiated with a split dose of 550 rads x 2 (1100 rads in total) in the mouse irradiation facility. Bone marrow reconstitution was then performed on the mice. These experiments were performed at Ludwig Institute for Cancer Research, Parkville, Victoria, Australia by Brendan J Jenkins. Further details can be found in this paper: (Ernst *et al.,* 2008).

2.2.4 Administration of LPS and blocking antibodies to mice

Systemic inflammation was induced *in vivo* by intraperitoneal (i.p.) injection of repurified LPS at 4mg/kg. At 0, 1.5, 3 and 6hrs, animals were culled,

and spleen, liver and blood were harvested (see section 2.2.5 below). The Mar-1 IFNAR1 blocking antibody (IgG1) (Sheehan *et al.,* 2006) (1mg) or isotype control antibody (IgG1) (1mg) were i.p co-injected into mice (along with LPS), whereas the 2B10 IL-6R blocking antibody (IgG1) (section 2.7 (1.5mg) or isotype control (IgG1) (1.5mg) antibody (Lissilaa *et al.,* 2010) were i.p. injected for 1hr prior to LPS administration. For studies involving administration of sgp130Fc (Nowell *et al.,* 2003), mice were pretreated with 150μg by i.p. injection for 16hrs prior to LPS administration. In survival studies, mice were monitored over 72hrs. In short-term studies (up to 6hrs), mice were initially cheek bled (0hr time point), and then injected with LPS.

2.2.5 Organ collection

Mouse spleen and liver was collected and frozen in liquid nitrogen and stored at -80^{o}C for storage prior/post LPS administration.

Blood collected before LPS injection via cheek bleeds involved puncture of the jaw bone in the submandibular area with a lancet. After LPS injection, blood was collected either via cheek bleeds (approximately 50-100μl) at specific time-points or via cardiac puncture (approximately 500μl l-1ml) following euthanasia. For platelet and white blood cell (WBC) determinations, approximately 50-100μl blood was collected in microvette tubes (500KE SARSTEDT, Germany) and mixed with 2mg/ml EDTA/ Phosphate Buffer Saline (PBS; Appendix II), following which samples were analysed on a Sysmex automated hematology analyser KX-21N (Roche). To prepare serum, the remaining blood collected was added to a separate eppendorf tube and then incubated at RT for approximately 10'. Blood clots were then removed with a pipette tip, samples centrifuged for 5' at 6,700 g and supernatants frozen at -80°C.

The peritoneal cavity of untreated mice was lavaged with ice-cold PBS, PBS was collected, centrifuged down at 15,500 g and supernatant was stored at -20°C.

2.2.6 Cytospins and staining

Total cell numbers of peritoneal lavage were determined using the Sysmex analyser. Cells ($5x10^4$ per sample) were centrifuged on a Cytospin III slide

(Shandon Scientific, Cheshire, UK), and then stained with DiffQuick (Dade Baxter) to assess morphology by light microscopy. A minimum of 200 cells per slide were counted.

2.2.7 Bone marrow macrophage (BMM) preparation

The femurs from culled mice were collected in 10ml PBS, then rinsed in ethanol then flushed with approximately 5ml Roswell Park Memorial Institute (RPMI)/10% FCS media (Appendix II) in a falcon 50ml tube. The cells were centrifuged at 15,000 g for 5', the supernatant was removed and the cells were then resuspended in RPMI/10% FCS and macrophage colony stimulating factor (M-CSF) at 50ng/ml (Peprotech, USA) into 4x10 centimetre $(cm)^2$ low adherence plates (SARSTEDT) (per 2 femurs). Plates were incubated at 37^{o}C, 5% carbon dioxide (CO_2) (Heraeus, Themo Fisher Scientific, Waltham, WA, USA) for a week.

2.2.8 Peritoneal macrophage (PM) preparation

PMs were isolated from the peritoneal cavity of mice by flushing with 2mls of ice cold PBS, and then plating cells out at approximately $1.5x10^6$ cells per well in a 24-well plate in RPMI/10% FCS. Media was changed 2-4hrs later to remove non-adherent cells and stimulations were performed the following day.

2.3 Tissue Culture

2.3.1 Thawing cells

A frozen cryotube (Sigma) of cell stocks (RAW-elam cells with NF-κB reporter) was taken out of liquid nitrogen storage and thawed in pre-warmed (37^{o}C) RPMI/10% media by slow, repeated up and down mixing inside the tube. The resuspended cells were then transferred to a 15ml falcon tube (BD Biosciences, Bedford, MA, USA) and centrifuged for 5' at 1,912 g to remove residual freezing media. Cell pellets were then resuspended into fresh warm growth media 10-15ml and plated into $75cm^2$ polystyrene tissue culture flasks

(BD Biosciences) for incubation at 37°C in a humidified atmosphere of 5% (v/v) CO_2 (Heraeus).

2.3.2 Cell culture maintenance

The immortalised cell line; RAW-elam cells were maintained in RPMI/10% media in 175cm^2 tissue culture flasks. Cells were prepared for passaging by removing the culture media, rinsing cells with 1x PBS (Gibco, Paisley, UK) by pipetting the media up and down to dislodge the cells. Approximately a tenth of this media was then transferred to a new culture flask and diluted with RPMI/10% media.

2.3.3 Freezing cells

Cells were frozen for long term storage in liquid nitrogen. Cells were initially passaged as described above, pelleted by centrifugation for 5' at 478 g and resuspended in 1ml of 5% DMSO/95% FCS freezing media. The cells were then transferred to a 1ml cryotube and stored O/N at -80°C, after which cryotubes were transferred to liquid nitrogen.

2.3.4 Viable cell counting

Confluent cells were passaged, and 100µl of the suspension removed and analysed on a Sysmex analyser.

2.3.5 MTT Assay

To determine cell viability, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assays were performed. This technique involved adding 20µl of MTT solution at 5mg/ml to the media of cells plated out in 96-well plates. Plates were placed on a shaker for 5' RT in foil (light sensitive) and incubated at 37°C for 4hrs. Media was then discarded and 100µl of DMSO was added to each well and incubated at RT for 10' on a shaker. Cell viability was then determined on the FLUROstar Optima plate-reader (BMG Labtech, Offenburg, Germany) at 560nm.

2.3.6 *In vitro* LPS stimulations

For all *in vitro* stimulations, cells were stimulated with repurified LPS in RPMI/10% media at 37^{o}C.

LPS

Approximately 1x10^{6} PMs from mice were plated into wells of 24-well plates in 500μl of media and stimulated with and without 10ng/ml LPS.

LPS and IL-10

Approximately 1x10^{6} PMs from mice were plated into wells of 24-well plates in 500μl of media and stimulated with and without 1ng/ml LPS and IL-10 (100ng/ml).

IFN blocking experiment

Approximately 1x10^{6} PMs from mice were plated into wells of 24-well plates and stimulated with and without 10ng/ml LPS. Isotype control (1000ng/ml) or interferon blocking antibody (Mar-1) (1000ng/ml) was also added in addition to LPS. In separate wells IFNα (1000IU/ml) was also added together with the Mar-1 or isotype control antibody to confirm the bioactivity of the Mar-1 antibody.

2.4 RNA preparation

2.4.1 Cell harvesting for RNA extraction

Cell culture supernatants were aspirated from the cells, cold PBS was added to each plate and the cells were dislodged with a cell scraper. The cells were then pelleted by centrifugation at 478 g for 5' at 4^{o}C and the supernatants discarded. The cell pellets were then lysed in 350μl of RLT buffer obtained from an RNeasy® mini kit (Qiagen). Cells were homogenised by passing the suspension through a 26 gauge needle fitted to an RNase-free syringe (BD Biosciences). The cell lysates were then stored at -20^{o}C until required.

2.4.2 Total RNA extraction from tissues

Approximately 50-100mg of spleen or liver was homogenised in 1ml of Trizol (Invitrogen) in flat bottom 5ml tubes. Homogenates were then transferred to 15ml tubes and centrifuged at 1,912 g (Biofuge, Germany) for 30' to pellet insoluble material at 4^{o}C. Supernatants were then transferred to clean tubes and left at RT for 5'. To each sample 200µl of chloroform (Labscan) was added, the tubes were vortexed and incubated at RT for 2-3'. Tubes were then centrifuged at 1,912 g for 45' at 4^{o}C and the upper aqueous phase was added to a new tube with 500µl of isopropanol (Labscan), vortexted and incubated at RT for 10'. Tubes were centrifuged at 1,912 g for 45' at 4^{o}C and supernatants were discarded and pellets washed in 500µl of 75% Ethanol/DEPC-treated water (Appendix II). Tubes were centrifuged at 1,912 g for 15' at 4^{o}C, supernatants discarded and pellets were air dried for approximately 15'. Pellets of RNA were then resuspended in 50µl DEPC-treated water, run on a 1% agarose gel to determine RNA quality and stored at -20^{o}C.

RNA concentrations were then determined on the Nanodrop analyser. To purify RNA, 100µg was added to 350µl of RLT (Qiagen)/1%β-mercaptoethanol (Sigma) mixed in a hood, and then 250µl of 100% ethanol was added. RNA solutions were then transferred onto an RNeasy column and centrifuged at 6,700 g for 30''. The flow through was then discarded and 350µl of RWI wash buffer added to the column, centrifuged at 6,700 g for 30'' and flow through discarded, then 10µl DNAse and 70µl of RDD buffer (Qiagen) was added to the sample membrane and incubated for 15' at RT. To each sample, 500µl of RWI buffer was added, centrifuged for 6,700 g for 30'' and then 500µl of 80% ethanol was then added. Tubes were centrifuged for 30'', flow through discarded and centrifuged again at maximum speed for 1'. Columns were then transferred into collection ependorf tubes and 30µl of RNAse-free water (Qiagen) added onto the membrane and incubated for 5' at RT. Tubes were centrifuged at 6,700 g for 1' and eluant (RNA) was collected. This last step was repeated to equal a total volume of 60µl RNA.

2.4.3 Total RNA extraction from cells

Cell lysates were thawed and mixed with 600µl of 70% ethanol made up in 0.1% DEPC-treated water. Samples were mixed and added to an RNeasy mini column (Qiagen) and centrifuged at 6,700 g for 30''. Flow through was discarded and 350µl RWI buffer was added to the column and centrifuged at 6,700 g for 30'', following which 10µl DNAse and 70µl of RDD buffer were mixed together and added to the column for 15' at RT. Buffer RWI (350µl) was added to the sample and centrifuged at 6,700 g for 30'' and the column was transferred into a new collection tube. RPE buffer (500µl) was added to the column and centrifuged at 6,700 g for 30'' and the flow through was discarded. Another 500µl RPE buffer was added to the column and centrifuged down at 6,700 g for 2'. The RNA was eluted in 30µl of DEPC-water, (incubating for 1' and spinning for 6,700 g for 1'). An additional 30µl of DEPC-water was added and incubated for 1' then centrifuged for 6,700 g for 1' and the two solutions mixed together to give a total volume of 60µl.

The quality of RNA recovered from tissues or extracted from cells was determined spectrophotometrically on the Nanodrop and the quality of the RNA determined by electrophoresis through a 1% agarose gel.

2.4.4 Reverse transcription reaction

RNA (1µg) from tissues was reverse transcribed using the Transcription High Fidelity cDNA synthesis kit (Roche), whereas the SuperScript III First-Strand Synthesis System (Invitrogen) was used to reverse transcribe RNA (140ng) from cells (as the latter was found to be the only kit to result in reproducible and reliable results from real time PCR on PM RNA).

Reverse transcription on cell RNA (Invitrogen): 1µl of dNTPs (Invitrogen) and 1µl of randomhexamers (Invitrogen) were added to each sample (140ng of RNA plus the required amount of DEPC-treated water (based on nanodrop readings), to make up 8µl total volume) and incubated for 5' at 65°C and pulse centrifuged. To each sample, 10µl of master mix containing; 2µl of 5 x Superscript First Strand buffer (Invitrogen), 2µl of dithiothreitol (DTT) (Invitrogen), 4µl of magnesium chloride, 1µl of RNase OUT (Invitrogen) and 1µl of SuperScript III Reverse

Transcriptase (Invitrogen) was added. A negative control reaction, which did not have any enzyme added, was performed concurrently to confirm that no contaminating DNA was present. All samples were incubated at RT for 10' and 50°C for 50'. The reactions were then heat inactivated by incubation at 85°C for 5'. Then 1µl RNase H (Invitrogen) was added to each tube on ice and pulsed down and incubated at 37°C for 20'. Samples were then stored at -20°C.

Reverse transcription on tissue RNA (Roche): 2µl of random hexamers (Roche) was added to each sample (1µg of RNA plus the required amount of DEPC-treated water (based on nanodrop readings), to make up 9.4µl total volume) and incubated for 10' at 65°C. Samples were then immediately cooled on ice and to each sample, 8.5µl of mastermix containing; 4µl of 5x Transcriptase Reaction Buffer (Roche), 0.5µl of RNase inhibitor (Roche), 2µl of dNTPs (Roche), 1µl of DTT (Roche) and 1µl of High Fidelity Reverse Transcriptase (Roche) was added. A negative control reaction, which did not have any enzyme added, was performed concurrently to confirm that no contaminating DNA was present. All samples were incubated at 55°C for 1hr, then incubated at 85°C for 5', centrifuged down and stored at -20°C.

2.4.5 Quantitative Real-Time PCR (qPCR)

The Applied Biosystems 7900HT Fast Real-time PCR system was used for qPCR. Two different technologies were used, depending on the genes examined: SYBR Green and Taq Man (the use of probes). All primer sequences used in conjunction with SYBR Green are in Appendix III.

SYBR Green: cDNA (2µl) was combined with a mastermix containing; 5µl of SYBR GREEN master mix, 2µl of nuclease free DEPC-treated water and 0.2µl of a gene-specific forward and reverse primer. Each reaction was performed in a total of 10µl in triplicate in a MicroAmp^{TM} Optical 384-well reaction plate (Applied Biosciences) and sealed with MicroAmp^{TM} Optical adhesive film. The expression of the gene of interest was normalised compared to *18S*, which was used throughout as a reference gene.

Taqman: multiplexed reaction mixes contained probes for the reference *18S* gene (VIC labelled) and the target gene (FAM labelled). cDNA (2µl) was combined with a mastermix containing; 0.5µl of *18S* probe, 0.5µl target probe, 5µl TaqMan® Universal PCR Master Mix and 2µl of nuclease free DEPC-treated water. Each reaction was performed in a total of 10µl in triplicate in a MicroAmp™ Optical 384-well reaction plate (Applied Biosciences) and sealed with MicroAmp™ Optical adhesive film.

For both SYBR and TaqMan qPCR, the thermal cycling protocol was as follows: initial degradation step of 95°C for 10' following with 40 cycles (95°C/15'', 60°C/1') and an additional dissociation step (for melt curve analysis during SYBR reactions only) (95°C/15''/60°C/15''/95°C/15''). Data acquisition and analyses were performed with the Sequence Detection System Version 2.3 software (Applied Biosystems). Cycle threshold (Ct) values for all probes and were exported and Ct values for each sample were calculated. Below is an equation used for comparing relative expression (R) data generated from qPCR:

$$R = 2^{-[\Delta Ct\ sample - \Delta Ct\ control]}$$

2.4.6 Statistical Analysis

Data are expressed as the mean ± SEM, and statistical analyses were performed using the GraphPad PRISM software (GraphPad Software, Inc., San Diego, California). Normally distributed data were analysed using a paired t-test, and if data did not show normal distribution, a Mann-Whitney test was performed. A one-way ANOVA was used to determine the differences between all genotypes for all normally distributed data. If data did not show normal distribution, then a Kruskal-Wallis one-way ANOVA on ranks was performed. A *P* value of less than 0.05 was considered statistically significant.

2.5 Protein manipulations

2.5.1 Lysate preparation

Protein extracts from snap frozen livers and spleen were prepared in ice-cold lysis buffer (Appendix II), where they were homogenized in flat bottom 5ml tubes. Lysate was then centrifuged at 15,500 g for 5' to pellet insoluble material at 4^{o}C, following which they were precleared of cellular debris by adding 20µl of Sepharose G beads (GE Healthcare, Australia) per 1ml of lysate and leaving at 4^{o}C for 30'. Following this incubation, samples were centrifuged at 15,500 g for 5' and supernatant was collected and transferred to a new tube. The lysates were precleared twice.

2.5.2 Lowry Protein Assay

Working reagent was prepared by adding (20µl of reagent S to each 1ml of reagent A (Appendix II). Standards were prepared at a concentration of 1mg/ml, 5mg/ml and 10mg/ml BSA stocks. Standards were then pipetted onto a flat bottom well 96-microtitre plate in the following way:

Table 2.1: Lowry plate layout

BSA stocks	1mg/ml	1mg/ml	1mg/ml	5mg/ml	10mg/ml	10mg/ml
Standard concentration	1ug	2ug	4ug	8ug	10ug	20ug
Stock	1µl	2µl	4µl	1.6µl	1µl	2µl
dH20	4µl	3µl	1µl	3.4µl	4µl	3µl
Final volume	5µl	5µl	5µl	5µl	5µl	5µl

Then 25µl of working reagent was added into each well and then 200µl of reagent B. The plate was gently agitated to mix the reagents and incubated at 15' RT in the dark, following which the plate was read at absorbance 490nm on the FLUROstar Optima plate-reader (BMG Labtech).

2.5.3 Sodium Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE protein gels (10%) were prepared with 5% upper stacking gel (Appendix II). Running buffer (Appendix II) was used to cover the wells of the upper gel. Protein markers were loaded into the first well, and samples (added to sample buffer (Appendix II) into subsequent wells. The gel was run at 80V until the samples ran into the lower gel and then allowed to run for approximately 1hr at 100V. The gel was then transferred onto a membrane using the iBlot (Invitrogen) (23V for 7'), and blocked for 1hr at RT on a shaker (XTRON, Australia) in Odyssey blocking buffer (OBB) (LI-COR, Lincoln, NE). Following blocking, the protein levels on the blot were detected using the Odyssey Infrared Imaging System. This involved probing the membranes with appropriate dilution of primary antibody (Appendix II) diluted in OBB with 5% (w/v) BSA and 0.01% Tween 20 (Sigma) O/N at 4°C and then washed with PBS/Tween on the shaker 3 times for 5' to remove excess antibody. Membranes were then incubated with the appropriate secondary antibody (AlexaFluor 680 (molecular probes)) and IRDye800CQ (Rockland, Boyertown, PA, USA) fluorescent labelled secondary antibodies (Appendix II) at a 1:3000 dilution in 0.1% PBST (Appendix II) with 20% OBB and 0.02% of 10% SDS. Membranes were then washed again as before and blots were visualised on the Odyssey flurimager (LI-COR).

2.5.4 Stripping Western membranes

Membranes requiring additional antibody detection were stripped using membrane stripping buffer (Appendix II) to strip the membrane of antibodies with 3x 5' washes at 55°C. Membranes were then washed in 1% PBST and blocked with OBB for 30'-1hr at RT using appropriate buffer as above. From this point on, antibody re-blotting followed the same procedure as outlined above.

2.5.5 Antibodies

Specific antibodies against ERK1/2 (Santa Cruz Biotechnology, Santa Cruz, CA), STAT1, STAT3, phosphoSTAT3-Tyr705 and phosphoSTAT1-Tyr701 (Cell Signaling Technology, Beverly, MA) were used for primary antibody solutions during Western blotting.

2.5.6 Cytokine Enzyme-linked Immunosorbent Assay (ELISA)

Murine IL-6 (Pharmingen, San Diego, CA), CCL5 (R&D Systems, Minneapolis, MN), TNFα (Becton Dickinson), Cxcl1 (R&D Systems) and IL-10 (R&D Systems) were quantified using commercial ELISA kits. Capture antibody (BD Biosciences) was diluted to a 2ug/ml in binding solution (Appendix II) and 100µl was added to wells of a 96-well F96 Maxisorp plate (Themo Fisher Scientific, Waltham, WA, USA) which was sealed and incubated O/N at 4°C. The plate was then brought to room temperature the next day and washed 3 times with 0.05% PBST (Appendix II) to remove any unbound antibody after which 200µl ELISA blocking buffer (Appendix II) was added to each well. The plate was sealed and incubated at RT for 1hr. The wells were then rinsed again as previously described and 100µl of appropriate standards (BD Biosciences) or samples (serum or cell supernatant) were added in triplicate to wells, the plate sealed and incubated for 2hrs at RT. A working standard of 4000pg/µl was serially diluted to 15.62pg/µl. Both standards and samples were diluted in ELISA blocking buffer as required. After incubation, wells were washed again as previously described. Anti-cytokine detection antibody (BD Biosciences) was diluted to 1ug/ml in ELISA Blocking Buffer and 100µl added to wells. The plate was sealed and incubated for 1hr at RT. The wells were then rinsed again as previously described and streptavidin-HRP (BD Biosciences) was added (0.5ug/ml) in ELISA Blocking Buffer, 100µl per well. The plates were sealed and incubated at RT for 30'. Wells were washed again as previously described and 100µl of Substrate Solution (Appendix II) was added to each well and colour was allowed to develop for 15' at RT. Stop solution (50µl) (Appendix II) was then added to stop the reaction and absorbance was measured on the plate-reader at 450nm and the level of cytokine determined from the standard curve.

2.6 Flow cytometry

2.6.1 Fixing cells

Flow cytometry was performed on BMMs to determine the levels of phosphorylated tyrosine 705 STAT3 after stimulation with IL-10 or IL-6. After cell preparation described previously, cells were incubated at 37°C in 1% FCS

with RMPI for 1–2hrs prior to stimulations. BMMs were then collected in 200µl aliquots at $1x10^7$ cells/ml and stimulated for desired time at 37°C; 100ng/ml for IL-10, 100ng/ml for IL-6 and 1/100 dilution of sIL-6R. Cells were then fixed by adding 1ml of pre-warmed (37°C) 1% Buffered Formalin and incubated for 10' at 37°C. Cold PBS was then added and cells are centrifuged at 478 g at 4°C, washed and centrifuged again. Excess supernatant was removed so that they were in minimal volume and 1ml ice cold 90% methanol was added while vortexing cells to permeabilise the cells. Cells were then incubated on ice for 20' then transferred to -80°C freezer for storage.

2.6.2 Staining cells

Cell staining buffer (Appendix II) was added after removing cells from -80°C to ice and cells were centrifuged at 478 g for 5' at 4°C. Cells were re-suspended in 2ml ice cold staining buffer and transferred to FACS tubes (BD Biosciences) and washed twice in ice cold staining buffer. Cells were resuspended in 240µl staining buffer and allowed to hydrate for 1hr, then transferred to fresh FACS tubes (containing 5 x 10^5 cells) for each stain. They were then stained with pY705STAT3 (Alexa Fluor® 647, BD Biosciences) in a total volume of 50µl for 60' on ice in the dark, washed in 1ml ice cold Staining Buffer and re-suspended in 400µl staining buffer for FACS analysis using the FACSCanto™ II Flow Cytometry (BD Biosciences).

2.7 Generation of anti-IL-6Rα mAb, 2B10.

Male Wistar rats (Charles River Laboratories, Wilmington, MA) were immunised by i. p. injection 3 times at 3 weekly intervals with 10^6 CHO cells expressing high levels of mouse membrane-IL-6Rα in MonoPhosphoryl Lipid A plus synthetic Trehalose DycorynoMycolate adjuvant (Sigma), followed by a subcutaneous hyperboost with 10µg of sIL-6Rα. After 3 days, a fusion was performed between splenocytes and the Sp2/0 myeloma fusion partner as previously described (Buell *et al.,* 1998). Subsequent screening of hybridomas was performed on mock transfected CHO cells, or CHO cells expressing mIL-6Rα, using the 8200 cellular detection system (Applied Biosystems, Zug, Switzerland). Positive clones were tested for their capacity to neutralise the IL-6-

dependant proliferation of the murine plasmacytoma cell line, T1165. This was performed by Rami Lissilaa at NovImmune SA, Geneva, Switzerland.

CHAPTER 3

Characterisation of gp130$^{+/+}$ versus gp130$^{F/F}$ mice in the context of experimental endotoxic shock

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3.1 Introduction of the role of STAT1 and STAT3 during inflammation and the hyper-active STAT1/3 mouse model (*gp130*$^{F/F}$)

A wealth of research over at least the last decade investigating the immunomodulatory roles of STAT molecules has revealed that STAT1 is pro-inflammatory, whereas STAT3 can have pro- or anti-inflammatory effects. Evidence for the pro-inflammatory role of STAT1 is derived from observations that mice deficient in SOCS1, the main negative regulator of STAT1, display hyper-activation of STAT1 and develop multi-organ inflammation as well as hypersensitivity to LPS (Alexander *et al.,* 1999). Furthermore, STAT1 deficiency in mice protects against LPS-driven endotoxic shock (Karaghiosoff *et al.,* 2003). The main driver of these pathologies was found to be excessive signalling via the pro-inflammatory cytokine IFNγ, which activates STAT1. The role for STAT3, however, is less clear, due to it acting as a signal transducer for a number of pro- and anti-inflammatory cytokines. Numerous innate immune cytokines including IL-6 and other gp130-acting cytokines (e.g. IL-11, leukemia inhibitory factor, oncostatin-M), the anti-inflammatory cytokine IL-10, and pro-inflammatory type I and II IFNs activate STAT3, which raises the conundrum of the mechanistic basis by which STAT3 can mediate both opposing pro- and anti-inflammatory responses.

In light of the embryonic lethality displayed by *Stat3*$^{-/-}$ mice (Takeda *et al.,* 1997), mouse strains with a conditional deletion of *Stat3* in macrophages/neutrophils or endothelial cells have been employed to identify the cell-type specific role of STAT3 in LPS-induced endotoxic shock (Takeda *et al.,* 1999; Kano *et al.,* 2003). The conditional deletion of *Stat3* in both cell types resulted in an increased susceptibility to LPS, associated with elevated production of pro-inflammatory cytokines which has been attributed to defective IL-10 signalling (Takeda *et al.,* 1999; Kano *et al.,* 2003; Matsukawa *et al.,* 2003). While

these studies imply a key role for STAT3 in mediating the potent anti-inflammatory effects of IL-10 in these cell types, the role of STAT3 in facilitating pro-inflammatory responses of IL-6 and other STAT3-activating cytokines remains ill-defined with current *Stat3* gene knock-out mouse models. Moreover, considering persistent STAT3 activation is a feature of numerous human inflammatory diseases (e.g. human ulcerative colitis, RA) (Shouda *et al.,* 2001; Li *et al.,* 2010), there is a growing need for genetically-defined mouse models displaying hyper-activated levels of endogenous STAT3 via specific activating pathways in order to investigate the mechanisms by which STAT3 promotes the pathogenesis of inflammatory diseases.

To genetically define the regulatory role of IL-6-mediated STAT1 and/or STAT3 signalling during LPS/TLR4-driven inflammation, studies presented in this thesis utilise the *gp130*$^{F/F}$ knock-in mice which demonstrate hyper-activation of STAT1 and STAT3 via the IL-6 family of cytokines, (Tebbutt *et al.,* 2002; Ernst, Jenkins, 2004) as described in Chapter 1. As well as spontaneous multi-organ inflammation (e.g. gastritis, peritonitis), these *gp130*$^{F/F}$ mice display additional pathologies, including gastric inflammation-associated tumours, thrombocytosis, neutrophilia, splenomegaly, and lymphadenopathy (Jenkins a *et al.,* 2005; Jenkins b *et al.,* 2005). In light of the striking inflammatory phenotype of these mice, it seemed reasonable to suggest that the gp130Y$_{757}$F mutation augments activation of key inflammatory signalling pathways. Accordingly, the aim of this section of the project was to characterise the response of the *gp130*$^{F/F}$ mice to LPS, the prototypical pro-inflammatory stimuli which signals via TLR4, and in doing so also determine whether hyper-activation of STAT1 and STAT3 via the gp130 signalling complex had an impact on the inflammatory response.

3.2 *Gp130*$^{F/F}$ mice are hypersensitive to LPS-induced endotoxic shock

The first step of this project was to administer re-purified LPS to mice so as to eliminate any impurities which might activate pathways other than TLR4, which in turn would complicate interpretation of these experiments. Purification involved phenol washes of *Escherichia coli* LPS and reconstitution in endotoxin-free triethylamine buffer, after which, the re-purified LPS was assayed for bioactivity. A sub-lethal dose (for control *gp130*$^{+/+}$ animals) of LPS (4mg/kg) was i.p. injected into both *gp130*$^{+/+}$ and *gp130*$^{F/F}$ mice, and the survival of mice was

monitored over 72hrs. As shown in Fig. 3.1, 100% of the *gp130*$^{+/+}$ control mice survived over 72hrs post-LPS administration. By contrast, *gp130*$^{F/F}$ mice displayed a remarkable hypersensitivity to LPS, with 100% of *gp130*$^{F/F}$ mice failing to survive beyond 72hrs (Fig. 3.1). These results reveal that *gp130*$^{F/F}$ mice were hypersensitive to LPS.

3.3 Hypersensitivity of *gp130*$^{F/F}$ mice to LPS-induced endotoxic shock is independent of sustained leukopenia and thrombocytopenia

Endotoxic shock in high-dose LPS mouse models can be associated with the rapid onset (within hrs) of leukopenia (Hinshaw, 1996), so I examined whether the hypersensitivity of *gp130*$^{F/F}$ mice correlated with marked leukopenia. To identify changes in the white blood cell (WBC) counts in mice challenged with LPS, a time-course experiment was undertaken. This involved administrating *gp130*$^{+/+}$ and *gp130*$^{F/F}$ mice with LPS (4mg/kg) via i.p. injection and collecting whole blood samples at 1.5, 3 and 6hrs post injection for analysis on a Sysmex KX-21N hematology analyser. For baseline measurements, blood was collected prior to injection via submandibular bleeding. Profiling of the circulating WBC count in mice at 1.5hrs after LPS administration revealed a similar drop in blood leukocyte numbers from baseline levels in *gp130*$^{F/F}$ (64%) and *gp130*$^{+/+}$ (58%) mice (Fig. 3.2A). At 3 and 6hrs following LPS exposure, the recovery of blood leukocyte numbers was also comparable between the two genotypes with WBC counts returning to baseline levels at 6hrs (Fig. 3.2A), suggesting the sensitivity of mice to shock was not the result of exacerbated leukopenia.

