CYTOKINE REGULATION OF MONOCYTIC CELL SUBSETS IN THE PERITONEAL CAVITY

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Summary

It is well known that Interleukin (IL)-6 is important in the regulation of both the chemokine directed recruitment and apoptotic removal of neutrophils and lymphocytes (Hurst *et al* 2001, McLoughlin *et al* 2003, 2005). However little is currently known about the role of IL-6 in governing the phenotype, activation and inflammatory trafficking of monocytic cells. In this thesis, an *in vivo* model of peritoneal inflammation was utilised in combination with *in vitro* studies using murine resident peritoneal leukocytes, to determine a role for IL-6 in the regulation of monocytic cell activity and trafficking during inflammation. In this respect, it was shown that resident peritoneal macrophages and dendritic cells (DC) were non-responsive to IL-6 signalling, which in DC was associated with an activation-induced loss of IL-6 receptor. This provides a potential mechanism by which DC are able to remove themselves from the immunosuppressive constraint imposed by IL-6, which is known to maintain immature DC (Park *et al* 2004), allowing them to mature into activated DC. Upon activation, resident DC did not display a change in chemokine receptor expression, suggesting that they may remain within the peritoneal cavity and undergo antigen presentation and lymphocyte activation locally. *In vivo* studies revealed that IL-6 did not regulate the inflammatory trafficking of resident macrophages, DC or recruited circulatory monocytes during acute peritonitis. However upon repeated inflammatory activation, IL-6 directed the increased recruitment of infiltrating monocytes to the peritoneal cavity, implicating them as a contributing factor to the peritoneal tissue damage and fibrosis associated with chronic disease progression. Later studies focused on IL-10, since there is a known interplay that exists between IL-6 and IL-10 (Niemand *et al* 2003, Fiorentino *et al* 1991, Chernoff *et al* 1995). *In vivo* studies defined an anti-inflammatory role for IL-10, whereby it was responsible for limiting inflammatory leukocyte recruitment *via* its control of chemokine expression, and the suppression of IL-17A secreting T cell development and recruitment. Therefore the pro-inflammatory and anti-inflammatory roles of IL-6 and IL-10 respectively must be finely balanced to allow competent cell mediated immunity against infection whilst preventing excessive tissue damage associated with disease progression.

Publications and presentations

Publications:

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G.W. Jones, R.M. McLoughlin, V.J. Hammond, C.R. Parker, J.D. Williams, R. Malhotra, J. Scheller, A.S. Williams, S. Rose-John, N. Topley and S.A. Jones (2009). "Loss of $CD4^+$ T-cell IL-6R expression during inflammation underlines a role for IL-6 trans-signaling in local Th-17 cell regulation". J. Immunol., manuscript accepted.

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Abbreviations

ADAM	Adamylsin and disintegrin-associated metalloprotease
APC	Antigen presenting cell
APC	Allophycocyanin
BAFF	B cell activating factor
BMDC	Bone marrow derived dendritic cells
cDC	Conventional dendritic cell
CFU	Colony forming units
CLP	Common myeloid precursor
CLD	C type lectin domain
CLR	C type lectin receptor
CMP	Common lymphoid precursor
CNTF	Ciliary neurotrophic factor
CRP	C reactive protein
CT-1	Cardiotrophin-1
DAMP	Damage-associated molecular pattern molecules
DC	Dendritic cell
DMEM	Dulbecco's modified medium
EGF	Epidermal growth factor
ELISA	Enzyme linked immunosorbent assay
EMT	Epithelial to mesenchymal transition
ERK	Extracellular signal regulated kinases
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
FITC	Fluoroscien isothiocyanate
FSC	Forward scatter
Gab	Grb2 associated binding protein
GDP	Guanosine diphosphate
GFR	Glomerular filtration rate
GPI	Glycosylphosphotidylinositol

GM-CSF	Granulocyte macrophage colony stimulating factor
Grb2	Growth factor receptor binding protein 2
GTP	Guanosine triphosphate
HPMC	Human peritoneal mesothelial cells
HRP	Horse radish peroxidase
ICAM	Intracellular adhesion molecule
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
IP	Inducible protein
JAK	Janus activated kinases
LDL	Low density lipoprotein
LFA	Lymphocyte function associated
LIF	Leukaemia inhibitory factor
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
M-CSF	Macrophage colony stimulating factor
MFU	Mean fluorescent units
MHC	Major histocompatibility complex
MIP	Macrophage inhibitory protein
MMP	Matrix metalloproteinase
MR	Mannose receptor
NADPH	nicotinamide adenine dinucleotide phosphate
NK	Natural Killer
NET	Neutrophil extracellular trap
NO	Nitric oxide
NOD	Nucleotide-binding oligomerisation domain
OSM	Oncostatin M
PAMP	Pathogen associated molecular pathogen

PD	Peritoneal dialysis
pDC	Plasmacytoid dendritic cell
PE	Phycoerythrin
PerCPCy5.5	Peridinin–chlorophyll–protein complex–cyanine 5.5
PI3K	Phosphotidylinositol-3-kinase
PMA	Phorbol 12-myristate 13-acetate
PMN	Polymorphonuclear cells
PPR	Pattern recognition receptor
RANKL	Receptor activator for nuclear factor κB ligand
SCF	Stem cell factor
SDF	Stromal derived factor
SES	*Staphylococcus epidermidis* cell free supernatant
SOCS	Suppressor of cytokine signalling
SOD	Superoxide dismutase
Sos	Son of sevenless
SR	Scavenger receptor
SSC	Side scatter
STAT	Signal transducer and activation of transcription
TAPI	TG-ase activating protease inhibitor
TGF	Transforming growth factor
Th	T helper
Tip DC	Tumour-necrosis factor and inducible nitric-oxide synthase-producing DC
TLR	Toll like receptor
TMB	3,3',5,5'-tetramethylbenzidine
TNF	Tumour necrosis factor
TRAIL	TNF related apoptosis inducing ligand
T-reg	T regulatory
TREM-1	Triggering receptors expressed by myeloid cells
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
WT	Wild type

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Chapter 1: Introduction

Chapter 2: Materials and Methods

CHAPTER 1

General Introduction

1.1 Thesis overview and objectives

Interleukin (IL)-6 is a vital regulator of the inflammatory response and is important for cellular proliferation, differentiation, lymphocyte activation, the acute phase response (Kishimoto *et al* 1995, Dienz *et al* 2009, Stockinger and Veldhoen 2007). Importantly, IL-6 is also essential for the transition from innate to acquired immunity (Jones 2005). In acute inflammation, this process is defined by a precise regulation of leukocyte recruitment and clearance, during which IL-6 is vital for the transition from a neutrophil to a mononuclear cell infiltrate (Hurst *et al* 2001, Jones 2005). Inappropriate regulation of this process can lead to a chronic inflammatory state, characterised by the retention of an activated mononuclear cell population within the affected tissue (Jones 2005). Research has shown that this is characteristic of a number of chronic inflammatory and autoimmune conditions including rheumatoid arthritis and Crohn's disease and a particular problem during persistent or recurrent bacterial peritoneal infections (known as peritonitis), which give rise to inflammation induced tissue damage. Bacterial peritonitis is a common complication in end stage renal failure patients receiving peritoneal dialysis treatment (Davies *et al* 1996). This treatment utilises the peritoneal membrane as a dialysing barrier to remove waste products from the blood (Venkat *et al* 2006). Infections are often triggered during treatment by the introduction of commensal bacteria such as *Staphylococcus spp.* via a catheter. Repeated infections lead to damage and thickening of the peritoneal membrane, compromising its ability to act as a dialysing barrier, and rendering the treatment ineffective (Davies *et al* 1996, Williams *et al* 2002, Di Paolo *et al* 1986, Devuyst *et al* 2002). In a murine model of repeated peritonitis, IL-6 deficient animals are protected from these fibrotic changes indicating that while IL-6 is important for the successful resolution of acute inflammation, its long-term activity may be detrimental to the underlining tissue (Fielding *et al* 2009, submitted, Hurst *et al* 2001, Jones 2005).

Much is known about the role of IL-6 in regulating the recruitment and apoptotic clearance of neutrophils and lymphocytes (Hurst *et al* 2001, McLoughlin *et al* 2003, 2005), however relatively little is known about the roles of IL-6 and related cytokines in determining monocyte activation and trafficking both during the resting state and

inflammation. The aims of this thesis were to characterise monocyte subsets found in the peritoneal cavity, with particular focus on macrophages and dendritic cells, and investigate how their phenotype, activity and trafficking were determined by IL-6 and related cytokines, both homeostatically, and during acute and recurrent peritoneal inflammation. In this respect, the aim was to determine how IL-6 progresses from being an important regulator for the resolution of acute inflammation, to becoming a detrimental cytokine driving the onset of recurrent inflammation induced tissue fibrosis.

1.2 The Immune System and Inflammation

The immune system has evolved into a highly orchestrated defence mechanism that protects the host from infection and disease by identifying and eliminating pathogens and tumor cells. Regulation of this response requires a complex array of specialized cells and biological mediators. The first and most simple are physical anatomical barriers. The main anatomical barrier is the epithelial layer of the skin, which is designed to prevent penetration by infectious agents. However, as organisms cannot be completely sealed against their environments, other systems act to protect the lining of the lungs, intestines, and the urinogenital tract. In the lungs, coughing and sneezing mechanically eject pathogens and other irritants from the respiratory tract. Cilia present in the airways and digestive tract direct a peristalsis motion to remove pathogens that become trapped in the secreted mucous layer, and the flushing action of tears and urine mechanically expel pathogens. Chemical barriers also protect against infection with the secretion of antimicrobial peptides by the skin and respiratory tract, and enzymes in saliva, tears, and breast milk, which all act to target bacterial infections. In the stomach, gastric acid and proteases serve as powerful chemical defenses against ingested pathogens. Finally, within the urinogenitary and gastrointestinal tracts, commensal flora serve as biological barriers by competing with pathogenic bacteria for food and space and, in some cases, by changing the local physiological environment, such as pH or available iron. This reduces the probability that pathogens will be able to reach sufficient numbers to cause illness (Murphy *et al* 2008).

Should pathogens penetrate any one of these multiple barriers, an inflammatory response is triggered. Inflammation can be defined as a complex cellular defence mechanism designed to combat infection or fibrotic damage by balancing a co-ordinate innate and adaptive immunological response. During inflammation, leukocytes migrate from the vasculature into damaged tissues to destroy the agents that potentially can cause tissue injury. When successfully controlled, inflammation results in minimal damage and complete clearance of cellular infiltrate, returning to homeostasis (Murphy *et al* 2008). Inflammation can be sub-defined as being acute or chronic. A single inflammatory episode that is successfully resolved is referred to as an acute inflammatory response.

This is a limited beneficial response. During the acute inflammatory response, the initial leukocyte infiltrate is mostly neutrophilic, which is replaced by a more sustained population of mononuclear cells. This mononuclear infiltrate includes both monocytic cells and lymphocytes (Jones 2005, Topley *et al* 1996). Chronic inflammation can arise as a result of repeated infection, but is also characteristic of many autoimmune conditions, arising when the immune system attacks the body's own tissue. It is defined as a persistent phenomenon that can lead to substantial host tissue injury or damage as a result of the immunological efforts to counteract the recurrent or persistent infections, which can be associated with leukocyte infiltrate retention and reduced resolution of the inflammatory response. Chronic inflammation is often defined by a much larger neutrophil infiltrate, which is much more closely followed by an elevated mononuclear cell infiltrate which is consequently retained for much longer periods of time (Gabay *et al* 2006).

The inflammatory response plays three essential roles in the combat of infection. First it allows the recruitment of additional effector molecules and cells to the site of infection to augment the killing of invading microorganisms. The second is to prevent dissemination of the infection to other tissues and the third is to promote tissue injury repair. Usually this response is coordinated both by a stromal compartment, which can release inflammatory mediators to attract effector cells, usually blood leukocytes, which comprise the haematopoietic compartment.

1.3 Innate and acquired immunity - two arms of the immune response

Generally, the immune response can be characterised by two stages termed the early innate and late acquired immune response. The innate and acquired arms of the immune response are in no way separate entities, but are linked, and interact to co-ordinate the clearance of bacteria, prevention of tissue damage, apoptotic removal of leukocytes and resolution of the infection, as well as promoting tissue injury repair.

Innate immunity provides a rapid yet generally non-specific response. This early innate immune response stems the rapid expansion of microbial infections and provides many

components for the induction of the more specific adaptive immune response. Here, the immune system "adapts" its response to one with an improved recognition of the specific pathogen, and this recognition is retained after the pathogen has been eliminated, which is known as immunological memory. This allows the immune system to mount a quicker and stronger response to the pathogen when it is re-encountered. The innate and acquired immune responses are linked, with recent studies endorsing a role for the innate immune system in driving acquired immunity, so that they work together to orchestrate an efficient and competent combat against infection. Both arms of the immune response are discussed in Sections 1.4 and 1.6.

1.4 Innate Immunity

The innate immune response is the most highly conserved mechanism of host defence, and basic innate immune type responses are observed in plants, fish, reptiles and insects. Innate immunity is therefore the initial host response to invading microbes, and prevents their further dissemination. The innate immune system is comprised of cellular components and secretory molecules, which play a role in antigen non-specific defence mechanisms. These innate immune regulators are fixed and do not change. The secretory components include cytokines and chemokines, which are discussed in Section 1.14 onwards. The cellular components of the innate immune system include phagocytic cells such as neutrophils, eosinophils, monocytes and macrophages, and cells that release inflammatory mediators such as eosinophils, neutrophils, macrophages, mast cells, basophils, and natural killer (NK) cells (Murphy *et al* 2008). Specific cells of the innate immune response are discussed further in Sections 1.5 and 1.10.

1.5 Polymorphonuclear cells: Neutrophils

Neutrophils are a common leukocyte in the blood and form an essential part of the innate immune system. They are characterized by their multi-lobed nuclei, which phenotypically distinguish polymorphonuclear cells. Originating from the bone marrow, maturing neutrophils are released into the blood where they circulate to monitor the micro-environment and peripheral tissues. Neutrophils have a short half-life and are replaced every three days. In this respect, once a neutrophil leaves the circulation and

enters the tissue, for example during an inflammatory response, they are programmed to die by apoptosis (Akgul *et al* 2001).

Neutrophils are recruited to the site of inflammation via trans-migration across the endothelial blood vessel wall into the underlining damaged or infected tissue. The initial contact between circulating neutrophils and the endothelium is established via selectins and their ligands, which results in neutrophils rolling along the activated endothelium. It is thought that this contact signals the neutrophils to mobilise their secretory vesicles, which then transport CD11b to the cell surface. This enhances firm adhesion of the neutrophil to the endothelium, arresting rolling, and allowing the neutrophil to migrate across the endothelial wall, a process referred to as diapedesis (Borregard and Cowland 1997).

Neutrophils possess highly effective microbicidal mechanisms for killing of pathogens. Neutrophils can phagocytose microbes, resulting in the formation of a phagosome enclosing the microbe, into which reactive oxygen species and hydrolytic enzymes are released to kill the micro-organism (Dale *et al* 2008, Borregard and Cowland 1997, Faurschou and Borregaard 2003). This killing is further enhanced by the secretion of additional anti-microbial enzymes, and peptides such as β-defensins. Neutrophils possess many granules, which start to form during neutrophil maturation, and contain anti-microbial proteins like those described above. The contents of these granules are secreted into the surrounding environment via a process known as degranulation, to combat infection (Akgul *et al* 2001).

