REGULATION OF ACUTE INFLAMMATION BY ONCOSTATIN M RECEPTOR-β

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Dedication

This thesis is dedicated to my family.

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There are many people to whom I owe a great deal of thanks for their help and support throughout the past three years.

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Summary

Although the interleukin (IL)-6 related cytokine Oncostatin M (OSM) affects a variety of inflammatory events associated with disease progression, the function of OSM in the face of an inflammatory challenge remains unclear. In this thesis a peritoneal model of inflammation, in association with in vitro studies using human primary cell lines, has been used to define the influence of OSM on chemokine-mediated leukocyte recruitment. When compared to wild type mice (WT) the induction of peritoneal inflammation in Oncostatin M receptor-β deficient mice (OSMR-KO) resulted in enhanced monocytic cell trafficking, with no differences in neutrophil or lymphocyte recruitment observed, suggesting that OSM control of leukocyte recruitment is functionally distinct from that of IL-6. Subsequent *in vitro* studies and an *in vivo* appraisal of inflammatory chemokine expression following peritoneal inflammation inferred that OSM regulation of CCL5 might account for the observed difference in monocytic cell trafficking. The OSM-mediated control of CCL5 is clearly distinct from the actions of IL-6, which acts as a more prominent *in vivo* regulator of CCL2 expression than OSM. Mechanistically, these studies inferred a hitherto unidentified interplay between OSM-mediated STAT signalling and NF-κB activation. In this respect, EMSA analysis of nuclear extracts from peritoneal membranes isolated during course of the inflammatory response showed that OSMR-KO mice display an enhanced profile of NF-κB activation as compared to WT mice. Initial *in vivo* appraisal of the role of OSMRβ-mediated signalling in repeated episodes of inflammation and associated tissue damage suggest that OSM continues to regulate monocytic cell trafficking throughout recurrent inflammatory episodes and does not play a significant role in inflammation-associated peritoneal tissue damage, again a finding clearly distinct from the observed effects of IL-6 in tissue injury. These findings suggest that activation of gp130 by IL-6 and OSM trigger distinct inflammatory responses to affect individual aspects of leukocyte trafficking.

Publications and Presentations

Publications

Hams E, Colmont CS, Dioszechy V, Hammond VJ, Fielding CA, Williams AS, Tanaka M, Miyajima A, Taylor PR, Topley N, Jones SA. Oncostatin M receptor-beta signaling limits monocytic cell recruitment in acute inflammation. *J Immunol.* 2008; **181(3)**: 2174-80.

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Presentations

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Abbreviations

ADAM	A Disintegrin and Metalloproteinase
APC	Antigen Presenting Cell
APC	Allophycocyanin
Bcl ($_{2/XL}$)	B-Cell Lymphoma ($_{2/XL}$)
BCR	B Cell Receptor
BDCA-2/4	Blood Dendritic Cell Antigen-2/4
BSA	Bovine Serum Albumin Fraction V
BSF-3	B-Cell Stimulating Factor-3
CD	Cluster of Differentiation
cDNA	Complementary DNA
CCR	CC Chemokine Receptor
CD40L	CD40 Ligand
CLC	Ciliary Neurotrophic Factor-Like Cytokine
CNTF	Ciliary Neurotrophic Factor
CNTFR	Ciliary Neurotrophic Factor Receptor
CRP	C Reactive Protein
CT-1	Cardiotrophin-1
Ctl	Unstimulated Control
CTLD	C-Type Lectin-Like Domain
CXCR	CXC Chemokine Receptor
DARC	Duffy Antigen Receptor for Chemokines
DC	Dendritic Cell
DC-SIGN	Dendritic Cell-Specific Intracellular Adhesion Molecule 3-Grabbing Nonintegrin
DD	Death Domain
DMEM	Dulbecco's Modified Eagle's Medium
dNTP	Deoxynucleotide Triphosphate
DS-sIL-6R	Differentially Spliced Soluble Interleukin-6 Receptor
DTT	Dithiothreitol
EAE	Experimental Autoimmune Encephalomyelitis
ECM	Extracellular Matrix

EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
EMSA	Electrophoretic Mobility Shift Assay
EMT	Epithelial-to-Mesenchymal Transition
ERF	Established Renal Failure
ERK1/2	Extracellular signal Related Kinase 1/2
FACS	Fluorescence Activated Cell Sorting
FCS	Fetal Calf Serum
FITC	Fluorescein Isothiocyanate
FoxP3	Forkhead box P3
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GATA3	GATA binding protein 3
G-CSF	Granulocye Colony Stimulating Factor
GDP	Guanosine 5'-Diphosphate
GLR	gp130-Like Receptor
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
gp130	glycoprotein 130
GPI	Glycosylphosphatidylinisotol
GROα	*gro* protein α
Grb2	Growth Factor Receptor Bound Protein 2
GTP	Guanosine 5'-Triphosphate
HHV	Human Herpesvirus
HIV	Human Immunodeficiency Virus
HPMC	Human Peritoneal Mesothelial Cell
HRP	Horseradish Peroxidase
Hrs	Hours
ICAM-1	Intracellular Adhesion Molecule-1
IFN-γ	Interferon-γ
IFNAR	Interferon α/β Receptor
IFNGR	Interferon γ Receptor
Ig	Immunoglobulin
IKK	Inhibitor of Nuclear Factor-κB Kinase
IκB	Inhibitor of Nuclear Factor-κB
IL-(1-31)	Interleukin-1-31

IL-6KO	Interleukin-6-deficient mice
IL-11KO	Interleukin-11-deficient mice
IL-(1-31)R	Interleukin-(1-31) Receptor
IL-1RAP	IL-1 Receptor Accessory Protein
iNOS	Inducible Nitric-Oxide Synthase
Ip	Intra-peritoneal
IP-10	10kDa Interferon-γ induced Protein
IRAK	IL-1-Receptor Associated Kinase
IRF3	Interferon-Regulatory Factor-3
I-TAC	Interferon-inducible T cell A Chemoattractant
iTreg	Inducible Regulatory T Cell
Jak	Janus Kinase
JNK	Jun N-terminal Kinase
LDL	Low Density Lipoprotein
LIF	Leukaemia Inhibitory Factor
LIFRβ	Leukaemia Inhibitory Factor Receptor β
LIFR-KO	Leukaemia Inhibitory Factor Receptor-β deficient mice
LPS	Lipopolysaccharide
LRR	Leucine Rich Repeat motif
LTA	Lipoteichoic Acid
MAPK	Mitogen Activated Protein Kinase
MCP	Monocyte Chemoattractant Protein
MFU	Mean Fluorescence Units
MHC	Major Histocompatibilty Complex
MIG	Monokine Induced by Interferon-γ
Mins	Minutes
MIP	Macrophage Inflammatory Protein
MMP	Matrix Metalloproteinase
MyD88	Myeloid Differentiation Primary-Response Protein 88
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NEMO	Nuclear Factor-κB Essential Modulator
NF-κB	Nuclear Factor-κB
NF-IL-6	Nuclear Factor-Interleukin-6
NK	Natural Killer Cell

NNT	Novel Neutotrophin-1
NO	Nitric Oxide
nTreg	Natural Regulatory T Cell
$O_2^{\cdot}$	Superoxide
OPG	Osteoprotegerin
OSM	Oncostatin M
OSMRβ	Oncostatin M Receptor β
OSMR-KO	Oncostatin M Receptor-β-deficient mice
PAF	Platelet Activating Factor
PAMP	Pathogen-Associated Molecular Pattern
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PC-sIL-6R	Proteolytic Cleaved Soluble Interleukin-6 Receptor
PD	Peritoneal Dialysis
PE	Phycoerythrin
PECy5	Phycoerythrin Cyano-5
PGN	Prostaglandin
PI3K	Phosphatidylinositol 3-Kinase
PMA	Phorbol 12-Myristate 13-Acetate
PMN	Polymorphonuclear Leukocytes
PMSF	Phenylmethylsulphonyl Fluoride
poly(dIdC)	Poly(2′-deoxyinosinic-2′-deoxycytidylic acid) sodium salt
PRR	Pathogen Recognition Receptor
RA	Rheumatoid Arthritis
RANKL	Receptor Activator for Nuclear Factor-κB Ligand
RANTES	Regulated upon Activation, Normal T cell Expressed and Secreted
ROR-γt	Retinoic Acid-Related Orphan Nuclear Hormone Receptor-γt
ROS	Reactive Oxygen Species
RT	Room Temperature
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SAA	Serum Amyloid A
SDF-1	Stromal Cell Derived Factor-1
SDS	Sodium Dodecyl Sulfate

SEM	Standard Error of the Mean
SES	*Staphylococcus epidermidis* Supernatant
sgp130	Soluble glycoprotein-130
SH2	Src Homology 2
Shc	SH2-and-Collagen-Homology-Domain-Containing Protein
SHP2	SH2-Domain-Containing Tyrosine Phosphatase
SIE	Sis-Inducible Element
sIL-6R	Soluble Interleukin-6 Receptor
SLC	Secondary Lymphoid Tissue Chemokine
SOCS	Suppressor of Cytokine Signalling
SOS	Son of Sevenless
SR-A	Scavenger Receptor-A
STAT	Signal Transducers and Activators of Transcription
SV40	Simian Virus 40
TAB	Transforming Growth Factor-β-Activated Kinase-Binding Protein
TACE	Tumour Necrosis Factor-α Cleavage Enzyme
TAK	Transforming Growth Factor-β-Activated Kinase
TARC	Thymus- and Activation-Regulated Chemokine
T_{CM}	Central Memory T Cells
TCR	T cell receptor
TECK	Thymus-Expressed Chemokine
T_{EM}	Effector Memory T Cells
TGF-β	Transforming Growth Factor β
Th cell	T helper Cell
TIMP	Tissue Inhibitor of Metalloproteinase
TIR	Toll/IL-1R domain
TLR	Toll-Like Receptor
TMB	3,3',5,5'-Tetramethylbenzidine
TNF-(αβ)	Tumour Necrosis Factor-α/β
TNFR	Tumour Necrosis Factor Receptor
Tr1	Type 1 Regulatory T Cell
TRAF	Tumour Necrosis Factor Receptor-Associated Factor 6
TRAIL	Tumour Necrosis Factor-Related Apoptosis Ligand
Treg	Regulatory T Cell

TYK2	Tyrosine Kinase 2
VCAM-1	Vascular cell adhesion Molecule
VEGF	Vascular Endothelial Growth Factor
WT	Wild Type

Table of Contents

List of Figures

Chapter 7 – General Discussion

List of Tables

Chapter 1

General Introduction

1.1 Overview of thesis objectives

The initiation of an immune response and ensuing inflammation is integral in clearance of any pathogen and returning the body to homeostasis. During these processes cytokines play an important role, not only in the initiation of an immune response, but also in the resolution of inflammation. Therefore understanding the role of individual cytokines during inflammation is important when considering potential therapeutic targets for chronic inflammatory conditions. One such factor is the inflammatory cytokine Interleukin (IL)-6, whose involvement in disease processes has led to the development of blocking agents (for example, the anti-IL-6 receptor antibody, Tocilizumab®) for therapeutic intervention. However, the contribution of other IL-6-related family members remains less well defined. Of particular interest in this study is the role of Oncostatin M (OSM). IL-6 and OSM elicit signalling through distinct receptor complexes, with IL-6 utilising gp130 homodimers and OSM utilising receptor complexes comprised of gp130 associated with alternative β-signal transducing subunits, OSMRβ or LIFRβ. Therefore there is a potential for OSM to have roles in inflammation and tissue injury unique from those elicited by IL-6. This thesis aims to delineate the role of OSM-mediated signalling in acute inflammation and compare these roles with those known to be elicited by IL-6, therefore assessing the potential for OSM as a therapeutic target for modulating inflammatory conditions.

1.2 Inflammation and the immune system

The immune system is a defence mechanism comprised of a variety of highly specialised defence cells, which utilise and respond to a series of communication molecules that work in unison to protect the body from infection and disease. This system enables the detection and subsequent removal of foreign pathogens with the aim of clearing the causative agent and restoring normal tissue architecture. Regulation of this immunological outcome is governed by activation of an inflammatory response.

Inflammation is a normal physiological response to infection, trauma or immunological challenge involving both innate and adaptive immunity, and can be characterised by the orderly recruitment of specific leukocyte subsets to sites of infection or tissue damage. The inflammatory response, initiated by inflammatory cytokines, involves a combination of cells, plasma components and cellular products,

the main aim of which is to regulate the recruitment, activation and the final clearance of leukocytes to bring about the effective resolution of the immune response. It is therefore a highly specialised communication system between leukocytes and stromal tissue cells local to the inflammatory insult or challenge.

During episodes of acute inflammation release of pro-inflammatory cytokines, predominantly IL-1 and tumour necrosis factor (TNF)-α, initiates the inflammatory process by inducing various cell types, including leukocytes and stromal cells to produce inflammatory mediators. At the onset of inflammation, leukocytes migrate to post-capillary venules surrounding the damaged tissue in a directional and selective manner, via chemotaxis. Many factors released by stromal cells and recruited leukocytes as well as the causative agent can act as chemoattractants, including phospholipid metabolites such as platelet activating factor (PAF), bacterial products (formylated N-terminal methionine groups), breakdown products of the complement cascade such as C5a, and possibly most importantly, the chemotactic cytokines (chemokines) (Hartmann *et al*, 1997; Zabel *et al*, 2006). Chemokines enable selective trafficking of individual leukocyte subsets dependent on the cell surface expression of specific chemokine-associated receptors. In addition to selective chemotaxis, chemoattractants perform other important roles during inflammation including leukocyte activation (eg. degranulation, NADPH oxidase activation), growth modulation of myeloid progenitor cells, induction of angiogenesis and sensitisation of cells to respond to sub-optimal levels of other inflammatory mediators (Baggiolini, 1998; Sallusto & Baggiolini, 2008).

After initiation of an immune response, the first cells to respond are typically neutrophils which initiate a rapid non-specific phagocytic response. This initial response is augmented by a secondary influx of monocytes and specific subsets of T and B cells, which accumulate at the site of inflammation and become activated to produce a more specialised or targeted immune response (Topley *et al*, 1996). The culmination of these activities comes from the orchestration of two immunological processes: innate immunity (a non-specific response to all potential foreign bodies), and acquired immunity (a specific response to a recognised pathogen).

1.2.1 Innate Immunity

Innate immunity is the first line of defence against all pathogens and involves not only a cellular response but also comprises anatomical and humoral barriers which act together to provide an initial rapid response. The innate defence mechanisms include the skin and mucosal membranes as well as leukocytes including neutrophils, macrophages, eosinophils and basophils. The innate response is a fast acting, non-specific response which does not change or evolve.

1.2.2 Polymorphonuclear neutrophils

Neutrophils are critical as a first line of anti-microbial defence and are the primary cell type involved in the innate immune response. Neutrophils differentiate from myeloid precursors in the bone marrow and are released into the periphery, where during incidences of infection or injury, they are rapidly recruited to the site of inflammation (Borregaard & Cowland, 1997). The initial phase in neutrophil recruitment is adhesion to the blood vessel wall, facilitating trans-migration from the circulation to the inflammatory site. Initially low affinity selectins facilitate neutrophil 'rolling' along stromal tissue cells, followed by firm attachment mediated by binding to adhesion molecules such as intracellular adhesion molecule (ICAM)-1. Tight adhesion is followed by extravasation into the damaged tissue (Faurschou & Borregaard, 2003). Neutrophils are trafficked to the site of inflammation by following a chemotaxis and can respond to a variety of stimuli including chemotactic agents such as C5a (a breakdown product of the complement cascade), lipid mediators (leukotrienes, eicosanoids) and neutrophil-activating chemokines including CXCL8 (formerly IL-8). Once at the site of inflammation, neutrophil activation leads to the phagocytosis of pathogens or cellular debris utilising degradative proteolytic enzymes and reactive oxygen species to clear the causative problem. In addition to phagocytosis, neutrophils also play an important role in the further recruitment of leukocytes to the site of inflammation through the release of cytokines and chemokines, including CXCL8 and receptor molecules including soluble CD62L (L-Selectin) and the soluble IL-6 receptor (sIL-6R) (Scapini *et al*, 2000).

Neutrophils are short-lived cells (typically 3 days) and are unable to proliferate but undergo constitutive apoptosis, which is vital not only in the regulation of basal neutrophil production but also in limiting excessive non-selective tissue inflammation

(Akgul *et al*, 2001). In this respect, self limiting shedding of CD62L and IL-6R reduces neutrophil recruitment and aids resolution (Venturi *et al*, 2003). Apoptotic neutrophils are recognised and phagocytosed by macrophages, depending on specific cell surface receptors expressed on the apoptotic cell.

1.3 Acquired Immunity

Adaptive or acquired immunity is a highly specific response involving recognition and targeting of the pathogen, then conferring 'immunologic memory' providing lasting protection against the targeted antigen. The adaptive immune response can be split into two categories; cell mediated, which is governed by T cells, and humoral, which is controlled by B cells, however, both these responses act in unison to convey host defence.

1.3.1 Cell mediated Immunity

Cell mediated immunity involves direct recognition and binding of T cells to antigen, via the T cell receptor (TCR), the composition of which is unique to an individual T cell. Antigens are classified as a specific peptide presented on the surface of a cell in association with an appropriate major histocompatibility complex (MHC) molecule. There are two classes of MHC; class I, which is expressed on all nucleated cells and is recognised by the TCR of $CD8^+$ cytotoxic T cells, and class II which are present only on the surface of antigen presenting cells (APC; for example macrophages, B-cells and dendritic cells (DCs)) and are recognised by $CD4^+$ T helper cells. $CD8^+$ T cells monitor MHC Class I expressed on the surface of all cells and are therefore able to distinguish 'self' antigens from virally infected cells (ie. the MHC presentation of virally-encoded antigens) or tumourigenic cells (Krogsgaard & Davis, 2005). $CD8^+$ T cells destroy infected cells by increasing membrane permeability and promoting apoptosis.

Following activation, peripheral $CD4^+$ T helper cells can differentiate into several distinct subsets, which are characterised by the secretion of a distinct profile of inflammatory cytokines. As a result, each T-helper subset is considered to convey a specific function.

1.3.2 $CD4^+$ T effector cell subsets

1.3.2.1 Th1 Cells

T cells activated by dendritic cells and macrophages induce cell-mediated inflammatory responses which are characterised by the type of cytokines they secrete and the expression of specific transcription factors. The Th1 subset can be characterised by high expression of interferon (IFN)-γ and require IL-12 for differentiation (Figure 1.1). IFN-γ signalling through STAT (signal transducers and activators of transcription)1 activates the T-box differentiation factor, T-bet, which in turn upregulates expression of IL-12Rβ2. IL-12, utilising IL-12R activates STAT4 thereby stabilising IFN-γ production and development of terminally differentiated Th1 cells (Murphy & Reiner, 2002; Szabo *et al*, 2000). Th1 cell responses are associated with eradication of intracellular pathogens, but are largely associated with the progression of chronic inflammation (Takeda *et al*, 1999; Harrington *et al*, 2006; Leonard *et al*, 1995).

1.3.2.2 Th2 Cells

Th2 cells recognise MHC class II expressed on antigen presenting cells and aid antibody secretion by activated B-cells (see Section 1.3.5). Th2 cells are associated with elimination of parasitic infections and the development of allergic responses. In this respect Th2 cells are characterised by production of IL-4, IL-5 and IL-13, which are potent activators of B-cell immunoglobulin (Ig)E production, eiosinophil recruitment and mucous production (Harrington *et al*, 2006). Differentiation of Th2 cells is initiated through IL-4 mediated induction of STAT6 and activation of the TCR, which promotes expression of the transcription factor GATA3, which in turn upregulates production of IL-4, IL-5 and IL-13, while suppressing Th1 differentiation by blocking STAT4 activation (Zhang & Flavell, 1997; Ouyang *et al*, 1998; Ouyang *et al*, 2000). Dysregulation of Th2 responses is associated with allergy and asthma (Larche *et al*, 2003).

1.3.2.3 Th17 Cells

An additional T-helper population was recently characterised by its capacity to secrete IL-17. These cells (termed Th17 cells) are characterised by production of IL-17A, IL-17F, IL-21, IL-22, IL-23 and expression of IL-23R (Harrington *et al*, 2005). IL-17 is a pleiotropic cytokine consisting of a number of isoforms which induces pro-

inflammatory cytokine and chemokine expression, angiogenesis and regulates neutrophil chemotaxis and dendritic cell maturation (Kolls *et al*, 2004).

Differentiation to a Th17 phenotype is dependent on several cytokines including IL-6, transforming growth factor (TGF)-β, IL-21 and IL-23, and can be blocked by the cytokines associated with promoting differentiation to either a Th1 or Th2 phenotype (McGeachy & Cua, 2008). Analysis of Jak-STAT pathway involvement in Th17 cell differentiation in response to IL-6 and TGFβ activation outlines a role for STAT3 and to a lesser extent STAT4, in the differentiation of Th17 cells. Further STAT3 driven signalling via IL-23 is subsequently needed to maintain the effector properties of Th17 cells (Yang *et al*, 2007; Mathur *et al*, 2006). Analysis of the transcription factors important in Th17 cell differentiation has shown a role for STAT3 driven activation of the transcription factor ROR-γt (Retinoic acid-related orphan nuclear hormone receptor-γt) (Ivanov *et al*, 2006).

Th17 cells are considered important in autoimmune responses as demonstrated in a variety of autoimmune conditions including murine models of rheumatoid arthritis and experimental autoimmune encephalomyelitis (EAE), where IL-17A deficient mice demonstrate decreased inflammation and tissue destruction and overexpression of IL-17 exacerbates disease severity (Nakae *et al*, 2003; Lubberts *et al*, 2001; Bettelli *et al*, 2007).

1.3.3 $CD4^+$ memory T cells

Memory T cells can be generated by clonal expansion and differentiation of effector T cells, the functions and migratory properties of which are imprinted during the interaction of T cells with antigen presenting cells. There are two general subsets of memory T cells; central memory cells (T_{CM}), which can be distinguished from a second subset, effector memory cells (T_{EM}), by the expression of CD27, CD62L and CCR7 (Campbell *et al*, 1998; Forster *et al*, 1999; Henger *et al*, 2003). T_{CM} provide reactive memory, they have little effector function but are homed to T cell areas within secondary lymphoid organs via CCR7 where they can readily proliferate and differentiate to effector cells in response to antigenic stimulation. Conversely, T_{EM} migrate to inflamed peripheral tissues and display immediate effector function (Lanzavecchia & Sallusto, 2000).

1.3.4 $CD4^+$ T cell regulatory subsets

In addition to effector T cells, $CD4^+$ T cells can be induced to differentiate into regulatory T cells (Treg), which are characterised by expression of CD25 (IL-2Rα) and the transcription factor FoxP3 (Figure 1.1) (Roncarolo *et al*, 2006; Chen *et al*, 2003). Tregs are widely accepted as immunological suppressor cells preventing autoimmune and chronic inflammatory conditions and their activity is governed by cytokines including IL-10, TGFβ and IL-6 (Vignali *et al*, 2008). In addition to inducible regulatory T cells, which are adapted from effector $CD4^+$ T cells, naturally occurring $FoxP3^+$ Tregs develop in the thymus and display a diverse TCR repertoire that is specific for self antigens (Fontenot *et al*, 2005). Antigen presenting cells, when exposed to a given pathogen in the periphery, initiate both effector T cell and natural Treg responses (Vignali *et al*, 2008). Critically IL-6 has been shown to suppress Treg activity and differentiation. In this respect Treg are considered to be derived from the same T cell lineage as Th17 cells (Bertelli *et al*, 2007; Pasare & Medzhitof, 2003).

Figure 1.1 Differentiation of T cell subsets

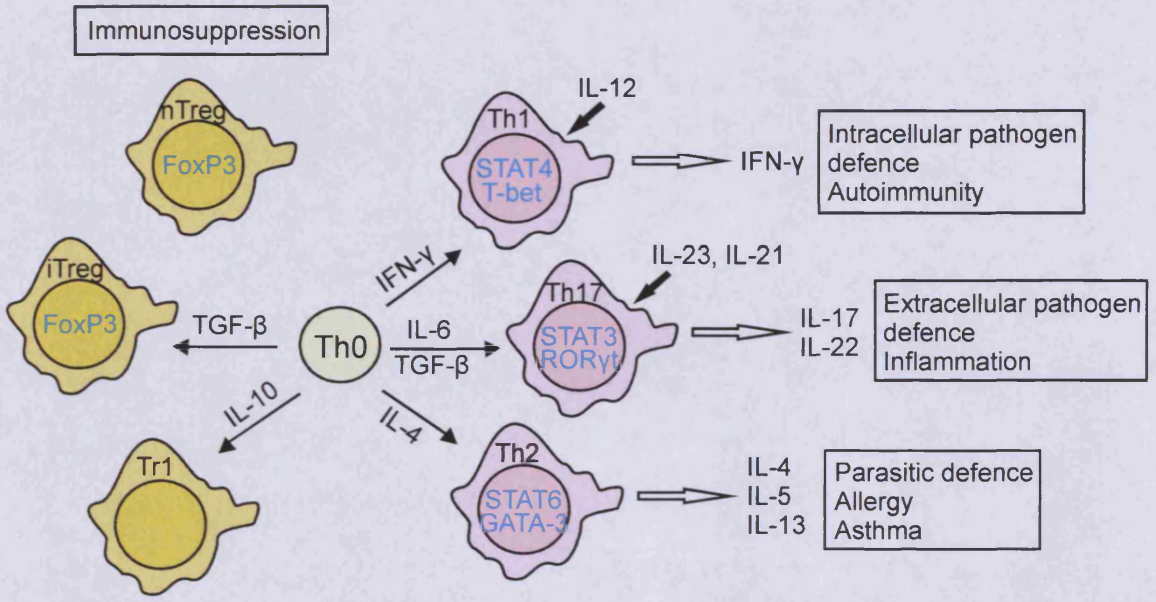

Fig 1.1. Differentiation of T cell subsets. Peripheral naïve $CD4^+$ T cells (Th_0 cells) can differentiate into at least three effector subsets (expressing unique transcription factors) dependent upon cytokine stimulation; Th1, Th2 and Th17, each of which have important but unique roles in host defence. In addition Th_0 cells can also differentiate into regulatory T cells including induced Treg (iTreg) cells and Tr1 cells (another class of regulatory T cell). Naturally occurring Tregs (nTreg) are generated from $CD4^+$ thymic T cell precursors. (Adapted from Bettelli *et al*, 2007).

1.3.5 Humoral Immunity

Humoral immunity involves the production of antibodies by B cells in response to the recognition of specific antigenic epitopes. Naïve B cells can be activated in either a T cell dependent or independent manner. Activation is through binding of antigens on the pathogen to a specific B cell receptor (BCR), which have a typical immunoglobulin (Ig) structure and resemble either IgD or monomeric IgM.

Naïve B cells are responsible for the primary humoral response, however, after exposure to cytokines including IL-4, IL-6, IL-10, IL-13, IL-21 and TGFβ, and repeated TCR activation, B cells can be induced to differentiate to give a memory phenotype. This involves Ig 'class switching' where naïve B cells, in response to specific stimuli, can be induced to switch Ig isotype from secretory IgM (either pentameric or heptameric) to IgA, IgG or IgE enabling a more robust specialised response (Tangye & Hodgkin, 2004). Memory B cells also demonstrate increased expression of MHC class II, CD80, CD86 and CD95 which enable them to act as antigen presenting cells for $CD4^+$ T cells (Lui *et al*, 1995) (see Section 1.3.2). Memory B cells can be induced to secrete Ig, and thus become antibody releasing cells after continued exposure to the T cell and certain regulatory molecules including CD40L (Arpin *et al*, 1995).

The primary source of serum antibodies are terminally differentiated plasma cells, which differ from memory B cells through alterations in cell surface marker expression. Plasma cells downregulate expression of membrane bound Ig and MHC Class II, but acquire expression of CD38 (Abney *et al*, 1978; Halper *et al*, 1978; Tangye & Hodgkin, 2004). Unlike memory B cells, plasma cells do not process and present antigen, instead the main function of plasma cells appears to be production and secretion of large quantities of specific antibody (Slifka *et al*, 1998).

1.4 Mononuclear phagocytes

Mononuclear phagocytes are integral to host immune defence but also play a crucial role in development and tissue homeostasis (Gordon, 1986; Gordon, 1998). Macrophages can be split into two very broad categories, 'resident' tissue macrophages and 'inflammatory' macrophages; however, both are derived from circulating bone marrow-derived monocytes (Volkman & Gowans, 1965; Gordon & Taylor, 2005). Monocytes can also give rise to antigen presenting dendritic cells (see Section 1.4.2) (Taylor *et al*, 2005). Tissue macrophages are highly heterogeneous populations, with differences in their activities dependent on the local environment and function they are required to perform (Taylor *et al*, 2005). In addition to lymphoid organs, many other organ systems including the liver, lung, nervous system, gut and epidermis have distinct populations of specialised tissue macrophages (Taylor *et al*, 2005). A major role of the macrophage in homeostasis and after infection or injury is the phagocytic removal of apoptotic and senescent cells (Fadok *et al*, 1998; Pickering *et al*, 2000). In response to immune stimulation or triggering of the inflammatory response, additional monocytes are recruited from the periphery where they adapt to their new microenvironment by altering cell surface receptor expression (Taylor *et al*, 2005).

Studies into human peripheral blood monocyte heterogeneity demonstrate that in addition to morphological heterogeneity, these cells can be characterised as two distinct subsets: 'inflammatory' $CD16^-$ monocytes, which show high expression of CD14, the classical human monocyte marker which forms part of the receptor for lipopolysaccharide (LPS), an endotoxin present in the cell wall of gram-negative bacteria; and 'resident' $CD14^+CD16^+$ monocytes, which in addition to expressing CD14, also express CD16, an Fc receptor for IgG1 (Passilick *et al*, 1989). Several phenotypic and physiological differences have been observed between these two subsets. Each demonstrates individual chemokine receptor expression, for example $CD14^{hi}CD16^-$ monocytes express CCR2, whereas $CD14^+CD16^+$ cells express CCR5 (Weber *et al*, 2000). The specific receptor expression for each subset is outlined in Table 1.1. Each subset also display a unique cytokine and chemokine profile, with 'inflammatory' monocytes associated with high expression of pro-inflammatory cytokines including IL-1, TNF-α and IL-6, whereas 'resident' monocytes are associated with the release of IL-12 and IFN-α (Akiyama *et al*, 1985; Elias *et al*,

1985; Fernandez *et al*, 1986; Szabo *et al*, 1990). As in humans, mice also exhibit two distinct monocyte subsets, each determined by unique receptor expression (Figure 1.2). Monocytes in mice can be identified as $F4/80^{+}CD11b^{+}$, but can be further divided by expression of CD62L (L-selectin), CCR2, CX_3C-chemokine receptor 1 (CX_3CR1) and Ly6C (Gordon & Taylor, 2005; Palframan *et al*, 2001). Studies have shown that $CCR2^{+}CD62L^{+}CX_3CR1^{low}Ly6C^{+}$ monocytes are functionally equivalent to $CD14^{hi}CD16^{-}$ 'inflammatory' monocytes in humans, whereas the $CCR2^{-}CD62L^{-}$ $CX_3CR1^{hi}Ly6C^{-}$ monocytes relate to human $CD14^{+}CD16^{+}$ 'resident' monocytes (Gordon & Taylor, 2005).

Table 1.1 Monocyte phenotype in humans and mice

	Human $CD14^{hi}CD16^{-}$ 'inflammatory' monocytes	Human $CD14^{+}CD16^{+}$ 'resident' monocytes	Mouse $CCR2^{+}$ CX_3CR1^{low} 'inflammatory' monocytes	Mouse $CCR2^{-}$ CX_3CR1^{hi} 'resident' monocytes
Chemokine receptors				
CCR1	+	-		
CCR2	+	-	+	-
CCR4	+	-		
CCR5	-	+		
CCR7	+	-		
CXCR1	+	-		
CXCR2	+	-		
CXCR4	+	++		
CX_3CR1	+	++	+	++
Other receptors				
CD4	+	+		
CD11a			+	++
CD11b	++	++	++	++
CD11c	++	+++	-	+
CD14	+++	+		
CD31	+++	+++	++	+
CD32	+++	+		
CD33	+++	+		+
CD43			-	-
CD49b			+	-
CD62L	++	-	+	
CD86	+	++		++
CD115	++	++	++	++
CD116	++	++	++	
F4/80			+	+
Ly6C			+	-
MHC Class II	+	+	-	-

Table 1.1. Cell surface marker expression of mouse and human 'inflammatory' and 'resident' monocytes (Adapted from Gordon & Taylor, 2005).

Figure 1.2 Maturation of mononuclear phagocytes in mice

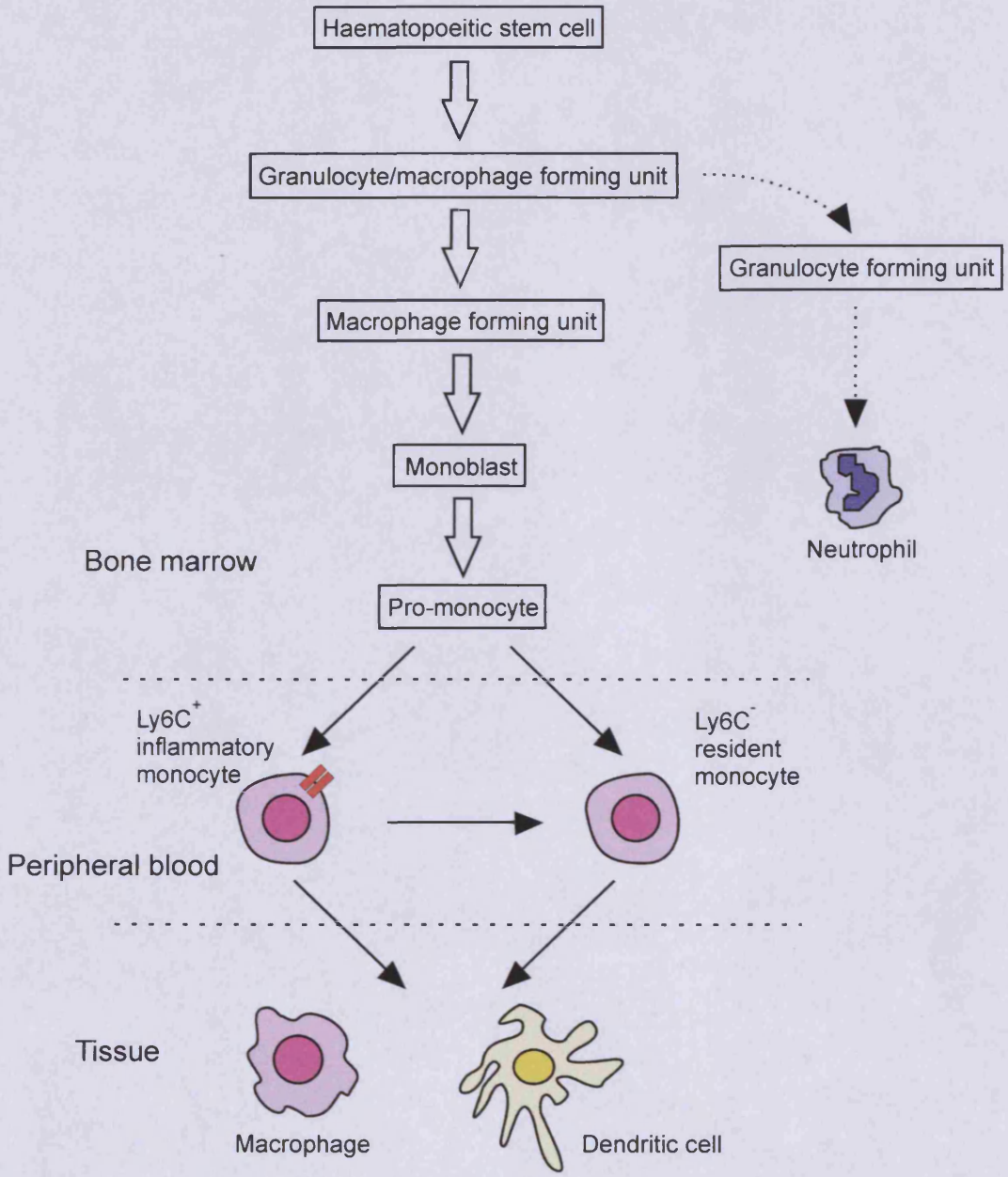

Fig 1.2. Maturation of mononuclear phagocytes in mice. Ly6C^{+} monocytes are generated from haematopoietic stem cells in the bone marrow and released into the peripheral blood, where they are thought to adopt a Ly6C^{mid} phenotype, both of which are able to respond to pro-inflammatory chemokines and are therefore recruited to sites of inflammation. Most 'inflammatory' monocytes differentiate into macrophages, but some migrate into draining lymph nodes where they acquire dendritic cell-like phenotypes. In the absence of inflammation, Ly6C^{-} monocytes enter the tissue where they replenish the resident macrophage and dendritic cell populations. (Adapted from Gordon & Taylor, 2005).

1.4.1 Macrophage activation

Cell mediated macrophage activation can follow two pathways: classical activation, which relies on IFN-γ, and alternative activation, which is triggered after stimulation by IL-4 or IL-13 (Nathan *et al*, 1983; Stein *et al*, 1992; Goerdt *et al*, 1999) (Figure 1.3).

Classical activation requires two signals, IFN-γ (Nathan *et al*, 1983) and a secondary signal which sensitises the macrophages to respond to IFN-γ. Both $CD4^+$ Th1 and $CD8^+$ cytotoxic T cells produce IFN-γ, Th1 cells provide the other signal by expressing CD40 ligand (CD40L) on their cell surface, which when bound to CD40 on the macrophage, sensitise the macrophage to respond to IFN-γ (Stout *et al*, 1996). Alternative secondary signals include LPS and other conserved pathogen-associated molecular patterns (PAMPs) binding to pathogen recognition receptors (PRRs) expressed on the surface of the macrophage (see Table 1.2) thus inducing TNF production (Taylor *et al*, 2005); and endogenous factors including heat shock protein (Van Ginderachter *et al*, 2006). Classical activation is associated with a pro-inflammatory response, inducing increased anti-microbial activity, increased cytotoxic activities, increased expression of pro-inflammatory cytokines including TNFα, IL-1, IL-6, IL-12 and IL-23, and stimulates increased expression of MHC class II and CD40L (Mytar et al, 1999; Stuehr & Nathan, 1989; Urban *et al*, 1986). Due to the cytokine profile exhibited by classically activated macrophages, this pathway is associated with promoting Th1 immune responses.

In addition to IFN-γ-induced regulation of macrophage function, a pro-inflammatory class of macrophages can be activated through stimulation with Toll-like receptor (TLR) ligands, such as LPS (Boldrick *et al*, 2002; Nau *et al*, 2002). This form of activation is associated with a rapid, innate immune response, rather than cell-mediated. Innate activation is associated with increased production of pro-inflammatory cytokines as well as upregulation of reactive oxygen species (ROS) and inducible nitric-oxide synthase (iNOS), therefore increasing the phagocytic activity of the macrophage (Gordon, 2003).

The targeted recognition of bacterial, apoptotic or necrotic cells for phagocytic removal is highly complex and dependent on a wide array of specific receptor

complexes. Receptors including TLRs, mannose and β-glucan receptors, fibronectins and integrins expressed on the surface of both classically and innate activated macrophage bind to specific molecular patterns expressed on the surface of the target. These include markers such as phosphatidyl serine expressed on the surface of apoptotic or senescent cells (Taylor *et al*, 2005). Once recognition has occurred, the particle is internalised and exposed to detrimental levels of reactive oxygen species including superoxide and nitric oxide, which together with the degranulation of degradative enzymes leads to the clearance of the pathogen or cellular debris.

Table 1.2 Pathogen associated molecular patterns (PAMPs) and associated pathogen recognition receptors (PRR)

PRR family	**Examples of associated PAMPs (PRR ligands)**
Scavenger (collagenous) eg. SR-A	Polyanionic ligands, LDL (low density lipoproteins)
Scavenger (noncollagenous) eg. CD36	Oxidised LDL, apoptotic cells
GPI-anchored eg. CD14	LPS, LTA (lipoteichoic acid), PGN (prostaglandin), apoptotic cells
Integrin eg. CD11b	Apoptotic cells
Ig superfamily eg. CD33	Siacylic acid
NK-like C-type lectin-like eg. Dectin-1	B-glucan polysaccharides
Multiple CTLD eg. Mannose receptor	Mannose, fucose, N-acetylglucosamine
Toll-like receptor eg. TLR2, TLR4	LPS, peptodoglycan

Alternative activation is mediated through the Th2 cytokines, IL-4 and IL-13 (Gordon, 2003; Stein *et al*, 1992). Macrophages activated by IL-4 and IL-13 display increased expression of both MHC class II and the mannose receptor, facilitating endocytosis and antigen presentation; and suppression of pro-inflammatory cytokines (such as IL-1 and IL-6). Instead, alternatively activated macrophages secrete anti-inflammatory cytokines including TGFβ and IL-10, and direct Th2-type responses through the control of $CCR4^+$ $CD4^+$ T cell recruitment via secretion of CCL22 and CCL17 (Bonnechi *et al*, 1998; Andrew *et al*, 1998; Imai *et al*, 1999; Gordon, 2003). The anti-inflammatory cytokine IL-10 (also released by Th2 cells) also serves to suppress macrophage function by limiting MHC class II expression and anti-inflammatory mediator production (Fiorentino *et al*, 1989). As a result, alternatively activated macrophages are associated with tissue repair and have a general anti-inflammatory phenotype.

Figure 1.3 Activation pathways of macrophages

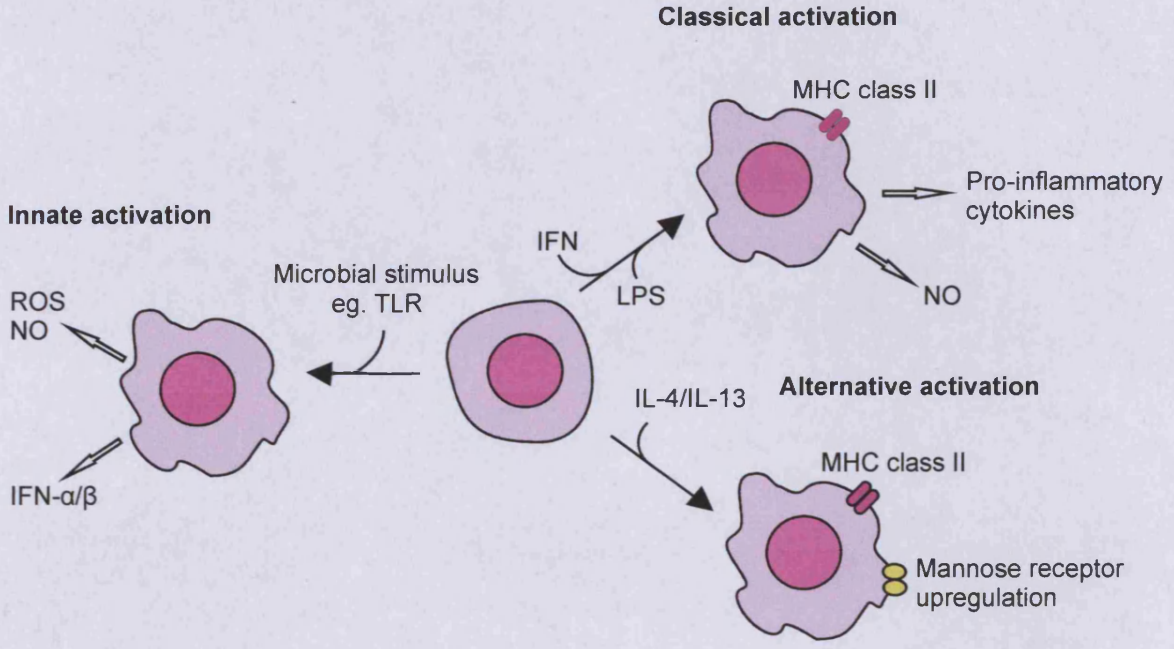

Fig 1.3. Activation pathways of macrophages. Innate activation occurs in response to recognition of microbial PAMPs (pattern-associated molecular patterns) by pattern-recognition receptors such as TLRs. Stimulation results in the release of pro-inflammatory cytokines such as IFNs, and reactive oxygen species (ROS) and nitric oxide (NO) thus promoting phagocytosis. Classical activation is mediated by IFN-γ followed by a secondary microbial stimulus such as LPS. Stimulation results in upregulation of MHC class II molecules, the release of pro-inflammatory cytokines such as IL-1 and IL-6 and initiation of the respiratory burst. The net effect of classical macrophage activation is microbicidal and conveys cellular immunity. Alternative activation is mediated by IL-4 and IL-13 and results in upregulation of MHC class II and Mannose receptor resulting in increased antigen endocytosis. Stimulation also results in the release of anti-inflammatory cytokines IL-10 and TGFβ, the net response of which is to mediate allergic and anti-parasitic responses and to induce tissue repair. (Adapted from Gordon, 2003).

1.4.2 Dendritic cells

Dendritic cells (DCs) are an additional class of antigen presenting cell, derived from circulating and bone-marrow derived monocytes. DCs are distinct from macrophages in their ability to present antigen to naïve T cells, whereas macrophages can only activate primed T cells (Inaba *et al*, 1990; Knight *et al*, 1985). There are two main categories of DC: plasmacytoid and conventional (also referred to as lymphoid and myeloid), which are distinguished by the presence of specific cell surface markers (Shortman & Naik, 2007). Plasmacytoid DCs produce large quantities of type-I IFN (IFNα, IFNβ) and express many lymphoid markers including CD123, CD45Rα, BDCA-2/4 (blood dendritic cell antigen-2/4), CCR6 and CCR7, whereas conventional DCs express CD11b, CD11c, CD1a and DC-SIGN (dendritic cell-specific intercellular adhesion molecule (ICAM) 3-grabbing nonintegrin) (Barchet *et al*, 2005). Numerous agents activate DCs including PAMPs (binding to PRRs), TLR signalling and damage-associated molecular pattern molecules (DAMPs), including heat shock proteins and uric acid expressed by dying cells (Janeway & Medzhitov, 2000; Calderwood *et al*, 2005; Pulendran, 2004). DCs secrete a wide array of inflammatory cytokines, chemokines and co-stimulatory molecules which aid the activation of naïve T cells, and their polarisation into Th1, Th2, Th17 or Treg cells.

1.5 Cytokines

Cytokines are a group of polypeptides produced and released by a variety of stromal cells and leukocytes. Cytokines act as intracellular and extracellular signalling mediators, which regulate cellular responses through juxtracrine, paracrine and autocrine mechanisms, affecting both homeostatic 'house-keeping' function and immunological processes. In this respect, cytokines are important regulators of the innate and adaptive immunity and affect proliferation, cellular differentiation, haematopoiesis and apoptotic regulation of cell survival or death (Yarden & Ullrich, 1988; Thomson, 1991). Proteins with cytokine-like properties include growth factors, interleukins, interferons and members of the TNF superfamily.

1.5.1 Interleukins

The interleukins (derived from communicates '*inter*' with leukocytes) refer to a group of cytokines able to provide communication signals between different populations of leukocytes (Aarden *et al*, 1979). There are currently 35 members of the interleukin family (Niedbala *et al*, 2007). Although the name implies that these factors only affect leukocytes, many interleukins are produced by non-haematopoietic cells and affect the functions of a diverse range of somatic cells. Of particular interest in this study are IL-6 and its related cytokines which will be discussed in further detail in Section 1.7.

1.5.2 Interferons

Interferons (IFN) are cytokines originally defined by their ability to inhibit virus replication and can be sub-divided into type I and type II interferons. Type I (IFNα/β) are induced by virally infected cells and can respond to almost any cell type (Roberts *et al*, 1998). In addition the type I IFNs have been shown to induce many activities linking innate and acquired immunity including dendritic cell maturation, B-cell differentiation and NK activation (Biron, 2001). Whereas type II IFN (IFNγ) is induced following antigenic stimulation of T cells and has a wide variety of actions including macrophage activation and upregulation of MHC class II (see Section 1.4.1) (Schroder *et al*, 2004).

1.5.3 TNF superfamily members

The tumour necrosis factor (TNF) superfamily currently consists of 19 ligands and orchestrates a wide range of biological functions including host defence, inflammation, cell death and development of the immune system (Zhang, 2004; Bodmer *et al*, 2002).

1.5.4 Functional redundancy within cytokine networks

Cytokines elicit similar or overlapping functions on the same cell types (functional redundancy). In this way a cytokine network is established, enabling the precise control of cellular proliferation, differentiation and activation of a variety of cell types, as well as controlling the further production and release of inflammatory mediators. Consequently cytokines provide an inflammatory cascade of signalling molecules, which function to fine-tune the inflammatory response dictating the duration and magnitude. This response therefore finely balances both beneficial and detrimental outcomes. As a result, the cytokine network is tightly regulated. Cytokines are only transiently expressed and are highly specific depending on cell surface expression of receptors corresponding to a particular cytokine. Other regulatory mechanisms exist including decoy and soluble receptors and specific cytokine antagonists which will be discussed in Section 1.6.6.