In a similar vein, the severity of the ensuing shock in response to LPS can also parallel the extent of platelets lost from the blood due to their rapid accumulation in the lungs (Shibazaki *et al.,* 1996). Whole blood samples obtained from the previous experiment were also analysed for platelet numbers using the Sysmex KX-21N hematology analyser. The reduction in circulating platelets from baseline levels at 3hrs following LPS exposure was less pronounced in *gp130*$^{F/F}$ mice (26%) compared to *gp130*$^{+/+}$ mice (45%) (Fig. 3.2B). At 6hrs post-LPS, I also observed a greater recovery of platelet numbers in *gp130*$^{F/F}$ mice (to 85% of baseline) compared to *gp130*$^{+/+}$ mice (70%) (Fig. 3.2B). Therefore, these data strongly imply that the LPS hypersensitivity of *gp130*$^{F/F}$ mice does not correlate with an impaired haematological profile. Consequently, the mortality of these

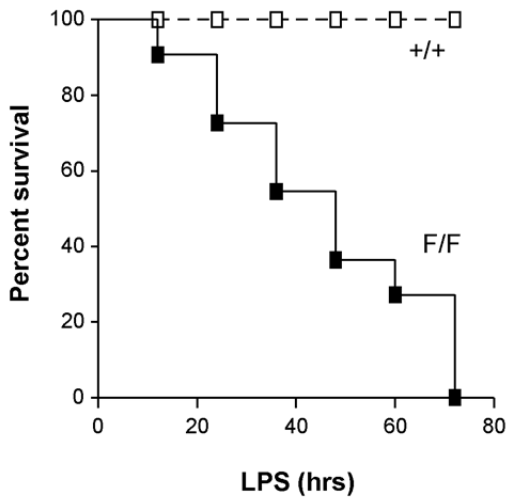

Figure 3.1. *Hypersensitivity of gp130$^{F/F}$ mice to LPS-induced endotoxic shock*. Survival of *gp130$^{+/+}$* (+/+, dashed line, n=9) and *gp130$^{F/F}$* (F/F, solid line, n=11) mice over 72hrs following i.p. administration of 4mg/kg LPS.

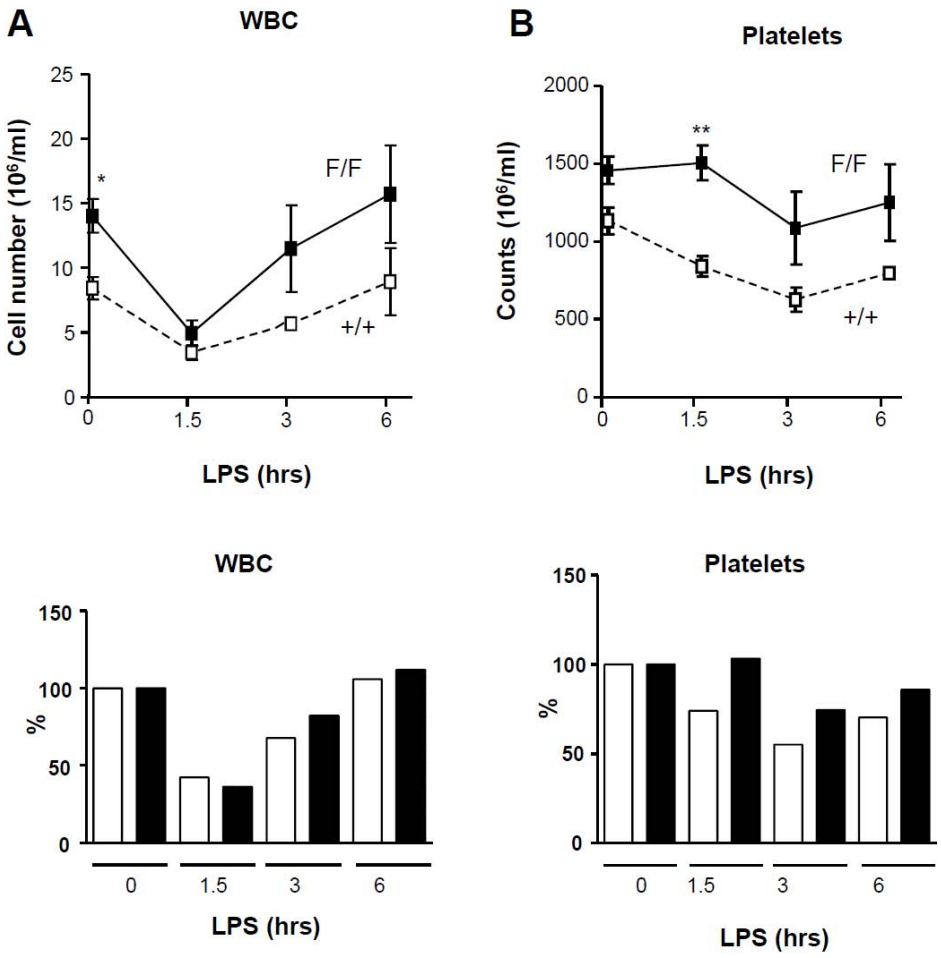

Figure 3.2. *Hypersensitivity of gp130$^{F/F}$ mice to LPS-induced endotoxic shock is independent of sustained leukopenia and thrombocytopenia.* **(A)** Total white blood cell (WBC) and **(B)** platelet counts of *gp130*$^{+/+}$ (+/+, dashed lines, white bars) and *gp130*$^{F/F}$ (F/F, solid lines, black bars) mice at indicated time points after i.p. administration of 4mg/kg LPS, relative to unstimulated samples. Data are from at least 3 mice of each genotype and are expressed as the mean ± SEM. * P<0.05, ** P<0.01, versus data from +/+ mice at the corresponding time point.

mice must be due to alternative mechanisms, potentially the increased production of an array of pro-inflammatory cytokines that overwhelm the host, leading to organ failure.

3.4 LPS hypersensitivity of *gp130*$^{F/F}$ mice correlates with elevated production of IL-6

It is well established that hypersensitivity to LPS is characterised by augmented TLR4-driven pro-inflammatory cytokine production (Galanos, Freudenberg, 1993; Freudenberg *et al.,* 2008). I therefore measured the levels of key TLR4-induced pro-inflammatory cytokines in the serum of mice up to 6hrs post-LPS administration. ELISA assays revealed that the serum concentration of IL-6 in LPS-treated *gp130*$^{F/F}$ mice was significantly elevated by approximately 3.5-fold (1.5hrs) and 2-fold (3 and 6hrs) compared to *gp130*$^{+/+}$ mice (Fig. 3.3A). By contrast, TNFα serum levels were comparable between LPS-challenged *gp130*$^{+/+}$ and *gp130*$^{F/F}$ mice (Fig. 3.3B). The levels of CCL5 in the serum of LPS treated *gp130*$^{F/F}$ mice was significantly reduced by approximately 2-fold at 1.5hrs, not significantly changed at 3hrs and significantly reduced by 1.5-fold at 6hrs post-LPS administration in comparison to *gp130*$^{+/+}$ mice (Fig. 3.3C).

To support these data, the levels of these cytokines in the spleens of the LPS-injected *gp130*$^{F/F}$ and *gp130*$^{+/+}$ mice were next measured by qPCR expression analyses. The spleen was chosen for these analyses as immune cells in this organ play a key role in the immune response. These results confirmed that *IL-6* mRNA was significantly induced to higher levels in *gp130*$^{F/F}$ compared to *gp130*$^{+/+}$ spleen tissues (17-fold at 1.5hrs and 12.5-fold at 3hrs) (Fig 3.4A). By contrast, *TNFα* mRNA levels were comparable between the two genotypes (Fig. 3.4B) as were those for *IL-1β* (Fig. 3.4C). Interestingly, and in contrast to the ELISA (CCL5) data, I observed significantly augmented expression of TLR4/MyD88-independent genes *IFNβ* (2.5-fold and 11-fold at 1.5hrs and 3hrs, respectively) and *CCL5* (7-fold at 6hrs) in LPS-treated *gp130*$^{F/F}$ compared to *gp130*$^{+/+}$ mouse spleen tissue (Fig. 3.4D,E).

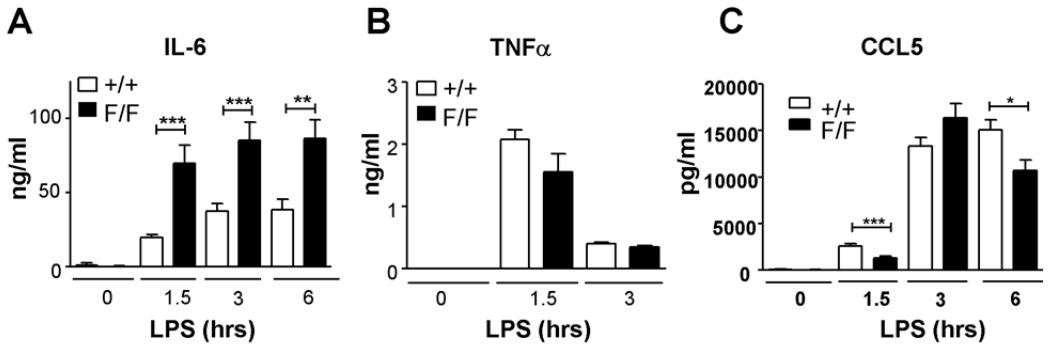

Figure 3.3. *Increased systemic IL-6 levels in gp130$^{F/F}$ mice in response to LPS-induced endotoxic shock.* Serum was collected from *gp130*$^{+/+}$ (+/+, white bars) and *gp130*$^{F/F}$ (F/F, black bars) mice at indicated time points following i.p. administration of 4mg/kg LPS and **(A)** IL-6, **(B)** TNFα, and **(C)** CCL5 ELISAs were performed. Data are from 3 mice of each genotype and are expressed as the mean ± SEM. **P*<0.05, ***P*<0.01, ****P*<0.001 versus data from +/+ mice at the corresponding time points.

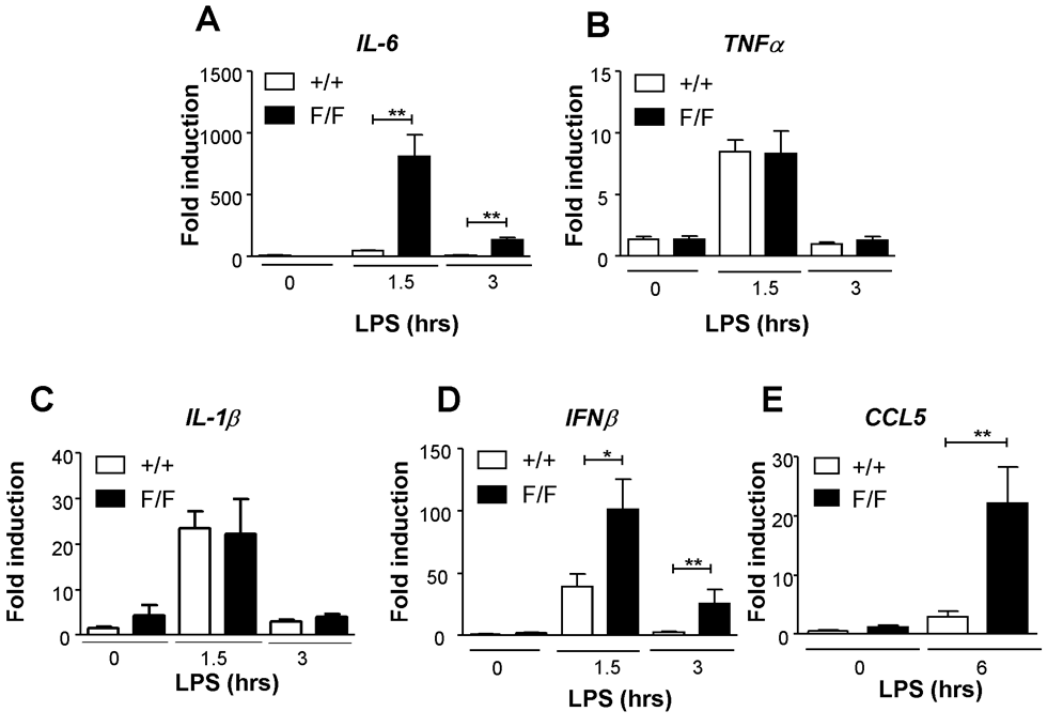

Figure 3.4. *Increase in specific pro-inflammatory cytokines in gp130$^{F/F}$ spleen tissue in response to LPS-induced endotoxic shock*. qPCR analyses on *gp130*$^{+/+}$ (+/+, white bars) and *gp130*$^{F/F}$ (F/F, black bars) mice for **(A)** *IL-6,* **(B)** *TNFα,* **(C)** *IL-1β* **(D)** *IFNβ* and **(E)** *CCL5* gene expression on cDNA derived from total RNA prepared from spleen tissue at indicated time points following i.p. administration of 4mg/kg of LPS. Expression data from 3 samples per genotype are shown following normalisation to *18S* expression and are presented from replicate analysis as the mean fold induction ± SEM relative to unstimulated samples. *P<0.05, **P<0.01, versus data from +/+ mice at the corresponding time points.

3.5 Local immune responses in the *gp130*$^{F/F}$ mice are augmented after LPS administration

An additional contributing factor to the hypersensitivity of the *gp130*$^{F/F}$ mice to LPS may be caused by alterations to the profile of infiltrating immune/inflammatory cells. Therefore, to examine the local inflammatory cellular profiles exhibited by *gp130*$^{+/+}$ and *gp130*$^{F/F}$ mice following LPS (4mg/kg) administration, mice were i.p. injected with LPS and peritoneal lavage was collected at 0, 1.5, 3 and 6hrs. The lavage fluid was then separated by centrifugation and cellular differentials were assessed by counting of cells on Wright-Giemsa stained slides using light microscopy. The results indicated that there was a slight (but not significant) increase in the numbers of basophils in the *gp130*$^{F/F}$ when compared to *gp130*$^{+/+}$ mice before and after LPS administration (Fig. 3.5A). In addition, there was an increase in the numbers of lymphocytes and macrophages consistently in pre- and post-LPS administration in the *gp130*$^{F/F}$ lavage fluid in comparison to *gp130*$^{+/+}$ controls (Fig. 3.5B,C). Notably, I observed that LPS induced a 4-fold greater influx of neutrophils into the peritoneal cavity of *gp130*$^{F/F}$ mice at 3hrs (Fig. 3.5D), which coincided with a significantly increased level (2-fold) in the production of the neutrophil-attractant KC/CXCL1 in the peritoneal lavage fluid of *gp130*$^{F/F}$ mice in response to LPS at 1.5hrs (Fig. 3.6A). The increase in CXCL1 levels in the *gp130*$^{F/F}$ lavage is not significantly different from *gp130*$^{+/+}$ controls at 3 or 6hrs (Fig. 3.6A).

To examine whether local TLR4-driven IL-6 production was also heightened in *gp130*$^{F/F}$ mice, I also measured the production of IL-6 locally at the site of LPS injection (peritoneal cavity). Indeed, IL-6 protein levels in the peritoneal lavage fluid of LPS-challenged *gp130*$^{F/F}$ mice were significantly elevated at 1.5hrs (4-fold), 3hrs (3-fold) and 6hrs (34-fold) compared to *gp130*$^{+/+}$ mice (Fig. 3.6B).

Collectively, the above data identify that a subset of TLR4-driven local and systemic inflammatory responses *in vivo* are augmented in *gp130*$^{F/F}$ mice.

3.6 LPS hypersensitivity of *gp130*$^{F/F}$ mice correlates with elevated activation of STAT3 in the liver

Among the numerous intracellular signalling cascades activated by IL-6 via the signal-transducing gp130 receptor subunit, the predominant pathway

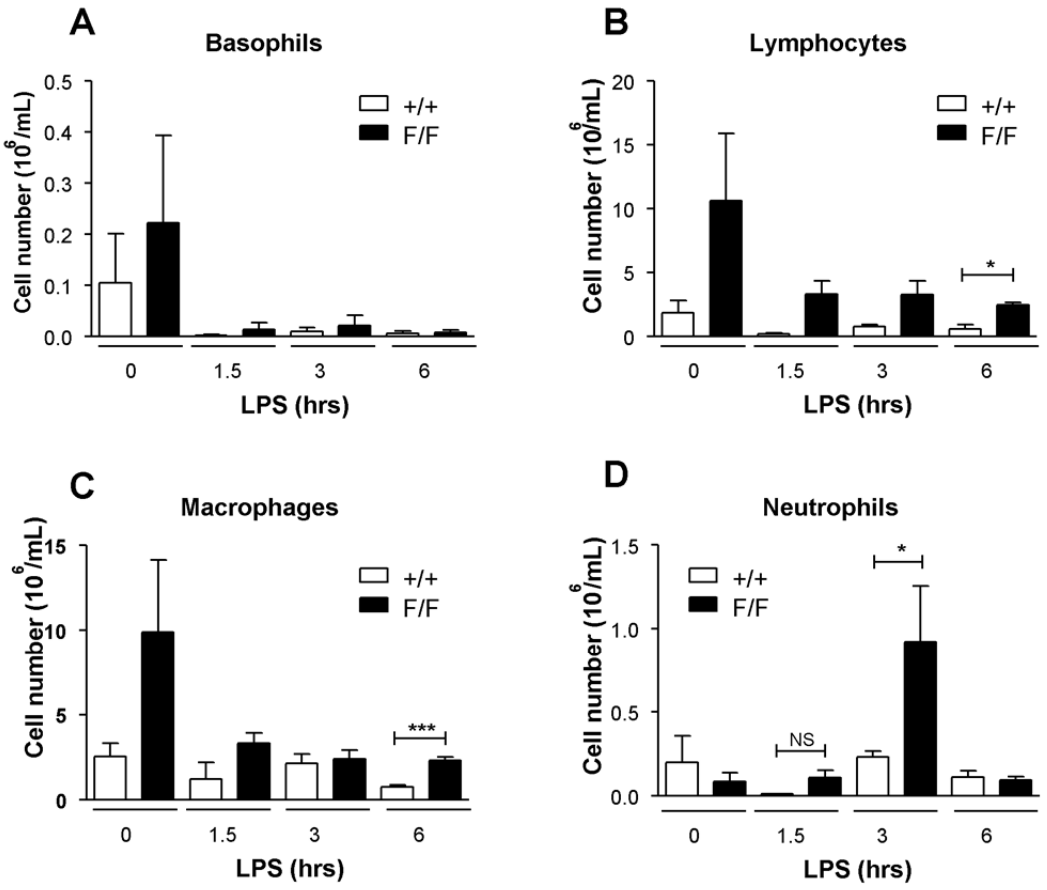

Figure 3.5. *Altered cellular blood profile of gp130$^{F/F}$ mice after LPS-induced endotoxic shock.* White blood cell differentials of peritoneal lavage fluid of *gp130*$^{+/+}$ (+/+, white bars) and *gp130*$^{F/F}$ (F/F, black bars) at indicated time points after i.p. administration of 4mg/kg LPS; **(A)** basophils, **(B)** lymphocytes, **(C)** macrophages and **(D)** neutrophils. Data are from at least 3 mice of each genotype and are expressed as the mean ± SEM. *P<0.05, ***P<0.001 versus data from +/+ mice at the corresponding time point.

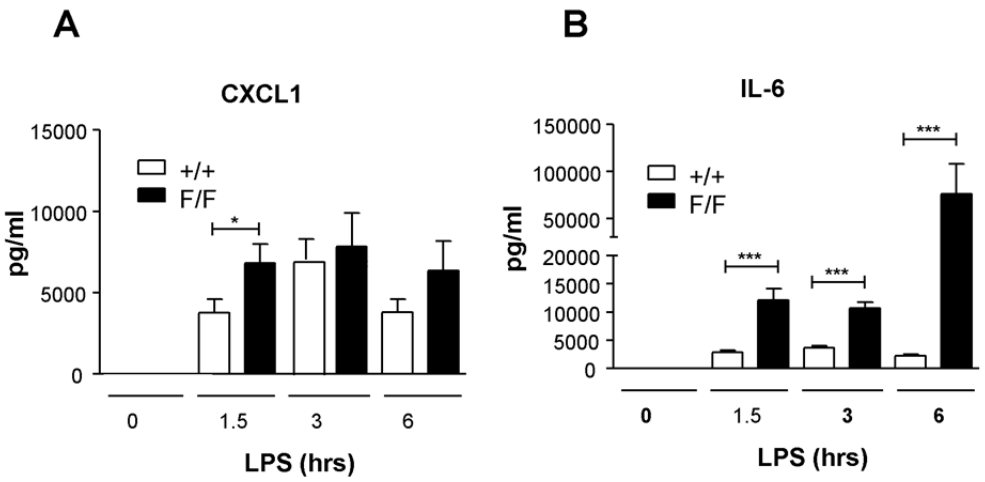

Figure 3.6. *Increased local CXCL1 and IL-6 production in gp130$^{F/F}$ mice in response to LPS-induced endotoxic shock.* Peritoneal lavage fluid was collected from *gp130*$^{+/+}$ (+/+, white bars) and *gp130*$^{F/F}$ (F/F, black bars) mice at the indicated time points following i.p. administration of 4mg/kg LPS, and ELISA for **(A)** CXCL1 and **(B)** IL-6 production were performed. Data are from 3 mice of each genotype and are expressed as the mean ± SEM . **P*<0.05, ****P<0.001* versus data from +/+ mice at the corresponding time points.

activated is Jak2-STAT3 (Heinrich *et al.,* 2003). The observation that LPS augmented IL-6 production in *gp130*$^{F/F}$ mice, therefore, led me to initially investigate whether STAT3 was systemically hyper-activated by LPS in *gp130*$^{F/F}$ mice. In addition to analysing the spleen as a surrogate source for immune cells, the liver was also analysed as it is the site for activation of IL-6/STAT3 driven acute phase response genes (Moshage, 1997). Liver and spleen were collected from LPS-injected (4 mg/kg) *gp130*$^{F/F}$ and *gp130*$^{+/+}$ mice and lysates prepared for Western blot analysis for the activated tyrosine phosphorylated form of STAT3 (pY-STAT3). These results demonstrated that STAT3 activation was exaggerated in spleen and liver tissue of *gp130*$^{F/F}$ compared to *gp130*$^{+/+}$ mice in response to LPS at all time points analysed (i.e. 1.5, 3 and 6hrs) albeit not significantly in the liver (Fig. 3.7A,B, 3.8A,B). In addition, the levels of total STAT3 were also increased in the *gp130*$^{F/F}$ spleen and liver lysates (Fig. 3.7A,B, 3.8A,B), consistent with previous observations (Jenkins b *et al.,* 2005) along with STAT3 being a target of itself (reviewed in Yang, Stark, 2008). To investigate whether STAT1 activation was augmented in the *gp130*$^{F/F}$ liver after LPS administration, levels of LPS-induced STAT1 tyrosine phosphorylation (pY-STAT1) were also examined by Western blotting and found to be augmented in comparison to *gp130*$^{+/+}$ controls (Fig. 3.9). Therefore, this hyper-activation of STAT1 and STAT3 occurs concurrently with LPS-induced IL-6 production, possibly contributing to the pro-inflammatory phenotype of the *gp130*$^{F/F}$ mice.

3.7 Hyper-responsiveness of *gp130*$^{F/F}$ mice to LPS is not associated with impaired production and/or activity of IL-10

IL-10, which also activates STAT3, is a potent anti-inflammatory cytokine that is induced by LPS to negate the inflammatory response by suppressing the release of pro-inflammatory mediators (reviewed in Moore *et al.,* 2001). I therefore investigated whether the hyper-responsiveness of *gp130*$^{F/F}$ mice to LPS could be explained by the impaired production and/or biological activity of IL-10. As shown in Figure 3.10, serum levels of IL-10 in LPS-injected *gp130*$^{+/+}$ and *gp130*$^{F/F}$ mice were comparable. Furthermore, *IL-10* gene expression in spleen tissue from LPS-challenged *gp130*$^{F/F}$ mice was significantly elevated at all measured time points compared to *gp130*$^{+/+}$ mice (Fig. 3.11), suggesting that LPS/TLR4-induced IL-10 production was not impaired in *gp130*$^{F/F}$ mice.

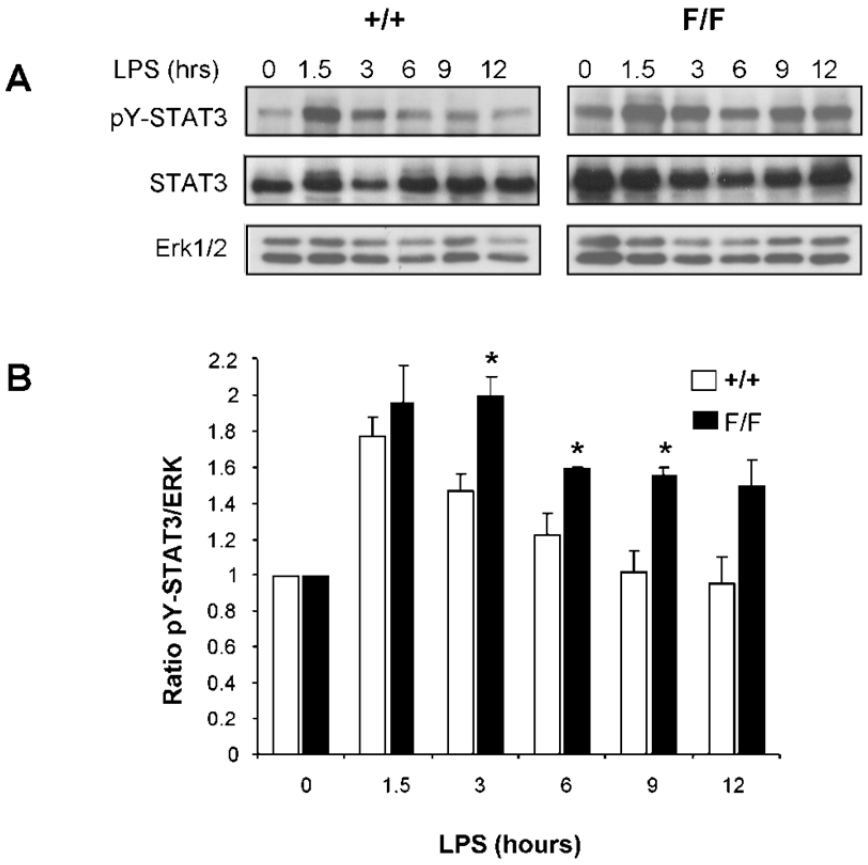

Figure 3.7. *Increased LPS-induced STAT3 tyrosine phosphorylation in spleen lysates from gp130$^{F/F}$ mice.* **(A)** *Gp130$^{+/+}$ (+/+,* white bars*)* and *gp130$^{F/F}$ (*F/F, black bars*)* mice were i.p. administered with LPS (4mg/kg), and at defined intervals STAT3 tyrosine phosphorylation, total STAT3 and Erk1/2 levels were measured by immunoblotting. Results shown are representative of 3 mice. **(B)** Densitometric quantitation of tyrosine-phosphorylated STAT3 in each of 3 representative samples per genotype per time point was performed and normalised against Erk1/2 protein levels. Data are presented as the mean fold induction ± SEM relative to expression in +/+ mice at the corresponding time point. **P<0.05.* (results from Ludwig Institute for Cancer Research- B.J Jenkins).

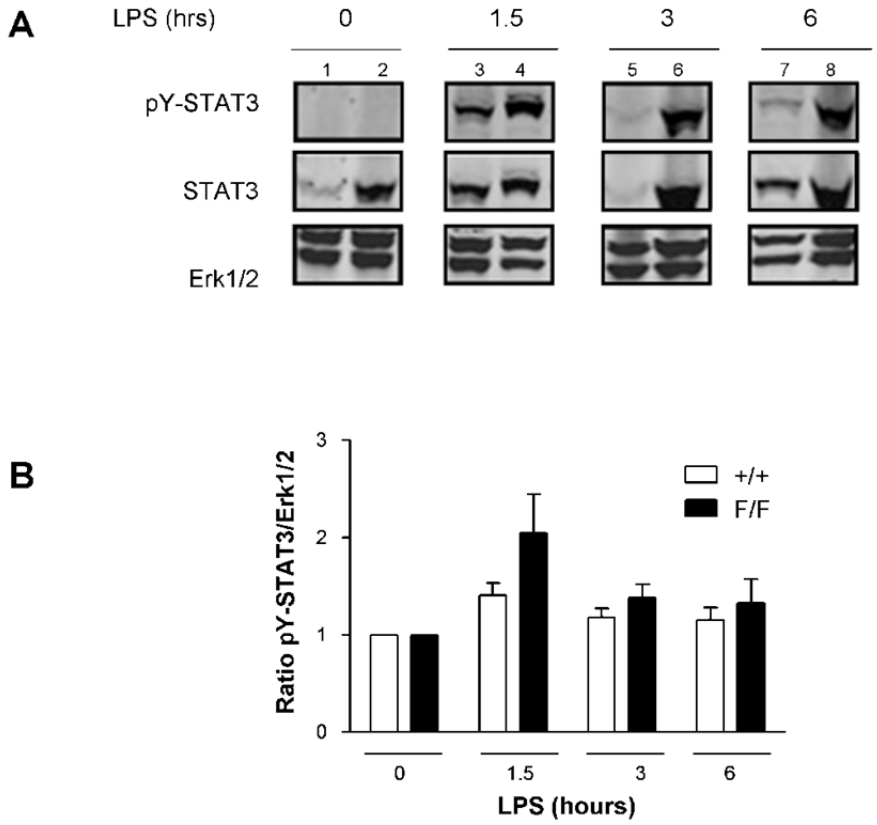

Figure 3.8. *Increased LPS-induced STAT3 tyrosine phosphorylation in liver lysates from gp130$^{F/F}$ mice* **(A)** *Gp130$^{+/+}$* (+/+; lanes 1, 3, 5, 7) and *gp130$^{F/F}$* (F/F; lanes 2, 4, 6, 8), mice were i.p. administered with LPS (4mg/kg), and at defined intervals STAT3 tyrosine phosphorylation, total STAT3 and Erk1/2 levels were measured by immunoblotting. Results shown are representative of 3 mice per genotype per time point. **(B)** Densitometric quantitation of tyrosine-phosphorylated STAT3 in each of 3 representative samples per genotype per time point was performed and normalised against Erk1/2 protein levels.