It has recently been observed that activation of neutrophils causes the release of web-like structures of DNA known as neutrophil extracellular traps (NETs), which comprise a web of fibres composed of chromatin and serine proteases that trap and kill microbes extracellularly. This activation induced release of NETs by neutrophils has been shown to be induced by TLR4 activated platelets, which have been seen to bind to the surface of the neutrophils, and result in NET formation (Clark *et al* 2007). It has been suggested that NETs provide a high local concentration of antimicrobial components and bind,

disarm, and kill microbes independent of phagocytic uptake. In addition to their possible antimicrobial properties, NETs may serve as a physical barrier that prevents further spread of pathogens (Brinkmann *et al* 2004).

In addition to their role in innate immunity, neutrophils also release a number of inflammatory cytokines, including Tumour Necrosis Factor (TNF)-α and Interleukin (IL)-1β, and chemokines such as IL-8, Macrophage Inhibitory Factor (MIP)-1α, MIP-1β and Interferon-γ-inducible Protein (IP)-10. The chemokines IL-8, MIP-1α, MIP-1β and IP-10 are responsible for the further recruitment of neutrophils, as well as the subsequent accumulation of monocytes, immature dendritic cells (DC) and T lymphocyte subsets (Scapini *et al* 2000). A review of cytokines and chemokines is found in Sections 1.14 and 1.15.

1.6 Acquired immunity

Acquired or adaptive immunity refers to the antigen *anti*body *gen*erating specific response by lymphocytes, providing the defence mechanism that develops a few days following infection. Cellular mechanisms involved in acquired immunity adapt to the pathogen or antigen and consequently take time to develop. This more specialized immune response triggers immunoglobulin production and drives "immunological memory" (Murphy *et al* 2008).

1.7 Cell mediated immunity

Induction of the adaptive immune response begins with the phagocytosis of a pathogen by an antigen presenting cell (APC) (e.g. Langerhans cells, dendritic cells, macrophages or B cells), and subsequent presentation of antigen on the cell surface. On doing this, the APC then migrates to the peripheral lymphoid tissues where it matures into an effective antigen-presenting cell. Here it presents antigen to naïve T lymphocytes, which, once stimulated, proliferate and differentiate into their fully functional form of T helper (Th) cells of which there are currently four main known subtypes, Th1, Th2, Th17 and T regulatory cells (Zhu and Paul 2008). While the T helper pool is currently sub-classified into distinct groups, it is thought that these subsets may display a degree of plasticity

suggesting that the environment may impact the expansion of these subsets (O'Shea *et al* 2009, Weaver *et al* 2009).

Antigens are presented on the surface of APC by Major Histocompatibility Complex (MHC) molecules. There are two different kinds of MHC molecules known as MHC class I and MHC class II, which obtain peptides at different cellular locations. MHC I molecules bind peptides from the cytosol such as viral and self-antigens, which are then recognized by $CD8^+$ T lymphocytes. MHC II molecules bind peptides generated in intracellular vesicles such as phagosomes, and these are recognized by $CD4^+$ T lymphocytes. MHC I is expressed on all nucleated cells whereas MHC II is only expressed on professional antigen presenting cells such as dendritic cells, macrophages and B cells (Chaplin 2003). This thesis deals exclusively with MHC II mediated antigen presentation to $CD4^+$ T cells.

1.8 $CD4^+$ T helper subsets within the immune system

1.8.1 T helper cells

T helper (Th) cells are a set of differentiated lymphocytes that play an important role in establishing and maximizing the capabilities of the immune system. These cells have no cytotoxic or phagocytic activity; they cannot kill infected host cells or pathogens, and without other immune cells they would usually be considered useless against an infection. T helper cells are involved in activating and directing other immune cells, and are particularly important in the immune system. They are essential in determining B cell antibody class switching, in the activation and growth of cytotoxic T cells, and in maximizing bactericidal activity of phagocytes such as macrophages (Murphy *et al* 2008).

1.8.2 T helper 1 (Th1) cells

Th1 cells mediate immune responses to intracellular pathogens and in humans are particularly important for the resistance to mycobacterial infections. Their differentiation and commitment from naive T helper cells is driven by IL-12 (Hsieh *et al* 1993).

The main cytokines produced by these cells are interferon-γ (IFN-γ), lymphotoxin α and Interleukin (IL)-2. IFN-γ is important for the activation of macrophages to increase their microbicidal activity, IL-2 is important for T cell proliferation (Moretta *et al* 1982), $CD4^+$ T cell memory, and IL-2 stimulation of CD8 cells during their priming phase, which is important for CD8 memory formation (Zhu and Paul, 2008). Th1 cells also contribute to autoimmune pathology, and are therefore an important target for the treatment of various autoimmune diseases.

1.8.3 T helper 2 (Th2) cells

Th2 cells mediate immune responses to extracellular pathogens such as helminths, and are also important in the induction and persistence of asthma and allergic diseases. Th2 cells produce cytokines such as IL-4, IL-5, IL-9, IL-10, IL-13 and IL-25. IL-4 mediates IgE class switching in B cells, IL-5 is crucial for recruitment of eosinophils, and IL-9 induces mucin production in epithelial cells during allergic reactions. IL-10 suppresses Th1 cells and dendritic cell function and IL-13 is responsible for helminth expulsion (Zhu and Paul, 2008). Both Th1 and Th2 cells can activate B cells to produce antibodies in what is known as humoral immunity (see Section 1.9)

1.8.4 T helper 17 (Th17) cells

IL-17-secreting T cells (Th17) are a recently discovered subset of T helper cells whose differentiation is driven by IL-6, Transforming Growth Factor (TGF) β and IL-1β (Bettelli *et al* 2006, 2007, Aliahmadi *et al* 2009), and their effector functions are known to be maintained by IL-23 (Veldhoen *et al* 2006). Th17 cells mediate immune responses against extracellular bacteria and fungi. These cells produce IL-17A and IL-17F, IL-21 and IL-22. IL-17A can induce many inflammatory mediators, cytokines and chemokines and so plays an important role in inducing the inflammatory response. IL-17A and IL-17F also recruit and activate neutrophils during the immune response to bacteria and fungi (Witowski *et al* 2000). IL-21 made by Th17 cells is a stimulatory factor for Th17 differentiation and serves as a positive feedback mediator as IFNγ does for Th1 cells and IL-4 does for Th2 cells (Zhu and Paul, 2008, Stockinger and Veldhoen 2007). IL-22 has been shown to act co-operatively with IL-17 to enhance the expression of anti-microbial

peptides important for host defence (Liang *et al* 2006). Importantly Th17 cells have been implicated in a number of autoimmune diseases with a direct link to tissue injury (Langrish *et al* 2005).

1.8.5 T regulatory cells (T-regs).

T regulatory cells are a specialised subpopulation of T cells, which play a critical role in maintaining self tolerance as well as regulating immune responses and maintaining immune system homeostasis. Natural regulatory T cells express the cell-surface marker CD25 and the transcriptional repressor FOXP3 (forkhead box P3). These cells mature and migrate from the thymus and constitute 5–10% of peripheral T cells in normal mice. Other populations of antigen-specific regulatory T cells can be induced from naive $CD4^+CD25^-$ or $CD8^+CD25^-$ T cells in the periphery under the influence of semi-mature dendritic cells, IL-10, TGFβ and possibly IFN-γ. The inducible populations of regulatory T cells include distinct subtypes of $CD4^+$ T cell: T regulatory 1 (T_R1) cells, which secrete high levels of IL-10, no IL-4 and no or low levels of IFN-γ; and T helper 3 (T_H3) cells, which secrete high levels of TGF-β (Mills 2004). The expansion and activity of T-regs is suppressed by IL-6, which is secreted from antigen presenting cells (Pasare and Medzhitov 2003). T-regulatory cells exert their suppressive functions through several mechanisms, some of which require cell-cell contact, and some are through their production of certain immunosuppressive cytokines such as TGF-β, IL-10 (Section 1.17.1) and IL-35 (Zhu and Paul, 2008).

1.9 Humoral immunity

The humoral immune response protects extracellular regions by the production of antibodies by B cells. Naïve B cells are activated by antigen and usually require the presence of helper T cells, which activate B cells that recognise the same antigen. B cells capture their cognate antigen through their B cell receptor, which is comprised of monomeric membrane immunoglobulin (Ig) in the form of IgD or IgM. Upon antigen binding B cells then internalise the antigen for intracellular processing and presentation on the cell surface, coinciding with an up-regulation of co-stimulatory molecules CD80 and CD86. T cells recognising the same specific antigen will bind, and signal

reciprocally to the B cell by the interaction of T cell CD40 ligand with B cell CD40. Upon activation the B cell differentiates and matures into an antibody secreting plasma cell. Signalling via the CD40 ligand and cytokines produced by the activating T cell are able to control the isotype of Ig produced by B cells (known as isotype switching). This is characterised by the conversion of IgM to either IgA, IgD, IgG or IgE. Isotype switching is strongly associated with the development of B cell memory, defined by the rapid induction of high levels of high affinity antibody following secondary antigen challenge (Chaplin 2003).

The antibodies secreted by these cells perform a number of functions. The first is called "neutralisation", which acts by antibodies binding to the pathogen to prevent them from binding to cell surface markers, which they do to invade cells. The second way antibodies act is by binding to bacteria outside the cell to prevent them from multiplying by facilitating their uptake by phagocytosis. This coating of the pathogen to enhance phagocytosis is known as opsonisation. The third mechanism of action by antibodies is the triggering of the complement cascade upon binding a pathogen, the terminal components of which can lyse the microorganism by the formation of pores in their membrane (Chaplin 2003).

B cells can also be activated independently of T cell help, by polymeric antigens with a repeating structure, which have the ability to cross link and cluster Ig molecules on the B cell surface. An example of such antigens is bacterial lipopolysaccharides (Chaplin 2003).

1.10 Mononuclear phagocytes: monocytes

Monocytes are a member of the mononuclear phagocyte system, which is important for non-specific defence against pathogens, tumour recognition and immunoregulation (Grage-Griebenow *et al* 2001). Monocytes are derived from dividing monoblasts ($CD34^+$ myeloid progenitors) within the bone marrow, and are released into the bloodstream as non-dividing cells (Geissman *et al* 2003). Newly produced monocytes circulate in the blood stream for 1-3 days and then enter tissues where they perform an integral role in local tissue defence (see Figure 1.1) (Taylor and Gordon 2003). Monocytes have the capacity to differentiate into either macrophages or dendritic cells (DC), following appropriate cytokine activation, suggesting that these cells probably constitute a considerable systemic reservoir of myeloid precursors. This theory is supported by the fact that 50% of circulating monocytes leave the circulation under steady state conditions each day (Geissman *et al* 2003).

1.10.1 Murine monocytic cells

Murine blood monocytes have bean shaped nuclei and are defined by the expression of F4/80, CD11b and CD11c. Adoptive transfer experiments in mice have shown that there are two distinct populations of blood monocytic cells which are equally represented in the blood: a short lived "inflammatory" subset that migrate to inflamed tissue where they can trigger immune responses, and a "resident" subset with a longer life that home to non-inflamed tissues (Geissman *et al* 2003). These subsets are further defined by the expression of particular markers, with the inflammatory subset characterised as $CX_3CR1^{lo}CCR2^+Gr1^+$, whilst the resident subset are $CX_3CR1^{hi}CCR2^-Gr1^-$. Gr1 refers to an antibody that recognises two epitopes, Ly6C and Ly6G, which are glycosylphosphotidylinositol (GPI) linked proteins, and are generally recognised as differentiation markers for certain cell types including monocytes. The monocytic subsets described have been defined as Gr1 positive or negative based on their expression of Ly6C, and have been referred to as either $Gr1^{+/-}$ or $Ly6C^{+/-}$ in subsequent literature (Geissman *et al* 2003, Gordon and Taylor 2005, Sunderkotter *et al* 2004). From herein, they will be referred to as $Gr1^{+/-}$ subtypes. Further characteristic features of these subsets are presented in Table 1.1.

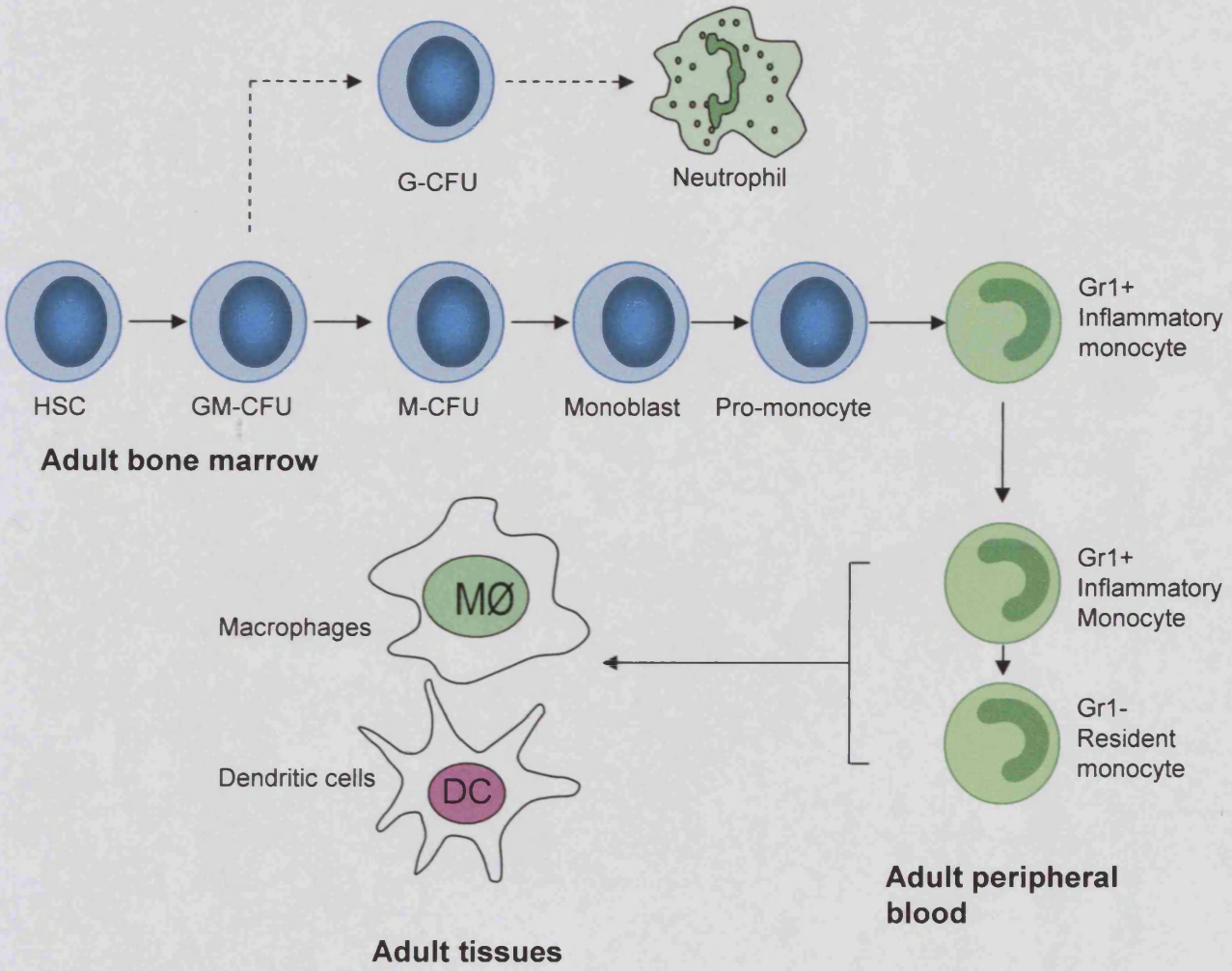


Figure 1.1: *Development of monocytes from bone marrow precursor cells, and subsequent differentiation into macrophages and dendritic cells.* HSC: Haematopoietic stem cell, GM-CFU: Granulocyte/monocyte colony forming unit, G-CFU: Granulocyte colony forming unit, M-CFU: monocyte colony forming unit.