1.6 Cytokine Receptors

Cytokine receptors are expressed on the membranes of a wide variety of cell types, including leukocytes and stromal cells, whist some are also present in soluble form. Their ability to alter signalling in a wide variety of cell types is vital to their role in physiological processes. Membrane bound cytokine receptors are transmembrane glycoproteins, comprising an extracellular amino-terminal ligand binding domain and an intracellular carboxy-terminal domain. Based on this structural organisation cytokine receptors can be split into families which dictate ligand specificity and biological function.

1.6.1 Type I cytokine receptors

Type I cytokine receptors are transmembrane glycoproteins comprising an extracellular ligand-binding domain, a short hydrophobic transmembrane region and a carboxy-terminal intracellular domain. All receptors belonging to this group share structural homology in a 210 amino acid region in the ligand-binding domain, including four highly conserved cysteine residues and a repeated tryptophan-serine motif separated by one random amino acid (WSXWS motif) proximal to the transmembrane domain (Hibi *et al*, 1990). There is little sequence homology in the intracellular domains, however, none of the type I receptors have intrinsic tyrosine kinase activity so rely on activation of cytoplasmic tyrosine kinases, the activity of which are discussed in Section 1.8. Many cytokines utilise type I receptors including members of the interleukin-related family of cytokines, therefore these receptors will be discussed in further detail in Sections 1.7 and 1.8.

1.6.2 Type II cytokine receptors

Type II cytokine receptors are tripartite single pass transmembrane proteins characterised by structural similarities in their extracellular domain, which includes the ligand binding motif. Similar to type I receptors, members of the type II cytokine receptor family lack intrinsic tyrosine kinase activity. However, type II receptors are distinguished from type I receptors by the absence of the carboxy terminal WSXWS motif. Instead class II receptors have a variety of sequences which appear to be important in preservation of the three-dimensional fold of the extracellular domain of the receptor (Langer *et al*, 2004). Ligands utilising the class II family of receptors include the type I interferons (IFN-α, IFN-β and IFN-κ) which utilise IFNAR-1 and

IFNAR-2 (Roberts *et al*, 1998), the type II interferon IFN-γ which utilises the IFNGR-1 and IFNGR-2 receptors (Schroder *et al*, 2004), IL-10 (and other related interleukins including IL-19, IL-22, IL-24 and IL-26) (Fickenscher *et al*, 2002) and factor VIIa, the ligand for tissue factor. Class II cytokines have varied roles in both innate and acquired immune responses including DC maturation, B cell differentiation, macrophage activation, T helper cell biasing and suppression of tumour development (Langer *et al*, 2004).

1.6.3 Tumour necrosis factor superfamily receptors

Tumour necrosis factor (TNF) family members and their corresponding receptors play a pivotal role in the development and function of the immune system. Due to the roles of TNF superfamily proteins in human disease, pharmaceuticals to inhibit TNF have been developed to treat inflammatory conditions such as rheumatoid arthritis and inflammatory bowel disease (Maini & Taylor, 2000; Papadakis & Targan, 2000). Members of the TNF superfamily include TNFα, CD40L and TRAIL (TNF-related apoptosis ligand). TNF family ligands exist as trimers, with each monomer composed of structurally conserved β strands organised in two layered sheets. Tumour necrosis family receptors (TNFR) are type I transmembrane proteins that form elongated structures on a scaffold of disulphide bridges forming cysteine-rich domains which are important in ligand recognition and binding (Locksley *et al*, 2001). The cytoplasmic domain of TNFRs functions as docking sites for signalling molecules. Signalling occurs through the engagement of adaptor proteins, either TRAFs (TNF receptor associated factors), or DD ('death domain') molecules, which are associated with receptors that generally result in caspase activation and subsequent cell death such as Fas. Association of TRAF proteins with TNF receptors results in activation of pathways such as NF-κB through direct interaction with IRAK (see Figure 1.4) (Muzio *et al*, 1998).

1.6.4 IL-1/Toll-like receptors

Toll-like receptors (TLRs) are type I integral membrane glycoproteins and on the basis of shared structural homology in the intracellular region, belong to a larger superfamily including IL-1 receptors. Conversely there is little homology in the extracellular domain, with the extracellular region of TLRs consisting of 19-25 tandem copies of a leucine-rich repeat (LRR) region, whereas the extracellular region

of IL-1 receptors contains three immunoglobulin-like domains. The LRR domains form a horseshoe structure, which is thought to be directly involved in pathogen recognition. Members of this superfamily share a conserved region of approximately 200 amino acids in the cytoplasmic region, the Toll/IL-1R (TIR) domain, which comprises three conserved boxes essential for signalling (Akira *et al*, 2001).

After ligand binding TLRs dimerise and undergo a conformational change enabling the binding of subsequent downstream signalling molecules as demonstrated in Figure 1.4. In the case of some TLRs including TLR2 and TLR4 signalling, TLR dimers associate with the adaptor molecule myeloid differentiation primary-response protein 88 (MyD88), which in turn recruits IL-1-receptor associated kinase (IRAK) proteins (Wesche *et al*, 1997; Li *et al*, 2002). Activation by phosphorylation initiates a cascade resulting in activation of both the inhibitor of nuclear factor-κB kinase (IKK) complex and mitogen-activated protein (MAP) kinase cascade (Wesche *et al*, 1997). NF-κB is present in the circulation in an inactive form bound to inhibitor proteins (inhibitor of NF-κB (IκB)), which are broken down by IKKs, allowing translocation of NF-κB subunits to the nucleus (Karin & Ben-Neriah, 2000). Other TLRs, including TLR4 also utilise a MyD88-independent pathway, instead activating IFN-regulatory factor (IRF3) and resulting in expression of IFNβ and IFN-inducible gene products (Fitzgerald et al, 2003; Hoebe *et al*, 2003).

Figure 1.4 TLR-mediated activation of the NF-κB pathway

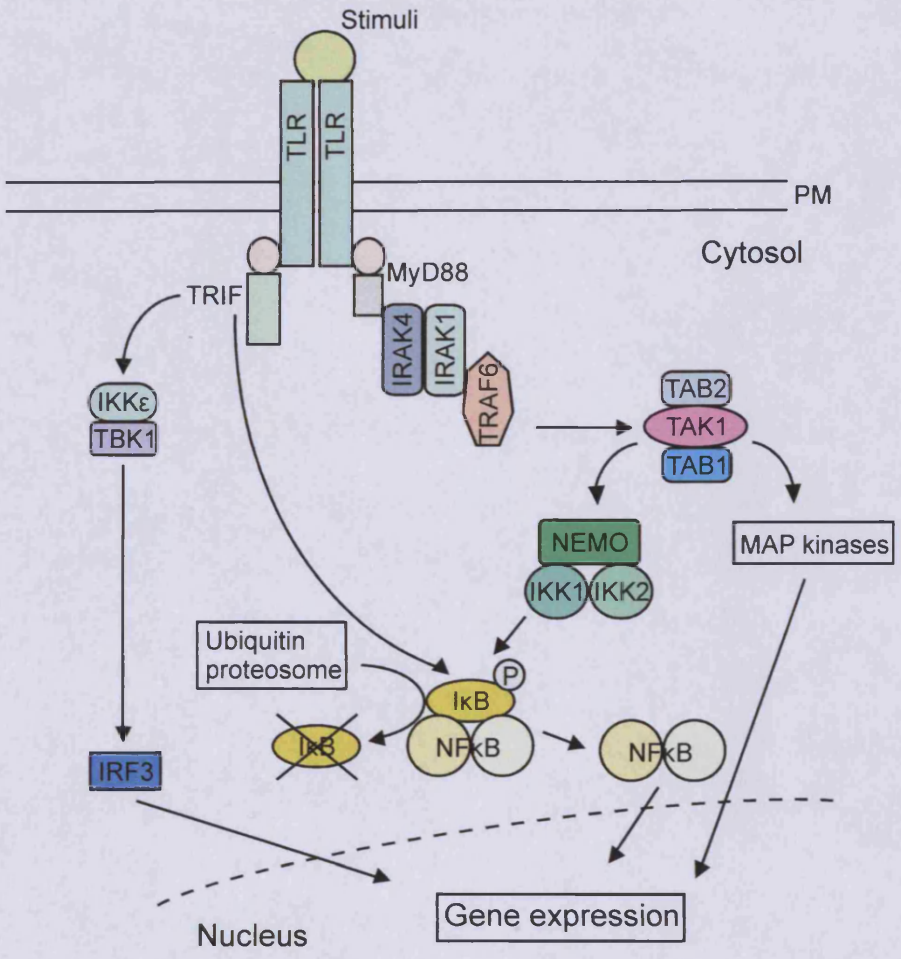

Fig 1.4. TLR mediated activation of the NF-κB pathway. A) MyD88 (myeloid differentiation primary response protein 88) activation involves the association of the MyD88 to the intracellular domain of TLRs and subsequent recruitment of IRAK4 (IL-1R-associated kinase 4), which induces phosphorylation of IRAK1 and additional association of TRAF6 (tumour-necrosis-factor-receptor-associated factor 6) with the receptor complex. Phosphorylated IRAK1 and TRAF6 dissociate from the receptor complex and associate with TAK1 (transforming-growth-factor-β-activated kinase), TAB1 (TAK1-binding protein 1) and TAB2 resulting in the phosphorylaytion of TAB2 and TAK1 and recruitment of ubiquitin ligases which ubiquitylate TRAF6, activating TAK1. TAK1 activates both the MAPK pathway and phosphorylates the IKK (inhibitor of nuclear factor κB (IκB)-kinase) complex (comprising NEMO, IKK1 and IKK2), which in turn phosphorylates IκB leading to its ubiquitylation and subsequent degredation. This allows NF-κB subunits to translocate to the nucleus. B) MyD88-independent pathways activate IRF3 and late-phase NF-κB. (Adapted from Akira *et al*, 2004; Li *et al*, 2002; Beinke *et al*, 2004).

1.6.5 Seven-transmembrane cytokine receptors

Seven-transmembrane receptors form the functional receptors for the family of chemotactic cytokines, referred to as chemokines.

1.6.5.1 Chemokines

Chemokines are a population of chemotactic cytokines that are characterised by four highly conserved cysteine residues within their amino acid sequence. There are two main families of chemokines, distinguished by the position of the first two cysteines which lie either adjacent to one another (CC chemokines) or are separated by one amino acid (CXC chemokines) (Baggiolini *et al*, 1997). Exceptions to this are fractalkine (CX3CL1), which has three amino acids separating the first two cysteine residues (CX3C) (Bazan *et al*, 1997) and lymphotactin, which has only two cysteines (C) (Houck *et al*, 1977). Chemokines were originally characterised by their capacity to direct leukocyte chemotaxis and activation (for example, degranulation, respiratory burst) during inflammation, however, it is now understood that chemokines also play roles in homeostasis, cell proliferation, haematopoiesis, angiogenesis and cancer metastasis (Bagglioni & Loetscher, 2000). Chemokines can be clustered into two subgroups, either constitutive or inducible; however, there is overlap between the two groups. Constitutive chemokines are responsible for the physiological control of leukocyte homing, this group includes CXCL12 (stromal cell derived factor-1; SDF-1), CCL12 (secondary lymphoid tissue chemokine; SLC), CCL17 (thymus- and activation-regulated chemokine; TARC) and CCL25 (thymus-expressed chemokine; TECK). Inducible chemokines are produced in response to immune or inflammatory signals and are responsible for the observed increase in leukocytic trafficking under these conditions. In this respect they are often termed 'inflammatory' chemokines, and their activities will be dealt with in more detail in subsequent sections.

1.6.5.2 Chemokine receptor interactions

Chemokine signalling is achieved through binding to specific G-protein coupled seven-transmembrane domain receptors (Kuang *et al*, 1996; Wu *et al*, 1993). Chemokine receptors function as allosteric molecular relays where chemokine binding to the extracellular portion of the receptor creates a conformational change allowing the intracellular portion to bind to and activate heteromeric G protein (Kuang *et al*, 1996). Heteromeric G proteins consist of α, β, and γ subunits, and ligand binding to

the receptor allows exchange of GDP for GTP on the alpha subunit, triggering its dissociation from the Gβγ complex subunit. Both dissociated subunits are able to activate further downstream signalling cascades including mobilisation of intracellular Ca^{2+} and the activation of the mitogen-activated protein kinase (MAPK) cascade, phosphatidylinositol 3-kinase (PI3K) and nuclear factor κB (NF-κB) pathways (Rossi & Zlotnik, 2000).

As demonstrated in Table 1.3, there is high redundancy in the chemokine family as multiple chemokines utilise the same receptor. In general CC chemokine receptors are more promiscuous than CXC receptors. Cellular distribution of each chemokine receptor is also regulated, with some receptors being cell-type specific and others expressed on multiple leukocyte subsets (Moser & Willimann, 2004). In this respect, receptors such as CXCR4 have a more widespread cellular distribution than chemokine receptors including CXCR3 which is restricted to defined T cell subsets. In addition to the functional signal transducing chemokine receptors, there are several 'decoy receptors' which bind chemokines with high affinity but do not elicit signal transduction (Mantovani *et al*, 2007), these are further discussed in Section 1.6.6.2. These regulatory features of the chemokine system allow for fine tuned specific responses and ensure that the effects of the chemokine are only short-lived.

Chemokine receptors	Ligands	Cellular distribution
CXCR1	CXCL6, CXCL8	Neutrophils
CXCR2	CXCL1-3, CXCL5-8	Neutrophils
CXCR3	CXCL9-11	Th1 cells
CXCR4	CXCL12	Neutrophils, monocytes, T cells, B cells, DCs
CXCR5	CXCL13	Memory B cells, memory T cells
CXCR6	CXCL16	T cells
CXCR7	CXCL11, CXCL12	Monocytes, B cells, T cells
CCR1	CCL3-5, CCL8, CCL14-16, CCL23	Monocytes, eosinophils, basophils, activated T cells
CCR2	CCL2, CCL7, CCL8, CCL13	Monocytes, basophils, activated T cells
CCR3	CCL5, CCL8, CCL11, CCL13, CCL24, CCL26	Th2 cells, eosinophils, basophils
CCR4	CCL17, CCL22	T cells, thymocytes
CCR5	CCL3-5, CCL7, CCL13	Th1 cells, monocytes
CCR6	CCL20	T & B cells, immature DCs
CCR7	CCL19, CCL21	Naïve T cells, mature DCs
CCR8	CCL1	Th2 cells
CCR9	CCL25	Memory T cells, B cells
CCR10	CCL27, CCL28	Memory T cells
XCR1	XCL1, XCL2	Neutrophils, B cells, T cells
CX3CR1	CX3CL1	T cells, mast cells

Table 1.3. Human chemokine receptors. Cellular distribution and ligands associated with each receptor.

1.6.5.3 CXC chemokines

In addition to the classification of chemokines based on the position of the cysteine residues, CXC chemokines can be functionally defined by the presence or absence of a three amino acid sequence (Glu-Leu-Arg (ELR) motif), in the NH_2-terminal portion directly preceding the first cysteine. Chemokines which possess the ELR motif (ELR^+ chemokines, CXCL1-8), signal through CXCR1 and CXCR2 which are chemokine receptors expressed on the surface of neutrophils. For this reason ELR^+ chemokines, such as CXCL8 (IL-8) and CXCL1 (*gro* protein α, GROα) are specifically neutrophil chemoattractants and also possess the ability to induce angiogenesis (Strieter *et al*, 2005). In addition to chemotaxis, CXC chemokines are also important in pathogenic destruction through their ability to promote degranulation and the production of superoxide (Jones *et al*, 1996; Jones *et al*, 1997). CXC chemokines that do not possess this motif (ELR^- chemokines, CXCL9-16) signal via a range of receptors expressed on T lymphocytes, this includes the

IFN-inducible chemokines CXCL9 (monokine induced by interferon-γ; MIG), CXCL10 (10kDa interferon-γ induced protein; IP-10), and CXCL11 (interferon-inducible T cell a chemoattractant; I-TAC), all of which signal via CXCR3 (Loetscher *et al.* 1996). An exception to this is the ELR$^-$ chemokine CXCL12 (stromal-derived factor-1α; SDF-1α) which utilises the chemokine receptor CXCR4 expressed on many cell types including neutrophils, monocytes, T cells and stromal cells. CXCL12 is involved in lymphocyte maturation and other homeostatic functions such as embryonic development of peripheral tissue, trafficking progenitor cells into the appropriate maturation sites in the bone marrow (Baggiolini, 1998; Lapidot *et al*, 2005).

1.6.5.4 CC chemokines

CC chemokines tend to be more promiscuous than their CXC counterparts exerting their effects on multiple leukocyte subsets including monocytes, basophils, eosinophils, T cells, dendritic cells and natural killer cells (Baggiolini, 1998). Constitutive homeostatic CC chemokines including CCL12, CCL17 and CCL25 are expressed in the thymus, lymph nodes and other lymphoid tissues and are responsible for coordination of T cell homing to and from the secondary lymph nodes and may aid T cell development (Kim *et al*, 1998). Inducible CC chemokines of note include the MCP (monocyte chemoattractant protein) family (comprising four members in humans and five in mice), which are potent monocyte chemoattractants utilising CCR2 (Charo *et al.* 1994) and CCL5 (regulated upon activation, normal T cell expressed and secreted; RANTES), which signals via a range of receptors (CCR1, CCR3 and CCR5), expressed on lymphocytes as well as other mononuclear cells (Baggiolini, 1998).

Chemokines, although potentially damaging in chronic inflammatory conditions due to their ability to induce monocytic cell trafficking into the affected area, provide potential therapeutic agents for certain cancers where increased leukocyte trafficking may be beneficial (Reckamp *et al*, 2008). Some chemokines, for example CCL3, CCL4 and CCL5, can also act as human immunodeficiency virus (HIV) suppressive factors, as HIV utilises certain chemokine receptors namely CXCR4 (T-cell tropic) and CCR5 (macrophage tropic) and to a lesser extent CCR2, CCR3 and CX_3CR1. Selective blockade of these specific chemokine receptors with suitable antagonists or

modified chemokines, may attenuate HIV progression (Princen & Schols, 2005). Anti-chemokine based therapy may also aid the management of chronic inflammatory conditions such as rheumatoid arthritis and Crohn's disease (Rossi & Zlotnik, 2000). However, to date clinical trials therapeutically targeting the chemokine receptor CCR2 in rheumatoid arthritis patients have been unsuccessful (Proudfoot, 2008).

1.6.6 Regulation of cytokine activity

1.6.6.1 Soluble receptors

Soluble forms of many cytokine receptors have been discovered both in culture supernatants and biological fluids. Once bound to their respective ligands, soluble receptors serve four primary functions: they can be either agonistic (eg. sIL-6R) or antagonistic (eg. sTNFR1), they can act as carrier molecules (eg. sIL-4R) and can protect the cytokine, thereby increasing the cytokines half life. In this respect, soluble cytokine receptors have been identified for IL-2, IL-4, IL-6, IL-7, IFNγ, TNFα, TNFβ and leukaemia inhibitory factor (LIF) (Rose-John & Heinrich, 1994). Soluble forms of conventional signalling receptor complexes can be generated through a variety of mechanisms including proteolytic cleavage, phospholipase C mediated cleavage and alternative splicing (Levine, 2008).

Soluble receptor complexes retain an ability to bind their ligand and can act as natural antagonists binding ligand in circulation and preventing its association with the membrane bound receptor and therefore inhibiting signalling. Examples of this include the soluble receptor for TNF (p75/type II TNFR), which is used therapeutically (for example Enbrel/Etanercept) to sequester and retain TNF in the circulation (Maini & Taylor, 2000). Soluble receptors can also act as agonists, an example of this is soluble IL-6 receptor (sIL-6R), which can facilitate IL-6 signalling in cells not expressing membrane bound IL-6R (Mackiewicz *et al*, 1992), this relationship will be further discussed in Section 1.8.

1.6.6.2 Decoy receptors

Decoy receptors possess high affinity binding for a specific ligand, but are structurally incapable of signalling (Mantovani *et al*, 2001). The primary role of decoy receptors, unlike soluble receptors, appears to be to fine-tune the action of primary inflammatory cytokines such as members of the IL-1 and TNF family and inflammatory

chemokines. Examples of decoy receptors include IL-1RII (Colotta *et al*, 1993), which competes with IL-1RI for IL-1 binding and also engages with IL-1R accessory protein (IL-1RAP) preventing signal transduction. Another example of a decoy receptor is osteoprotegerin (OPG), which binds members of the TNF family and is associated with the regulation of osteoclastogenesis by RANKL (Simonet *et al*, 1997). Decoy receptors in the chemokine system can either be 'silent' which are structurally incapable of signalling (such as DARC (Duffy antigen receptor for chemokines)) (Mantovani *et al*, 2001), or 'functional' which are structurally identical to signalling receptors but are induced to dissociate from the signal transducing subunits (D'Amico *et al*, 2000). This latter response is highly regulated and cytokines like IL-10 induce functional decoys for the CC chemokine receptors CCR1, CCR2 and CCR5 on dendritic cell and monocytes (D'Amico *et al*, 2000).

The role of soluble cytokine receptors in inhibiting the actions of pro-inflammatory cytokines has been exploited by several viruses which synthesise homologues of mammalian cytokine receptors in order to evade host defence mechanisms. Viruses including poxviruses and herpes viruses encode proteins that mimic either cytokines or cytokine receptors to modulate cytokine activity during infection (Alcami, 2003). Examples of cytokines mimicked by viruses include viral IL-6 encoded by human herpesvirus 8 (HHV8), which is a structural and functional homologue of human IL-6 (Müllberg *et al*, 2000; Fielding *et al*, 2005). Viral IL-6 is able to activate the Jak-STAT pathway in a similar manner to that exhibited by human IL-6 and is thought to be of significance in several conditions associated with viral infection including Kaposi's sarcoma (Molden *et al*, 1997).

1.7 Interleukin (IL)-6 related cytokines

IL-6 and several other pleiotropic cytokines are related through their utilisation of receptor complexes composed of at least one subunit of the signal transducing protein, glycoprotein 130 (gp130). This family includes Oncostatin M (OSM), IL-11, leukaemia inhibitory factor (LIF), IL-6, IL-27, ciliary neurotrophic factor (CNTF), granulocyte colony-stimulating factor (G-CSF), cardiotrophin-1 (CT-1) and B-cell stimulating factor-3 (BSF-3 also known as NNT; novel neurotrophin-1 and CLC; CNTF-like cytokine) (Heinrich *et al*, 2003; Senaldi *et al*, 1999; Pflanz *et al*, 2004). All IL-6-related cytokines are polypeptides with molecular weights of approximately 20kDa, comprising four α-helices connected by loop domains to allow two anti-parallel bundles (Heinrich *et al*, 1998). Members of this family have both pro- and anti-inflammatory properties as well as being involved in neuronal differentiation and development, haematopoiesis and remodelling of the extra-cellular matrix (Heinrich *et al*, 2003).

1.7.1 Interleukin-6

Interleukin (IL)-6 was originally characterised through its ability to induce the differentiation and proliferation of B-cells, and as such was termed B-cell growth factor or B-cell differentiation factor. Cloning of these factors in 1986 lead to the discovery of a novel cytokine (originally named B-cell stimulatory factor-2, hepatocyte stimulating factor and Interferon β_2, later re-named IL-6) able to induce acute phase reactions and induce the differentiation of myeloid precursor cells (Kishimoto, 2006). Further studies demonstrated IL-6 as a pleiotropic cytokine that influences antigen-specific immune responses and inflammatory reactions (Kopf *et al*, 1994). The specific roles of IL-6 in inflammation will be further discussed in Section 1.9. IL-6 signalling follows one of two pathways: in cells expressing membrane bound IL-6R, signalling follows the classical pathway; whilst cells not expressing IL-6R can also respond to IL-6 signalling through its soluble receptor.

As discussed in Section 1.2, inflammation is characterised by alterations in the concentration of a group of plasma proteins, termed acute phase proteins defined as a group of plasma proteins whose concentrations deviate by at least 25% during inflammatory progression. These hepatocyte-derived proteins are important regulators of inflammation and include C reactive protein (CRP), serum amyloid A

(SAA) and fibrinogen. Cytokines induced during the onset of inflammation including IL-1β, TNFα, IFNγ and TGFβ are capable of stimulating the release of acute phase proteins, however IL-6 is the primary stimulator of many of the acute phase proteins (Andus *et al*, 1988).

Studies using IL-6 deficient (IL-6KO) mice have demonstrated a role for IL-6 in the resolution of acute inflammation through the control of chemokine-mediated leukocyte trafficking, control of pro-inflammatory cytokines (Schindler *et al*, 1990; Romain *et al*, 1997) and promoting the release of the anti-inflammatory mediators IL-1 receptor antagonist and the soluble p55 TNFα receptor (Tilg *et al*, 1994), thereby directing transition from neutrophil to mononuclear cell recruitment (Hurst *et al*, 2001). In addition, IL-6 deficient mice demonstrate impaired viral and bacterial clearance (Onogawa, 2005; Ramshaw *et al*, 1997). However, in instances of chronic inflammation IL-6 signalling is detrimental, as demonstrated by the resistance of IL-6 deficient mice to a variety of experimental autoimmune conditions (Nowell *et al*, 2003; Ohshima *et al*, 1998; Mihara *et al*, 1998).

1.7.1.1 IL-6R classical signalling

Classical IL-6 receptor signalling is initiated by cytokine binding to IL-6Rα (CD126), a non-signalling α-chain. IL-6 binding to IL-6Rα is essential for interaction with the signal transducing β-chain gp130 and subsequent receptor homodimerisation, which is required for signal transduction. Although gp130 is ubiquitously expressed, the cellular distribution of the membrane-bound IL-6R is largely restricted to hepatocytes and leukocyte subsets (Rose-John *et al*, 2006).

1.7.1.2 IL-6 trans-signalling

As outlined in Section 1.6.6.1, soluble forms for many cognate α-receptor chains have been identified in serum and urine (Rose-John & Heinrich, 1994). Although many of these soluble receptors act in an antagonistic manner, sequestering free cytokine in the circulation and preventing association with the respective signal transducing β chains (see Section 1.6.6.1), soluble forms of IL-6Rα form an agonistic complex with IL-6 in the circulation which binds to membrane bound gp130 to initiate signalling (Rose-John *et al*, 2006). As gp130 is ubiquitously expressed, the trans-signalling pathway enables cell types typically unresponsive to IL-6 to become activated by IL-6. The ability of IL-6 to signal via two distinct pathways is responsible for its role in the 'switch' between innate and adaptive immunity (Hurst *et al*, 2001; Jones, 2005). IL-6 trans signalling is important in the pathophysiology of many chronic inflammatory disorders including rheumatoid arthritis, inflammatory bowel disease and some types of cancer (Atreya *et al*, 2000; Nowell *et al*, 2003; Richards *et al*, 2006; Becker *et al*, 2004). However, IL-6 trans-signalling is also vital during development as embryonic stem cells, early haematopoietic progenitor cells and neural cells are only responsive to IL-6 in the presence of soluble IL-6Rα (Humphrey *et al*, 2004; Peters *et al*, 1997; Marz *et al*, 1999).

1.7.1.3 Generation of soluble IL-6R

Soluble IL-6 receptor subunits lacking the cytoplasmic domains have been detected in biological fluids of both healthy individuals and in various disease states (Muller-Newen *et al*, 1996). Two mechanisms for the generation of soluble receptors exist: proteolytic cleavage of the cognate IL-6R (receptor shedding) (PC-sIL-6R) and differential IL-6R mRNA splicing (DS-sIL-6R) (Jones *et al*, 2001). Although both forms are structurally related, the differentially spliced isoform possesses a novel 10 amino acid C terminal sequence (GSRRRGSCGL), which is introduced during the splicing process (Horiuchi *et al*, 1994).

During an inflammatory response, chemotactic agents including CXCL1, CXCL8, C5a and CRP promote IL-6R shedding from the infiltrating neutrophils (Jones *et al*, 1999; Marin *et al*, 2001; McLoughlin *et al*, 2004). In addition apoptosis is a natural stimulus of IL-6R shedding, due to upregulation of the metalloproteinase ADAM17 (a disintegrin and metalloproteinase 17) which acts as an IL-6R cleavage enzyme

(Chalaris *et al*, 2007). Conversely, differential mRNA splicing appears to be responsible for basal production of sIL-6R in certain cell lines, including monocytic THP-1 cells (Jones *et al*, 1998). During episodes of acute inflammation however, emergence of differentially spliced sIL-6R corresponds with the influx of mononuclear cell infiltration suggesting a potential role for DS-sIL-6R in later stages of the immune response (Hurst *et al*, 2001).

A soluble isoform of gp130 exists as a natural antagonist to IL-6 trans signalling (Jostock *et al*, 2001). Soluble gp130 (sgp130) specifically binds to sIL-6R, once bound with IL-6, leaving membrane bound 'classical' IL-6 signalling unaffected. As such sgp130 provides a potential therapeutic agent for chronic inflammatory conditions such as Crohn's disease and rheumatoid arthritis, where IL-6 trans-signalling is damaging (Rose-John, 2006). The application of the inhibitory properties of sgp130 has been instrumental in defining the physiological involvement of IL-6 trans-signalling *in vivo*.

Figure 1.5 Classical IL-6R signalling and trans-signalling pathways utilised by IL-6

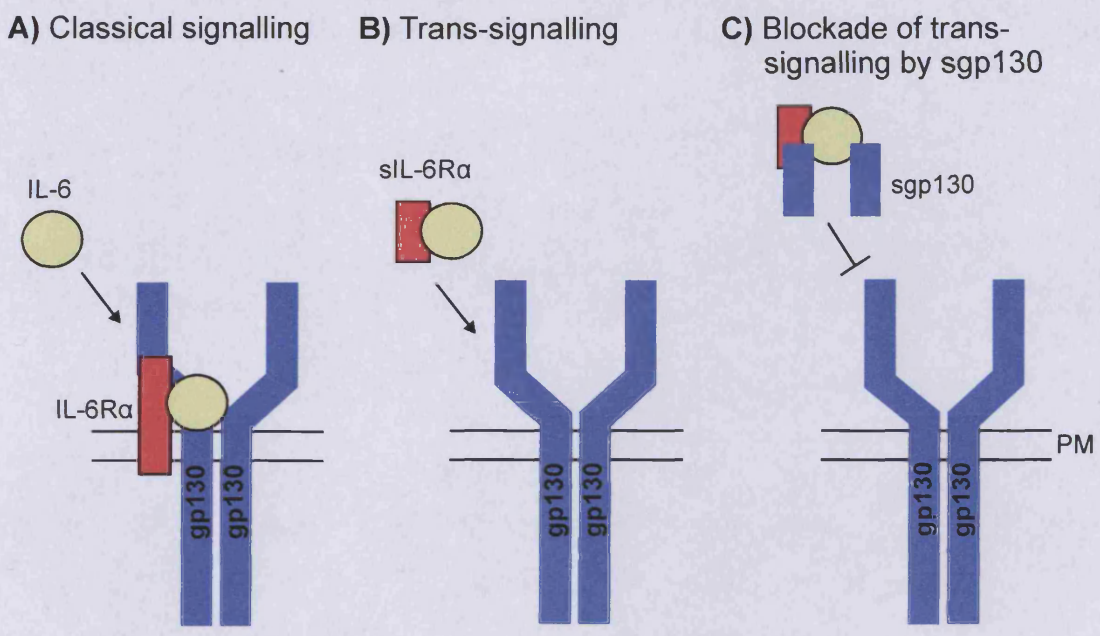

Fig 1.5. IL-6 utilises two signalling pathways, classical (A) and trans-signalling (B). Classical signalling involves IL-6 binding to membrane-bound IL-6Rα, which then associates with membrane-bound gp130, resulting in receptor complex dimerisation. Trans-signalling involves IL-6 binding to soluble IL-6Rα (either released by proteolytic cleavage or differential mRNA splicing), which binds to membrane-bound gp130 again resulting in receptor dimerisation and subsequent signal transduction (as demonstrated in figures 1.8 and 1.9). The trans-signalling pathway can be selectively blocked by soluble gp130 binding to sIL-6Rα/IL-6 complexes, thereby preventing its association with membrane-bound gp130 (C). Soluble gp130 lacks the signal transducing subunits, therefore binding of IL-6/sIL-6Rα complexes prevents IL-6 trans-signalling. (Adapted from Rose-John *et al*, 1994; Rose-John, 2003).

1.7.2 Oncostatin M

Oncostatin M (OSM) is a multifunctional cytokine belonging to the IL-6 superfamily. OSM has a diverse range of biological functions including the ability to modulate tumour cell growth, regulate the inflammatory response, affect re-modelling of the extracellular matrix and modulate haematopoiesis (Zarling *et al*, 1986; Zhang *et al*, 1994; Modur *et al*, 1997; Richards *et al*, 1993; Tanaka *et al*, 1999).

Human OSM (hOSM) is a 28KDa secreted glycoprotein monomer consisting of four α-helical chains and three inverting helix regions (Hoffman *et al*, 1996). Initially isolated from PMA-stimulated human hystiocytic lymphoma U937 cells (Zarling *et al*, 1986), it was originally identified by its ability to inhibit the proliferation of various tumour cells including the A375 human melanoma line (Zarling *et al*, 1986). Human OSM cDNA encodes a precursor protein of 252 amino acids with a short signal peptide of 25 amino acids (Malik *et al*, 1989). Subsequently it was shown that the C-terminal region of 31 residues is cleaved from the precursor to form a mature protein of 196 amino acid residues (Linsley *et al*, 1990).

The corresponding murine OSM gene was cloned in 1996 and found to be a cytokine-inducible gene; specifically regulated by IL-2, IL-3 and erythropoietin through the Jak-STAT5 pathway (Yoshimura *et al*, 1996).

OSM is closely structurally and functionally related to LIF, sharing 27% sequence homology in humans. In addition the genes for both cytokines are closely sited on the same chromosomal locus (22q12, in humans) (Rose & Bruce, 1991; Jeffery *et al*, 1993; Nicola *et al*, 1993). LIF and OSM share some biological functions, however OSM has several unique functions, potentially due to activation through the OSM specific receptor (gp130 bound to OSMRβ) (Mosely *et al*, 1996). These unique activities include growth inhibition of A375 melanoma cells (Bruce *et al*, 1992), autocrine growth stimulation of AIDS-related Kaposi's sarcoma cells (Miles *et al*, 1992) and upregulation of α1-proteinase inhibitor in lung derived epithelial cells (Cichy *et al*, 1998).

1.7.2.1 Oncostatin M receptor complexes

In humans two types of functional receptor exist for OSM: Type I is identical to the high affinity LIF receptor consisting of LIFRβ and gp130 (Gearing *et al*, 1992); Type II is comprised of gp130 and OSMRβ, which also forms the β-receptor subunit for another cytokine, IL-31 (Mosley *et al*, 1996). OSMRβ has been found on a wide variety of cell types including endothelial cells, keratinocytes, hepatic cells, lung cells and many tumour cell lines (Linsley *et al*, 1989; Mosley *et al*, 1996). However, under basal conditions peripheral blood leukocytes do not express either OSMRβ or LIFRβ (Hurst *et al*, 2002; Godard *et al*, 1992).

1.7.2.2 Murine OSM receptor complex

In mice OSM is unable to signal through the LIF receptor, relying solely on signal transduction through OSMRβ (Tanaka *et al,* 1999). This implies that there is only one functional receptor for OSM in mice, composed of gp130 and murine OSMRβ. Such differences may explain the observed differences in function between mouse and human OSM (Ichihara *et al,* 1997). It has unique roles in mouse embryo development relating to hematopoiesis, including maturation of hepatic cells (Kamiya *et al*, 1999; Kinoshita *et al,* 1999), none of which are exhibited by human OSM or LIF. Instead these activities are governed by other human gp130 activating cytokines (Tanaka *et al*, 1999). In this regard, human OSM is unable to signal via murine OSMRβ, but instead activates cells through LIFRβ (Lindberg *et al*, 1998).

1.7.3 Interleukin-31 and IL-31Rα

Interleukin (IL)-31 is a novel cytokine derived from Th2 cells, which has been implicated in allergic responses (Dillon *et al*, 2004). Transgenic mice over-expressing IL-31 develop severe dermatitis (Dillon *et al*, 2004). However, mice deficient in IL-31Rα show increased type 2 inflammation in lung epithelial cells (Perrigoue *et al*, 2007). In a similar manner to OSM, cellular activation by IL-31 has been shown to induce the release of pro-inflammatory cytokines, chemokines and matrix metalloproteinases (MMPs) (Yagi *et al*, 2007; Ip *et al*, 2007).

IL-31 signals through a receptor comprising a specific IL-31Rα subunit bound to the OSM receptor OSMRβ. IL-31Rα (also referred to as gp130-like receptor, GLR) is a member of the type I group of cytokine receptors, sharing many of their structural

motifs including the cytokine receptor homology domain comprising two pairs of conserved cysteine residues and a WSXWS sequence in the extracellular domain. IL-31Rα is closely related to gp130, sharing 28% sequence homology. In humans both are located in tandem on chromosome 5 with opposite transcriptional translation, suggesting the evolution of IL-31Rα as a result of gene duplication (Ghilardi *et al*, 2002; Diveu *et al*, 2003). Cells expressing IL-31Rα include monocytic cells, activated $CD4^+$ and $CD8^+$ T cells and skin and lung epithelial cells (Dreuw *et al*, 2004). Expression of OSMRβ, which as previously stated forms the β-subunit of the receptor complex, has been detected on a variety of cell types including skin and lung epithelial cells, and can be induced on monocytic cells (Dillon *et al*, 2004). Cellular expression of IL-31Rα is closely related to the apparent role of IL-31 in allergic responses, in particular asthma and allergic dermatitis (Dreuw *et al*, 2004; Dillion *et al*, 2004).

Signalling through IL-31Rα utilises the Jak-STAT pathway, primarily activating STAT3 and STAT5 through tyrosine residues 721 and 652 respectively, but only weakly transducing STAT1 (Dreuw *et al*, 2004). Conversely activation of the MAP kinase cascade appears to require IL-31Rα heterodimerisation with OSMRβ, as IL-31Rα cannot directly phosphorylate ERK1/2 or recruit either SHP2 or Shc (Dreuw *et al*, 2004). Therefore, when considering the role of OSMRβ signalling *in vivo*, it is important to note the contribution of both IL-31 and OSM.

1.8 Signalling of IL-6 related cytokines

Receptor-mediated activation leads to gp130-dimerisation or gp130 interaction with its related receptors LIFRβ or OSMRβ. This leads to signalling events via extrinsic tyrosine kinase regulation. The following sections summarise details relating to these activation processes.

1.8.1 The signal transducing protein, gp130

Glycoprotein 130 (gp130) is a ubiquitously expressed type I cytokine receptor, which acts as the signal transducing β-receptor subunit for all IL-6-related cytokines. Signal transduction through gp130 is essential in development, as the phenotype of gp130 deficient mice is lethal *in utero* as evidenced by the requirement of LIF in maintaining embryonic cell totipotency (Ernst & Jenkins, 2004; Shellard *et al*, 1996; Yoshida *et al*, 1996). As described in Section 1.6.1 all members of this family share several common features in the extracellular domain: four conserved cysteine residues in the amino-terminal portion and a WSXWS motif adjacent to the transmembrane domain (Hibi *et al*, 1990). The conserved cysteine residues are critical in maintaining the structural and functional integrity of the receptor, with the WSXWS motif essential for facilitating interaction between ligand and receptor, as such this domain is termed the cytokine-binding module (Cosman, 1993; Bravo *et al*, 1998). The extracellular region of gp130 consists of an N-terminal immunoglobulin-like domain followed by the cytokine binding module and three additional fibronectin type III-like domains, which appear necessary for recognition of the non-signalling cognate α-subunit of the receptor complex (Heinrich *et al*, 1998; Kurth *et al*, 2000).

Signalling through gp130 is integral to the IL-6 family of cytokines. Receptor complexes can be subdivided into two classes: those which utilise non-signalling ligand-specific α-receptors (including IL-6Rα, IL-11Rα, IL-27Rα/WSX-1 and CNTFRα), and signal transducing β-receptors (including OSMRβ and LIFRβ) (Heinrich *et al*, 2003). Cytokine binding to its receptor leads to either the homodimerisation or heterodimerisation of gp130 and subsequent signal transduction.

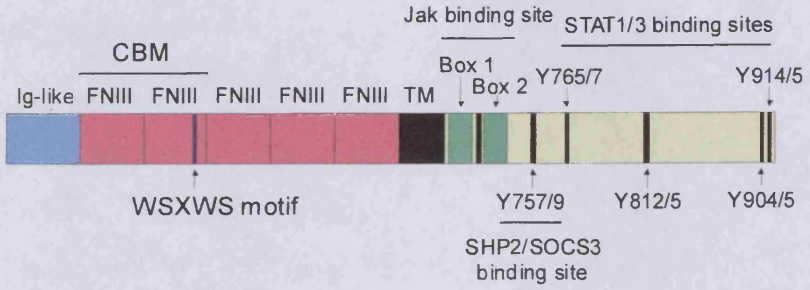

Fig 1.6. Structural organisation of the gp130 protein. The extracellular domain comprises an Ig-like domain and two fibronectin-like domains (FNIII) which comprise the cytokine binding module containing several conserved cysteine residues and the highly conserved WSXWS motif which is integral in all type I receptors, gp130 also has three additional fibronectin-like domains. The intracellular domain comprises box 1 and box 2 motifs which form the Jak (Janus kinase) binding site. In addition there are several tyrosine residues that are phosphorylated upon Jak association including $Y^{757/759}$ (mouse/human respectively) which is integral for SHP2 (SH2-domain-containing tyrosine phosphatase) and SOCS3 (suppressor of cytokine signalling 3) binding; and $Y^{765/7}$, $Y^{812/5}$, $Y^{904/5}$ and $Y^{914/5}$, which form important STAT1 and STAT3 binding sites. TM – transmembrane. (Adapted from Heinrich *et al*, 2003; Ernst & Jenkins, 2004).

1.8.1.1 Formation of gp130 homodimers

Cytokines which signal utilising receptor complexes comprising gp130 homodimers include IL-6, IL-11 and IL-27. These cytokines initially bind specifically to their related non-signalling α-chains (IL-6Rα, IL-11Rα and IL-27Rα/WSX-1 respectively), which is a vital step in receptor complex formation as neither the ligand or α-chain can effectively associate with gp130 separately (Pflanz *et al*, 2004; Heinrich *et al*, 2003). The function of integral binding to α-chains is to exert some control over cellular response to certain cytokines, although due to the presence of soluble forms for many of these receptors, in particular soluble IL-6Rα, cell types not expressing membrane bound forms of the α-subunit receptor are still able to elicit responses to some of these cytokines (Hibi *et al*, 1990; Yasukawa *et al*, 1990) (see Section 1.8.5.2). Mutagenesis studies have identified three specific 'sites' important for receptor complex formation. Site I binds to the cytokine binding module conferring

specificity for the α-chain, site II and site III interact with two gp130 subunits (Grotzinger *et al*, 1997; Simpson *et al*, 1997; Heinrich *et al*, 1998).

1.8.1.2 Formation of gp130 heterodimers

In addition to the signal transducing receptor gp130, two other gp130-like β-chains exist in the IL-6-related family of cytokine receptors which are capable of signal transduction, LIFRβ and OSMRβ. In both cases ligand binding induces association of either LIFRβ or OSMRβ with one subunit of gp130 via interactions between site II and site III (Layton *et al*, 1994; Deller *et al*, 2000). LIF and OSM directly induce receptor heterodimerisation, however, several other cytokines (for example CNTF) utilise the LIFRβ:gp130 receptor complex through first binding to a non-signalling α-chain in a similar manner to that described for IL-6 and IL-11 (Davis *et al*, 1991).

Figure 1.7 Formation of gp130 homo- and heterodimers.

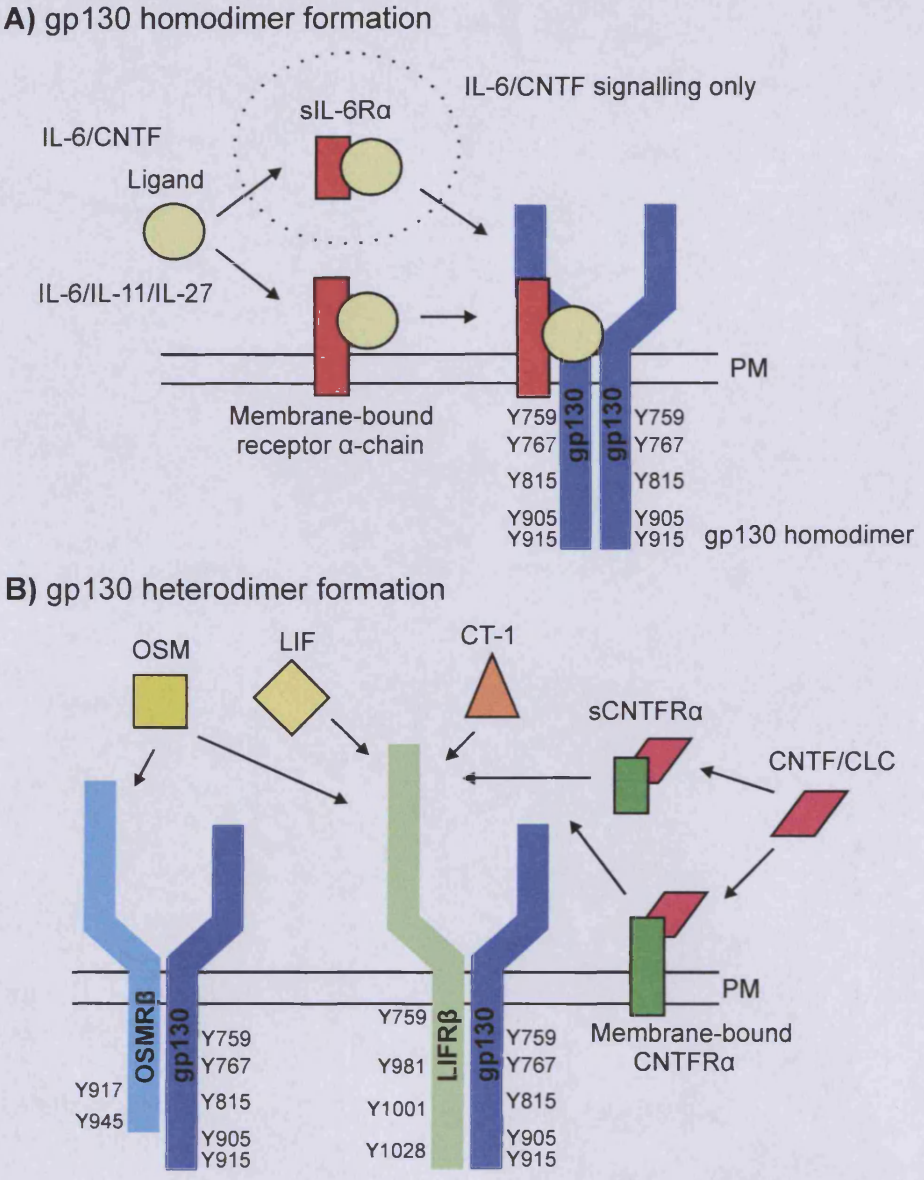

Fig 1.7. (A) Homodimer formation requires the binding of pre-formed ligand/non-signalling α-chain receptor complexes. These can be either membrane-bound α-chains (IL-6R, IL-11R or WSX-1 for IL-6, IL-11 and IL-27 respectively) or for IL-6, can be soluble. (B) Heterodimer formation involves the association of gp130 with either LIFRβ or OSMRβ (both signal-transducing β-chains). Signal transduction can either involve direct cytokine binding to the receptor (OSM, LIF) or through association of the gp130 heterodimer with the non-signalling α-chain CNTF in either a membrane bound or soluble form, in this way gp130:LIFRβ acts as the receptor complex for CNTF and CLC. (Adapted from Ernst & Jenkins, 2004; Schuster *et al*, 2003).

1.8.2 Jak-STAT mediated signalling

Due to the lack of intrinsic tyrosine kinase activity, IL-6-related cytokine receptor signalling is mediated via the Janus kinases (Jaks) (Silvennoinen *et al*, 1997; Heinrich *et al*, 1998). Jaks are intracellular tyrosine kinases of approximately 120-140kDa. There are four identified mammalian Jaks, of which Jak1, Jak2 and tyrosine kinase 2 (Tyk2) are of particular importance in IL-6-related cytokine signalling (Stahl *et al*, 1994). Of these, it is known that Jak1 plays an essential role. Studies using IL-6 and IL-11 in combination with their associated soluble receptors in Jak1 negative cells demonstrated a lack of signalling (Guschin *et al*, 1995) and Jak1 deficient mice fail to elicit efficient IL-6-related signalling *in vivo* (Rodig *et al*, 1998).