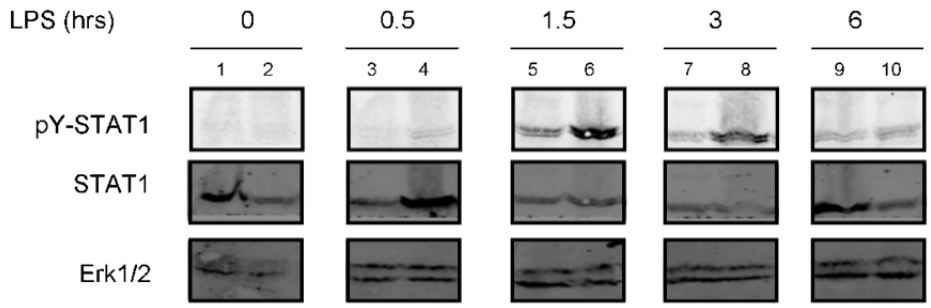

Figure 3.9. *Increased LPS-induced STAT1 tyrosine phosphorylation in liver lysates from gp130$^{F/F}$ mice. Gp130$^{+/+}$* (+/+; lanes 1, 3, 5, 7, 9) and *gp130$^{F/F}$* (F/F; lanes 2, 4, 6, 8, 10), mice were i.p. administered with LPS (4mg/kg), and at defined intervals STAT1 tyrosine phosphorylation, total STAT1 and Erk1/2 levels were measured by immunoblotting. Results shown are representative of 3 mice per genotype per time point.

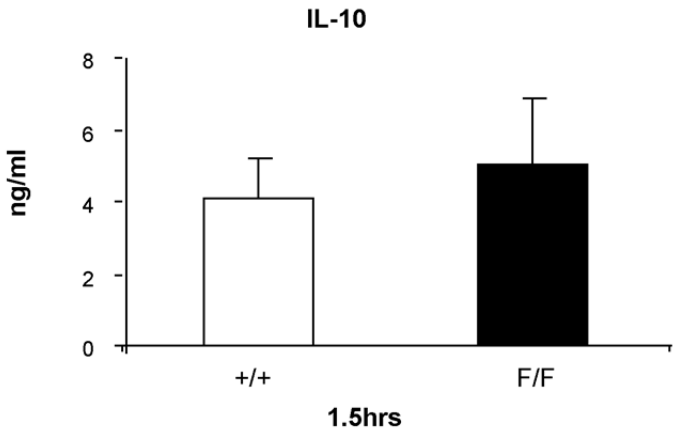

Figure 3.10. *Production of IL-10 is unaffected in gp130$^{F/F}$ mice following LPS-induced endotoxic shock.* IL-10 ELISA was performed on serum collected from *gp130*$^{+/+}$ (+/+, white bars) and *gp130*$^{F/F}$ (F/F, black bars) mice at 1.5 hrs following i.p. administration of 4mg/kg LPS. Data are from 5 mice of each genotype, and are expressed as the mean ± SEM. (results from Ludwig Institute for Cancer Research- B.J Jenkins).

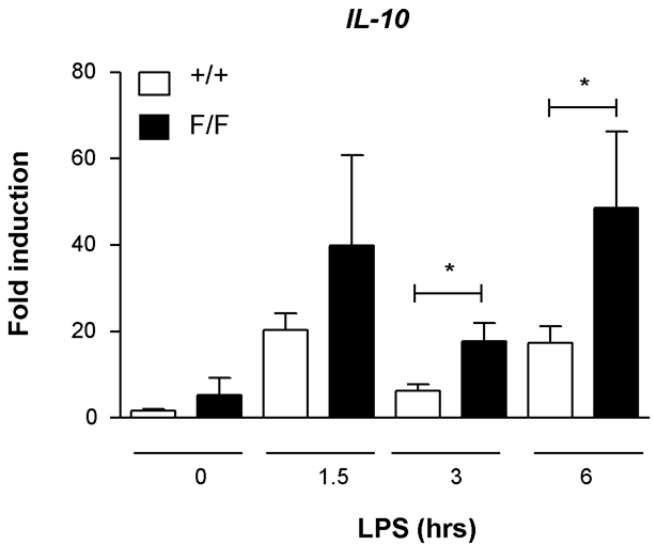

Figure 3.11. *Production of IL-10 is unaffected in spleen of gp130$^{F/F}$ mice following LPS-induced endotoxic shock.* Spleen tissue from g*p130*$^{+/+}$ (*+/+,* white bars*)* and *gp130*$^{F/F}$ *(*F/F, black bars) mice following i.p. administration of 4mg/kg LPS was performed at the indicated time points and qPCR analyses of *IL-10* gene expression was performed. Expression data from 3 samples per genotype are shown following normalisation to *18S* expression, and are presented from replicate analysis as the mean fold induction ± SEM. **P*<0.05 versus data from +/+ mice at the corresponding time points.

I next determined whether the ability of IL-10 to inhibit TLR4-driven pro-inflammatory cytokine production was impaired by the gp130Y_{757}F mutation. However, treatment of *gp130*$^{+/+}$ and *gp130*$^{F/F}$ peritoneal macrophages with LPS (1ng/ml) in the presence of IL-10 (100ng/ml) led to comparable inhibition of LPS-induced *IL-6* and *TNF*α mRNA expression (Fig. 3.12A,B).

To identify if IL-10-induced STAT3 activation was altered in the *gp130*$^{F/F}$ macrophages, flow cytometric analyses of pY-STAT3 levels following IL-10-stimulation of *gp130*$^{+/+}$ and *gp130*$^{F/F}$ macrophages were performed. The results revealed a comparable induction in the intracellular levels of pY-STAT3 in response to IL-10, thus confirming that IL-10 signalling was intact in *gp130*$^{F/F}$ cells (Fig. 3.13A). As a positive control for this data, macrophages were also stimulated with IL-6 (100ng/ml) and levels of pY-STAT3 levels assessed. There were increased pY-STAT3 levels in IL-6-treated *gp130*$^{F/F}$ macrophages (Fig. 3.13B), as I would expect since IL-6-dependent STAT3 activation is elevated in *gp130*$^{F/F}$ mice (Tebbutt *et al.,* 2002; Ernst, Jenkins, 2004; Jenkins b *et al.,* 2005).

Collectively, these data therefore suggest that augmented TLR4-mediated inflammatory responses in *gp130*$^{F/F}$ mice are not due to the impaired production or activity of IL-10.

3.8 Discussion

The data presented in this chapter demonstrate that *gp130*$^{F/F}$ mice are hypersensitive to LPS/TLR4-driven local and systemic inflammatory responses, as evidenced by, for example, the augmented local and systemic production of certain pro-inflammatory cytokines, especially IL-6. Importantly, these *gp130*$^{F/F}$ mice build upon the current paucity of genetically-defined mouse models to directly investigate the mechanistic basis by which over-activated endogenous IL-6/STAT3 signalling promotes chronic inflammation. For instance, *Socs3*$^{+/-}$ mice, which presumably would display global elevated IL-6/STAT3 signalling akin to *gp130*$^{F/F}$ mice due to deletion of one allele of the IL-6 signalling negative regulator, are also hypersensitive to LPS (Yasukawa *et al.,* 2003). This observation is supported by studies demonstrating that global over-expression of SOCS3 (and therefore suppression of IL-6/STAT3 signalling) *in vivo* either via gene delivery (Fang *et al.,* 2005) or intracellular protein delivery (Jo *et al.,* 2005) protects mice against LPS challenge. These studies are therefore consistent with

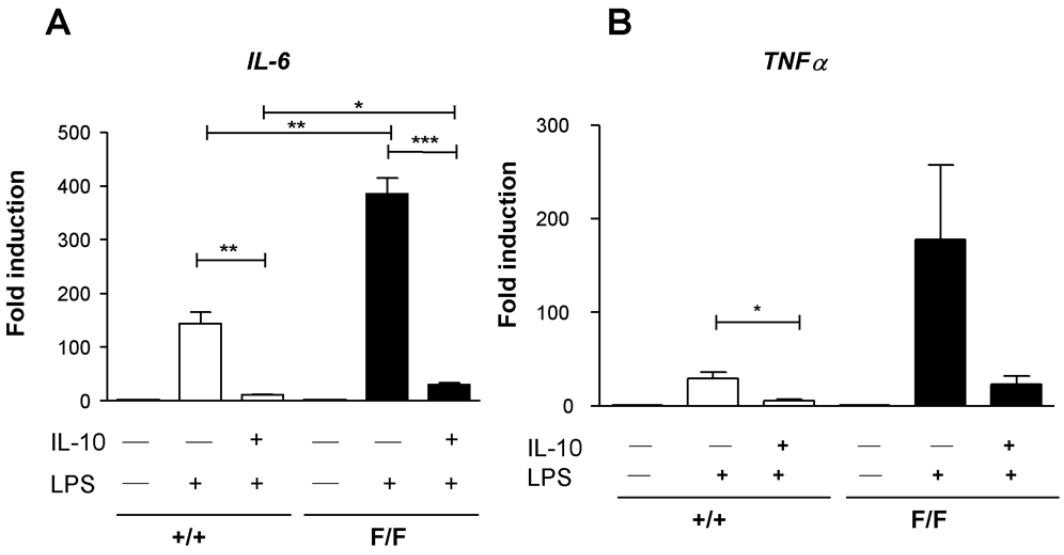

Figure 3.12. *Activity of IL-10 is unaffected in gp130$^{F/F}$ mice following LPS stimulation.* Peritoneal macrophages were collected from *gp130*$^{+/+}$ (+/+, white bars) and *gp130*$^{F/F}$ (F/F, black bars) mice and stimulated for 0 and 3hrs with 1ng/ml of LPS alone or in combination with IL-10 (100ng/ml) and qPCR analyses of **(A)** *IL-6* and **(B)** *TNFα* gene expression was performed. Expression data from 3 samples per genotype are shown following normalisation to *18S* expression, and are presented from replicate analysis as the mean fold induction ± SEM relative to unstimulated samples. **P*<0.05, ***P*<0.01, ****P*<0.001.

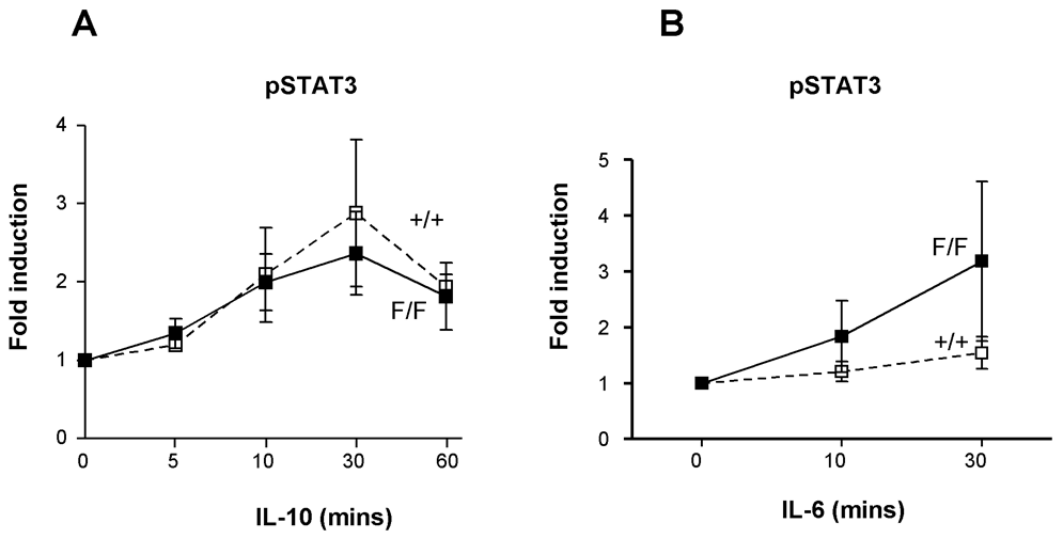

Figure 3.13. *Activity of IL-10 is unaffected in gp130$^{F/F}$ mice.* **(A)** *gp130$^{+/+}$* (+/+, dashed line) and *gp130$^{F/F}$* (F/F, solid line) macrophages were stimulated with IL-10 (100ng/ml) or **(B)** IL-6 (100ng/ml) and phospho-flow cytometry was performed to assess the level of tyrosine phosphorylated STAT3 (pSTAT3). Data are from 3 mice per genotype and are expressed as the mean fold induction ± SEM relative to unstimulated samples.

my own observations that global elevated IL-6 production and STAT3 activation promotes LPS hypersensitivity.

Contradictory to these findings, however, are studies which have indicated an anti-inflammatory role for hepatic gp130 signalling in septic inflammatory responses (Sander *et al.,* 2010). In this regard, mice with hepatocyte-specific deletion of gp130 were highly susceptible to sepsis associated mortality induced by cecal ligation and puncture (CLP; non-TLR4 specific) and were characterised by elevated systemic levels of IL-6 and TNFα. Conversely, knock-in mice harbouring the $gp130_{Y757F}$ mutation exclusively in hepatocytes were protected against CLP-induced mortality (Sander *et al.,* 2010). However, the mice in the latter experiments were not challenged with LPS, thus preventing a direct comparison being made with my study on $gp130^{F/F}$ mice. Furthermore, despite the observation that STAT3 was reduced in the hepatic gp130 knock-out mice (which is a deletion mutant where exon 16 in the transmembrane region is flanked by LoxP sites) and elevated in the liver lysates of the hepatocyte-specific gp130 mutant mice, the authors didn't investigate a causal role for STAT3 signalling via IL-6 contributing to the septic phenotype. An obvious explanation for the disparity between these observations and those of my current study is the differences in experimental models (polymicrobial, non-TLR4-specific versus LPS, TLR4-specific) used to trigger sepsis. It is also noteworthy that another conditional mouse model in which *Socs3* was deleted in macrophages, thus indirectly leading to elevated macrophage-specific IL-6/STAT3 activation, was shown to be largely protected against LPS-mediated endotoxic shock (Yasukawa *et al.,* 2003). A possible explanation for why this finding contradicts my current study is most likely the use by Yasukawa and colleagues of the low dose LPS model, which in contrast to my study on $gp130^{F/F}$ mice (high dose LPS model) involves macrophage-mediated acute liver injury in mice upon D-galactosamine (D-Gal) sensitisation (Yasukawa *et al.,* 2003). Nonetheless, collectively these studies involving conditional mouse mutants imply that the artificial compartmentalisation of IL-6/STAT3 hyper-activation to a specific subset of mutant cells (e.g. SOCS3-deficient macrophages or $gp130_{Y757}$-bearing hepatocytes only) may elicit an alternate anti-inflammatory environment in response to systemic inflammatory insults (Yasukawa *et al.,* 2003; Sander *et al.,* 2010).

LPS can induce activation of STATs through the activation of the type I or II IFN signalling pathway (reviewed in, Heinrich *et al.,* 1998; Kovarik *et al.,* 1998), the IL-6 signalling cascade (reviewed in, Heinrich *et al.,* 1998), or possibly through more direct mechanisms (Rhee *et al.,* 2003; Samavati *et al.,* 2009). Consequently, STATs can then behave as transcription factors, and activate target gene expression (reviewed in, Heinrich *et al.,* 1998). This activation of STATs is via tyrosine or serine phosphorylation, however, STATs have also shown to activate gene transcription in their non-phosphorylated state (Yang *et al.,* 2007; Yang, Stark, 2008). My studies illustrated the LPS-induced STAT1 and STAT3 tyrosine phosphorylation (i.e. activation) in *gp130*$^{F/F}$ and *gp130*$^{+/+}$ mice (liver and spleen). These results are consistent with previous studies which show STAT1 and STAT3 are activated following LPS administration in liver nuclear extracts from mice, and are consistent with the role of activated STAT3 in the acute phase response in the liver (Alonzi *et al.,* 2001). Interestingly, this LPS-induced STAT1/3 tyrosine phosphorylation was augmented and sustained in the *gp130*$^{F/F}$ mice in my studies, which could be a result of the observed augmented systemic IL-6 production in these mice. It is important to note that previous studies on these mice observed an augmented STAT1 and STAT3 tyrosine phosphorylation following IL-6 stimulation in the liver in comparison to *gp130*$^{+/+}$ controls (Jenkins b *et al.,* 2005). Limited attempts during my studies to assess serine phosphorylation in the g*p130*$^{F/F}$ and *gp130*$^{+/+}$ mice (liver and spleen) have been unsuccessful. Notably, previous results show that the levels of STAT3 serine phosphorylation following IL-6 stimulation in the liver is not thought to be affected by the gp130$_{Y757F}$ mutation (Jenkins b *et al.,* 2005). However, LPS may have different kinetics, so future studies on LPS-induced serine phosphorylation in the *gp130*$^{F/F}$ liver would be necessary. Moreover, my results also show an increase in total STAT3 protein levels in the *gp130*$^{F/F}$ liver lysates following LPS administration. As mentioned in Chapter 1, non-phosphorylated STAT3 co-operates with NF-κB to bind to the promoter of pro-inflammatory genes such as IL-6 to induce their transcription (Yang *et al.,* 2007). Consequently, this increase in total STAT3 in the *gp130*$^{F/F}$ mice may be contributing to the LPS-induced IL-6 induction and LPS hypersensitivity in these mice. In addition, non-phosphorylated STAT1 can act as a transcription factor (Marg *et al.,* 2004; Yang, Stark, 2008) and it has been shown to exist in this form in the nucleus independent of stimulation

(Yang, Stark, 2008). Interestingly, my results do not indicate an elevation in total STAT1 levels in the *gp130*$^{F/F}$ liver lysates in comparison to *gp130*$^{F/F}$ controls following LPS administration. This may be due to STAT3 being activated on a greater number of tyrosine gp130 residues than STAT1 following IL-6 stimulation (which in this case is triggered by LPS) (Stahl *et al.,* 1995; Gerhartz *et al.,* 1996; Kuropatwinski *et al.,* 1997; Heinrich *et al.,* 1998; reviewed in Heinrich *et al.,* 2003). This would result in a greater amount of activated STAT3 relative to STAT1, and consequently, a stronger induction of *Stat3* than *Stat1* gene expression. In addition, it has been shown that STAT1 requires phosphorylation for its interaction with importin-α5 and consequential nuclear import (Sekimoto *et al.,* 1996; Sekimoto *et al.,* 1997), whereas STAT3 binds to importin-α3 and importin-α6 independently of its phosphorylation (Liu *et al.,* 2005). Consequently, the increase in non-phosphorylated STAT3 could further upregulate its own expression more readily than STAT1 in the livers of the LPS administered *gp130*$^{F/F}$ mice.

Another important finding of this chapter was the preferential upregulation of IL-6 after LPS stimulation compared to TNFα in the *gp130*$^{F/F}$ mice. Although the mechanistic rationale for this observation remains unclear, it is likely to reflect subtle differences in the transcriptional regulation of specific pro-inflammatory genes produced via TLR4 signalling cascades. For instance, activation of p38 MAPK is required for the LPS/TLR4-induced expression of TNFα, but not IL-6 (Horwood *et al.,* 2006). Moreover, consistent with *in vivo* data, *in vitro* studies have shown that blocking STAT3 activity preferentially inhibits LPS-mediated IL-1β and IL-6 production, but not TNFα, in RAW264.7 cells (Samavati *et al.,* 2009), and STAT3 activation does not directly regulate LPS-induced TNFα production in human monocytes (Prele *et al.,* 2007). Furthermore, it has been shown in human monocytes that IL-6 inhibits TNFα production in response to LPS (Aderka *et al.,* 1989). This form of regulation may also explain, at least in part, why TNFα is not increased, but rather maintained at *gp130*$^{+/+}$ levels, in the serum and spleen of LPS treated *gp130*$^{F/F}$ mice where IL-6 levels are increased.

Another explanation for the differential regulation of IL-6 and TNFα could be due to alternate NF-κB heterodimer combinations, which affect the activation of the canonical (p50/RelA(p65) and p50/c-Rel) and the non-canonical

(p52/RelB) NF-κB pathways. These pathways can then have differing effects on the regulation of certain LPS-induced cytokines after LPS. For instance, p50 knock-out macrophages (which do not signal via the canonical pathway) had reduced IL-6 and IL-12 production in response to LPS stimulation (Sha *et al.,* 1995; Bohuslav *et al.,* 1998; Kanters *et al.,* 2004), whereas TNFα responses remained unaltered. This, however, contrasted the reduced LPS-induced TNFα expression observed in p50 knock-out DCs, (Lamhamedi-Cherradi *et al.,* 2003), indicating this mechanism is cell type specific. In addition, macrophages from molecule containing ankyrin repeats induced by LPS (MAIL)-deficient mice have ablated production of IL-6 following LPS stimulation (Yamamoto *et al.,* 2004), and MAIL-deficient monocytes have suppressed production of LPS-induced IL-6 (Seshadri *et al.,* 2009). This is due to MAIL being a positive regulator of LPS-induced IL-6 production by binding to the p50 subunit of NF-κB in both humans and mice (Yamamoto *et al.,* 2004; Seshadri *et al.,* 2009). Therefore, it is likely that the specific composition of NF-κB heterodimers in the *gp130*$^{F/F}$ mice, and which cell type is being observed, could also impact on the expression of certain genes. While not within the scope of this study, the role of the canonical and non-canonical NF-κB pathways in the *gp130*$^{F/F}$ mice may be addressed by performing electrophoretic mobility shift assay (EMSA)s/supershift assays on the nuclear extracts of *gp130*$^{+/+}$ and *gp130*$^{F/F}$ macrophages that have been stimulated with/without LPS using a probe for the NF-κB consensus sequence, in addition to antibodies against NF-κB p50 and p65 subunits. Moreover, relative amounts of the NF-κB subunits in these mice may be assessed using Western blotting. My studies did attempt to determine NF-κB activation and IκBα levels by Western blotting in the *gp130*$^{+/+}$ and *gp130*$^{F/F}$ mice following LPS administration, however, the results were inconclusive.

My study also provided evidence that *gp130*$^{F/F}$ mice had basal thrombocytosis, which is in agreement with previous studies indicating that *gp130*$^{F/F}$ mice had haemopoietic abnormalities which were STAT3 dependant (Jenkins b *et al.,* 2005). My group has previously showed that *gp130*$^{F/F}$ mice surpass the STAT3 threshold necessary for megakaryocyte expansion from immature progenitor cells, since circulating platelets from *gp130*$^{F/F}$ mice were significantly elevated compared to *gp130*$^{+/+}$ mice (Jenkins b *et al.,* 2005).

However, further increases in the severity of thrombocytosis post-LPS administration were not evident, ruling this condition out as the detrimental culprit during *gp130*$^{F/F}$ LPS hypersensitivity. Consequently, it was interesting to compare differences in local cellular responses between genotypes pre- and post-LPS administration.

With respect to local cellular responses to LPS, an increase in neutrophils in the *gp130*$^{F/F}$ peritoneal lavage compares favourably with previous studies indicating an initial peak in neutrophil number in the *gp130*$^{F/F}$ peritoneal cavity following *Staphylococcus epidermidis* cell-free supernantant (SES)-induced peritoneal inflammation (Fielding *et al.,* 2008). This was followed by a more rapid clearance of neutrophils coincident with the down-regulation of CXCL1/KC, a key neutrophil chemo-attractant, in comparison to *gp130*$^{+/+}$ controls (Fielding *et al.,* 2008). This process was found to be driven by IL-6 via STAT3, as *gp130*$^{F/F}$:*IL-6*$^{-/-}$ and *gp130*$^{F/F}$:*Stat3*$^{+/-}$ mice corrected the rapid clearance of neutrophils and reduction in CXCL1 production. Furthermore, the proposed molecular basis for this finding was the ability of IL-6/STAT3 to regulate leukocyte infiltration (and hence neutrophil clearance) by suppressing the production of CXCL1 and augmenting neutrophil apoptosis (Fielding *et al.,* 2008). The rapid clearance of neutrophils in *gp130*$^{F/F}$ mice observed after SES treatment was not observed in the current studies in response to LPS, possibly due to mechanistic differences (e.g. dose, kinetics) resulting from different models of inflammation (i.e. SES which activates TLR2- and possibly other PRRs- versus re-purified LPS which only activates TLR4). Such differences most likely also explain the contrast in levels of CXCL1 following SES (reduced in *gp130*$^{F/F}$ mice at 3hrs in comparison to *gp130*$^{+/+}$ controls) (Fielding *et al.,* 2008) and LPS (comparable between *gp130*$^{F/F}$ and *gp130*$^{+/+}$ mice at 3hrs). Overall, however, the increased numbers of *gp130*$^{F/F}$ macrophages and lymphocytes following LPS administration could be contributing to damage of the inflamed tissues and the observed augmented pro-inflammatory cytokine (i.e. IL-6) production. Interestingly, there were increased macrophages and lymphocytes basally in the *gp130*$^{F/F}$ mice, perhaps priming them for an increased inflammatory response following LPS administration.

In this chapter it was also important to address the role of IL-10 in the hypersensitivity of the *gp130*$^{F/F}$ mice to LPS. In particular, it is important to note

that gp130$^{F/F}$ mice show increased basal expression of *Socs3* in the liver (Jenkins b *et al.,* 2005) and there is evidence showing that SOCS3 may negatively regulate IL-10 anti-inflammatory signalling (Berlato *et al.,* 2002). However, analysis of the production of IL-10 and the ability of IL-10 to suppress cytokine expression in response to LPS were unaffected in macrophages derived from *gp130*$^{F/F}$ mice compared to *gp130*$^{+/+}$ mice, thus eliminating impaired IL-10-mediated anti-inflammatory activity as contributing to the LPS hypersensitivity phenotype of *gp130*$^{F/F}$ mice.

Of further interest would be the investigation of potential impairment of other anti-inflammatory cytokines such as IL-4, IL-13 and TFGβ in the *gp130*$^{F/F}$ mice, following LPS administration. This is especially relevant considering the IL-4/IL-13 receptor may signal via STAT3 (Orchansky *et al.,* 1999) and that altering the amount of STAT activation (as is evident in *gp130*$^{F/F}$ mice) during cytokine signalling can affect their function, e.g. IL-6 signalling can activate IFN specific genes when *Stat3* is ablated (Costa-Pereira *et al.,* 2002). Therefore the hyper-activation of STAT3 in *gp130*$^{F/F}$ mice could be potentially altering the output of these signalling pathways during inflammation.

Collectively, the results from this Chapter indicate that *gp130*$^{F/F}$ mice are hypersensitive to LPS and that there is elevated production of specific pro-inflammatory cytokines, in particular IL-6, both systemically and locally in comparison to *gp130*$^{+/+}$ controls. These mice also have an altered haematological profile, however, this did not appear to contribute to LPS hypersensitivity. The augmented production of pro-inflammatory cytokines was not a result of impaired anti-inflammatory actions of IL-10, indicating that the observed hypersensitivity was most likely due to aberrant signalling via IL-6/STAT3/STAT1. Considering the consistent increase in IL-6 production in the *gp130*$^{F/F}$ mice following LPS administration, the next step of this project was to investigate whether or not IL-6 had a pathological role during this process.

CHAPTER 4

The causal role of IL-6 in the hypersensitivity of gp130$^{F/F}$ mice to endotoxic shock

4.1 Introduction to the role of IL-6 during endotoxic shock

The lack of success of treatments for sepsis, such as neutralising TNFα antibodies, has provoked researchers to identify novel pro-inflammatory mediators that when reduced in expression and/or activity, will consistently alleviate disease. One such candidate is the cytokine IL-6 which has been a hallmark of many human inflammatory states, including sepsis (Hack *et al.,* 1989) , rheumatoid arthritis (RA) (Hirano *et al.,* 1988) and inflammatory bowel disease (IBD)/colitis (Atreya *et al.,* 2000). Moreover, in mice, experimentally-induced RA and IBD are attenuated by abrogating IL-6 signalling with a neutralising antibody against the IL-6R (Takagi *et al.,* 1998; Atreya *et al.,* 2000), and IL-6 deficient mice *(IL-6*$^{-/-}$*)* are resistant to experimentally-induced colitis and arthritis (Alonzi *et al.,* 1998; Gay *et al.,* 2006; Nowell *et al.,* 2009). While these observations support the notion that IL-6 is a critical cytokine contributing to these inflammatory symptoms, by contrast, the use of *IL-6*$^{-/-}$ mice to investigate the role of IL-6 in response to local and systemic LPS-induced inflammatory responses has been controversial. For instance, in the context of LPS-induced endotoxic shock, IL-6 has been assigned either a non-essential (Fattori *et al.,* 1994) or an anti-inflammatory (Xing *et al.,* 1998) role.

The diverse portfolio and often opposing roles of IL-6 during the inflammatory response might be explained, at least in part, by its ability to initiate 2 modes of signalling: the first involving the membrane-bound IL-6Rα subunit (classical signalling) and the other mediated by sIL-6Rα (trans-signalling) (reviewed in, Jones b *et al.,* 2005). Although a pro-inflammatory role for IL-6 trans-signalling has been suggested in various chronic inflammatory diseases and cancer (Atreya *et al.,* 2000; Hurst *et al.,* 2001; Nowell *et al.,* 2009), the role of IL-6 trans-signalling in the pathogenesis of sepsis, and more specifically LPS/TLR4-mediated endotoxic shock, is ill-defined.

In light of the striking augmented local and systemic expression of IL-6 in the *gp130*$^{F/F}$ mice during LPS-induced endotoxic shock, my next avenue of research focussed on assessing the role of IL-6 in this model. Accordingly, I aimed to evaluate the role of IL-6 classical signalling and trans-signalling during LPS hypersensitivity in the *gp130*$^{F/F}$ mice.