Both subsets have been shown to have the potential to differentiate into dendritic cells *in vivo*, with the inflammatory subset being able to do so in inflamed tissue where they can initiate naïve T cell proliferation (Geissman *et al* 2003). It is also believed, however not supported, that both subsets have the capacity to generate tissue resident or inflammatory macrophages. The 'inflammatory' subset derived its name following experiments by Geissman *et al* in 2003 who showed that when this subset was adoptively transferred into mice with experimentally induced peritonitis, it infiltrated the peritoneal cavity, and often acquired CD11c expression, while MHC II expression by these cells was shown *in vitro*, demonstrating their differentiation into antigen presenting DC (Geissman *et al* 2003). When the inflammatory subset was adoptively transferred into naïve mice however, they remained undetectable in nearly all tissues except the spleen, unlike the 'resident' subset, which were widely disseminated in the blood, spleen, lung, liver and brain of recipient mice for several days, demonstrating an ability by the resident subset to replenish tissue resident myeloid populations. The distribution of the adoptively transferred resident subset did not greatly alter in mice with induced peritonitis, reinforcing its identification of a resident monocytic subset (Geissman *et al* 2003). Geissman and colleagues also showed that some of the donor resident monocytic cells recovered from the spleen had developed a DC-like phenotype following adoptive transfer into naïve mice (Geissman *et al* 2003), consequently resident monocytic cells can enter tissues and acquire a DC like phenotype under steady state conditions. As a result it has been proposed that the 'resident' subpopulation traffic into tissues in the resting state in a CX_3CR1 dependent manner, where they differentiate into either resident macrophages or DC under the control of the local cytokine network (Geissman *et al* 2003).

The resident monocyte population has also recently been assigned another function whereby they have been observed to 'patrol' healthy tissues through long range crawling on the resting endothelium lining the blood vessels, allowing for a rapid monocyte response following damage or infection. This patrolling behaviour is dependent on the integrin LFA-1 and the chemokine receptor CX_3CR1 and is required for rapid tissue invasion by the "resident" monocytes at the site of infection or tissue damage (Auffrey *et al* 2007). Once in the tissue, an immune response is initiated, and they differentiate into

macrophages (Auffrey *et al* 2007). Activation of an inflammatory response therefore gives rise to waves of monocyte recruitment, with patrolling resident monocytes arriving prior to the recruitment of $Gr1^+$ 'inflammatory' monocytes which then go on to differentiate into inflammatory macrophages or DC (Auffrey *et al* 2007).

Work by Sunderkotter *et al* has uncovered a third population of monocytic cells in the circulation. This group identified a rare population of Gr1 intermediate monocytes, which they have defined as $CCR2^+$ $Gr1^{int}$. The work done by this group suggests that the $CCR2^-$ $Gr1^-$ "resident" monocyte population may derive from the $CCR2^+$ $Gr1^+$ "inflammatory" population, via this intermediary $CCR2^+$ $Gr1^{int}$ subset (Figure 1.2) (Sunderkotter *et al* 2004). This was concluded from observations that the first of the monocyte subsets to appear in the circulation is the $CCR2^+$ inflammatory subset and in the absence of inflammatory stimuli, these monocytes differentiate into the resident subset, via the intermediate subset they describe (Sunderkotter *et al* 2004). This intermediate subset can also respond to pro-inflammatory cues and migrate to inflamed tissues and differentiate into macrophages or DC. This population is associated with selective expression of CCR7 and CCR8 and retention of CCR2, making them uniquely disposed to emigrate to the draining lymphatic system, where they have the potential to acquire DC properties (Qu *et al* 2004).

1.10.2 Human monocytic cells

Humans also have two subsets of blood monocytes, which are believed to be equivalent to the mouse subsets described in Section 1.10.1. These populations are defined by the differential expression of CD14 (a co-receptor associated with TLR4 activation by lipopolysaccharide) and CD16 (an Fc-gamma receptor FcγRIII). The so-called "classical" $CD14^+CD16^-$ monocytes represent approximately 95% of the monocytic cells in a healthy individual, with a "non-classical" $CD14^{lo}CD16^+$ population comprising the remainder. A further characteristic description of these cells is presented in Table 1.1.

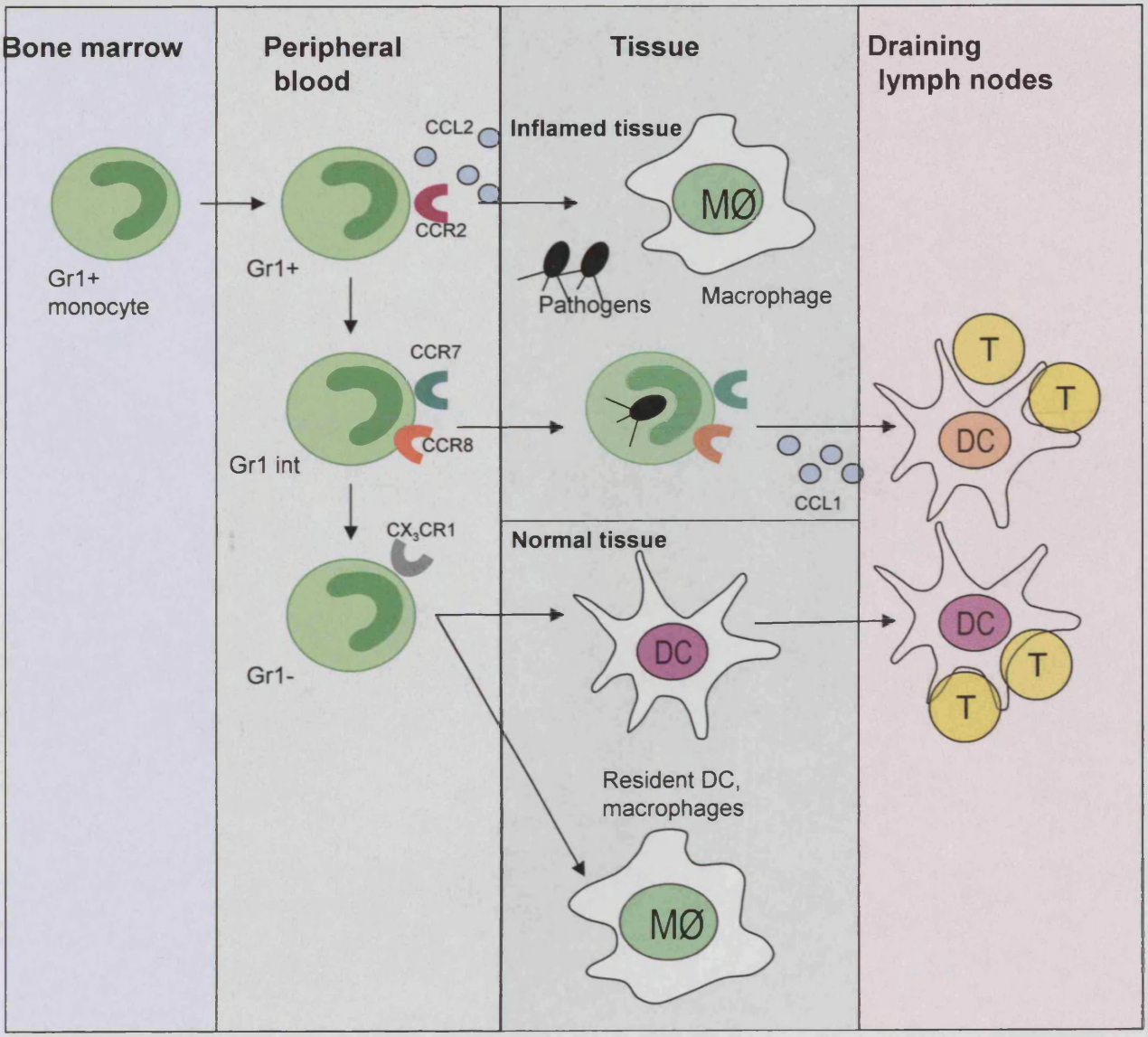


Figure 1.2: *Differentiation of resident and inflammatory macrophages and dendritic cells from Gr1$^+$ monocytes.* Resident DC and macrophages differentiate from Gr1$^-$ monocytes that develop from Gr1$^+$ monocytes via a Gr1^{int} intermediate. Gr1$^{+/int}$ monocytes give rise to inflammatory macrophages and DC upon inflammatory stimuli. Adapted from Gordon and Taylor, 2005.

These subsets differ in a number of aspects including adhesion molecule and chemokine receptor expression. $CD14^+CD16^-$ monocytes express CCR2, CD62L and low CX_3CR1 and are thought to be the human equivalent of the murine $CX_3CR1^{lo}CCR2^+Gr1^+$ subset, whereas the $CD14^{low}CD16^+$ monocytes lack CCR2 and have higher levels of MHCII and CX_3CR1, and are equivalent to the murine $CX_3CR1^{hi}CCR2^-Gr1^-$ subset (Tacke and Randolph 2006, Geissman *et al* 2003). The $CD14^+CD16^-$ population and the murine $CX3CR1^{lo}CCR2^+Gr1^+$ counterpart both have a high forward and side scatter profile when analysed by flow cytometry, whereas the $CD14^{low}CD16^+$ population and its murine $CX_3CR1^{hi}CCR2^-Gr1^-$ equivalent are smaller and less granular (Geissman *et al* 2003).
There is evidence to suggest that the $CD14^{low}CD16^+$ population are precursors of DC given their predisposition to become DC *in vitro* compared to the $CD14^+CD16^-$ population (Randolph *et al* 2002). This does not however rule out the $CD16^-$ population acting as DC precursors *in vivo*. A further intermediary $CD14^+CD16^+$ population of human monocytes was also recently identified, which is characterised by the expression of CCR5 (Tacke and Randolph 2006).

Interest in $CD16^+$ monocytes originally arose due to their elevation in the blood during inflammatory conditions, hence these cells are often referred to as inflammatory monocytes (Ziegler-Heitbrock 2007). This was compounded by the fact that this population produce lower levels of IL-10 in response to Toll-like Receptor (TLR)-4 stimulation, and higher levels of TNF-α in response to TLR-2 and -4 stimulation (Frankenberger *et al* 1996, Belge *et al* 2002). However this remains controversial since data presented by Zeigler-Heitbrock *et al* showed that the $CD14^{low}CD16^+$ subset produce less TNFα, IL-1 and IL-6 than the $CD16^-$ subset of human monocytes (Zeigler-Heitbrock *et al* 1992). Consequently, many refrain from defining the $CD16^+$ monocytic subset as an inflammatory population until further characterisation of these cells *in vivo* has been undertaken, especially since the $CD16^+$ population do not express CCR2 (Weber *et al* 2000) and have an enhanced capacity for transendothelial migration (Randolph *et al* 2002). This level of caution reflects the contrasting function displayed by their murine equivalent, "resident" $CX_3CR1^{hi}CCR2^-Gr1^-$ population, which show limited migration into sites of inflammation *in vivo* as compared to the $CX_3CR1^{lo}CCR2^+Gr1^+$ subset (Tacke

and Randolph 2006). Based on this, many refer to the $CD14^{+}CD16^{-}$ population as the inflammatory subset due to their murine equivalent being the "inflammatory" $CX_3CR1^{lo}CCR2^{+}Gr1^{+}$ population.

Antigen	Human $CD14^{hi}$ $CD16^{-}$ 'inflammatory' monocytes	Human $CD14^{+}$ $CD16^{+}$ 'resident' monocytes	Mouse $CCR2^{+}$ $Gr1^{+}$ CX_3CR1^{lo} 'inflammatory' monocytes	Mouse $CCR2^{-}$ $Gr1^{-}$ CX_3CR1^{hi} 'resident' monocytes
CCR1	+	-	ND	ND
CCR2	+	-	+	-
CCR4	+	-	ND	ND
CCR5	-	+	ND	ND
CCR7	+	-	ND	ND
CD11b	++	++	++	++
CD11c	++	+++	-	+
F4/80	ND	ND	+	+
CD14	+++	+	ND	ND
CD80	ND	ND	ND	ND
CD86	+	++	ND	ND
Ly6C	ND	ND	+	-
7/4	ND	ND	+	-
MHC II	+	++	-	-
CD62L	++	-	+	-

Table 1.1: *Comparison of human and mouse subsets by their phenotypic characterisation.* Data have been assigned arbitrary symbols: – refers to no expression of the antigen, + refers to low expression, ++ refers to medium expression, +++ refers to high expression and N.D refers to expression not determined. Adapted from Gordon and Taylor 2005.

1.11 Mononuclear phagocytes: Macrophages and dendritic cells

Traditionally, the cellular network comprising peripheral mononuclear phagocytes has been subdivided into macrophages and dendritic cells. Both of these populations are known to consist of multiple subsets that are defined upon their distinct anatomical location and their phenotypes. Macrophages include tissue specific representatives in the intestinal lamina propria, alveolar, and interstitial spaces of the lung, brain (microglia),

bone (osteoclasts), liver (kupffer cells) and kidneys. Dendritic cells have been divided into lymphoid-resident DC, pre-DC, conventional DC and inflammatory DC (Shortman and Naik 2007).

1.11.1 Macrophage and dendritic cell origin and differentiation

Circulating monocytes give rise to a variety of tissue resident macrophages as well as specialised DC, in both the steady and inflammatory states, which contribute to host defence, tissue remodelling and repair (Gordon and Taylor 2005, Geissman *et al* 2003, Sunderkotter *et al* 2004). As previously described, murine monocytes, identified by their F4/80 and CD11b expression, can be subdivided into two subsets: inflammatory ($CX_3CR1^{lo}CCR2^{+}Gr1^{+}$), and resident ($CX_3CR1^{hi}CCR2^{-}Gr1^{-}$) subsets. The bone marrow derived monocytes ($CCR2^{+}CX_3CR1^{lo}Gr1^{+}$ "inflammatory" subset) are released into the circulation and are recruited to an inflammatory site in the case of injury or infection, where they are believed to differentiate into either macrophages or DC (Geissman *et al* 2003). In the absence of infection, these cells are believed to alter their functional and phenotypic characteristics, passing through an intermediate phenotype ($CCR2^{+}CCR7^{+}CCR8^{+}Gr1^{mid}$), which is also able to migrate to an inflammatory site through CCL1, to the resident population of monocytes ($CCR2^{-}Gr1^{-}CX_3CR1^{hi}$). These are then able to enter tissues and are suggested to replenish tissue resident macrophage and DC populations (Sunderkotter *et al* 2004, Gordon and Taylor 2005). There is also evidence to suggest that many tissue resident populations of macrophages are replenished by local proliferation (Gordon and Taylor, 2005).

Dendritic cells are not only derived from myeloid cells, but also from lymphoid–committed progenitor cells (Figure 1.3). Bone marrow derived haematopoietic stem cells give rise to multipotent progenitors, which then form common myeloid and lymphoid progenitor cells. Myeloid progenitors go on to form granulocytes and monocytes, which, as previously stated, can give rise to macrophages and DC. Lymphoid precursor cells go on to form T and B lymphocytes and NK cells, but can also differentiate into DC (Wu and Liu 2007, Shotman and Naik 2006). DC subtypes are discussed further in Section 1.13.