Receptor association of the Jak proteins is mediated through binding to the membrane-proximal box1/box2 regions which are present on gp130, LIFRβ and OSMRβ (Gearing *et al*, 1991; Mosley *et al*, 1996; Murakami *et al*, 1991). The box 1 sequence comprises a short proline-rich sequence that is essential for Jak association, whereas the box 2 region, comprising a short sequence of hydrophobic residues followed by several positively charged residues, is not always required (Heinrich *et al*, 1998). Jak activation occurs in response to receptor dimerisation because two Jaks are bought into close proximity, thus allowing trans-phosphorylation between the two Jak proteins. Activated Jaks phosphorylate highly conserved tyrosine residues on the proximal intracellular domain of the receptor, creating docking sites for STAT (signal transducers and activators of transcription) proteins. However, in addition to activation of STAT proteins, phosphorylation of a single tyrosine residue in gp130 (Y^{759} in humans, Y^{757} in mice) governs the recruitment and activation of the SH2-domain-containing tyrosine phosphatase, SHP2, and subsequent activation of the Ras-ERK1/2 MAP kinase cascade (Ernst & Jenkins, 2004). This tyrosine residue, however, serves a dual function and also binds suppressor of cytokine signalling (SOCS)-3, which is a negative suppressor of gp130-mediated STAT3 signalling (Ernst & Jenkins, 2004).

STATs have conserved structural organisation consisting of several identified domains including a leucine zipper-like domain at the N-terminus, a DNA-binding domain in the middle and a C-terminal SH2 domain, which is responsible for both binding to the tyrosine phosphorylated Jak proteins, and dimerisation with other

phosphorlyated STAT proteins (Heinrich *et al*, 1998). All IL-6 type cytokines activate STAT3 and STAT1 (although to a lesser extent) via binding to phosphorylated YXXQ motifs on the signal transducing β-receptor chain. In human gp130 these are present at positions Y^{767}RHQ, Y^{815}FKQ, Y^{905}LPQ and Y^{915}MPQ; in LIFRβ Y^{981}QPQ, Y^{1001}KPQ and Y^{1028}RPQ; and in OSMRβ at Y^{917} and Y^{945} (Hermanns *et al*, 2000; Chattopadhyay *et al*, 2007). Activated STAT proteins form homo- (STAT1:STAT1, STAT3:STAT3) or heterodimers (STAT1:STAT3). In addition, OSM is also able to trigger STAT5 signalling, however the mechanism of STAT5 activation requires further clarification as it is unclear whether this occurs *via* a specific tyrosine residue within the OSMRβ subunit or *via* direct interaction of STAT5 with Jaks (Heinrich *et al*, 1998). The receptor bound STAT proteins are subsequently phosphorylated on single tyrosine residues (Y^{701} in STAT1 and Y^{705} in STAT3) in the SH2 domain, which enables STAT homo- or hetero-dimerisation, which is integral for translocation to the nucleus (Heinrich *et al*, 2003). Once in the nucleus, dimerised STATs bind specific regulatory sequences to either activate or repress transcription of target genes, including acute phase proteins and transcription factors (Heinrich *et al*, 1998).

1.8.3 Activation of the MAPK cascade

IL-6-type cytokines not only signal via the Jak-STAT pathway but can also induce the mitogen activated protein kinase (MAPK) signalling cascade (Heinrich *et al*, 2003). MAP kinases are a group of serine/threonine kinases including ERK1/2, which have roles in cell survival and the control of the stress-activated members of the MAPK family p38 and JNK. Activation of the MAPK cascade is dependent on Jak phosphorylation creating binding sites for adaptor proteins such as SHP2 and SH2-and-collagen-homology-domain-containing (Shc) proteins. Phosphorylated SHP2 binds the adaptor growth factor receptor bound protein 2 (Grb2) through a specific phosphorylated tyrosine (Y^{304}) in the SH2 domain of SHP2. This receptor complex activates the guanine nucleotide releasing protein son of sevenless (SOS), which activates the GTPase ras allowing initiation of the Ras-Raf-MAPK cascade (Stancato *et al*, 1998; Heinrich *et al*, 2003).

OSMRβ does not recruit SHP2, instead recruiting Shc through a specific phosphorylated tyrosine residue (Y^{861}) in the OSMRβ chain (Figure 1.10) (Thoma *et*

al, 1994; Hermanns *et al*, 2000). Activated Shc also recruits the adaptor protein Grb2 through a phosphorylated tyrosine residue on Shc (Y^{317}). Recruitment of SOS to the receptor complex allows Ras activation and subsequent initiation of the Ras-Raf-MAPK cascade (Stancato *et al*, 1998; Heinrich *et al*, 2003).

1.8.4 Mechanisms of signal termination and SOCS proteins

In addition to extracellular regulatory mechanisms designed to sequester and antagonise cytokine activity in the circulation, several mechanisms exist to terminate signal transduction. These include protein tyrosine phosphatases, which dephosphorylate activated tyrosine residues thereby terminating the signal, PIAS (protein inhibitor of activated STAT) proteins, which are important transcriptional co-regulators of the Jak-STAT pathway and SOCS (suppressors of cytokine signalling) proteins (Heinrich *et al*, 2003). Most SOCS proteins are induced by cytokines, therefore providing a classical negative-feedback system to control cytokine-induced signal transduction. There are eight members of the SOCS family, each comprising a central SH2 domain, a variable N-terminal domain and a C-terminal 40 amino acid module, the SOCS box (Yoshimura *et al*, 2007). Of importance in IL-6-related cytokine signalling are SOCS1, which binds directly to phosphorylated Jak1 to terminate STAT1 signalling, and SOCS3 which is specific for STAT3 (Ernst & Jenkins, 2004). SOCS3 is recruited to the phosphorylated tyrosine residue ($Y^{757/759}$) required for SHP2 binding in gp130 and LIFRβ (Nicholson *et al*, 1999; Schmitz *et al*, 2000).

Figure 1.8 Activation of the Jak-STAT pathway

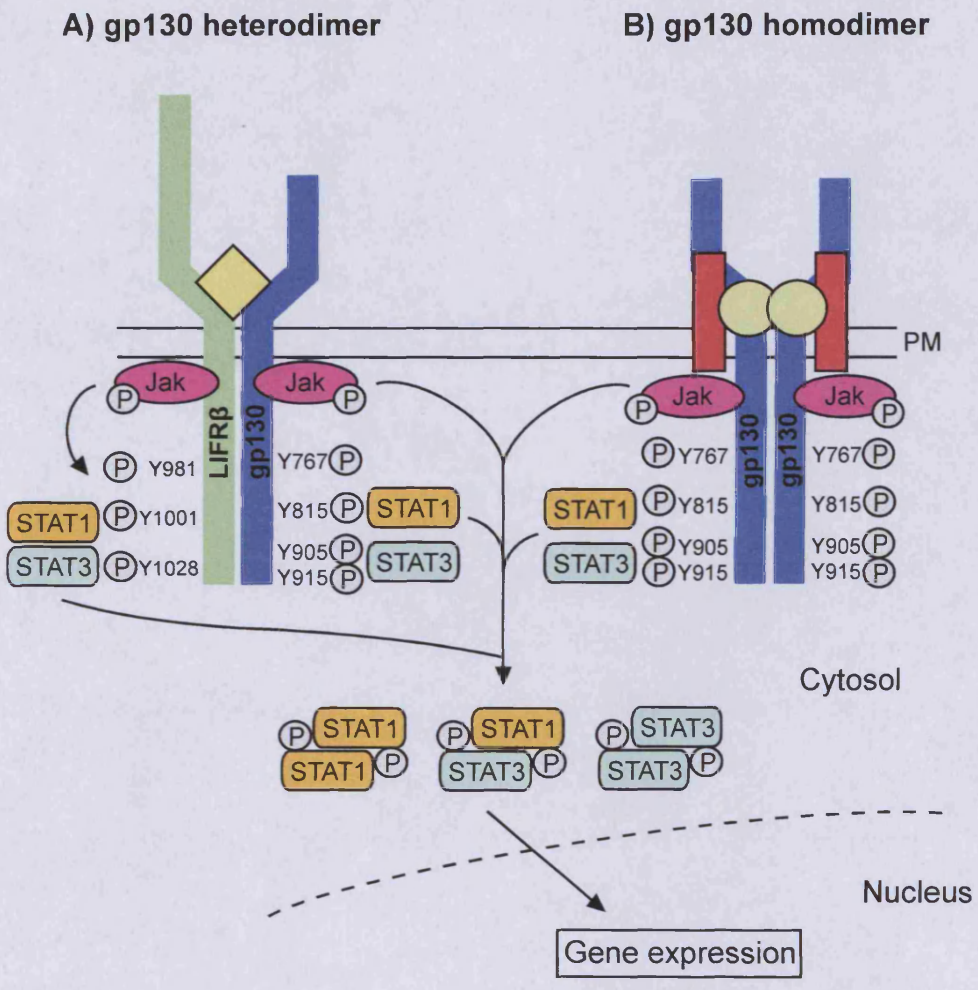

Fig 1.8. Activation of the Jak-STAT pathway through gp130-medaited pathways in humans. Cytokine binding induces homo- or heterodimerisation of each receptor subunit (as demonstrated in Fig 1.7) resulting in binding and activation of JAKs. Activated JAKs phosphorylate tyrosine residues on the intracellular domain of the receptor, creating docking sites for STAT proteins. Homo- or hetero-dimerization of the STAT proteins occurs after phosphorylation of the monomers, once again by the JAKs, which enables translocation and accumulation of the STATs in the nucleus. (Adapted from Heinrich *et al*, 2003; Ernst & Jenkins, 2004).

Figure 1.9 Activation of the MAPK pathway

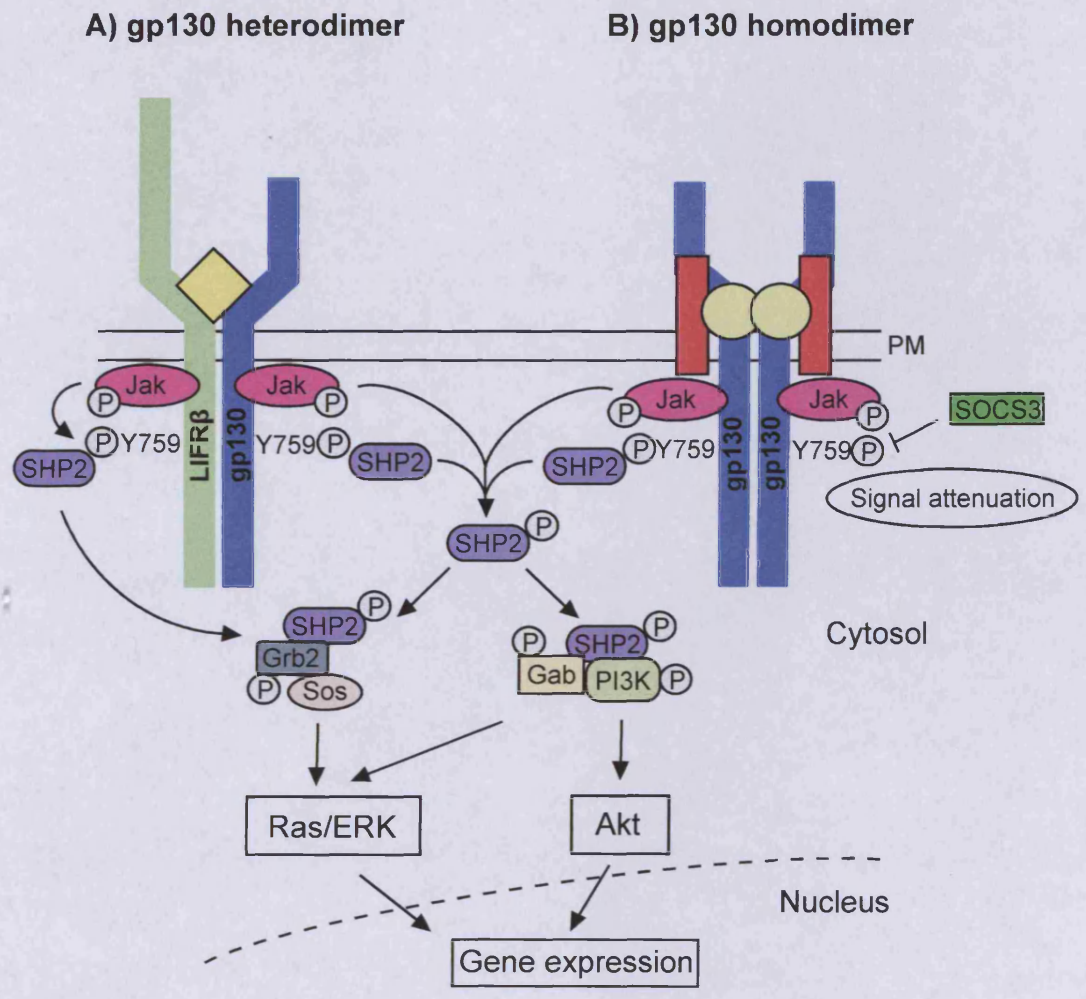

Fig 1.9. Activation of the MAP kinase pathway by IL-6-related cytokines in humans. SHP2 is recruited to the receptor subunit (either gp130 or LIFRβ) a site containing the phosphorylated tyrosine residue 757, which results in its phosphorylation and its subsequent association with the adaptor protein Grb2 or activation of the PI3K cascade. Association with Grb2 recruits SOS allowing activation of the Ras-Raf-MAPK cascade. Tyrosine 757 is also the binding site for SOCS3, which negatively regulates cytokine signalling. (Adapted from Ernst & Jenkins, 2004; Schmitz *et al*, 2000).

Figure 1.10 Signalling pathways initiated by OSMRβ-mediated signalling

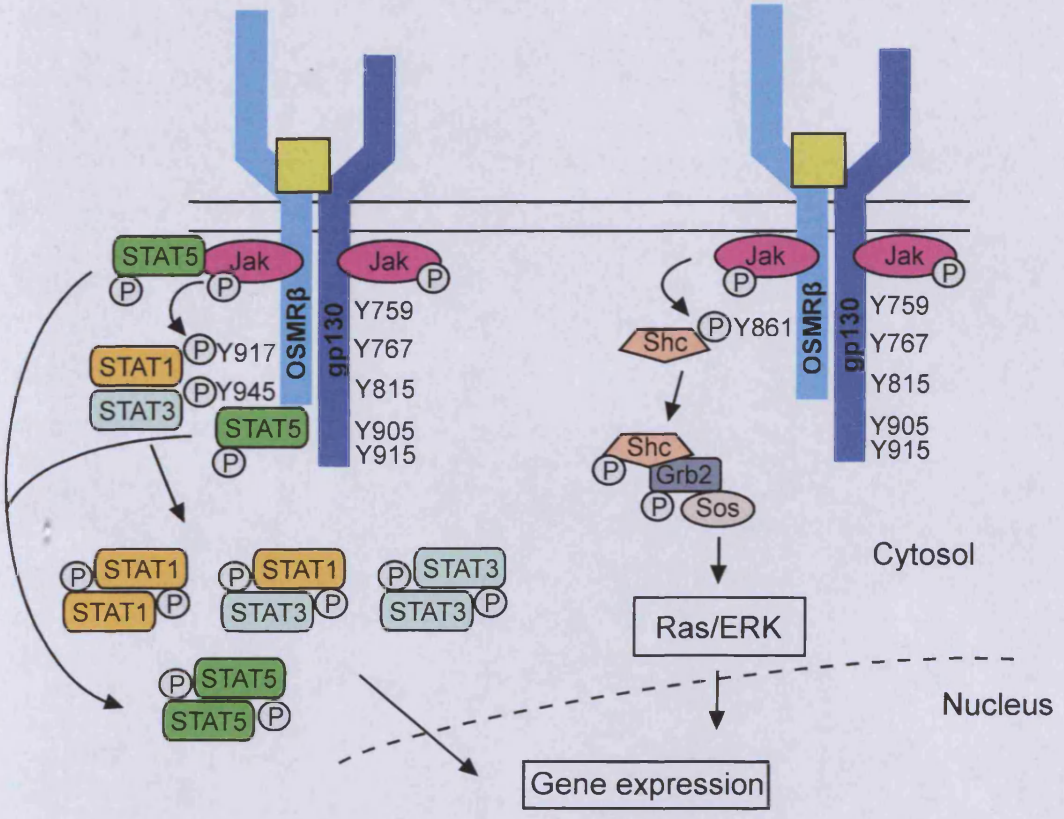

Fig 1.10. Signalling through OSMRβ:gp130 heterodimers initiates both Jak/STAT and MAPK pathways. The Jak/STAT pathway is initiated as for all IL-6-related cytokines resulting in the formation of STAT1:STAT3 homo- and heterodimers. In addition OSMRβ also induces activation of STAT5 potentially through direct binding to Jak2 and the OSMRβ subunit. Unlike gp130 and LIFRβ, OSMRβ does not recruit SHP2, instead activation of the MAPK pathway is *via* a Shc cascade; phosphorylated Shc bound to OSMRβ recruits Grb2, SOS binds to this adaptor protein allowing activation of the Ras-Raf-MAPK cascade. (Adapted from Heinrich *et al*, 2003; Hermanns *et al*, 2000).

1.9 The role of IL-6-related cytokines in inflammation

A variety of studies have implicated overproduction of IL-6 in the pathogenesis of various autoimmune and chronic inflammatory disorders. During episodes of acute inflammation IL-6 is involved in the switch between early and late inflammation, acting to promote the clearance of neutrophils, activate and promote expansion of T cells and induce differentiation of B cells. However, if IL-6 activity becomes exaggerated, as is evident in many chronic inflammatory conditions, its effects become detrimental. In this regard IL-6 has been implicated in the retention of inflammatory cells, including effector T cells, at sites of disease and has been linked with tissue/cell proliferation and damage.

1.9.1 IL-6 in acute inflammation

During acute inflammation the initial neutrophil influx is replaced by a more sustained population of mononuclear cells, this marks a transition from an initial innate-type immune response to acquired immunity. IL-6 trans-signalling is pivotal in this transition (see Section 1.7.1.2) through suppressing chemokine-directed neutrophil trafficking and steering T-cell recruitment and activation, promoting neutrophil apoptosis (in a mechanism involving caspase-3) and inducing B cell differentiation and subsequent antibody production (McLoughlin *et al*, 2003, McLoughlin *et al*, 2005; La Flamme & Pearce, 1999; Xing *et al*, 1998; Romani *et al*, 1996).

Stromal cells typically lack cognate IL-6Rα expression instead relying on IL-6 trans-signalling to respond to IL-6. Therefore local sIL-6Rα concentrations determine the magnitude of the IL-6 response. During an acute inflammatory response, shedding of membrane bound IL-6Rα from infiltrating neutrophils (in response to chemotactic agents) is the source of sIL-6Rα (Hurst *et al*, 2001). Interestingly, infiltrating neutrophils are also the predominant source of OSM during an acute immune response (Hurst *et al*, 2002), suggesting a potential link between these two IL-6-related cytokines. IL-6 driven transition from neutrophil to mononuclear cell trafficking is mediated through attenuation of the IL-1 and TNFα induced chemokines (which are predominantly neutrophil chemoattractants) and direct enhancement of mononuclear cell chemoattractants (including CCL2 and CCL8), complemented by additional upregulation of adhesion molecules. In this regard the role of IL-6 appears

to be critical for resolution of acute inflammation and is evidenced by the inability of IL-6KO mice to effectively clear both bacterial and viral infection (Kopf *et al*, 1994; Onogawa *et al*, 2005; Longhi *et al*, 2008).

1.9.2 IL-6 in chronic inflammation

The transition from neutrophil to mononuclear cell recruitment is essential for the successful resolution of the immune response; however, if the mononuclear cell infiltrate (primarily T-cell) is not effectively cleared a chronic inflammatory state can ensue. Overproduction and increased signalling of IL-6 has been associated with several chronic inflammatory and autoimmune conditions (including rheumatoid arthritis, Crohn's disease, psoriasis and systemic lupus eryhthematosus), which through an undefined dysregulation of chemokine-mediated recruitment and leukocyte apoptosis, is potentially detrimental (Gross *et al*, 1992; Houssiau *et al*, 1988; Swaak *et al*, 1989; Grossman *et al*, 1989). In this respect, IL-6KO mice showed limited histological signs of disease in various models of experimental autoimmunity (Kallen *et al*, 2002).

As previously mentioned, IL-6 trans-signalling is able to enhance production of several mononuclear cell chemoattractants from resident tissue cells (Jones, 2005). In addition to this, IL-6 also has anti-apoptotic effects on T cells, which can result in retention of mononuclear cells at sites of inflammation as observed in the synovium of RA patients and in the lamina propria of Crohn's disease patients (Salmon *et al*, 1997; Atreya *et al*, 2000). Both *in vitro* and *in vivo* studies have demonstrated the ability of IL-6 trans-signalling to 'rescue' T cells from apoptosis through a mechanism involving STAT3 driven expression of the anti-apoptotic regulators Bcl-2 and Bcl-x_L (Teague *et al*, 2000; Atreya *et al*, 2000).

The detrimental effects of IL-6 trans-signalling have been confirmed in many inflammatory models using IL-6 deficient mice, which are protected against a variety of chronic inflammatory diseases including experimental models of arthritis Crohn's disease, nephritis, uveitis and experimental autoimmune encephalomyelitis (Boe *et al*, 1999; de Hooge *et al*, 2000; Kallen *et al*, 2002; Mihara *et al*, 1998). As such pharmaceutical agents have been developed to target IL-6 signalling, including the anti-IL-6 receptor blocking antibody MRA (Atlizumab, Tocilizumab), which have

shown promise in the treatment of both Crohn's disease and inflammatory arthritis (Choy *et al*, 2002; Ito *et al*, 2004). However, as previously mentioned, IL-6 signalling is integral in the resolution of acute inflammation and also protects against septic shock (Barton *et al*, 1993; Ulich *et al*, 1991) so completely blocking IL-6 signalling may have an undefined deleterious effect on the host's ability to resolve bacterial infections (Onogawa *et al*, 2005). Such findings emphasise the need to define the balance between classical IL-6R signalling and IL-6 trans-signalling and underline the importance of developing second generation anti-IL-6 blockers. In this respect, use of soluble gp130 (sgp130) as a therapeutic agent may provide a suitable alternative as sgp130 is specific for IL-6 trans-signalling, potentially leaving the classical signalling pathway unaffected (see Section 1.7.1.2).

1.9.3 The role of OSM in disease

OSM is a modulator of the inflammatory response, and as such has been demonstrated to be present in a variety of inflammatory disorders. OSM is below the level of detection in the serum or tissue of healthy individuals (Robak *et al*, 1997). However, OSM can be detected in biological fluids obtained from patients with certain inflammatory conditions. For example, OSM has been found in the synovium of patients with rheumatoid arthritis (Okamoto *et al*, 1997), the bronchial lavage fluid of patients with pneumonia (Grenier *et al,* 2001) and in the peritoneum of patients with acute bacterial peritonitis (Hurst *et al*, 2002) suggesting a potential role for OSM in these inflammatory conditions. OSM also has an important role in regulating the growth of certain cancers; inhibiting the growth of melanoma cells (Zarling *et al*, 1986), breast and lung cancer cells (Lui *et al*, 1997) and glioma cells (Halfter *et al*, 1998). In contrast OSM stimulates growth of AIDS-related Kaposi's sarcoma cells (Miles *et al*, 1992). However, similarities in the biological action of IL-6 and OSM *in vitro* have lead investigation to consider the therapeutic potential of modulating OSM in disease.

1.9.4 Inflammatory responses mediated via OSM

Although OSM affects processes associated with disease progression, the specific function of OSM in the face of an inflammatory challenge remains unclear. *In vitro* studies illustrate that OSM and IL-6 share a similar capacity to control inflammatory processes, however, it remains to be established whether their control of common

biological activities provides a cytokine hierarchy with one factor over-riding the properties of the other. In this regard, the *in vivo* function of OSM in inflammation is not as well defined as IL-6. However, several pro- and anti-inflammatory responses have been observed *in vitro* (Hurst *et al*, 2002; Wahl *et al*, 2001; Wallace *et al*, 1999; Modur *et al*, 1997). The anti-inflammatory effects of OSM include regulation of tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-3 in several cell types (Cross *et al*, 2004; Li *et al*, 2001). The TIMP family are responsible for controlling the action of the MMPs, a group of enzymes that regulate extracellular matrix composition (Li *et al*, 2001). MMPs play a vital role in tissue remodelling under both physiological and pathological conditions, however, excessive activation, induced by pro-inflammatory cytokines such as IL-1 and TNFα, is associated with ECM (extra-cellular matrix) destruction in inflammatory conditions such as arthritis (Lark *et al*, 1997). The TIMP proteins form complexes with MMPs, thereby preventing their action on the ECM, in addition many are also anti-tumourigenic and have anti-angiogenic and pro-apoptotic characteristics (Brew *et al*, 2000). Therefore, through the upregulation of TIMPs, OSM is able to protect against degradation of the ECM by MMPs.

OSM is also able to inhibit the production of the neutrophil chemoattractant CXCL8 by the pro-inflammatory cytokine IL-1 in lung fibroblasts, in a similar manner to that of IL-6, suggesting that OSM may also promote the clearance of neutrophils (Cross *et al*, 2004). Similarly IL-6 and OSM are equally capable of promoting expression of the ELR^+ CXC-chemokines CXCL5 and CXCL6 (Modur *et al*, 1997; McLoughlin *et al*, 2004). However, the function of CXCL5 and CXCL6 remains unclear and may not represent frontline PMN chemoattractants (McLoughlin *et al*, 2004).

Pro-inflammatory effects of OSM include the induction of adhesion and chemotaxis in neutrophils and increased production of chemokines in endothelial cells, synovial fibroblasts and human peritoneal mesothelial cells (HPMC) (Cross *et al*, 2004; Hurst *et al*, 2002). In a similar manner to IL-6 trans-signalling, OSM has been shown to induce CCL2 production in mesothelial cells (Hurst *et al*, 2002) and fibroblasts (Brown *et al*, 1991; Nowell *et al*, 2006), as well as having as additive effect on CCL2 expression after co-stimulation with the pro-inflammatory mediators IL-1 and TNFα. In addition OSM is also able to induce IL-6 expression in a variety of cell types

(Brown *et al*, 1991; Bernard et al, 1999; Hurst *et al*, 2002). The observed effects of OSM on mononuclear chemoattractants, in addition to its ability to regulate the production of IL-6 suggest that OSM may be important in the progression of the immune response by promoting mononuclear cells activities.

1.9.5 OSM as a therapeutic target

Pro-inflammatory cytokines such as TNFα, IL-1β and IL-6 have been identified as promising therapeutic targets in the treatment of chronic inflammatory conditions (Maini & Taylor, 2000; Papadakis & Targan, 2000; Choy *et al*, 2002; Ito *et al*, 2004; Verbsky & White, 2004). As discussed in Section 1.9.4, OSM appears to have both pro- and anti-inflammatory roles in inflammation, therefore it is currently unclear whether it represents a significant therapeutic target (Pelletier & Martel-Pelletier, 2003). However, studies using experimental models of arthritis have demonstrated some success of antibodies against OSM in limiting disease development, suggesting OSM may present a therapeutic target (Plater-Zyberk *et al*, 2001; Milner *et al*, 2003).

1.10 Peritoneal dialysis associated infection and inflammation

Studies outlined within this thesis will adopt a model of inflammation designed to mimic inflammatory events observed during episodes of bacterial peritonitis seen in the peritoneal cavity of end-stage renal failure patients receiving peritoneal dialysis (PD). PD is a treatment strategy in cases of established renal failure (ERF).

1.10.1 Peritoneal dialysis

PD is an alternative treatment to haemodialysis, which involves the use of a highly concentrated sugar solution to remove solutes from the blood using the peritoneal lining as a dialysing membrane. Due to the highly concentrated nature of the fluid, an osmotic and chemical gradient is developed between dialysis fluid and the blood capillaries of the peritoneum. Water and small solutes including uremic metabolites move from the blood into the dialysate through a combination of osmotic pressure and hydrostatic ultrafiltration.

1.10.2 The peritoneal cavity

The peritoneal membrane is composed of two distinct layers: an epithelial layer known as the mesothelium; and an underlying connective tissue, the interstitium. These two layers are separated by a discontinuous basement membrane, composed of proteoglycans, type IV collagen and glycoproteins, which supports the mesothelium (Nagy & Jackman, 1998). The peritoneal membrane is highly vascularised to traffic molecules to and from the tissues and organs of the abdominal cavity (Nagy & Jackman, 1998). A lymphatic system is also present, which maintains the small volume of fluid present in the normal peritoneal cavity and has an important role in host defence within the peritoneum (Nagy & Jackman, 1998).

1.10.2.1 The mesothelium

The mesothelium lining the peritoneal cavity is composed of a monolayer of flattened mononuclear mesenchymal cells (human peritoneal mesothelial cells, HPMC). The major function of the mesothelium is to maintain normal homeostasis within the peritoneal cavity (Mutsaers, 2004); however, mesothelial cells also play an important role in regulating the inflammatory response after peritoneal infection or injury (Topley *et al*, 1993). Mesothelial cells represent the principle population lining the peritoneal cavity and serve a prominent role during inflammation providing the major

source of inflammatory cytokines, chemokines and other mediators (Topley *et al*, 1993; Lanfrancone *et al*, 1992).

1.10.3 Peritoneal dialysis associated peritonitis

Peritonitis, a generalised infection of the peritoneal cavity, is a major complication of PD treatment and recurrent bouts of infection can lead to progressive alterations in tissue architecture including vasculopathy, fibrosis and membrane thickening (Williams et al, 2002). Repeated episodes of peritonitis are directly related with increased susceptibility to further peritoneal infection due to the loss of the mesothelium and subsequent attenuation of host cell defence (Williams *et al*, 2002; Davies *et al*, 1996). The most common pathogens associated with PD peritonitis are gram-positive Staphlococci, including *Staphylococcus epidermidis* and *Staphylococcus aureus*.

Peritoneal infections trigger an immune response through the TLR-mediated production of pro-inflammatory cytokines from both HPMC and the small population of resident peritoneal macrophages (Park *et al*, 2007; Kato *et al*, 2004).

1.10.3.2 The inflammatory role of the mesothelium

In response to pathogen recognition and subsequent activation of both NF-κB and MAP kinase pathways, mesothelial cells and resident peritoneal macrophages express a variety of inflammatory mediators which serve to regulate the recruitment and activation of inflammatory leukocytes. As previously discussed in Section 1.2, the initial cells to respond during an immune assault are neutrophils, which are trafficked into the peritoneal cavity from the circulation in response to secretion of neutrophil chemoattractants such as mesothelial-derived CXCL8 (Topley *et al*, 1993; Visser *et al*, 1995). Other inflammatory mediators are released from both the mesothelial cells and infiltrating neutrophils, including members of the IL-6 family (Topley *et al*, 1993; Hurst *et al*, 2002) which aid the progression of the immune response by inducing mesothelial secretion of mononuclear cell chemoattractants and the upregulation of specific cell surface adhesion molecules. Adhesion molecules including selectins, ICAM-1 and vascular cell adhesion molecule (VCAM)-1 provide an interaction between the leukocyte and activated mesothelial cell enabling transmigration through the vascular endothelial cell layers into the peritoneal cavity.

1.10.4 Peritoneal dialysis associated fibrosis

In acute episodes of bacterial peritonitis the influx of leukocytes efficiently clears the pathogen, thereby allowing resolution of inflammation. However, repeated episodes of peritonitis and long-term exposure to peritoneal dialysis fluids result in longitudinal changes in both peritoneal membrane structure and function (Williams *et al*, 2002). Examples of these changes include, thickening of the submesothelial tissue due to the deposition of collagen (fibrosis or sclerosis) and alterations in mesothelial cell integrity, including reduced regenerative capacities (Di Paulo *et al*, 1986; Williams *et al*, 2002; Devuyst *et al*, 2002). In cases of membrane thickening or 'fibrosis' the effectiveness of the cavity as a dialysing membrane is compromised, which ultimately leads to treatment failure.

Figure 1.11 Peritoneal membrane sections demonstrating normal and fibrosed morphology

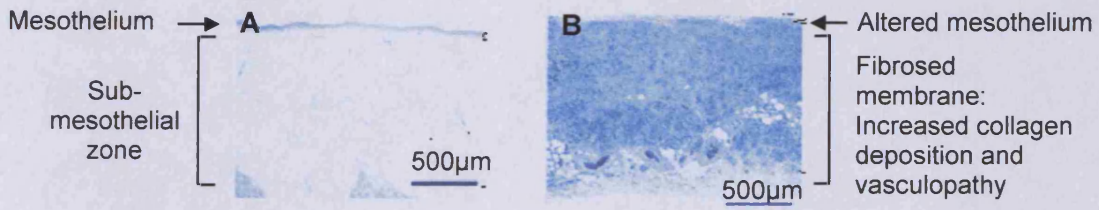

Fig 1.11. A comparison of peritoneal membrane sections taken from a patient prior to therapy (A) showing normal morphology; the mesothelial layer covers a submesothelial zone consisting of collagen fibres as well as lymphatic and blood vessels. B shows a membrane section taken from a patient on long term PD demonstrating denuded mesothelium and increased vasculopathy and collagen deposition (Adapted from Williams *et al*, 2002)

1.10.5 The role of IL-6 related cytokines in peritoneal inflammation

Analysis of peritoneal dialysis fluid from patients with PD associated peritonitis has shown raised levels of both OSM and IL-6 during bacterial infection coinciding with the influx of neutrophils into the peritoneal cavity (Hurst *et al*, 2002; Hurst *et al*, 2001). Levels of LIF have also been analysed and shown to remain undetectable through the course of infection (Hurst *et al*, 2002). This therefore suggests that both IL-6 and OSM are of potential importance in the progression of bacterial peritonitis.

OSM and IL-6 (via IL-6 trans-signalling) induces the release of mononuclear cell chemoattractants such as CCL2 from HPMC and can upregulate the production of adhesion molecules, further enhancing the trafficking of mononuclear cells (Hurst *et al*, 2001) (Section 1.10.3.2). Unpublished work has shown that IL-6 deficient mice, in addition to being protected against experimental models of autoimmunity, are also resistant to membrane thickening in experimental models of peritonitis and show an impaired ability to clear bacteria (Fielding *et al*, unpublished data; Coles *et al*, unpublished data). Due to the similarities between IL-6 and OSM in the chemokine driven profile, this study will aim to delineate the role of OSM in leukocyte trafficking during experimental models of peritonitis.

1.10.6 The potential role of OSM in peritoneal fibrosis

As discussed in Section 1.10.4, the primary reason for failure of peritoneal dialysis in the treatment of end-stage renal failure is fibrosis of the peritoneal membrane. This occurs in part due to the bio-incompatible composition of peritoneal dialysis fluid but predominantly due to repeated incidences of bacterial peritonitis (see Section 1.10.3). In addition to the observed roles of OSM in the progression of inflammation, it has also been implicated in the development of fibrosis (O'Hara *et al*, 2003; Nightingale *et al*, 2004). Currently no work has been published on the potential role of OSM in peritoneal epithelial to mesenchymal transition (EMT). However, a role for OSM in renal and lung EMT has been implicated (Nightingale *et al*, 2004; Pollack *et al*, 2007; O'Hara *et al*, 2003). The process of EMT involves attenuation of cell-to-cell and cell-to-extracellular matrix interactions, which enable epithelial cells to move through the extracellular matrix, where they take on a more mesenchymal phenotype. Such alterations are typically characterised by expression of smooth muscle α-actin. This process is integral in early embryonic development, however, dysregulation of this

process in fully differentiated tissues can lead to damage and fibrosis (Radisky, 2005). Induction of EMT is cell type specific, however, in peritoneal fibrosis the major cause is upregulation of TGF-β (Margetts *et al*, 2005).

Project aims

The observed *in vitro* similarities between the action of OSM and IL-6 (via IL-6 trans-signalling) suggest that OSM may also play a pivotal role in the progression from innate to acquired immunity, either by augmenting the response of IL-6 (through its upregulation), or through its direct effect on chemokine-driven leukocyte recruitment. This, in addition to the observed role of OSM in the progression of fibrosis, makes it a potential target for therapeutic blocking (Pelletier & Martel-Pelletier, 2003).

This PhD project aims to investigate the inflammatory role of OSM in governing leukocyte recruitment within the peritoneal cavity, using *in vitro* and *in vivo* model systems.

Objective 1. Determine which cell populations are likely to be affected by OSM by defining OSMRβ expression on HPMC and human leukocyte subsets.

Objective 2. Investigate the role of OSMRβ (and IL-31) in leukocyte trafficking and adhesion using both *in vivo* and *in vitro* methods.

Objective 3. Compare the activities of OSM with responses known to be governed by IL-6 using *in vivo* approaches.

Objective 4. Examine the OSMRβ signalling events controlling leukocyte recruitment.

Objective 5. Investigate a potential role for OSMRβ signalling in progressive inflammation, using an *in vivo* simulation of recurrent peritoneal inflammation.

Objective 6. Define what effect OSMRβ deficiency has on tissue damage.

Chapter 2

Materials and Methods

2.1 Reagents

All chemicals were purchased from Sigma-Aldrich unless otherwise stated. All chemicals were of analytical grade or higher.

2.2 Tissue culture

2.2.1.1 Isolation of human peritoneal mesothelial cells

Human peritoneal mesothelial cells (HPMC) were isolated and cultured from omentum tissue derived from consenting patients undergoing elective abdominal surgery using an adapted version of Stylianou's original method (Stylianou *et al*, 1990; Beavis *et al*, 1995). Briefly, tissue sections were washed in sterile PBS (Dulbecco's phosphate-buffered saline; 2.5mM KCl, 1.5mM KH_2PO_4, 137mM NaCl, 8mM Na_2HPO_4 pH7.4) (Gibco, Invitrogen, Paisley, UK), then digested in 20ml 0.1% (w/v) trypsin/ 0.02% (w/v) EDTA diluted in PBS and incubated for 15 minutes at 37°C with continuous rotation. HPMC were harvested by centrifugation at 800g for 6 minutes. Pelleted cells were suspended in growth medium.

2.2.1.2 HPMC growth conditions

Cells were cultured in complete Earle's buffered 199-medium (Gibco, Invitrogen, Paisley, UK) containing 10% (v/v) heat-inactivated foetal calf serum (Hyclone, Perbio Science Ltd, Cranlington, UK), supplemented with 2mM L-glutamine, 100U/ml penicillin, 100μg/ml streptomycin, 5μg/ml transferrin, 5μg/ml insulin and 0.4μg/ml hydrocortisone (Li *et al*, 1998). Cell monolayers were grown (incubated at 37°C, 5% CO_2) in T25 Falcon culture flasks (Beckton Dickinson, Oxford, UK) until confluent, once cells reach confluence they were passaged at a ratio 1:3.

2.2.1.3 Monolayer sub-culture

On reaching confluence in the primary culture flask, cells were initially transferred into T75 flasks before subsequent passage into the appropriate culture vessel for experimental purposes. Sub-culture involved the use of trypsin to remove the cells from the bottom of the plate. Growth media was removed by aspiration and the cells washed with sterile PBS, before trypsinisation with 10% (v/v) trypsin/EDTA diluted in sterile PBS. Cell detachment was monitored by light microscopy following brief incubation at 37°C. Following detachment 10mls of the appropriate growth medium supplemented with 10% (v/v) FCS was added to neutralise the trypsin. The cell

suspension was transferred to a 50ml universal tube and spun at 600g for 6 minutes, the supernatant removed and the cell pellet resuspended in fresh FCS supplemented media. Cells were transferred to the appropriate culture vessel and incubated at 37°C, 5% CO_2.

2.2.1.4 Growth arrest and cell stimulation

Prior to each experiment, the growth phase of the cells was synchronised by growth arrest in the appropriate serum free media. Briefly, growth medium was removed, the cells washed to remove all FCS then the media replaced with serum free medium. Under these conditions cells remain viable for up to 96 hours and were typically used after 48 hours growth arrest (Topley *et al*, 1991). For cell stimulation the growth arrest media was removed and the cells were washed with serum free medium. Stimulation was performed by incubation of the cells with the desired cytokine (eg. OSM, IL-1) as outlined within the specific Figure Legends.

2.2.1.5 Characterization of HPMC

The morphology of HPMC monolayer cultures was examined using an inverted light microscope (Axiovert 25, Carl Ziess Ltd, Hertfordshire, UK). Confluent HPMC were typically polygonal in shape having approximately 5 sides and displaying the classical 'cobblestone' appearance as demonstrated in Figure 2.1. Cells adopting this morphology were growth arrested (Section 2.2.1.4) to be used in experiments. Confluent HPMC were also characterised by their expression of cell surface markers including Cytokeratin 8/18, Vimentin and lack of Factor VIII related antigen expression (Stylianou *et al*, 1990; Zhang *et al*, 1999).

Figure 2.1 Morphology of HPMC monolayers.

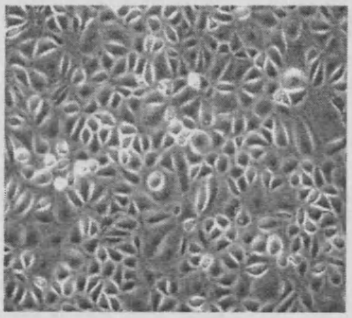

Fig 2.1. Confluent monolayer of HPMC showing polygonal shape and typical 'cobblestone' appearance after growth arrest (magnification x250).

2.2.2 SV40 transformed HPMC

SV40 transformed HPMC (kindly provided by Jean-Phillipe Rougier, Department of Nephrology and Dialysis, Tenon Hospital, Paris, France) are derived from a primary HPMC line transformed with a plasmid containing a modified SV40 sequence, containing a deletion in the late region and a 1bp insertion, which disrupts the origin of replication site within the SV40 genome. These cells retain many of the phenotypic characteristics of primary HPMC including morphology and the expression of Cytokeratins 8 and 18 and Vimentin (Fischereder *et al*, 1997).

2.2.2.1 SV40 HPMC growth conditions

SV40 transformed HPMC were cultured in DMEM (Dulbecco's modified medium) (Gibco, Invitrogen, Paisley, UK) with 10% (v/v) foetal calf serum, supplemented with 2mM L-glutamine, 100U/ml penicillin, 100μg/ml streptomycin, 5μg/ml transferrin, 5μg/ml insulin and 0.4μg/ml hydrocortisone. Cell monolayers were then grown (incubated at 37°C, 5% CO_2) in T25 Falcon culture flasks until confluent and passaged as previously described for HPMC (Section 2.2.1.3).

2.2.3 Isolation and culture of peripheral blood leukocytes

Peripheral blood mononuclear cells (PBMC) were isolated from venous blood taken from non-smoking healthy volunteers (Ulmer *et al*, 1984; Kanof *et al*, 2001). Briefly, blood was layered onto Dextran 70 (6% in 0.9% sodium chloride) with citrate (76mM citric acid, 169mM sodium citrate, pH5.6) and left to sediment for 1 hour at 37°C. The straw-coloured plasma was removed, leaving the red blood cells undisturbed. Leukocytes within the plasma were pelleted by centrifugation, then washed in PBS (pH7.4) and re-suspended in 5ml PBS (pH7.4). This was layered on Ficoll-Paque PLUS (Ficoll PM400 and sodium diatrizoate with a specific density of 1.077g/ml) then centrifuged at 400g for 35 minutes with no braking. PBMC sediment at the interface, whereas neutrophils (PMN) pellet at the bottom. PBMC were collected and washed in PBS, then re-suspended at a concentration of $2x10^6$ cells/ml in serum-free RPMI-1640 medium (Sigma-Aldrich, Poole, UK) supplemented with 2mM L-glutamine, 100U/ml penicillin, 100µg/ml streptomycin. Cells were cultured to remove all non-adherent cells. PMN were also collected, washed in PBS (pH7.4) and retained for flow cytometric analysis.

2.2.3.1 Stimulation of PBMC

After incubation for 2 hours, any non-adherent cells were removed by washing with PBS. Cells were subsequently stimulated overnight in the absence of serum with various cytokines and inflammatory mediators (as described in the appropriate Figure Legends). Culture supernatant samples were rendered cell-free by centrifugation and stored at -70°C for subsequent cytokine analysis. Cells were collected for flow cytometric analysis or extraction of total RNA (using methods described in section 2.5.1).

2.3 Animal experiments

Experiments were performed on 6-12 week C57/Blk6 wild type (WT) and Oncostatin M Receptor β deficient (OSMR-KO) mice. Procedures were carried out following Home Office approval under project license number PPL-30/2269 'Regulation of leukocyte trafficking and fibrosis'.

2.3.1 Generation of OSMRβ deletion

OSMR-KO mice (kindly provided by Minoru Tanaka, Institute of Molecular and Cellular Biosciences, University of Tokyo, Tokyo, Japan) were generated as previously described (Tanaka *et al*, 2003). Briefly, disruption of the OSMRβ gene was achieved through replacement of the proximal region of the initiation codon with the LacZ gene and neomycin resistance cassette, resulting in deletion of the N-terminal region of the gene.

2.3.2 Genotyping OSMR-KO animals

Approximately 1mm was removed from the end of the tail of mice at between 2-4 weeks old using a scalpel blade. This was incubated with 750µl tail buffer (50mM Tris-HCl (pH7.4), 100mM EDTA, 100mM NaCl, 1% SDS, in 100ml distilled H_2O) and 10µl Proteinase K (20mg/ml) overnight at 55°C. This was mixed with 5M NaCl and separated by centrifugation (13,000g, 25 minutes). The lower phase was collected and mixed with isopropanol (1:1.6), precipitated DNA was pelleted by centrifugation (13,000g, 10 minutes). The DNA was washed in 70% (v/v) ethanol and resuspended in sterile double distilled H_2O prior to use.

Polymerase chain reaction (PCR) was performed using 2µl cDNA per reaction. PCR reactions were performed as described in Section 2.6.1. Amplification conditions were as follows:

Initial denaturation	94°C	2 mins	
	94°C	1 min	35 cycles
	60°C	1 min	
	72°C	90 secs	
Final extension	72°C	10 mins	

DNA banding was visualised on 2% agarose gels using ethidium bromide (5mg/ml) (Tanaka *et al*, 2003).

Primer Sequences	Annealing Temp	%GC content
5'-GTAATCAGACCAATGGCTTTCTC-3'	70	43
5'-GATCCAACACAATCATGAAGC-3'	72	45
5'-GCACATAACTTCAGC-3'	65	50

Table 2.1. Genotyping was performed by polymerase chain reaction (PCR) using the outlined primers from genomic DNA isolated from tail tips. Wild type mice generate a 364bp product, whereas OSMR-KO mice generate a 750bp product. Heterozygous mice show both fragments (Tanaka *et al*, 2003).

Figure 2.2 Genotyping OSMR-KO mice

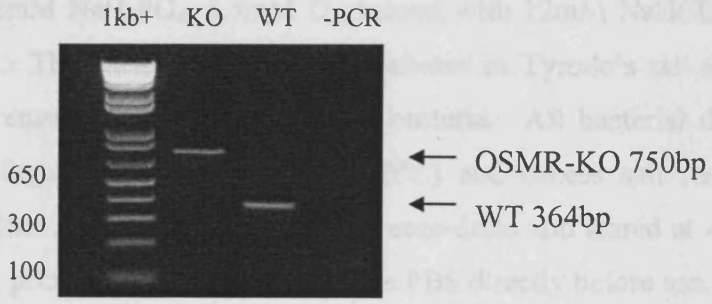

Fig 2.2. cDNA was isolated from tail tips of WT and OSMR-KO mice and PCR performed using primers as outlined in Table 2.1 (Section 2.3.2). Wild type mice generate a 364bp product, whereas OSMR-KO mice generate a 750bp product.

2.3.3 Determination of baseline leukocyte numbers

Differential cell counts and FACS analysis were performed on peripheral blood and peritoneal leukocytes from WT and OSMR-KO animals. Peripheral blood was collected by cardiac puncture into tubes containing EDTA (0.5M) as an anti-coagulant. All red blood cells were removed by hypertonic lysis (155mM NH_4Cl, 7mM K_2CO_3, 0.1mM EDTA pH 7.2). To collect peritoneal leukocytes, healthy

animals were sacrificed by schedule 1. The peritoneal cavity was lavaged with 2ml ice cold sterile PBS. Leukocytes were collected by centrifugation (2000g, 5 minutes) and resuspended in PBS. Cell numbers were determined using a Coulter counter. Slides for differential cell counting were prepared by cytospin (Thermo Fisher Scientific, Waltham, MA, USA) and stained with GIEMSA (Sigma, Poole, UK). Leukocyte subsets were counted according to cell morphology as visualised by light microscopy. Baseline leukocyte numbers were also quantified by flow cytometric analysis using pan-leukocyte markers as described in Section 2.4.3.

2.3.4 Generation of peritoneal inflammation using *Staphylococcus epidermidis* cell-free supernatant

A lyophilized cell free supernatant (SES) was prepared from *Staphylococcus epidermidis* following previously stated protocol (Hurst *et al*, 2001). Briefly, *S.epidermidis* taken from a clinical isolate, was grown to stationary phase in nutrient broth 2 (Oxoid, Basingstoke, UK) at 37°C in a shaking incubator overnight. The cell pellet was collected by centrifugation (1800g, 20mins, 20°C) and re-suspended in Tyrode's salt solution (1.8mM $CaCl_2 \cdot 2H_2O$, 1mM $MgCl_2 \cdot 6H_2O$, 2.6mM KCl, 137mM NaCl, 0.42mM NaH_2PO_4, 5.5mM D-glucose, with 12mM $NaHCO_3$) diluted to an OD_{600} of 0.5. The bacterial pellet was incubated in Tyrode's salt solution at 37°C overnight to ensure complete lysis of the bacteria. All bacterial debris was removed by centrifugation (5000g, 30 mins, 20°C) and excess salt removed by dialysis against water. Aliquoted samples were freeze-dried and stored at -80°C as a lyophilised powder, prior to reconstitution in sterile PBS directly before use.