4.2 Genetic ablation of IL-6 rescues *gp130*$^{F/F}$ mice from LPS-induced endotoxic shock

In order to provide genetic evidence for a causative pathologic role of IL-6 in the LPS/TLR4-induced hypersensitivity of *gp130*$^{F/F}$ mice, I used *gp130*$^{F/F}$ mice in which IL-6 had been genetically ablated (Jenkins *et al.,* 2007). Notably, in contrast to *gp130*$^{F/F}$ mice, all *gp130*$^{F/F}$:*IL-6*$^{-/-}$ mice were completely resistant to LPS-induced shock (Fig. 4.1), thus indicating that IL-6 is a primary gp130-acting pro-inflammatory cytokine which promotes LPS hypersensitivity in *gp130*$^{F/F}$ mice.

4.3 Rescue of LPS hypersensitivity in *gp130*$^{F/F}$:*IL-6*$^{-/-}$ mice does not correlate with a reduction in pro-inflammatory cytokine production

To investigate whether the LPS hypersensitivity rescue of *gp130*$^{F/F}$ mice upon depletion of IL-6 correlates with a reduction in systemic pro-inflammatory cytokine production after LPS administration, I measured the levels of key pro-inflammatory cytokines in the serum of *gp130*$^{F/F}$:*IL-6*$^{-/-}$ mice up to 6hrs post-LPS administration. ELISA revealed that the serum concentration of TNFα in LPS treated *gp130*$^{F/F}$:*IL-6*$^{-/-}$ mice was significantly elevated by approximately 1.5-2-fold (1.5hrs) and 2.5-3-fold (3hrs) compared to *gp130*$^{F/F}$ and *gp130*$^{+/+}$ mice (Fig. 4.2A). In addition, *gp130*$^{F/F}$:*IL-6*$^{-/-}$ serum levels of CCL5 were comparable to *gp130*$^{F/F}$ levels at 1.5hrs, significantly reduced at 3hrs (1.5-fold) and significantly elevated at 6hrs (2-fold) (Fig. 4.2B). Considering that neither of these pro-inflammatory mediators were reduced consistently in *gp130*$^{F/F}$:*IL-6*$^{-/-}$ mice in response to LPS, these data therefore suggest that they do not play a role in promoting LPS hypersensitivity of *gp130*$^{F/F}$ mice.

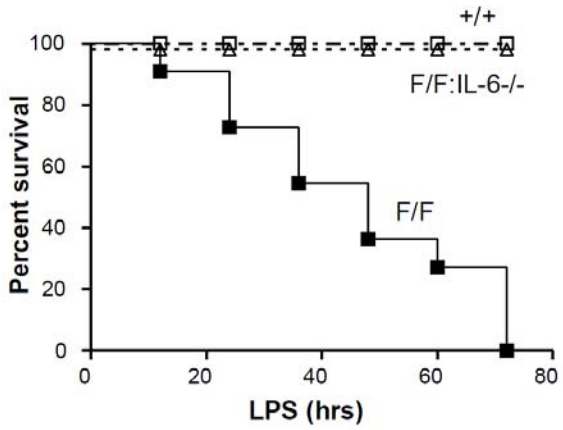

Figure 4.1. *Genetic ablation of IL-6 rescues gp130$^{F/F}$ mice from LPS-induced endotoxic shock.* Survival of *gp130$^{+/+}$* (+/+, dashed line), *gp130$^{F/F}$* (F/F, solid line) and *gp130$^{F/F}$:IL-6$^{-/-}$* (F/F:IL-6-/-, dotted line) mice over 72hrs following i.p. administration of 4mg/kg LPS.

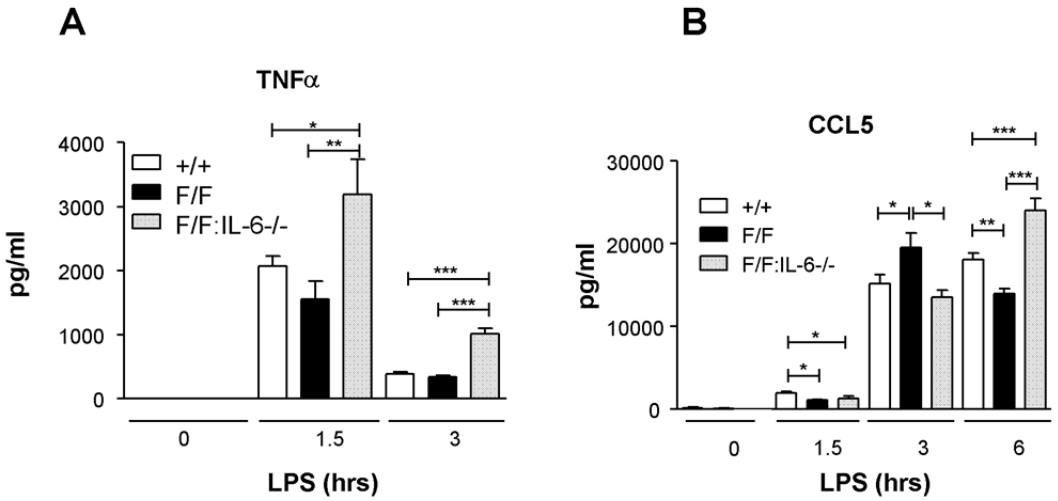

Figure 4.2. *Genetic ablation of IL-6 in the gp130$^{F/F}$ mice results in altered systemic pro-inflammatory cytokine production in response to LPS-induced endotoxic shock.* Serum from *gp130*$^{+/+}$ (+/+, white bars), *gp130*$^{F/F}$ (F/F, black bars) and *gp130*$^{F/F}$*:IL-6*$^{-/-}$ (FF:IL-6-/-, grey bars) mice was collected at the indicated time points following i.p. administration of 4mg/kg LPS, and **(A)** TNFα and **(B)** CCL5 ELISAs were performed. Data are from 3 mice of each genotype and are expressed as the mean ± SEM. * *P*<0.05, ***P*<0.01, ****P*<0.001.

4.4 IL-6 trans-signalling promotes LPS/TLR4-induced hyperinflammatory responses in *gp130*$^{F/F}$ mice

To further demonstrate that IL-6 signalling is responsible for the LPS hypersensitivity of *gp130*$^{F/F}$ mice, I next employed a preventative approach by utilising the 2B10 antibody raised against the ligand-binding IL-6Rα subunit which abolishes IL-6-dependent signalling. Accordingly, *gp130*$^{F/F}$ mice were i.p. injected for 1hr prior to LPS administration (4mg/kg) with 2B10 or isotype control antibody (1.5mg/mouse) and monitored over 72hrs. As shown in Fig. 4.3A, *gp130*$^{F/F}$ mice pretreated with the isotype control antibody remained hypersensitive to LPS-induced mortality, whereas all *gp130*$^{F/F}$ mice pretreated with the 2B10 IL-6R antibody were resistant to LPS-induced lethality.

To examine whether trans-signalling is playing a causative role in the LPS hypersensitivity exhibited by *gp130*$^{F/F}$ mice, I pretreated LPS-challenged *gp130*$^{F/F}$ mice with sgp130Fc, a recombinant version of soluble gp130 that specifically antagonises IL-6 trans-signalling (Nowell *et al.,* 2009). As shown in Fig. 4.3B, *gp130*$^{F/F}$ mice pretreated with sgp130Fc were completely resistant to LPS hypersensitivity over 72hrs. Taken together, these data reveal that IL-6 trans-signalling exacerbates TLR4-dependent inflammatory responses in *gp130*$^{F/F}$ mice. Moreover, the general applicability of IL-6 trans-signalling as a key pro-inflammatory mechanism in LPS-mediated endotoxic shock in normal mice was demonstrated by the complete protection of *gp130*$^{+/+}$ mice against a lethal dose (6mg/kg) of LPS upon sgp130Fc pretreatment (Fig. 4.3C).

4.5 Genetic reduction of IL-6 in *gp130*$^{F/F}$ mice reduces STAT3 activity in the liver after LPS administration

My observations that STAT3 was systemically hyper-activated by LPS in *gp130*$^{F/F}$ mice led me to investigate if LPS-induced pY-STAT3 was altered in *gp130*$^{F/F}$:*IL-6*$^{-/-}$ mice. Liver was collected from LPS-injected (4mg/kg) mice and lysates prepared for Western blot analysis. These results demonstrated that the augmented levels of pY-STAT3 in *gp130*$^{F/F}$ compared to *gp130*$^{+/+}$ mice (Fig. 3.8A,B) in response to LPS were dramatically reduced in *gp130*$^{F/F}$:*IL-6*$^{-/-}$ mice at all time points analysed (i.e. 1.5, 3 and 6hrs) (Fig. 4.4A,B), thus revealing a major role for IL-6 in promoting STAT3 tyrosine phosphorylation in response to LPS. In a similar vein, I assessed the levels of pY-STAT1 in *gp130*$^{F/F}$:*IL-6*$^{-/-}$ liver lysates

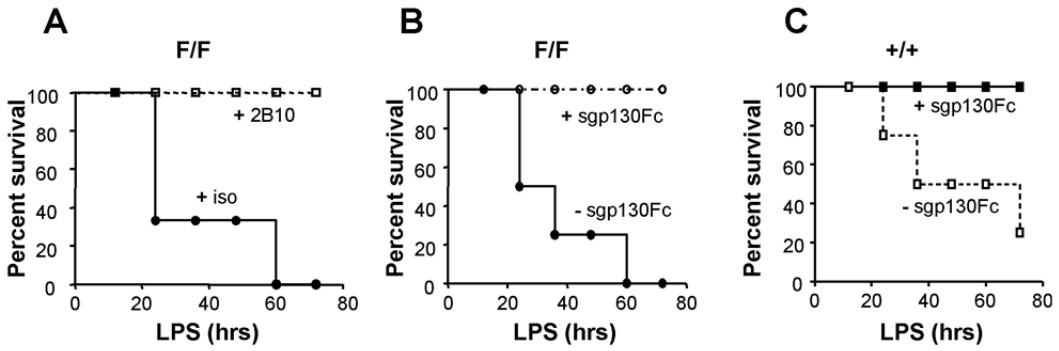

Figure 4.3. *Therapeutic abrogation of IL-6 trans-signalling in mice protects against LPS hypersensitivity.* Survival over 72hrs of **(A)** *gp130*$^{F/F}$ (F/F) mice pretreated with IL-6Rα blocking antibody 2B10 (dotted line; n = 4) or isotype control (iso; solid line; n = 3) followed by i.p. injection of LPS (4mg/kg), and **(B)** F/F mice untreated (solid line; n = 4) or pretreated with sgp130Fc (dotted line; n = 4) followed by i.p. injection of LPS, and **(C)** *gp130*$^{+/+}$ (+/+) mice untreated (dotted line; n = 4) or pretreated with sgp130Fc (solid line; n = 4) followed by i.p. injection of LPS (6mg/kg).

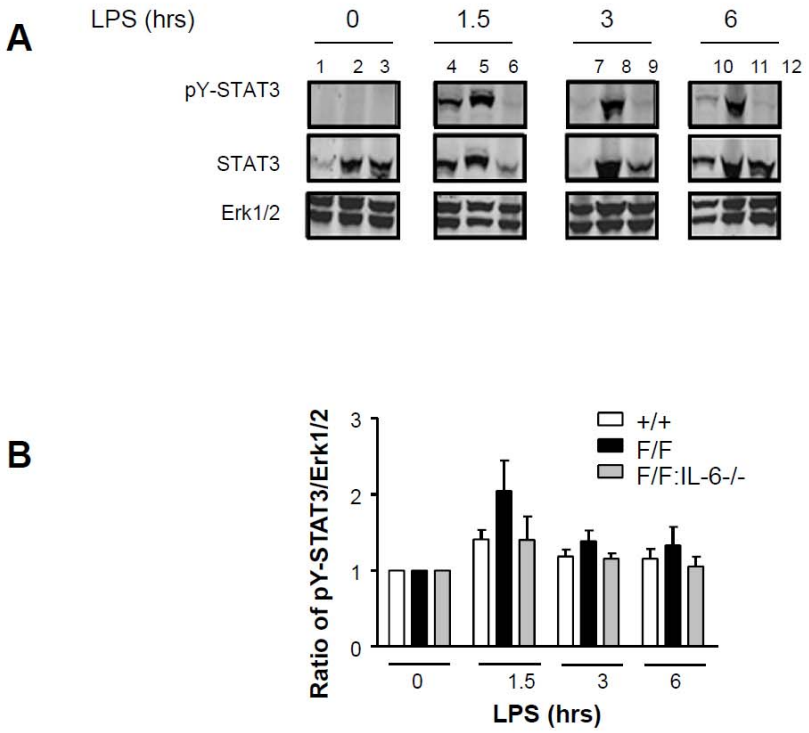

Figure 4.4. *Genetic reduction of IL-6 in gp130$^{F/F}$ mice reduces LPS-induced STAT3 activity.* **(A)** *Gp130$^{+/+}$* (+/+; lanes 1, 4, 7, 10), *gp130$^{F/F}$* (F/F; lanes 2, 5, 8, 11) and *gp130$^{F/F}$:IL-6$^{-/-}$* (F/F:IL-6-/- ; lanes 3, 6, 9, 12) mice were i.p. administered with LPS (4mg/kg) and at defined intervals STAT3 tyrosine phosphorylation, total STAT3 and Erk1/2 levels were measured by immunoblotting on liver lysates. Results shown are representative of 3 mice per genotype per time point. **(B)** Densitometric quantitation of tyrosine-phosphorylated STAT3 in each of 3 representative samples per genotype per time point was performed and normalised against Erk1/2 protein levels.

and found that they were also substantially lower after LPS administration compared to *gp130*$^{F/F}$ and *gp130*$^{+/+}$ mice (Fig. 4.5). Consequently, IL-6 also plays a major role in promoting STAT1 tyrosine phosphorylation in response to LPS.

4.6 Discussion

Overall, this chapter has provided evidence that IL-6 plays an essential pro-inflammatory role during LPS-induced endotoxic shock in the *gp130*$^{F/F}$ mice. My results not only contribute to a growing body of evidence that IL-6 is pro-inflammatory during sepsis (Hack *et al.,* 1989), as mentioned in the introduction to this chapter, but are also consistent with previous research illustrating that anti-IL-6 antibodies improved survival to cecal ligation and puncture (CLP) in mice (Riedemann *et al.,* 2003). In this regard, it is also important to note that monoclonal IL-6 antibody administration has been beneficial in protecting against numerous experimentally-induced pro-inflammatory mouse models of lethality, such as *E.coli*-induced bacteremia, TNFα challenge (Starnes *et al.,* 1990), as well as lethal endotoxemia (Heremans *et al.,* 1992). Additional proof of the ability of IL-6 to be pro-inflammatory comes from human trials, where blockade of IL-6 signalling with a humanised anti-IL-6R antibody has alleviated disease severity for inflammatory diseases. For instance, in RA disease activity was reduced (reviewed in, Nishimoto *et al.,* 2004), along with inhibition in progression of joint damage (Nakahara, Nishimoto, 2006), and in Castleman's disease the severity of clinical symptoms (e.g. lymphadenopathy) were improved (Nishimoto *et al.,* 2000). Furthermore, humanised anti-IL-6R antibody is thought to improve Crohn's disease, as suppression of the acute phase response was achieved (Ito *et al.,* 2004; Ito, 2005). Considering the data in this chapter illustrated that using an anti-IL-6R antibody also protected *gp130*$^{+/+}$ mice against LPS hypersensitivity, my findings therefore add further credibility to potential future clinical trials of targeting IL-6 during endotoxic shock treatment.

Despite this established pro-inflammatory role for IL-6 during endotoxic shock, several other studies have conflicted with these results. For instance, it has been proposed that IL-6 plays a non-essential role during the pathogenesis of sepsis, since genetic ablation of IL-6 does not alter the induction of acute phase proteins, or body weight change of mice in response to systemic injection of LPS (Fattori *et al.,* 1994). Differences in the inflammatory nature of IL-6 in these

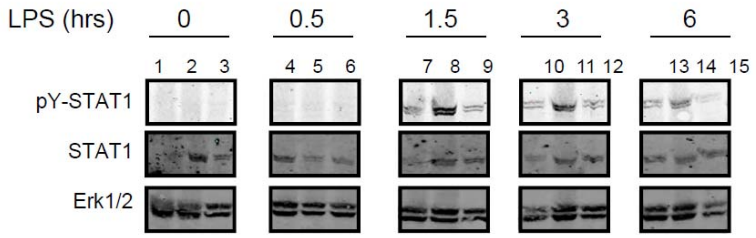

Figure 4.5 *Genetic reduction of IL-6 in gp130$^{F/F}$ mice reduces LPS-induced STAT1 activity.* *Gp130$^{+/+}$* (+/+; lanes 1, 4, 7, 10, 13), *gp130$^{F/F}$* (F/F; lanes 2, 5, 8, 11, 14) and *gp130$^{F/F}$:IL-6$^{-/-}$* (F/F:IL-6-/-, lanes 3, 6, 9, 12, 15) mice were i.p. administered with LPS (4mg/kg), and at defined intervals STAT1 tyrosine phosphorylation, total STAT1 and Erk1/2 levels were measured by immunoblotting on liver lysates. Results shown are representative of 3 mice per genotype per time point.

results and those of my current study could be due to the dose of LPS administrated (1mg/kg v 4mg/kg), or the fact that the LPS was not re-purified and hence, could be activating PRR pathways other than TLR4. Conversely, previous research has also deemed IL-6 as being anti-inflammatory. Specifically, wild-type mice were significantly protected from LPS-induced (plus D-Gal) mortality when pretreated with both recombinant IL-6 protein and anti-TNFα antibody (Barton, Jackson, 1993). The differences between these results and my own presented in this chapter could be explained by the models used, because D-Gal affects UDP ribosylation in the liver leading to macrophage-mediated acute liver injury in mice, whereas the high dose LPS-induced endotoxic shock model I have employed causes multi-organ damage due to systemic production of pro-inflammatory cytokines. Moreover, *IL-6*$^{-/-}$ mice have shown elevated levels of circulating cytokines such as TNFα, IFNβ and MIP-2 following 4μg/g of LPS administration (Xing *et al.,* 1998) which are reduced in the *IL-6*$^{-/-}$ mice when administered with recombinant IL-6, thus suggesting IL-6 can be anti-inflammatory (Xing *et al.,* 1998). Also, Xing et al. (1998) found that the survival of *IL-6*$^{-/-}$ mice was reduced by 50% when compared to wild-type mice following administration with 20μg/g of LPS. A possible explanation for this finding which contradicts my current survival studies could be due to the differences in LPS dose (20μg/g v 4mg/kg) and the fact that the LPS was not re-purified and hence, could be activating other PRRs.

Interestingly, despite the overwhelming pro-inflammatory role of IL-6 in the *gp130*$^{F/F}$ mouse model, my results suggested that IL-6 may suppress the production of certain pro-inflammatory mediators in response to LPS. For instance, the production of TNFα and CCL5 was increased in the serum of *gp130*$^{F/F}$:*IL-6*$^{-/-}$ mice in comparison to *gp130*$^{F/F}$ levels following LPS administration. In this regard, this observation is consistent with the previous report that IL-6 can negatively regulate TNFα production. Specifically, this inhibition occurred when U937 cells were co-incubated with GM-CSF (to induce differentiation) and IL-6 followed by LPS stimulation at various concentrations *in vitro* (Aderka *et al.,* 1989). Furthermore, TNFα production has been elevated in the serum of *IL-6*$^{-/-}$ mice 1.5hrs after administered with LPS (Barton, Jackson, 1993). This was also observed by Xing *et al.,* (1998) on *IL-6*$^{-/-}$ mice discussed

above, as TNFα production was elevated significantly above wild type levels at 1.5 and 6hrs after LPS administration. Moreover, mice with hepatocyte specific deletion of gp130 and high susceptibility to sepsis-associated mortality induced by CLP, had a higher production of serum TNFα (Sander *et al.,* 2010). It is thought that this increase in TNFα levels is a compensatory mechanism in the absence of IL-6 (Fattori *et al.,* 1994). However, considering that TNFα levels do not correlate with LPS hypersensitivity, this leads to the conclusion that TNFα does not contribute to the pathogenesis of LPS-induced endotoxic shock hypersensitivity in the *gp130*$^{F/F}$ mice.

A key finding of my current work is the novel discovery that IL-6 trans-signalling via the sIL-6R is the primary mode of signalling that elicits the potent pro-inflammatory actions of IL-6 during LPS/TLR4-driven endotoxic shock. This finding contributes to emerging evidence implicating a key role for IL-6 trans-signalling in the pathogenesis of numerous experimentally-induced inflammation models such as chronic intestinal inflammation, peritoneal inflammation and experimental arthritis (Atreya *et al.,* 2000; Hurst *et al.,* 2001; Nowell *et al.,* 2003). Specifically, treatment with sgp130Fc has been shown to suppress disease activity in mice with experimental colitis (Atreya *et al.,* 2000). These findings were thought to be a result of IL-6s-IL6R conferring resistance of T cells against apoptosis, which was ablated as a result of sgp130 treatment. Furthermore, sgp130Fc has been shown to suppress the progression and histopathological hallmarks of antigen-induced arthritis in wild type mice when co-administered alongside methylated BSA (Nowell *et al.,* 2003). Conversely, research has shown that the administration of sgp130Fc into mice to eliminate IL-6 trans-signalling does not significantly rescue these mice from a model of haemorrhage and subsequent sepsis (triggered by CLP) (Mees *et al.,* 2009). The differences between this study and my own can be attributed to the different treatments utilised to induce inflammation (i.e. Hemorrhage and CLP v LPS). As discussed previously, CLP activates multiple TLRs, whereas LPS specifically activates signalling via TLR4. Based on the previous studies, it seems sgp130Fc has the potential to treat inflammatory and autoimmune diseases in which IL-6 is thought to play a role. An advantage of administration of sgp130Fc as opposed to targeting 'global' IL-6 signalling (i.e. trans-signalling and classical signalling)

with antibodies against IL-6 or the IL-6R, is that it does not suppress the classical IL-6 signalling, which is important in triggering the acute phase response (Tenhumberg *et al.,* 2008). For instance, previous studies have shown that despite its beneficial effects, global blockade of IL-6 in RA has resulted in some patients suffering from infections (reviewed in, Nishimoto *et al.,* 2004; Tenhumberg *et al.,* 2008). Furthermore, it has been shown that targeting global IL-6 signalling in the treatment of inflammatory disorders can lead to unwanted side effects such as obesity and glucose intolerance (Wallenius *et al.,* 2002). Instead, sgp130Fc specifically targets only the pro-inflammatory actions of IL-6 trans-signalling. Consequently, the understanding of which cell types are signalling via sIL-6R during sepsis would be vital to the understanding of this disease. Despite these observations, a potential complication associated with the treatment of inflammatory diseases with sgp130Fc is the generation of neutralising auto-antibodies. Indeed this was shown to be a problem with RA (Tanaka *et al.,* 2000), and will need to be investigated in the future during potential sepsis treatment with sgp130.

In addition to IL-6, it may be potentially interesting to assess the role of other gp130-acting cytokines, such as IL-27 and IL-11 during endotoxic shock in the *gp130*$^{F/F}$ mice. The role of IL-27 during inflammation is controversial (reviewed in, Carl, Bai, 2008). For instance, WSX-1 (which, along with gp130, form the receptor for IL-27) knock-out mice are hyper-susceptible to experimental autoimmune encephalomyelitis (Batten *et al.,* 2006), indicating IL-27 plays an anti-inflammatory role. Furthermore, IL-27 p28 (one of two subunits that make up IL-27, the other being Epstein-Barr virus-induced gene 3) has shown to be an antagonist of gp130/IL-6 signalling, as it has shown to reduce IL-6/gp130 interaction and IL-6-induced STAT activation (Stumhofer *et al.,* 2010). Conversely, p28 blockade resulted in suppression of ongoing adjuvant-induced arthritis (Goldberg *et al.,* 2004), indicating a pro-inflammatory role for IL-27. Therefore, to help determine the role of this cytokine in the context of inflammation, I could examine whether IL-27 levels were increased in response to LPS in the *gp130*$^{F/F}$ mice. If research indicates this was the case, *gp130*$^{F/F}$ mice could be crossed with the WSX-1 knock-out mice that are currently available in my laboratory, and perform LPS survival experiments. Moreover, although IL-11 (like IL-6), has a role in activating the acute phase response, the role of IL-11

during sepsis appears to be anti-inflammatory. For instance, pretreatment with IL-11 in a mouse model of toxic shock syndrome (induced by administration of 20mg of D-Gal and 25µl of *Staphylococcus aureus* i.p.) results in reduced mortality (Barton *et al.,* 1996). Furthermore, in a rabbit model of endotoxemia, IL-11 treatment inhibits hypotension and decreases gastro-intestinal mucosal damage induced by LPS (Misra *et al.,* 1996). This anti-inflammatory role of IL-11 is thought to be due to the inhibition of pro-inflammatory mediators produced by macrophages (Trepicchio *et al.,* 1996). Moreover, IL-11 administration together with granulocyte colony stimulating factor has been successful at treating experimentally-induced Gram-negative bacterial sepsis in rats (Opal *et al.,* 1999). Interestingly, IL-11 does not trans-signal, potentially explaining why it has an anti-inflammatory role as oppose to the pro-inflammatory role of IL-6. This could possibly be because IL-6 can exert its effects by trans-signalling in specific cell sets which cannot perform IL-11 signalling, or perhaps because IL-6 trans-signalling in addition to classical signalling results in a stronger net signal output when compared to IL-11. Therefore, this stronger signal could be over the threshold needed for gp130 signalling to change from anti-inflammatory to pro-inflammatory. It is a known fact that cytokines can signal via the same receptors but have differing outputs (reviewed in, Ernst, Jenkins, 2004). Consequently research examining the response of *gp130*$^{F/F}$ mice lacking IL-11R (Jenkins *et al.,* 2007) to LPS would help to further understand the role of gp130 signalling during inflammation.

Considering the pro-inflammatory role for IL-6 signalling in the *gp130*$^{F/F}$ mice during LPS/TLR4-induced endotoxic shock, the next line of investigation was to examine which components of the TLR4 pathway are impacted upon by the detrimental IL-6 signalling.

CHAPTER 5

The role of TLR4 pathway components during endotoxic shock in the gp130$^{F/F}$ mice

5.1 Introduction of TLR4 pathway components in the context of endotoxic shock

TLR4 plays a key role in triggering the LPS-induced inflammatory response (reviewed in, Palsson-McDermott, 2004). LPS engagement of TLR4 initiates a cascade of signalling events via intracellular TIR signalling domains, which involves the primary recruitment of the Mal adaptor protein and its subsequent association with MyD88 to ultimately activate the NF-κB transcriptional complex and induce the production of many pro-inflammatory cytokine genes (i.e. TLR4/MyD88-dependent pathway), such as *IL-6*, *TNFα* and *IL-1β* (Fitzgerald *et al.,* 2001; Horng *et al.,* 2002; Palsson-McDermott, O'Neill, 2004). Host immune responses triggered by TLR4 also involve the recruitment of other intracellular signalling adaptors, in particular TRIF and TRAM, which also facilitate activation of NF-κB and IRF3 (i.e. the TLR4/MyD88-independent pathway), the latter of which promotes the transcription of pro-inflammatory type I IFN genes (Fitzgerald *et al.,* 2003). Expression of IFNβ is driven via the TLR4/MyD88-independent pathway, and results in the activation of STATs through the IFNβ-bound type I IFN receptor complex, which comprises IFNAR1 and IFNAR2 (Karaghiosoff *et al.,* 2003; de Weerd *et al.,* 2007). STAT1, and to a lesser extent, STAT3 are activated by type I IFNs and facilitate many of their biological actions (reviewed in, Darnell *et al.,* 1994).

My data showing that *IFNβ* and *CCL5* mRNA levels were elevated in LPS-challenged *gp130*$^{F/F}$ mice suggests that type I IFN signalling could also contribute to the LPS/TLR4-driven hypersensitivity of *gp130*$^{F/F}$ mice (Fig. 3.4E,F). Furthermore, the augmented STAT1 and STAT3 activation in *gp130*$^{F/F}$ mice may further up-regulate type I IFN signalling. To address these issues, in this Chapter I investigated the role of the TLR4/MyD88-dependent and/or

TLR4/MyD88-independent pathways during hypersensitivity of the *gp130*$^{F/F}$ mice by employing both genetic and pharmacological intervention approaches.

5.2 The TLR4/MyD88-independent pathway is not responsible for LPS hypersensitivity in *gp130*$^{F/F}$ mice

To address the role of the TLR4/MyD88-independent pathway in the LPS hypersensitivity phenotype of *gp130*$^{F/F}$ mice, I initially used an *in vivo* antibody based approach to suppress the biological actions of type I IFNs (i.e. IFNα/β) in *gp130*$^{F/F}$ mice challenged with LPS. Specifically, *gp130*$^{F/F}$ mice were co-injected with LPS and either a type I IFN blocking antibody (Mar-1), at a dose previously shown to block the actions of IFNβ (1mg/mouse) (Sheehan *et al.,* 2006), and therefore the TLR4/MyD88-independent pathway, or its isotype control. In response to LPS, all *gp130*$^{F/F}$ mice treated with the isotype control antibody failed to survive past 72hrs, and only 25% of *gp130*$^{F/F}$ mice treated with the Mar-1 antibody survived (Fig. 5.1), thus suggesting that *in vivo* targeting of the type I IFN pathway did not substantially alleviate the LPS hypersensitivity of *gp130*$^{F/F}$ mice. To confirm the neutralising activity of the Mar-1 antibody on type I IFN signalling in *gp130*$^{F/F}$ mice, peritoneal macrophages derived from *gp130*$^{F/F}$ mice were stimulated with either LPS or IFNα together with the Mar-1 antibody or isotype control over 5hrs, following which the expression of the IFN/STAT-dependent target gene *ISG15* was examined by qPCR. Stimulation of isotype control antibody treated *gp130*$^{F/F}$ macrophages with LPS or IFNα led to an approximate 14- and 18-fold induction, respectively, of *ISG15* mRNA (Fig. 5.2A). By contrast, treatment with the Mar-1 antibody suppressed the induction of *ISG15* mRNA by LPS or IFNα by approximately 5-fold. Notably, blocking the biological activity of type I IFNs failed to have any effect on the levels of IL-6 protein induced by LPS in *gp130*$^{F/F}$ macrophages (Fig. 5.2B).