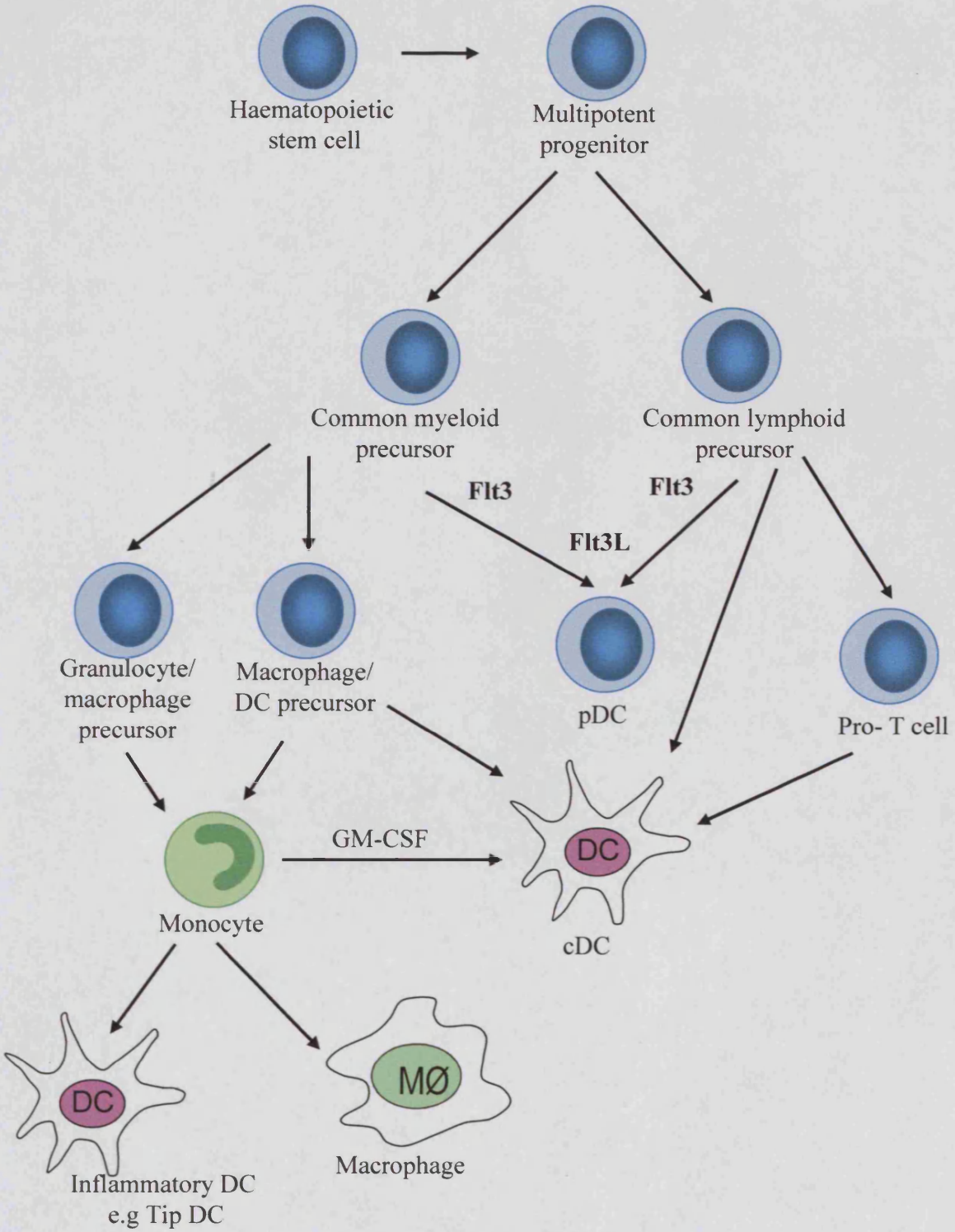


Figure 1.3: *DC subtypes arise from specific precursors.* DC can be both myeloid and lymphoid derived, giving rise to pre-DC such as plasmacytoid DC, and conventional DC as well as monocyte derived DC and Tip DC (see Section 1.13.1).

1.12 Macrophages

Metchnikoff (1893) was the first person to use the term macrophage to describe a large cell able to take up microorganisms. Macrophages are mononuclear phagocytes, which orchestrate the initiation and resolution of the inflammatory response, playing a central role in acute and chronic inflammation. They also play a broad homeostatic role in the clearance of senescent cells and in tissue remodelling and repair following injury and infection (Gordon 1995, Gordon 2003, Gordon and Taylor 2005).

Macrophages are an important part of the innate immune system as illustrated by their ability to recognise, engulf and destroy many potential pathogens including bacteria, pathogenic protozoa, fungi and helminths. They also recognise and are involved in the clearance of tumour cells, some viral infected cells and cells undergoing programmed cell death or apoptosis. Macrophages can also recruit haematopoietic cells to local sites of inflammation and regulate their activities through the secretion of chemokines and cytokines respectively (Gordon 1998). As well as their involvement in primary innate immunity, macrophages also play an important role in adaptive immunity and act as antigen presenting cells. Macrophages therefore provide a link between innate and acquired immunity and the physiological changes that contribute to humoral and cell mediated immunity (Gordon 1998). The specific roles of macrophages are discussed in Section 1.12.2.

1.12.1 Macrophage heterogeneity

Macrophages have long been known as a vastly heterogeneous population with many subsets arising as a result of the specialisation of tissue macrophages in particular micro-environments (Gordon and Taylor 2005). Table 1.2 details the different macrophage populations that are currently known.

Tissue	Specific macrophage Name	Function
Bone	Osteoclast	Bone remodelling and providing a stem cell niche
	Bone marrow macrophage	Erythropoiesis
Brain	Microglial cell	Neuronal survival and connectivity, repair after injury
Epidermis	Langerhans cell	Immune surveillance
Eye	NA	Vascular remodelling
Intestine	Crypt macrophage	Immune surveillance
Kidney	NA	Ductal development
Liver	Kupffer cells	Clearance of debris from blood and liver tissue regeneration after damage
Mammary gland	NA	Branching morphogenesis and ductal development
Ovary	NA	Steroid hormone production and ovulation
Pancreas	NA	Islet development
Testis	NA	Steroid hormone production
Uterus	Uterine DC	Angiogenesis and decidualisation
	Uterine macrophage	Cerival ripening
Peritoneum	Peritoneal macrophage	Immune surveillance

Table 1.2: *Tissue macrophage heterogeneity and function.* Adapted from Pollard 2009.

1.12.2 Macrophage immune function

Macrophages recognise and engulf pathogens *via* a process called phagocytosis using opsonins (antibody, complement) or non-opsonic pattern recognition receptors (PPRs) e.g. Mannose receptors (Examples of membrane bound and intracellular PPRs and their functions are summarised in Table 1.3) (Taylor *et al* 2005). Pattern recognition receptors recognise a limited set of conserved molecular patterns on microbes referred to as pathogen-associated molecular patterns (PAMPs). Macrophages express a range of plasma membrane bound and intracellular receptors that mediate their interaction with natural and altered self-components as well as a range of micro-organisms. Recognition is followed by surface changes, uptake, signalling, and altered gene expression, which all contribute to homeostasis, host defence, innate effecter mechanisms, and the induction of acquired immunity (Taylor *et al* 2005).

Receptor Family	Example	Function (example)
Scavenger (collagenous)	SR-A (Scavenger receptor A)	Phagocytosis of bacteria and apoptotic cells, endocytosis of modified Low Density Lipoprotein (LDL), adhesion.
Scavenger (non-collagenous)	CD36	Phagocytosis of apoptotic cells, diacyl lipid recognition of bacteria.
GPI-anchored	CD14	LPS-binding protein/interactions MD2/MyD88, TLR signalling, apoptotic cell recognition.
Integrin	CR3 (Complement Receptor 3) (CD18/11b)	Complement receptor (C3bi) mediated phagocytosis, adhesion to endothelium
Ig Superfamily	FcR (ITAM/ITIM-Immunoreceptor Tyrosine-based Activation/Inhibition Motif) TREM-1 (Triggering receptors expressed by myeloid cells) (ITAM)	Antibody dependent binding, uptake, killing Regulation of inflammation
Seven transmembrane	CCR2 C5aR (Complement factor) EMR2 (EGF module containing mucin-like hormone receptor, part of the EGF-TM7-seven transmembrane receptor family) f-MLP (formyl Met Leu Phe)	Receptor for MCP-1 Chemotaxis, degranulation, myeloid cell adhesion, chondroitin sulphate binding Formyl peptide receptors
NK-like C-type lectin-like	Dectin-1 (ITAM-like)	β-glucan receptor, fungal particle ingestion, TNFα release/interaction TLR2
C-type lectin (single CTLD)	DC-sign	Mostly DC, pathogen recognition, ICAM adhesion
Multiple CTLD	Mannose receptor (MR)	Clearance, alternative activation, antigen transport?
Toll-like receptors (leucine rich repeats)	TLR2 (Toll-like Receptor) TLR4	Response to peptidoglycan Response to LPS
NOD like receptors	NOD1 (Nucleotide-binding Oligomerisation Domain) NOD2	Intracellular receptors recognising peptidoglycan

Table 1.3: *An overview of macrophage pattern recognition and innate immune receptors implicated in immune recognition.* Adapted from Taylor *et al* 2005.

Surface receptors on macrophages regulate their differentiation, growth, survival, adhesion, migration, activation, phagocytosis and cytotoxicity (Taylor *et al* 2005).

Following phagocytosis, macrophages degrade the proteins and process the antigens for presentation to T cells via MHC class II molecules. In addition to their role in antigen presentation, macrophages also possess microbicidal and tumoricidal activities characterised by their capacity to produce reactive-oxygen and reactive nitrogen metabolites, which kill ingested pathogens by the release of these metabolites into the phagosome. Bacteria are engulfed into a phagosome, a vacuole formed around the ingested material, which then fuses with a lysosome, an organelle containing digestive enzymes. Subunits of the enzyme NADPH (nicotinamide adenine dinucleotide phosphate) oxidase assemble in the lysosome membrane forming an active enzyme. This enzyme then catalyzes the synthesis of superoxide anions, and this activity produces a large increase in oxygen consumption, referred to as the "respiratory burst". Superoxide anions themselves are extremely toxic to pathogens. Superoxide dismutase (SOD) then converts these anions into oxygen and the highly reactive oxygen species, hydrogen peroxide, which kills the engulfed bacteria (Nathan *et al* 1977, Nathan *et al* 1979, Sasada *et al* 1980).

Reactive nitrogen species are produced by the enzyme nitric oxide synthase, which catalyses a reaction between L-arginine, oxygen and NADPH to form nitric oxide, which possesses potent anti-microbial activities (Nathan *et al* 1991). These nitrogen metabolites are released into the phagosome in the same way as reactive oxygen species. Once the ingested bacteria have been neutralised, residual and non-digested material within the phagosome is then expelled by exocytosis. When phagocytosis cannot occur, macrophages are able to release antimicrobial agents into the surrounding space to fight the infection.

Macrophages not only ingest pathogens, they also assist in the clearance of apoptotic cells, both homeostatically and during inflammation. This includes the clearance of

apoptotic neutrophils and other cells that accumulate at sites of inflammation (Fadok *et al* 1998). This process is often associated with the induction of macrophage derived anti-inflammatory mediators, which dampen the inflammatory response before it becomes detrimental to the host (Fadok *et al* 1998). Without this mechanism in place, the failure to clear apoptotic cells and debris at sites of inflammation would lead to exacerbation of inflammation and a predisposition to the development of autoimmunity (Pickering *et al* 2000).

Finally, macrophages are involved in wound healing and tissue repair. Interleukin (IL)-4 and IL-13 are important for this process, and have been shown to stimulate arginase activity allowing macrophages to convert arginine to ornithine, which is a precursor of polyamines and collagen, thereby contributing to the production of extracellular matrix (Deodhar *et al* 1997). Macrophages also contribute to fibrin dissolution, the removal of dead tissue, and the ingrowth of new blood vessels (angiogenesis), as well as regulating fibroblast recruitment, growth and connective tissue remodelling (Gordon 2003).

While macrophages have many beneficial roles that contribute to the clearance of microbial pathogens and tumourogenic cells, tissue homeostasis, remodelling and repair following injury, they have also been shown to have detrimental effects such as causing much of the pathology of infectious, inflammatory and malignant diseases. Many chronic inflammatory conditions are characterised by an increased and retained mononuclear infiltrate (Jones 2005, Nowell *et al* 2003, Nowell *et al* 2009), including monocytes/macrophages, which have been shown to contribute to tissue damage as a result of their production of reactive oxygen species and nitric oxide (Duffield *et al* 2000, Duffield *et al* 2001, Kipari *et al* 2002, Duffield *et al* 2001).

1.12.3 Peritoneal "resident" and "inflammatory" macrophages

As described in Section 1.12.2, macrophages display a high degree of functional and phenotypic heterogeneity, with a wide range of phenotypes observed *in vivo*, each of which adapt to their local environment to perform specific functions.

In the peritoneal cavity, a population of macrophages exists known as the "resident" macrophages. These adapt to their local environment to perform specific functions, which include tissue homeostasis through the clearance of senescent cells and remodelling and repair of tissues following inflammation (Taylor *et al* 2005). Peritoneal macrophages are the most studied primary macrophages in mice because they are easily isolated by peritoneal lavage. During a mild acute inflammatory response, resident peritoneal macrophages have been shown to combat infection by providing the first line of defence against the invading pathogen (Dunn *et al* 1985). They have also been shown to be essential for sensing peritoneal injury, and initiating the recruitment of polymorphonuclear cells through the production of neutrophil activating CXC chemokines (Cailhier *et al* 2005).

During a more severe acute inflammatory response however, or when a disturbance occurs in the tissue normality, be it infection, normal cell turnover, wounding, immune response or malignancy, infiltrating monocytes are also recruited to the cavity. These monocytes go on to differentiate into inflammatory macrophages or DC, contributing to host defence, tissue remodelling and repair (Gordon 2007, Gordon and Taylor 2005). This process is facilitated by resident macrophages, which constitute an important source of inflammation-related factors such as chemokines and Matrix Metalloproteinase (MMP)-9, and coordinate leukocyte extravasation and trafficking into the peritoneal cavity (Kolaczkowska *et al* 2007). The rapidly recruited macrophages are appropriately named recruited or activated macrophages. These recruited macrophages often exhibit many phenotypic differences from resident tissue macrophages, and this depends on the nature of the recruiting stimulus and the tissue location and environment (Gordon 1995). Recruited macrophages are generally ascribed a greater destructive potential than resident macrophages (Gordon 1995), and are important for the propagation and resolution of inflammation (Taylor *et al* 2003).

It is currently unclear what happens to recruited macrophages once the recruiting stimulus has been resolved. One possibility is that many of the recruited macrophages die *in situ*. Apoptotic cells are a stimulus for macrophage recruitment (Messmer 2000),

and it could be considered these macrophages may be cleared by newly elicited resident macrophages. Inflammatory macrophages also enter the draining lymphatics where they could either enter apoptosis or acquire antigen-presenting functions (Bellingan *et al* 1996). There is no current evidence of macrophages being recycled back into the circulation as reverted monocyte-like cells.