Prior to use the activity of each batch of SES was assayed using SV40 HPMC. Monolayers of SV40 HPMC were growth arrested (Section 2.2.2.1) prior to stimulation with a serial dilution of SES reconstituted in serum free DMEM. Cells were incubated for 24 hours, culture supernatant collected and assayed for CXCL8 using ELISA (Section 2.9). Production of CXCL8 in the range of 2500±500pg/ml in response to undiluted SES was used for all experiments.

For each experiment groups of mixed sex, age-matched WT and OSMR-KO animals were used. Induction of peritoneal inflammation was achieved by intra-peritoneal injection of SES (500µl). At designated time intervals of between 30 minutes and 48

hours, mice were sacrificed by schedule 1 and the peritoneal cavity lavaged with 2ml of ice cold sterile PBS. Sections of the peritoneal cavity were removed and snap frozen in liquid nitrogen for analysis by EMSA (Section 2.8).

Samples of peritoneal membrane were taken for histology from mice after resolution of four episodes of SES-induced inflammation. Groups of four OSMR-KO and five WT mice received four injections of SES (500μl at weekly intervals) and were sacrificed 21 days after the final episode to allow for resolution. Sections of peritoneal membrane were removed and pinned flat in PBS, exposing the mesothelium. Sections were fixed overnight at 4°C in 10% neutral buffered formal saline (Sigma, Poole, UK) prior to embedding in paraffin. Fixed sections were cut (5-6μm thickness) and stained with haematoxylin and eosin or primary antibodies against collagen I or collagen III (Santa-Cruz Biotechnology, Santa Cruz, CA) in combination with an HRP-conjugated secondary antibody. All histology was performed by Central Biotechnology Services, Cardiff University.

Peritoneal leukocytes were collected and counted using methods outlined in Section 2.3.3. Slides were prepared by cytospin and stained for differential cell count as described in Section 2.3.3. Remaining leukocytes were stained for flow cytometric analysis of macrophage specific markers (F4/80 and CD11b) and CCR5 expression as described in Section 2.4.4. Peritoneal lavage supernatant was retained and stored for future analysis by ELISA (Sections 2.9 and 2.9.1).

2.4 FACS Analysis

2.4.1 Use of FACS Calibur 4CA flow cytometer

Flow cytometric analysis enables information on the relative size, granularity and phenotypic expression of defined cell markers.

2.4.1.1 Analysis of individual cell subsets utilising forward vs side scatter

Size and granularity can be assessed using plots of forward scatter (FSC, reflects particle size) versus side scatter (SSC, reflects particle granularity), thus enabling individual cell subsets to be identified and analysed further. As cells pass though the laser beam light is deflected and refracted by the cells in the stream. The scattered light is collected by the FSC and SSC photodiodes, which convert the light signal into electronic signals that are expressed as a plot of points on an axis corresponding to the morphology of the cells, as illustrated in Figure 2.3.

Figure 2.3 FSC vs SSC dot plot of human peripheral blood leukocytes

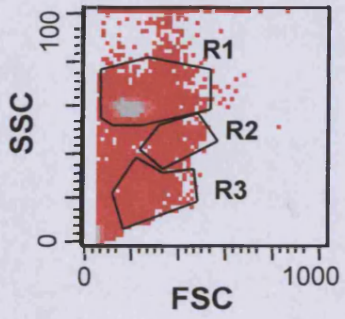

Figure 2.3. Density plot demonstrating forward vs side scatter of freshly isolated human peripheral blood leukocytes. Due to the characteristics of individual leukocyte subsets, each can be gated for further analysis. R1 are neutrophils, which are relatively small but demonstrate granular morphology. R2 are monocytes, which are large, relatively granular cells. R3 are lymphocytes which are smaller and less granular than neutrophils and monocytes.

2.4.1.2 Analysis of receptor expression using single colour staining

When using a single fluorescent dye conjugated to a monoclonal antibody, histogram analysis can be used to gauge the relative distribution of a surface marker. The amount of fluorescent signal detected by the machine (electronic events) is proportional to the number of flourochrome molecules.

Specific alterations in the expression of cell markers can be calculated by monitoring the mean fluorescence units (MFU) associated with receptor expression. Markers can be placed on a histogram to specify a range of events for a single parameter. Negative isotype controls are used to place the marker for positive events as illustrated in Figure 2.4. Any events to the right of the end of the negative isotype peak are considered to be positive.

Figure 2.4 Histogram analysis of cells stained with a FITC positive stain and an isotype control

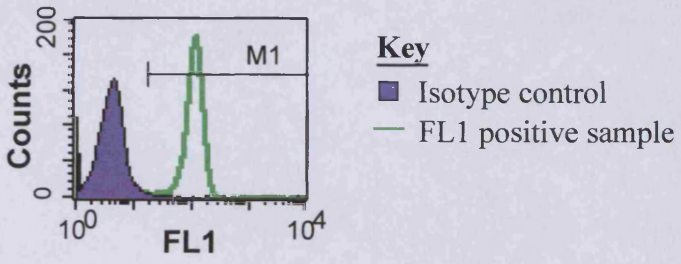

Fig 2.4. Isolated neutrophils were stained with a FITC conjugated antibody against CXCR1 and a FITC conjugated isotype control. The marker is set to enclose the positive sample but not the isotype control.

By utilising the statistics function on CellQuest Pro software, the number of positive events (those enclosed by the marker M1) can be determined and the MFU calculated using the formula below. Calculation of the MFU enables the effect of cell stimulation on the relative receptor expression to be assessed, where sample 1 is the stimulated cell population and sample 2 is the unstimulated control cell population.

$$\text{MFU (from M1)} = \frac{\text{events from positive sample 1} - \text{events from negative IgG}}{\text{events from positive sample 2} - \text{events from negative IgG}}$$

2.4.1.3 Analysis of multiple receptors using more than one fluorochrome

More than one fluorochrome can be analysed simultaneously if the wavelengths required for excitation are similar and the peak emission wavelengths are far enough apart to be detected by separate detectors. However, the emission spectra of the fluorochromes used can overlap; this overlap can be compensated for by altering the

optical filters within the machine. To set the correct compensation, control samples stained with negative isotype and positive controls for FITC (FL1), PE (FL2), PECy5 (FL3) and APC (FL4) (see Table 2.2) were analysed to ensure no detection of fluorochromes in any other detector than that stated but that there was sufficient positive detection by the appropriate detector.

Detector	Flourochrome	Abbreviation
FL1	Fluoroscein / Fluoroscien isothiocyanate	FITC
FL2	Phycoeryhtrin	PE
FL3	Phycoerythrin cyano-5	PECy5
FL4	Allophycocyanin	APC

Table 2.2. Fluorochromes used for flow cytometric analysis within this report and associated detectors.

Dot plots can be used to analyse two parameters together, enabling receptor expression to be determined on cell subsets based on additional expression of specific pan-leukocyte markers (see Section 2.4.3) and further quantification of specific cell subsets (see Section 2.4.5). Quadrant markers can be used to divide two parameter plots into four sections to determine populations which are negative, single positive or double positive. As demonstrated in Figure 2.5, isotype controls are used to set the position of the quadrant marker. Cells within the lower left quadrant are considered negative for both parameters, cells within the lower right quadrant are positive only for the x axis parameter (positive for the FITC conjugated stain). The upper left quadrant contains cells positive only for the y axis parameter (positive for the PE conjugated stain). The upper right quadrant contains cells that are positive for both parameters (double positive for FITC and PE). Using the quadrant statistics function on Cellquest Pro software, the percentage of cells in each quadrant can be calculated.

Figure 2.5 Dot plots of isotype control and PE and FITC positive cells with quadrant markers

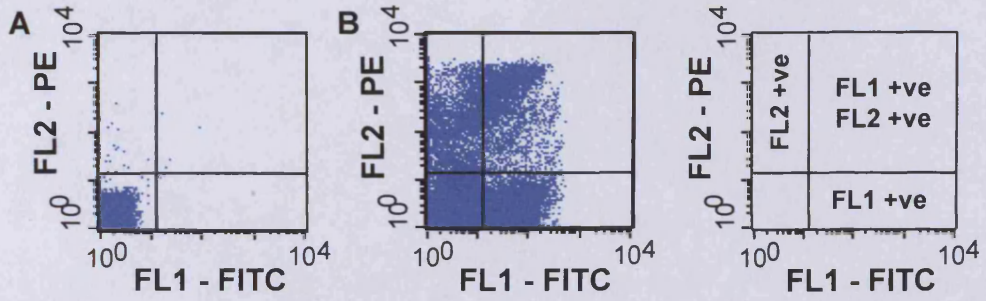

Fig 2.5. A, isotype controls are used to set the quadrant positions. B, cells positive for only the FITC stain are in the lower right quadrant, cells positive for only the PE stain are in the upper left quadrant, cells in the upper right quadrant are positive for both stains.

2.4.2 Flow cytometric analysis of receptor expression on HPMC

Flow cytometric analysis was used to assess the expression of a variety cytokine receptors on the surface of mesothelial cells. Confluent HPMC were growth arrested as previously described (Section 2.2.1.4) and collected by gently scraping into PBS. Cells were pelleted by centrifugation and resuspended in FACS buffer (0.5% BSA, 7.5mM NaN_3, 5mM EDTA in PBS, pH 7.4). To reduce non-specific binding, cells were incubated for 5 minutes in FACS blocking buffer (50% FACS buffer, 25% human antibody serum (HD Supplies, Aylesbury, UK), 25% rabbit serum (DAKO, Cambridgeshire, UK)). HPMC were assessed for cell surface expression of IL-6-related receptors, adhesion molecules and IL-31Rα by incubation for 1 hour with antibodies against the extracellular portions of named receptors as outlined in Table 2.3.

Cells incubated with antibodies against OSMRβ and IL-31Rα required additional incubation with fluorochrome-associated secondary antibodies (phycoerythrin goat-$F(ab')_2$ fragment was used in conjunction with antibodies against OSMRβ and its associated isotype control, whilst anti-goat IgG FITC conjugate was used in for antibodies against IL-31Rα and its associated isotype control, Table 2.3). Cells were washed and resuspended in FACS buffer prior to analysis. Cell staining was quantified using a FACS Calibur 4CA cell sorter (see Section 2.4.1). Data was

acquired from 10,000 gated events for each sample. Cells stained with secondary antibody alone were used as controls to eliminated non-specific binding, whilst non-labelled cells were used to monitor the degree of autofluorescence. All antibody concentrations used and incubation periods were chosen in response to prior optimisation. Data was analysed using CellQuest-pro software, as described in Section 2.4.1.

2.4.3 Flow cytometric analysis of receptor expression on peripheral blood leukocytes

Neutrophils and mononuclear cells were separated using Ficoll density centrifugation (see Section 2.2.3). Cells were diluted to a concentration of $2x10^6$ cells/ml in FACS buffer. To identify individual leukocyte subsets, cells were stained with FITC-conjugated antibodies against the extracellular portions of specific pan-leukocyte cell markers: CD14 (monocytes), CXCR1 (neutrophils) and CD3 and CD4 (T cells) (conditions as described in Table 2.2).

Each subset was co-stained with an antibody against either receptor subunits for IL-6-related cytokines (OSMRβ, gp130 or LIFRβ) or IL-31Rα or related isotype controls (conditions as described in Section 2.3.2 and Table 2.3). To reduce non-specific antibody binding, cells were incubated with FACS blocking buffer for 30 minutes at 4°C in the dark, before the addition of antibody (specific antibody combinations used are described in the relevant chapters) and further 1 hour incubation (in the dark, 4°C). Cells were washed in FACS buffer then resuspended in secondary antibody (if required, see Section 2.4.2) and incubated for 30 minutes. Antibody-labelled cells were washed and resuspended in FACS buffer before acquisition (20,000 gated events). Autofluorescence and antibody labelling controls were included as before (Section 2.4.2), and the data analysed as per Section 2.4.1.

Antibody	Clone	Isotype	Fluoro-chrome	Working dilution	Source
OSMRβ	AN-A2	Mouse IgG_1	N/A	5μg/ml	Santa-Cruz Biotechnology
LIFRβ	7G7	Mouse IgG_1,κ	PE	2μg/ml	BD Pharmingen
IL-6Rα	M5	Mouse IgG_1,κ	PE	2μg/ml	BD Pharmingen
gp130	28126.111	Mouse IgG_1	PE	2.5μg/ml	R&D Systems
IL-31Rα	N-16	Goat IgG	N/A	5μg/ml	Santa-Cruz Biotechnology
ICAM-1	BBIG-I1	Mouse IgG_1	Fluoro-scein	2μg/ml	R&D Systems
VCAM-1	BBIG-V3	Mouse IgG_{2a}	Fluoro-scein	1μg/ml	R&D Systems
CD3	UCHT1	Mouse IgG_1,κ	FITC	1/100	BD Pharmingen
CD4	RPA-T4	Mouse IgG_1,κ	PECy5	1/100	BD Pharmingen
CD14	M5E2	Mouse IgG2a,κ	FITC	1/100	BD Pharmingen
CXCR1	5A12	Mouse IgG2b,κ	FITC	5μg/ml	BD Pharmingen
Mouse IgG_1	N/A	N/A	N/A	5μg/ml	Santa-Cruz Biotechnology
Anti-goat IgG	N/A	N/A	FITC	1/400	Sigma-Aldrich
RPE $F(ab')_2$	N/A	N/A	PE	0.1μg/ml	Dako

Table 2.3. Primary and secondary antibodies and isotype controls used for FACS analysis of human peripheral blood cells and HPMC.

2.4.4 Quantification of baseline leukocyte numbers in WT and OSMR-KO mice

Murine peritoneal and blood leukocytes were isolated as described in Section 2.3.3. Cells were re-suspended to a concentration of 2 x 10^6 cells/ml in Fc block (4μg/ml Rat anti-mouse CD16/CD32 FcγR1 monoclonal antibody (BD Pharmingen, Oxford, UK) in FACS buffer) to reduce non-specific antibody binding, and incubated in the dark at 4°C for 30 minutes. Samples were diluted to a concentration of 10^5 cells per sample in FACS buffer and incubated (in the dark, 4°C, 1 hour) with Phycoerythrin-conjugated antibodies against the extracellular portion of CD3 (T cells), B220 (B cells) and F4/80 (monocytes) as well as a related isotype control (conditions as outlined in Table 2.4). Expression of these cell markers was quantified from a total of 20,000 gated events using a FACS Calibur 4CA flow cytometer, (Section 2.4.1).

2.4.5 Flow cytometric analysis of CCR5 expression on murine peritoneal macrophages

Peritoneal leukocytes were isolated from inflammatory challenged mice and counted (Section 2.3.4). Cells were re-suspended in blocking buffer and incubated for 30 minutes at 4°C (Section 2.4.4). To characterise individual monocytic subsets, cells were labelled (1hr incubation) with a FITC-conjugated anti-F4/80 antibody (Serotec AbD Ltd, Oxford, UK) in combination with an APC-conjugated anti-CD11b (BD Pharmingen, Oxford, UK) (resident-like monocytes are characterised as $F4/80^{high}CD11b^{high}$, inflammatory monocytes are characterised as $F4/80^{low}CD11b^{+}$, see Chapter 5.3) and CCR5 expression evaluated using a PE-conjugated anti-CCR5 antibody (BD Pharmingen, Oxford, UK). Specific information relating to antibody concentrations used is presented in Table 2.4; again, all antibody concentrations were used dependent on prior optimisation. Cells were washed and resuspended in FACS buffer prior to analysis. Expression of cell markers was quantified from a total of 20,000 gated events using a FACS Calibur 4CA flow cytometer, as described in Section 2.4.1.

Antibody	Clone	Isotype	Fluoro-chrome	Working dilution	Source
CD16/CD32 (Fc block)	2.4G2	Rat $IgG_{2b}\kappa$	N/A	4μg/ml	BD Pharmingen
CD3	GK1.5	Rat $IgG_{2b}\kappa$	FITC	5μg/ml	BD Pharmingen
B220	RA3-6B	Rat $IgG_{2a}\kappa$	FITC	5μg/ml	BD Pharmingen
F4/80	CI:A3-1	Rat IgG_{2b}	FITC	5μg/ml	Serotec
CD11b	M1/70	Rat $IgG_{2b}\kappa$	APC	1μg/ml	BD Pharmingen
CCR5	C34-3448	Rat $IgG_{2c}k$	PE	2μg/ml	BD Pharmingen
Rat IgG2	A95-1	Rat $IgG_{2b}\kappa$	FITC	5μg/ml	BD Pharmingen
Rat IgG2	A95-1	Rat $IgG_{2b}\kappa$	APC	2μg/ml	BD Pharmingen
Rat IgG2	A95-1	Rat $IgG_{2b}\kappa$	PE	2μg/ml	BD Pharmingen

Table 2.4. Primary and secondary antibodies and isotype controls used for FACS analysis of murine peritoneal leukocytes.

2.5 RNA Analysis

2.5.1 Chloroform/Isopropanol RNA extraction

Total cellular RNA was extracted using TRI reagent (Sigma-Aldrich, Poole, UK). Cell monolayers established in 6-well microtitre plates were incubated for 5 mins with 1ml TRI reagent per well. The cell lysate was collected, chloroform added (1/10 to lysate) and samples incubated for 5 mins to allow separation of the aqueous phase. Samples were centrifuged (12,000g, 15 mins, 4°C with no braking), the colourless upper aqueous phase was collected, and the lower solvent phase and the interface were discarded. An equal volume of isopropanol was added to the aqueous phase to precipitate total cellular RNA at -20°C overnight. The RNA was pelleted by centrifugation and washed in 75% (v/v) ice cold ethanol. Samples were air dried and resuspended in 10µl of sterile H_2O.

2.5.2 Determination of RNA concentration

RNA concentrations were determined by spectrophotometric analysis (Beckman UV-DU64 spectrophotometer, Beckman Instruments Ltd, High Wycombe, UK). In an appropriate cuvette 1µl of RNA was diluted in 54µl of sterile water. The absorbance was read at 260 and 280nm. A 260/280 ratio of above 1.8 was indicative of a sufficiently pure sample. The total RNA concentration was calculated using the following formula:

[RNA] (µg/ml) = OD_{260} x molar extinction coefficient x dilution factor

Molar extinction coefficient for RNA = 40
Dilution factor = 55

An OD_{260} of between 0.1 – 1.0 was required to be in the linear range of the Beer-Lambert law, which states that there is a linear relationship between absorbance and the concentration of an absorbing substance, relying on both the distance light travels through the substance (path length) and the absorption coefficient of the substance. Samples were diluted where necessary and discarded if $OD_{260} < 0.1$.

2.5.3 RNA quantification

RNA integrity was determined by flat bed electrophoresis using a mini-gel system (Thermo Life Sciences Ltd, Basingstoke, UK) through a 2% (w/v) agarose gel (composed of 1g electrophoresis grade agarose (Ultrapure agarose, Gibco/BRL), 50ml of 1X TAE (40mM Tris-Acetate, 1mM EDTA) buffer (Promega, Southampton, UK) and 5µl ethidium bromide (5mg/ml). Into a single well, 1µg RNA was loaded with 5µl of loading buffer (15% (v/v) Ficoll Type 400 (GE Healthcare, Chalfont St Giles, UK) in H_2O, with 0.25% (w/v) Orange G). RNA integrity was visualised under ultra violet light in the ChemiDoc™ gel documentation system (Bio-Rad Laboratories, Hemel Hempstead, UK). The quality of the RNA was assessed by an observed ethidium bromide staining pattern for 28S and 18S ribosomal RNA.

Figure 2.6 Representative RNA gel demonstrating expected ethidium bromide staining pattern for 28S and 18S ribosomal RNA.

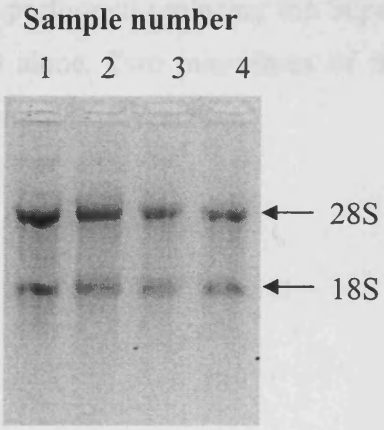

Fig 2.6. RNA was isolated from four individual primary HPMC isolates (Section 2.5.1). RNA integrity was determined by gel electrophoresis (2% (w/v) agarose gel), 1µg RNA was loaded per well, staining was visualised using ethidium bromide under ultra violet light.

2.5.4 Semi-quantitative reverse transcription

Reverse transcription was performed using the random hexamer method (Topley *et al*, 1993). Briefly, 1μg total RNA in a total volume of 20μl comprising 100μM random hexamers (GE Healthcare, Chalfont St Giles, UK), 5mM mixed deoxynucleotides (2'deoxyadenosine 5'triphosphate (dATP), 2'deoxyguanidine 5'triphosphate (dGTP), 2'deoxycytidine 5'triphosphate (dCTP), 2'deoxythymidine 5'triphosphate (dTTP)) (GE Healthcare, Chalfont St Giles, UK), 1X PCR buffer (20mM Tris-HCl, pH 8.4, 50mM KCl) (Invitrogen, Paisley, UK) and 1mM DTT (Dithiothreitol) (Invitrogen, Paisley, UK) was heated to 95°C for 5 mins to linearise the RNA. Following immediate cooling on ice, 40U of recombinant ribonuclease inhibitor (RNAsin, Promega, Southampton, UK) and 200U Superscript II Rnase H-ve reverse transcriptase (Invitrogen, Paisley, UK) was added. The RNA was heated to 20°C for 10 mins to enable primer annealing to the linearised RNA, before transferring to 42°C for 60 mins to allow primer extension from the random hexamers by reverse transcription. The reaction was stopped by denaturation at 95°C for 5 mins. Negative control reactions were also performed replacing the Superscript reverse transcriptase enzyme with distilled H_2O alone. Two microlitres of the resulting complementary DNA (cDNA) was used for each PCR reaction.

2.6 DNA Analysis

2.6.1 Polymerase Chain Reaction (PCR)

Each 50µl reaction comprised 2µl cDNA, 1X PCR buffer containing 1.5mM $MgCl_2$ (10mM Tris-HCl, pH8.3, 50mM KCl, 0.001% (w/v) gelatin) (Applied Biosystems, Warrington, UK), 5mM mixed deoxynucleotides (dATP, dCTP, dGTP and dTTP) (GE Healthcare, Chalfont St Giles, UK), 2.5U AmpliTaq Gold (Applied Biosystems, Warrington, UK) and 1mM oligonucleotide primers (5'-3' and 3'-5', forward and reverse) (Invitrogen, Paisley, UK). Negative control reactions were also performed, replacing the cDNA with 2µl of distilled water. The standard PCR protocol used in this study is as follows:

Step 1 – Initial Linearisation

94°C 2 mins

Step 2 – Denaturation, primer annealing and extension

94°C 30 secs
55°C 1 min
72°C 1 min
} 26 – 38 cycles (dependent on primers)

Step 3 – Final extension and proof reading

68°C 15 mins

All PCR reactions were carried out in a GeneAmp PCR System 9700 Thermo-cycler (Applied Biosystems Ltd).

2.6.2 Primer design

All primers were designed using the internet based Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer 3/primer3) and purchased from Invitrogen Ltd. Primers were designed to have a GC content of 50-60% and an annealing temperature of approximately 60°C. All primers were reconstituted in sterile water to give a stock concentration of 200µM. Detailed sequences for forward and reverse primers are shown in Table 2.5.

Gene		Sequence	Tm (°C)	%GC	Cycle No.	Product length (bp)
β-actin	F	5'-ATCCCCCAAAGTTCACAA-3'	65	44	26	198
	R	5'-CTGGGCCATTCTCCTTAG-3'	65	55		
GAPDH	F	5'-CGAGATCCCTCCAAAATCAA-3'	58	45	28	330
	R	5'-ATCCACAGTCTTCTGGGTGG-3'	58	55		
IL-31Rα	F	5'-TGGTGGAGGCCTTCTTATTC-3'	60	50	35	180
	R	5'-CACAGAGTCATCAGACTCCTTCA-3'	60	48		
IL-11Rα	F	5'-CCAACCCTGTAGAGGACCCA-3'	70	55	32	230
	R	5'-CGTTCCTTGAGCAGAACTCC-3'	70	55		
IL-31	F	5'-TGGACCTCGCACTAAAATCATTG-3'	69	43	32	60
	R	5'-CGAAAGGAAGAGATGGCCTTAA-3'	69	45		

Table 2.5. Human primer sequences used in PCR reactions, outlining the annealing temperature (Tm), GC content, the required cycle number and resulting product size. All PCR reactions were performed using the amplification conditions specified in Section 2.6.1. Product length was estimated by comparison to banding exhibited by 1kb+ DNA ladder. (F - forward sequence, R - reverse sequence).

2.6.3 Sizing of PCR products

All PCR products were run on a 2% agarose gel (as described in Section 2.5.3). 5μl of PCR product was loaded onto the gel along with 5μl of loading buffer (as in section 2.4.3) and separated by electrophoresis at 75V for 45-60 mins in TAE (Tris-Acetic Acid, EDTA) buffer (Promega, Southampton, UK). The DNA banding pattern was visualised/photographed using a ChemiDoc™ gel documentation system, and the size of product compared against molecular weight standards (1kb+ DNA ladder; Invitrogen, Paisley, UK).

2.7 CCL5 promoter construct analysis

CCL5 promoter sequences (outlined in Table 2.6) were kindly provided by Lisa M. Schweibert (Department of Physiology and Biophysics, University of Alabama, Birmingham, Alabama, USA) and inserted into the SmaI and KpnI sites of the multiple cloning region of pGL2 basic luciferase reporter vector (Promega, Southampton, UK). Briefly, a 1.4kb 5' noncoding sequence upstream of the CCL5 gene was cloned as described by Moruichi *et al*, 1997 and found to contain the published promoter sequence and approximately 0.4kb sequence further upstream (R1.4). Additional sequences were generated via 5' deletions and site directed mutations within putative cis-acting elements (Moruichi *et al*, 1997). Plasmids were inserted and stored in competent *E.coli*.

Putative cis-acting element	Position	Base change in site-directed mutagenesis	Promoter sequence length
NFIL-6	-92	TCCGTT**GCA**TGCAATT	1.4kb
NF-κB (KB1)	-44	GGAAACTT**A**CGTAGG	1.4kb

Table 2.6 CCL5 promoter constructs. Additional CCL5 promoter constructs were generated to analyse putative cis-acting elements by site-directed mutagenesis. The base changes involved in site-directed mutagenesis are shown in bold type, positions are relative to the transcription start site (Moruichi *et al*, 1997).

2.7.1 Plasmid purification

Plasmids were isolated from competent cells (*E.coli*; Bioline, London, UK) using QIAGEN plasmid midi kits (Qiagen, Crawley, UK) following the manufacturers' protocol. Briefly, 20µl cell stock (as stored in LB medium and glycerol, -80°C) was used to inoculate 50ml LB medium (10g/l tryptone, 5g/l yeast extract, 0.17M NaCl, pH7.0, heat sterilised) and incubated overnight with constant shaking. Cells were collected by centrifugation (6,000g, 15 mins) and lysed (using 200mM NaOH-1% (w/v) SDS buffer) resulting in the release of the cell contents. Neutralisation of the lysate causes SDS to precipitate, trapping chromosomal DNA and cellular debris but leaving plasmid DNA in solution. The precipitated cell debris was removed by centrifugation (13,000g, 15 mins), the plasmid DNA was bound to resin columns (QIAGEN-tips) and washed to remove all contaminating RNA and proteins. Plasmid DNA was eluted from the column, precipitated by the addition of isopropanol and collected by centrifugation. DNA was washed in 70% ethanol and resuspended in 1ml distilled H_2O.

The concentration of plasmid DNA isolated was quantified by UV spectrophotometry as described in Section 2.5.2. Briefly, 1µl DNA was diluted in 54µl H_2O in an appropriate cuvette. The absorbance was read at 260 and 280nm. The DNA concentration was calculated using the following formula:

[DNA] (µg/ml) = OD_{260} x molar extinction coefficient x dilution factor
Molar extinction coefficient for DNA = 50

2.7.2 Transient transfection

Transient transfection of SV40 HPMC was performed using the lipofection agent FuGene 6 (Roche Diagnostics, Lewes, UK). SV40 HPMC were grown as described in Section 2.2.2.1. When cells reached 70% confluence in 6 well plates, the growth medium was removed and the cell monolayer washed with PBS. Cells were transfected with 0.9µg pGL2 containing the CCL5 promoter sequence (Section 2.7) and 0.1µg of *Renilla* luciferase vector (Promega, Southampton, UK) in accordance with the manufacturer's protocol for the application of FuGene 6. All transfections were carried out in serum free medium. After 24 hours the transfection medium was

replaced with fresh medium containing the necessary stimulus for the experiment and incubated for a further 18 hours.

2.7.3 Reporter gene analysis

The medium was removed and the cells washed in PBS before the addition of 500µl of lysis buffer (supplied in Dual-Glo luciferase assay kit (Promega, UK)) per well. After a 15 minute incubation at room temperature, adherent cells were removed by scraping and the resultant supernatant transferred to an eppendorf tube, where each sample was vortexed to ensure complete lysis. 20µl of each cell lysate was transferred to a white luminmetric 96-well plate, luciferase activity was assayed using the Dual-Glo luciferase assay kit as outlined in the manufacturer's protocol. Briefly, 100µl of Luciferase Assay Reagent II was added to each sample, immediately after this the luminescence of each well was read for 10 seconds using a luminometer (FLUOSTAR Optima, BMG Labtechnologies GmbH, Offenburg, Germany). 100µl Stop n' Glo reagent was then added to each well and *Renilla* luminescence recorded as before.

2.8 Electrophoretic mobility shift assays (EMSA)

2.8.1 Generating nuclear extract from HPMC

HPMC were grown to confluence in T25 tissue culture flasks, growth arrested (Section 2.2.1.4) then treated with the desired stimulants over a designated time course. Cells were washed and scraped into ice cold PBS and pelleted by centrifugation. The cells were resuspended in buffer A (10mM HEPES, 1.5mM $MgCl_2$, 10mM KCl, 0.8μM PMSF, 40 μM Na_3VO_4, 0.2mM NaF, 2μM DTT, 0.025% protease inhibitor cocktail (containing 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), pepstatinA, E-64, bestatin, leupeptin, and aprotinin) (Sigma-Aldrich, Poole, UK), 0.01% (v/v) NP-40) and incubated on ice for 10 minutes. Samples were centrifuged (13,000g, 5mins, 4°C), the supernatant collected (cytosolic extract), and the pellet resuspended in buffer B (20mM HEPES, 40mM NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 25% glycerol, 0.8μM PMSF, 40 μM Na_3VO_4, 0.2mM NaF, 2μM DTT, 0.025% (w/v) protease inhibitor cocktail (Sigma-Aldrich, Poole, UK)) and incubated on ice for 20 minutes for high salt extraction of nuclear proteins. Cell debris was removed by centrifugation (conditions as before); the supernatant (nuclear extract) was stored at -70°C. Protein concentrations were determined as outlined in Section 2.8.3.

2.8.2 Generating nuclear extract from murine peritoneal membrane sections

Nuclear protein was extracted from snap frozen peritoneal membrane sections taken during *in vivo* experiments. Membrane sections were kept frozen on dry ice, each was submerged in liquid N_2 in a mortar and ground to a powder with the pestle. The tissue extracts were collected and suspended in buffer A and incubated on ice for 30 minutes. Samples were centrifuged (13,000g, 5mins, 4°C), the supernatant collected and the pellet resuspended in buffer B, before being incubated on ice for 30 minutes. Cell debris was removed by centrifugation; the supernatant (nuclear extract) was stored at -70°C.

2.8.3 Estimation of protein concentration

The protein concentration of the nuclear extract was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Hemel Hempstead, UK). Protein concentration was determined by direct comparison with BSA (bovine serum albumin fraction V) standards diluted in buffer B, ranging from 0-1mg/ml. Protein standards and samples

diluted appropriately in buffer B (10µl total volume), were loaded in duplicate on a 96-well plate before addition of 40% (v/v) dye binding reagent (Bio-Rad Laboratories, Hemel Hempstead, UK). Absorbance was read at 450nm (FLUOSTAR Optima, BMG Labtechnologies GmbH, Offenburg, Germany), concentrations were deduced using the logarithmic relationship between the standards and their absorbencies.

2.8.4 Radio-labelling double-stranded oligonucleotide probes with ^{32}Phosphorous

All work involving radioactive material was performed behind a perspex shield. Double stranded oligonucleotide primers (sequences outlined in Table 2.7) were labelled using an end-labeling technique. Complementary oligonucleotide sequences were annealed so as to create overhanging 5' single stranded sequences (identified by lower case in 5'-3' sequences described in Table 2.7). Primers were annealed at a concentration of 100ng/ml in the presence of 10% 1M NaCl at 95°C for 10 minutes. The annealed probe was cooled slowly overnight and stored at -20°C.

Transcription factor	Orientation	Sequence
NFκB	Forward	5'-gaTCCATGG**GGAATTCCC**C-3'
	Reverse	5'-gaG**GGGAATTCC**CCATGGA-3'
SIE(M67)	Forward	5'-cgaCAT**TTCCCGTAA**ATCG-3'
	Reverse	5'-cgaCGAT**TTACGGGAA**ATG-3'
NF1	Forward	5'-gaTCTTTTGGATTGAAG**CCAAT**ATGATAA-3'
	Reverse	5'-gaTCTTATCAT**ATTGGC**TTCAATCCAAAA-3'

Table 2.7. Oligonucleotide sequences used for probe preparation for all EMSA analysis. Transcription factor binding motifs are shown in bold type.

The labeling reaction was prepared as follows: oligonucleotide probe (25ng), dNTP mix (dATP, dCTP, dGTP) (2.5mM each), 10X Klenow buffer (500mM Tris-HCl, pH7.2, 100mM $MgSO_4$, 1mM DTT) (Promega, Southampton, UK), NaCl (1M) made up to 46µl with RNA grade H_2O. Binding of 30µCi of α-[^{32}P]dTTP (GE Healthcare, Chalfont St Giles, UK) (30µCi) was catalysed by 2U of Klenow fragment (DNA

Polymerase I Large) (Promega, Southampton, UK). The reaction was incubated at room temperature for 1 hour, after which time the reaction was stopped by the addition of EDTA (0.5M, pH8) and STE (100mM NaCl, 10mM Tris-HCl, 1mM EDTA, pH8). Probes were purified from any free contaminating radioactivity using size exclusion columns (GE Healthcare, Chalfont St Giles, UK) and stored at -80°C.

2.8.5 Gel preparation

All samples were run on vertical 8% (w/v) polyacrylamide gels (cast and run on Hoeffer Sturdier gel apparatus), the composition of which are outlined in Table 2.8. Loading wells were washed with 0.5X TBE (Tris-Boric Acid EDTA, running buffer) prior to use.

Distilled H_2O	44.25ml
40% Acrylamide/Bis-acrylamide (29:1)	15ml
5X TBE*	7.5ml
50% Glycerol	7.5ml
10% Ammonium Persulphate (APS)	0.75ml
TEMED	0.06ml

* 5X TBE (0.45M Tris-HCl, 0.45M Boric acid, 0.01M EDTA, pH8)

Table 2.8. Composition of 8% (w/v) polyacrylamide gels.

2.8.6 Analysis of DNA-protein complexes by electrophoresis

Experiments evaluated formation of protein-DNA complexes which showed restricted electrophoretic mobility. The binding reaction consisted of 5X binding buffer (50mM HEPES, 250mM KCl, 50% glycerol, 1mg/ml acetylated BSA, 5mM DTT, 1mM PMSF) (1/5 final volume), poly dI/dC (1mg/ml), nuclear extracts (2μg HPMC, 10μg peritoneal membrane extract) and RNA grade H_2O (to final volume minus 2μl). After a 10 minute incubation (RT) to allow blocking of non-specific binding sites, radiolabelled oligonucleotide probe containing a binding motif for a transcription factor was added. Samples were incubated with the probe for a further 20 minutes prior to loading onto the gel. Gels were run at 180V for 3hrs 30 minutes, then transferred to 3M Whatman paper and dried on a vacuum drier at 80°C for 2 hrs.

Dried gels were exposed to X-ray film (GE Healthcare, Chalfont St Giles, UK) (6hrs to overnight at -80°C) and developed by autoradiography.

2.8.6.1 Supershift analysis of individual subunits

Supershift analysis utilises antibodies to define the protein composition of individual transcription factor subunits in the DNA:protein complex. If the subunit is present, antibody binding to the protein bound with the probe will either directly block DNA binding or result in formation of a larger complex and will migrate slower upon gel electrophoresis (the supershift). Briefly, nuclear extracts were incubated (20 mins, RT) with antibodies against NF-κB subunits and STAT subunits (as described in Table 2.10) prior to the addition of radio-labelled probe.

Transcription factor	Subunit	Clone	Concentration
NF-κB	p50	NL2	20ng/ml
	p52	C5	20ng/ml
	p65	A	20ng/ml
	c-Rel	N-466	20ng/ml
SIE(M67)	STAT1	M-22	20ng/ml
	STAT3	C-20	20ng/ml
	STAT5	C-17	20ng/ml

Table 2.10. Antibodies used for human and murine supershift analysis and optimal concentrations used. All antibodies were purchased from Santa-Cruz Biotechnology, Santa Cruz, CA.

2.8.7 Densitometry and standardisation of temporal changes

Quantification of banding intensity was performed for each individual time point using the Bio-Rad gel documentation system (Section 2.5.3) and QuantityOne software. For HPMC stimulations, temporal changes in IL-1β-mediated transcription factor activation following co-stimulation with OSM is expressed as a ratio of the banding intensity of IL-1β stimulation alone. *In vivo* transcription factor activation in OSMR-KO mice was expressed as a ratio of the banding intensity seen in WT mice at each time point.

2.9 Enzyme-linked immunosorbent assays (ELISA)

Cell culture supernatant and murine peritoneal lavage fluid were assayed for cytokine and chemokine concentrations in accordance with the BD OptEIA™ (murine CCL2, KC/CXCL1, IL-6, IL-1β; human CCL2, CXCL8, CXCL9 and CXCL10) or the R&D Systems Duoset (murine CCL3, CCL4 and CCL5; human IL-6, CCL5 and CXCL11) ELISA protocols. For specific antibody dilutions and concentrations see Table 2.11.

Species	Chemokine /Cytokine	Primary capture Ab concentration /dilution	Secondary detection Ab concentration /dilution	Top standard concentration	Limit of detection
Human	CCL2	1/250	1/500	500pg/ml	7.8pg/ml
	CCL5	1μg/ml	20ng/ml	1000pg/ml	15.1pg/ml
	CXCL8	1/250	1/250	200pg/ml	3.1pg/ml
	CXCL9	1/250	1/250	2000pg/ml	31.3pg/ml
	CXCL10	1/250	1/250	500pg/ml	7.8pg/ml
	CXCL11	1μg/ml	200ng/ml	500pg/ml	7.8pg/ml
	IL-6	2μg/ml	200ng/ml	600pg/ml	9.1pg/ml
Murine	CCL2	1/250	1/250	1000pg/ml	15.1pg/ml
	CCL3	0.4μg/ml	100ng/ml	500pg/ml	7.8pg/ml
	CCL4	2μg/ml	100ng/ml	1000pg/ml	15.1pg/ml
	CCL5	2μg/ml	400ng/ml	2000pg/ml	31.3pg/ml
	CXCL1	2μg/ml	200ng/ml	1000pg/ml	15.1pg/ml
	IL-1β	1/250	1/250	2000pg/ml	31.3pg/ml
	IL-6	1/250	1/250	1000pg/ml	15.1pg/ml

Table 2.11. Antibody concentrations and samples used for commercial ELISA kits. Kits supplied by R&D Systems and BD Biosciences.

Briefly a 96-well micro-titre plate (Immulon 4HBX, Thermo Life Sciences Ltd) was coated with specific monoclonal capture antibody (100μl per well) and incubated overnight. After each step the plate was washed using 0.05% (v/v) Tween 20 in PBS

on a wellwash 4 automatic plate washer (Thermo Life Sciences Ltd). Plates were blocked for 1 hour with assay diluent (3% (w/v) BSA in PBS) (200µl per well), then washed prior to incubation with the samples and standards, followed by a 2 hour incubation at room temperature (samples were diluted to an appropriate concentration). Each well was washed again before addition of working detection solution (specific biotinylated monoclonal antibody and streptavidin-horseradish peroxidase conjugate) (100µl per well) and further 1 hour incubation. Immuno-reactivity was detected by the addition of substrate solution (Sureblue TMB (3,3',5,5'-tetramethylbenzidine) substrate, KPL), once the required colour change had been observed, the reaction was stopped by the addition of 2M H_2SO_4. The absorbance of each sample was read at 450nm (FLUOSTAR Optima, BMG Labtechnologies GmbH, Offenburg, Germany). Concentrations were deduced using the logarithmic relationship between the standards and their absorbencies.

2.9.1 Murine OSM ELISA development

Murine OSM levels were quantified using an ELISA system developed using 0.8µg/ml goat anti-mouse OSM polyclonal IgG (AF-495-NA, R&D Systems, Abingdon, UK) as a capture antibody and 200ng/ml biotinylated goat anti-mouse OSM polyclonal IgG (BAF495, R&D Systems, Abingdon, UK) as a secondary antibody. Concentrations of OSM were quantified against murine OSM (495-MO-025, R&D Systems, Abingdon, UK) as a protein standard. Antibody concentrations were optimised using a 'chequerboard' design, as outlined in Figure 2.6. The ELISA protocol was followed as described in Section 2.9.

Figure 2.6 Optimisation of murine OSM ELISA using individual antibodies supplied by R&D Systems.

	50ng/ml detection Ab						100ng/ml detection Ab					
	0.2µg/ml capture		0.4µg/ml capture		0.8µg/ml capture		0.2µg/ml capture		0.4µg/ml capture		0.8µg/ml capture	
control	0	0	0	0	0	0	0	0	0	0	0	0
1ng/ml OSM	1	1	1	1	1	1	1	1	1	1	1	1
2ng/ml OSM	2	2	2	2	2	2	2	2	2	2	2	2
4ng/ml OSM	4	4	4	4	4	4	4	4	4	4	4	4
control	0	0	0	0	0	0	0	0	0	0	0	0
1ng/ml OSM	1	1	1	1	1	1	1	1	1	1	1	1
2ng/ml OSM	2	2	2	2	2	2	2	2	2	2	2	2
4ng/ml OSM	4	4	4	4	4	4	4	4	4	4	4	4

200ng/ml detection Ab **400ng/ml detection Ab**

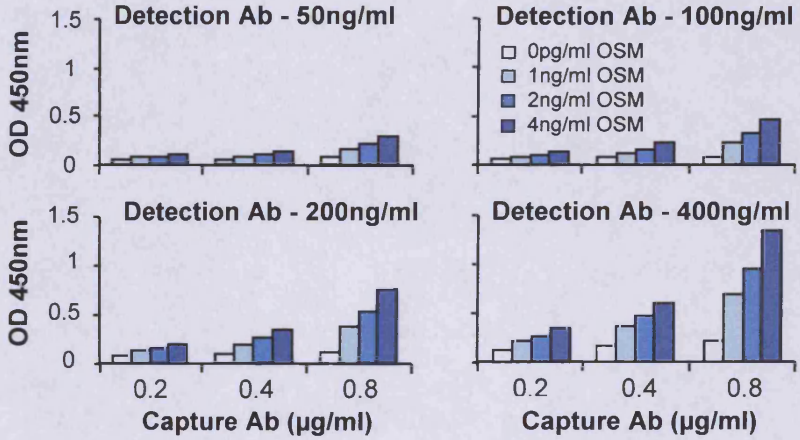

Fig 2.6. Murine OSM ELISA design. A plate was coated with goat anti-mouse polyclonal IgG diluted to the designated concentrations (0.2, 0.4 and 0.8µg/ml) in PBS and incubated at RT overnight. The plate was washed prior to the addition of recombinant murine OSM diluted to 1, 2 and 4ng/ml in 1% (w/v) BSA in PBS and incubated at RT for 2 hours. Again, the plate was washed prior to incubation with biotinylated goat anti-mouse polyclonal IgG diluted to the designated concentrations (50, 100, 200 and 400ng/ml) in 1% (w/v) BSA in PBS for 2 hours. Immuno-reactivity was quantified by the addition of streptavidin-

horseradish peroxidase conjugate and TMB substrate. The absorbance of each sample was read at 450nm. As demonstrated, optimum conditions were observed using 0.8µg/ml capture antibody and 200ng/ml detection antibody.

2.10 Statistical analysis

Statistical analyses were performed using the Student's *t*-test. A *P*-value of <0.05 was considered significant. All data represent mean ± SEM. The number of repeats for each experiment is shown in the appropriate figure legends.

Chapter 3

Defining the receptor signature for gp130-related cytokines in mesothelial cells and leukocyte subsets

3.1 Introduction

Previous work has demonstrated a role for the Interleukin (IL)-6-related cytokine OSM in stromal cell activation, leukocyte recruitment and tissue injury (Wahl *et al*, 2001; Wallace *et al*, 1999; Modur *et al*, 1997; Ihn *et al*, 2000), however, the role of OSM in the progression of acute inflammation has not been extensively studied. This Chapter aims to examine the ability of OSM to mediate expression of chemokines and adhesion molecules by both HPMC and peripheral blood leukocytes.

To examine the cytokine network governing leukocyte trafficking during acute inflammation studies adopted the peritoneal cavity as a site in which to investigate the role of OSM. The peritoneum represents an easily accessible cavity, which can periodically become susceptible to bacterial infections (peritonitis) as a consequence of perforated appendicitis, rupture of the cavity wall or complications from gastric ulcers. However, bacterial peritonitis is a frequent complication in end-stage renal failure patients undergoing peritoneal dialysis treatment (Section 1.10). The primary cell types involved in driving the initial inflammatory response within the peritoneal cavity are peritoneal mesothelial cells lining the peritoneum, and smaller populations of resident macrophages and lymphocytes which play an important role in immune surveillance and initiation of an inflammatory response (Goldstein *et al*, 1984; Topley *et al*, 1996).

The role of IL-6 in the progression of acute inflammation, has been extensively studied and shown to be mechanistically reliant upon IL-6 trans-signalling and the transcription factor STAT3 (Hurst *et al*, 2001; McLoughlin *et al*, 2003; McLoughlin *et al*, 2005; Fielding *et al*, 2008). During episodes of acute inflammation, the primary source of sIL-6R is from infiltrating neutrophils which shed membrane-bound receptor as a consequence of apoptosis or inflammatory activation by chemokines and other chemotactic regulators (Hurst *et al*, 2001; Marin *et al*, 2001; Chalaris *et al*, 2007). In addition infiltrating neutrophils produce and secrete OSM suggesting OSM may also be important in the progression of the immune response (Hurst *et al*, 2002). Although less extensively studied, previous work has shown that OSM can induce expression of pro-inflammatory mediators including IL-6 and CCL2, and also regulate expression of IL-1-induced neutrophil chemoattractants such as CXCL5, CXCL6 and

CXCL8 in a similar manner to that exhibited by IL-6 (Modur *et al*, 1997; Hurst *et al*, 2002; Cross *et al*, 2004; McLoughlin *et al*, 2004).

Due to the short-lived and synergistic nature of the cytokine network, many cytokines are subject to a functional hierarchy with certain factors eliciting comparable outcomes. In this respect, several of the IL-6-related family of cytokines have been shown to promote similar activities. However, each individual cytokine also has its own set of biological properties. This redundancy may be explained by the cellular expression of each specific receptor subunit. Although gp130 is ubiquitously expressed, cognate receptor subunits to the various cytokine family members including LIFRβ and OSMRβ show distinct expression patterns (Ernst & Jenkins, 2004). Previous work using peritonitis as an inflammatory model has demonstrated a lack of LIFRβ expression on both peripheral blood leukocytes and HPMC, however HPMC express OSMRβ (Hurst *et al*, 2002). This, taken in combination with the release of OSM from infiltrating neutrophils, suggests a role for OSM in the progression of peritoneal inflammation potentially in a similar manner to that seen in response to IL-6 trans-signalling due to the previously observed similarities in chemokine regulation between the two cytokines (Langdon *et al*, 1997; Hartner *et al*, 1997; Hurst *et al*, 2002).