My observation that a small number of *gp130*$^{F/F}$ mice pretreated with the Mar-1 antibody survived suggested that the antibody only partially blocked the *in vivo* TLR4-mediated actions of type I IFNs and/or type I IFNs do contribute (at least in part) to the impaired survival of *gp130*$^{F/F}$ mice to LPS. I therefore performed genetic complementation studies to provide definitive evidence that the LPS/TLR4-driven hypersensitivity of *gp130*$^{F/F}$ mice occurred independently of type I IFN signalling. Specifically, compound mutant *gp130*$^{F/F}$:*Ifnar2*$^{-/-}$ mice were

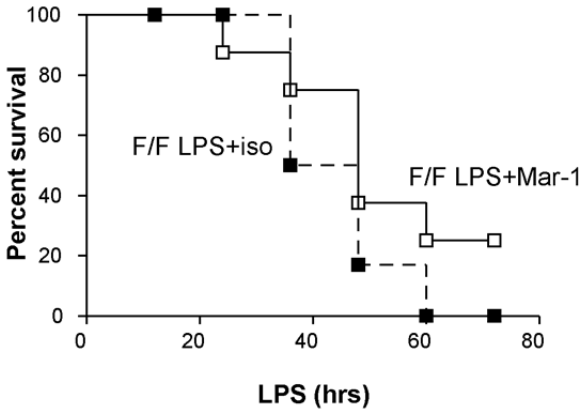

Figure 5.1. *Blocking type 1 IFN signalling does not protect gp130$^{F/F}$ mice from LPS hypersensitivity.* **(A)** Survival over 72hrs of *gp130$^{F/F}$* (F/F) mice co-injected with LPS (4mg/kg) and Mar-1 antibody (solid line; n = 8) or isotype control (iso; dashed line; n = 6) (1mg/mouse).

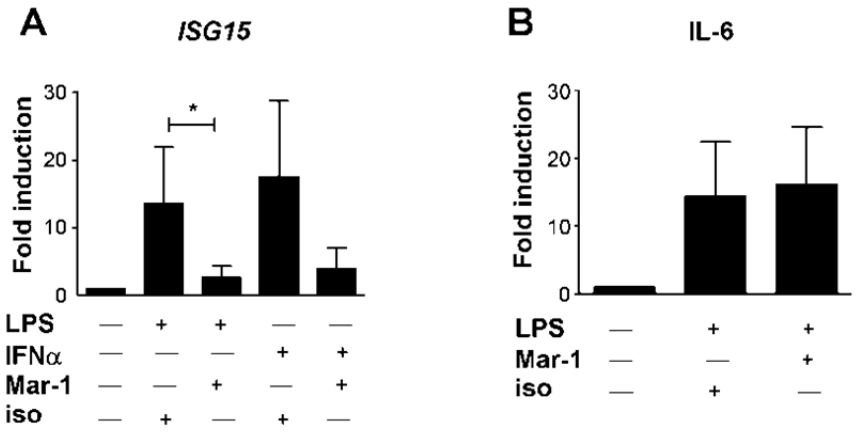

Figure 5.2. *Blocking type 1 IFN signalling does not suppress IL-6 production from LPS-treated gp130$^{F/F}$ macrophages.* **(A)** qPCR was performed on *gp130*$^{F/F}$ (F/F) peritoneal macrophages to determine *ISG15* mRNA levels at 5hrs after stimulation with LPS (1ng/ml) or IFNα (1000IU/ml) together with the Mar-1 antibody (1µg/ml) or isotype control (iso; 1µg/ml). Expression data from 2 samples are shown following normalisation to *18S* expression, and are presented from replicate analysis as the mean fold induction ± SEM. **(B)** IL-6 protein levels were assessed after 3hrs by ELISA. Data are representative of 3 individual experiments and are expressed as the mean ± SEM relative to unstimulated macrophages. *P<0.05.

generated in which the IFNAR2 receptor that is required for type I IFN signalling (Fenner *et al.,* 2006) was genetically ablated. As shown in Fig. 5.3, no *gp130*$^{F/F}$:*Ifnar2*$^{-/-}$ mice survived beyond 72hrs upon challenge with 4mg/kg LPS, therefore confirming that the complete ablation of type I IFN signalling does not protect mice from LPS hypersensitivity.

5.3 TLR4-driven IFNβ production is not responsible for elevated IL-6 production in serum of *gp130*$^{F/F}$ mice in response to LPS

To examine the potential effect of IFN-STAT activity on the production of IL-6 and other pro-inflammatory cytokines, I next investigated the production of pro-inflammatory cytokines in the serum following LPS administration to *gp130*$^{F/F}$ and *gp130*$^{F/F}$:*Ifnar2*$^{-/-}$ mice. IL-6 and CCL5 were not reduced in the serum of the *gp130*$^{F/F}$:*Ifnar2*$^{-/-}$ compared to *gp130*$^{F/F}$ mice (Fig. 5.4A,B).

I next examined the levels of these cytokines in the spleens of the *gp130*$^{F/F}$ and *gp130*$^{F/F}$:*Ifnar2*$^{-/-}$ mice. In accordance with the serum results, mRNA levels of *IL-6* and *CCL5* were not decreased in the spleens of the *gp130*$^{F/F}$:*Ifnar2*$^{-/-}$mice compared to *gp130*$^{F/F}$ mice (Fig. 5.5A,B). Instead, *IL-6* levels were significantly increased by approximately 5-fold at 1.5hrs and 3hrs following LPS administration in the *gp130*$^{F/F}$:*Ifnar2*$^{-/-}$ spleen, and *CCL5* was significantly increased by 2-fold at 1.5hrs and approximately 3-fold at 3hrs compared to *gp130*$^{F/F}$ levels (Fig. 5.5A,B).

Collectively, these data suggest that the modulation of LPS/TLR4-dependent inflammatory responses in *gp130*$^{F/F}$ mice occurs independently of type I IFN production and signalling via IFNAR2.

5.4 The TLR4/MyD88-dependent pathway promotes systemic hyperinflammatory responses in *gp130*$^{F/F}$ mice to LPS

The Mal signalling adaptor acts as a crucial bridge by recruiting MyD88 to TLR4 to facilitate LPS-induced activation of the NF-κB transcription factor and induction of pro-inflammatory cytokine production (TLR4/MyD88-dependent pathway (Fitzgerald *et al.,* 2001). To determine whether gp130 signalling modulated LPS/TLR4-induced responses via Mal, *gp130*$^{F/F}$ mice lacking Mal were generated (*gp130*$^{F/F}$*:Mal*$^{-/-}$) and then subjected to LPS challenge. Notably,

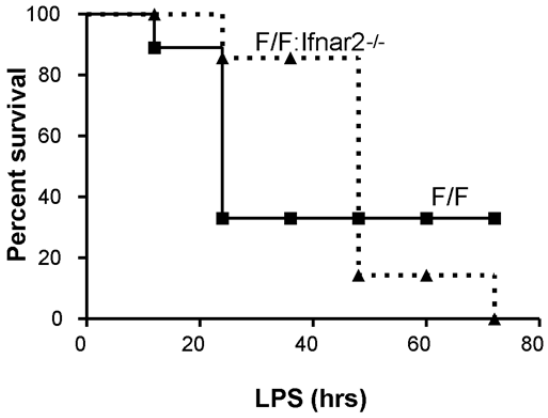

Figure 5.3. *Genetic ablation of type 1 IFN signalling does not protect gp130$^{F/F}$ mice from LPS hypersensitivity.* Survival of *gp130*$^{F/F}$ (F/F, solid line; n=8) and *gp130*$^{F/F}$*:Ifnar2*$^{-/-}$ (FF:Ifnar2-/-, dotted line; n=7) mice administrated i.p. with LPS (4mgkg) and monitored over 72hrs.

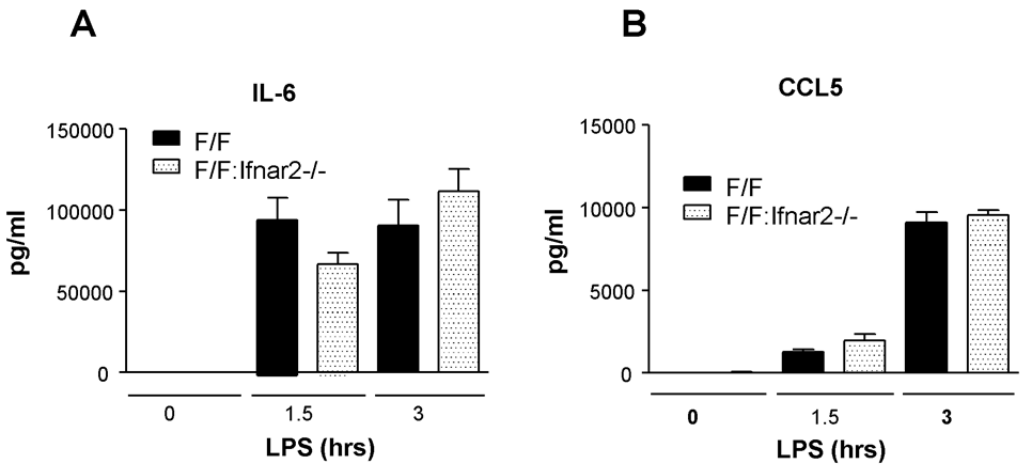

Figure 5.4. *Genetic ablation of type I IFN signalling in gp130$^{F/F}$ mice does not alter serum protein production of pro-inflammatory cytokines in response to LPS-induced endotoxic shock.* Serum from *gp130$^{F/F}$* (F/F, black bars) and *gp130$^{F/F}$:Ifnar2$^{-/-}$* (F/F:Ifnar2-/-, dotted bars) mice was collected at the indicated time points following i.p. administration of 4mg/kg LPS, and **(A)** IL-6 and **(B)** CCL5 ELISA assays were performed. Expression data from 3 samples per genotype are presented from replicate analysis as the mean fold induction ± SEM.

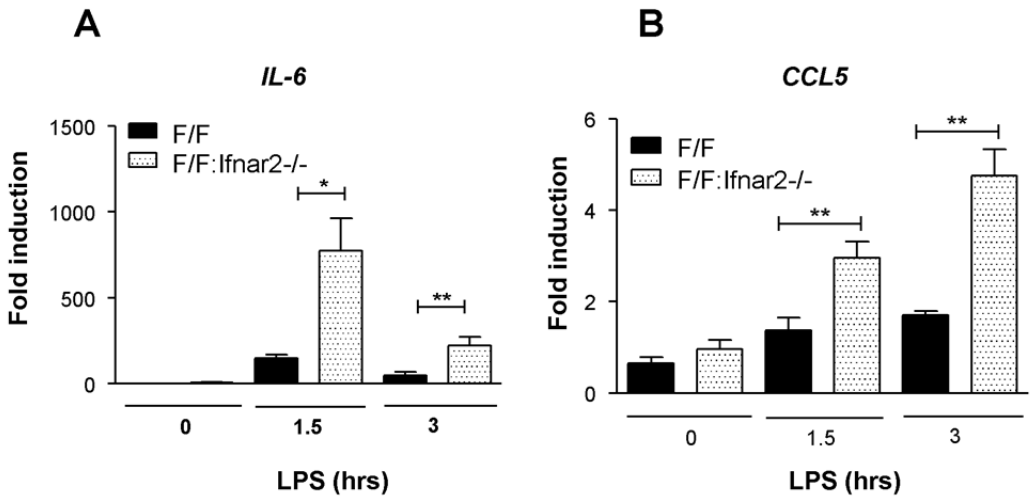

Figure 5.5. *Genetic ablation of type I IFN signalling in gp130$^{F/F}$ mice does not decrease pro-inflammatory mRNA production in the spleen in response to LPS.* qPCR analyses of **(A)** *IL-6* and **(B)** *CCL5* gene expression in whole spleen tissue from g*p130*$^{F/F}$ (F/F, black bars) and *gp130*$^{F/F}$:*Ifnar2*$^{-/-}$ (F/F:Ifnar2-/-, dotted bars) mice at the indicated time points following i.p. administration of 4mg/kg LPS. Expression data from 3 samples per genotype are shown following normalisation to *18S* expression, and are presented from replicate analysis as the mean fold induction ± SEM relative to unstimulated samples. **P*<0.05, ***P*<0.01 versus data from F/F mice at the corresponding time points.

the genetic ablation of Mal in *gp130*$^{F/F}$*:Mal*$^{-/-}$ mice dramatically ameliorated their hypersensitivity to LPS (Fig. 5.6).

5.5 The TLR4/MyD88-dependent pathway is responsible for elevated IL-6 production in serum of *gp130*$^{F/F}$ mice in response to LPS

Consistent with the vital role for Mal in facilitating the induction of TLR4/NF-κB-dependent pro-inflammatory cytokines, I also observed that IL-6 serum levels were significantly reduced by 2-fold in *gp130*$^{F/F}$*:Mal*$^{-/-}$ mice at 3hrs (Fig. 5.7A), and TNFα production was also reduced significantly at 3hrs in comparison to *gp130*$^{F/F}$ levels (Fig. 5.7B). By contrast, serum levels of CCL5 were slightly elevated, although not significantly, between the genotypes (Fig. 5.7C). Regarding this latter observation, mRNA levels for the MyD88-independent genes *IFNβ* and *CCL5* were also significantly increased in the *gp130*$^{F/F}$:*Mal*$^{-/-}$ splenocytes (16-fold at 1.5hrs and 12-fold at 3hrs for *IFNβ*, and 4-fold at 6hrs for *CCL5*) (Fig. 5.8A,B). Nonetheless, collectively the above data suggest a key role for the TLR4/Mal signalling axis, rather than the TLR4/MyD88-independent pathway, in augmenting systemic LPS-driven IL-6-specific inflammatory responses in *gp130*$^{F/F}$ mice.

5.6 The TLR4/MyD88-dependent pathway appears to modulate STAT3 activation in *gp130*$^{F/F}$ liver in response to LPS

Previous research has indicated that MyD88 has the potential to affect STAT activation following LPS administration (Yamawaki *et al.,* 2010). Considering the STAT3 tyrosine phosphorylation (i.e. activation) was exaggerated in *gp130*$^{F/F}$ compared to *gp130*$^{+/+}$ mice in response to LPS, I next investigated whether or not STAT3 activation levels were altered in *gp130*$^{F/F}$:*Mal*$^{-/-}$ mice. The activation of STAT3 (pY-STAT3) was reduced in the *gp130*$^{F/F}$:*Mal*$^{-/-}$ compared to *gp130*$^{F/F}$ liver lysates after LPS administration (Fig. 5.9A,B). This reveals that the TLR4/MyD88-dependent pathway is possibly involved in the regulation of STAT3 activation in response to LPS in the livers of *gp130*$^{F/F}$ mice.

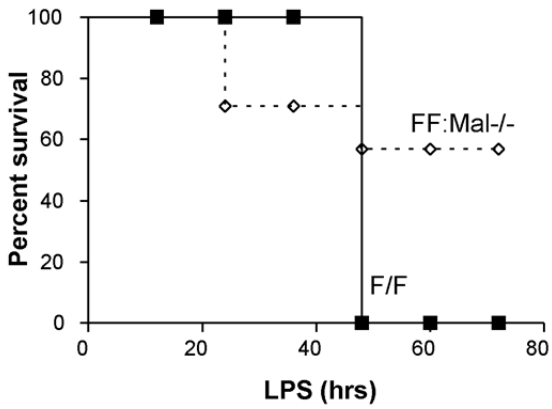

Figure 5.6. *Genetic ablation of Mal alleviates LPS hypersensitivity of gp130$^{F/F}$ mice.* Survival of *gp130*$^{F/F}$ (F/F, solid line; n = 5) and *gp130*$^{F/F}$:*Mal*$^{-/-}$ (F/F:Mal-/-, dotted line; n = 7) mice over 72hrs following i.p. administration of 4mg/kg LPS.

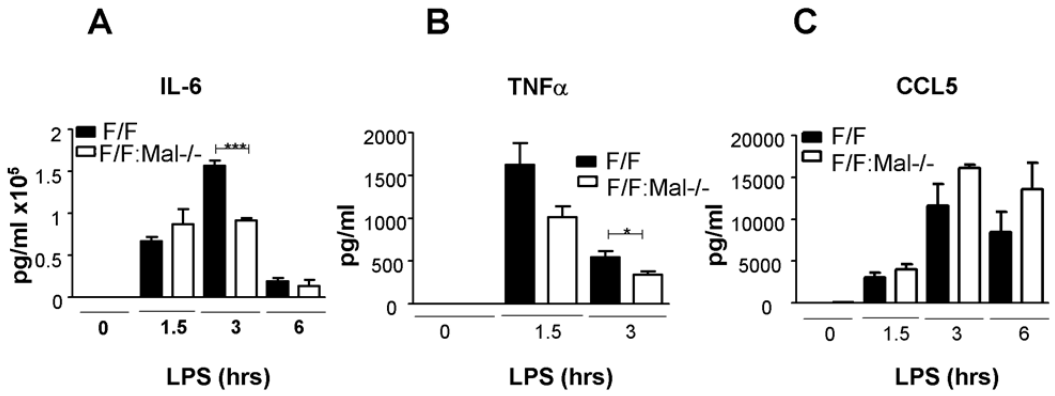

Figure 5.7. *Genetic ablation of Mal in gp130$^{F/F}$ mice reduces systemic production of specific pro-inflammatory cytokines in response to LPS-induced endotoxic shock.* Serum from *gp130$^{F/F}$* (F/F, black bars) and *gp130$^{F/F}$:Mal$^{-/-}$* (F/F:Mal-/-, white bars) mice was collected at the indicated time points following i.p. administration of 4mg/kg LPS, and **(A)** IL-6, **(B)** TNFα and **(C)** CCL5 ELISAs were performed. Data are from at least 3 mice of each genotype, and are expressed as the mean ± SEM. ***P<0.001 versus data from F/F mice at the corresponding time point.

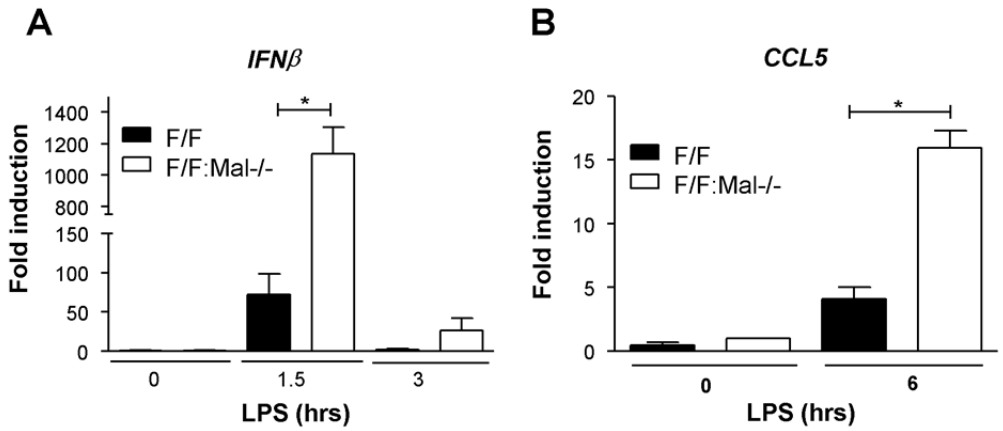

Figure 5.8. *Genetic ablation of Mal in the gp130$^{F/F}$ mice results in increased MyD88-independent pro-inflammatory cytokine production in the spleen in response to LPS-induced endotoxic shock.* qPCR analyses of **(A)** *IFNβ* and **(B)** *CCL5* gene expression in whole spleen tissue from *gp130$^{F/F}$* (F/F, black bars) and *gp130$^{F/F}$:Mal$^{-/-}$* (F/F:Mal-/-, white bars) mice at the indicated time points following i.p. administration of 4mg/kg LPS. Expression data from 3 samples per genotype are shown following normalisation to *18S* expression, and are presented from replicate analysis as the mean fold induction ± SEM relative to unstimulated samples.

* $P<0.05$ versus data from F/F mice at the corresponding time points.

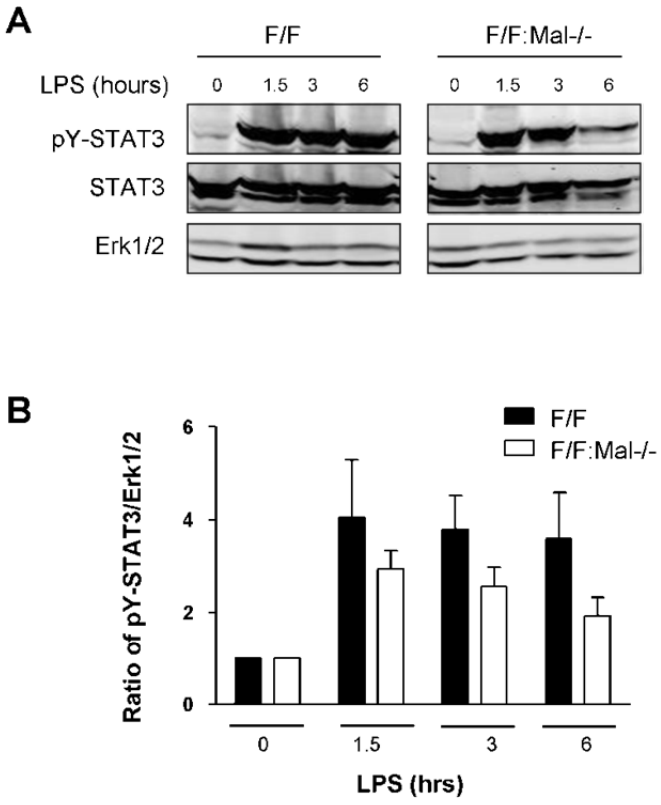

Figure 5.9. *Genetic ablation of Mal in gp130$^{F/F}$ mice decreases LPS-induced STAT3 activation.* **(A)** *Gp130$^{F/F}$* (F/F; lanes 1-4) and *gp130$^{F/F}$:Mal$^{-/-}$* (F/F:Mal-/-, lanes) mice were administered with LPS (4mg/kg), and at defined intervals STAT3 tyrosine phosphorylation, total STAT3 and Erk1/2 levels were measured by immunoblotting. Results shown are representative of 3 mice per genotype per time point. **(B)** Densitometric quantitation of tyrosine-phosphorylated STAT3 in each of 3 representative samples per genotype per time point was performed and normalised against Erk1/2 protein levels.

5.7 Discussion

Collectively, the results of this chapter suggest that the modulation of LPS/TLR4-dependent inflammatory responses in *gp130*$^{F/F}$ mice occurs independently of type I IFN production and signalling via IFNAR2. Rather, they suggest a key role for the TLR4/Mal signalling axis in augmenting LPS-driven IL-6-specific inflammatory responses in *gp130*$^{F/F}$ mice.

Previous studies on Mal knock-out mice indicate that although they were resistant to LPS-induced shock, and the production of cytokines such as IL-6, TNFα and IL-1β was suppressed in response to LPS, they were still able to induce late phase NF-κB, MAPK and IRF3 activation triggered by the TLR4/MyD88-independent pathway (Yamamoto a *et al.,* 2002). These results compliment my own findings on the *gp130*$^{F/F}$:*Mal*$^{-/-}$ mice, as they were partially protected from LPS hypersensitivity and maintained production of certain pro-inflammatory cytokines (albeit significantly reduced for IL-6) following LPS administration, which is most likely initiated by the TLR4/MyD88-independent pathway. To further confirm the role of the TLR4/MyD88-dependent pathway during LPS hypersensitivity in the *gp130*$^{F/F}$ mice, future experiments could include LPS survival experiments with *gp130*$^{F/F}$:*MyD88*$^{-/-}$ mice, which would completely inactivate the MyD88-dependent arm of the TLR4 pathway.

Interestingly, it has been shown that Mal can inhibit TLR3 signalling in BMMs (Kenny *et al.,* 2009). This inhibition has been proposed to be due to the sequestering of IRAK2 away from TLR3 (which signals via the MyD88-independent pathway) by Mal, however, this needs to be clarified (Kenny *et al.,* 2009). Therefore, there is a possibility that Mal may also inhibit the MyD88-independent arm of the TLR4 pathway in *gp130*$^{F/F}$ mice following LPS administration. Such inhibition could provide a plausible explanation for the elevated levels of *IFNβ* and *CCL5* produced from the TLR4/MyD88-independent arm of the TLR4 pathway following LPS administration in the spleen tissues of *gp130*$^{F/F}$:*Mal*$^{-/-}$ mice. To further investigate the inhibition of Mal on MyD88-independent TLR4 signalling in the *gp130*$^{F/F}$ mice, levels of IRF3 activation and IκBα degradation activated by LPS could be assessed in the *gp130*$^{F/F}$:*Mal*$^{-/-}$spleen tissues.

Interestingly, the lack of importance of the TLR4/MyD88-independent pathway in promoting the LPS hypersensitivity of *gp130*$^{F/F}$ mice conflicts with research by Karaghiosoff *et al*. (2003). Specifically, they demonstrated that IFNβ knock-out mice are resistant to lethal endotoxemia, compared to wild-type mice, induced by high doses of LPS, thus indicating a vital role for type I IFN signalling in endotoxic shock (Karaghiosoff *et al.,* 2003). A likely explanation for these contrasting observations by Karaghiosoff *et al*. and those presented in this chapter are differences in the experimental design, including the amount of LPS administered to the mice (50mg/kg v 4mg/kg). These authors illustrated that a high dose of LPS (such as 50mg/kg) results in a Tyk2- and IFNβ-dependent transition to a strong type I IFN response which would therefore bias the dependence of the study by Karaghiosoff *et al.* on type I IFN biology (Karaghiosoff *et al*., 2003). In addition, the mice that they used were on a C57BL/6 background, unlike our mice which were a mixed genetic background and the LPS they used was not re-purified. Interestingly, the levels of IL-6 were not assessed in the IFNβ knock-out mice (Karaghiosoff *et al.*, 2003). It is also noteworthy that IFNAR1 knock-out mice are protected against LPS, however, this study used 200μg of LPS (Mahieu *et al.,* 2006), which is also higher than the amount used in my study (4mg/kg). Moreover, the LPS used to inject into the mice from this study did not appear to be re-purified, which could potentially be activating TLR pathways other than TLR4.

The role of the MyD88-dependent pathway during LPS hypersensitivity in the *gp130*$^{F/F}$ mice opens up the potential for therapeutic targeting of components of this signalling pathway for the treatment of septic shock patients. Indeed, expanding research into the use of antagonistic reagents that target TLRs and their signalling components has provided opportunities for the treatment of diseases such as sepsis (reviewed in, O'Neill, 2003; Wiersinga, van der Poll, 2005). For instance, currently it is possible to block signalling through TLRs such as TLR4. Auranofin (an anti-rheumatic gold compound), cinnamaldehyde (major constituent of the essential oil of cinnamon bark) and acrolein (present in cigarette smoke) are all molecules that prevent TLR homodimerisation (Youn *et al.,* 2006; Lee, J. S. *et al.,* 2008; Youn *et al.,* 2008). Furthermore, molecules that inhibit the binding of MyD88 to TLR are also being researched (Lee, H. K. *et al.,* 2007), as

well as cell penetrating peptides that attach to BB loop sequences on TLR4 and inhibit LPS signalling (Toshchakov, Vogel, 2007). However, there may be prospective disadvantages to this strategy which could result in the broad paralysis of the innate immune response.

Considering there are only 13 TLRs and even fewer adaptors and kinases involved in TLR signalling, (reviewed in, Rock *et al.,* 1998; Takeuchi *et al.,* 1999; Du *et al.,* 2000; Hemmi *et al.,* 2000; Chuang, Ulevitch, 2001; Zhang *et al.,* 2004) blocking any one of these molecules to reduce signalling via a particular TLR could affect the immune system's responses to other microbes, which may not be desirable (reviewed in, Beutler, 2004). Therefore, targeting a component of the TLR4 signalling network that will block only one arm of signalling would be advantageous. For example, a splice variant of TRAM has been found; TRAM adapter with gold domain, which is located in endosomes and displaces TRIF from TRAM (Palsson-McDermott *et al.,* 2009), therefore specifically blocking the MyD88-independent signalling pathway. However, based on my studies, targeting the TLR4/MyD88-dependent pathway may provide greater protection against certain cases of endotoxic shock. In this respect, the MyD88 inhibitor, ST2825, is a heptapeptide analog that specifically inhibits MyD88 dimerisation (Loiarro *et al.,* 2007). In addition, ST2825 has been shown to inhibit IL-1β-induced expression of IL-6 when administered to mice *in vivo* (Loiarro *et al.,* 2007). Furthermore, a low molecular weight MyD88 mimic, 'compound 4a', has the potential to interfere with the interaction between MyD88 and the TIR domain (Bartfai *et al.,* 2003). Moreover, small molecules which inhibit MyD88 binding to TLR4 are emerging and have been shown to inhibit LPS stimulated cytokine production in RAW264.7 cells (Lee, H. K. *et al.,* 2007). Although these molecules have great potential for the treatment of sepsis, a thorough understanding of the complexities of the TLR4 pathway and its cross-talk with other pathways such as the Jak/STAT cascade would be necessary before any specific interventions were considered in the clinic.

Collectively, my results support the notion of cross-talk between the MyD88-dependent arm of the TLR4 pathway and the Jak/STAT pathway. Having determined the importance of the MyD88-dependent pathway during LPS hypersensitivity in the *gp130*$^{F/F}$ mice, in the next chapter I will investigate the role of macrophages during this condition.

CHAPTER 6

The non-essential role of macrophages during endotoxic shock in the gp130$^{F/F}$ mice

6.1 Introduction to investigating the role of macrophages in the LPS hypersensitivity phenotype of *gp130*$^{F/F}$ mice

While the previous chapters have focussed on uncovering the molecular mechanisms underlying the LPS hypersensitivity of *gp130*$^{F/F}$ mice, my next aim was to identify the cell type that was responsible. I decided to focus on macrophages since they 1) are a major component of the innate immune response against pathogens (reviewed in, Chaplin, 2010), 2) play a key role in the resolution of inflammation (Porcheray *et al.*, 2005), and 3) are easily isolated for *in vitro* experimentation (Fujiwara, Kobayashi, 2005; Chawla, 2010). Furthermore, macrophages are a major cellular source of cytokine production in response to LPS (reviewed in, Cavaillon, Adib-Conquy, 2005), and thus are thought to be indirectly involved in the clinical manifestations of systemic bacterial infections (reviewed in, Sweet, Hume, 1996).