1.12.4 Macrophage activation

In vitro studies have shown the ability of elicited macrophages to acquire distinct phenotypes and physiological activities (Figure 1.4). There are currently four suggested modes of macrophage activation that have been defined *in vitro*: 1) **Classical activation** is induced by lipopolysaccharide (LPS) and interferon (IFN)-γ, and is associated with high microbicidal activity, pro-inflammatory cytokine production and cellular immunity; 2) **Innate activation**, triggered by TLR ligands, is associated with microbicidal activity and pro-inflammatory cytokine production; 3) **Alternative activation**, triggered by IL-4 or IL-13 stimulation, is associated with tissue repair and humoral immunity; 4) **Deactivation**, triggered by IL-10 or TGF-β, or ligation of inhibitory receptors such as CD200 and CD172a, is associated with anti-inflammatory cytokine production, and reduced MHCII expression (Mosser 2003, Gordon 2003, Gordon and Taylor 2005, Taylor *et al* 2005).

1) Classically activated macrophages are activated by IFN-γ and TNF, and they have enhanced microbicidal or tumouricidal capacity and secrete high levels of pro-inflammatory cytokines and mediators. IFNγ is produced by innate and adaptive immune cells, with NK cells being an important innate early source of this cytokine. The IFN-γ produced by NK cells primes macrophages to secrete pro-inflammatory cytokines, and produce increased levels of superoxide anions and oxygen and nitrogen radicals, which increases their microbial killing ability (Dale *et al* 2008). The production of IFN-γ by NK cells is generally short lived and they cannot sustain a population of activated macrophages. Consequently an adaptive immune response is usually required to maintain these classically activated macrophages and provide prolonged host defence against invading pathogens (Mosser and Edwards 2008).

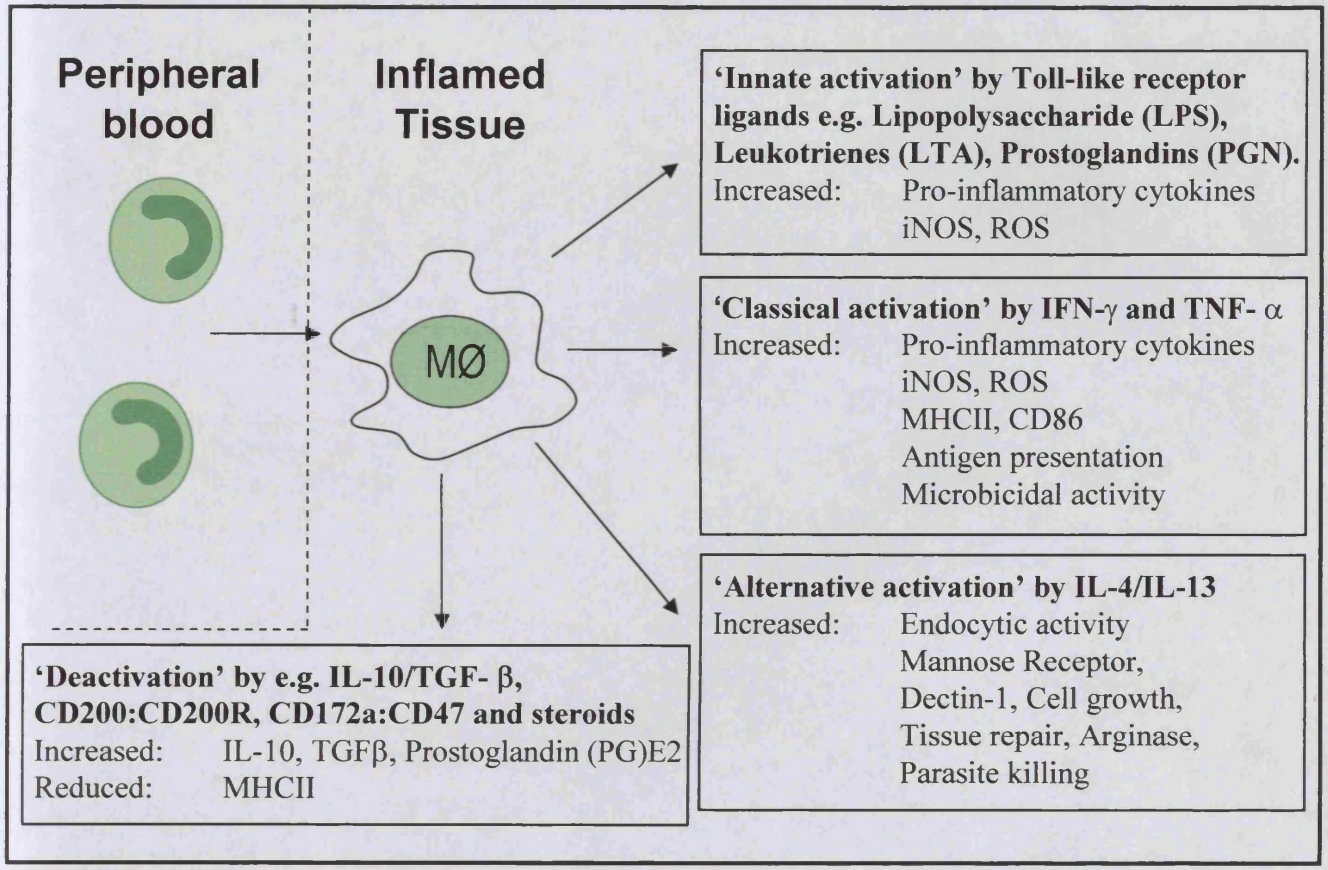


Figure 1.4: *Macrophages exhibit four modes of activation in vitro, upon inflammatory stimuli.* Innate activation is driven by TLR stimulation, triggering a pro-inflammatory macrophage action. Classical activation is driven by IFN-γ and TNF-α stimulation, and also triggers a pro-inflammatory macrophage response. Alternative activation is driven by IL-4/13 stimulation, triggering tissue repair and parasite killing. Deactivation is driven by IL-10/TGF-β and CD200:CD200R stimulation, leading to an anti-inflammatory effect on the macrophage. Adapted from Gordon and Taylor 2005.

The second signal, TNF is usually released endogenously as a result of macrophage TLR ligation. Thus, classically activated macrophages are developed in response to IFN-γ in combination with exposure to a microbe or microbial product such as LPS (Mosser 2003). These macrophages produce IL-12 and IL-23 upon IFN-γ stimulation, which are involved in the Th1 cell mediated immune resolution of infection (Gordon 2003). They are also easily identified by their production of nitric oxide. These activated macrophages migrate to sites of inflammation where they encounter pathogens and degrade them. They have enhanced capacity to kill and degrade intracellular microorganisms, accomplished by an increase in the production of toxic oxygen species and nitric oxide (Mosser 2003). Unfortunately, whilst the pro-inflammatory cytokines produced by classically activated macrophages are an important component of host defence, they can also cause extensive host damage. IL-1, IL-6 and IL-23 produced by these cells are important for the development and expansion of Th17 cells which produce IL-17, a cytokine that is associated with a high level of PMN recruitment to tissues, which can contribute to inflammatory autoimmune pathologies (Langrish *et al* 2005).

2) Innate activation of macrophages is triggered when microbial stimuli are recognised by pattern recognition receptors such as TLRs, CD14/LPS binding protein and a range of non-opsonic receptors. These stimuli induce the production of pro-inflammatory cytokines such as IFN-α/β, reactive oxygen species and nitric oxide, followed by a regulated anti-inflammatory response. Enhanced co-stimulatory surface molecules also favor antigen presentation, and scavenger receptor-A and mannose receptor promote phagocytosis and endocytosis of host and exogenous ligands (Gordon 2003).

3) Alternatively activated macrophages are generally produced in Th2 type responses, particularly in allergic, cellular and humoral responses to parasitic and extracellular pathogens. IL-4 and IL-13 up-regulate expression of mannose receptor and MHC class II in macrophages, which stimulates endocytosis and antigen presentation, and they induce the expression of selective chemokines and intracellular enzymes such as arginase, which are implicated in cell recruitment and granuloma formation (an organised collection of macrophages). Alternatively activated macrophages have impaired ability to produce

nitrogen radicals, making them relatively poor at killing intracellular pathogens, and even though they have up-regulated MHCII expression, they do not exhibit efficient antigen presentation, and in many instances, they inhibit T cell proliferation (Mosser 2003). Alternatively activated macrophages have been shown to produce a number of components involved in the synthesis of the extracellular matrix, including fibronectin and matrix associated proteins, as well as promoting fibrogenesis from fibroblastoid cells, and so it is thought that their primary role is involved in fibrosis and tissue repair (Gordon 2003, Mantovani *et al* 2004, Mosser 2003).

4) Deactivation of macrophages can be either innate or acquired in origin. The uptake of apoptotic cells or lysosomal storage of host molecules will generate anti-inflammatory responses. Certain pathogens can also deactivate macrophages by various mechanisms (Gordon 2003).

While these four modes of activation have been described *in vitro*, it is thought that the activity of macrophages displays a vast degree of plasticity *in vivo*.

1.13 Dendritic cells

Dendritic cells (DC) were first discovered in 1972 when experiments were designed to characterise "accessory" cells that enhanced immunity in response to antigen. This led to the discovery of unusual stellate cells in the murine spleen, which had the ability to form and retract dendrites and as a result were named dendritic cells (Steinman 2007).

In the steady state, DC reside in both peripheral tissues and lymphoid organs and also circulate in the blood. Dendritic cells are antigen-sampling cells of the peripheral tissue and specialise in antigen capture, processing and presentation to T cells. The function of DC is therefore to prime the expansion of antigen specific T cell responses (Novak *et al* 2008). Dendritic cells are crucial for the development of innate and adaptive immunity against infection, and for the maintenance of immune tolerance to self antigens (immune homeostasis) (Wu and Liu 2007, Steinman *et al* 2003). Dendritic cells represent a sparsely distributed population of bone marrow derived cells and whilst they share many

common features, multiple subsets of DC have been identified with distinct life spans and immunological functions. This heterogeneity arises from the subset-specific expression of distinct surface markers, inflammatory cytokines, and by their location. Tissue micro-environments appear to have a major impact on the function of resident DC, and their localisation determines their phenotype so that they can become highly specialised for their specific function, their trafficking to defined chemokine profiles, and their dependence on inflammatory stimuli for their generation (Wu and Lui 2007 and Wu and Dakie 2004).

1.13.1 Dendritic cell types and subtypes

Dendritic cells are derived from both a Common Myeloid Progenitor (CMP) and a Common Lymphoid Progenitor (CMP), which arise from the bone marrow (Shortman and Naik 2007). Both myeloid and lymphoid progenitors give rise to defined DC subsets (Figure 1.3). Classification of DC subsets is controversial, and many groups describe them differently. Dendritic cell sub-classification is presented here in Sections 1.13.1.2-1.13.1.5, based on descriptions by Shortman and Naik, 2007.

1.13.1.1 Pre-Dendritic Cells (pre-DC)

Pre-Dendritic cells are so named because they are precursors of DC, and represent the last precursor phenotype in DC differentiation. This subset are often found in the circulation and may sometimes be referred to as circulatory DC, often considered as a pool of bystander DC which may be rapidly recruited where required (Novak and Bieber 2007). Different pre-DC can produce distinct subsets of DC, with some developing in the steady state, whilst other require further microbial or inflammatory stimulation. An example of a pre-DC is the plasmacytoid DC (pDC), which are instrumental in the defence against viral infections through their production of type I IFN (Cao and Liu 2007, Shortman and Naik 2007). They have unique MHC class I compartments which directs the presentation of viral antigens to cytotoxic $CD8^+$ T cells. In addition, they are also able to take up allergens *via* high affinity receptors for IgE and have the capacity to cross present allergens to T cells (Ueno *et al* 2007, Novak and Beiber 2007).

1.13.1.2 Conventional Dendritic Cells (cDC)

Conventional DC (cDC) are fully differentiated and can be subdivided into migratory cDC and lymphoid-tissue-resident cDC (Sections 1.13.1.3 and 1.13.1.4).

1.13.1.3 *Migratory cDC*

Migratory cDC serve as antigen sampling sentinels in the circulation and peripheral tissues. Upon antigen capture they migrate through the lymph to the lymph nodes, and this may occur rapidly upon encounter of inflammatory stimulus, or at a lower rate in the steady state. Here they present antigen to T cells. An example of the migratory cDC is the Langerhans cell, which can be found in the epidermis, and the epithelia of the intestine and the respiratory and reproductive tracts. It is likely that most DC in the peripheral non-lymphoid tissues fall into this group (Shortman and Naik 2007). Migratory cDC may also be found in the blood, however their physiological role is so far unclear, but may represent a pool of precursor cells that migrate into the tissues to replenish the resident DC population, and also act as a sensor for blood borne pathogens (Ueno et al 2007).

1.13.1.4 *Lymphoid-tissue resident cDC*

Unlike migratory cDC, lymphoid-tissue-resident DC do not travel to the lymphoid organs from the lymphatic system, but instead they collect and present antigen within the lymphoid organ (Spleen, thymus, lymphatic system) itself. The lymphoid tissue DC subset includes the majority of thymic and splenic DC, as well as approximately 50% of DC found in the lymph nodes (Lymph-Node resident DC). This subset can be further divided into $CD8^+$ and $CD8^-$ lymphoid tissue resident cDC, which differ in immune functions, including cytokine production and antigen presentation (Shortman and Naik 2007). Lymphoid tissue resident DC generally have an immature phenotype and are active in antigen uptake and processing, unlike the migratory DC, which have a mature phenotype upon reaching the draining lymph node, with their antigen uptake abilities suppressed (Wilson *et al* 2003) (Shortman and Naik 2007). The source of lymphoid-tissue-resident DC is controversial with some reports suggesting that DC colonise the lymphoid organs directly from the blood. These are then regarded as a static population

of cells that form a network of secondary lymphoid organ-resident APC, able to phagocytose soluble antigen in the lymphoid organ itself or receive antigen carried to them by migratory DC (Carbone *et al* 2004). However this was challenged by other reports that showed evidence of DC proliferation in lymphoid organs, suggesting this population of DC may be replenished *in situ* (Kabashima *et al* 2005). In the steady state, lymphoid-tissue-resident DC capture self-antigens and induce tolerance (Ueno et al 2007).

1.13.1.5 Inflammatory DC

Inflammatory DC are not present in the steady state, but appear as a consequence of inflammation or microbial stimuli. An example of inflammatory DC are the monocyte-derived DC. These inflammatory DC are derived from the $CX_3CR1^{lo}CCR2^{+}Gr1^{+}$ blood monocytes and are recruited to the peritoneal cavity and spleen upon inflammatory challenge (Geissman *et al* 2003). These monocyte-derived DC can be distinguished from steady state splenic cDC by their intermediate rather than high expression of CD11c and their high level of expression of CD11b (Naik *et al* 2006). Monocyte-derived DC are thought to be essential for the induction of effective defence mechanisms against infection (Lopez-Bravo and Ardavin 2008). Another example of an inflammatory DC is the tumour-necrosis factor and inducible nitric-oxide sythase-producing DC, termed Tip DC. These inflammatory Tip-DC were first discovered in mice infected with *Listeria monocytogenes* (Serbina *et al* 2003, Wu and Lui 2007 and Wu and Dakie 2004).