This chapter aims to examine expression of IL-6-related cytokine specific α-chains, LIFRβ, OSMRβ and IL-31Rα on HPMC, as the primary cell type orchestrating the immune response within the peritoneal cavity. Expression of OSMRβ and IL-31Rα on peripheral blood leukocytes before and after activation with IFN-γ and LPS (Nathan *et al*, 1983) will be determined. In addition the effect of OSM and IL-31 on HPMC and peripheral blood leukocytes before and after LPS and IFN-γ activation will be examined. This will provide a basis for examining the role of OSM in peritoneal inflammation and also determine any potential role for IL-31 during acute inflammation.

3.2 Materials and Methods

3.2.1 Flow cytometric analysis of receptor expression on unstimulated HPMC

HPMC were isolated from consenting patients undergoing elective surgery as described in Section 2.2.1.1. Monolayers were grown to confluence prior to growth arrest (Section 2.2.1.4). Cells were collected by scraping into ice cold PBS, washed and resuspended in FACS buffer (Section 2.4). To determine the expression of IL-6-related cytokine receptor expression, cells were incubated with antibodies against the extracellular portion of IL-6Rα, LIFRβ, gp130, OSMRβ or an appropriate isotype control (Section 2.4.2). Cells stained with antibodies against OSMRβ and the isotype control were washed then incubated for a further 30 minutes with a phycoerythrin-conjugated secondary antibody. Cells were washed and resuspended in FACS buffer before cell surface receptor expression analysis by flow cytometry (Section 2.4.1).

3.2.2 Flow cytometric analysis of receptor expression on freshly isolated peripheral blood leukocytes

Blood leukocytes were isolated from healthy volunteers as described in Section 2.2.3. Isolated neutrophils and PBMC were resuspended in FACS buffer at a concentration of $2x10^6$ cells/ml, prior to blocking for 30 minutes with block buffer (see Sections 2.4.1 and 2.4.2). Leukocyte subsets were identified by expression of specific pan leukocyte cell markers (CD3 and CD4 (T cells), CD14 (monocytes) and CXCR1 (neutrophils)) (see Section 2.4.2). In addition to the leukocyte-specific markers, cells were co-stained with antibodies against the extracellular portion of LIFRβ, gp130, OSMRβ or a specific isotype control, as outlined in Table 3.2.1 (conditions of use outlined in Table 2.2). Again, cells were washed and resuspended in FACS buffer before cell surface receptor expression analysis by flow cytometry (Section 2.4.4).

Leukocyte subset	Pan-leukocyte marker	Flouro-chrome	Co-stain	Flouro-chrome	Secondary antibody
Monocyte	CD14	FITC	OSMRβ	N/A	RPE-goat (ab')2
			LIFRβ	PE	N/A
			gp130	PE	N/A
			Mouse IgG_1	N/A	RPE-goat (ab')2
Neutrophil	CXCR1	FITC	OSMRβ	N/A	RPE-goat (ab')2
			LIFRβ	PE	N/A
			gp130	PE	N/A
			Mouse IgG_1	N/A	RPE-goat (ab')2
Lymphocyte	CD3	FITC	OSMRβ	N/A	RPE-goat (ab')2
			LIFRβ	PE	N/A
			gp130	PE	N/A
			Mouse IgG_1	N/A	RPE-goat (ab')2
T cell	CD4	PE-Cy5	OSMRβ	N/A	RPE-goat (ab')2
			LIFRβ	PE	N/A
			gp130	PE	N/A
			Mouse IgG_1	N/A	RPE-goat (ab')2

Table 3.2.1. Combinations of antibodies used to determine gp130, OSMRβ and LIFRβ expression on leukocyte subsets. Cell staining was quantified by FACS analysis, performed as previously described (Section 2.4.1). (FITC - flourescein isothiocyanate, PE - phycoerythrin, PECy5 – phycoerythrin cyano-5).

3.2.3 Reverse-transcription PCR analysis of receptor expression on unstimulated HPMC

Total cellular RNA was isolated from growth arrested HPMC using TRI reagent and reverse transcribed into complementary DNA (cDNA) (Section 2.5). PCR was performed using forward and reverse primers for IL-11Rα and IL-31Rα as outlined in Section 2.6.

3.2.4 Stimulation of HPMC with OSM and subsequent quantification of induced cytokines and chemokine production

Growth arrested HPMC (Sections 2.2.1.1, 2.2.1.2 and 2.2.1.4) were stimulated (37°C, 5% CO_2) with defined doses of human recombinant OSM (R&D Systems, Abingdon, UK) in serum free medium. Dose- and time-dependent experiments were performed as outlined in the appropriate Figure Legends. Cells stimulated with recombinant human IL-1β (R&D Systems, Abingdon, UK) (50pg/ml, chosen based on the levels quantified *in vivo* and was shown to elicit robust chemokine induction *in vitro*) or IFN-γ (500U/ml, chosen in response to previous dose response experiments (Robson *et al*, 2001)) (R&D Systems, Abingdon, UK) were used as a positive control. Unstimulated controls (cells treated with fresh serum free medium) provided baseline measurements for all mediators tested. Following stimulation culture supernatant was removed and rendered cell free by centrifugation. Levels of CCL2, CCL5, CXCL8, CXCL9, CXCL10, CXCL11 and IL-6 were quantified by ELISA (Section 2.9).

3.2.5 *In vitro* activation of PBMC with a combination of LPS and IFN-γ

PBMC were isolated from whole blood (Section 2.2.3) and resuspended in serum free supplemented medium at a concentration of $2x10^6$ cells/ml. Cells were allowed to adhere for 2 hours in 12 well plates and subsequently activated overnight with a combination of LPS (10ng/ml) and IFN-γ (500U/ml).

To assess the induction of OSMRβ on 'LPS/IFN-γ-activated' PBMC, cells were stimulated overnight with either OSM (30ng/ml) or IL-31 (50ng/ml). CCL2 levels in cell-free culture supernatant were quantified by ELISA (Section 2.9). Cells were collected by scraping into ice cold PBS and flow cytometry used to assess surface expression of OSMRβ and IL-31Rα (Section 2.4.2). Total cellular RNA was extracted for PCR analysis of IL-31Rα expression (Sections 2.5 and 2.6).

3.2.6 Analysis of adhesion molecule expression on stimulated HPMC

Confluent HPMC were growth arrested (Section 2.2.1.4) and stimulated overnight with OSM (30ng/ml), IL-1 (50pg/ml) or TNFα (100pg/ml). Cells were harvested and incubated with antibodies against the extracellular portions of ICAM-1 and VCAM-1 and an appropriate isotype control (conditions used are outlined in Table 2.2) for 1 hour. Cell staining was quantified by flow cytometry (Section 2.4.1).

3.3 Results

3.3.1 Receptor expression on HPMC at basal levels

Due to the central importance of HPMC in orchestrating cytokine production during peritoneal inflammation, cytokine receptor expression on HPMC was evaluated to determine which IL-6-related cytokines potentially govern mesothelial cell responses during inflammatory processes.

IL-6-related cytokines signal through a receptor system utilising at least one subunit of the ubiquitously expressed signal transducing receptor gp130. As outlined in Section 1.8.1, signalling via gp130 can either occur through a gp130 homodimer complex, or via interaction with a secondary subunit as a heterodimeric receptor complex. To examine the potential of HPMC to trigger signalling events through these distinct modes of gp130 activation, studies evaluated the expression of receptor subunits that direct gp130-mediated responses in HPMC. As previously determined, HPMC do not express IL-6Rα (Hurst *et al*, 2001), however expression of IL-11Rα was detected (Figure 3.3.1). In addition to gp130 homodimers, two additional receptor complexes utilising gp130 exist, comprising one subunit of gp130 associated with either OSMRβ or LIFRβ. A variety of IL-6-related cytokines utilise LIFRβ:gp130 receptor complexes, including OSM, LIF, CNTF and CT-1, however, HPMC do not express LIFRβ (Figure 3.3.1) therefore are unable to respond to these cytokines. As previously demonstrated HPMC express OSMRβ (Figure 3.3.1) and are therefore able to respond directly to OSM.

Figure 3.3.1 IL-6-related receptor expression on HPMC

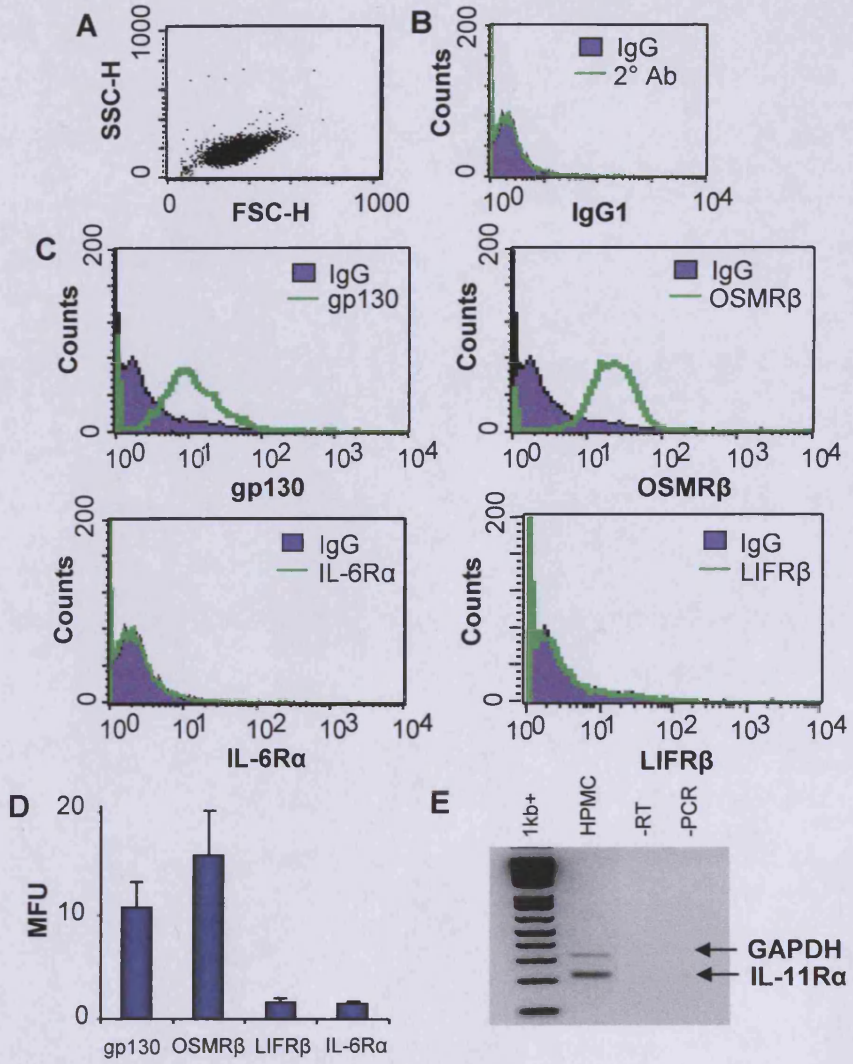

Fig 3.3.1. IL-6-related receptor expression on HPMC. A, demonstrates forward/side scatter of HPMC at passage 2. Stained samples were compared to isotype controls and secondary antibody controls (B). Growth arrested HPMC were incubated with antibodies against gp130, OSMR, IL-6R and LIFR (C), the calculated mean fluorescence for each receptor, compared to the isotype control, can be seen in panel D. IL-11Rα expression was quantified by RT-PCR (E). Data represents mean +/- SEM of three independent primary isolates.

3.3.2 OSM selectively regulates inflammatory chemokine production, but not that of IL-6

It has previously been reported that OSM, released from infiltrating neutrophils during episodes of acute inflammation, is able to regulate secretion of IL-6 (Brown *et al*, 1991), which has been suggested as a contributing mechanism for OSM involvement in the control of leukocyte trafficking (Hurst *et al*, 2002). However, when IL-6 production in response to OSM was assayed, no significant increase in IL-6 was observed in either a dose- or time-dependent manner (Figure 3.3.2).

Studies have previously demonstrated the ability of OSM to upregulate expression of the mononuclear cell chemoattractant CCL2 in HPMC (Hurst *et al*, 2002). Figure 3.3.3 confirms these previous observations and shows that OSM treatment stimulates both a dose- and time-dependent upregulation of CCL2, which due to the magnitude and reproducibility of this response will be used as a positive control for further experiments. To further examine the potential ability of OSM to regulate mononuclear cell trafficking, production of two additional mononuclear chemoattractants, CCL3 and CCL5, was assayed. However, no increase in production of either CCL3 or CCL5 was observed in response to OSM (Figure 3.3.3). OSM stimulation also had minimal effects on the T cell chemoattractants CXCL9, CXCL10 and CXCL11 (Figure 3.3.4), with only CXCL10 production observed in response to high concentrations of OSM. The induction of CXCL10 by OSM, was however minimal when compared with the archetypal activator of CXCL10, IFN-γ (Figure 3.3.4). In addition OSM does not induce expression of the neutrophil chemoattractant CXCL8 (Figure 3.3.5).

Figure 3.3.2 OSM does not regulate IL-6 expression in HPMC

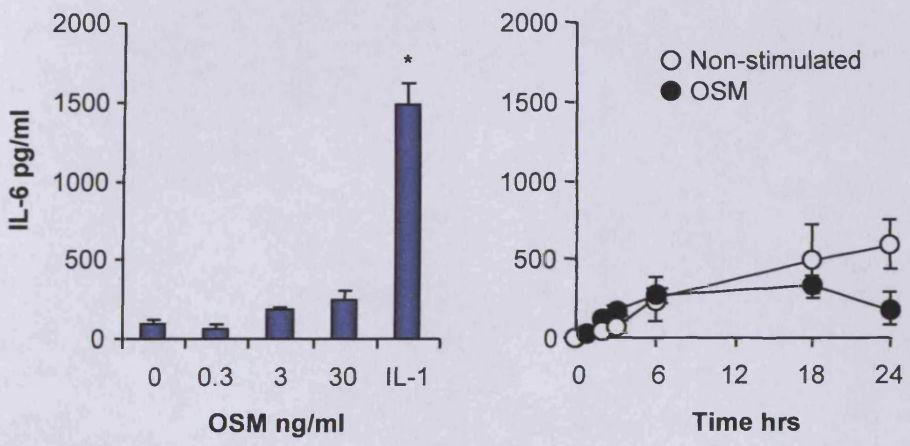

Fig 3.3.2. Growth arrested HPMC were stimulated with increasing doses of OSM for 18 hours, or with 30ng/ml OSM for increasing time periods. IL-6 expression was quantified in the culture supernatant by ELISA, OSM treated cells were compared to IL-1β (50pg/ml) stimulated cells or non-stimulated control cells. Data represents mean +/- SEM of three independent primary isolates (*$p<0.05$).

Figure 3.3.3 OSM selectively increases expression of the monocyte chemoattractant CCL2 in HPMC

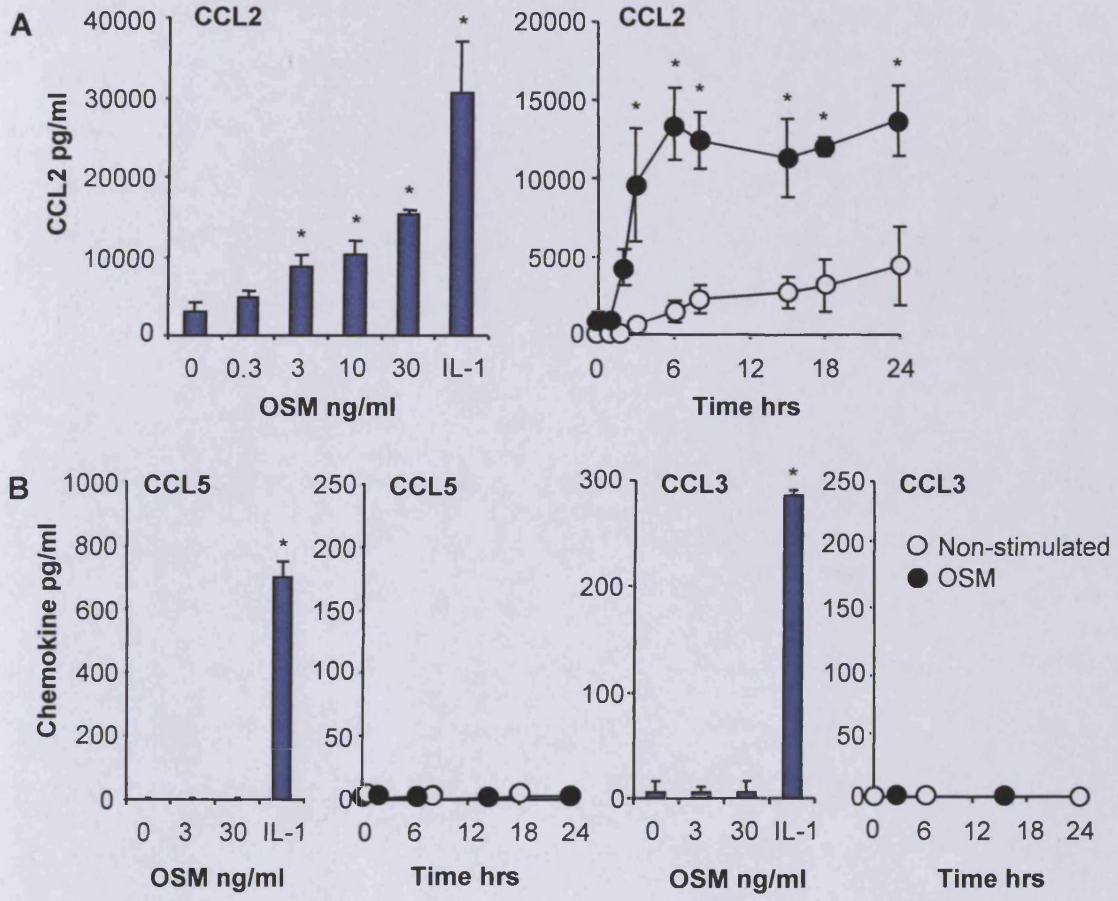

Fig 3.3.3. OSM selectively induces expression of CCL2. Growth arrested HPMC were stimulated with increasing doses of OSM for 18 hours, or with 30ng/ml OSM for increasing time periods. CCL2 (A), CCL3 and CCL5 (B) expression was compared to IL-1β (50pg/ml) stimulated cells or non-stimulated control cells. Chemokine expression in the culture supernatant was quantified by ELISA. Data represents mean +/- SEM of three independent primary isolates (*$p<0.05$).

Figure 3.3.4 OSM selectively increases expression of the T cell chemoattractant CXCL10 in HPMC

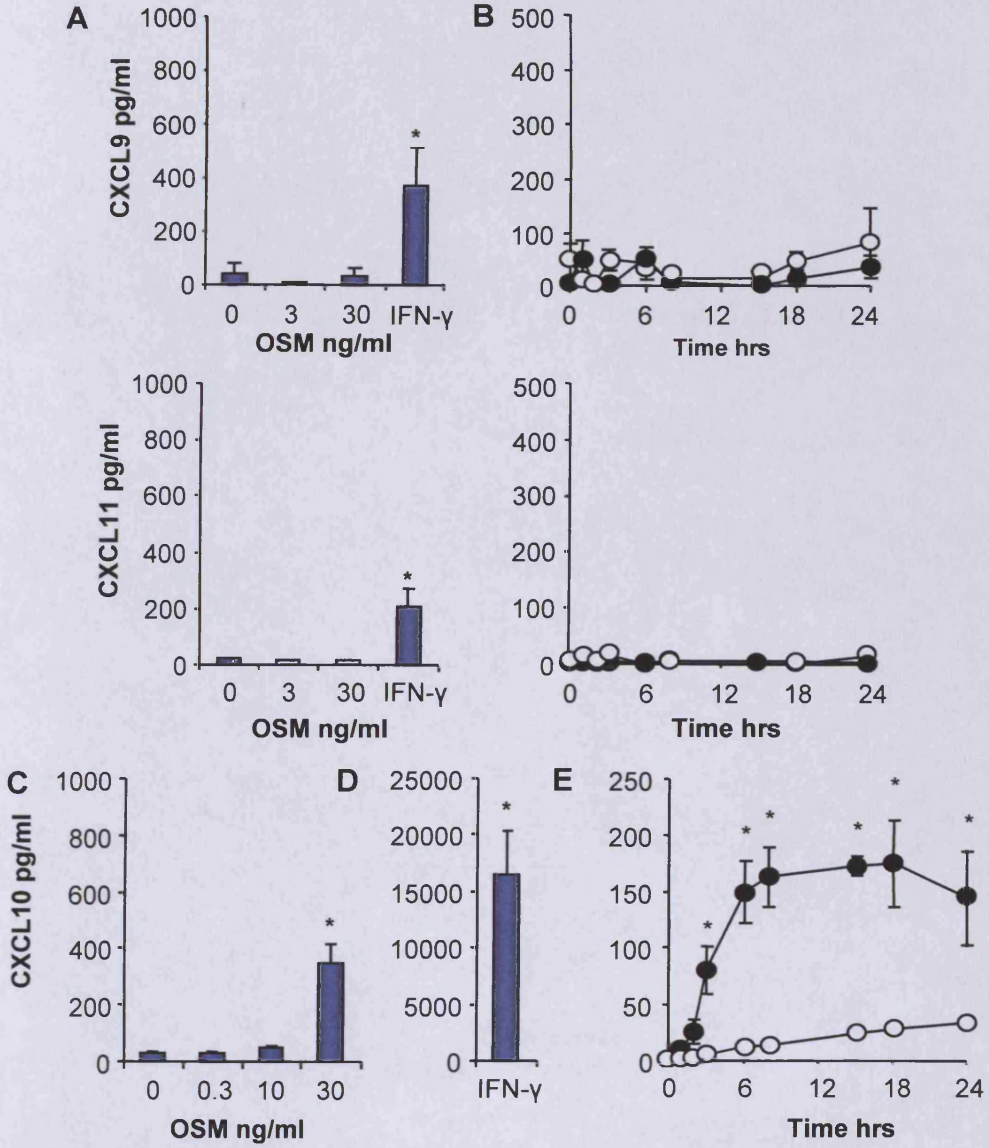

Fig 3.3.4. OSM control of ELR⁻ CXC chemokines. CXCL9, CXCL10 and CXCL11 expression in HPMC was quantified after overnight stimulation with OSM (0-30ng/ml) or IFN-γ (500U/ml) (A, C, D), or OSM (30ng/ml) compared to non-stimulated control cells over a time course (B, E). Data represents mean +/- SEM of three independent primary isolates (* $p<0.05$).

Figure 3.3.5 OSM does not increase expression of the neutrophil chemoattractant CXCL8 in HPMC

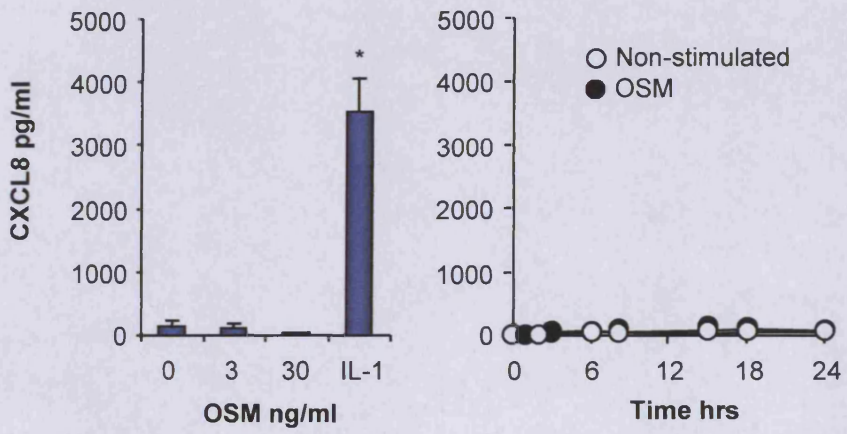

Fig 3.3.5. Growth arrested HPMC were stimulated with increasing doses of OSM for 18 hours, or with 30ng/ml OSM for increasing time periods. CXCL8 expression in the culture supernatant was quantified by ELISA, OSM treated cells were compared to IL-1β (50pg/ml) stimulated cells or non-stimulated control cells. Data represents mean +/- SEM of three independent primary isolates (*$p<0.05$).

3.3.3 OSM selectively promotes expression of the adhesion molecule VCAM-1

In addition to regulating chemokine expression, IL-6-related cytokines can also control leukocyte trafficking by controlling expression of adhesion molecules (Modur *et al*, 1997; Oh *et al*, 1998). Previous work has illustrated OSM induced upregulation of intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) in endothelial cells (Modur *et al*, 1997 Fritz *et al*, 2006; Fearon *et al*, 2006). Stimulation with OSM significantly increases surface expression of VCAM-1 but not ICAM-1 on HPMC, which remains comparable to that observed in unstimulated cells (Figure 3.3.6). VCAM-1 is associated with the adhesion of a variety of mononuclear cells including monocytes and T cells, whereas ICAM-1 is more closely associated with the adherence of neutrophils (Liberek *et al*, 1996). The ability of OSM to specifically upregulate expression of VCAM-1 on HPMC provides further evidence for a role in mononuclear cell trafficking.

Figure 3.3.6 OSM selectively upregulates expression of the adhesion molecule VCAM-1 in HPMC

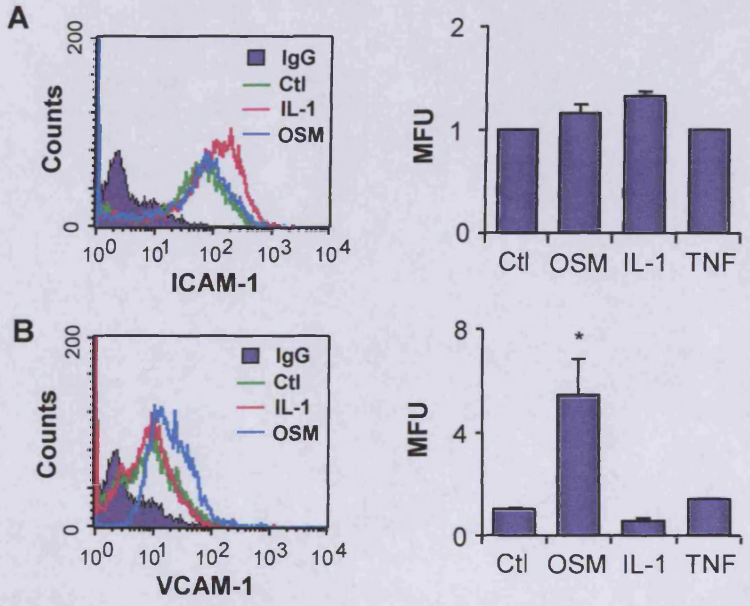

Fig 3.3.6. Growth arrested HPMC were treated with OSM (30ng/ml), IL-1β (50pg/ml) or TNFα (100pg/ml). Cells were stained with FITC conjugated antibodies against ICAM-1 (A) or VCAM-1 (B), compared back to a FITC conjugated IgG control. Data is representative of three independent primary isolates (* $p<0.05$).

3.3.4 IL-6 related receptor expression on peripheral blood leukocytes

Inflammation is a highly orchestrated communication system, which links responses between stromal tissue cells local to the initial insult and infiltrating leukocytes. Studies outlined thus far illustrate that OSM is likely to affect mesothelial cell responses within the peritoneal cavity. To examine the potential impact of OSM on infiltrating cells, peripheral blood leukocytes were examined for OSMRβ expression. As shown in Figure 3.3.7, freshly isolated peripheral blood leukocytes do not express either OSMRβ or LIFRβ and are unable to respond to OSM stimulation (assessed by production of both CCL2 and IL-6) (Figure 3.3.8) confirming PBMC do not express a functional receptor complex for OSM. However, PBMC produce RNA for the cognate IL-11Rα (Figure 3.3.7) and have previously been shown to express IL-6Rα (McLoughlin *et al*, 2004), implying signalling is only mediated through gp130 homodimers on peripheral blood mononuclear cells.

During an inflammatory response macrophages are activated through several distinct mechanisms (Section 1.4.1), one of which is the classical pathway where activation is achieved through IFN-γ and additional TLR activation by microbial products such as LPS. Stimulation of peripheral blood leukocytes with LPS in combination with IFN-γ, promoted expression of OSMRβ on the surface of PBMC but not PMN (Figure 3.3.9). This process is confined to signalling through TLR4 however, as co-stimulation with IFN-γ and the TLR2 agonist SES, (a cell-free supernatant derived from the gram positive bacterium *Staphylococcus epidermidis*), failed to induce OSMRβ expression on either PBMC or PMN (Figure 3.3.9). The TLR4-driven expression of OSMRβ was functional, and OSM stimulation of these pre-activated PBMC promoted CCL2 production (Figure 3.3.9).

Figure 3.3.7 Freshly isolated PBMC express IL-11Rα but not LIFRβ or OSMRβ

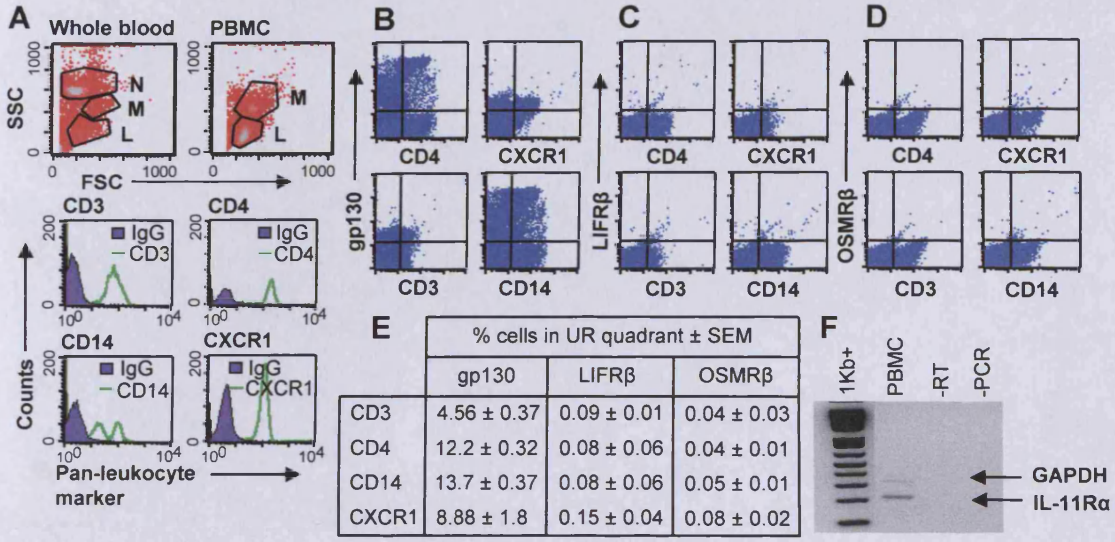

Fig 3.3.7. Leukocyte subsets were gated dependent on their forward-side scatter (N-neutrophils, M-monocytes, L-lymphocytes) and expression of pan-leukocyte markers (A). To identify leukocyte specific expression PBMC were incubated with FITC-conjugated antibodies against pan-leukocyte markers in combination with PE-conjugated antibodies against either gp130 (B), LIFRβ (C) or OSMRβ (D), the percentage of cells expressing these receptors (cells in the upper right (UR) quadrant) is demonstrated in Panel (E). RT-PCR was performed using primers for GAPDH and IL-11R on cDNA isolated from growth arrested PBMC (32 cycles) (F). Data is representative of three individual donors.

Figure 3.3.8 Freshly isolated PBMC are unable to respond to direct OSM stimulation

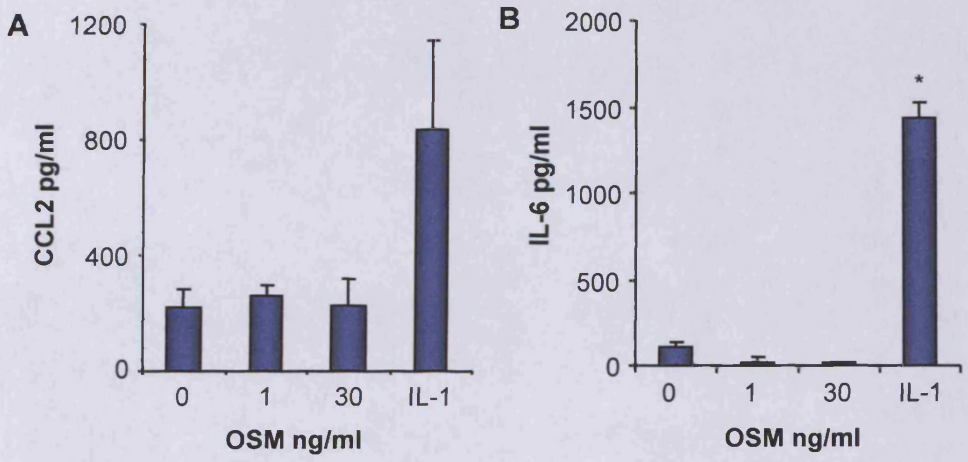

Fig 3.3.8. Isolated PBMC were stimulated with increasing concentrations of OSM (0-30ng/ml) for 18 hours. Cell culture supernatants were assayed for CCL2 (A) and IL-6 (B) compared to IL-1β (50pg/ml) stimulated cells. Data represents mean +/- SEM of three independent PBMC isolates (*$p<0.05$).

Figure 3.3.9 Induction of OSMRβ expression on PBMC

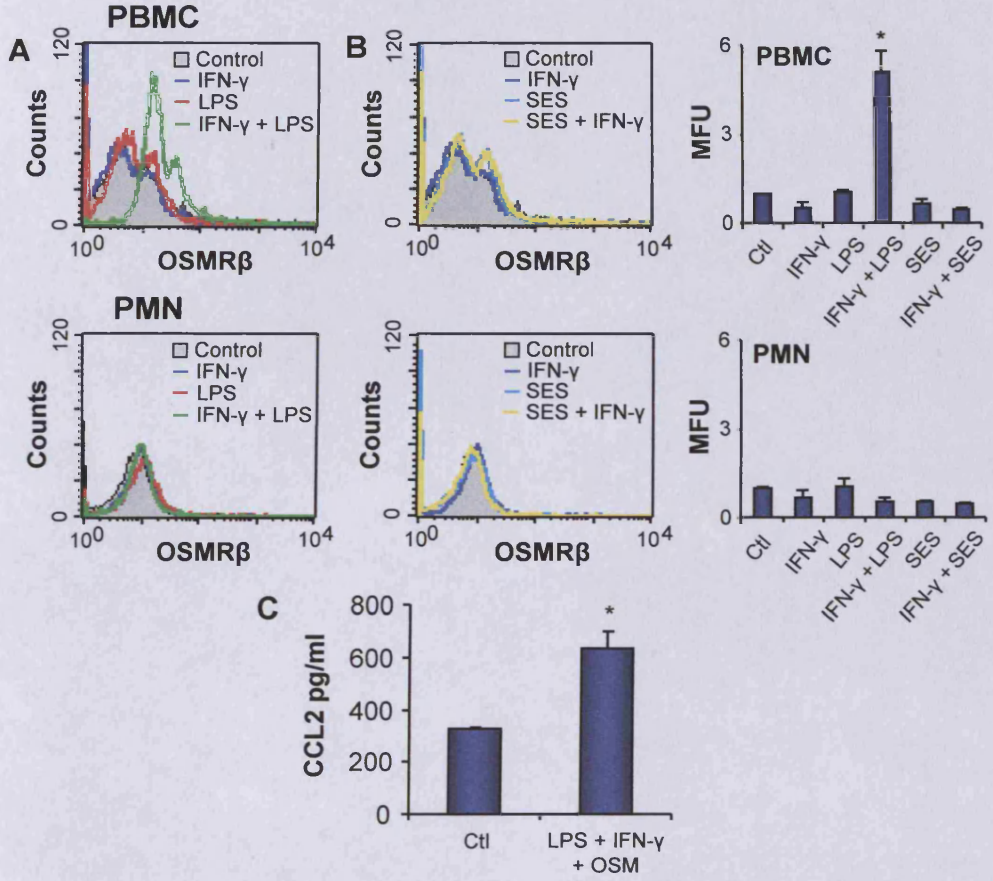

Fig 3.3.9. Isolated PBMC and PMN were treated with LPS (50ng/ml) or IFN-γ (500U/ml) individually and in combination overnight (A). Panel B shows an identical experimental set up in which LPS has been replaced with the TLR2 agonist SES. Changes in OSMRβ were evaluated by flow cytometry and compared to isotype and secondary antibody controls. C, isolated PBMC were stimulated with LPS and IFN-γ alone and in combination with OSM (30ng/ml). CCL2 in the culture supernatants was assayed by ELISA. All data represents mean +/- SEM of three independent PBMC isolates (*p<0.05).

3.3.5 IL-31Rα expression on HPMC

In addition to the association with gp130, OSMRβ also interacts with the cognate IL-31Rα to form a functional receptor complex for IL-31 (Dillon *et al*, 2004). IL-31 plays an important role in allergic responses (Dillon *et al*, 2004; Perrigoue *et al*, 2007), with IL-31Rα expression detected in several cell types including keratinocytes, lung epithelial cells, activated monocytic cells and T lymphocytes (Dreuw *et al*, 2004). Basal IL-31 expression was detected at low levels in OSM stimulated HPMC (Figure 3.3.10), indicating a potential role for IL-31 in peritoneal inflammation. However, RT-PCR analysis showed HPMC do not express IL-31Rα, and was confirmed by an inability of HPMC to produce CCL2 in response to IL-31 (Figure 3.3.10) To control for the activity of IL-31, a similar set of experiments were performed using THP-1 cells (a leukaemic monocytic cell line). As shown in Figure 3.3.10, IL-31 promoted CCL2 production by THP-1 cells and subsequent RT-PCR analysis on cDNA isolated from THP-1 cells exhibited low level IL-31Rα expression (Figure 3.3.10).

HPMC were however shown to upregulate IL-31Rα expression following exposure to IFN-γ, which was further enhanced by additional stimulation with the TLR2 and TLR4 agonists SES and LPS respectively (Figure 3.3.11). To substantiate the functional relevance of this induction, IL-31-mediated regulation of CCL2 was examined in LPS and IFN-γ treated HPMC (Figure 3.3.11). As predicted from prior RT-PCR and flow cytometry studies (Figure 3.3.11), IFN-γ and LPS rendered HPMC susceptible to IL-31 stimulation (Figure 3.3.11).

Figure 3.3.10 HPMC do not express IL-31Rα under basal conditions

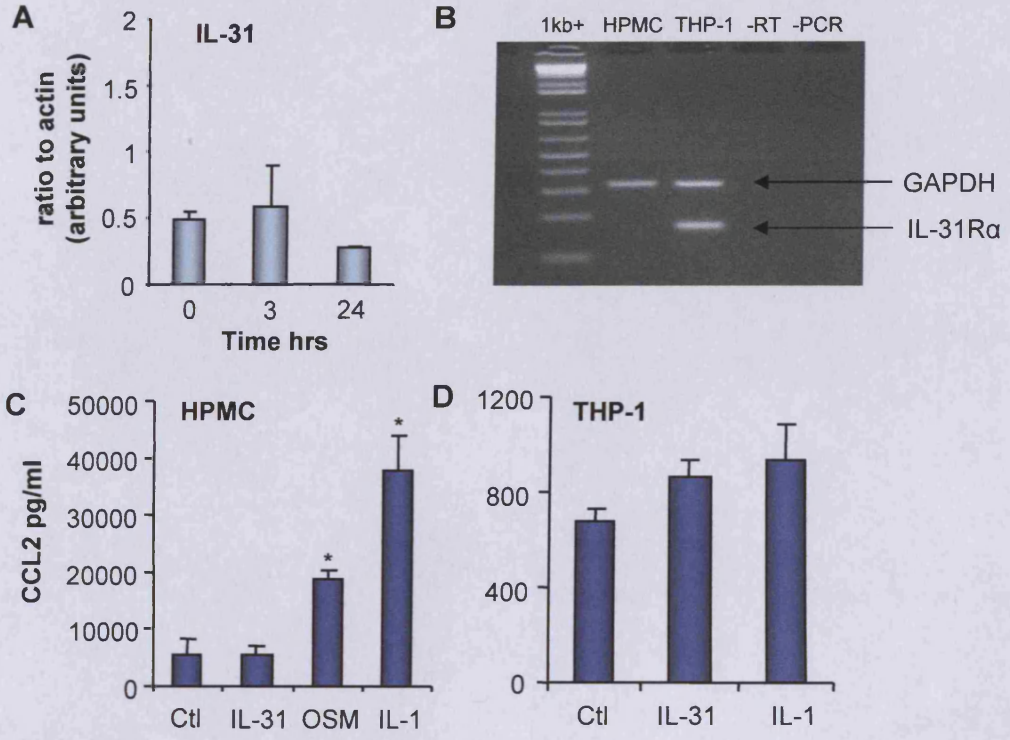

Fig 3.3.10. Regulation of IL-31 responsiveness in HPMC (A). RT-PCR was performed using primers for IL-31 on cDNA isolated from OSM stimulated HPMC (30 cycles). Densitometry was performed comparing each band to the corresponding β-actin control. Expression of IL-31Rα on growth arrested HPMC and THP-1 cells was quantified by RT-PCR using primers for GAPDH and IL-31Rα (35 cycles) (B). CCL2 expression was assayed in the culture supernatants following IL-31 (50ng/ml), IL-1β (50pg/ml) and OSM (30ng/ml) stimulation overnight (C, HPMC; D, THP-1). Data represents mean +/- SEM of three independent primary isolates (*p<0.05).

Figure 3.3.11 Induction of IL-31Rα expression on HPMC

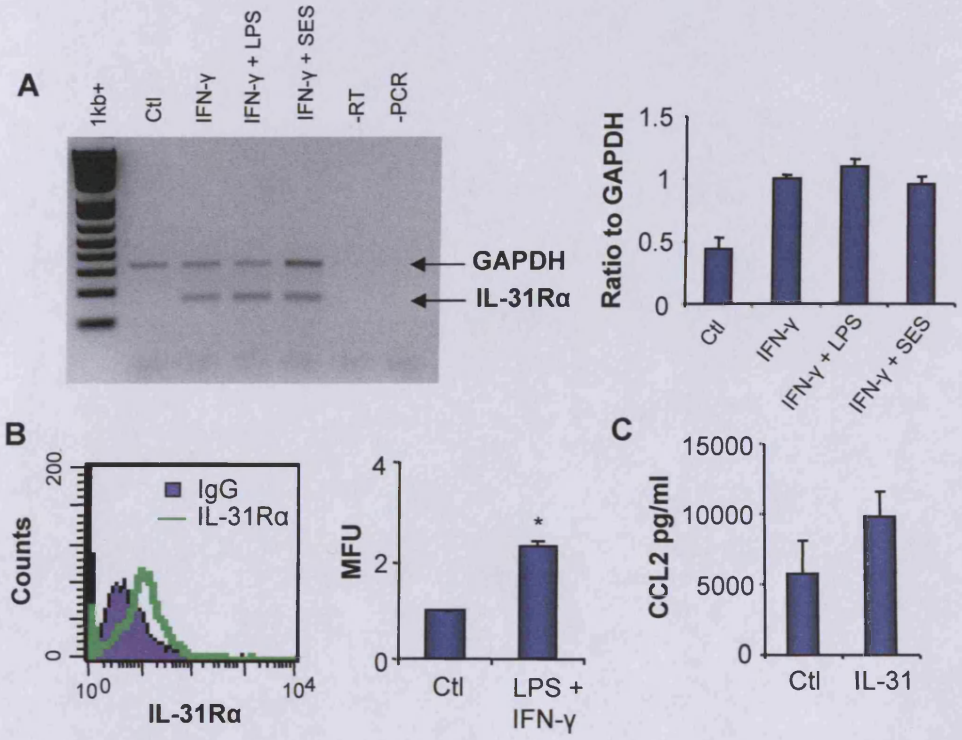

Fig 3.3.11. Expression of IL-31Rα on HPMC stimulated with IFN-γ (500U/ml) alone or in combination with either LPS (50ng/ml) or SES overnight was quantified by RT-PCR (35 cycles) (A) and flow cytometry (B). CCL2 expression was assayed in the culture supernatants, following pre-activation with LPS and IFN-γ and subsequent IL-31 stimulation (C). Data is representative of three independent primary isolates (*$p<0.05$).

3.3.6 Analysis of IL-31Rα expression on PBMC and PMN

Studies on the role of IL-31 in allergic responses have demonstrated expression of IL-31Rα on activated mononuclear cells, corresponding with expression of OSMRβ but at substantially reduced levels (Dillon *et al*, 2004). RT-PCR demonstrated that freshly isolated PBMC do not express IL-31Rα, however expression can be induced by stimulation with IFN-γ (Figure 3.3.12). Changes in IL-31Rα expression on PBMC were confirmed by flow cytometry (Figure 3.3.12). Conversely, PMN show no alteration in IL-31Rα expression following IFN-γ and LPS stimulation (Figure 3.3.12).

Figure 3.3.12 Induction of IL-31Rα expression on PBMC

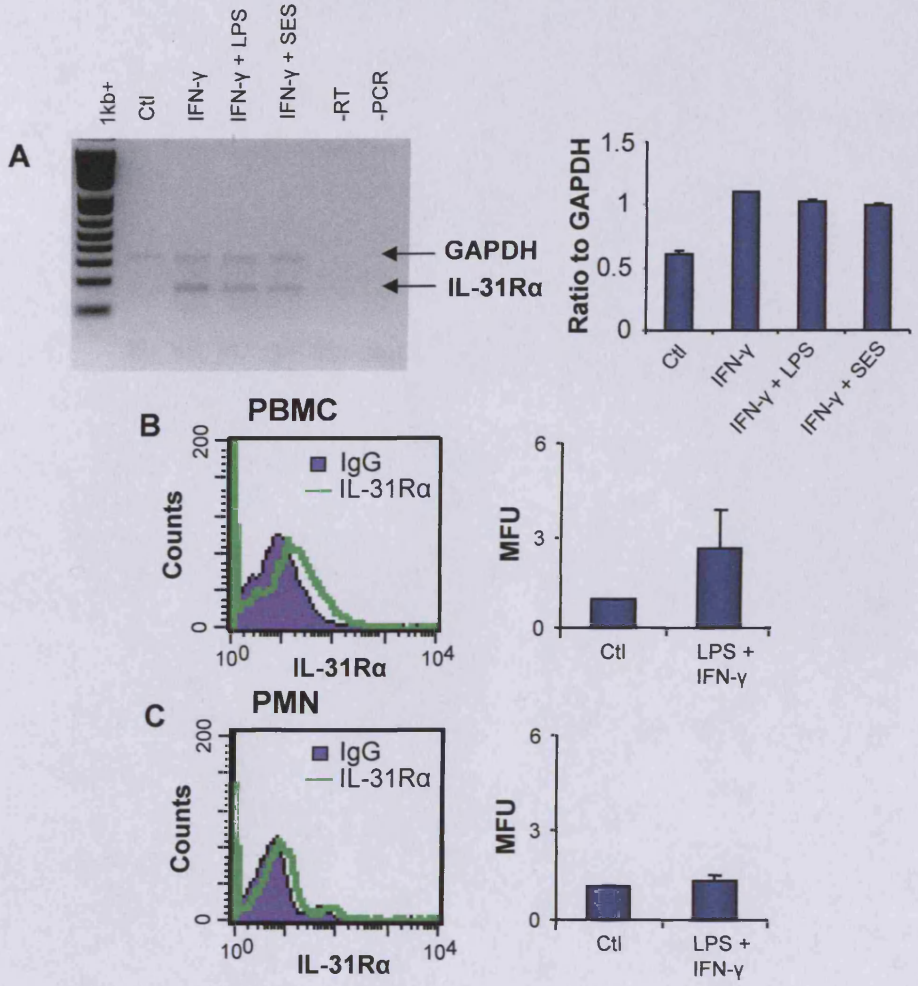

Fig 3.3.12. Expression of IL-31Rα on PBMC stimulated with IFN-γ (500U/ml) alone or in combination with either LPS (50ng/ml) or SES overnight was quantified by RT-PCR (35 cycles) (A) and flow cytometry (B). Expression of IL-31Rα on PMN was quantified by flow cytometry after stimulation with LPS and IFN-γ (C). Data is representative of three independent PBMC isolates.

3.4 Discussion

Previous studies have outlined a potential role for the gp130-activating cytokine OSM in the development of acute inflammation (Hurst *et al*, 2002). Using bacterial peritonitis as a model in which to study acute inflammation, *in vitro* studies described in this Chapter suggest a role for OSM upregulating expression of inflammatory mediators and adhesion molecules thereby aiding leukocyte recruitment in a similar manner to that observed in response to IL-6 and its soluble receptor (Hurst *et al*, 2001).

Previous studies have demonstrated that IL-6 or OSM can regulate chemokine expression in a variety of stromal cells. In many instances, these two gp130-activating cytokines are similar in their capacity to control these inflammatory regulators (Modur *et al*, 1997; Hurst *et al*, 2002; McLoughlin *et al*, 2004; Langdon *et al*, 1997). For example, both cytokines demonstrate the ability to upregulate expression of the mononuclear cell chemoattractant CCL2, which in the case of IL-6 aids the progression of inflammation by mediating recruitment of mononuclear cells (Hurst *et al*, 2001; Romano *et al*, 1997). This shared role taken in combination with the observed ability of OSM to upregulate expression of adhesion molecules suggests OSM may be of importance in orchestrating leukocyte recruitment and activation during acute inflammation.