The increased numbers of macrophages in *gp130*$^{F/F}$ mice in comparison to *gp130*$^{+/+}$ controls before and after LPS administration (Fig. 3.5C) also indicated the need to investigate the LPS-induced pro-inflammatory cytokine production from these cells. Expression of the macrophage colony stimulating factor (M-CSF) receptor, c-fms, is reduced in bone marrow from *gp130*$^{F/F}$ compared to *gp130*$^{+/+}$ mice (Jenkins *et al.*, 2004), resulting in the hypo-responsiveness of *gp130*$^{F/F}$ bone marrow cells during the *ex vivo* M-CSF-driven expansion of BMMs (Jenkins *et al.*, 2004). For this reason, I avoided the use of BMMs in experiments investigating LPS/TLR4-induced pro-inflammatory cytokine production. Instead, I used PMs as they do not require M-CSF to grow in culture. These cells are well studied, do not require *ex vivo* manipulation and are easily accessible from the peritoneal cavity of mice (Zhang *et al.*, 2008).

6.2 Increased specific pro-inflammatory cytokine production from LPS-treated *gp130*$^{F/F}$ macrophages

I initially investigated whether the production of IL-6 and other pro-inflammatory cytokines was augmented in PMs from *gp130*$^{F/F}$ mice. For this purpose, equal numbers of PMs from *gp130*$^{F/F}$ mice and *gp130*$^{+/+}$ controls were plated out and stimulated with LPS (10ng/ml), following which cellular RNA and culture supernatants were harvested at 0 and 3hrs for qPCR and ELISA analyses, respectively. Surprisingly, the expression levels of *IL-6* mRNA were comparable between genotypes (Fig. 6.1A). By contrast, I observed a significant 4.5-fold increase in *TNFα* expression, and a 2-fold (not significant) increase in *IL-1β* and *IFNβ* mRNA at 3hrs post LPS in the *gp130*$^{F/F}$ compared to *gp130*$^{+/+}$ PMs (Fig. 6.1B-D). Protein levels of TNFα were increased 2-fold in the *gp130*$^{F/F}$ macrophages, albeit not significant for the sample size examined (Fig. 6.2). Protein levels of IL-6 were unable to be determined from LPS-treated PMs by ELISA in these experiments due to the unexplained high readings of basal IL-6 protein levels specifically in untreated *gp130*$^{+/+}$ PMs only that were comparable to those following LPS stimulation. Nonetheless, based on my previous *in vivo* data, I have observed a correlation between *gp130*$^{+/+}$ and *gp130*$^{F/F}$ IL-6 mRNA and protein levels measured by qPCR and ELISA (Fig. 3.3, 3.4), respectively, and expect that this would be the case for protein levels of IL-6 produced from PMs following LPS stimulation. These results therefore demonstrate that specific pro-inflammatory cytokines are augmented in the *gp130*$^{F/F}$ macrophages following LPS stimulation. Most notably, the pattern of pro-inflammatory cytokines induced in macrophages contrasted my *in vivo* results. For instance, the levels of IL-6 were not elevated in *gp130*$^{F/F}$ PMs following LPS stimulation, which indicates that these cells may not be the main cell type responsible for LPS hypersensitivity.

6.3 Deletion of Mal in macrophages does not significantly alter *gp130*$^{F/F}$ hypersensitivity to LPS-induced shock

The data I presented in Chapter 5 revealed that TLR4 signalling via the Mal adapter played a crucial role in the hypersensitivity of *gp130*$^{F/F}$ mice to LPS (e.g. Fig. 5.6). To confirm that macrophages did not play a causal role in LPS hypersensitivity of the *gp130*$^{F/F}$ mice, I used *gp130*$^{F/F}$ mice crossed with *LysM-*

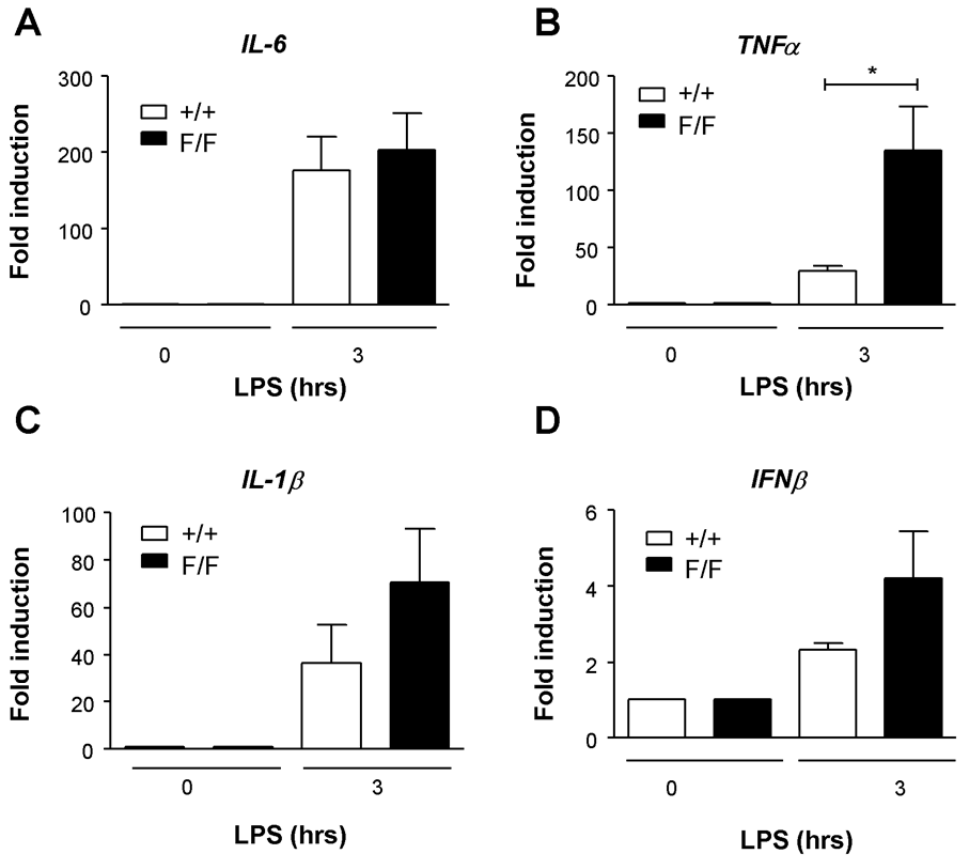

Figure 6.1. *Increased expression of specific pro-inflammatory cytokines in LPS-stimulated gp130$^{F/F}$ macrophages.* Peritoneal macrophages were collected from *gp130*$^{+/+}$ (+/+, white bars) and *gp130*$^{F/F}$ (F/F, black bars) mice and stimulated for 0 and 3hrs with 10ng/ml LPS. qPCR gene expression analysis was performed for **(A)** *IL-6,* **(B)** *TNFα*, **(C)** *IL-1β,* and **(D)** *IFNβ.* Expression data from at least 3 samples per genotype are shown following normalisation to *18S* expression and are presented from replicate analysis as the mean fold induction ± SEM relative to unstimulated samples of the same genotype. * $P<0.05$.

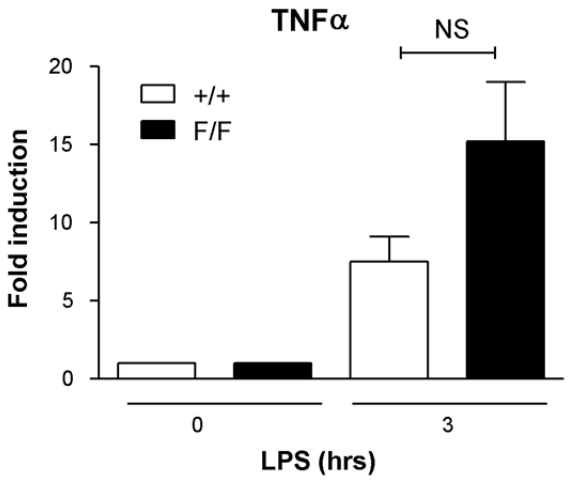

Figure 6.2. *Increased TNFα protein production in LPS-stimulated gp130$^{F/F}$ macrophages.* Peritoneal macrophages were collected from *gp130$^{+/+}$* (+/+, white bars) and *gp130$^{F/F}$* (F/F, black bars) mice and stimulated for indicated time points with 10ng/ml of re-purified LPS, and ELISA was performed on supernatants for TNFα. Data was made relative to cell numbers deduced from MTT assays, and are representative of at least 3 individual experiments. Data are expressed as the mean ± SEM relative to unstimulated samples.

Cre/*Mal* mice (*gp130*$^{F/F}$:*LysM*-Cre/*Mal* mice) in which *Mal* was conditionally deleted in macrophages (and neutrophils (previously validated by PCR)). Considering the importance of the TLR4/Mal pathway during LPS hypersensitivity of *gp130*$^{F/F}$ mice, its ablation in macrophages would be predicted to protect these mice from LPS-induced mortality if this cell type was playing a key role in the pathogenesis of this condition.

A sub-lethal dose (for control *gp130*$^{+/+}$ animals) of LPS (4mg/kg) was i.p. injected into both *gp130*$^{F/F}$ and *gp130*$^{F/F}$:*LysM*-Cre/*Mal* mice, and the survival of mice was monitored over 72hrs. As shown in Fig. 6.3, survival of the *gp130*$^{F/F}$ and *gp130*$^{F/F}$:*LysM*-Cre/*Mal* mice was comparable (57% of *gp130*$^{F/F}$ and 68% of *gp130*$^{F/F}$:*LysM*-Cre/*Mal*) over 72hrs following LPS administration. The higher survival rate observed in these *gp130*$^{F/F}$ mice in comparison to Chapters 3-5 is due to the particular batch of LPS used in these experiments which exhibited reduced bioactivity (as determined by LPS bioassay) upon repurification. Nonetheless, these results therefore reveal that Mal signalling in macrophages does not play a role in the LPS hypersensitivity of the *gp130*$^{F/F}$ mice.

6.4 The genotype of bone marrow-derived macrophages in mice does not alter their survival rate following LPS-induced mortality

To further verify that macrophages, and more generally haemopoietic-derived cells, did not play a role in the LPS hypersensitivity of the *gp130*$^{F/F}$ mice, a bone marrow reconstitution experiment was performed which involved the transfer of *gp130*$^{+/+}$ bone marrow into *gp130*$^{F/F}$ mice, and vice versa (Fig. 6.4A). As controls for this experiment, bone marrow from *gp130*$^{+/+}$ mice was transferred into *gp130*$^{+/+}$ mice, and *gp130*$^{F/F}$ bone marrow transplanted into *gp130*$^{F/F}$ mice. In addition, naïve *gp130*$^{+/+}$ and *gp130*$^{F/F}$ mice not subjected to reconstitution were also used. These mice were then subjected to LPS (4mg/kg) administration and survival was monitored over 72hrs. These results indicated that *gp130*$^{+/+}$ recipient mice were resistant to the low dose of LPS and demonstrated 100% survival regardless of whether the donor bone marrow genotype was *gp130*$^{F/F}$ or *gp130*$^{+/+}$ (Fig. 6.4A). Furthermore, *gp130*$^{F/F}$ mice remained hypersensitive to LPS regardless of their bone marrow genotype, demonstrating that bone marrow cells do not influence the LPS hypersensitivity (Fig. 6.4A).

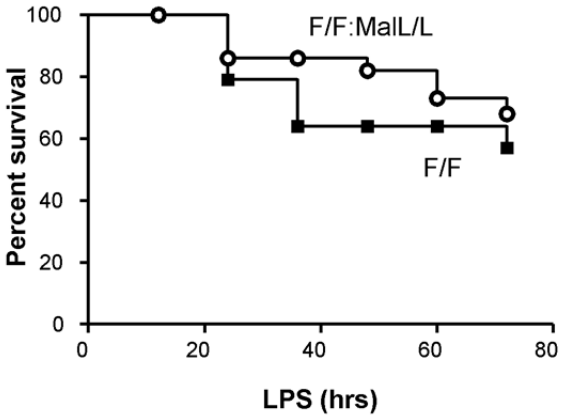

Figure 6.3. *Genetic deletion of Mal in the gp130$^{F/F}$ macrophages does not protect against LPS hypersensitivity.* Survival of *gp130*$^{F/F}$ (F/F, solid line with squares, n=14) and *gp130*$^{F/F}$*:LysM/LysMMal*, (F/F:MalL/L, solid line with circles, n=22) mice over 72hrs following i.p. administration of 4mg/kg LPS.

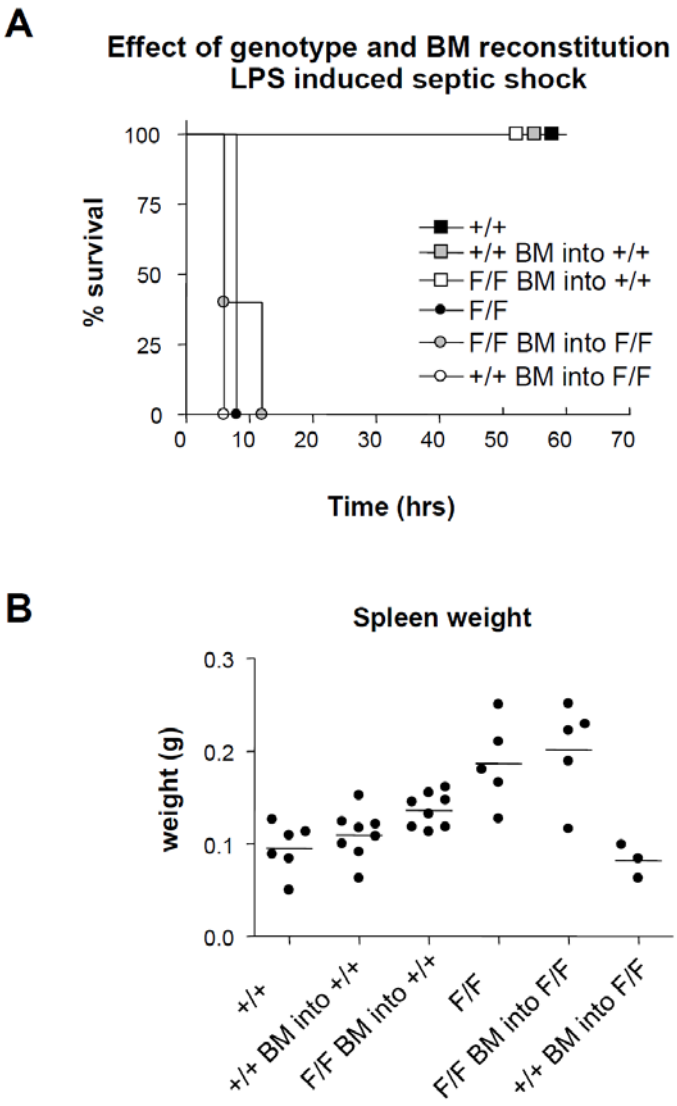

Figure 6.4. *The bone marrow compartment is not involved in the LPS hypersensitivity of the gp130$^{F/F}$ mice.* **(A)** Bone marrow from *gp130*$^{+/+}$ (+/+) and *gp130*$^{F/F}$ (F/F) mice were reconstituted into irradiated +/+ (n =6-8) and F/F mice (n =3-5), and survival was monitored over 72hrs following i.p. administration of 4mg/kg LPS. **(B)** Spleen weights of mice following reconstitution were obtained (results from Ludwig Institute for Cancer Research- B.J Jenkins).

It has been shown previously that *gp130*$^{F/F}$ mice develop splenomegaly which is associated with an expansion of all haematopoietic lineages (Jenkins *et al.*, 2007). Therefore, to verify the successful reconstitution of bone marrow into the mice, the mass of their spleens were measured. As expected, naïve *gp130*$^{F/F}$ mice, *gp130*$^{+/+}$ mice reconstituted with donor *gp130*$^{F/F}$ bone marrow, and *gp130*$^{F/F}$ mice reconstituted with donor *gp130*$^{F/F}$ bone marrow all had larger spleen weights compared to *gp130*$^{+/+}$ controls (Fig. 6.4B) indicative of splenomegaly. Conversely, *gp130*$^{+/+}$ mice, *gp130*$^{+/+}$ mice reconstituted with donor *gp130*$^{+/+}$ bone marrow and *gp130*$^{F/F}$ mice reconstituted with donor *gp130*$^{+/+}$ reconstitution had spleens of lower weight, the latter representing a rescue of the splenomegaly phenotype (Fig. 6.4B). Collectively, these data not only indicate that the reconstitution of bone marrow into these mice was successful, but moreover, they further imply that bone marrow macrophages were not responsible for the LPS hypersensitivity of the *gp130*$^{F/F}$ mice.

6.5 Discussion

Considering macrophages do not play a vital role in the LPS hypersensitivity of the *gp130*$^{F/F}$ mice, this suggests the involvement of another cell type. This is consistent with previous studies which have indicated that macrophage-deficient mice (homozygous for the osteopetrosis mutation and are genetically deficient in M-CSF/CSF-1 resulting in defective differentiation and function of macrophages) have intact neutrophil responses to LPS (Jiang *et al.*, 2000), suggesting the action of other cell types during TLR4-driven inflammation. Likely candidates are endothelial cells, as most parenchymal cells (epithelium/endothelium) have been shown to express and respond to TLR4 (Abreu *et al.*, 2003; Dauphinee, Karsan, 2006). Moreover, studies on chimeric mice lacking TLR4 in the bone marrow suggest the recruitment of neutrophils to the lungs in the first 4hrs following LPS exposure is as efficient as wild type mice, suggesting important roles for parenchymal cells in the inflammatory response (Andonegui *et al.*, 2003). Specifically, the endothelium lines blood vessels and are the first cells to be exposed to LPS that has disseminated into the blood stream, and therefore, are likely to play an important role during the inflammatory response. Moreover, previous studies have indicated that mice with TLR4 expressed exclusively on the endothelium had sufficient neutrophil recruitment to

the peripheral tissues following LPS administration, as well as clearance of Gram-negative bacterial infection (Andonegui *et al.,* 2009).

Another reason why endothelial cells are a likely candidate for the production of pro-inflammatory cytokines (especially IL-6) that drive LPS hypersensitivity in the *gp130*$^{F/F}$ mice is because they are only responsive to IL-6 when the sIL-6R is present (Romano *et al.,* 1997). Research has shown that endothelial cells are responsive to IL-6/sIL-6R as measured by STAT3 activation levels and chemokine expression, and this was suppressed by a IL-6R antagonist (anti-IL-6R MAb 15A7) (Romano *et al.,* 1997). Therefore, endothelial cells are responsive to IL-6 only via trans-signalling, which I have proven to be responsible for the LPS hypersensitivity in the *gp130*$^{F/F}$ mice. In addition, low levels of TLR4 and MD2 were shown to be expressed on human colonic epithelial cells and lamina propria cells, as well as on intestinal epithelial cell lines, indicating these cells may also be involved in TLR4-driven inflammation in the *gp130*$^{F/F}$ mice (Abreu *et al.,* 2003). Indeed, it has been recently shown that IL-6 trans-signalling by colonic epithelial cells is essential to the development of colitis-associated premalignant cancer in a murine model (Matsumoto *et al.,* 2010).

Consequently, the investigation into the role of endothelial cells and/or epithelial cells in the *gp130*$^{F/F}$ mice following LPS exposure will be of interest in the future. This could be achieved by harvesting endothelial and epithelial cells from *gp130*$^{F/F}$ mice (for example from the blood vessels) and stimulating them with LPS *in vitro* and analysing pro-inflammatory cytokine outputs. Alternatively, an *in vivo* approach would involve for example, creating *gp130*$^{F/F}$ mouse models with Mal or IL-6 conditionally-deleted in endothelial cells (using the TIE2–kinase promoter) and examining their survival after LPS exposure. Interestingly, mice with STAT3 conditionally deleted in endothelial cells have been previously generated (Kano *et al.,* 2003), and these mice demonstrated an increased susceptibility to LPS and elevated serum production of pro-inflammatory cytokines in response to LPS, indicating an anti-inflammatory role for "endothelial" STAT3 (Kano *et al.,* 2003). However, the work by Kano et al. (2003) used LPS that was not re-purified and at a slightly different dose to what I administered to the *gp130*$^{F/F}$ mice. Moreover, despite the serum levels of IL-10 in the STAT3 endothelial-specific conditional mutant mice being higher in comparison to wild type mice, the authors did not investigate whether the activity

of IL-10, which predominantly signals via STAT3, was impaired (Kano *et al.*, 2003). Therefore, the impairment of IL-10 activity could be responsible for the pro-inflammatory phenotype of the STAT3 endothelial-specific knock-out mice following LPS exposure.

Overall, the results of this chapter indicated that macrophages did not appear to be involved in the LPS hypersensitivity of the *gp130*$^{F/F}$ mice. The next aim of this project was to investigate the roles of STAT1 and STAT3 during *gp130*$^{F/F}$ LPS hypersensitivity.

CHAPTER 7

The role of STAT1 and STAT3 in the gp130$^{F/F}$ mouse model of endotoxic shock

7.1 Introduction of STATs in the context of endotoxic shock

In vitro and *in vivo* analyses have assigned both anti- and pro-inflammatory roles for STAT3 in the context of LPS/TLR4-induced inflammatory responses (Takeda *et al.*, 1999; Kano *et al.*, 2003; Samavati *et al.*, 2009), although the mechanistic basis for these opposing roles remains poorly understood. By contrast, the role of STAT1 in promoting inflammation has been associated with its activation during IFN signalling (Gingras *et al.*, 2004). The data presented in this thesis provide strong evidence for a crucial role for STAT1 and STAT3 in endotoxic shock, with hyperactivation of both STATs coincidental with LPS hypersensitivity in *gp130*$^{F/F}$ mice compared to *gp130*$^{+/+}$ mice in response to LPS (Fig. 3.1). Moreover, the observation that IL-6/gp130-mediated STAT1 and STAT3 activation is augmented in the liver of LPS-treated *gp130*$^{F/F}$ mice (Fig. 3.9) also led me to investigate the role of STAT1 and STAT3 hyperactivation during inflammation in this mouse model. Furthermore, the multi-organ inflammation (e.g. gastritis, peritonitis) driven by hyperactivation of STAT3 and to a lesser extent STAT1 in these mice is another reason to investigate their role in LPS-mediated inflammation in the *gp130*$^{F/F}$ mice (Ernst *et al.*, 2008; Jenkins a *et al.*, 2005). To formally define the contribution of endogenous STAT1 and STAT3 activation to the LPS/TLR4-driven hyper-inflammatory phenotype of *gp130*$^{F/F}$ mice, I utilised *gp130*$^{F/F}$:*Stat1*$^{-/-}$ and *gp130*$^{F/F}$:*Stat3*$^{+/-}$ mice in which the levels STAT1 and STAT3 has been genetically reduced (Jenkins a *et al.*, 2005).

7.2 Genetic reduction of STAT3 protects against LPS/TLR4-induced hypersensitivity in *gp130*$^{F/F}$ mice

To investigate the role of STAT3 during LPS hypersensitivity in the *gp130*$^{F/F}$ mice, I performed LPS survival experiments using *gp130*$^{F/F}$:*Stat3*$^{+/-}$ mice. Compared to *gp130*$^{F/F}$ mice, the genetic reduction of STAT3 activity in

gp130$^{F/F}$:*Stat3*$^{+/-}$ mice dramatically ameliorated the LPS hypersensitivity, with 78% of *gp130*$^{F/F}$:*Stat3*$^{+/-}$ mice challenged with LPS surviving over 72hrs (Fig. 7.1).

7.3 Genetic reduction of STAT3 suppresses LPS-induced augmented systemic IL-6 production in *gp130*$^{F/F}$ mice

In further support of my earlier data that indicates IL-6 production correlates strongly with the LPS-induced hypersensitivity of *gp130*$^{F/F}$ mice (Fig. 3.1,3.3A), the improved survival of LPS challenged *gp130*$^{F/F}$:*Stat3*$^{+/-}$ mice correlated with a reduction in serum protein levels of IL-6 as measured by ELISA (Fig. 7.2A). The levels of IL-6 were significantly decreased in the *gp130*$^{F/F}$:*Stat3*$^{+/-}$ mice by approximately 2-fold at 1.5hrs and 3-fold at 6hrs compared to *gp130*$^{F/F}$ mice, and their levels were comparable to *gp130*$^{+/+}$ controls (Fig. 7.2A). At 3hrs the levels of IL-6 protein were comparable between *gp130*$^{F/F}$:*Stat3*$^{+/-}$ and *gp130*$^{F/F}$ mice (Fig. 7.2A) illustrating a bimodal mechanism by which STAT3 promotes LPS hypersensitivity. By contrast, the levels of TNFα in *gp130*$^{F/F}$:*Stat3*$^{+/-}$ mice were comparable to *gp130*$^{+/+}$ and *gp130*$^{F/F}$ levels at 1.5hrs and 3hrs in response to LPS (Fig. 7.2B).

In addition to the serum data, mRNA levels of LPS-induced pro-inflammatory cytokines in the spleens were assessed in mice injected with LPS. *IL-6* levels in the *gp130*$^{F/F}$:*Stat3*$^{+/-}$ spleen were reduced in comparison to *gp130*$^{+/+}$ controls (7-fold at 1.5hrs and 5.5-fold at 3hrs) and compared to *gp130*$^{F/F}$ mice were significantly decreased by approximately 120-fold at 1.5hrs, and by 70-fold at 3hrs following LPS administration (Fig. 7.3). Overall these results confirm that in *gp130*$^{F/F}$ mice, STAT3 promotes the LPS hypersensitivity and augmented IL-6 production.

7.4 Genetic reduction of STAT3 suppresses LPS-induced augmented local IL-6 production in *gp130*$^{F/F}$ mice

To further investigate the impact of genetically reducing STAT3 activation levels, I next measured the production of IL-6 locally at the site of LPS injection (peritoneal cavity) by ELISA. After LPS injection, IL-6 levels were significantly higher in the peritoneal lavage fluid of *gp130*$^{F/F}$ compared to *gp130*$^{+/+}$ mice, and this increase was largely reversed in the *gp130*$^{F/F}$:*Stat3*$^{+/-}$ mice (decreased 1.6-fold

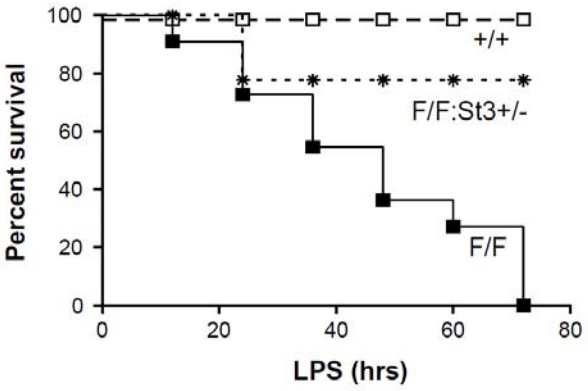

Figure 7.1. *Genetic reduction of STAT3 in gp130$^{F/F}$ mice protects against LPS hypersensitivity.* Survival of *gp130*$^{+/+}$ (+/+, dashed line, n =9), *gp130*$^{F/F}$ (F/F, solid line, n =11) and *gp130*$^{F/F}$:*Stat3*$^{+/-}$ (F/F:St3+/-, dotted line, n =9) mice over 72hrs following i.p. administration of 4mg/kg LPS.

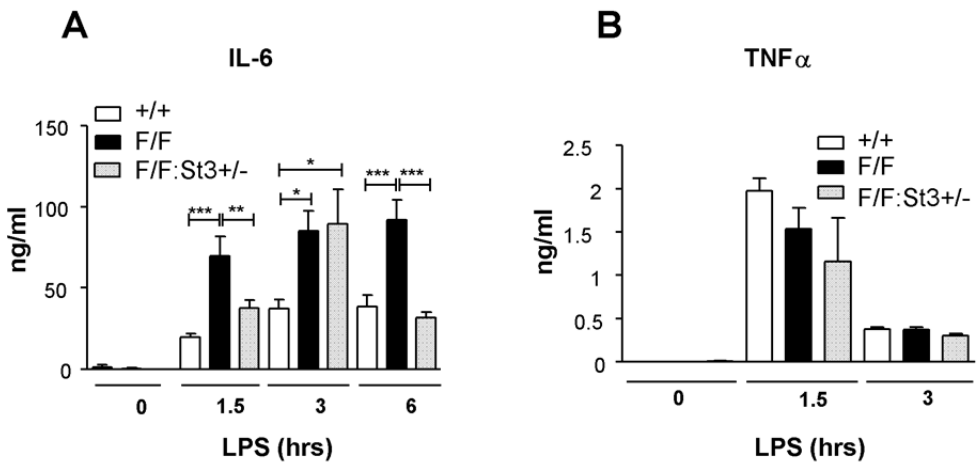

Figure 7.2. *Genetic reduction of STAT3 in gp130$^{F/F}$ mice suppresses the augmented systemic production of specific pro-inflammatory cytokines in response to LPS-induced endotoxic shock.* Serum was collected from *gp130*$^{+/+}$ (+/+, white bars), *gp130*$^{F/F}$ (F/F, black bars) and *gp130*$^{F/F}$:*Stat3*$^{+/-}$ (F/F:St3+/-, grey bars) mice at the indicated time points following i.p. administration of 4mg/kg LPS, and ELISA for **(A)** IL-6 and **(B)** TNFα was performed. Data are from 3 mice of each genotype and are expressed as the mean ± SEM. **P*<0.05, ***P*<0.01, ****P*<0.001.