1.13.2 DC trafficking

Circulating DC and their precursors exit the blood in response to tissue specific homing signals that are displayed on the vascular wall. These signals may emanate from sites of inflammation, or from normal tissues that recruit precursor DC during initial seeding and subsequent turnover of tissue resident DC (Rot and Von Andrian 2004). Circulating DC can only follow recruitment signals by engagement with selectin molecules on the endothelium, which promote trans-endothelial migration into the underlining tissue. Homeostatic and inflammatory trafficking and tissue specific homing of DC occurs *in vivo via* a broad range of chemokine receptors expressed on their surface (Rot and Von

Andrian 2004). Once DC are tethered to and rolling on the endothelium, they encounter a chemoattractant stimulus which is often, but not always, presented on the surface of the endothelium (Rot and Von Andrian 2004). The presentation of chemokines on the endothelium triggers the clustering of integrin molecules on the venular surface, such as LFA (lymphocyte function associated)-1, VLA-4 and Mac-1, which facilitates DC engagement with endothelial cell surface integrin ligands including ICAM-1, ICAM-2 and VCAM (vascular cell adhesion molecule)-1 (Springer 1994). This process is often called firm arrest, and trans-endothelial migration into target tissue is subsequently driven by localised chemoattractant gradients.

Access by immature DC to non-lymphoid peripheral tissues is usually co-ordinated by chemokine receptor-ligand pathways such as CCR2-CCL2 (Geissman *et al* 2003), CCR5-CCL5 (Stumbles *et al* 2001) and CCR6-CCL20 (Merad *et al* 2004). CXCR4 is also expressed by monocytes, and may be responsible for low level constitutive recruitment in response to stromal cell-derived factor (SDF)-1 (Sallusto *et al* 1998). Once DC become mature upon capture of antigen, they down-regulate their responsiveness to inflammatory chemokines, and traffic to the draining lymph nodes by up-regulating homeostatic chemokine receptors such as CCR7, which responds to ligands CCL19 and CCL21 (Sozzani *et al* 1998). These chemokines are produced by peripheral lymphatic endothelial cells and lymph node stromal cells, and guide DC to downstream lymph nodes, where they can undergo antigen presentation to naive T cells (Martin-Fontecha *et al* 2003).

Recruitment of DC to peripheral organs is much more effective during inflammation suggesting that there is a pool of by-standing DC in the circulation which are ready to be rapidly recruited to the site of inflammation where antigen processing is required.
Recruitment of monocyte-derived DC have been characterised by Geissman *et al* 2003, and Auffray *et al* 2007, who showed that the $Gr1^{hi}$ CX_3CR1^{lo} $CCR2^{+}$ "inflammatory" monocytes are preferentially recruited to inflamed tissues in a CCR2-CCL2 dependent manner, whereas the $Gr1^{-}$ CX_3CR1^{hi} $CCR2^{-}$ "resident" monocytes interact with

fractalkine (CX_3CL1) which is a transmembrane chemokine on resting endothelium (Geissman *et al* 2003, and Auffray *et al* 2007).

1.13.3 DC activation and function

In the periphery, DC reside in an immature state, specialised for antigen uptake *via* receptor mediated endocytosis (phagocytosis, pinocytosis) through pattern recognition receptors (PPRs) which are activated by a limited set of conserved molecular patterns on microbes referred to as pathogen-associated molecular patterns (PAMPs). Three distinct families of PPRs have been identified: 1) C-type lectin receptors (CLR); 2) Toll like receptors (TLR) and 3) Intracytoplamsic-nucleotide Oligomerisation Domain (NOD) like receptors (NLR). In the context of allergy however, multivalent cross-linking of the high affinity receptors for IgE may also act as a deactivation event. Toll-like receptors are also capable of recognising modified host structures, and DC have the capacity to phagocytose antigens (Novak and Beiber 2007 and Ueno *et al* 2007).

Dendritic cells are activated by numerous agents derived from microbes, apoptotic cells, and cell mediators derived from the innate or adaptive immune system. Conventional DC create cellular processes in the peripheral organs to create a large surface area for antigen sensing (Ueno *et al* 2007). Microbes can either directly activate DC through PPR, or indirectly by the capture of apoptotic or necrotic products of other cells dying in response to microbial exposure or damage. This indirect mode of DC activation occurs through the recognition of endogenous activating molecules by damage-associated molecular pattern molecules (DAMP) (Ueno *et al* 2007). These factors include heat shock proteins, high-mobility group box-1 containing polypeptides, β-defensins and uric acid. Microbes can also induce cells such as epithelial cells, fibroblasts, and cells of the innate immune system to produce cytokines that are capable of directing DC responses (Ueno *et al* 2007). Different PPR are specific for, and recognise different PAMPs, which therefore deliver distinct molecular signals, thereby yielding the maturation of DC into distinct subsets, which influence specific immune responses to defined immunological challenge (Ueno *et al* 2007).

Following antigen-uptake, processed antigens enter the endocytic compartments and antigenic peptides are linked to MHC molecules and presented on the cells surface. These events are associated with migration of DC to the T cell area of the draining lymph nodes. This chemokine directed process is accompanied with a series of maturation events and the down-regulation of antigen capturing capacity (Novak and Beiber 2007). These changes include:

- Alterations in adhesive structures, cytoskeleton reorganisation and acquisition of high cellular motility.
- Loss of endocytic/phagocytic receptors and decreased antigen uptake.
- Secretion of chemokines in coordinated waves according to the stimulus.
- Up-regulation of co-stimulatory molecules such as CD40, CD80 and CD86.
- Translocation of MHC class II molecules to the cell surface and increased half-life of MHC-peptide complexes.
- Altered chemokine receptor expression.
- Secretion of cytokines that direct the differentiation and polarisation of effector T cells. (Ueno *et al* 2007 and Wu and Dakie 2004).

Activated DC can orchestrate a variety of cells from the innate and adaptive immune systems. Dendritic cells secrete sets of chemokines in three waves, corresponding to the three stages of an immune response to a microbe: during the first wave, chemokines are produced within 2-4 hours of activation and include CXCL1, CXCL2, CXCL3 and CXCL8 which attract innate effector cells such as NK cells, and neutrophils with the aim of limiting the spread of infection. The second wave produced within 4-8 hours is CXCL9-11 and CCL3-5, which attract activated memory T cells, and monocytes that could replenish the pool of DC or tissue macrophages. Finally at 12 hours and later, when mature DC arrive in the draining secondary lymphoid organs, they secrete CXCL13, which attracts B cells and T cells specialised for humoral responses (Follicular T helper cells), CCL19 and CCL21, which attract naïve T cells and CCL22 which attracts T-regs that will finally facilitate the termination of the immune response. Consequently

DC act in a co-ordinated manner in order to orchestrate the immune response against infection (Piqueras *et al* 2006, Ueno *et al* 2007).

In the draining lymph nodes, DC present antigen to and promote the expansion of naïve T cells into specific T helper populations (Th1, Th2, Th17), as well as tolerogenic and regulatory T-cell subtypes (T-reg) (Novak and Beiber 2007). DC perform this role with the aid of three families of molecules: Cytokines, B7 family members and TNF family members. Cytokine release by activated DC serves to determine the differentiation fate of naive T helper cells for example IL-12 drives Th1 cell differentiation (Heufler *et al* 1996) whereas IL-6 and IL-23 drive Th17 differentiation (Langrish *et al* 2005). The B7 family includes the co-stimulatory molecules CD80 and CD86, which bind to CD28 on naive T helper cells and deliver the signal for T cells to become functional effector cells. Finally, TNF (Caux *et al* 1992) and CD40 ligation (Caux *et al* 1994) were shown to activate DC with CD40/CD40L interaction promoting DC to up-regulate CD80 and CD86 and to secrete IL-12.

As well as activating T cells, DC have also been shown to interact with and activate cells of the innate immune system such as NK cells, NKT cells and γδ T cells (Lucas *et al* 2007, Fujii *et al* 2002). The cytokines secreted by these activated innate immune cells in return act to activate immature DC to develop into mature cells with distinct phenotypes, which will then go on to induce distinct types of T-cell immunity. In addition, DC have been shown to activate B cells resulting in the development of a humoral immune response against antigens presented by DC (Ueno *et al* 2007).

1.14 Cytokines

Cytokines are a group of pleiotropic regulatory polypeptides that are produced by haematopoietic and non-haematopoietic cell types to govern activities associated with homeostasis or inflammatory activation. These factors are rapidly induced following appropriate activation and elicit their activities *via* autocrine, paracrine and juxtacrine mechanisms (Heinrich *et al* 1998). Cytokines are critical to the development and functioning of both the innate and adaptive immune response and include members of the interleukin, interferon, growth factor and tumour necrosis factor like superfamilies. They are often secreted by immune cells that have encountered a pathogen, thereby activating and recruiting further immune cells to increase the system's response to the pathogen (Heinrich *et al* 1998). These soluble mediators are important for the communication between cells, and act through transmembrane cell surface receptors eliciting a cellular response by the activation of an intracellular signal transduction pathway (Yarden and Ullrich 1988). In general, cytokines elicit four functions- induction of gene expression, proliferation, differentiation, and survival (pro and anti-apoptotic) to co-ordinate effector functions of cells (Thomson 1991, Heinrich *et al* 1998). The role of cytokines is not however limited to the immune system and they are also involved in several developmental processes during embryogenesis.

1.14.1 Interleukins

The interleukins (IL) are members of the cytokine family, so named because they co-ordinate intercommunications between leukocytes. Interleukins act as messenger molecules transmitting signals between non-haematopoietic stromal cells and cells of the immune system. There are currently 36 known interleukins, which belong to a number of families, each playing different roles depending on the context of their expression.

1.14.2 Interferons

Interferons are a family of closely related cytokines that possess potent anti-viral, anti-microbial, tumoricidal and immunoregulatory activities (Biron 2001). Interferons are produced by a wide variety of cells in response to the presence of double-stranded RNA, a key indicator of viral infection. They assist the immune response by inhibiting viral

replication within host cells, activating natural killer (NK) cells and macrophages, increasing antigen presentation to lymphocytes, and inducing the resistance of host cells to viral infection (Biron 2001). Interferons are classified into type I and type II according to their receptor specificity and sequence homology (Schroder *et al* 2004). Type I interferons are structurally related and bind a common heterodimeric receptor. This group includes IFN-α and IFN-β, which are both important for viral immune responses (Schroder *et al* 2004). Interferon-γ, the only type II interferon, is structurally unrelated to the type I interferons and binds a different receptor. This cytokine is similarly important for innate and adaptive immunity against viral and intracellular bacterial infections, as well as tumour control (Schroder *et al* 2004).

1.14.3 Tumour necrosis factor (TNF) super-family

The tumor necrosis factor super-family refers to a group of cytokines that control and orchestrate the immune and inflammatory responses (Bodmer *et al* 2002). Examples of these functions include the activation of immune precursor cells to fully competent effector cells, which is known to be dependent on several TNF family ligands, such as BAFF (B cell activating factor) and CD40L for B lymphocytes, and CD40L and RANKL (receptor activator for nuclear factor B ligand) for dendritic cells (Bodmer *et al* 2002). TNF can also regulate T cell activation, expansion and survival (Locksley *et al* 2001). Alternatively, some members of the TNF family (e.g. TNF, Fas ligand and TNF related apoptosis inducing ligand (TRAIL)) are pro-apoptotic and usually stimulate apoptosis through activation of the cysteine protease family of caspases (Locksley *et al* 2001). In this way, TNF members contribute to the function of cytotoxic effector cells and participate in the homeostasis of the lymphoid compartment by evoking activation-induced cell death in immune effector cells that have fulfilled their function (Locksley *et al* 2001, Bodmer *et al* 2002). Excessive or inappropriate control of TNF expression has been associated with acquired acute or chronic inflammatory conditions such as septic shock and rheumatoid arthritis (Bodmer *et al* 2002).

1.15 Chemokines

Chemokines are a group of small structurally related molecules ranging from 8-14kDa, that regulate the trafficking of various leukocyte subsets via interactions with a subset of seven-transmembrane, G protein-coupled receptors (Baggiolini and Loetscher 2000). Inflammatory chemokines mainly act on neutrophils, monocytes, lymphocytes and eosinophils and are known to play a crucial role in host defence. Chemokines have also been shown to have a number of other roles that expand beyond the immune system such as their role in development and homeostasis (Zlotnik and Yoshie 2000). Chemokines can be divided into four main subsets determined by the arrangement of their two N-terminal cysteine residues. These subsets are named CXC chemokines (CXCL1, CXCL2 etc), CC chemokines (CCL2, CCL5 etc), CX_3C chemokines (CX_3CL1, also termed fractalkine) or XC chemokines (Lymphotactin), depending on whether the first two cysteine residues are adjacent, or have amino acids separating them (Baggiolini *et al* 1997, Baggiolini 1998). Fractalkine (CX_3CL1), which has three amino acids separating the first two cysteine residues, is the only known membrane-bound chemokine, attached by a mucin stalk (Bazan *et al* 1997). Each chemokine nomenclature is then ended with an R referring to the receptor, or an L referring to the ligand. Table 1.4 summarises many examples of chemokines, their receptors and their functions.

Receptor	Ligands	Cellular Distribution
CXCR1	CXCL6, CXCL8	Neutrophils
CXCR2	CXCL1-3, CXCL5-8	Neutrophils
CXCR3	CXCL9, CXCL10, CXCL11	T cells (Th1)
CXCR4	CXCL12	Neutrophils, monocytes, T cells, B cells, DC, thymocytes
CXCR5	CXCL13	Memory B cells, follicular B-helper T cells, circulating memory T cells
CXCR6	CXCL16	T cells
CXCR7	CXCL11, CXCL12	Monocytes, B cells, T cells
CCR1	CCL3, CCL5, 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	Eosinophils, basophils, Th2 cells
CCR4	CCL17, CCL22	T cells (Th1, Th2), memory T cells, thymocytes
CCR5	CCL3-5, CCL7, CCL13	Monocytes, Th1 cells
CCR6	CCL20	T cells, B cells, immature DC
CCR7	CCL19, CCL21	Naive T cells, mature DC
CCR8	CCL1	Th2 cells
CCR9	CCL25	Memory T cells, B cells, immature thymocyte
CCR10	CCL27, CCL28	Memory T cells
XCR1	XCL1, XCL2	Neutrophils, B cells, T cells
CX_3CR1	CX_3CL1	T cells, mast cells, monocytes

Table 1.4: *A summary of chemokines receptors, their ligands and their cellular distribution.*

1.16 Interleukin-6

The IL-6 family of cytokines include IL-6, leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), IL-11 and cardiotrophin-1 (CT-1). An overview of these cytokines is detailed in Table 1.5.

Family Member	Receptor	Source	Target Cells	Functions
IL-6	IL-6R (CD126) + gp130 (CD130)	Monocytes, T cells, Fibroblasts, Endothelial cells.	B, T, plasma cells Thymocytes Hepatocytes	Antigen specific immune responses. T and B cell growth, activation and differentiation. Acute phase protein production. Inflammatory reactions. Fever.
OSM	OSMR or LIFR, gp130	T cells, Monocytes/ Macrophages.	Endothelial cells. Mesothelial cells.	Liver development. Haematopoiesis. Inflammation. Limits monocyte trafficking,
IL-11	IL-11R + gp130	Bone marrow stromal fibroblasts. Mesenchymal cells.	Megakaryocytes. Pre-adipocytes. Haematopoietic progenitors.	Synergistic actions with IL-3 and IL-4 in haematopoiesis. Induction of acute phase proteins by hepatocytes.
LIF	LIFR + gp130	Bone marrow stromal cells. Fibroblasts. Endothelial cells.	Neural tissue. Leukemia cells. Monocytes.	Maintains embryonic stem cells. Inflammation. Influences PMN recruitment.
CNTF	CNRFRα, gp130, LIFR	Glial cells	Neuronal cell types.	Survival and differentiation of neural tissues. Reduces inflammatory tissue damage.