Acute inflammation is a beneficial response to infection or injury ensuring successful resolution of the condition and a return to tissue homeostasis. Pivotal to the resolution of inflammation is the transition from innate to acquired immunity, which is defined by precise regulation of leukocyte recruitment, activation and clearance (Jones, 2005). Previous work using an *in vivo* model of acute bacterial peritonitis has demonstrated the importance of IL-6 in this transition, through control of chemokine-driven leukocyte recruitment and regulation of apoptosis (Hurst *et al*, 2001; McLoughlin *et al*, 2003; McLoughlin *et al*, 2004; McLoughlin *et al*, 2005). However, previous work based on OSM reconstitution studies in experimental models of disease has been unable to determine whether OSM has a beneficial or detrimental effect *in vivo* (Wallace *et al*, 1999; Langdon *et al*, 2000; Plater-Zyberk *et al*, 2001; De Hooge *et al*, 2003).

Regulation of OSM activity is governed by the cellular expression of OSMRβ. In this context, we identified two central events affecting OSM responsiveness. During acute inflammation, the primary cell types expressing OSMRβ and therefore able to respond to OSM are stromal cells, with leukocyte subsets not expressing OSMRβ or LIFRβ (Hurst *et al*, 2002). However, in response to activation with LPS and IFN-γ, OSMRβ expression can be induced on mononuclear cells, suggesting that in more progressive disease OSM may have two distinct modes of action.

As previously mentioned this project uses a model of bacterial peritonitis in which to study the inflammatory response. In addition to *in vitro* analysis of HPMC, subsequent studies will also utilise an *in vivo* mouse model which uses a cell-free supernatant derived from *S. epidermidis* to induce acute peritoneal inflammation. The application of OSMRβ-deficient mice will help to define the inflammatory role of both OSM and IL-31 to disease progression, although *in vitro* studies suggest that IL-31 is not involved in chemokine-directed leukocyte trafficking during acute inflammation. However, because factors including IFN-γ and LPS can induce IL-31Rα expression, IL-31 may serve an additional role in more progressive forms of disease as demonstrated by the involvement of IL-31 in dermatitis and type 2 lung inflammation (Dillon *et al*, 2004; Perrigoue *et al*, 2007).

In summary, studies outlined in this Chapter implicate OSM, acting through OSMRβ:gp130 heterodimers, in the progression of an inflammatory response by upregulating mononuclear cell chemoattratants and adhesion molecules. These effects are similar to those observed by IL-6 and its soluble receptor. Future chapters will aim to delineate the regulatory abilities of OSM, in comparison to those previously observed by IL-6 and any differences in signalling pathways initiated through activation of gp130 hetero- and homodimers.

Chapter 4

OSM-mediated regulation of inflammatory chemokine expression in HPMC

4.1 Introduction

Studies outlined in Chapter 3 demonstrate the ability of OSM to selectively upregulate expression of inflammatory mediators specific for trafficking of mononuclear cells. Many of these activities are shared by IL-6, with IL-6 trans-signalling known to govern neutrophil clearance and mononuclear cell recruitment (McLoughlin *et al*, 2004; Hurst *et al*, 2001). This Chapter aims to compare the ability of OSM to regulate chemokine expression with those observed by IL-6.

The ability of OSM to modulate chemokine activation has previously been outlined in several cell types (Hurst *et al*, 2002; Richards *et al*, 1996; Langdon *et al*, 1997). In many instances, these activities closely mirror those exhibited by IL-6 when utilising gp130 homodimeric receptor complexes (McLoughlin *et al*, 2004; Hurst *et al*, 2001). *In vivo* studies using IL-6 deficient mice have shown that IL-6-mediated control of these processes is pivotal for successful resolution of neutrophil infiltration and the development of acquired immunity through increased T cell recruitment (Hurst *et al*, 2001; McLoughlin *et al*, 2005; Jones, 2005). The contribution of OSM to this immunological switch, however, has not previously been examined.

Both *in vitro* and *in vivo* studies have illustrated the ability of IL-6 signalling, to selectively inhibit the IL-1β-induced production of the neutrophil chemoattractants CXCL1 and CXCL8, while enhancing secretion of other inflammatory chemokines including CCL2 and CCL8 thereby aiding the resolution of the neutrophil infiltration and promoting mononuclear cell recruitment (Romani *et al*, 1997; Hurst *et al*, 2001; McLoughlin *et al*, 2003; McLoughlin *et al*, 2004; Jones, 2005). Similar *in vitro* analysis has demonstrated that OSM is able to inhibit IL-1β-directed production of CXCL8 and to mediate production of CCL2 (Hurst *et al*, 2002), consequently OSM has the potential to regulate leukocyte trafficking in a similar manner to that of IL-6. However, in contrast to IL-6, OSM is able to selectively increase expression of the adhesion molecule VCAM-1 (Chapter 3). This data, taken in combination with the ability of OSM to upregulate mononuclear cell chemoattractants including CCL2 and CXCL10 (Chapter 3) suggests a distinct role for OSM in governing mononuclear cell recruitment.

Studies outlined within this Chapter aim to further examine the ability of OSM to regulate the expression inflammatory chemokines. In addition, the ability of OSM to regulate downstream signalling pathways will be studied, with an aim to outline the mechanisms by which OSM may regulate chemokine-driven leukocyte recruitment.

4.2 Materials and Methods

4.2.1 Stimulation of HPMC and subsequent quantification of induced cytokines and chemokine production

To examine the effects of OSM stimulation on the IL-1β- and IFN-γ-induced production of inflammatory mediators, growth arrested HPMC (Section 2.2.1.4) were stimulated with human recombinant IL-1β (R&D Systems, Abingdon, UK) (at concentrations between 0 and 50pg/ml) or human recombinant IFN-γ (R&D Systems, Abingdon, UK) (at concentrations between 0 and 500U/ml) both alone and in combination with 30ng/ml human recombinant OSM (R&D Systems, Abingdon, UK). To investigate the effects of IL-31 on IL-1β-induced chemokine production, HPMC were first treated with LPS (50ng/ml) and IFN-γ (500U/ml) (Section 3.2.5) for 18 hours and subsequently stimulated with human recombinant IL-1β (at concentrations between 0 and 50pg/ml) either alone or in combination with 50ng/ml human recombinant IL-31. Unstimulated controls (cells treated with fresh serum free medium) were used for all experiments to provide baseline chemokine production.

Following stimulation (37°C, 5% CO_2) culture supernatants were removed and rendered cell free by centrifugation. Analysis of CCL2, CCL5, CXCL8, CXCL9, CXCL10, CXCL11 and IL-6 was performed using commercial ELISA (Section 2.9).

4.2.2 Luciferase analysis of CCL5 promoter constructs

SV40 HPMC were grown to 70% confluence (Section 2.2.2) and transiently transfected overnight with 0.9μg CCL5 luciferase promoter constructs in pGL2 and 0.1μg *Renilla* luciferase vector (Section 2.7.2). Cells were washed with PBS then stimulated for 24 hours with a combination of PMA (15ng/ml) and Ionomycin (1μM) or IL-1β (1ng/ml). Medium was removed and the cells lysed prior to luciferase analysis (Section 2.7.3).

4.2.3 EMSA analysis of STAT and NF-κB activation in response to IL-1β and OSM stimulation of HPMC

Nuclear extracts were isolated from HPMC stimulated with IL-1β (50pg/ml) and OSM (30ng/ml) alone or in combination (Section 2.8.1). Prior to EMSA analysis, the protein concentration was determined as described in Section 2.8.3. EMSA using [α-^{32}P]dTTP labelled oligonucleotide probes containing consensus sequences for NF-κB and total STAT were performed using 2μg of nuclear extract (Section 2.8.6.1). Supershift analyses were performed using antibodies against individual transcription factor subunits as outlined in Section 2.8.6.2. Quantification of the banding intensity was performed for each time point as described in Section 2.8.7.

4.3 Results

4.3.1 OSM selectively regulates inflammatory cytokine and chemokine production

IL-6 signalling through its soluble receptor has been shown to inhibit IL-1β-induced production of several ELR^+ CXC chemokines, including CXCL1 and CXCL8. In accordance with this observation, IL-6 deficient mice show significantly enhanced KC and MIP-2 (the functional murine homologues of CXCL1 and CXCL8) expression in comparison to wild type animals following induction of peritoneal inflammation, which results in increased trafficking of neutrophils into the peritoneal cavity of IL-6 deficient animals (McLoughlin *et al*, 2004). We therefore assessed whether OSM could elicit similar activities *in vitro*. Although prior studies had shown that OSM could induce IL-6 expression (Brown *et al*, 1991; Hurst *et al*, 2002), studies outlined in Chapter 3 showed no significant increase in IL-6 expression in HPMC in response to OSM. In addition OSM was also unable to regulate the IL-1β-induced production of IL-6 (Figure 4.3.1).

Studies in Chapter 3 showed that OSM governs CCL2 expression but not that of CXCL8, CCL3 or CCL5. We therefore tested whether OSM could manipulate the IL-1β-mediated control of inflammatory chemokines. Consistent with previous studies OSM significantly inhibits IL-1β-mediated CXCL8 expression (Figure 4.3.1; Hurst *et al*, 2002; Langdon *et al*, 1997; Richards *et al*, 1996). In addition OSM selectively controlled expression of mononuclear cell chemoattractants, synergistically upregulating CCL2 production by IL-1β (Figure 4.3.1), whilst inhibiting IL-1β-induced production of CCL5 (Figure 4.3.1). The observed ability of OSM to control expression of CCL5 was in direct contrast to IL-6, which had no effect on IL-1β-induced CCL5 expression (Figure 4.3.1).

Figure 4.3.1 OSM regulates IL-1β-induced chemokine expression

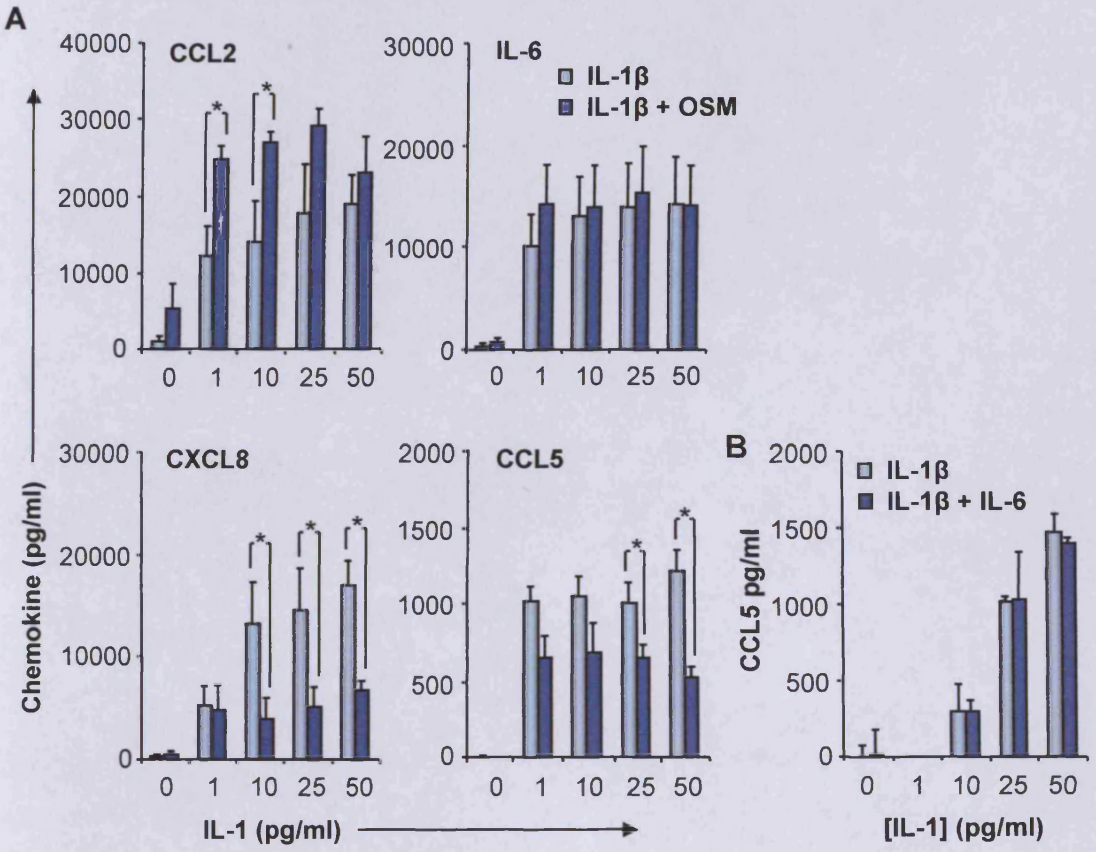

Fig 4.3.1. Growth arrested HPMC were stimulated with increasing doses of IL-1β (0-50pg/ml), either alone or in combination with OSM (30ng/ml) for 18 hours. Expression of CCL2, IL-6, CXCL8 and CCL5 in the culture supernatant was quantified by ELISA (A). Growth arrested HPMC were stimulated with IL-1β (0-50pg/ml) either alone or in combination with IL-6 (10ng/ml) and sIL-6R (50ng/ml) for 18 hours (B). CCL5 expression in the culture supernatant was quantified using ELISA. Data represents mean +/- SEM of three independent primary isolates (*p<0.05).

4.3.2 IL-31 is unable to regulate expression of inflammatory chemokines

As mentioned in Chapter 3, the related cytokine IL-31 also utilises OSMRβ as a β-signalling unit following binding to a cognate IL-31 receptor (Dillon *et al*, 2004). Due to the shared use of the OSMRβ subunit by OSM and IL-31 it was important to assess the role of IL-31 to fully appreciate the contribution of OSMRβ signalling. Stimulation of HPMC with IFN-γ and LPS induced IL-31Rα expression enabling cells to respond to IL-31 signalling (Chapter 3, Figure 3.3.10). However, unlike OSM, IL-31 is unable to suppress IL-1β-induced expression of either CXCL8 or CCL5 by HPMC (Figure 4.3.2), suggesting that IL-31 does not share the ability of OSM to govern chemokine-driven leukocyte trafficking in this context.

Figure 4.3.2 IL-31 does not regulate IL-1β-induced chemokine expression

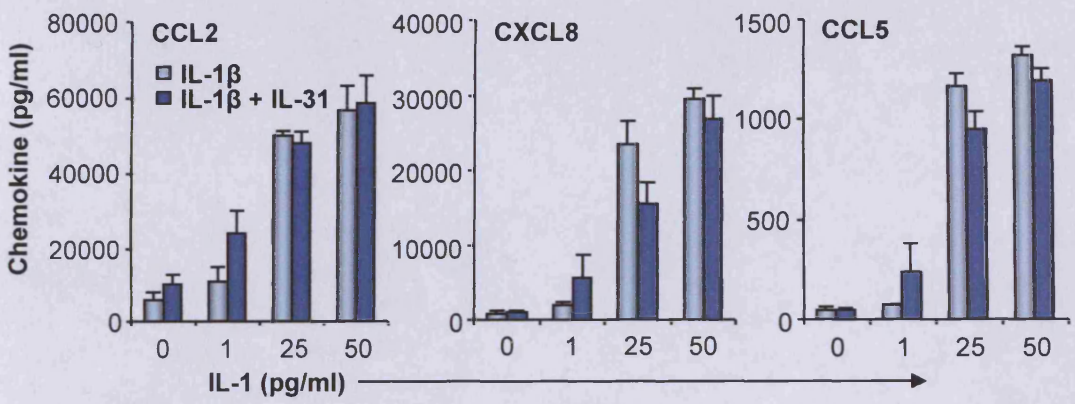

Fig 4.3.2. Growth arrested HPMC were stimulated with increasing doses of IL-1β (0-50pg/ml) alone or in combination with IL-31 (50ng/ml) for 18 hours. Chemokine expression was quantified in the culture supernatant by ELISA. This data represents mean +/- SEM of three independent primary isolates.

4.3.3 OSM significantly impairs IL-1β-mediated NF-κB signalling

The inhibition of IL-1β-driven expression of CCL5 by OSM infers that OSM signalling may inhibit NF-κB signalling. To assess this, EMSA analysis was performed using oligonucleotide probes containing the consensus sequence for NF-κB. Analysis of nuclear extracts isolated from HPMC stimulated with IL-1β and OSM demonstrated the ability of OSM to suppress the IL-1β-mediated activation of NF-κB (Figure 4.3.3). Stimulation of HPMC with OSM alone did not result in NF-κB activation (Figure 4.3.3). Supershift analysis of the protein-DNA complex from IL-1β stimulated cells demonstrated classical activation of the NF-κB pathway, with evidence of both p50 and p65 subunits (Figure 4.3.3). OSM-mediated suppression of NF-κB activation was not specific for an individual subunit, but globally affected both p50 and p65 subunits (Figure 4.3.3).

Conversely, IL-1β had no effect on OSM-mediated STAT activation (Figure 4.3.4). Analysis of OSM-induced STAT activation in nuclear extracts isolated from OSM and IL-1β stimulated HPMC demonstrated no alteration in total STAT activation in comparison to that stimulated with OSM alone (Figure 4.3.4). Supershift analysis of the STAT subunits identified a predominant activation of STAT3, whilst STAT1 and STAT5 were activated to a lesser extent (Figure 4.3.4).

Figure 4.3.3 OSM inhibits IL-1β-induced NF-κB activation

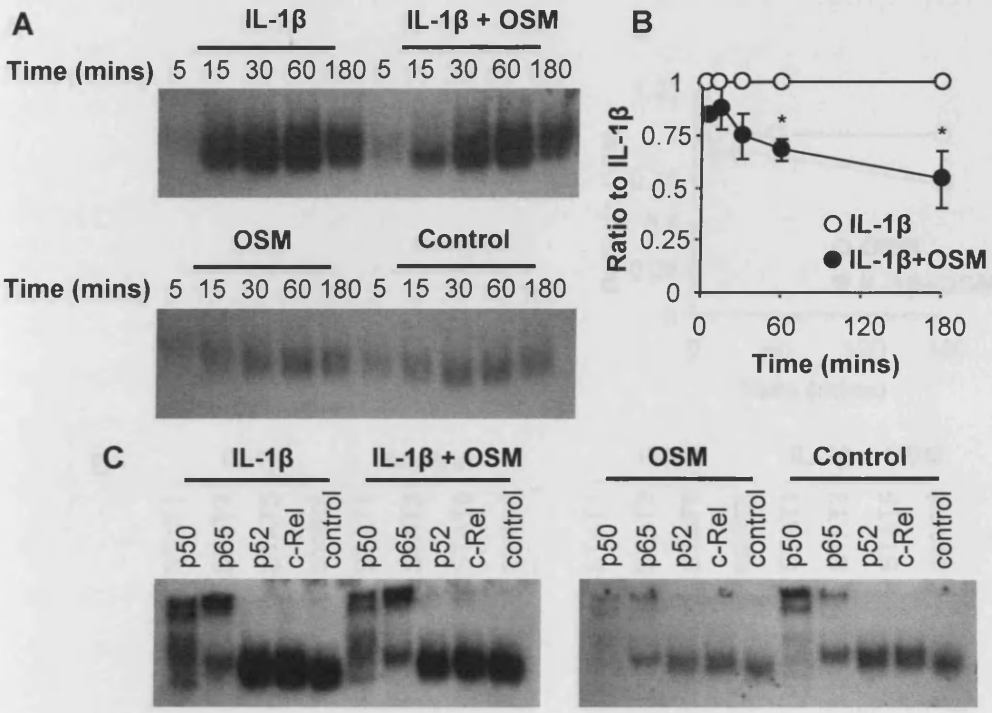

Fig 4.3.3. Temporal activation of NF-κB in nuclear extracts derived from HPMC incubated with IL-1β (50pg/ml) and OSM (30ng/ml) alone or in combination was monitored by EMSA (A). Alteration in NF-κB induction was quantified by densitometry by calculating the ratio of activation elicited by OSM and IL-1β, which was compared to activation with IL-1β alone (B). Composition of the NF-κB complex was verified by supershift analysis of nuclear extracts derived from HPMC cultures. Cells were stimulated for 30 minutes as indicated and supershift performed with antibodies against p50, p52, p65 and c-Rel subunits (C). Data is representative of mean +/- SEM of three individual primary isolates (*$p<0.05$).

Figure 4.3.4 OSM stimulation upregulates STAT activation in HPMC

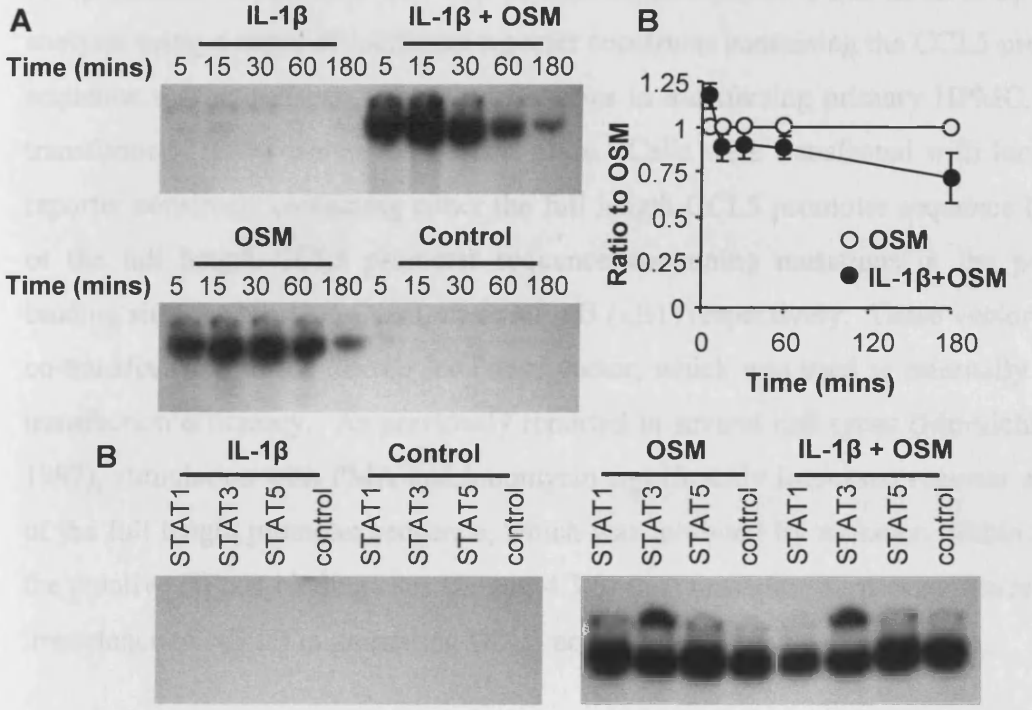

Fig 4.3.4. Temporal activation of STAT in nuclear extracts derived from HPMC incubated with IL-1β (50pg/ml) and OSM (30ng/ml) alone or in combination was monitored by EMSA (A). Alterations in STAT activation were quantified by densitometry by calculating the ratio of activation elicited by OSM and IL-1β, which was compared to activation with OSM alone (B). Composition of the STAT complex was verified by supershift analysis of nuclear extracts derived from HPMC. Cells were stimulated for 30 minutes as indicated and supershifts performed using antibodies against STAT1, STAT3 and STAT5 (C). Data is representative of mean +/- SEM of three individual primary isolates.

4.3.4 CCL5 promoter construct analysis

Studies into the molecular mechanism of CCL5 expression utilising CCL5 promoter sequences have demonstrated that NF-κB upregulates the promoter activity of CCL5 (Moruichi *et al*, 1997). Due to the observed OSM-mediated inhibition of IL-1β-induced NF-κB activation and parallel suppression of CCL5 in HPMC, further analysis using a series of luciferase reporter constructs containing the CCL5 promoter sequence was undertaken. Due to difficulties in transfecting primary HPMC, SV40 transformed HPMC were used in their place. Cells were transfected with luciferase reporter constructs containing either the full length CCL5 promoter sequence (R1.4), or the full length CCL5 promoter sequence containing mutations in the putative binding sites for NF-IL-6 (NFIL-6) or NF-κB (κB1) respectively. These vectors were co-transfected with the *Renilla* luciferase vector, which was used to internally assess transfection efficiency. As previously reported in several cell types (Moruichi *et al*, 1997), stimulation with PMA and ionomycin significantly increased reporter activity of the full length promoter sequence, which was inhibited by mutation within one of the putative NF-κB binding sites (Figure 4.3.5) thus providing further evidence of the importance of NF-κB in promoting CCL5 activity.

Previous work using CCL5 promoter constructs has also shown upregulation of CCL5 promoter activity in response to stimulation with IL-1β, which is inhibited by mutation within the κB1 site (Moruichi *et al*, 1997). However, IL-1β stimulation does not appear to significantly upregulate CCL5 promoter activity in SV40 transformed HPMC, when compared with unstimulated controls (Figure 4.3.6).

Figure 4.3.5 Effects of site-directed mutations of CCL5 promoter sequences on the promoter activity

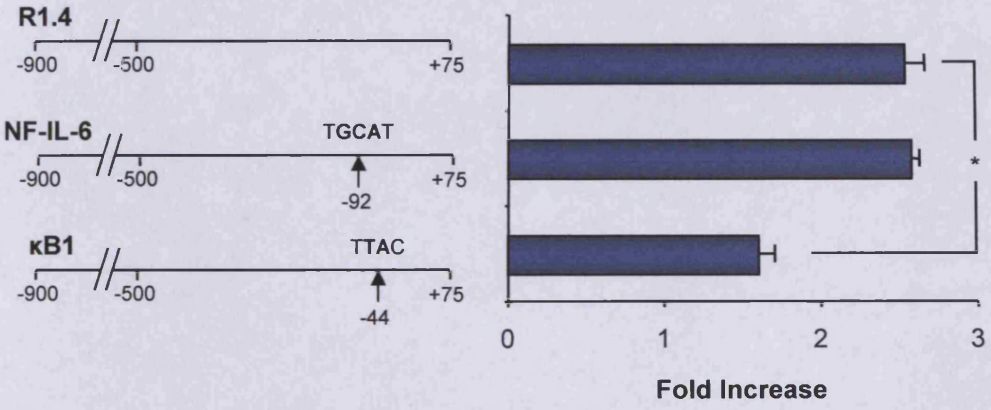

Fig 4.3.5. SV40 transformed HPMC were transfected overnight with 0.9μg of the indicated plasmid. Cells were either unstimulated or stimulated with PMA (15ng/ml) and ionomycin (1μM) for 24 hours. The fold increase of luciferase activity of stimulated compared to unstimulated cells was calculated. Data is representative of mean +/- SEM of three individual experiments performed in duplicate (*$p<0.05$).

Figure 4.3.6. The effect of IL-1β stimulation on CCL5 promoter activity

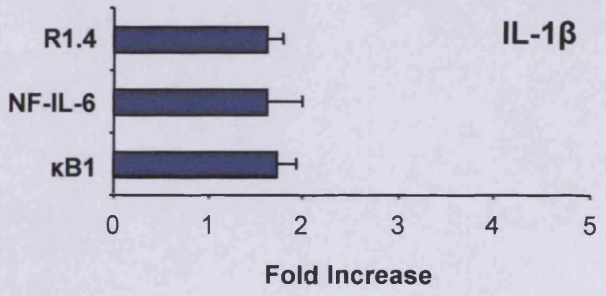

Fig 4.3.6. SV40 transformed HPMC were transfected overnight with 0.9μg of the indicated plasmid. Cells were either unstimulated or stimulated with IL-1β (1ng/ml) for 24 hours. The fold increase of luciferase activity of stimulated compared to unstimulated cells was calculated. Data is representative of mean +/- SEM of three individual experiments performed in duplicate.

4.3.5 OSM selectively regulates expression of IFN-γ-inducible chemokines

The ability of OSM to regulate expression of CCL5 suggests that in addition to aiding monocyte trafficking, OSM may also affect the trafficking of lymphocytes in particular lymphocytes with a Th1 phenotype. As previously mentioned, CCL5 utilises chemokine receptors present on a variety of mononuclear cells including Th1 cells, which express CCR5 (Loetscher *et al*, 1998). Th1 cell trafficking is also reliant on another group of chemokines which utilise CXCR3, also expressed on the surface of Th1 lymphocytes (Loetscher *et al*, 1998). This group of ELR^- CXC chemokines are reliant on induction by IFN-γ and include CXCL9, CXCL10 and CXCL11. HPMC can be induced to selectively express one of these chemokines, CXCL10, in response to OSM stimulation (Chapter 3, Figure 3.3.4). In addition, OSM was able to significantly inhibit IFN-γ-induced production of all three chemokines (Figure 4.3.7). These observations provide evidence that OSM, although unlikely to induce T cell trafficking, may play some accessory role in controlling the migration of certain T cell subsets, again this will require further study *in vivo*.

Figure 4.3.7 OSM inhibits IFN-γ-induced chemokine production

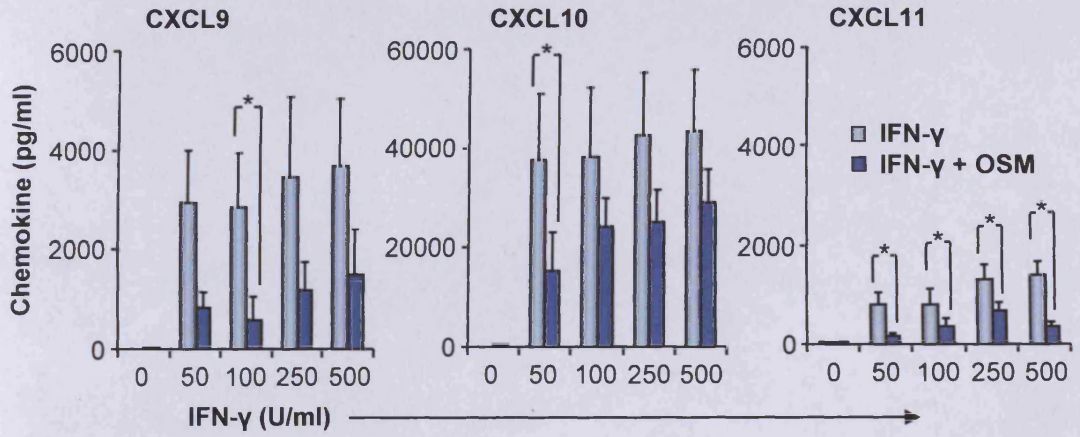

Fig 4.3.7. Growth arrested HPMC were stimulated with increasing doses of IFN-γ (0-500U/ml) alone or in combination with OSM (30ng/ml). Chemokine expression was quantified in the culture supernatant by ELISA. Data represents mean +/- SEM of three independent primary isolates (* $p<0.05$).

4.4 Discussion

Studies outlined in this Chapter demonstrate the ability of OSM to regulate IL-1β-mediated chemokine expression. Although both IL-6 and OSM suppress CXCL8 expression, OSM also drives suppression of IL-1β-mediated CCL5 production potentially through attenuation of NF-κB activation.

The role of IL-6 in the progression from innate to acquired immunity has been well documented (Jones, 2005). Both *in vitro* and *in vivo* studies utilising IL-6KO mice emphasise that IL-6 trans-signalling, aids the clearance of neutrophils by suppressing expression of the neutrophil chemoattractants CXCL1 and CXCL8 (Hurst *et al*, 2001; McLoughlin *et al*, 2004). As previously shown, OSM is also able to inhibit IL-1β-mediated CXCL8 expression, therefore suggesting a similar role for OSM in aiding resolution of the neutrophil influx. However, in direct contrast to IL-6, OSM also inhibits IL-1β-mediated expression of the mononuclear cell chemoattractant CCL5. CCL5 signals via several receptors including CCR1, CCR3 and CCR5 present on the surface of monocytes, eosinophils, basophils and activated T cells (Loetscher *et al*, 1998). Therefore the ability of OSM to control expression of CCL5 may affect mononuclear cells globally or be specific for certain subsets. This will be explored in more detail in Chapter 5. In addition OSM inhibits the IFN-γ-induced expression of CXCL9, CXCL10 and CXCL11, which signal via CXCR3 expressed on the surface of T cells again implying a potential role for OSM in governing chemokine-driven mononuclear cell trafficking.

Studies have outlined a potential mechanism for the OSM-mediated control of CCL5 expression through attenuation of NF-κB signalling. Parallel EMSA show no significant effect of IL-1β on OSM induced STAT activation. Supershift analysis of the DNA-protein complex confirms IL-1β mediates classical activation of NF-κB and suggests that OSM globally knocks down both the p50 and p65 subunits. It is mechanistically unclear whether this response results from co-operative manipulation of NF-κB signalling, competition for DNA binding within overlapping consensus sites for NF-κB and STAT factors, or NF-κB regulation via cytokine mediated increases in unphophorylated STAT3, all of which have been implicated in the gp130

control of NF-κB-mediated events (Zhang & Fuller, 1997; Yu *et al*, 2002; Yang *et al*, 2007).

Transcriptional studies using CCL5 reporter constructs will hopefully help to fully define the nature of the interaction between OSMRβ-mediated signalling and NF-κB activation. Initial studies outlined within this Chapter demonstrated the potential importance of NF-κB in promoting CCL5 activity. However, transcriptional studies were unsuccessful in response to IL-1β stimulation, therefore preventing further analysis. Although it is unclear why this was unsuccessful, it could potentially be due to the cells used for this experiment. SV40 transformed HPMC retain many of the characteristics of primary HPMC (Fischereder *et al*, 1997), however, the process of immortalisation is likely to result in alteration in the cellular responses to cytokine stimulation. Therefore these experiments would need to be performed in primary HPMC, but due to difficulties in transfecting these cells, this is not currently possible.

In vitro analysis of the regulatory abilities of OSM suggest its ability to govern chemokine-driven leukocyte trafficking. Although signalling through OSMRβ shares some functions with those previously attributed the IL-6-mediated gp130 homodimer activation, for example down-regulation of CXCL8, the ability of OSM to suppress the IL-1β-induced expression of CCL5 is clearly distinct from that of IL-6. This, taken in combination with the ability of OSM to regulate expression of the Th1 chemokines CXCL9, CXCL10 and CXCL11 suggests a role for OSM in governing mononuclear cell trafficking. The next chapter will utilise OSMRβ deficient mice to study the effects of OSMRβ-mediated signalling on leukocyte recruitment during episodes of acute peritoneal inflammation.

Chapter 5

OSMRβ-mediated control of leukocyte trafficking during acute inflammatory challenge

5.1 Introduction

Previous chapters have emphasised a potential role for OSM in governing leukocyte trafficking through the *in vitro* upregulation of mononuclear cell chemoattractants (CCL2), adhesion molecules (VCAM-1) and the control of IL-1β-directed expression of inflammatory chemokines. Using OSMRβ deficient (OSMR-KO) mice, this chapter aims to further examine the role of OSM-mediated signalling during an acute inflammatory response.

Signalling of IL-6-related cytokines is mediated through either gp130 homodimers, initiated by IL-6, IL-11 and IL-27 binding to non-signalling receptor subunits, or through gp130 heterodimerised with either LIFRβ or OSMRβ, which can be initiated by other IL-6-related cytokines including LIF, OSM, CT-1 and CNTF. Unlike human OSM, which can utilise both gp130:LIFRβ and gp130:OSMRβ receptor complexes, murine OSM has only one functional receptor complex comprised of gp130 and the selective OSMRβ, therefore, animals deficient in OSMRβ are unable to respond to OSM-mediated signalling (Tanaka *et al*, 1999).

In vitro analysis has highlighted functions for OSM in stromal cell activation, leukocyte recruitment and tissue injury (Wahl *et al*, 2001; Modur *et al*, 1997). In many instances, the effects of OSM are similar to those observed in response to the gp130 activating cytokine IL-6. However, due to the differences in signalling, with OSM utilising gp130 heterodimer complexes and IL-6 utilising gp130 homodimers, it would be expected that activation would result in distinct biological activities. In this respect, *in vitro* studies presented in Chapter 4 emphasise defined differences in the control of CCL5 by IL-6 and OSM. Indeed, distinct differences are evident in the control of haematopoiesis in OSMR-KO mice, when compared with IL-6KO, IL-11KO and LIFR-KO mice (Tanaka *et al*, 2003; Nandurkar *et al*, 1997; Escary *et al*, 1993). However, such differences are not well defined during inflammation.

Acute inflammation is characterised by an initial influx of neutrophils, which are later replaced by a more sustained population of mononuclear cells. The role of IL-6 in the progression of inflammation has been well characterised; IL-6, acting via its soluble receptor (IL-6 trans-signalling) is responsible for the switch from an innate to an

acquired response (Jones, 2005). Both *in vitro* and *in vivo* studies have demonstrated the ability of IL-6 to suppress chemokine-mediated neutrophil recruitment and concurrently promote neutrophil clearance in addition to preventing the apoptosis of T cells and upregulating production of mononuclear cell chemoattractants (Hurst *et al*, 2001; McLoughlin *et al*, 2005). The contribution of OSMRβ signalling in this immunological switch, however, has not been examined. This Chapter demonstrates OSM-mediated signalling is able to selectively control monocytic cell trafficking, but does not alter clearance and recruitment of neutrophils or lymphocytes. These results highlight a clear distinction between gp130 activation by OSMRβ and IL-6R during acute inflammation.

5.2 Materials and Methods

5.2.1 Generation of SES-induced acute peritoneal inflammation

All animal experiments were performed under Home Office License PPL-30/2269 on 6-12 week-old WT C57/Bl6 mice and OSMR-KO mice (see Section 2.3.1).

Peritoneal inflammation was induced using a cell-free supernatant derived from a clinical isolate of *Staphylococcus epidermidis* (SES) as described in Section 2.3.4. Experiments were performed on groups of 4-6 age-matched, mixed sex, WT and OSMR-KO mice. Peritoneal inflammation was induced by intraperitoneal injection of 500µl SES, groups of mice were sacrificed at designated intervals and the peritoneal cavity lavaged with 2ml ice-cold PBS. Peritoneal membrane sections were also collected for subsequent EMSA analysis.

5.2.2 Determination of leukocyte numbers by differential cell count

Leukocytes were isolated from the peritoneal lavage fluid and peripheral blood of mice either prior to or after induction of inflammation (Section 2.3.3). Cytospin slides were prepared and stained as described in Section 2.3.3. A total of 200 leukocytes were counted per slide, the percentage of each cell type was multiplied by the total number of cells isolated from the peritoneal cavity (as determined by Coulter count) to give the relative number of each leukocyte subset.

5.2.3 Flow cytometric analysis of peritoneal leukocytes

Peritoneal leukocytes were isolated (Section 2.3.3) and stained for expression of macrophage specific markers (F4/80 and CD11b) and CCR5 as outlined in Section 2.4.5. Peritoneal monocytic cells were identified and gated according to forward vs side scatter profiles as shown in Figure 5.2.1. To assess the relative cell number of each population, the percentage of events present in each gate was multiplied by the total number of peritoneal leukocytes isolated from the mouse. CCR5 expression was quantified on $F4/80^{+}CD11b^{+}$ monocytic cells by gating the upper right quadrant and assessing CCR5 expression on those cells. All flow cytometry data was compared with appropriate isotype controls.

Figure 5.2.1 Identification of 'resident-like' and 'inflammatory' peritoneal monocytes

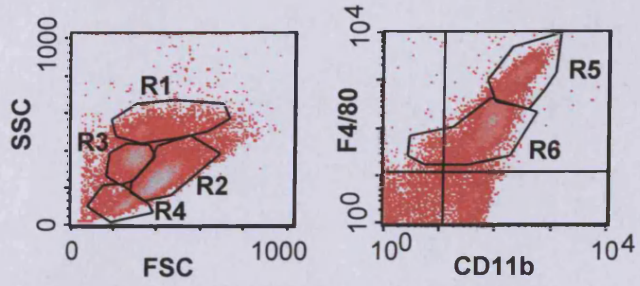

Fig 5.2.1. Murine peritoneal leukocyte subsets isolated in response to SES-induced peritoneal inflammation. Forward vs side scatter enables identification of individual leukocyte subsets (R1 – macrophages, R2 – monocytes, R3 – neutrophils, R4 – lymphocytes). CD11b and F4/80 staining was quantified on peritoneal monocytes (cells in gate R2). Two distinct populations are identified, F4/80^{high}CD11b^{high} 'resident-like' monocytes (R5) and F4/80^{low}CD11b^{+} 'inflammatory' monocytes (R6).

5.2.4 ELISA quantification of inflammatory mediators expressed in response to SES-induced peritoneal inflammation

Following SES-induced peritoneal inflammation, commercial ELISA was used to quantify expression of IL-1β, IL-6, CXCL1/KC, CCL2, CCL3, CCL4 and CCL5 in the peritoneal lavage fluid (Section 2.9). Levels of murine OSM were quantified using an ELISA system developed using goat anti-mouse OSM polyclonal IgG as a capture antibody and biotinylated goat anti-mouse OSM polyclonal IgG as a detection antibody (Section 2.9.1).

5.2.5 EMSA analysis of STAT and NF-κB activation in peritoneal membrane sections in response to SES-induced peritoneal inflammation

Nuclear extracts from peritoneal membrane sections were harvested following SES-induced peritoneal inflammation (Section 2.8.2). Prior to EMSA analysis, the protein concentration was determined as described in Section 2.8.3. EMSA using [α-^{32}P] dTTP labelled oligonucleotide probes containing consensus sequences for NF-κB and total STAT (SIE m67) were performed using 10μg of nuclear extract as described in Section 2.8.6.1. Supershift analyses were performed on the blank (time zero) and 6 hour time point groups of both WT and OSMR-KO mice using antibodies against individual transcription factor subunits (Section 2.8.6.2). Densitometry of the banding intensity was quantified for each time point as outlined in Section 2.8.7.

5.3 Results

5.3.1 OSMR-KO mice show increased monocytic cell trafficking following inflammatory challenge

To examine the role of OSMRβ signalling during acute inflammatory challenge, peritoneal inflammation was induced in WT and OSMRβ-deficient (OSMR-KO) mice using SES (Section 5.2.1). Prior to assessing the role of OSMRβ mediated signalling during an acute inflammatory response, baseline leukocyte numbers in both the peritoneal cavity and peripheral blood of OSMR-KO mice were assessed and shown to be comparable to WT mice (Figure 5.3.1).

As shown in Figure 5.3.2, administration of SES causes an initial influx of neutrophils into the peritoneal cavity that was later replaced by a more sustained population of lymphocytes and monocytes. Comparative analysis between WT and OSMR-KO mice demonstrated that an absence of OSMRβ signalling led to a significant increase in monocytic cell trafficking into the peritoneal cavity. However, deficiency in OSMRβ signalling did not appear to affect trafficking of neutrophils or lymphocytes. This is in contrast to the response exhibited by IL-6 deficient mice, which display increased neutrophil migration but no alteration in monocytic cell trafficking (Hurst *et al*, 2001; McLoughlin *et al*, 2003; McLoughlin *et al*, 2005; Hams *et al*, 2008).

The observed increase in monocyte trafficking was substantiated by flow cytometric analysis of $F4/80^{+}CD11b^{+}$ cells (Figure 5.3.3). Two distinct populations of monocytic cells were identified within the peritoneal cavity: resident-like $F4/80^{high}CD11b^{high}$ cells, which are the predominant cell type in the peritoneal cavity of mice prior to inflammatory challenge, and inflammatory $F4/80^{low}CD11b^{+}$ cells, which emerge following inflammatory activation. SES stimulation results in an exodus of the resident-like monocytes together with in influx of the inflammatory monocytes. However, a re-emergence of the resident-like monocytes was observed as inflammation progressed (24hrs post-induction). This pattern of monocytic cell trafficking was observed in both WT and OSMR-KO mice, however, both populations were significantly increased in OSMR-KO mice.

Figure 5.3.1 Leukocyte numbers are comparable between WT and OSMR-KO animals prior to inflammatory challenge

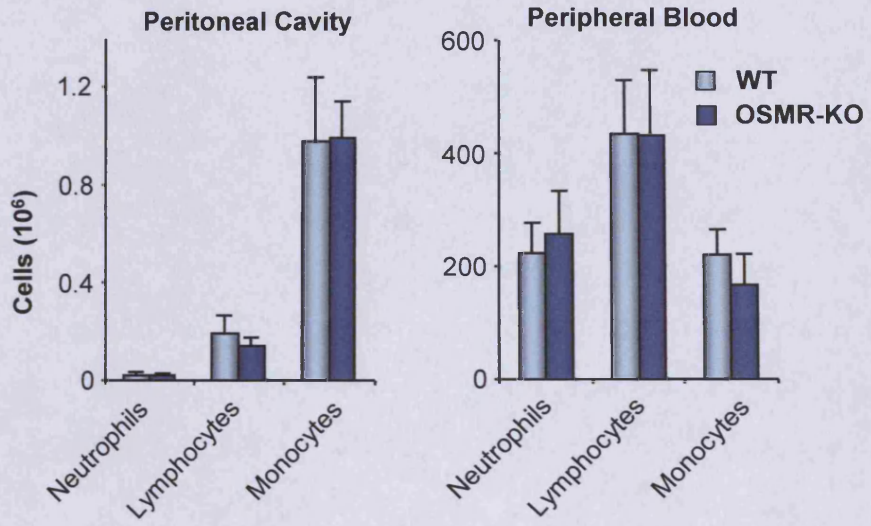

Fig 5.3.1. Baseline leukocyte numbers in WT and OSMR-KO mice. Differential cell counts were performed on peripheral blood and peritoneal leukocytes isolated from healthy animals. Data represents mean +/- SEM of four WT and four OSMR-KO animals.

Figure 5.3.2 OSMR-KO mice show increased monocyte infiltration during SES-induced peritoneal inflammation

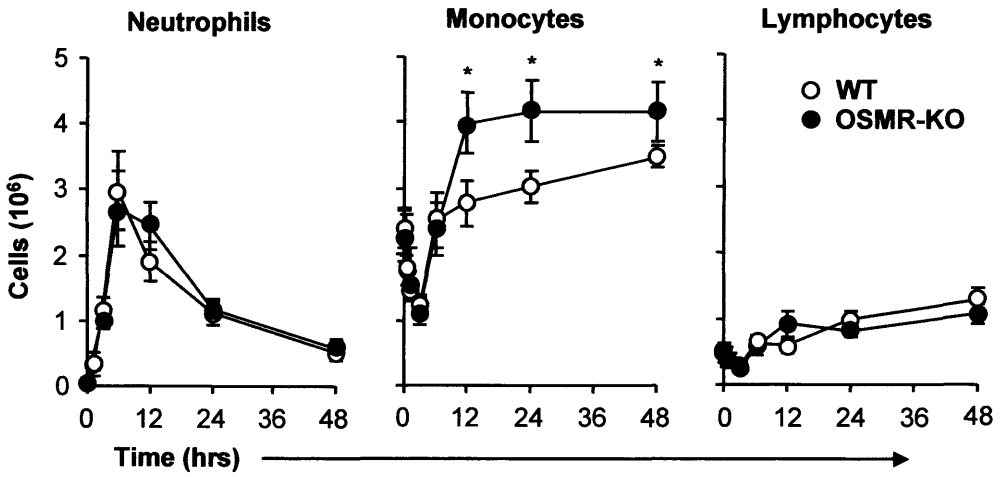

Fig 5.3.2. Peritoneal inflammation was induced in WT and OSMR-KO animals by i.p. injection of SES, mice were sacrificed at designated time points and differential cell counts performed on peritoneal leukocytes isolated from peritoneal lavage fluid. Data represents mean ± SEM of twelve WT and twelve OSMR-KO mice for each time point (*$p<0.05$).