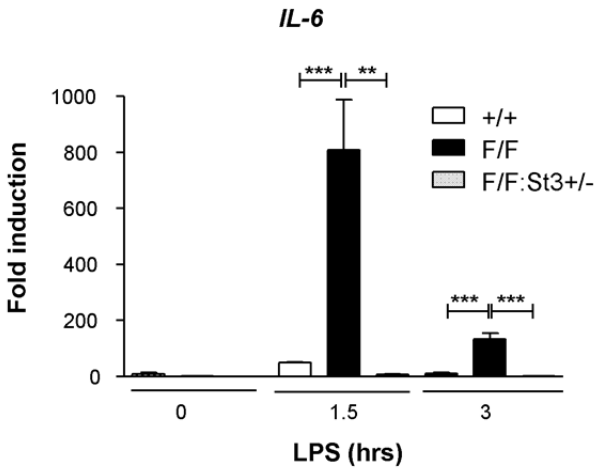

Figure 7.3. *Genetic reduction of STAT3 in gp130$^{F/F}$ mice suppresses augmented expression of IL-6 in the spleen in response to LPS-induced endotoxic shock.* qPCR analyses of *IL-6* gene expression in whole spleen tissue from *gp130$^{+/+}$* (+/+, white bars), *gp130$^{F/F}$* (F/F, black bars) and *gp130$^{F/F}$:Stat3$^{+/-}$* (F/F:St3+/-, grey bars) mice at the indicated time points following i.p. administration of 4mg/kg LPS. Expression data from 3 samples per genotype are shown following normalisation to *18S* expression and are presented from replicate analysis as the mean fold induction ± SEM relative to unstimulated samples. ***P*<0.01, ****P*<0.001.

at 3hrs and 7-fold at 6hrs in *gp130*$^{F/F}$: *Stat3*$^{+/-}$ compared to *gp130*$^{F/F}$ mice) (Fig. 7.4). Notably, this rescue was not complete, as the levels of IL-6 remained significantly increased in the *gp130*$^{F/F}$:*Stat3*$^{+/-}$ compared to *gp130*$^{+/+}$ peritoneal cavity by, for example, 4-fold at 1.5hrs and 2-fold at 3hrs post LPS exposure (Fig. 7.4). These results therefore provide further evidence that STAT3 is responsible for the augmented IL-6 production of *gp130*$^{F/F}$ mice following LPS administration.

7.5 Confirmation of reduced LPS-induced STAT3 activation in *gp130*$^{F/F}$:*Stat3*$^{+/-}$ mice

Following my initial observations of LPS-induced hyperactivation of STAT3 in the liver and spleen of *gp130*$^{F/F}$ mice (Fig. 3.7, 3.8), I investigated the levels of activated STAT3 in the livers of *gp130*$^{F/F}$:*Stat3*$^{+/-}$ mice. Western blot analyses of liver lysates from LPS-treated mice demonstrated that STAT3 tyrosine phosphorylation was dramatically reduced in LPS-treated *gp130*$^{F/F}$:*Stat3*$^{+/-}$ mice in comparison to *gp130*$^{F/F}$ levels and comparable to (if not less than) *gp130*$^{+/+}$ mice (Fig. 7.5A, B). Collectively, these results confirm the prediction that decreasing the genetic pool of STAT3 would result in a lower level of STAT3 activation following LPS administration.

7.6 Genetic deletion of STAT1 protects against LPS/TLR4-induced hypersensitivity in *gp130*$^{F/F}$ mice

To investigate the role of STAT1 in the LPS hypersensitivity of the *gp130*$^{F/F}$ mice, I utilised the *gp130*$^{F/F}$:*Stat1*$^{-/-}$ mice and analysed their survival following exposure to LPS (4mg/kg). Compared to *gp130*$^{F/F}$ mice, the genetic deletion of STAT1 in these mice prevented LPS hypersensitivity, as 100% of *gp130*$^{F/F}$:*Stat1*$^{-/-}$ mice challenged with LPS survived over 72hrs (Fig. 7.6). This suggests a pro-inflammatory role for STAT1 in this context.

7.7 Genetic reduction of STAT1 suppresses LPS-induced augmented systemic IL-6 production in *gp130*$^{F/F}$ mice

Additional evidence that there is a correlation between IL-6 production and LPS-induced hypersensitivity of the *gp130*$^{F/F}$ mice comes from the *gp130*$^{F/F}$:*Stat1*$^{-/-}$ mice, which display reduced serum IL-6 levels following LPS

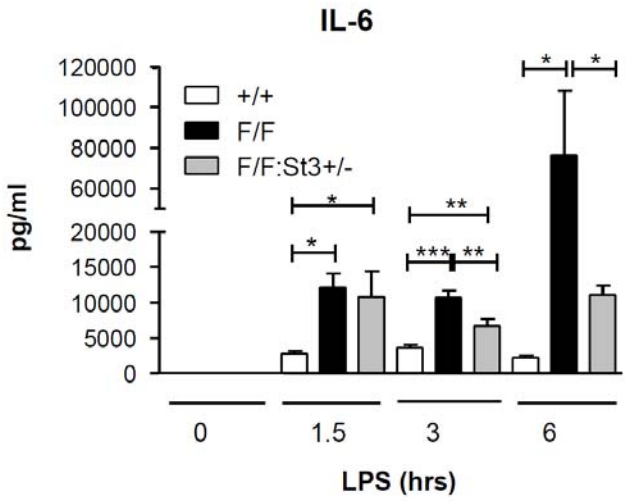

Figure 7.4. *Genetic reduction of STAT3 in gp130$^{F/F}$ mice suppresses augmented local IL-6 levels in response to LPS-induced endotoxic shock.* IL-6 ELISAs were performed on peritoneal lavage from *gp130*$^{+/+}$ (+/+, white bars), *gp130*$^{F/F}$ (F/F, black bars) and *gp130*$^{F/F}$:*Stat3*$^{+/-}$ (F/F:St3+/-, grey bars) mice at the indicated time points following i.p. administration of 4mg/kg LPS. Data are from 3 mice of each genotype and are expressed as the mean ± SEM. **P*<0.05, ***P*<0.01, ****P*<0.001.

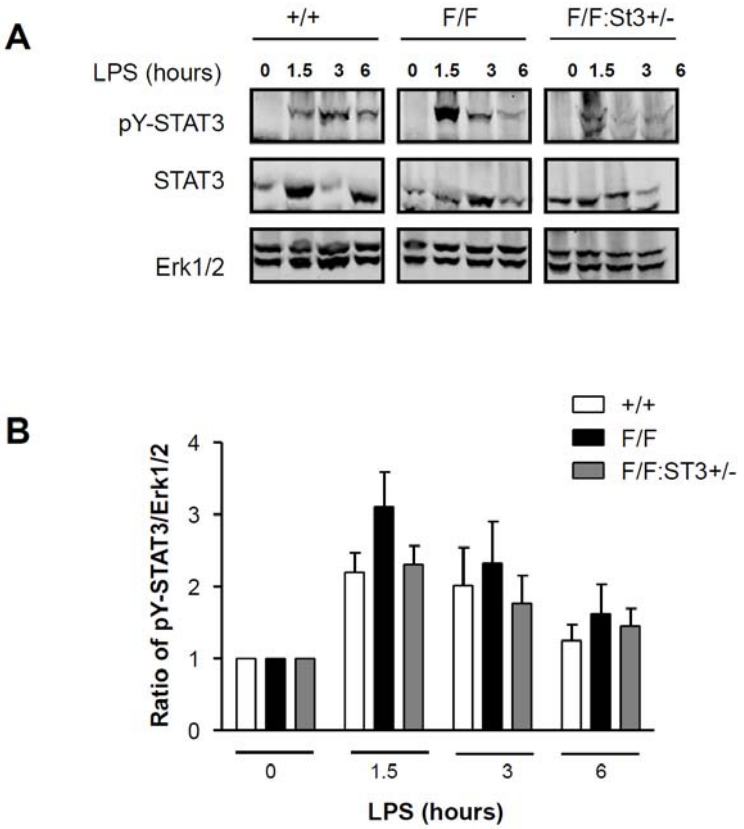

Figure 7.5. *Genetic reduction of STAT3 in gp130$^{F/F}$ mice suppresses augmented STAT3 activity in liver lysates after LPS-induced endotoxic shock. Gp130$^{+/+}$* (+/+), *gp130$^{F/F}$* (F/F) and *gp130$^{F/F}$:Stat3$^{+/-}$* (F/F:St3+/-) mice were i.p. administered with LPS (4mg/kg) and at defined intervals STAT3 tyrosine phosphorylation, total STAT3 and Erk1/2 levels were measured by immunoblotting. Results shown are representative of 3 mice per genotype per time point. **(B)** Densitometric quantitation of tyrosine-phosphorylated STAT3 in each of 3 representative samples per genotype per time point was performed and normalised against Erk1/2 protein levels.

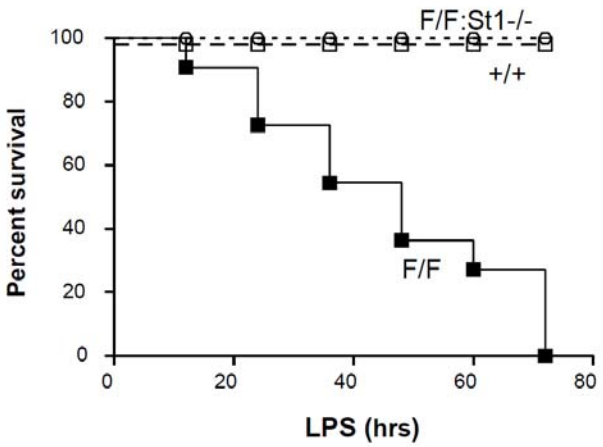

Figure 7.6. *Genetic reduction of STAT1 in gp130$^{F/F}$ mice protects against LPS hypersensitivity.* Survival of *gp130*$^{+/+}$ (+/+, dashed line, n =9), *gp130*$^{F/F}$ (F/F, solid line, n =11) and *gp130*$^{F/F}$:*Stat1*$^{-/-}$ (F/F:St1-/-, dotted line, n =4) mice over 72hrs following i.p. administration of 4mg/kg LPS.

administration (Fig. 7.7A). Compared to *gp130*$^{F/F}$ sera there was a significant 2-fold decrease of serum IL-6 levels in the *gp130*$^{F/F}$:*Stat1*$^{-/-}$ mice at 1.5hrs following LPS stimulation, no difference at 3hrs and a significant 23-fold decrease at 6hrs (Fig. 7.7A). This illustrates a bimodal mechanism by which STAT1 promotes LPS hypersensitivity. The levels of IL-6 in *gp130*$^{F/F}$:*Stat1*$^{-/-}$ sera were comparable to *gp130*$^{+/+}$ controls at 1.5hrs and 3hrs following LPS administration, however there was a significant 10-fold reduction at 6hrs (Fig. 7.7A). By contrast to these observations, the levels of TNFα were comparable among genotypes following LPS administration (Fig 7.7B).

In addition to the serum data, *IL-6* mRNA levels in the spleen of LPS-treated mice were significantly decreased in the *gp130*$^{F/F}$:*Stat1*$^{-/-}$ mice in comparison to *gp130*$^{F/F}$ mice by 2-fold at 1.5hrs, and 8-fold at 3hrs (Fig. 7.8). In comparison to *gp130*$^{+/+}$ spleen tissue, the levels of *IL-6* in the spleens of *gp130*$^{F/F}$:*Stat1*$^{-/-}$ mice were significantly increased at 1.5hrs by 3-fold following LPS exposure, however remained similar at 3hrs (Fig. 7.8). These data provide evidence that STAT1 is pro-inflammatory and is also contributing to the augmented LPS-induced IL-6 production in the *gp130*$^{F/F}$ mice.

7.8 Genetic reduction of STAT1 suppresses LPS-induced augmented local IL-6 production in *gp130*$^{F/F}$ mice

To investigate the impact of genetically reducing STAT1 on the local inflammatory response, the production of IL-6 at the site of LPS injection (peritoneal cavity) was assessed in the *gp130*$^{F/F}$:*Stat1*$^{-/-}$ mice. The levels of IL-6 protein were significantly decreased to wild-type levels in peritoneal lavage fluid from *gp130*$^{F/F}$:*Stat1*$^{-/-}$ mice compared to *gp130*$^{F/F}$ mice by approximately 4-fold at 1.5hrs, and 2-fold at 3hrs and 6hrs (Fig. 7.9). These data therefore further support a pro-inflammatory role for STAT1 in this model, and indicate that there is functional redundancy between STAT1 and STAT3 in the LPS regulation of IL-6 production both systemically and locally.

7.9 Genetic reduction of STAT1 suppresses augmented STAT3 activation in *gp130*$^{F/F}$ mice liver in response to LPS

Since increased STAT3 activity was shown to promote LPS hypersensitivity in *gp130*$^{F/F}$ mice (Fig. 3.7, 3.8), I next examined whether the

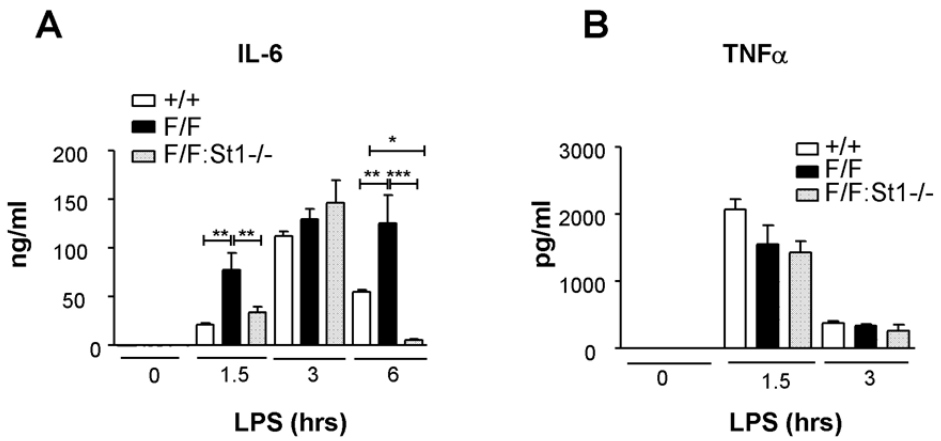

Figure 7.7. *Genetic reduction of STAT1 in gp130$^{F/F}$ mice suppresses augmented systemic production of specific pro-inflammatory cytokines in response to LPS-induced endotoxic shock.* Serum was collected from *gp130*$^{+/+}$ (+/+ white bars), *gp130*$^{F/F}$ (F/F, black bars) and *gp130*$^{F/F}$:*Stat1*$^{-/-}$ (F/F:St1-/-, grey bars) mice at the indicated time points following i.p. administration of 4mg/kg LPS, and ELISA for **(A)** IL-6 and **(B)** TNFα was performed. Data are from 3 mice of each genotype and are expressed as the mean ± SEM. **P*<0.05, ***P*<0.01, ****P*<0.001.

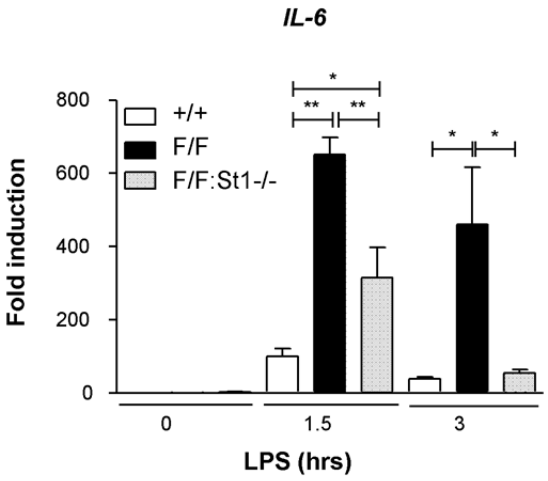

Figure 7.8. *Genetic reduction of STAT1 in gp130$^{F/F}$ mice suppresses augmented production of IL-6 cytokine levels in the spleen in response to LPS-induced endotoxic shock.* qPCR analyses of *IL-6* gene expression in whole spleen tissue from *gp130*$^{+/+}$ (+/+, white bars), *gp130*$^{F/F}$ (F/F, black bars) and *gp130*$^{F/F}$:*Stat1*$^{-/-}$ (F/F:St1-/-, grey bars) mice at the indicated time points following i.p. administration of 4mg/kg LPS. Expression data from 3 samples per genotype are shown following normalisation to *18S* expression and are presented from replicate analysis as the mean fold induction ± SEM relative to unstimulated samples. *P<0.05, **P<0.01.

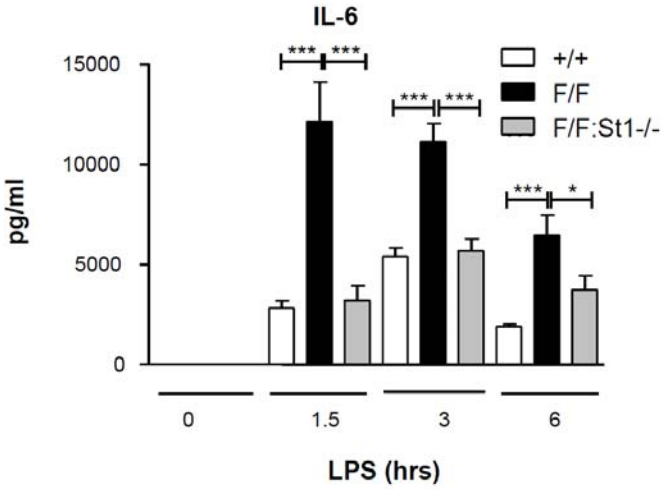

Fig 7.9. *Genetic reduction of STAT1 in gp130$^{F/F}$ mice reduces augmented local IL-6 levels in response to LPS-induced endotoxic shock.* IL-6 ELISAs were performed on peritoneal lavage from *gp130*$^{+/+}$ (+/+, white bars), *gp130*$^{F/F}$ (F/F, black bars) and *gp130*$^{F/F}$:*Stat1*$^{-/-}$ (F/F:St1-/-, grey bars) mice at the indicated time points following i.p. administration of 4mg/kg LPS. Data are from 3 mice of each genotype and are expressed as the mean ± SEM. **P*<0.05, ****P*<0.001 versus data from F/F mice at the corresponding time points.

rescue of the LPS hypersensitivity phenotype in *gp130*$^{F/F}$:*Stat1*$^{-/-}$ mice may have been due to a concomitant reduction in STAT3 activation in the absence of STAT1. Western blot analyses of liver lysates from LPS-treated mice showed that STAT3 tyrosine phosphorylation was dramatically suppressed in LPS-treated *gp130*$^{F/F}$:*Stat1*$^{-/-}$ mice compared to *gp130*$^{F/F}$ mice, and was also decreased in comparison to *gp130*$^{+/+}$ mice (Fig. 7.10A, B). The reduction in pY-STAT3 levels in *gp130*$^{F/F}$:*Stat1*$^{-/-}$ mice was not a consequence of reduced total STAT3 proteins levels, since Western blotting revealed comparable levels of total STAT3 protein in *gp130*$^{F/F}$ and *gp130*$^{F/F}$:*Stat1*$^{-/-}$ lysates (Fig. 7.10A, B). These data therefore suggest that the total levels of STAT1 protein may influence the activation status of STAT3 in response to LPS.

7.10 Discussion

The data presented in this chapter show a clear role for STAT1 and STAT3 in mediating the LPS hypersensitivity of *gp130*$^{F/F}$ mice, and provide evidence that this is due to the promotion of the pro-inflammatory response (in particular via the upregulation of IL-6).

My data reveal that heterozygous deletion of STAT3 partially rescued the LPS hypersensitivity phenotype of *gp130*$^{F/F}$ mice, suggesting either a gene dosage effect whereby sufficient levels of pro-inflammatory signals are still able to be transduced from the gp130Y$_{757}$F receptor in a STAT3 heterozygous state, or alternatively that there are signalling mediators downstream of gp130Y$_{757}$F other than STAT3 which can potentiate LPS-induced inflammatory responses. Regarding the latter scenario, a likely candidate is STAT1, which has potent pro-inflammatory activities and plays a significant role in the pathogenesis of LPS/TLR4-induced endotoxic shock (Alexander *et al.*, 1999; Karaghiosoff *et al.*, 2003). Although previously, the link between STAT1 and TLR4 signalling was proposed to be due to type I IFN-induced hyperactivation of STAT1 via the MyD88-independent pathway (Gingras *et al.*, 2004), my current data points to a pro-inflammatory role of IL-6-driven STAT1 during endotoxic shock. To further address the role of STAT3 during inflammation in the *gp130*$^{F/F}$ mice, I could cross them onto a STAT3 conditional knock-out background (e.g. endothelial cells - see section 6.5) to discover which cell types are involved in the LPS hypersensitivity.

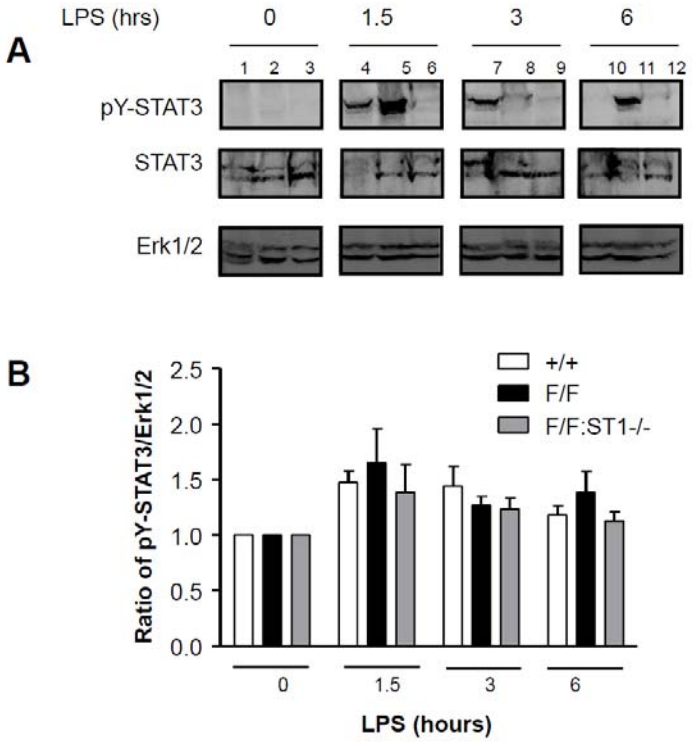

Figure 7.10. *Genetic reduction of STAT1 in gp130$^{F/F}$ mice suppresses augmented STAT3 activity in liver lysates after LPS-induced endotoxic shock. Gp130$^{+/+}$* (+/+; lanes 1, 4, 7, 10), *gp130$^{F/F}$* (F/F; lanes 2, 5, 8, 11), *gp130$^{F/F}$:Stat1$^{-/-}$* (F/F:St1-/-; lanes 3, 6, 9, 12) mice were administered i.p. with LPS (4mg/kg) and at defined intervals STAT3 tyrosine phosphorylation, total STAT3 and Erk1/2 levels were measured by immunoblotting. Results shown are representative of 3 mice per genotype per time point. **(B)** Densitometric quantitation of tyrosine-phosphorylated STAT3 in each of 3 representative samples per genotype per time point was performed and normalised against Erk1/2 protein levels.

Interestingly, my results indicate a lower activation of STAT3 in the liver of *gp130*$^{F/F}$:*Stat3*$^{+/-}$ mice in comparison to *gp130*$^{+/+}$ mice. This is consistent with the study by Jenkins (b *et al.,* 2005) which showed that activated STAT3 levels in the liver of *gp130*$^{F/F}$:*Stat3*$^{+/-}$ mice were lower at certain time points (i.e. 180') following IL-6 administration when compared with *gp130*$^{+/+}$ controls (Jenkins b *et al.,* 2005). This could possibly be due to a threshold effect, whereby the *gp130*$^{F/F}$:*Stat3*$^{+/-}$ mice have a lower amount of STAT3 than *gp130*$^{+/+}$ controls (as apparent at 1.5 and 6hrs following LPS administration (Fig. 7.5A)), resulting in lower STAT3 activation levels following LPS administration.

An interesting observation from this chapter was that the levels of STAT3 activation were lower in the *gp130*$^{F/F}$:*Stat1*$^{-/-}$ mice compared with *gp130*$^{F/F}$ following LPS administration, indicating that STAT1 may positively regulate STAT3 tyrosine phosphorylation. In this regard, an example of regulation between STAT molecules has been reported whereby STAT3 activated via IFNα attenuated the functions of STAT1 during IFNα signalling in myeloid cells (Ho, Ivashkiv, 2006). The proposed mechanism involved STAT3 sequestering STAT1 and suppressing the formation of STAT1 homodimers, leading to down-regulation of STAT1-dependent gene expression levels (Ho, Ivashkiv, 2006). While this unique suppression mechanism of STAT3 illustrated by Ho, Ivashkiv (2006) provides insights into the regulation of STATs, *in vivo* studies in mouse models (instead of *in vitro*) would help understand whether such mechanisms have any patho-physiological relevance (Ho, Ivashkiv, 2006). Nonetheless, whether such a mechanism accounts for the regulation between STAT1 and STAT3 in the *gp130*$^{F/F}$ mouse model is currently unclear and warrants further investigation. For instance, it would be interesting to investigate the relative amounts of STAT1 and STAT3 homodimers and/or heterodimers by EMSA in the *gp130*$^{F/F}$ mice in response to LPS, and their effect on gene transcription during inflammatory processes in differing cell types. Furthermore, it would be worthwhile investigating the levels of activated STAT1 in the *gp130*$^{F/F}$:*Stat3*$^{+/-}$ mice following LPS administration to determine whether the levels of total STAT3 protein have an impact on STAT1 activation (tyrosine phosphorylation). Previous studies have, however, shown that activation of STAT1 is comparable between *gp130*$^{F/F}$:*Stat3*$^{+/-}$ and *gp130*$^{F/F}$ liver lysates following IL-6 stimulation, indicating there is no affect of STAT3 protein levels on STAT1 activation at least in response to direct

stimulation of gp130 (Jenkins b *et al.,* 2005). Finally, it should be noted that serine phosphorylation levels of STAT1 and STAT3 also warrant investigation despite a previous study implying that the levels of phospho-serine (pS)STAT3 were not affected by the gp130Y_{757}F mutation following IL-6 stimulation (Jenkins b *et al.,* 2005). The importance of investigating pS-STAT1 and pS-STAT3 is also underscored by work currently being undertaken in my lab, which indicates that LPS rapidly induces pS-STAT1 and pS-STAT3 in HEK 293 (epithelial) and RAW (macrophage) cells (Mansell, Luu, personal communication).

The ability of STATs to dimerise in the absence of tyrosine phosphorylation had led to speculation that these unphosphorylated dimers may be able to facilitate subsequent activation of signal transduction pathways and gene networks (Braunstein, Brutsaert *et al.,* 2003). Therefore, the increased levels of total STAT3 in the *gp130*$^{F/F}$ mice (which I illustrated were reduced to wild type levels in the *gp130*$^{F/F}$:*Stat3*$^{+/-}$ mice and coincided with a partial rescue from LPS hypersensitivity) may consist of significant amounts of unphosphorylated STAT3, which has the potential to alter the inflammatory output. This idea is consistent with the observation that unphosphorylated STAT3 can interact with NF-κB components to initiate pro-inflammatory gene transcription, such as IL-6 (Yang *et al.,* 2007).

To date, direct exploration of the role STAT3 plays during the inflammatory response has largely relied upon a host of genetic mouse models in which STAT3 has been conditionally-deleted in a cell type- or organ-specific manner, due to the embryonic lethality associated with the global genetic ablation of STAT3 in mice (Takeda *et al.,* 1997). For instance, mice harbouring macrophage/neutrophil- or endothelial cell-specific deletion of *Stat3* have assigned an anti-inflammatory role for STAT3, irrespective of the initiating mode of "septic" inflammation (e.g. LPS, CLP) (Takeda *et al.,* 1999; Kano *et al.,* 2003; Matsukawa *et al.,* 2003) as a consequence of the inability of IL-10, which predominantly signals via STAT3, to mount a potent anti-inflammatory response. In contrast to this research, my studies address the role of IL-6/gp130-driven STAT3 activation during LPS-induced endotoxic shock in the *gp130*$^{F/F}$ mice (which have functional IL-10 signalling). Furthermore, my studies indicate that STAT1 and STAT3 activated via IL-6 trans-signalling are pro-inflammatory, however the cell types involved in this process are unknown. Nonetheless, my

results mirror previous studies showing that STAT1 and STAT3 are pro-inflammatory, for instance, the persistent STAT3 activation found in numerous human inflammatory diseases (e.g. human ulcerative colitis, RA) (Shouda, Yoshida *et al.,* 2001; Li, de Haar *et al.,* 2010) and the role of STAT1 in promoting endotoxic shock (Alexander *et al.,* 1999; Karaghiosoff *et al.,* 2003). Moreover, this research and my own correlates with that done by Pena *et al.,* (2010), who showed that inhibition of STAT3 phosphorylation in mice (by 'stattic,' a well-characterized inhibitor of STAT3 phosphorylation) results in prevention of systemic inflammation, as well as an increase in survival following experimental sepsis (Pena, Cai *et al.,* 2010). Collectively, my data demonstrate for the first time that IL-6 trans-signalling via STAT1 and STAT3 is a critical modulator of LPS-driven pro-inflammatory responses. This correlates with previous research which has shown that when STAT3 driven IL-6 trans-signalling is targeted by sgp130Fc, arthritis severity is alleviated in a murine inflammatory arthritis model (Nowell *et al.,* 2009).

Overall, the results of this chapter indicate a pro-inflammatory role for STAT1 and STAT3 in the *gp130*$^{F/F}$ mice after administration of LPS.

CHAPTER 8

Summary, Discussion and Conclusions

8.1 Summary

This thesis aimed to increase our understanding of the roles that IL-6/gp130-activated STAT1 and STAT3 play during inflammation by utilising the *gp130*$^{F/F}$ mouse model which displayed hyperactive STAT1 and STAT3, as well as a range of abnormalities, including basal multi-organ inflammation (Tebbutt *et al.,* 2002; Jenkins a *et al.,* 2005). Specifically, I investigated 1) the contribution of gp130-dependent STAT1 and STAT3 hyperactivation in the *gp130*$^{F/F}$ mice to LPS-induced endotoxic shock, 2) which TLR4 pathway impacts on the hypersensitivity of *gp130*$^{F/F}$ mice, and 3) whether impairment of anti-inflammatory IL-10 responses were playing a role in the LPS-induced inflammatory phenotype of the *gp130*$^{F/F}$ mice.