OSM: Oncostatin M
LIF: Leukemia inhibitory factor
CNTF: Ciliary Neurotrophic Factor

Table 1.5: *A table highlighting details of some members of the IL-6 family of cytokines.* CT-1 (Cardiotrophin) and CLC (Cardiotrophin like cytokine) are also members of the IL-6 family, but are not described in this table.

The IL-6 family cytokines exhibit pleiotropy and redundancy in biological activities and all share the common signal transducing receptor gp130 (Taga and Kishimoto 1997). Interleukin (IL)-6 is a pleiotropic cytokine that regulates various aspects of the immune response, acute phase reaction, liver regeneration and haematopoiesis (Kishimoto, T. *et al* 1989, 1995). The main producers of IL-6 are macrophages, DC, T cells, B cells, fibroblasts, endothelial cells, keratinocytes and most stromal fibrotic cells. IL-6 exerts a variety of effects during innate and acquired immunity, which can be defined as both pro- and anti-inflammatory. Following activation of TLR, IL-1β and TNFα production induces a rapid release of IL-6 (Topley *et al* 1993), which subsequently stimulates the production of acute phase proteins in the liver, factors that are important for mediating the immune response to pathogens. IL-6 then plays a role in leukocyte recruitment, specifically the resolution of neutrophil infiltration and the recruitment of mononuclear cells (Hurst *et al* 2001, McLoughlin *et al* 2003 and 2005). IL-6 mediates these changes by the regulation of chemokine expression (McLoughlin *et al* 2005), and leukocyte apoptosis. In this respect, IL-6 rescues T cells from apoptosis by the regulation of anti-apoptotic regulators such as Bcl-2 and Bcl-XL (Atreya *et al* 2000, Teague *et al* 1997). Conversely, IL-6 deficiency is associated with impaired Caspase-3 activity in neutrophils, suggesting IL-6 may orchestrate the apoptotic clearance of neutrophils (McLoughlin *et al* 2003). By regulating leukocyte recruitment and apoptosis in this way, IL-6 has become widely known as a key regulator for the transition from innate to acquired immunity, characterised by the successful clearance of neutrophils, replacing them with a more sustained mononuclear cell population as the inflammatory episode evolves (Jones 2005).

In addition to its role in leukocyte trafficking and apoptosis, IL-6 is also important for the determination of B and T cell differentiation fate and function (Bettelli *et al* 2006, Veldhoen *et al* 2006, Dienz *et al* 2009). Recently, IL-6 has emerged as a key regulator of Th17 cells, and it has become increasingly evident that this subset of T helper cells is involved in the development of autoimmune diseases (Betelli *et al* 2006). IL-6 in combination with TGF-β drives Th17 expansion and prevents the expansion of T-regs (Bettelli *et al* 2007, Veldhoen *et al* 2006, Pasare and Medzhitov 2003). This allows increased T cell activity and the onset of increased inflammation, which is damaging to

the host, and characteristic of autoimmune disease (Langrish *et al* 2005, Bettelli *et al* 2006).

IL-6 is an important growth factor for B cells, and is involved in the differentiation of proliferating B cells into antibody producing plasma cells. IL-6 has also recently been identified as a key promoter of B cell antibody production. IL-6 promotes the B cell helper capabilities of $CD4^+$ T cells by inducing an increased production of IL-21 by these cells, which is required for IL-6 to promote B cell antibody production *in vitro*. This identifies IL-6 as a potential co-adjuvant to enhance humoral immunity (Dienz *et al* 2009).

Dysregulation of IL-6 signalling has been implicated in chronic inflammatory conditions, and sustained circulating levels of IL-6 have been documented extensively in autoimmune diseases (Jones *et al* 2001). In this respect, IL-6 deficient mice are resistant to the induction of a number of experimental autoimmune conditions (Nowell *et al* 2003, Mihara *et al* 1998, Kallen *et al* 2002), while agents that inhibit IL-6, such as the blocking anti-IL-6 receptor antibody MRA (Atlizumab, Tocilizumab) have proven highly effective in the treatment of chronic inflammatory autoimmune diseases such as rheumatoid arthritis and Crohn's disease (Choy *et al* 2002, Ito *et al* 2004). IL-6 has also recently been implicated in cancer, being the cause of between 15 and 20% of cancers (Schafer *et al* 2007, Rose-John *et al* 2006, 2009, Scheller *et al* 2006).

1.16.1 IL-6 signalling

IL-6 is able to signal *via* two different pathways (Figure 1.5). **Classical signalling** occurs when IL-6 binds to its cell surface bound cognate receptor known as IL-6R (CD126).
IL-6R is a non-signalling receptor, normally present only on hepatocytes and certain leukocyte subsets such as neutrophils, monocytes, and T and B lymphocytes. This complex then becomes associated with the signal transducing β-receptor gp130 (CD130), which acts as the signal transducing receptor for all IL-6 related cytokines (see Section 1.16.3) (Rose-John *et al* 1994, Jones 2005).

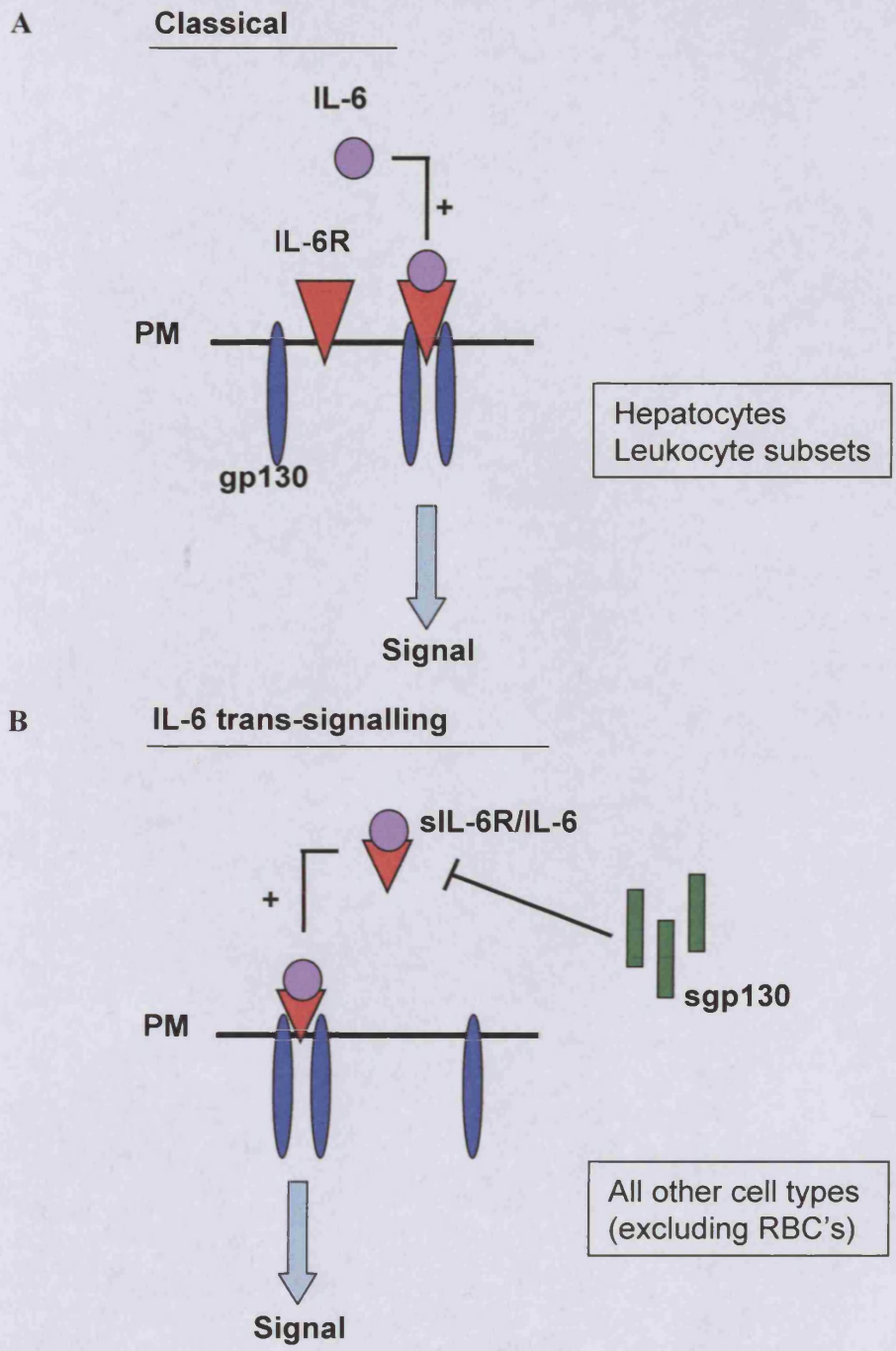


Figure 1.5: *Modes of IL-6 signalling* (A) Classical signalling occurs when IL-6 binds to its cognate surface bound IL-6R, which then associates with gp130 dimers, and initiates the down stream signalling pathway. (B) Trans-signalling occurs independently of the surface bound IL-6R, whereby IL-6 binds to its soluble receptor, which then binds gp130 as a complex, to initiate the downstream signalling pathway. This pathway can be blocked by soluble gp130 (sgp130), which binds and immobilizes the IL-6/sIL-6R complex.

An alternative signalling pathway also exists whereby IL-6 signals *via* its soluble receptor. This pathway is referred to as **IL-6 trans-signalling**. The soluble receptor binds IL-6 with similar affinity to that of the cognate receptor, and is produced either by proteolytic shedding from the surface of cells by the sheddase Adamylsin and Disintegrin-associated Metalloprotease (ADAM)-17 and to a lesser extent ADAM-10, or by differential mRNA splicing. Both these mechanisms of sIL-6 generation exist in humans, however in mice, the soluble receptor is generated exclusively by shedding. In this mode of activation, IL-6 simply binds to its non-surface bound receptor, which then binds to gp130 as a complex. This then activates the signalling pathway in exactly the same way as in classical signalling. This form of IL-6 signalling allows activation of cell types that do not express the surface bound IL-6R and since gp130 is ubiquitously expressed, this includes most cell types, excluding red blood cells (Rose-John *et al* 1994, Jones 2005).

1.16.2 Intracellular signalling pathways of IL-6

In the case of both IL-6 classical signalling and IL-6 trans-signalling, the ligand bound IL-6R becomes associated with surface bound gp130. Ligand-induced β subunit gp130 dimerisation then leads to the activation of JAK (Janus activated kinases), specifically JAK1, JAK2 and Tyk2 in IL-6 signalling, by tyrosine phosphorylation. JAK then phosphorylate gp130 at the four C terminal tyrosine residues and this facilitates the recruitment of STATs (signal transducer and activation of transcription) 1 and 3, mediated by their SH2 domain. STAT1/3 bind to the phosphorylated YxxQ signalling motifs on gp130, where they then become phosphorylated by JAKs. This phosphorylation leads to the formation of active STAT dimers (STAT 1 and 3 homodimers and less commonly, heterodimers), also mediated by their SH2 domains. These STAT dimers are subsequently transported to the nucleus where they activate gene expression (Figure 1.6) (Heinrich *et al* 2003).

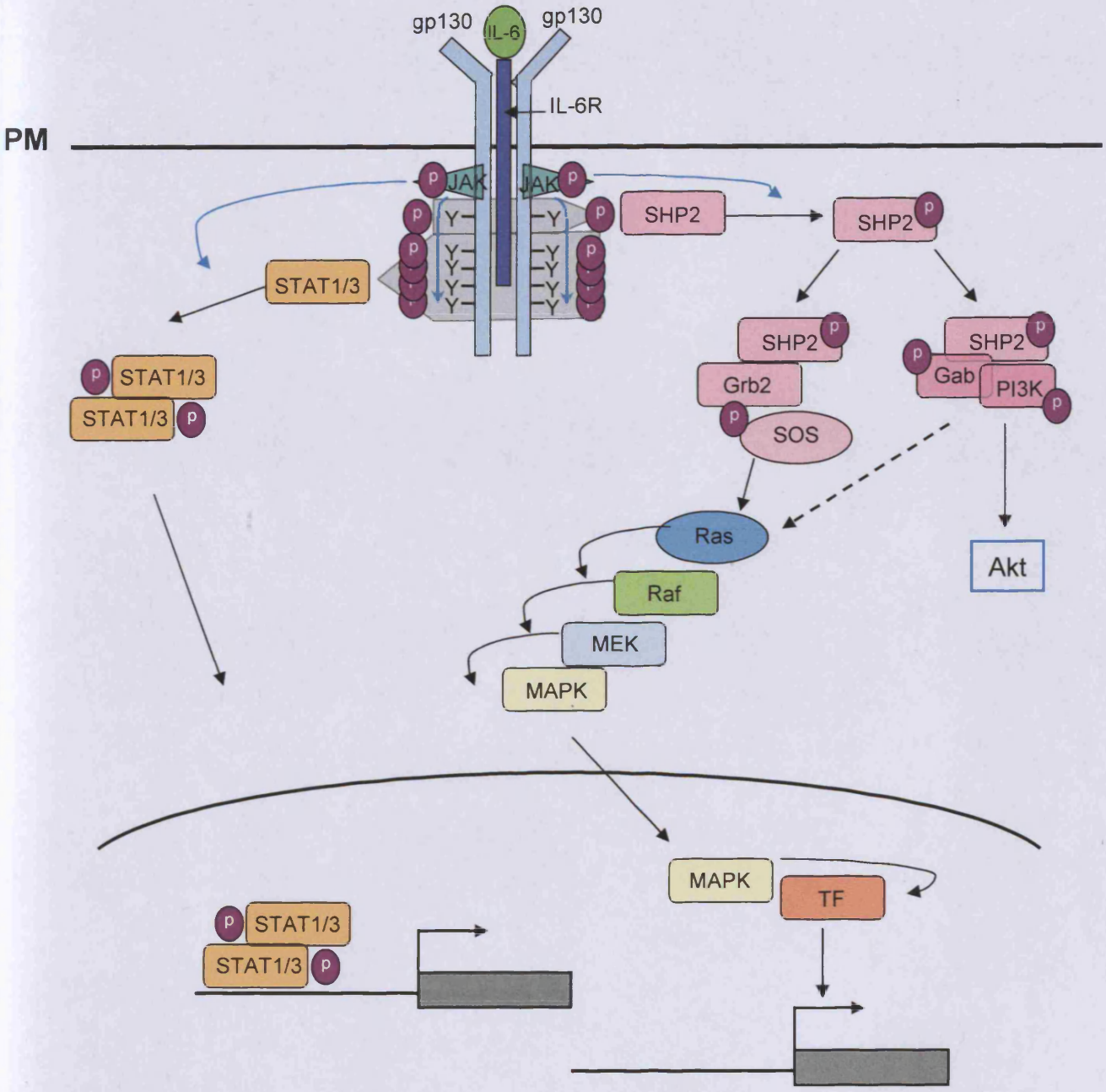


Figure 1.6: *IL-6 signalling via the STAT and SHP2 pathways*. Upon binding of IL-6 to its receptor, it becomes associated with gp130 dimers. Gp130 dimerisation leads to the activation of JAKs (Janus activated kinases), by tyrosine phosphorylation, which then phosphorylate gp130 at the four C terminal tyrosine residues, facilitating the recruitment of STATs (signal transducer and activation of transcription) 1 and 3. The STATs then become phosphorylated by JAKs, which leads to the formation of active STAT dimers, which are subsequently transported to the nucleus where they activate gene expression (Heinrich *et al* 2003). IL-6 signalling also can activate the mitogen-activated protein kinase (MAPK) pathway. This occurs when activated JAKs tyrosine phosphorylate receptor bound SHP2, which then binds the adaptor protein growth factor receptor binding protein 2 (Grb2), which is constitutively associated with the GDP/GTP Ras exchange factor Sos, and GTP bound Ras triggers ERK activation, which leads into the MAPK pathway. Phosphorylated SHP2 can also bind with scaffolding proteins Gab1/2 and phosphotidylinositol-3-kinase (PI3K) which leads to the activation of the AKT pathway (Heinrich *et al* 2003, Ernst and Jenkins 2004, Naugler and Karin 2008).