Figure 5.3.3 OSMR-KO mice show significantly increased monocyte trafficking during acute inflammation

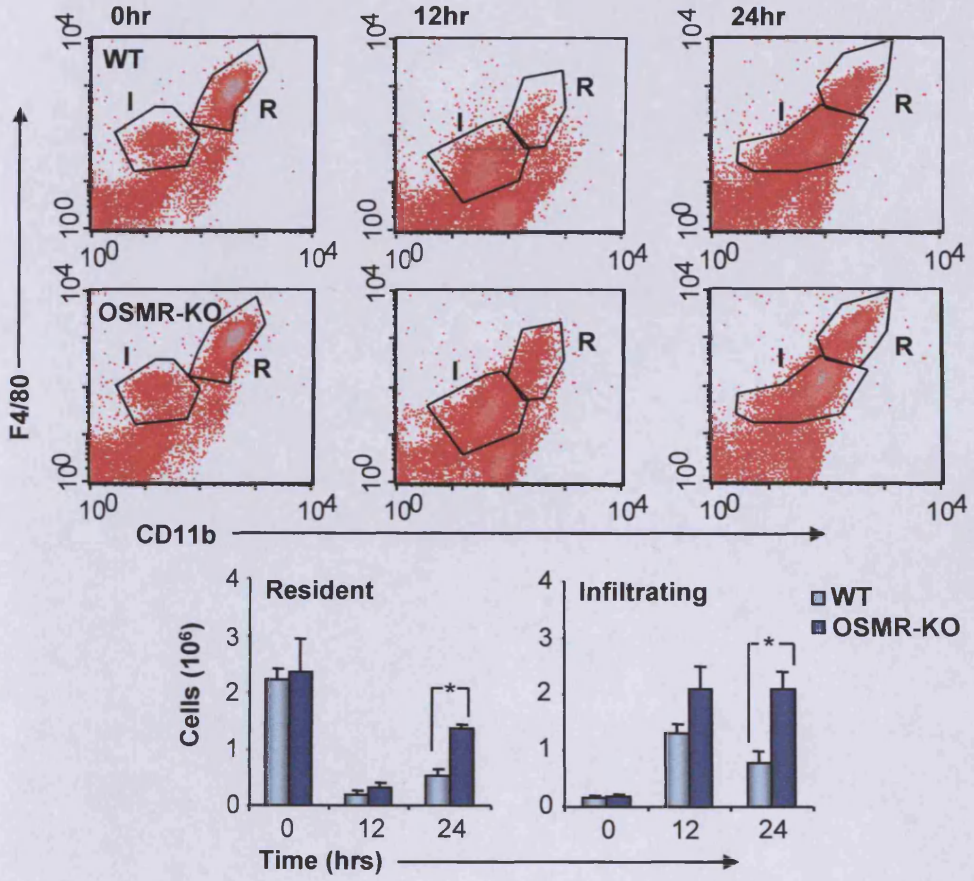

Fig 5.3.3. SES-induced peritoneal inflammation was established in WT and OSMR-KO mice. At designated intervals FACS analysis was performed on leukocytes lavaged from the peritoneal cavity, using antibodies against F4/80 and CD11b. Representative flow cytometry plots for WT and OSMR-KO mice together with temporal changes in the number of resident-like F4/80^{high}CD11b^{high} monocytic cells (R) and infiltrating F4/80^{low}CD11b^{+} monocytic cells (I) are shown. Data represents mean +/- SEM of four WT and four OSMR-KO mice for each time point (*p<0.05).

5.3.2 OSMRβ mediated signalling selectively controls expression of inflammatory chemokines

OSM has previously been detected in the peritoneal fluid of patients with clinical peritonitis, the source of which was shown to be infiltrating neutrophils (Hurst *et al*, 2002). Analysis of OSM in the peritoneal cavity of WT and OSMR-KO mice following SES-induced peritoneal inflammation showed that the initial increase of OSM corresponds with the neutrophil influx and OSM levels are comparable between WT and OSMR-KO mice (Figure 5.3.4). Expression of IL-1β and IL-6, two principal regulators of inflammatory chemokines, were determined in response to SES-induced inflammation. Expression of both increased at between 1 and 3 hours post inflammatory induction, with levels comparable between WT and OSMR-KO mice, implying that any observed effect of OSM was not due to an aberrant control of these cytokines (Figure 5.3.5).

To evaluate the ability of OSMRβ signalling to control expression of inflammatory chemokines, CXCL1, CCL2 and the CCR5 ligands CCL3, CCL4 and CCL5 were quantified in response to SES-induced inflammatory challenge. Expression of CCL5 was significantly increased in OSMR-KO mice in response to SES challenge; however, CCL3 and CCL4 remained consistent between WT and OSMR-KO mice (Figure 5.3.6). Studies presented in Chapter 3 highlighted the *in vitro* control of CCL2 by OSM. However, no significant difference in CCL2 was observed between WT and OSMR-KO mice (Figure 5.3.6). Expression of CXCL1/KC was also significantly decreased in OSMR-KO mice in comparison to WT mice (Figure 5.3.7), however as previously mentioned, no corresponding alteration in neutrophil trafficking was observed.

Figure 5.3.4 OSM production in the peritoneal cavity is comparable between WT and OSMR-KO mice

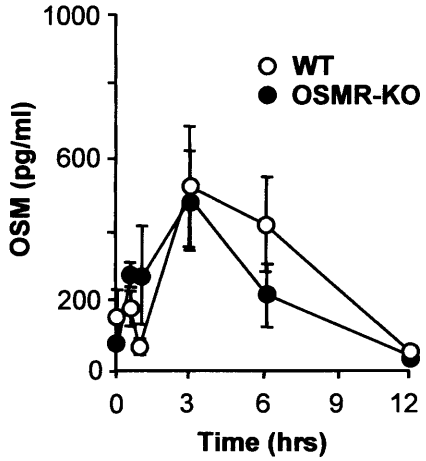

Fig 5.3.4. SES-induced inflammation was established in WT and OSMR-KO mice, and OSM levels in peritoneal lavage fluid quantified using ELISA. Data represents mean ± SEM of fifteen WT and fifteen OSMR-KO mice.

Figure 5.3.5 The temporal expression of IL-1β and IL-6 is unaltered in OSMR-KO mice

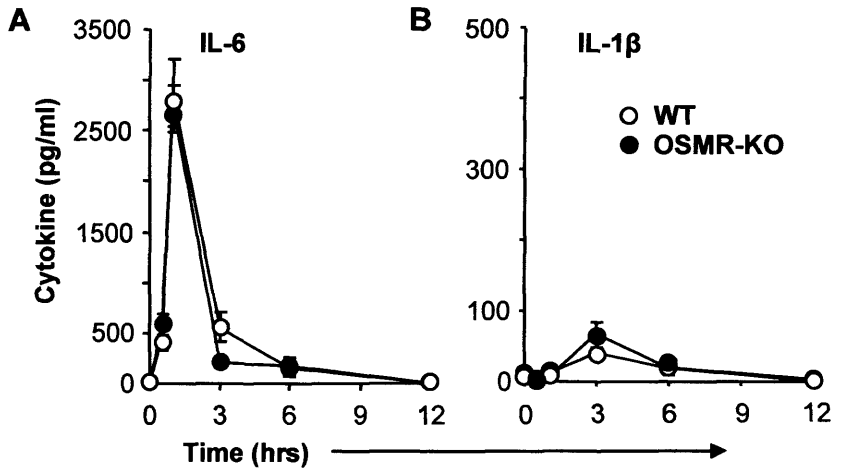

Fig 5.3.5. SES-induced inflammation was established in WT and OSMR-KO mice. Levels of IL-6 (A) and IL-1β (B) in peritoneal lavage fluid were quantified using ELISA. Data represents mean ± SEM of twelve WT and twelve OSMR-KO mice.

Figure 5.3.6 OSMR-KO mice display a selective increase in CCL5 expression following inflammatory activation

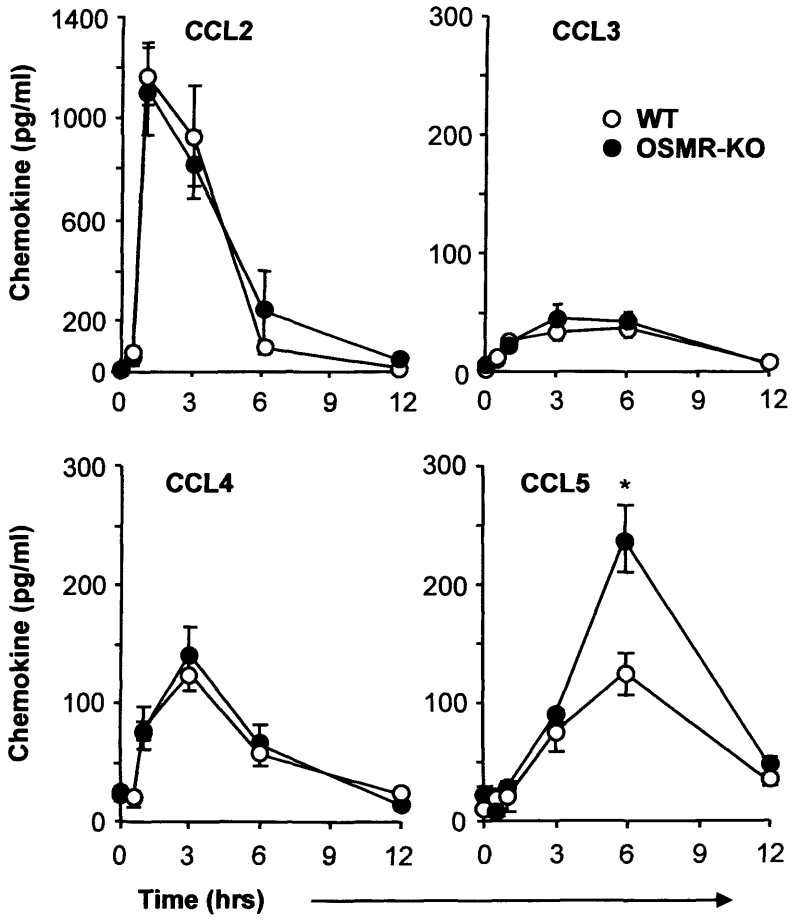

Fig 5.3.6. SES-induced inflammation was established in WT and OSMR-KO mice. ELISA was used to quantify CCL2, CCL3, CCL4 and CCL5 levels in peritoneal lavage fluid at designated time points. Data represents the mean ± SEM of twelve WT and twelve OSMR-KO mice (*$p<0.05$).

Figure 5.3.7 OSMR-KO mice show decreased CXCL1/KC expression in response to inflammatory challenge

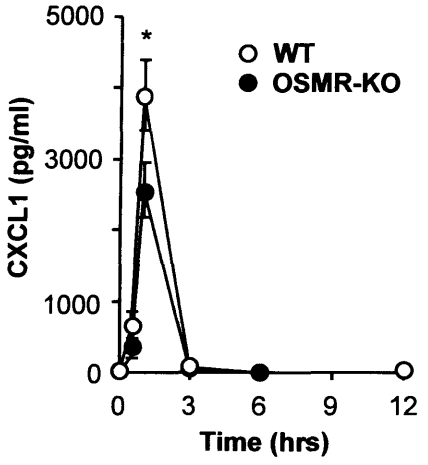

Fig 5.3.7. SES-induced inflammation was established in WT and OSMR-KO mice. ELISA was used to quantify levels of CXCL1/KC in the peritoneal lavage fluid at designated time points. Data represents mean +/- SEM of twelve WT and twelve OSMR-KO mice (*p<0.05).

5.3.3 OSMRβ signalling does not alter expression of the inflammatory chemokine receptor CCR5

To determine whether the observed increase in CCL5 corresponds to increased monocytic expression of CCR5, flow cytometric analysis was performed on isolated peritoneal leukocytes after SES-induced inflammation. Prior to the induction of inflammation, resident-like $F4/80^{high}CD11b^{high}$ monocytes are either $CCR5^{low}$ or $CCR5^{null}$. During the progression of inflammation, $F4/80^{+}CD11b^{+}$ monocytes increase expression of CCR5, implying that activation of monocytes induces expression of CCR5. However, no difference in CCR5 expression was observed between WT and OSMR-KO mice (Figure 5.3.8).

Figure 5.3.8 CCR5 expression is comparable on peritoneal monocytes of WT and OSMR-KO mice

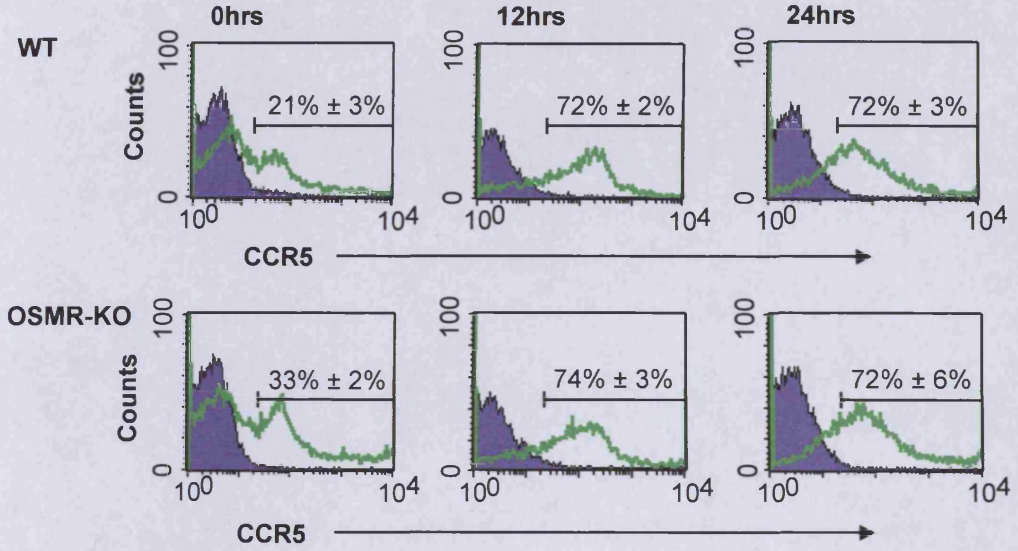

Fig 5.3.8. SES-induced peritoneal inflammation was established in WT and OSMR-KO mice. At designated intervals FACS analysis was performed on the peritoneal leukocyte population using antibodies against F4/80, CD11b and CCR5. Monocytes were identified and gated through cell surface expression of F4/80 and CD11b. CCR5 expression (—) on monocytes is compared to corresponding isotype controls (■). Data is representative of mean +/- SEM of four WT and four OSMR-KO mice.

5.3.4 OSMR-KO mice demonstrate decreased STAT activation

As previously discussed, OSM signalling predominantly activates the Jak-STAT pathway, through association of Jak subunits with the OSMRβ:gp130 receptor complex. Therefore mice deficient in OSMRβ would be expected to demonstrate decreased STAT signalling. Analysis of SES-induced STAT activation in nuclear extract isolated from peritoneal membranes of OSMR-KO and WT mice demonstrated decreased peritoneal STAT activation in OSMR-KO mice (Figure 5.3.9). Supershift analysis of the STAT/DNA complex showed evidence of STAT3, and to a lesser extent STAT1. Prior studies have shown that OSM-mediated signalling can induce STAT5 activation (Heinrich *et al*, 1998), however, this was not seen in this experiment. The decreased STAT activation observed in OSMR-KO mice was not however specific for an individual STAT transcription factor, but globally affected both STAT1 and STAT3 activation.

Figure 5.3.9 OSMR-KO mice show decreased STAT activation

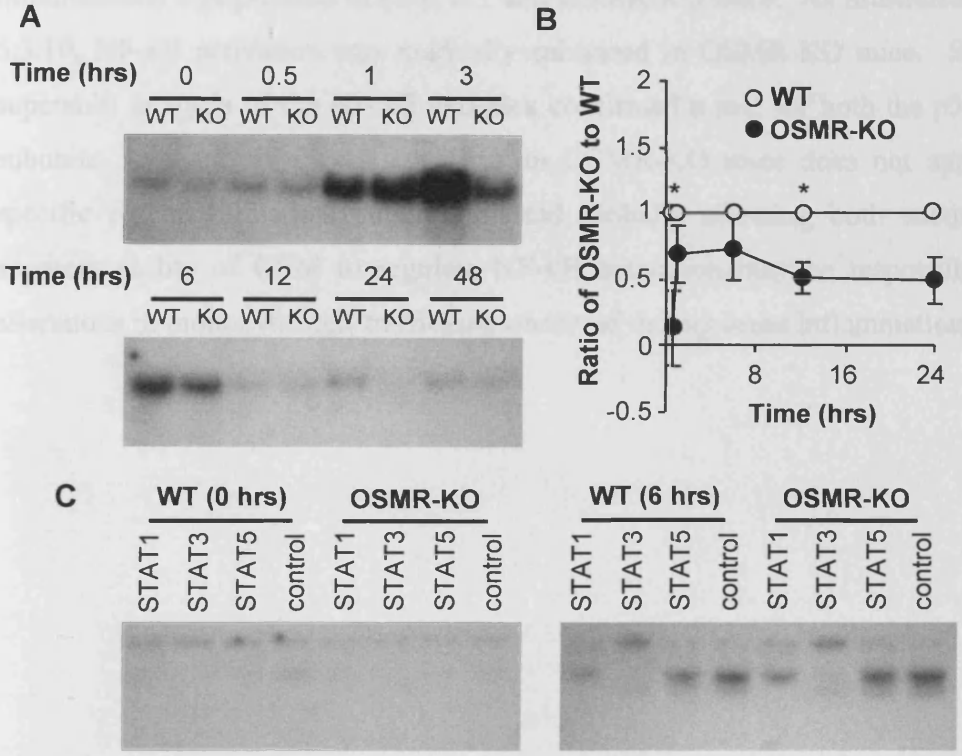

Fig 5.3.9. Temporal activation of STAT signalling in nuclear extracts derived from peritoneal membranes of WT and OSMR-KO mice was monitored by EMSA following SES activation (A). Alterations in STAT activation were quantified by densitometry by calculating the ratio of activation elicited in OSMR-KO mice, which was compared to activation in WT mice (B). Composition of the STAT complex was verified by supershift analysis of nuclear extracts isolated from both non-challenged mice and mice exposed to SES for 6 hours using antibodies against STAT1, STAT3 and STAT5 subunits (C). Data is representative of three WT and three OSMR-KO mice (*$p<0.05$).

5.3.5 OSMR-KO mice demonstrate altered NF-κB activity

As discussed in Chapter 4, EMSA analysis of nuclear extracts isolated from HPMC highlight the potential for OSM to block NF-κB activation (Section 4.3.4). To substantiate these observations, NF-κB activation following SES-induced inflammation was profiled in both WT and OSMR-KO mice. As illustrated in Figure 5.3.10, NF-κB activation was markedly enhanced in OSMR-KO mice. Subsequent supershift analysis of the NF-κB complex confirmed a role for both the p50 and p65 subunits. Enhanced NF-κB signalling in OSMR-KO mice does not appear to be specific for an individual subunit, instead globally affecting both subunits. The apparent ability of OSM to regulate NF-κB activation may be responsible for the alterations in monocytic cell trafficking observed during acute inflammation.

Figure 5.3.10 OSMR-KO mice show increased NF-κB activation

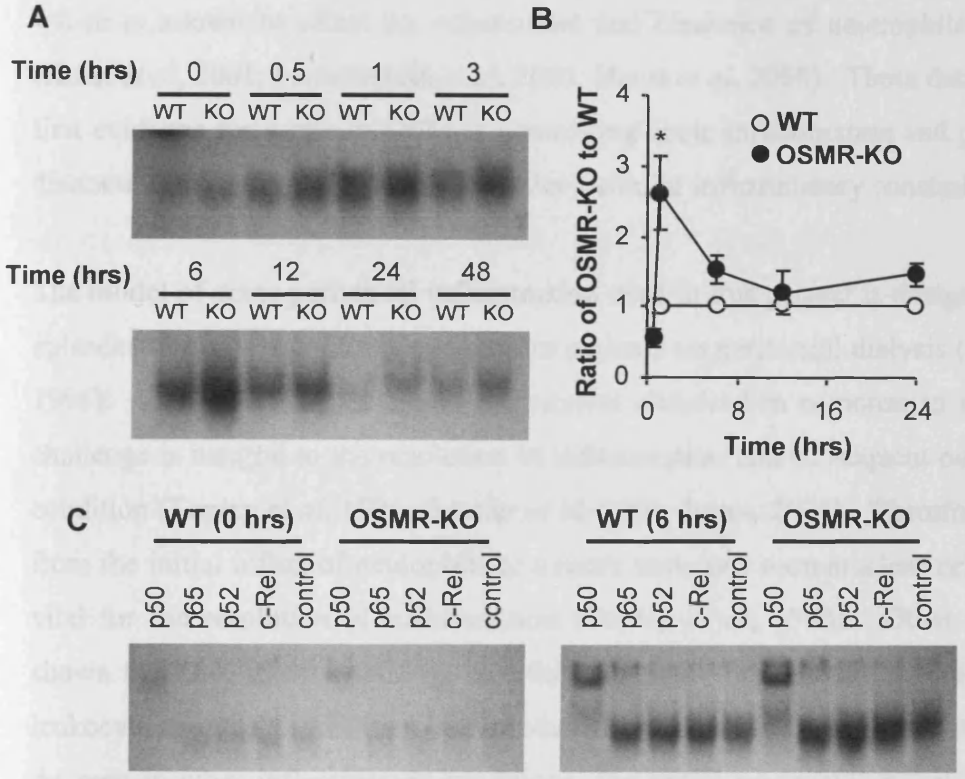

Fig 5.3.10. Temporal activation of NF-κB in nuclear extracts derived from peritoneal membranes of WT and OSMR-KO mice was monitored by EMSA following SES activation (A). Alterations in NF-κB activation were quantified by densitometry by calculating the ratio of activation elicited in OSMR-KO mice, which was compared to activation in WT mice (B). Composition of the NF-κB complex was verified by supershift analysis of nuclear extracts isolated from both non-challenged mice and mice exposed to SES for 6 hours using antibodies against p50, p52, p65 and c-Rel subunits (C). Data is representative of three WT and three OSMR-KO mice (*$p<0.05$).

5.4 Discussion

Studies outlined in this Chapter suggest that OSMRβ signalling selectively affects the inflammatory trafficking of monocytic cells. This is distinct from the action of IL-6, which is known to affect the recruitment and clearance of neutrophils and T cells (Hurst *et al*, 2001; McLoughlin *et al*, 2005; Hams *et al*, 2008). These data provide the first evidence for a role to OSM in controlling acute inflammation and point to clear distinction from the activity of IL-6 under a similar inflammatory constraint.

The model of acute peritoneal inflammation used in this project is designed to profile episodes of clinical peritonitis as seen in patients on peritoneal dialysis (Topley *et al*, 1996). The pattern of leukocyte recruitment observed in response to inflammatory challenge is integral to the resolution of inflammation and subsequent outcome of the condition (Topley *et al*, 1996; Beutler *et al*, 2004; Jones, 2005). Therefore the switch from the initial influx of neutophils to a more sustained mononuclear cell infiltrate is vital for the resolution of inflammation (Topley *et al*, 1996). Other studies have shown that IL-6 trans-signalling, aids this transition through differentially regulating leukocyte apoptosis and chemokine-mediated leukocyte trafficking (Hurst *at al*, 2001). As seen in other inflammatory conditions, the initial neutrophil influx is associated with an increase in the expression of a variety of inflammatory cytokines (Kasama *et al*, 2005; Corvol *et al*, 2003). Previous studies have shown that neutrophils are the primary source of sIL-6Rα (McLoughlin *et al*, 2004; Jones *et al*, 1999; Hurst *et al*, 2001; Marin *et al*, 2002). Clinical evaluation of OSM in acute peritonitis has illustrated a direct correlation between OSM levels and neutrophil numbers (Hurst et al, 2002). In this present study we demonstrate a similar trend with the profile of OSM closely resembling the neutrophil influx.

Previous *in vitro* analysis on HPMC demonstrate significant similarities between IL-6 and OSM-mediated signalling. However, as outlined in this Chapter, there appears to be a hierarchy in the ability of each cytokine to control the *in vivo* expression of inflammatory chemokines and to affect recruitment of leukocytes. IL-6-mediated signalling may override the ability of OSM to affect neutrophil clearance, whereas OSMRβ-mediated signalling selectively governs monocytic cell recruitment. This could potentially explain the ability of OSM to regulate expression of neutrophil

activating chemokines both *in vitro* and *in vivo*, but lack of effect on neutrophil clearance observed in response to acute inflammatory challenge. In this respect, we show a marginal effect of OSM on CXCL1/KC expression, yet it is unclear whether OSM or IL-6 control of other neutrophil activating chemokines (for example MIP-2/ CXCL8 or CXCL5) could affect this outcome (Hurst *et al*, 2001; Modur *et al*, 1997; McLoughlin *et al*, 2004).

The OSMRβ-mediated control of monocytic cell trafficking appears to centre on the ability of OSM to control expression of CCL5. Previous *in vitro* analyses have demonstrated than OSM can suppress the IL-1β induced expression of CCL5 by HPMC, whilst CCL5 expression in response to SES-induced inflammation is significantly increased in OSMRβ deficient mice. This is in direct contrast to that of IL-6, which is unable to promote expression of CCL5 *in vitro* or *in vivo* (McLoughlin *et al*, 2005; Hams *et al*, 2008). However, IL-6 represents a principle regulator of CCL2 *in vivo*, a chemokine upregulated by OSM *in vitro* but not affected by a lack of OSM-mediated signalling *in vivo* (Hams *et al*, 2008). Despite the ability of IL-6 to regulate expression of CCL2, no significant alteration in monocytic cell recruitment has been observed in IL-6KO mice (Hams *et al*, 2008; McLoughlin *et al*, 2005; Nowell *et al*, 2008 submitted). In this respect, CCL2 may predominantly affect T cell trafficking since IL-6KO mice display impaired T cell migration in acute inflammation (McLoughlin *et al*, 2005). Such findings highlight functional differences in the gp130-mediated control of the mononuclear cell chemoattractants CCL2 and CCL5. Studies outlined herein infer an important role for OSMRβ-mediated control of CCL5 in selectively governing monocytic cell recruitment. However, further work would be required to evaluate the potential role of other chemokines and adhesion molecules involved in monocytic cell recruitment before confirming OSM-mediated control of CCL5 as the primary mechanism orchestrating the control of monocytic cell recruitment.

Studies examining differential cellular changes in expression of inflammatory chemokine receptors provide evidence for the potential importance of CCL5 in governing monocytic cell recruitment. For example, monocytic cell CCR2 expression, one of the receptors for CCL2, defines a population of monocytes actively recruited to sites of inflammation. However, terminally differentiated macrophages lose

expression of CCR2, whilst increasing expression of the CCL5 receptors CCR1 and CCR5 (Fantuzzi *et al*, 1999; Kaufmann *et al*, 2001). The gp130-activating cytokines IL-6, LIF and OSM have been shown to regulate various aspects of monocyte differentiation, including macrophage formation, induction of receptors for G-CSF and CM-CSF and the activation and expansion of dendritic cells (Tanigawa *et al*, 1995; Clutterbuck *et al*, 2000; Chomarat *et al*, 2000; Jenkins *et al*, 2004). However, these activities are not universally regulated by all three gp130-activating cytokines (Tanigawa *et al*, 1995), suggesting OSMRβ-mediated signalling may have a unique role in monocytic cell trafficking.

OSM-mediated control of monocytic cell trafficking appears to rely on the ability of OSM to regulate NF-κB signalling. Work in Chapter 4 demonstrated the regulation of IL-1β-induced NF-κB activation by OSM. Such signalling interplay may be responsible for the OSM regulation of inflammatory chemokine production *in vivo*. Nuclear extracts from the peritoneal membrane of OSMR-KO mice showing significantly increased NF-κB activation as compared to WT animals. Comparative analysis using OSMR-KO and IL-6KO mice imply the regulation of NF-κB activation is specific for OSMRβ-mediated signalling and is not regulated by gp130-homodimeric receptor complexes (Hams *et al*, 2008). As expected, OSMR-KO mice show impaired STAT signalling due to the lack of gp130:OSMRβ heterodimers. However, significant decreases in STAT-activated chemokines such as CCL2 were not observed, again suggesting a hierarchy of gp130-activating cytokines. It would therefore be important to monitor IL-6-mediated STAT signalling in OSMR-KO mice.

Studies outlined in this Chapter highlight a unique role for OSMRβ-mediated signalling in controlling monocytic cell recruitment during acute inflammation. Regulation of this response was associated with a signalling interplay between OSM and NF-κB. However, it remains unclear whether these signalling mechanisms underpin the inflammatory control of chemokines including CCL5. Further transcriptional studies would be required to fully define the nature of this interaction. The next aim is to investigate whether these activities observed during acute inflammation impact progression of a chronic inflammatory state. This may have significant bearing on the potential modulation of OSMRβ signalling for therapeutic benefit.

Chapter 6

Defining a role for OSMRβ-mediated signalling in recurrent inflammatory challenge and tissue injury

6.1 Introduction

Studies outlined in Chapters 3-5 have demonstrated the ability of OSM both *in vitro* and *in vivo* to regulate chemokine-mediated leukocyte trafficking during acute inflammation. Using repeated episodes of inflammation (Section 6.2.1) to simulate the consequence of recurrent bouts of peritonitis, this Chapter aims to gauge whether OSM-mediated control of inflammation becomes modified in more progressive inflammatory processes.

As mentioned previously, leukocyte recruitment in acute inflammation is characterised by an initial influx of neutrophils, which are subsequently replaced by populations of inflammatory monocytes and T cells (Jones, 2005). Inappropriate regulation of leukocyte recruitment can lead to impaired neutrophil clearance and increased tissue damage due to the accumulation and retention of activated leukocytes at the site of immunological challenge including inflammatory bowel disease, sepsis, renal injury and chronic peritonitis (Brannigan *et al*, 2000; Brown *et al*, 2006; Heinzelmann *et al*, 1999).

Previous studies have illustrated a distinction in the role IL-6-related cytokines perform during acute and chronic inflammation. For example, although IL-6 is integral for the resolution of acute inflammation, its activities give rise to more detrimental consequences during more progressive inflammatory conditions. For example, in rheumatoid arthritis IL-6 has been shown to induce proliferation of synovial fibroblasts and promote cartilage damage by decreasing aggrecan and collagen II production in chondrocytes (Mihara *et al*, 1995; Legendre *et al*, 2003). These deleterious effects of IL-6-mediated signalling have been confirmed *in vivo*, where IL-6 deficient mice are protected against development of chronic inflammatory conditions including collagen-induced arthritis and colitis (Alonzi *et al*, 1997; Yamamoto *et al*, 2000; Kallen, 2002). In addition to these observations, the therapeutic use of an antibody against IL-6 receptor (Tocilizumab, Actemra) has proven effective in the treatment of rheumatoid arthritis, Castleman's disease and Crohn's disease (Nishimoto *et al*, 2003; Nishimoto *et al*, 2005; Ito *et al*, 2004).

However, the role of IL-6-related cytokines in chronic inflammatory conditions appears to be dependent on the particular gp130-activating cytokine. *In vivo* studies using experimental arthritis have suggested that IL-6 is the primary gp130-activating cytokine governing these inflammatory processes as both IL-11Rα- and OSMRβ-deficient mice develop disease hallmarks comparable with those observed in WT mice (Wong *et al*, 2006). However, OSM has also been implicated in tissue damage associated with recurrent inflammation, including epithelial-to-mesenchymal transition (Bamber *et al*, 1998; Goren *et al*, 2006; Nightingale *et al*, 2004; Pollack *et al*, 1997), which could be of potential significance in the development of peritoneal fibrosis (Masunaga *et al*, 2003; Kalluri & Neilson, 2003; Topley *et al*, 1996; Lai *et al*, 2000). However, it is unclear whether these activities are disease (tissue) specific, whilst the coordination of these responses in disease is ill-defined.

This Chapter will use recurrent episodes of SES-induced peritoneal inflammation, with an aim to simulate the progression of chronic peritoneal inflammation and fibrosis as is associated with recurrent episodes of bacterial peritonitis. Using repeated intraperitoneal introduction of SES at weekly intervals allows for clearance of the resulting leukocyte infiltrate between episodes thereby simulating the effect of repeated episodes of clinical peritonitis, and allowing the potential role for OSM-mediated signalling in the progression of chronic inflammatory conditions to be examined.

6.2 Materials and Methods

6.2.1 Generation of SES-induced acute and chronic peritoneal inflammation

All animal experiments were performed under Home Office License PPL-30/2269 on 6-12 week-old WT C57/Bl6 mice and OSMR-KO mice (see Section 2.3.1).

Peritoneal inflammation was induced using a cell-free supernatant derived from a clinical isolate of *Staphylococcus Epidermidis* (SES) as described in Section 2.3.4. Experiments were performed on groups of 4-5 age-matched, mixed sex, WT and OSMR-KO mice. Recurrent peritoneal inflammation was induced by four sequential intraperitoneal injections of 500µl SES at 7 day intervals. After the final injection groups of five WT and four OSMR-KO mice were sacrificed at designated time intervals and peritoneal membrane sections and lavage fluids obtained. In addition, a group of WT and OSMR-KO mice were treated with four recurrent doses of SES (as described above) and left for 21 days after the final injection. At which time peritoneal membranes were taken and prepared for histological analysis (Section 2.3.4). Inflammatory parameters and leukocyte recruitment was monitored as outlined in Chapter 5.

Figure 5.2.1 Flow diagram of recurrent inflammation model procedure

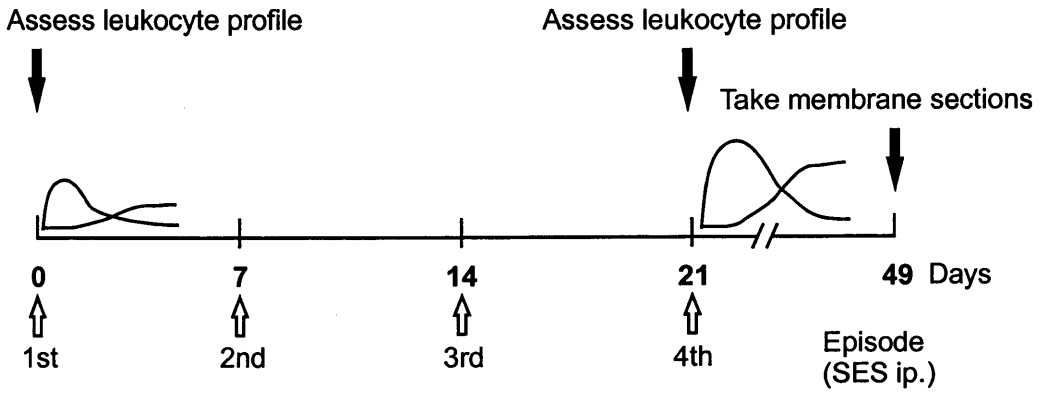

Fig 5.2.1. To simulate recurrent peritoneal inflammation, groups of WT and OSMR-KO mice received four ip injections of SES at weekly intervals. Peritoneal leukocytes were sampled after the first and fourth episodes of inflammation. Peritoneal membrane sections were recovered from mice that had received 4 episodes of inflammation, 49 days after the initial SES administration.

6.3 Results

6.3.1 Recurrent episodes of peritoneal inflammation heightens the increase in monocytic cell infiltration seen in OSMR-KO mice

Under basal conditions in both WT and OSMR-KO mice, the peritoneal leukocyte population consists predominantly of mononuclear cells. However, comparative analysis of neutrophil, monocytic cell and lymphocyte numbers in the peritoneal cavity prior to the induction of a fourth SES challenge shows a significant increase in the retention of neutrophils in addition to mononuclear cells. Moreover a direct comparison between WT and OSMR-KO mice showed increased retention of lymphocytes in the peritoneal cavity of WT mice (Figure 6.3.1, Table 6.3.1). The pattern of leukocyte recruitment associated with repeated episodes of peritoneal inflammation was also characterised by an initial influx of neutrophils, subsequently replaced by a mononuclear cell infiltrate (Figure 6.3.1). However, leukocyte trafficking was significantly enhanced after four inflammatory episodes.

Table 6.3.1 WT and OSMR-KO mice show increased retention of leukocytes within the peritoneal cavity after repeated episodes of inflammation

	Neutrophils ($x10^6$)			Macrophages ($x10^6$)			Lymphocytes ($x10^6$)		
	WT	OSMR-KO	*P*	WT	OSMR-KO	*P*	WT	OSMR-KO	*P*
Episode 1	0.06±0.04	0.01±0.01	0.12	2.32±0.17	2.44±0.35	0.38	0.47±0.09	0.33±0.06	0.14
Episode 4	0.50±0.46	0.44±0.22	0.45	4.24±0.29	4.86±1.28	0.33	1.72±0.41	0.93±0.16	0.06
P	0.19	0.07		0.00	0.07		0.01	0.01	

Table 6.3.1. Peritoneal leukocytes were collected from WT and OSMR-KO mice prior to the first and fourth episode of SES-induced peritoneal inflammation. The relative composition of the resident population was compared by differential cell count. Data represents mean ± SEM of five WT and four OSMR-KO mice.

Studies outlined in Chapter 5 demonstrated that during a single acute inflammatory episode, an absence of OSMRβ signalling significantly enhances monocytic cell recruitment into the peritoneal cavity, whilst appearing not to alter trafficking of either neutrophils or lymphocytes (Chapter 5, Figure 5.3.1). To examine whether recurrent sequential bouts of inflammation modified this response profile, leukocyte

recruitment was compared in mice that received a single dose of SES and mice that had received four repeated SES challenges (7 days apart). Consistent with the data presented in Chapter 5, OSMR-KO mice showed significantly increased monocytic cell recruitment when compared with WT mice both after one and four episodes of SES-induced inflammation (Figures 6.3.1 and 6.3.2).

Flow cytometric analysis of $F4/80^{+}CD11b^{+}$ cells, confirmed significant increases in both the 'resident-like' $F4/80^{high}CD11b^{high}$ cells and 'inflammatory' $F4/80^{low}CD11b^{+}$ cells in OSMRβ deficient mice (Figure 6.3.2). The magnitude of the inflammatory response was significantly enhanced in both murine strains following four episodes of inflammation (Figure 6.3.2).

Figure 6.3.1 Leukocyte infiltration into the peritoneal cavity of WT and OSMR-KO mice following acute and recurrent inflammation

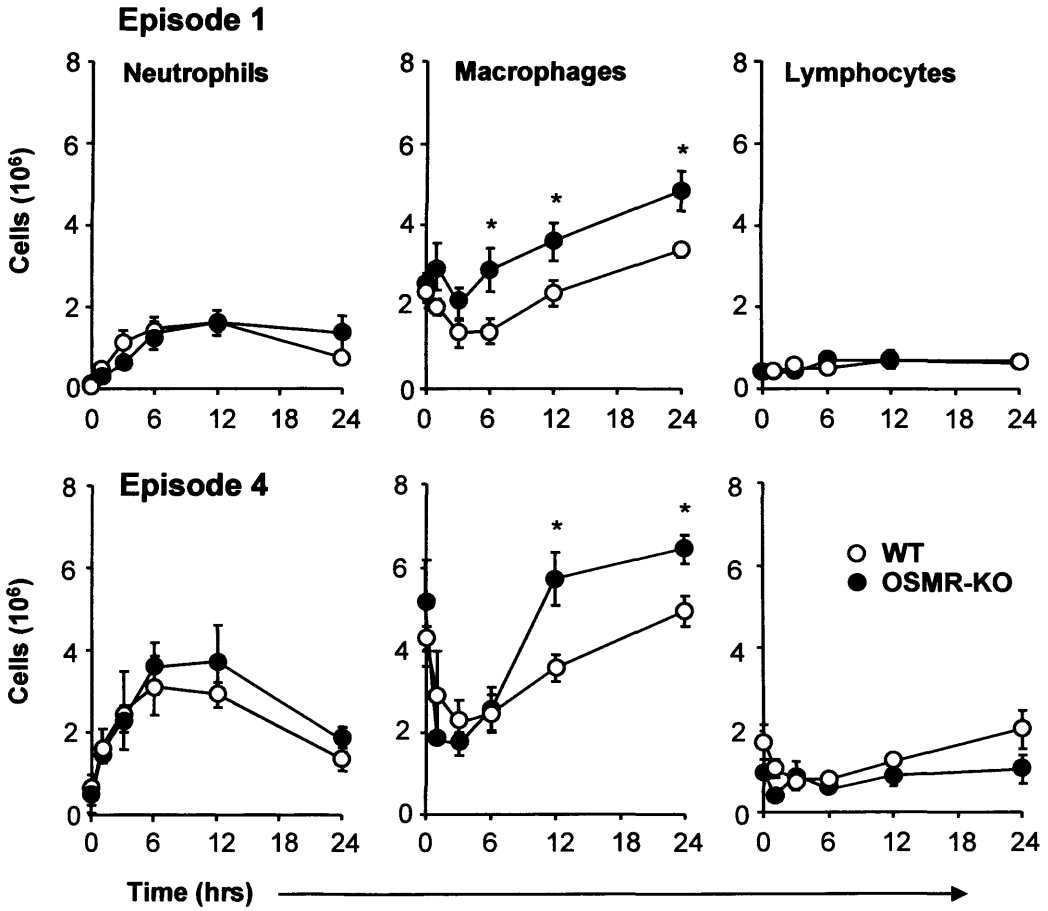

Fig 6.3.1. SES-induced inflammation was established in WT and OSMR-KO mice. Data is presented for mice receiving a single SES dose and mice treated with four individual doses of SES at weekly intervals. At designated time points the peritoneal cavity was lavaged and the composition of the inflammatory infiltrate compared by differential cell count. Data represents mean ± SEM of five WT and four OSMR-KO mice for each time point (*$p<0.05$).

Figure 6.3.2 OSMR-KO mice show increased monocytic cell infiltration during both acute and repeated inflammation

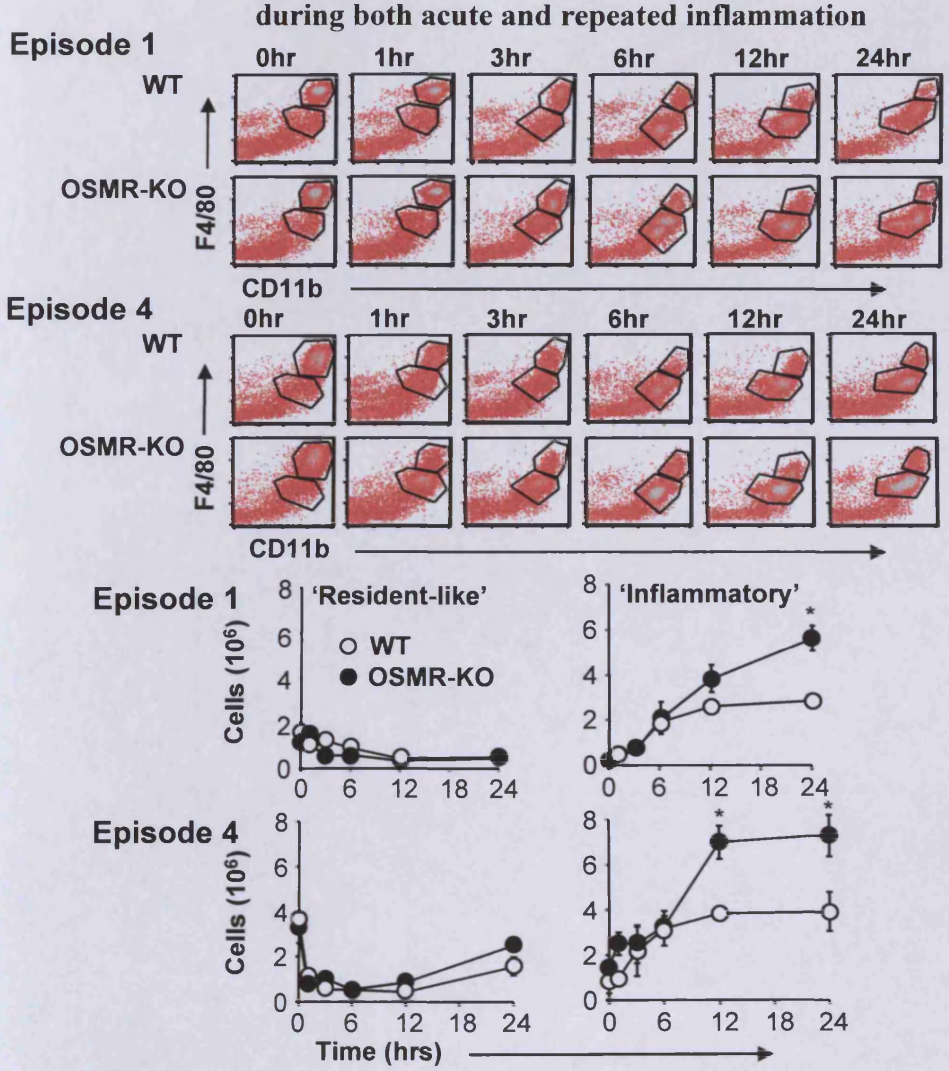

Fig 6.3.2 Single (Episode 1) and recurrent (Episode 4) SES-induced peritoneal inflammation was established in WT and OSMR-KO mice. At designated intervals FACS analysis was performed on peritoneal leukocytes to determine numbers of resident-like F4/80^{high}CD11b^{high} monocytic cells and infiltrating F4/80^{low}CD11b^{+} monocytic cells. Data represents mean +/- SEM of five WT and four OSMR-KO mice (*p<0.05).

6.3.2 OSMRβ mediated signalling selectively regulates CCL5 expression during recurrent inflammatory challenge

Analysis of OSM expression during acute inflammatory conditions has defined neutrophils as the primary source of OSM (Hurst *et al*, 2002), however, during chronic inflammation the cellular source of OSM is predominantly activated macrophages and T cells (Modur *et al*, 1997; Langdon *et al*, 2000). Mice exposed to an acute SES-induced inflammatory challenge demonstrated low levels of OSM expression within the peritoneal cavity, which coincided with the neutrophil influx (Figure 6.3.3), confirming observations outlined in Chapter 5 (Figure 5.3.4). This profile was somewhat delayed and more sustained as a consequence of repeat SES challenge, and may reflect the more prolonged neutrophil infiltration observed in response to recurrent inflammation (Figure 6.3.3). Furthermore, OSM levels were observed to be higher in WT mice, however, due to the low expression and low number of replicates, this would need to be substantiated by additional studies.

Expression of the mononuclear chemoattractants CCL2 and CCL5 in response to acute and repeated episodes of inflammation was also quantified. Interestingly the profile of CCL2 expression is comparable between acute and repeated inflammation (Figure 6.3.4). Due to the increased and sustained mononuclear infiltrate, increased expression of mononuclear cell chemoattractants would be expected with repeated inflammation. However, analysis of CCL2 levels in the peritoneal cavity demonstrated only slightly enhanced expression in response to repeated inflammatory assault, with OSMR-KO mice demonstrating significantly higher CCL2 in comparison to WT mice (Figure 6.3.4).

In vivo and *in vitro* experiments previously outlined in Chapters 4 and 5 illustrated an ability of OSM to control CCL5 expression (Figure 4.3.1; Figure 5.3.7). The profile of CCL5 expression is similar between single and repeated episodes of inflammation, however, the observed profile of CCL5 expression was notably more sustained following repeated SES activation, with significantly elevated CCL5 levels detected 24 hours post SES activation (Figure 6.3.5). In response to repeated episodes of inflammation, OSMR-KO mice demonstrate significantly increased CCL5 expression in comparison to WT animals (Figure 6.3.5), thereby suggesting a role for OSMRβ-mediated signalling in regulating CCL5 in recurrent and acute inflammation.

Figure 6.3.3 OSM expression in response to acute and repeated episodes of inflammation in WT and OSMR-KO mice

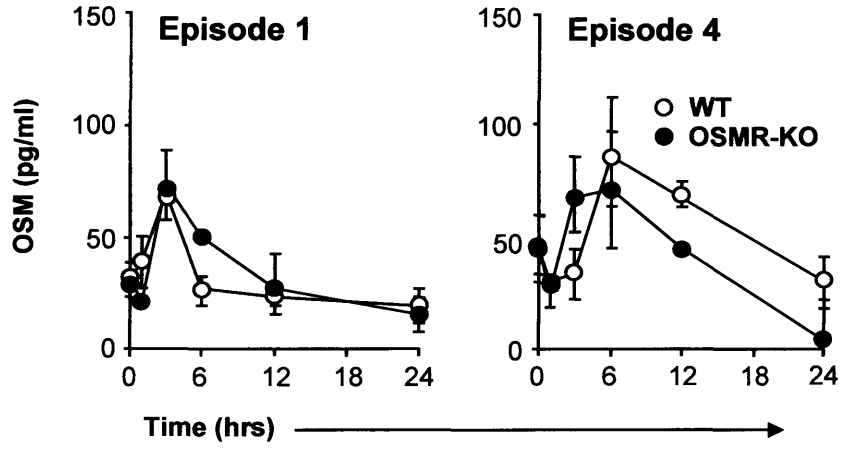

Fig 6.3.3. Single (Episode 1) and recurrent (Episode 4) SES-induced inflammation was established in WT and OSMR-KO mice. ELISA was used to quantify OSM levels in peritoneal lavage fluid at designated time points. Data represents the mean ± SEM of five WT and four OSMR-KO mice.

Figure 6.3.4 CCL2 expression in response to acute and repeated episodes of inflammation in WT and OSMR-KO mice

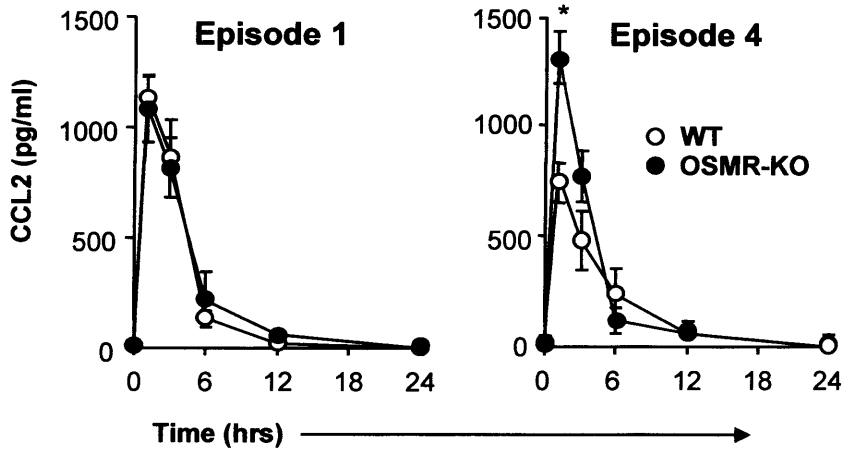

Fig 6.3.4. Single (Episode 1) and recurrent (Episode 4) SES-induced inflammation was established in WT and OSMR-KO mice. Temporal changes in CCL2 concentration were quantified in peritoneal lavage fluid using ELISA. Data represents the mean ± SEM five WT and four OSMR-KO mice (*p<0.05).