The immunomodulatory roles of STAT molecules have been studied in depth over the last decade, revealing that STAT1 has a pro-inflammatory role, whereas STAT3 can have pro- or anti-inflammatory effects (as discussed in Chapter 1). While the pro-inflammatory role of STAT1 has been aligned to its role in IFNγ signalling, the role for STAT3 during inflammation, however, is not thoroughly understood, due to it acting as a signal transducer for a number of pro- and anti-inflammatory cytokines such as IL-6, IL-11, IL-10 and type I and II IFNs. This therefore raises the conundrum of the mechanistic basis by which STAT3 can mediate both opposing pro- and anti-inflammatory responses.

In Chapter 3, the role of STAT1 and STAT3 during inflammation was assessed by analysing the responses of *gp130*$^{F/F}$ mice (with elevated STAT1/3 activation) to LPS-induced endotoxic shock. These studies suggested a pro-inflammatory role for both STAT1 and STAT3, as *gp130*$^{F/F}$ mice were hypersensitive to a sub-lethal dose of LPS and displayed specific augmented local and systemic IL-6 production when compared to *gp130*$^{+/+}$ mice following LPS administration. The activation of STAT1 and STAT3 was augmented in the livers of the *gp130*$^{F/F}$ mice in comparison to *gp130*$^{+/+}$ mice following LPS administration, as were total STAT3 protein levels. These studies highlight the

potential for non-phosphorylated STAT3 protein, which accumulates over time, to act as a transcription factor in the *gp130*$^{F/F}$ mice following LPS administration. For instance, as mentioned in Chapter 1, non-phosphorylated STAT3 co-operates with NF-κB to bind to the promoter of pro-inflammatory genes such as IL-6 to induce their transcription (Yang *et al.,* 2007).

The role of IL-10 following LPS-induced endotoxic shock in the *gp130*$^{F/F}$ mice was addressed in Chapter 3. It was found that IL-10 was adequately induced and fully functional when compared to *gp130*$^{+/+}$ controls. These results prompt further investigations into the role of other anti-inflammatory cytokines such as TGFβ, IL-4 or IL-13 during LPS hypersensitivity of the *gp130*$^{F/F}$ mice.

Based on the observation that IL-6 production was augmented in the *gp130*$^{F/F}$ mice following LPS administration, I investigated a potential pathological role of this cytokine during this process. This was addressed in Chapter 4 by utilising *gp130*$^{F/F}$ mice lacking IL-6, as well as therapeutic targeting of IL-6R (2B10 antibody) and trans-signalling (sgp130Fc) in *gp130*$^{F/F}$ mice, prior to LPS administration. My results indicated that IL-6 1) is the primary gp130-acting pro-inflammatory cytokine which promotes LPS hypersensitivity in *gp130*$^{F/F}$ mice, and 2) plays a major role in promoting STAT1 and STAT3 tyrosine phosphorylation in response to LPS, further emphasising the importance of this cytokine during the inflammatory response. Moreover, I demonstrated that IL-6 trans-signalling exacerbates TLR4-dependent inflammatory responses leading to the hypersensitivity of *gp130*$^{F/F}$ mice.

Following on from these results, in Chapter 5 I examined which components of the TLR4 pathway have an impact on detrimental IL-6 signalling, and identified the TLR4/MyD88-dependent pathway involving the Mal signalling adapter was a vital player in the LPS hypersensitivity of the *gp130*$^{F/F}$ mice. These results suggested a potential cross-talk between this pathway and the IL-6/gp130/STAT pathway. Therefore, components of the MyD88-dependent pathway could be potential therapeutic targets during sepsis.

Having established the vital role of IL-6 and the MyD88-dependent pathway during LPS hypersensitivity of the *gp130*$^{F/F}$ mice, in Chapter 6 I next investigated the role of macrophages as the primary cell type promoting LPS hypersensitivity. My results indicated that macrophages were not the key cell type responsible for the LPS hypersensitivity of the *gp130*$^{F/F}$ mice, as the production of

IL-6 in PMs from *gp130*$^{F/F}$ mice was not augmented in comparison to *gp130*$^{+/+}$ macrophages. Moreover, the deletion of Mal in the macrophages (and neutrophils) of *gp130*$^{F/F}$ mice did not rescue them from LPS hypersensitivity. Furthermore, reconstitution experiments with bone marrow indicated that the genotype of heaemopoietic cells in mice does not alter their survival rate following LPS-induced mortality. These results indicate future studies are needed to identify which cell type (possibly endothelial cells) is important in this LPS hypersensitivity.

Lastly, in Chapter 7 I demonstrated potential redundancy in the role of STAT1 and STAT3 in mediating the LPS hypersensitivity of *gp130*$^{F/F}$ mice. These studies also indicated that reducing the level of STAT1 resulted in a suppression of activated STAT3 levels in the *gp130*$^{F/F}$ liver. As a consequence, the levels of STAT1 may be impacting on the levels of activated STAT3 following LPS administration in these mice, a regulation mechanism that needs further investigation. Furthermore, the relative abundance of STAT3α versus STAT3β in the *gp130*$^{F/F}$ mice following LPS administration would be vital, considering the latter has been shown to have anti-inflammatory properties (Yoo *et al.,* 2002). In this regard, preliminary studies indicate that the levels of *Stat3β* in the *gp130*$^{F/F}$ spleen tissue is reduced in comparison to *gp130*$^{+/+}$ (Fig. 8.1). However, experiments may involve examining the level of *Stat3β* in other organs of the *gp130*$^{F/F}$ mice, such as the spleen, as immune cells in this organ play a key role in the immune response.

Collectively, these data lead me to propose a novel mechanism whereby IL-6 trans-signalling via STAT1/3, activated downstream of TLR4 in response to LPS, feeds back into the Mal/NF-κB pathway to specifically modulate TLR4/LPS-driven IL-6 production and therefore the inflammatory response (Greenhill *et al.*, 2010) (Fig. 8.2).

8.2 Discussion and Conclusions

The novel discovery of my work was that IL-6 trans-signalling via the sIL-6R is the primary mode of signalling that elicits the potent pro-inflammatory actions of IL-6 during LPS/TLR4-driven endotoxic shock. Importantly, these results validate the *gp130*$^{F/F}$ mouse as a unique preclinical model for further

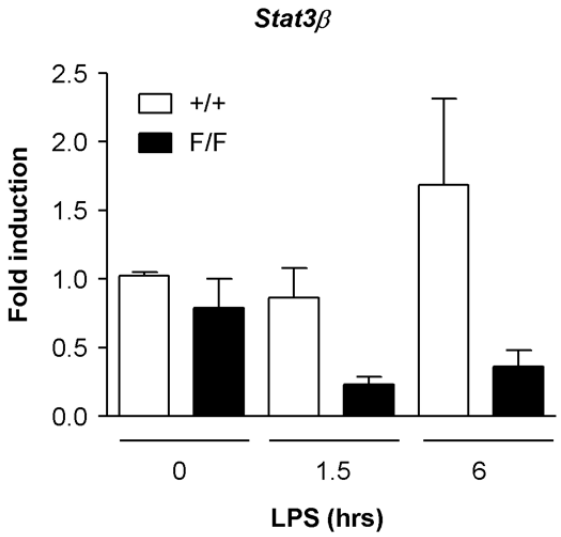

Figure 8.1. *Levels of Stat3β in gp130$^{F/F}$ spleen tissue in response to LPS-induced endotoxic shock.* qPCR analyses of *Stat3β* gene expression in whole liver tissue from *gp130$^{+/+}$ (+/+,* white bars*)* and *gp130$^{F/F}$* (F/F, black bars) mice at indicated time points following i.p. administration of 4mg/kg LPS. Expression data from at least 2 samples per genotype are shown following normalisation to *18S* expression and are presented from replicate analysis as the mean fold induction ± SEM relative to unstimulated samples.

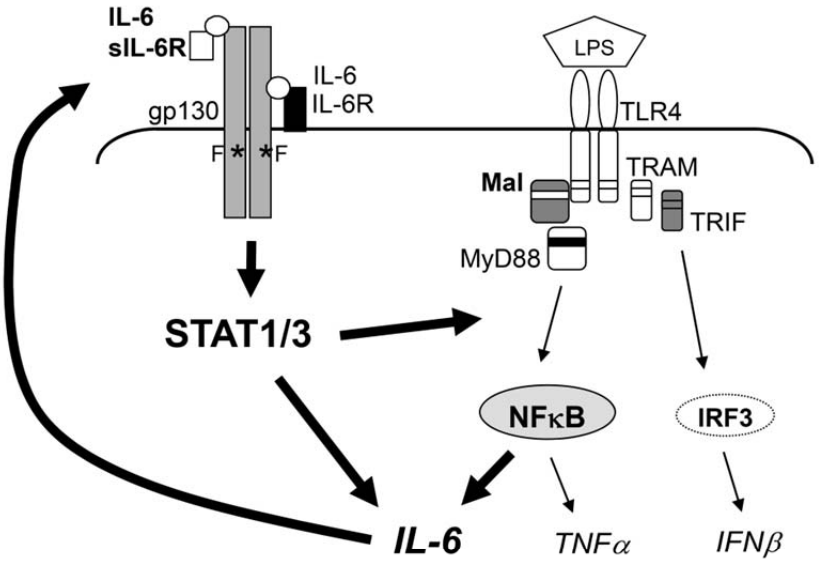

Figure 8.2. *Proposed model for regulation of LPS/TLR4 signalling by IL-6 and STAT1/3.* Prior to LPS engagement of TLR4, basal IL-6-mediated STAT1 and/or STAT3 activation selectively modulates the TLR4/Mal signalling axis for potentiated production of IL-6. Upon LPS-induced activation of TLR4/Mal (and NF-κB) signalling, upregulated IL-6 complexes with sIL-6R (trans-signalling) and the gp130Y_{757}F receptor to augment STAT1/3 activation. Hyperactivation of STAT1/3 further upregulates IL-6 production, both directly as well as via TLR4/Mal signalling.

translational research into the potential therapeutic benefits of targeting IL-6 trans-signalling in patients exhibiting symptoms of bacterial shock. Since up-regulated production of IL-6 and elevated activation of STAT3 are common traits of human chronic inflammatory states, RA and IBD, my study is also likely to provide important mechanistic insights of potential clinical relevance to such disorders driven by microbial and/or endogenous TLR ligands. For instance, TLR4 on host cells has been shown to recognise various Gram-negative pathogens including *Neisseria meningitides*, *E. coli*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, and *Brucella abortus* (reviewed in Schnare *et al.,* 2006). Moreover, treatment of the *gp130*$^{F/F}$ mice exposed to other inflammatory models (such as cecal ligation and puncture (CLP), *Staphylococcus epidermidis* cell-free supernantant (SES) and colon ascendens stent peritonitis (CASP)) with sgp130Fc would determine the importance of trans-signalling during sepsis triggered by TLR ligands other than LPS. Interestingly, the related TLR2 has been thought to play a role in infections from Gram-positive pathogens such as *S. pneumoniae* meningitis, group B streptococcus, *Bacillus subtilise* and *L. monocytogenes*, as well as in atherosclerosis (reviewed in, O'Neill *et al.,* 2009). Furthermore, SES-induced peritonitis is thought to be TLR2 driven, as *Staphylococcus epidermidis* is recognised by TLR2 (Stevens *et al.,* 2009; Strunk *et al.,* 2010). Previously, research has shown that *gp130*$^{F/F}$ mice have increases in activated STAT3 and neutrophil clearance in response to SES (Fielding *et al.,* 2008), as well as elevated T cell recruitment to the peritoneal cavity (McLoughlin *et al.,* 2005). Hence it would be interesting to investigate the role of trans-signalling in this inflammatory condition. Moreover, to confirm the importance of trans-signalling during LPS hypersensitivity of the *gp130*$^{F/F}$ mice, they could be crossed with sgp130 transgenic mice (Chalaris *et al.,* 2010) and assessed for survival in response to LPS.

Although LPS is an accepted experimental model for establishing sepsis in mice, there are limitations to its use as a model for human sepsis (reviewed in, Doi *et al.,* 2009). For instance, it has been observed that polymicrobial sepsis in humans or rodents is not TLR4-dependent (McMasters *et al.,* 1994; Weighardt *et al.,* 2002; Feterowski *et al.,* 2003; Dear *et al.,* 2006). Although outside the scope of this thesis, it would be interesting to complete experiments on the *gp130*$^{F/F}$ mice using other these other models of sepsis (CLP, CASP or infusion or

instillation of exogenous bacteria) and assess survival, local and system production of IL-6, and whether sgp130Fc administration impacts the survival of these mice. These results would assess whether trans-signalling is driving the inflammatory phenotype of the *gp130*$^{F/F}$ mice across multiple sepsis models. Furthermore, it would be critical to analyse the importance of the TLR4/MyD88-dependent pathway in driving the inflammatory response in these models, by undertaking survival studies with the *gp130*$^{F/F}$:*Mal*$^{-/-}$ mice.

Previous attempts to more closely correlate mouse models of sepsis with human disease have been undertaken with successful outcomes (reviewed in, Doi *et al.,* 2009). For instance, replicating genetic heterogeneity of the human population that typically have sepsis, by using various different mouse backgrounds, as well as mimicking the treatments patients receive in hospital (such as antibiotics) can result in improved models of sepsis (reviewed in, Doi *et al.,* 2009). The significance of the general applicability of IL-6 trans-signalling as a key pro-inflammatory mechanism in LPS-mediated endotoxic shock in wild type mice was addressed by my results in Chapter 4, which revealed that *gp130*$^{+/+}$ mice are protected against a lethal dose of LPS when administered with sgp130Fc. These results give more strength to the importance of trans-signalling during this disease.

Translating mouse experiments into a clinical setting presents many challenges, due mostly to differences in genetic makeup between these two organisms. For instance, rodents are less sensitive to LPS than humans (reviewed in, Doi *et al.,* 2009). Although outside the scope of this thesis, future experiments could include investigating the molecular mechanisms involved in human sepsis, as at present there is no data linking short nuclear polymorphisms in STAT3 with human sepsis, or whether this transcription factor is associated with disease onset. For instance, determining the levels of STAT1 and STAT3 in sepsis patient samples would be vital. This could involve utilising peripheral blood mononuclear cells harvested from normal and sepsis patients, and then treated *ex vivo* with/without LPS. Following stimulation, the levels of activated STAT1/3 could be assessed using phospho-flow cytometry and IL-6 production determined by ELISA. The potential for treatment with sgp130Fc to reduce the IL-6 production and STAT1/3 activation in these cells could also be investigated.

Although not experimentally-tested in my thesis, a potential molecular mechanism contributing to, at least in part, the inflammatory phenotype observed in the *gp130*$^{F/F}$ mice following LPS administration may stem from the inability of TGF-β1 to signal. Previous studies in the *gp130*$^{F/F}$ mice indicate that augmented activation of STAT3 up-regulates expression of Smad7, the negative regulator of TGF-β1, thus preventing TGF-β1 from signalling in the gastric compartment of mice (Jenkins a *et al.,* 2005). In addition, it was shown that TGF-β1 signalling is reduced in the haemopoietic compartment of the *gp130*$^{F/F}$ mice (Jenkins a *et al.,* 2005). As discussed earlier in Chapter 1, TGF-β1 is a potent anti-inflammatory factor that is induced in response to LPS to counteract the production of pro-inflammatory cytokines. Furthermore, mice lacking TGF-β1, or the TGF-β1 signalling molecule Smad3, had increased expression of TLR4 and augmented production of pro-inflammatory cytokines following LPS administration (McCartney-Francis *et al.,* 2004). Accordingly, impaired TGF-β1 signalling in the *gp130*$^{F/F}$ mice may contribute to their LPS hypersensitivity. To formally address whether there was any functional interaction between TGF-β1 and gp130/STAT3 signalling in endotoxemia in *gp130*$^{F/F}$ mice, genetic complementation studies could be employed using *gp130*$^{F/+}$ mutant mice heterozygous for either *TGF-β1* or *Smad3* (mice which individually display "normal" response to LPS) to determine whether the resulting *gp130*$^{F/+}$:*Tgfb1*$^{-/+}$ or *gp130*$^{F/+}$:*Smad3*$^{-/+}$ mice become LPS hypersensitive akin to *gp130*$^{F/F}$ mice. Notably, *gp130*$^{F/F}$ mice cannot be crossed onto a Smad7-deficient background as the majority of these mice are not viable, and those that are have impaired cardiac functions and severe arrhythmia (Chen *et al.,* 2009). Furthermore, investigating whether TGF-β1 reduces the level of IL-6 produced in the *gp130*$^{F/F}$ mice would be vital as IL-6 is the main driver of LPS hypersensitivity in these mice. Future experiments could include treating endothelial/epithelial cells from *gp130*$^{+/+}$ and *gp130*$^{F/F}$ mice with LPS in the presence/absence of TGF-β1 and examining IL-6 levels. Moreover, *gp130*$^{F/F}$ mice could be injected with LPS and TGF-β1 and the levels of IL-6 in the serum analysed and survival monitored. Furthermore, examining the levels of TGF-β1 itself in the LPS-treated *gp130*$^{F/F}$ mice would determine whether it's down-regulation leads to a suppression in anti-inflammatory signalling. Additionally, investigating the levels of Smad7 in the *gp130*$^{F/F}$ mice (for instance in

endothelial/epithelial cells) would also be beneficial in understanding the role of TGF-β1 signalling in endotoxic shock in *gp130*$^{F/F}$ mice.

Another form of TGF-β1 regulation relates to SOCS3, which has been shown to inhibit TGF-β1/Smad3 signalling in macrophages (Liu *et al.,* 2008). Specifically, it was found that SOCS3 could interact with Smad3 and inhibit its nuclear translocation and transcriptional activity (Liu *et al.,* 2008). Therefore, the basal increase in SOCS3 in *gp130*$^{F/F}$ mice (Jenkins b *et al.,* 2005), while no longer able to downregulate gp130 signalling, may be playing a role in the inhibition of TGF-β1 signalling.

Previously, it has been shown that IFN-γ signalling through the Jak1/STAT1 pathway increases the expression of Smad7, which inhibits the TGF-β-mediated Smad3 phosphorylation and consequently, reduced TGF-β signalling (Ulloa *et al.,* 1999). Accordingly, another experiment may include examining the role of hyperactivated STAT1 in the inhibition of TGF-β1 signalling in the *gp130*$^{F/F}$ mice by stimulating *gp130*$^{F/F}$:*Stat1*$^{-/-}$ epithelial/endothelial cells with LPS in with/without TGF-β1 and examining pro-inflammatory outputs.

Perhaps the most obvious future experiments implicated by my research are the need to uncover STAT1/3-regulated genes and/or interacting protein partners whose expression and/or activity is influenced by IL-6 trans-signalling in the *gp130*$^{F/F}$ mouse model. I note that ongoing DNA microarray and proteomic approaches in my laboratory are designed to reveal this information. For instance, studies are examining the global gene expression profile in livers of LPS-treated *gp130*$^{F/F}$ mice to look for candidate pathways and genes that may lead to hypersensitivity. Following the identification of the cell type involved in the LPS hypersensitivity of the *gp130*$^{F/F}$ mice, microarrays on these cells following LPS treatment could be carried out, as well as qPCR validation. Moreover, chromatin immunoprecipitation (ChIP) experiments will be undertaken to identify if STAT1 and STAT3 bind to the promoter of a particular gene, following LPS administration in the *gp130*$^{F/F}$ mouse cells. Moreover, confirmation of the role of the suspected interacting protein partners during LPS hypersensitivity in the *gp130*$^{F/F}$ mice would be achieved by generating mice genetically-deficient in this protein and crossing them onto *gp130*$^{F/F}$ mice for future LPS survival studies. However, if this interacting protein was in fact repressed by STAT1 and/or

STAT3, then over-expressing it in a transgenic mouse would help confirm its role during LPS hypersensitivity. These experiments will shed light on the possible therapeutic interventions that could be made during sepsis. In this respect, it is tempting to speculate that such a candidate may include NF-κB, which can form a transcriptional complex with STAT3 to induce a specific subset of genes, albeit in immortalised human mammary epithelial cells, including IL-6 (Yang *et al.,* 2007).

In summary, my results indicate a novel mechanism of cross-talk which involves TLR4-activated IL-6 trans-signalling via STAT1/3 regulating the Mal/NF-κB pathway to specifically modulate TLR4/LPS-driven IL-6 production and therefore the inflammatory response. Once the STAT3-regulated genes and/or interacting protein partners whose expression and/or activity is influenced by IL-6 trans-signalling in the *gp130*$^{F/F}$ mouse model have been revealed, they will provide a platform for further research and potentially be a therapeutic target, especially in Gram-negative cases of sepsis/septic shock characterised by elevated IL-6 production.

APPENDIX I

Accepted Manuscripts Generated from this Thesis

Part of the work in this thesis was presented as the following journal article:

Greenhill, C. J., S. Rose-John, R. Lissilaa, W. Ferlin, M. Ernst, P. J. Hertzog, A. Mansell and B. J. Jenkins (2010). IL-6 Trans-Signaling Modulates TLR4-Dependent Inflammatory Responses via STAT3. *J Immunol* **186**(2): 1199-208.

APPENDIX II

Buffers and Solutions

50x TAE:

2 M Tris-HCl, 50 mM EDTA, 5.7% (v/v) glacial acetic acid, adjusted to pH 8.0.

Tail Buffer (1L):

0.05M Tris, 0.025M EDTA, 0.05M NaCl, 0.005% SDS

T.E Buffer:

10 mM Tris-HCl, 1 mM EDTA, adjusted to pH 8.0.

Diethyl Pyrocarbonate (DEPC-treated water):

0.1% DEPC (Sigma). Made up with 1L with MilliQ water. Incubated O/N at 37^{o}C then autoclaved

PCR Buffer

250mM KCl/100mM Tris pH 8.3/15mM MgCl2 (10x)

dNTP stock:

25% of each nucleotide (adenine, thymine, guanine, cytosine) stock (100 mM). Stored in aliquots at -20ºC.

1.5% Agarose:

1.5 g of electrophoresis LE analytical grade agarose (Promega), 100ml of stock 1 X TAE, gently heat and agitate until agarose has dissolved.

Ethidium Bromide:

0.1 ml stock of 10 µg/ml Ethidium Bromide, adjust volume to 250 ml.

DNA Loading Dye:

0.25% Bromophenol Blue, 0.25% Xylene Cyanol, 10 mM EDTA, 20% Glycerol, adjusted to pH 8.0.

10x Phosphate Buffered Solution (PBS; pH 7.4):

14 mM NaCl, 3 mM KCl, 10 mM $Na_2HPO_4.12H_2O$, 1.8 mM KH_2HPO_4, adjusted to pH 7.4.

RPMI/10% FCS media

((Roswell Park Memorial Institute (RPMI) 10% (v/v) heat inactivated foetal bovine calf serum (FCS) (Gibco) and 1% (v/v) Penicillin/Streptomycin (Gibco)]

KalB Lysis Buffer:

50 mM Tris, 150 mM NaCl, 1% Trition X-100 and 1 mM EDTA, adjusted to pH 7.4 and stored at 4ºC. Prior to use 1% (w/v) NaF, 1% $NaVO_4$, 1% of 100 mM Phenylmethylsulphonyl fluoride (PMSF) and 1 tablet of protease inhibitor cocktail (EDTA-free) (Roche) per 50 ml was added.

Reagents for Lowry

Reagent A- an alkaline copper tartrate solution

Reagent B- a dilute Folin Reagent

Reagent S

SDS-PAGE Resolving Gel Buffer (RGB):

2 M Tris-HCl and 10% SDS, adjusted to pH 8.8.

SDS-PAGE Stacking Gel Buffer (SGB):

1 M Tris-HCl and 10% SDS, adjusted to pH 6.8.

Tris-glycine SDS-Polyacrylamide Gel (2x gels):

Stacking gel (5%)/ Lower (10%)

H_2O (ml)- 2.45/4

30% Polyacrylamide (ml)- 0.80/3.3

1M Tris(pH6.8/8.8)& 10% SDS (ml)- 1.25/2.5

TEMED (µl)- 4/30

10% Ammonium persulfate (µl)- 100/100

2x SDS PAGE Sample Buffer:

2.5% (v/v) SDS, 25% Glycerol, 0.04% (w/v) Bromophenol blue, 0.125 M Tris-HCl, adjusted to pH 6.8. Prior to use add 5% (v/v) β-Mercaptoethanol.

1X SDS-PAGE Running Buffer:

25 mM Tris-HCl, 192 mM Glycine and 0.1% (w/v) SDS.

Dilutions of antibodies for Western blotting-

Antibody	Dilution	Secondary antibody
Ptyr705 STAT3	1:2000	Anti-rabbit - IRDye800CQ
Total STAT3	1:500	Anti-rabbit -AlexaFluor 680
Erk1/2	1:1000	Anti-rabbit- AlexaFluor 680
Ptyr705 STAT1	1:1000	Anti-rabbit- IRDye800CQ
Total STAT1	1:1000	Anti-rabbit- AlexaFluor 680

0.1% PBST

1ml of 1% (v/v) Tween 20 was added to 1itre of PBST

Membrane Stripping Buffer:

0.2 M Glycine, 0.05% (v/v) Tween 20 and 100 mM β-Mercaptoethanol, adjusted to pH 2.5.

ELISA for TNFα and IL-6

-Binding solution- 0.1M Sodium Carbonate, pH 9.5

-Blocking buffer- PBS with 10% FCS, pH 7

-Substrate solution- Tetramethylbenzidine and Hydrogen Peroxide. The BD PharmigenTM TMB Substrate Reagent Set

-Stop solution- 1M H_3PO_4

ELISA for CCL5/Cxcl1/IL-10

-Binding solution- PBS

-Blocking buffer- 1% (w/v) BSA in PBS, pH7.2-7.4

-Substrate solution- 1:1 mixture of Colour Reagent A (H_2O_2) and Colour Reagent B (Tetramethylbenzidine) (R&D Systems Catalog #DY999)

-Stop solution-1M H_3PO_4

0.05% PBST

0.5ml of 1% (v/v) Tween 20 was added to 1itre of PBST

Cell Staining Buffer-

(0.5% BSA, 100mM NaF, 1mM NaV, 10mM B-Glycerophosphoric acid, 4.5mM Na Pyrophosphate)

APPENDIX III

Primer Sequences

IFNAR2 Genotyping PCR:

AR2 forward-

5' GCAGGAAGTATGCCTAGCGAGG

AR2 reverse-

5' AGAGAACAAGTCTGGCCCACCC

Sequences of qRT primers

Qm18S F	GTAACCCGTTGAACCCCATT	**Qm18S R**	CCATCCAATCGGTAGTAGCG
QmCCL5F	ATATGGCTCGGACACCACTC	**QmCCL5R**	GTGACAAACACGACTGCAAGA
QmCD14F	GGCGCTCCGAGTTGTGACT	**QmCD14R**	TACCTGCTTCAGCCCAGTGA
QmIFNβF	ATGAGTGGTGGTTGCAGGC	**QmIFNβR**	TGACCTTTCAAATGCAGTAGATTCA
QmIL-1βF	CAACCAACAAGTGATATTCTCCATG	**QmIL-1βR**	GATCCACACTCTCCAGCTGCA
QmIL-6F	ATGGATGCTACCAAACTGGAT	**QmIL-6R**	TGAAGGACTCTGGCTTTGTCT
QmIL-10F	GGTTGCCAAGCCTTATCGGA	**QmIL-10R**	ACCTGCTCCACTGCCTTGCT
QmTNFαF	AGCCCACGTCGTAGCAAACCA	**QmTNFαR**	CGGGGCAGCCTTGTCCCTTG

Taqman qRT probes

Were designed and ordered from Applied Biosciences:

-*ISG15* (Label: FAM, Code: Mm01705338_s1)

-*18S* control (Label: VIC, Code: 4319413E)

APPENDIX IV

Suppliers

Applied Biosystems	Foster City, CA, USA
Applied Biosystems	Zug, Switzerland
BD Bioscience	Bedford, MA, USA
Beckman Instruments	Fullerton, CA, USA
Becton Dickinson	Franklin Lakes, NJ, USA
Biofuge	Germany
BioRad Gel Doc	Richmond, CA, USA
BMG Lab Technologies	Offenburg, Germany
Cell Signaling Technology	Beverly, MA, USA
Crown Scientific	Australia
Eppendorf	Hamburg, Germany
GE Healthcare	Australia
Gibco-BRL	Paisley, UK
GraphPad Software, Inc.	San Diego, California
Invitrogen	San Diego, CA, USA
Leica Instruments	Nussloch, Germany
LI-COR	Lincoln, NE
Millipore	Bedford, MA, USA

Nanodrop Technologies	Wilmington, DE, USA
Peprotech	USA
Pharmingen	San Diego, CA
Promega	Madison, WI, USA
Qiagen	Germany
R&D Systems	Minneapolis, MN
Roche Molecular Biochemicals	Mannheim, Germany
Rockland	Boyertown, PA, USA
Santa Cruz Biotechnology	Santa Cruz, CA
SARSTEDT	Germany
Shandon Scientific	Cheshire, UK
Sigma-Aldrich	Saint Louis, MO, USA
Themo Fisher Scientific	Waltham, WA, USA
XTRON	Australia

APPENDIX V

Equipment

Agarose gel electrophoresis	Mini-sub Cell GT, Bio-Rad
Centrifuges	J2-21M/E, Beckman Biofuge stratos, Heraeus instruments
Cell incubator	HERA Cell, Heraeus instruments
Flow Cytometry	BD FACS Canto II Flow Cytometry, BD Pharmigen
Homogeniser	Ika Ultra Turrax T25, Crown Scientific
Light Microscope	Leica DME, Leica Microsystems Inc.
Luminescence Reader	Fluostar Optima, BMG Labtech
My Cycler ™Thermal Cycler	Bio-Rad Laboratories
qRT-PCR cycler	7900HT Real-Time PCR System, Applied Biosystems
Spectrophotometer	ND-100, Nanodrop Technologies
UV illuminator	Gel Doc 1000, Bio-Rad

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