Figure 6.3.5 CCL5 expression in response to acute and repeated episodes of inflammation in WT and OSMR-KO mice

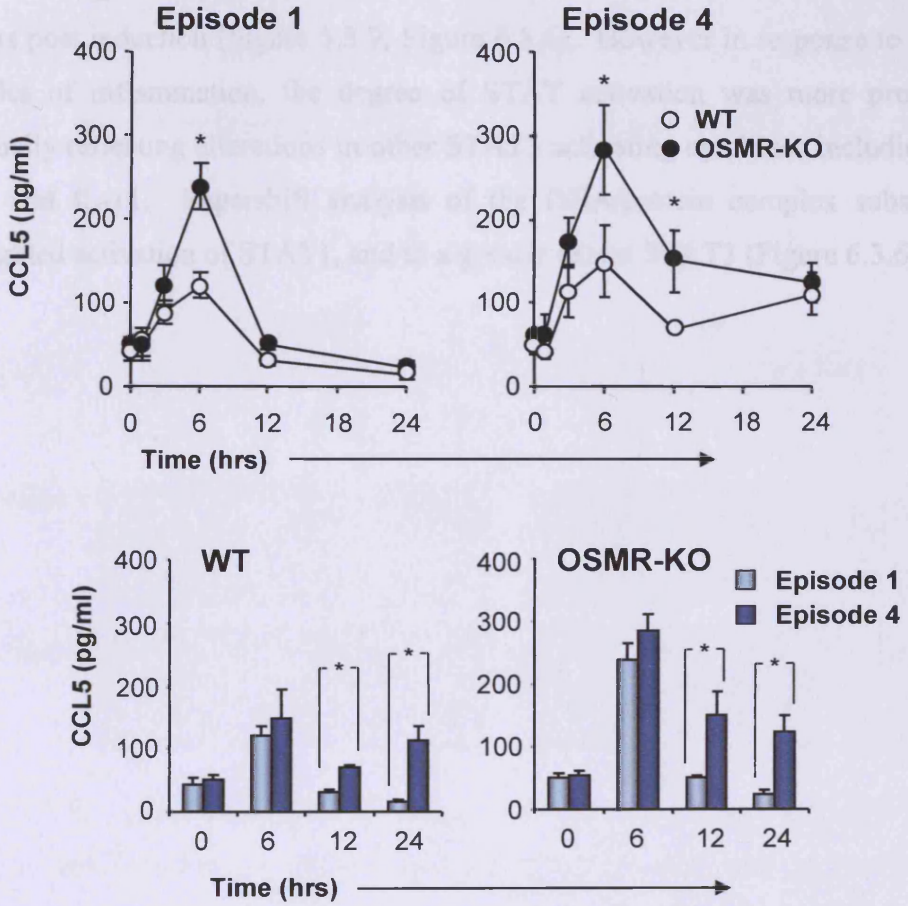

Fig 6.3.5. Single (Episode 1) and recurrent (Episode 4) SES-induced inflammation was established in WT and OSMR-KO mice. Temporal changes in CCL5 concentration were quantified in peritoneal lavage fluid using ELISA. Data represents the mean ± SEM five WT and four OSMR-KO mice (*p<0.05).

6.3.3 OSMR-KO mice demonstrate decreased STAT activation

As predicted by the data generated following a single acute inflammatory challenge OSMR-KO mice demonstrate decreased STAT activation (Figure 5.3.9). Analysis of SES-induced STAT activation in nuclear extracts isolated from peritoneal membranes of OSMR-KO and WT mice after both single and repeated episodes of SES-induced inflammation demonstrated decreased STAT activation in OSMR-KO mice (Figure 6.3.5). During acute inflammation the peak of STAT activation occurs between 1 and 3 hours post induction (Figure 5.3.9; Figure 6.3.6). However in response to repeated episodes of inflammation, the degree of STAT activation was more pronounced potentially reflecting alterations in other STAT3 activating cytokines including IL-6 , IL-10 and IL-11. Supershift analysis of the DNA/protein complex subsequently highlighted activation of STAT1, and to a greater extent STAT3 (Figure 6.3.6).

Figure 6.3.6 STAT activation is enhanced in response to recurrent episodes of SES-induced inflammation

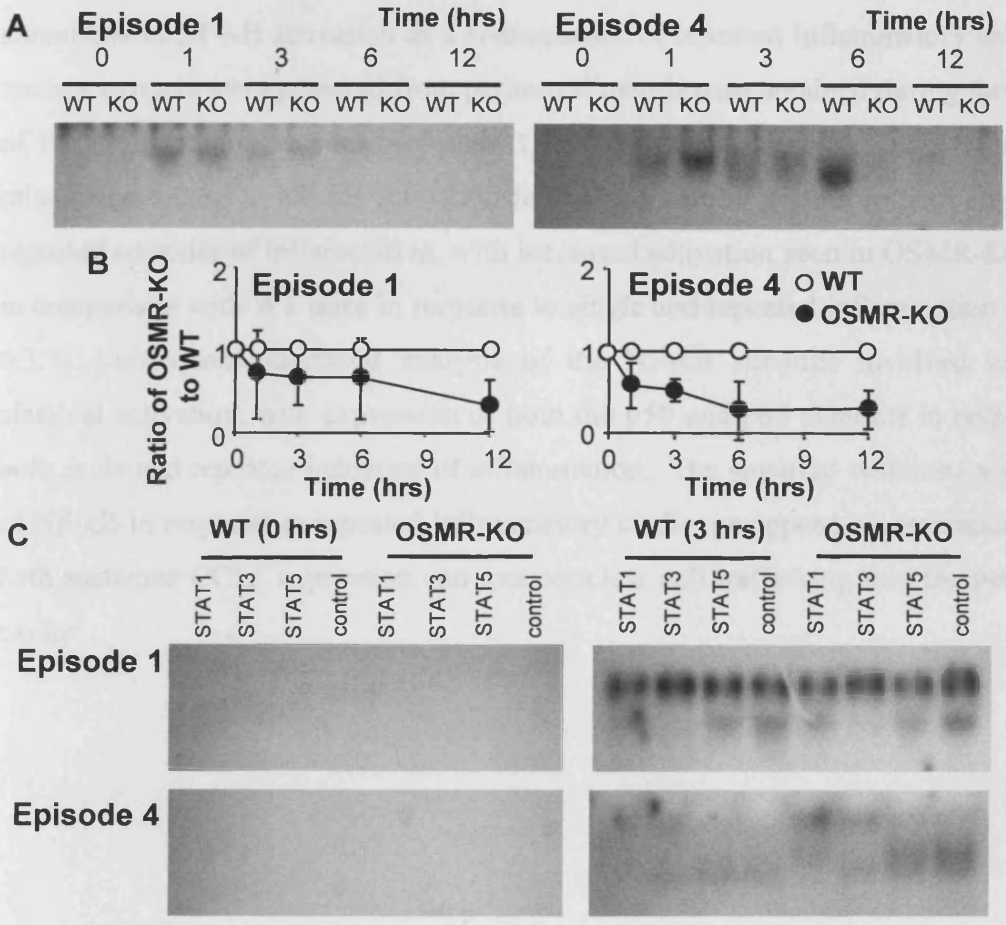

Fig 6.3.6. (A) Temporal activation of STAT in nuclear extracts from peritoneal membranes of WT and OSMR-KO mice was monitored by EMSA following acute (Episode 1) and repeated episodes of peritoneal inflammation (Episode 4). Banding intensity was quantified by densitometry by calculating the ratio of activation elicited in OSMR-KO mice, which was compared to activation in WT mice (B). Composition of the STAT/DNA complex was verified by supershift analysis of extracts isolated from unchallenged mice and mice exposed to SES for 3 hours (C). Data is representative of EMSA performed using nuclear extracts from three WT and three OSMR-KO mice.

6.3.4 OSMR-KO mice demonstrate altered NF-κB activity

Studies outlined in Chapters 4 and 5 demonstrated an ability of OSM to regulate NF-κB activation both *in vitro* (Chapter 4, Figure 4.3.3) and during an acute inflammatory challenge *in vivo* (Chapter 5, Figure 5.3.10). To examine potential alterations in NF-κB activation as a consequence of repeated inflammatory challenge nuclear extracts were prepared from peritoneal membranes obtained during the course of the inflammatory response (episode 1 and episode 4) from WT and OSMR-KO mice (Figure 6.3.7). NF-κB activation followed a similar pattern in both single and repeated episodes of inflammation, with increased activation seen in OSMR-KO mice in comparison with WT mice in response to single and repeated inflammation (Figure 6.3.7). Subsequent supershift analysis of the NF-κB subunits involved confirms classical activation, with expression of both the p50 and p65 subunits in response to both acute and repeated induction of inflammation. The apparent sustained activation of NF-κB in response to repeated inflammatory challenge appears to correspond with both sustained CCL5 expression and mononuclear cell trafficking into the peritoneal cavity.

Figure 6.3.7 OSMR-KO mice demonstrate increased NF-κB signalling in response to acute and recurrent inflammatory challenge

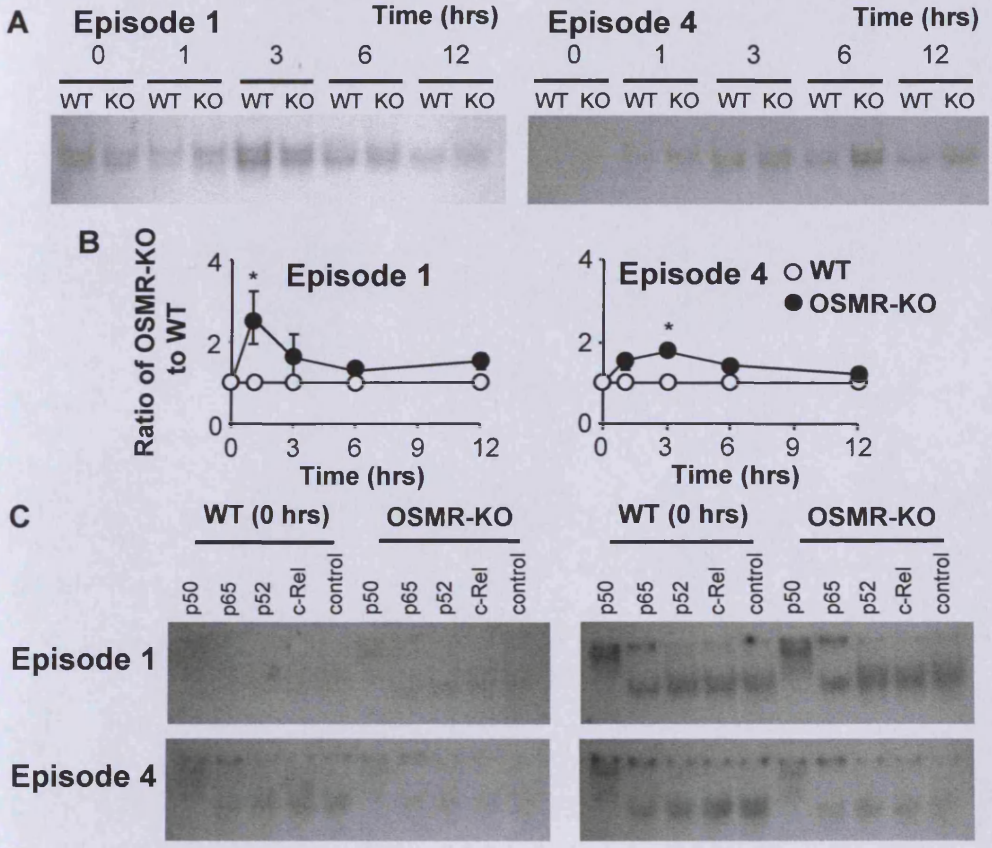

Fig 6.3.7. (A) Temporal activation of NF-κB in nuclear extracts from peritoneal membranes of WT and OSMR-KO mice was monitored by EMSA following acute (Episode 1) and repeated episodes of peritoneal inflammation (Episode 4). Banding intensity was quantified by densitometry by calculating the ratio of activation elicited in OSMR-KO mice, which was compared to activation in WT mice (B). Composition of the NF-κB complex was verified by supershift analysis of extracts isolated from unchallenged mice and mice exposed to SES for 3 hours (C). Data is representative of EMSA performed using nuclear extract from three WT and three OSMR-KO mice (*$p<0.05$).

6.3.5 OSMR-KO mice develop peritoneal membrane thickening comparable to that observed in WT mice

Chronic inflammatory conditions are often characterised by accumulation of a persistent leukocyte infiltrate at the site of inflammation (Buckley, 2003). In this respect, peritoneal membrane biopsies taken from PD patients who had encountered repeated episodes of bacterial peritonitis display increased evidence of histological abnormalities including membrane thickening and EMT (Suassuna *et al*, 1994). As illustrated in Figure 6.3.8, mice exposed to repeated episodes of SES-induced inflammation develop significantly increased thickening of the peritoneal membrane, potentially due to increased leukocyte infiltration and deposition of collagen. OSMR-KO mice appear to develop membrane thickening comparable to that observed in WT mice, however, OSMR-KO mice appear to have thickened peritoneal membranes prior to induction of peritoneal inflammation, although the reasons for this remain unclear (Figure 6.3.8).

Figure 6.3.8 OSMR-KO mice develop peritoneal membrane thickening comparable to that observed in WT mice

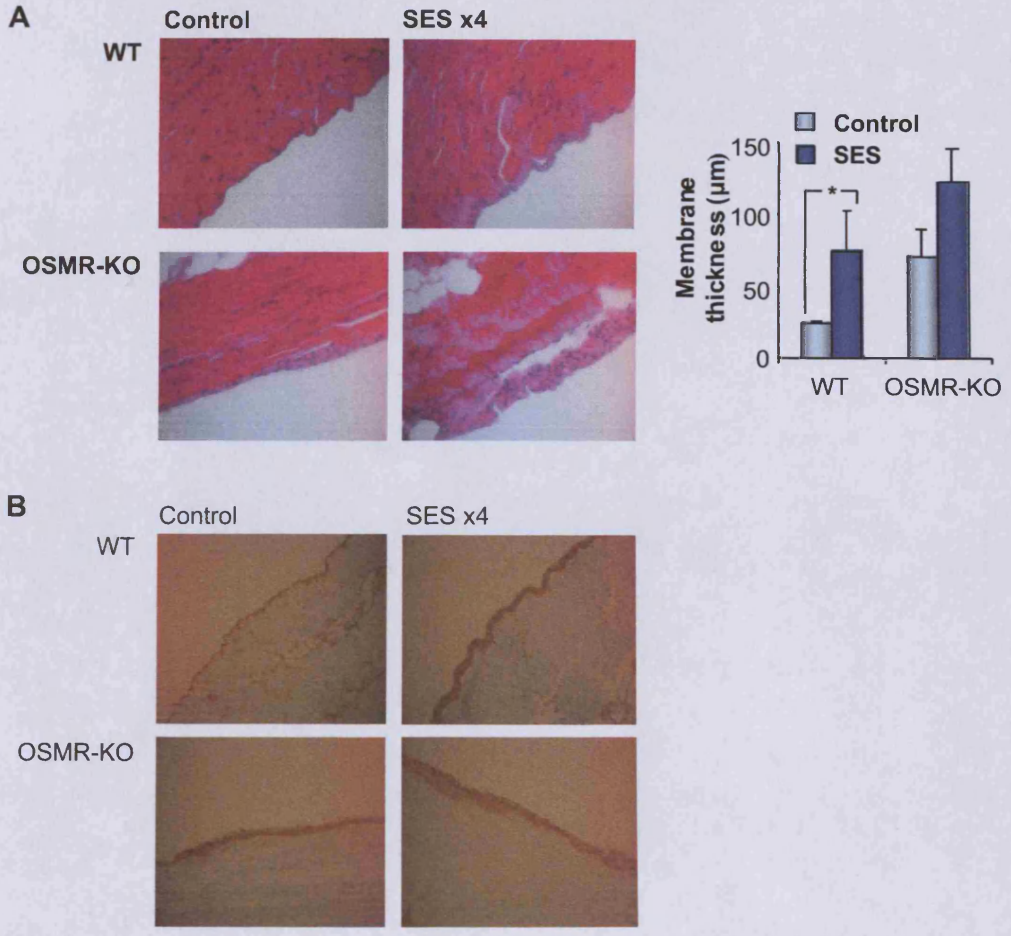

Fig 6.3.8. SES-induced inflammation was established in WT and OSMR-KO mice using four individual doses of SES at weekly intervals (SES x4). Peritoneal membrane sections were taken 49 days after the initial dose of SES and compared to membrane sections taken from unchallenged, age-matched mice. Sections were stained with haematoxylin and eosin (A) and an antibody against collagen I (B). The thickness of the mesothelium was assessed and the average thickness for each section calculated. Data is representative of three WT and three OSMR-KO mice (*$p<0.05$).

6.4 Discussion

Studies outlined in this Chapter suggest that OSMRβ-mediated signalling plays an important role in maintaining control of monocytic cell trafficking during recurrent episodes of inflammation. As indicated by the results outlined in previous chapters, the ability of OSM to selectively control monocytic cell trafficking may be due to enhanced NF-κB activation as evidenced by changes in CCL5 expression.

Resolution of inflammation is dependent on the effective clearance of the leukocyte infiltrate, which if not cleared can lead to chronic inflammation and tissue damage (Buckley, 2003). In accordance with this, both WT and OSMR-KO mice demonstrate an increased population of resident peritoneal leukocytes after repeated inflammatory episodes. Corresponding to the sustained neutrophil influx, OSM expression within the peritoneal cavity also appears increased and sustained. Collectively, these data infer that OSM activity is associated with the control of leukocyte retention within inflamed tissue, with OSM expression becoming heightened and more sustained.

As discussed in Chapter 5, IL-6 and OSM regulate distinct roles in governing leukocyte trafficking during acute inflammation, with IL-6 primarily affecting the neutrophil and T cell populations, and OSM controlling monocytic cell recruitment (McLoughlin *et al*, 2005; Hurst *et al*, 2001; Hams *et al*, 2008). However, both cytokines appear to be of significant importance in the transition of innate to acquired immunity (Jones, 2005; Hams *et al*, 2008). This pattern is maintained in response to recurrent inflammatory challenge, suggesting that during progressive inflammation OSM remains the predominant gp130-activating cytokine controlling monocytic cell recruitment. Analysis of the inflammatory chemokines CCL2 and CCL5 suggests that OSMRβ-mediated control of CCL5 expression is maintained in response to repeated inflammatory challenge and be a principle regulator of monocytic cell trafficking.

As previously discussed, although pivotal in bacterial clearance, IL-6 is also associated with tissue damage as exemplified in a variety of chronic inflammatory disorders, with IL-6KO mice protected against a variety of experimental inflammatory conditions (Alonzi *et al*, 1998; Yamamoto *et al*, 2000; Onogawa, 2005; Ramshaw *et al*, 1997). In addition, IL-6 deficient mice are also resistant to membrane thickening

in experimental models of peritonitis (Fielding *et al*, unpublished data). However, a deficiency in OSM-mediated signalling does not exhibit this protective phenotype, with OSMR-KO mice developing experimental arthritis (Wong *et al*, 2006). As demonstrated within this Chapter, OSMR-KO mice also develop peritoneal membrane thickening as associated with chronic peritoneal inflammation. This also implies that within the inflammatory setting used in this project, OSM-mediated signalling does not have a significant effect on EMT as has previously been reported in renal fibrosis (Pollack *et al*, 2007; Nightingale *et al*, 2004), however, further analysis of the typical markers of EMT in OSMR-KO mice in response to recurrent inflammatory stimulation would be required to support this idea.

These results suggest that during repeated episodes of peritoneal inflammation OSM continues to direct CCL5-mediated monocytic cell recruitment in a similar manner to that observed in response to a single episode of acute inflammation. This upregulation of chemokine expression, taken in combination with a more sustained profile of OSM expression, due to the delayed clearance of the neutrophil influx, may prove to be detrimental resulting in an increased population of monocytic cells within the peritoneum. However, studies outlined within this Chapter were unable to find any evidence for a role for OSM in membrane thickening or tissue injury associated with repeated inflammatory assault, suggesting that OSM does not play an important role in tissue damage within this inflammatory context.

Chapter 7

General Discussion

7. Discussion

Previous *in vitro* and *in vivo* investigations have outlined potential roles for the gp130-activating cytokine OSM in inflammation, tissue remodelling and haematopoiesis (Zarling *et al*, 1986; Modur *et al*, 1997; Langdon *et al*, 2000; Tanaka *et al*, 2003; Hurst *et al*, 2002; Richards *et al*, 1996). However, these investigations have not evaluated the *in vivo* contribution of OSM during acute inflammation. Using an *in vivo* model of acute bacterial peritonitis, supported by *in vitro* studies, this thesis illustrates that OSMRβ signalling selectively affects the inflammatory trafficking of monocytic cells (Hams *et al*, 2008). This OSM-mediated response is distinct from that elicited by IL-6, which predominantly regulates recruitment and clearance of neutrophils and lymphocytes (Hurst *et al*, 2001; McLoughlin *et al*, 2005; Jones, 2005; Hams *et al*, 2008). Such findings highlight a distinction between the inflammatory activities controlled by a gp130 homodimer receptor complex and those governed by OSMRβ:gp130 heterodimeric receptor.

Studies outlined in Chapters 3 and 4 illustrate an ability of OSM to selectively upregulate inflammatory chemokines and adhesion molecules *in vitro* when utilising a OSMRβ:gp130 receptor complex expressed on HPMC. In addition, OSM can selectively control IL-1β-mediated chemokine production, suppressing expression of the neutrophil chemoattractant CXCL8 and the mononuclear cell chemoattractant CCL5. The potential importance of OSM-mediated control of CCL5 was confirmed *in vivo*, where OSMR-KO mice demonstrated significantly enhanced CCL5 expression following acute inflammatory activation. Such coordinated regulation of inflammatory chemokine expression, as highlighted by the impact of OSM on CCL5 secretion, may represent a potential mechanism for the OSMRβ-mediated control of monocytic cell recruitment. Subsequent examination of transcriptional events highlighted a role for OSM in negatively regulating the level of NF-κB activation. The orchestrated control of NF-κB signalling by OSM may explain the regulation of CCL5 (and CXCL8 production), and provides an additional mechanism for the OSM-mediated control of mononuclear cell trafficking. However, the specific interplay between OSM/OSMRβ and NF-κB activation requires further analysis.

7.1 The differential roles of IL-6 and OSM in leukocyte recruitment during acute inflammation.

The leukocyte profile following an acute inflammatory challenge can be characterised by an initial influx of neutrophils, which are subsequently replaced by a more sustained population of activated mononuclear cells (Topley *et al*, 1996). This transition marks a switch from innate to acquired immunity, which is vital for the effective resolution of inflammation (Jones, 2005; Beutler, 2004). The importance of IL-6 in this transition has been well documented in several recent review articles (Jones, 2005; Rose-John *et al*, 2006), however, the roles of other gp130-activating cytokines are less well defined. Analysis of effluent from peritoneal dialysis patients with acute bacterial peritonitis has shown expression of both IL-6 and OSM, but not LIF, suggesting that within this inflammatory context the two primary gp130-activating cytokines of potential importance are IL-6 and OSM (Hurst *et al*, 2002). However, it should be noted that other IL-6-related cytokines including IL-11 and IL-27 have prominent inflammatory roles as defined by their involvement in other models of inflammation. For example, IL-11 has been described as the principle cytokine promoting STAT1 and STAT3 mediated gastric-inflammation and tumourigenesis, whilst IL-27 plays a pivotal role in governing T-cell effector functions (Ernst *et al*, 2008; Villarino *et al*, 2003; Artis *et al*, 2004). Collectively, these studies emphasise a hierarchy of gp130-activating cytokines in governing specific inflammatory activities. While other family members such as LIF, CLC and CNTF may provide homeostatic functions not typically associated to immunological processes (Taga & Kishimoto, 1997; Nakashima & Taga, 1998).

The archetypal gp130-activating cytokine IL-6 plays an integral role in the transition from innate to acquired immunity by regulating both the chemokine-driven recruitment and apoptotic clearance of both neutrophils and T cells (Hurst *et al*, 2001; Jones, 2005). Initial *in vitro* investigations outlined both within this project and in previous studies have documented similarities between OSM and IL-6 in their capacity to regulate chemokine-driven responses (Hurst *et al*, 2002; Nowell *et al*, 2006; Modur *et al*, 1997; McLoughlin *et al*, 2004; Richards *et al*, 1996). For example both cytokines exhibit an ability to suppress expression of the neutrophil chemoattractant CXCL8, although unlike IL-6KO mice, OSMR-KO mice do not display any alteration in neutrophil influx (Chapters 4 and 5; McLoughlin *et al*, 2003;

McLoughlin *et al*, 2004; Fielding *et al*, 2008; Hams *et al*, 2008). Likewise, although *in vitro* analysis has suggested the potential for OSM to regulate T cell trafficking through the control of mononuclear cell chemoattractants, including CCL2, CCL5 and CXCL10, *in vivo* analysis illustrated OSMRβ-mediated signalling does not control lymphocyte recruitment (Chapters 4 and 5; Hams *et al*, 2008). Consequently *in vitro* studies would infer a role for OSM in the control of neutrophil and T cell trafficking. However, this is not borne out *in vivo* suggesting that a hierarchy exists where IL-6 is of more importance in orchestrating certain chemokine-mediated events.

In direct contrast to IL-6, OSM appears to be of importance in the recruitment of monocytic cells. *In vitro* and *in vivo* analysis illustrates that IL-6 is the principle gp130-activating cytokine involved in governing CCL2 expression. In this respect OSMR-KO mice show a comparable profile of CCL2 production to WT mice following inflammatory activation (Chapter 5). CCL2 was originally described as a monocytic cell chemoattractant, however, IL-6 deficiency has no significant impact on monocytic cell recruitment (Hurst *et al*, 2001; McLoughlin *et al*, 2005; Hams *et al*, 2008). It is therefore questionable as to whether CCL2 is a more prominent regulator of activated T cells (Loetscher *et al*, 1996). A principle mechanism for controlling monocytic cell recruitment appears to be the OSM-mediated control of CCL5 and regulation of NF-κB signalling. These specific activities are distinct from the action of IL-6 (Chapters 4 and 5; McLoughlin *et al*, 2005; Hams *et al*, 2008). These findings suggest that during acute inflammation the OSM-mediated regulation of CCL5 may override IL-6-mediated control of CCL2 when recruiting monocytic cells to the site of inflammation. Such observations may support the notion that CCL2 preferentially control T cell recruitment, whilst CCL5 governs monocyte trafficking.

This contrasting ability of OSM and IL-6 to differentially regulate monocytic and T cell chemoattractants may further relate to studies into the receptors known to bind CCL2 and CCL5. Monocytic cells expressing CCR2, the primary functional receptor for CCL2, are defined as 'inflammatory' monocytes, which are actively recruited to sites of inflammation. Conversely, terminally differentiated macrophages lose cellular CCR2 expression, and instead upregulate CCR1 and CCR5, both of which act as receptors for CCL5 (Fantuzzi *et al*, 1999; Kaufmann *et al*, 2001). Previous studies have demonstrated the importance of gp130-activating cytokines in the differentiation

of monocytes, with OSM and IL-6 having unique roles in macrophage formation and the expansion of dendritic cells (Tanigawa *et al*, 1995; Chomarat *et al*, 2000; Kitamura *et al*, 2005). Therefore, differential regulation of CCL2 and CCL5 by IL-6 and OSM may mediate the controlled recruitment of specific subpopulations of monocytic cells during inflammation. Initial studies into the monocyte subsets recruited into the peritoneal cavity following inflammatory challenge suggest that OSMRβ-mediated signalling does not result in an alteration of the proportion of CCR5 expressing infiltrating monocytes, implying that the increase in CCL5 expression does not increase the recruitment of terminally differentiated macrophages (Chapter 5). However, CCL5 also utilises CCR1 and to a lesser extent, CCR3, therefore to further understand the implications of CCL5 on mononuclear trafficking, cellular expression of these receptors would also have to be determined.

As previously mentioned, the OSMRβ-mediated control of NF-κB signalling has been suggested as the principle mechanism regulating CCL5 expression (Moriuchi *et al*, 1997), which may be in part responsible for the observed alteration in monocytic cell recruitment. This was demonstrated *in vitro*, where OSM inhibition of the IL-1β-induced expression of CCL5 was associated with suppression in NF-κB activity (Chapter 4). Similarly, *in vivo* activation of NF-κB as a consequence of SES-driven TLR2 activation suggests that OSM may also regulate TLR-mediated NF-κB signalling (Figure 7.1). As TLR activation is associated with the innate immune response (Beutler & Rietschel, 2003), the OSM-mediated control of NF-κB suggests the potential for OSM to regulate innate immune activation, in addition to regulating mononuclear cell recruitment associated with an acquired immune response. The observed suppression of NF-κB in inflammatory activated OSMR-KO mice is unique and is not seen in IL-6KO mice. Since IL-6 and OSM utilise gp130, changes in NF-κB activation may arise from signals triggered via OSMRβ. However, the shared effects of OSM and IL-6, for example the ability of both cytokines to upregulate CCL2 and control the IL-1β-induced expression of CXCL8 (Chapters 3 and 4; Hurst *et al*, 2001), are potentially mediated through gp130.

Figure 7.1 OSMRβ-mediated signalling regulates NF-κB activity

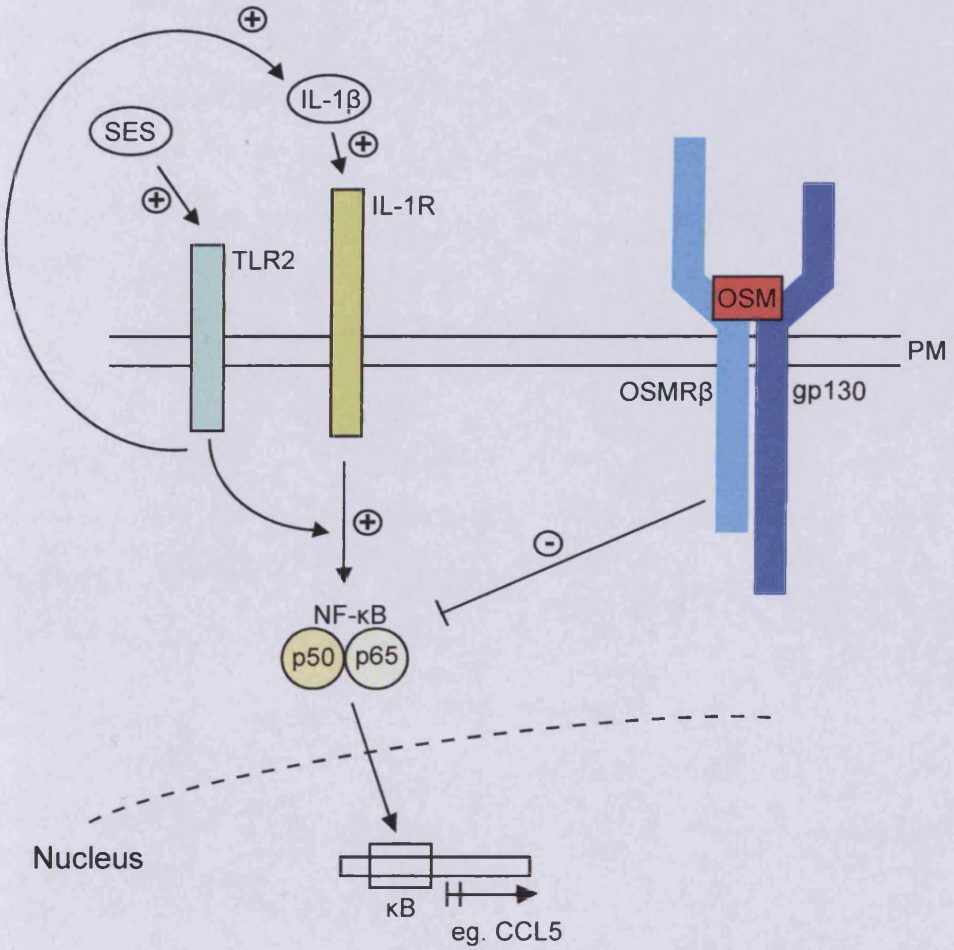

Fig 7.1. OSM-mediated signalling regulates both IL-1β- and TLR2-mediated activation of NF-κB. Both *in vivo* and *in vitro* analysis has illustrated the ability of OSM to suppress NF-κB activation, resulting in the inhibition of chemokines such as CCL5. This mechanism is unique to OSM, suggesting it is mediated through the OSMRβ subunit rather than gp130. This ability of OSM to regulate NF-κB activation may be responsible for regulation of monocytic cell recruitment observed in response to OSMRβ-mediated signalling. It remains unclear whether OSM acts on both signalling events (TLR and IL-1β) or if OSM acts solely on IL-1β responses which are triggered through TLR activation.

Figure 7.2 IL-6 and OSM have unique roles in the transition from innate to acquired immunity

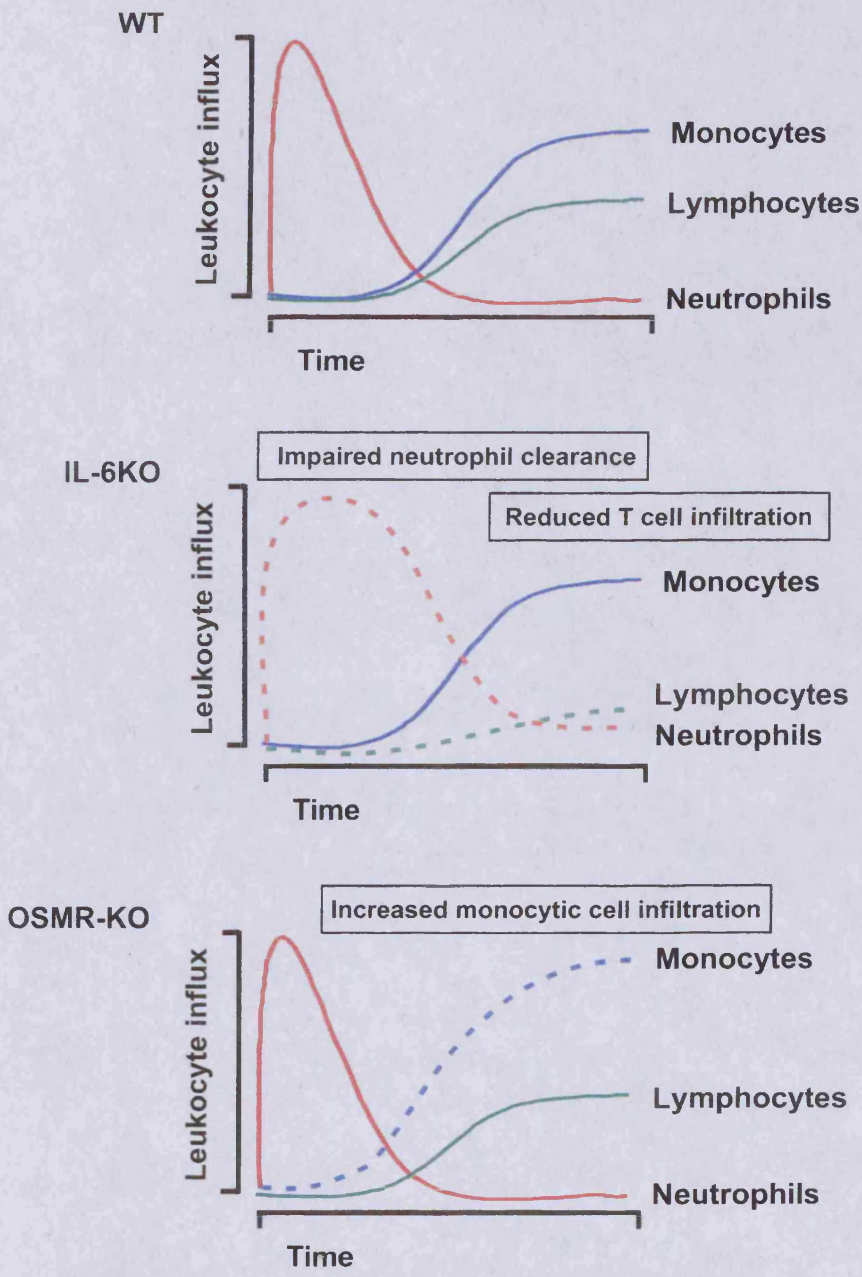

Fig 7.2. The leukocyte profile generated in response to SES-induced peritoneal inflammation in WT, IL-6KO and OSMR-KO mice demonstrates the ability of IL-6 to regulate both neutrophil and lymphocyte recruitment and clearance. Conversely, OSMR-KO mice demonstrate neutrophil and lymphocyte trafficking comparable to that seen in WT mice, however, OSM selectively regulates trafficking of monocytes, in an independent fashion.

Taken in combination, this data suggest that while IL-6 critically governs the pattern of neutrophil and T cell recruitment, monocytic cell trafficking is co-ordinated by OSM/OSMRβ (Figure 7.2). However, these studies suggest that OSM is also of significance in the transition from innate to acquired immunity and resolution of inflammation as increased monocytic cell infiltration may lead to enhanced phagocytic clearance of dying neutrophils (Savill *et al*, 2002; Brown & Savill, 1999). Further functional studies will be required to define the role of OSM in governing the clearance of apoptotic cells.

7.2 Differential roles for OSM and IL-6 in chronic inflammation and tissue injury

Chronic inflammatory conditions are characterised by an increased retention of activated mononuclear cells at sites of inflammation. The maintenance of a persistent leukocyte infiltrate reflects an imbalance between factors that promote cellular recruitment and those which promote cell death (Buckley, 2003). Chronic inflammation is often associated with tissue injury, for example articular cartilage damage in rheumatoid arthritis and development of fibrosis, characterised by excessive deposition of extracellular matrix components (Wynn, 2008; Arend & Dayer, 1990; Feldmann *et al*, 1990). As previously mentioned, gp130-activating cytokines are integral in governing leukocyte recruitment during acute inflammation, it is therefore conceivable that dysregulation of these cytokines may play a role in chronic inflammation.

This assumption has proved correct for IL-6, which has been shown to be detrimental in chronic inflammatory conditions, encouraging mononuclear cell infiltrate both through increasing expression of mononuclear cell chemoattractants and protecting T cells from apoptotic clearance (Salmon *et al*, 1997; Atreya *et al*, 2000; Teague *et al*, 2000; Nowell *et al*, 2008 in press). The detrimental effects of IL-6 have been demonstrated in several experimental models of chronic inflammatory disease, where IL-6KO mice are protected against models of arthritis, EAE and renal fibrosis (Alonzi *et al*, 1998; Yamamoto *et al*, 2000; Kallen, 2002). Initial studies have suggested that these activities are unique to IL-6 as OSMRβ and IL-11Rα deficient mice develop classic hallmarks of experimental arthritis (synovial hyperplasia, leukocyte infiltration and joint erosion), comparable to those observed in WT mice (Wong *et al*, 2006). In

addition, OSMR-KO mice are not protected against membrane thickening in response to repeated peritoneal inflammation, unlike IL-6KO mice (Chapter 6; Fielding *et al*, unpublished data). This data further suggests that there is a hierarchy in the activities of gp130-activating cytokines, with IL-6 playing the primary role in governing leukocyte retention in chronic inflammation, which may contribute to excessive tissue damage. However, OSM has previously been shown to be of importance in tissue injury associated with fibrosis and EMT (Pollack *et al*, 2007; Nightingale *et al*, 2004), therefore suggesting potentially unique roles during chronic inflammatory conditions which require further analysis.

Studies have detected expression of OSM in chronic inflammatory conditions, however, it appears that the primary source switches from the infiltrating neutrophils to activated macrophages and T cells (Cawston *et al*, 1998). This suggests that unlike its involvement in acute inflammatory events, where OSM expression is transient, expression is more sustained at the site of inflammation. This implies that OSM may play a role in the pathogenesis of chronic inflammatory conditions, however, studies have demonstrated both pro- and anti-inflammatory roles for this cytokine in tissue injury (Nagata *et al*, 2003; Sohara *et al*, 2002; Li *et al*, 2001; Plater-Zyberk *et al*, 2001; Langdon *et al*, 2000). These contrasting roles for OSM may relate to changes in the cellular distribution of OSMRβ. In this respect stromal cells, but not inflammatory cells express OSMRβ (Chapter 3). Here again, we see that OSMRβ expression has the potential to be modulated on both HPMC and leukocytes, with IFN-γ and bacterial agonists affecting the repertoire of cells responsive to OSM (Chapter 3).

One interesting observation from this study is the apparent upregulation of OSMRβ expression on monocytic cells in response to activation with LPS and IFN-γ (Chapter 3; Dillon *et al*, 2004). Stimulation with LPS and IFN-γ is associated with an enhancement in macrophage responsiveness (Nathan *et al*, 1983; Taylor *et al*, 2005), suggesting that during more progressive forms of disease where there is an accumulation of activated mononuclear cells, OSM may perform additional inflammatory roles as a consequence of OSMRβ induction on activated monocytic cells. These activities could be potentially distinct from the cellular responses directed by OSM in stromal cells. However, the observed upregulation of OSMRβ on

monocytic cells also corresponds to an increase in expression of IL-31Rα, both of which form the functional receptor complex for IL-31 (Chapter 3). Although initial studies have found no involvement for IL-31 in the *in vitro* control of chemokine-mediated leukocyte trafficking, previous studies have implicated both IL-31 and OSM in more chronic inflammatory conditions such as dermatitis and asthma (Dreuw *et al*, 2004; Dillion *et al*, 2004; Boniface *et al*, 2007). Further differential analysis of both IL-31 and OSM signalling in activated monocytes would be required to establish any additional role this pathway may have.

It still remains unclear whether OSM plays an important role in the progression of chronic inflammation and associated tissue injury. Studies outlined in this thesis taken in combination with the observation that OSMR-KO mice do not share the protective phenotype exhibited by IL-6KO mice in experimental models of autoimmunity suggest that during chronic inflammation, IL-6 overrides OSMRβ-mediated signalling. However, further analysis of OSMRβ-mediated signalling in experimental models of chronic inflammation would be necessary to fully determine the inflammatory role of OSM. In this respect fibrosis following repeated SES-induced inflammatory stimulation is mild, a more aggressive model of fibrosis would be required to fully assess the role of OSM.

7.3 Is OSM pro- or anti-inflammatory?

Previous studies have demonstrated the therapeutic benefits of selectively blocking certain pro-inflammatory cytokines, for example the use of pharmaceuticals to inhibit both TNF (eg. Enbrel, Etanercept) and IL-6 (eg. Atlizumab, Tocilizumab) (Maini & Taylor, 2000; Papadakis & Targan, 2000; Choy *et al*, 2002; Ito *et al*, 2004). There is evidence for OSM to have both beneficial and detrimental roles in inflammation and from the information currently available through published literature and studies outlined in this project, it still remains unclear whether OSM represents a suitable therapeutic target. This is therefore an avenue for future studies.

However, the apparent ability of OSM to selectively regulate monocytic cell trafficking may prove to be of importance as more is discovered about the specific roles of monocytes and macrophages during inflammatory activation. Although the roles of lymphocytes during progressive inflammation are well defined, the potential

role of the macrophage is less clearly defined. Therefore, as more is understood about the potential implications of macrophage activation, the therapeutic benefits of OSM in regulating monocytic cell recruitment may become apparent. Although effective, concerns are now being raised about the use of anti-TNF therapies (such as Etanercept) due to the increased risk of severe bacterial and viral infections associated with the effects of blocking TNF on the immune system (Desai & Furst, 2006; Domm *et al*, 2008; Winthrop, 2006). Therefore it may be advantageous to investigate the use of anti-cytokine therapies which are more specific, such as the potential therapeutic use of soluble gp130, which specifically blocks IL-6 trans-signalling whilst leaving classical IL-6R signalling unaffected (Rose-John *et al*, 2007; Richards *et al*, 2006).

Studies have thus far emphasised both pro- and anti-inflammatory roles for OSM in the progression of an immune response (Chapters 3-6; Modur *et al*, 1997; Wahl & Wallace, 1999). Both *in vitro* and *in vivo* analysis have shown OSM to promote wound healing and to upregulate expression of acute phase proteins and protease inhibitors (eg. TIMP1 and TIMP3), which have been implicated in modulating cytokine function and limiting tissue damage at sites of inflammation (Wallace et al, 1995; Cross *et al*, 2004; Li *et al*, 2001; Goren *et al*, 2006). In addition, OSM can regulate inflammatory chemokine expression, and may aid the resolution of inflammation (Chapters 3-6). Such data suggest the potential for the therapeutic use of OSM in inflammatory disorders. Initial studies have demonstrated that OSM can protect against LPS-induced toxicity and reduce inflammation in experimental models of arthritis and inflammatory bowel disease (Wallace et al, 1999; Wahl & Wallace, 2001; Loy et al, 1999). However, these studies were performed using human OSM, which in mice utilises the LIF receptor complex (Yoshimura *et al*, 1996), and as such do not appropriately reflect the potential contribution or impact of OSM via OSMRβ.

Subsequent studies have also implicated OSM as a pro-inflammatory mediator able to upregulate expression of inflammatory cytokines and tissue destruction both *in vitro* and *in vivo* (Langdon *et al*, 2000; Brown *et al*, 1991; Nowell *et al*, 2006; Plater-Zyberk *et al*, 2001). In accordance with these observations, the use of antibodies against OSM has shown some success in inhibiting joint destruction in experimental models of arthritis (Plater-Zyberk *et al*, 2001). In addition, OSM has been implicated in renal EMT, which is fundamentally linked to the pathogenesis of fibrosis (Pollack

et al, 2007; Nightingale *et al*, 2004). Although studies outlined herein fail to demonstrate a role for OSM in EMT within the peritoneum, future studies using a more aggressive model of peritoneal fibrosis may better illustrate a defined role for OSM in this process.

7.4 Future directions

Although this project has clearly outlined a role for OSM in governing monocytic cell recruitment during acute inflammation there are several questions that remain unanswered:

- Does OSM regulation of CCL5 represent the primary mechanism orchestrating the OSMRβ-mediated control of monocytic cell recruitment? To answer this question a full evaluation of inflammatory mediators involved in governing monocytic cell trafficking in response to OSM would be required. However, this is currently restricted by the lack of suitable blocking agents for murine OSM or OSMRβ. In addition, further analysis of the interplay between OSMRβ-mediated signalling and the inhibition of NF-κB using transcriptional analysis with suitable reporter assays would be required to fully understand the interaction between the two pathways.
- Does IL-31 play any role in the progression of acute or progressive peritoneal inflammation? Although *in vitro* analysis suggests that during acute inflammation IL-31 does not share the ability of OSM to regulate inflammatory chemokine expression, the observation that IL-31Rα expression can be induced on HPMC in response to IFN-γ stimulation implies that IL-31 may play an additional role in more progressive forms of disease. The use of OSM-KO mice in comparison with OSMRβ-KO mice within the same inflammatory model could be used to outline any potential role of IL-31 in the observed effects of OSMRβ-mediated signalling during peritoneal inflammation.
- Does OSMRβ-mediated signalling on activated monocytic cells play a role in progressive inflammatory disorders? The apparent induction of OSMRβ expression on monocytic cells activated with LPS and IFN-γ suggests the potential for unique signalling events in chronic inflammatory conditions, either mediated by OSM or IL-31. The use of mice deficient in stromal

OSMRβ expression could be used to assess the potential importance of OSMRβ-mediated signalling solely on monocytic cells.

7.5 Concluding comments

Studies outlined within this thesis suggest a role for OSM in regulating monocytic cell trafficking during inflammation, potentially through altering NF-κB activity thereby inhibiting production of specific downstream chemoattractants. Monocytes play an important role in the immune response, both in the removal of pathogen and in the clearance of apoptotic cells (Fadok *et al*, 1998; Savill *et al*, 2002), however, accumulation of activated monocytes and macrophages at inflammatory sites is associated with tissue damage (Buckley, 2003). These observations suggest OSM-mediated regulation of monocytic cells may have a beneficial role during progressive inflammatory conditions. However, OSM control of mononuclear cell chemoattractants appears to be limited to CCL5 *in vivo* suggesting the effects of OSM may be confined to specific subsets of monocytic cells. In this respect the impact of OSM on NF-κB may be more pertinent. These observations and their implications would require further analysis *in vivo*. Studies into the potential role of OSM in peritoneal fibrosis were inconclusive and would require further analysis in a more robust fibrosis model, however, it would appear from previous studies that OSM does not represent the primary gp130-activating cytokine involved in tissue damage (Wong *et al*, 2006). From studies outlined within this thesis it remains unclear whether OSM would provide a suitable therapeutic target, however, results have demonstrated unique and previously undefined roles for OSMRβ-mediated signalling in the progression of inflammation.

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