As well as activating the STAT pathway, IL-6 signalling also can activate the mitogen-activated protein kinase (MAPK) pathway (Figure 1.6). This occurs when activated JAKs phosphorylate a further tyrosine residue (Y757 in mouse, Y759 in humans). Subsequently, these JAKS phosphorylate receptor bound SHP2 (a protein tyrosine phosphatase), which then binds the adaptor protein Growth factor Receptor Binding protein 2 (Grb2), which is constitutively associated with the Guanosine diphosphate (GDP)/Guanosine triphosphate (GTP) Ras exchange factor Son of Sevenless (Sos). GTP bound Ras triggers Extracellular signal Regulated Kinases (ERK) activation, which leads into the MAPK pathway. Phosphorylated SHP2 can also bind with scaffolding proteins Grb2 Associated Binding protein (Gab)-1/2 and phosphotidylinositol-3-kinase (PI3K), which leads to the activation of the AKT pathway (Heinrich *et al* 2003, Ernst and Jenkins 2004, Naugler and Karin 2008). Signalling via the STAT1/3 pathway is associated with modulation of cellular differentiation, apoptosis and the G1-S cell cycle transition (Yamanaka *et al* 1996, Fukada *et al* 1996), whereas the activation of the SHP2/ERK MAPK pathways is known to mediate cell cycle progression (Fukada *et al* 1996).

1.16.3 Regulation of IL-6 signalling

IL-6 trans-signalling is facilitated by the ligation of IL-6 to the soluble form of the receptor, which allows the regulation of cell types that do not express the cognate surface bound IL-6 receptor, through gp130. However, due to the ubiquitous cellular expression of gp130, IL-6 trans-signalling has the potential to stimulate all cell types in the body. Consequently it is important that this mode of IL-6 signalling is tightly regulated. This regulation is facilitated in a number of ways: 1) Regulation can be provided by a soluble variant of gp130 (sgp130), which is detected at high levels circulating in human sera (1-300ng/ml), and is known to counteract IL-6 signalling *via* its soluble receptor. Antagonism by sgp130 only occurs once the IL-6/sIL-6R complex has formed and so it binds the complex as opposed to the individual components. Murine studies have demonstrated that sIL-6R levels increase before that of sgp130 suggesting individual regulation of each protein, and although gp130 shedding has been investigated as a source of sgp130 *in vitro*, it is predicted that there is a more active role for differential mRNA splicing of the gp130 gene *in vivo* (Jones 2008, Diamant *et al* 1997). 2) As part

of the transcriptional events controlled by IL-6, a series of negative regulators including suppressor of cytokine signalling (SOCS) 1 and SOCS 3 are induced. SOCS3 counteracts STAT3 activation by interaction with a tyrosine residue that also directs SHP2 signalling. Specifically SOCS3 binds via its SH2 domain to tyrosine residue 757 of gp130 in mice and 759 in humans (Nicholson *et al* 2000, Schmitz *et al* 2000). 3) The third mode of regulation is facilitated by auto-antibodies specific for IL-6. These exist in humans and are able to bind to and immobilise this cytokine *in vivo* (Suzuki *et al* 1994). 4) IL-6 trans-signalling is also regulated by the rate of sIL-6 receptor production. 5) Regulation also occurs by the control of the balance between classical and trans-signalling and 6) the balance between IL-6 directed STAT1 and STAT3 signalling.

1.16.4 IL-6 in acute inflammation

As previously described, IL-6 is critically required for the resolution of innate immunity and the development of an acquired immune response (Jones 2005). In acute inflammation, this process is defined by a precise regulation of leukocyte recruitment and clearance, during which IL-6 is vital for the transition from a neutrophil to a mononuclear cell infiltrate (Jones 2005). This pattern of leukocyte infiltration is regulated by IL-6 and its soluble receptor, which suppresses neutrophil infiltration and promotes mononuclear cell recruitment and activation, through the modulation of inflammatory chemokine expression and apoptotic control processes (Hurst *et al* 2001, McLoughlin *et al* 2003, 2005, Atreya *et al* 2000). This is discussed further in Section 1.20.6. IL-6 has also been shown to be a key regulatory signal in the development of Th17 cells (Harrington *et al* 2005, Betelli *et al* 2006, Veldhoen *et al* 2006). This subset of T helper cells produce the cytokines IL-17A and IL-17F, which are important for the innate immune response against extracellular bacteria (Bettelli *et al* 2007).

By directing and regulating the acute inflammatory response, IL-6 allows the successful clearance of the pathogen, and subsequent resolution of inflammation. This has been demonstrated effectively by Coles *et al* who has shown that IL-6 deficient mice exhibit impaired bacterial clearance during an experimental model of bacterial peritonitis (Coles *et al* unpublished). In support of this, it was recently shown that the reconstitution of

IL-6 trans-signalling in IL-6 deficient mice subjected to bacterial infection, improved the rate of mortality and led to reduced neutrophilia and increased bacterial clearance in these mice (Onogawa 2005). This IL-6-driven outcome may be particularly relevant in septic shock, where IL-6 has proven to be protective (Barton *et al* 1993, Diao *et al* 2005). Finally, IL-6 deficient mice also display impaired viral clearance (Kopf *et al* 1994).
Therefore, IL-6 is clearly important for the regulation of cellular immunity against infections. The protective properties displayed by IL-6 during infection must however be balanced against the detrimental aspects of IL-6 biology seen in chronic inflammatory disease. Dysregulation of this cytokine has been largely implicated in chronic inflammatory and autoimmune disease and this is discussed in more detail in Section 1.16.5.

1.16.5 IL-6 in chronic inflammation

As previously described, a pivotal role in the resolution of any inflammatory episode is the transition from innate to acquired immunity. Inappropriate regulation of this process can lead to a chronic inflammatory state, characterised by the retention of an activated mononuclear cell population within the affected tissue (Jones 2005).

Overproduction and increased signalling of IL-6 has been associated with chronic inflammatory and autoimmune conditions (rheumatoid arthritis, systemic lupus erythematosus, psoriasis, colitis, diabetes and various other inflammatory bowel diseases), which through an undefined dysregulation of chemokine-mediated recruitment and leukocyte apoptosis, is potentially detrimental (Grossman *et al* 1989, Swaak *et al* 1989, Houssiau *et al* 1988, Naugler and Karin 2008). In this respect, increased levels of IL-6 in the synovial fluid within the inflamed joints of rheumatoid arthritis patients has been associated with joint pathology, with a series of *in vivo* studies using models of experimental arthritis having endorsed a role for IL-6 in joint destruction, leukocyte recruitment, apoptosis and T cell activation (Kallen 2002, Nowell *et al* 2003, Ohshima *et al* 1998, Alonzi *et al* 1998). The importance of IL-6 in chronic inflammatory diseases is reinforced by the fact that IL-6 deficient mice are resistant to the induction of experimental encephalitis, colitis, lupus nephritis and rheumatoid arthritis (Atreya, R. *et*

al 2000, Nowell, M.A. *et al* 2003, Mihara, M. *et al* 1998). IL-6 deficient mice are also resistant to the induction of experimental chronic peritonitis and the associated membrane thickening, which has implications for peritoneal dialysis patients (see Sections 1.20.1-7) (Fielding *et al* 2009, submitted).

IL-6 trans-signalling is able to enhance production of several mononuclear cell chemoattractants from resident tissue cells, driving the recruitment of monocytic cells and lymphocytes to the inflammatory site (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 lamina propria of Crohn's disease patients, and the synovium of arthritic joints (Atreya *et al* 2000, Yamamoto *et al* 2000, Kikly *et al* 2006, Nowell *et al* 2003, 2009). 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). *In vivo* blockade of IL-6R signalling by administration of an anti-IL-6R monoclonal antibody or soluble gp130 therefore confers protection against these conditions, which are specifically associated with the IL-6 mediated suppression of T cell apoptosis. This is important since inflammatory T helper cells such as the recently characterised Th17 cells have been increasingly associated with the development of many autoimmune and chronic inflammatory conditions (Atreya *et al* 2000, Yamamoto *et al* 2000, Kikly *et al* 2006, Nowell *et al* 2003, 2009).

These findings have led to the development of strategies that therapeutically inhibit IL-6 responses, and a number of anti-IL-6 drugs are already available. The humanised blocking anti-IL-6 receptor antibody MRA (Tocilizumab, Atlizumab) is an example that has been shown to be highly effective in advanced clinical trials for the treatment and management of rheumatoid arthritis and Crohn's disease (Choy *et al* 2002, Ito *et al* 2004).

It is important to remember that the detrimental consequences of IL-6 activity in chronic disease is balanced by its capacity to protect against septic shock (Barton *et al* 1993),

coordinate microbial clearance (Onogawa 2005, Coles *et al* unpublished) and to direct resolution of acute inflammation (Jones 2005). Completely blocking IL-6 signalling may have an undefined deleterious effect on the host's ability to resolve bacterial infections. It is currently unknown what causes the conversion from a protective to a detrimental IL-6 biology.

1.17 Interleukin-10

IL-10 is a pleiotropic cytokine, which plays an important role in the early immune response, and displays suppressive activity toward a number of cell types in the immune system including macrophages, DC, neutrophils, eosinophils and Th1 cells. IL-10 is important in regulating the severity and duration of inflammatory responses, making it important for obtaining the right balance between inducing the immune response to fight the pathogen, and inhibiting the immune response to prevent collateral tissue damage (Ho *et al* 1994).

IL-10 is produced by antigen presenting cells upon TLR-4 activation by LPS. This TLR induced production of IL-10 is also further increased by the presence of damaged cells, which usually arise during the inflammatory response (Byrne *et al* 2002). IL-10 is a potent stimulator of NK cells (Zheng *et al* 1996), which is important for the clearance of the pathogen and the acquisition of antigen from dead cells for cross priming by APC, which increases antigen availability and induces further activation of APC by induction of apoptosis/necrosis of damaged cells (Albert *et al* 1998). This facilitates the induction of an adaptive immune response, providing a link between innate and acquired immunity (Belardelli *et al* 2002). These combined allow IL-10 to contribute to the destruction of the pathogen, while limiting collateral tissue damage by simultaneously dampening the immune response, which it does in a number of ways discussed below.

IL-10 regulates the expression of cytokines, chemokines and co-stimulatory molecules on myeloid cells, having important effects on their ability to activate and sustain the inflammatory response (Moore *et al* 2001). In this respect, IL-10 suppresses the ability of APC to prime effector T cells by dampening the expression of inflammatory

chemokines, co-stimulatory molecules and preventing further APC maturation (Moore *et al* 2001). IL-10 suppresses the release of monocyte inhibitory protein-1α (MIP-1α), MIP-1β, CCL2, CCL5 and KC (Berkman *et al* 1995, Marfaing-Koka *et al* 1996, Kopydlowski *et al* 1999, Olszyna *et al* 2000), TNF-α, IL-1α, IL-1β, IL-6 and IL-8 by activated APC (Fiorentino *et al* 1991, de Waal Malefyt *et al* 1991, Moore *et al* 2001). IL-10 also inhibits the expression of MHCII, CD80 and CD86 on monocytes (de Waal Malefyt *et al* 1991, Ding *et al* 1993). In this respect, IL-10 indirectly inhibits T cell proliferation, by the suppression of antigen presenting cells (Chernoff *et al* 1995, de Waal Malefyt *et al* 1991). This is most likely a mechanism in place in order to restrict the extent of an inflammatory process, and prevent any collateral damage. By inhibiting APC maturation, IL-10 conserves their ability for antigen uptake and delays their migration to the draining lymph nodes. If the pathogen is not cleared in the early response, further signals are provided which lead to APC activation, maturation and the onset of the adaptive immune response (Brossart *et al* 2000).

Similar anti-inflammatory properties of IL-10 includes its ability to inhibit microbicidal activities in macrophages, and blocking IL-10 increases their killing capacity (Fleming and Campbell 1995). The same is true for neutrophils, where studies have shown that IL-10 inhibited bacterial phagocytosis and attenuated neutrophil microbicidal activity towards internalised bacteria (Laichalk *et al* 1996). In this respect, IL-10 plays a central role in facilitating bacterial outgrowth and dissemination.

Finally, IL-10 production by $CD4^+$ T cells serves to halt the later phases of the inflammatory response when the pro-inflammatory stimuli induced by the pathogen is eliminated by the antigen specific effector mechanisms (Moore *et al* 2001). In particular IL-10 is thought to promote intracellular infection by disabling Th1 responses and deactivating parasitised tissue macrophages (Murray *et al* 2002). In this respect, IL-10 has been shown to induce anergy or non-responsiveness in T cells (Groux *et al* 1996).

1.17.1 IL-10 signalling

The receptor for IL-10 has two subunits called IL-10 Receptor (R) α and IL-10 Receptor (R) β. As the ligand-binding α chains interact with IL-10 they dimerise and become associated with the two secondary β chains. Receptor assembly leads to activation of the Janus kinases JAK1 and Tyk2 and phosphorylation of tyrosine residues on the IL-10 R1. This leads to the recruitment and phosphorylation of STAT3, which forms homodimers and translocates to the nucleus to activate a wide range of IL-10 -responsive genes (Figure 1.7) (Riley *et al* 1999, Ho *et al* 1993, 1994).

1.18 Interferon (IFN)-γ

Interferon (IFN)-γ co-ordinates a diverse array of cellular programs through the transcriptional regulation of immunologically important genes. It was at first thought that IFN-γ was solely produced by $CD4^+$ Th1 lymphocytes, $CD8^+$ cytotoxic T lymphocytes and NK cells, but further investigation has shown that B cells, NKT cells, and professional APC all produce IFN-γ (Schroder *et al* 2004).

Initially, IFN-γ orchestrates the trafficking of leukocytes by the up-regulation of adhesion molecule and chemokine expression. IFN-γ and Nitric Oxide (NO) produced at the site of inflammation cause blood vessels in the vicinity to dilate thereby increasing "leaky vessels" and maximizing blood flow to the inflammatory site. IFN-γ then up-regulates the expression of various chemokines such as MCP-1, MIP-1α/β and RANTES, and also the expression of adhesion molecules ICAM-1 and VCAM-1, which in combination maximize trans-endothelial migration of leukocytes to the site of inflammation (Boehm *et al* 1997). IFN-γ then has the ability to direct the activities of numerous cell types: IFN-γ is an important stimulator of macrophages, inducing direct antimicrobial and antitumour mechanisms in these cells. IFN-γ stimulation of macrophages increases pinocytosis and receptor-mediated phagocytosis, and enhances their microbial killing abilities by the induction of the NADPH-dependent phagocyte oxidase system (respiratory burst) and by priming for nitric oxide (NO) production (Decker *et al* 2002). IFN-γ also up-regulates macrophage antigen processing and presentation pathways.

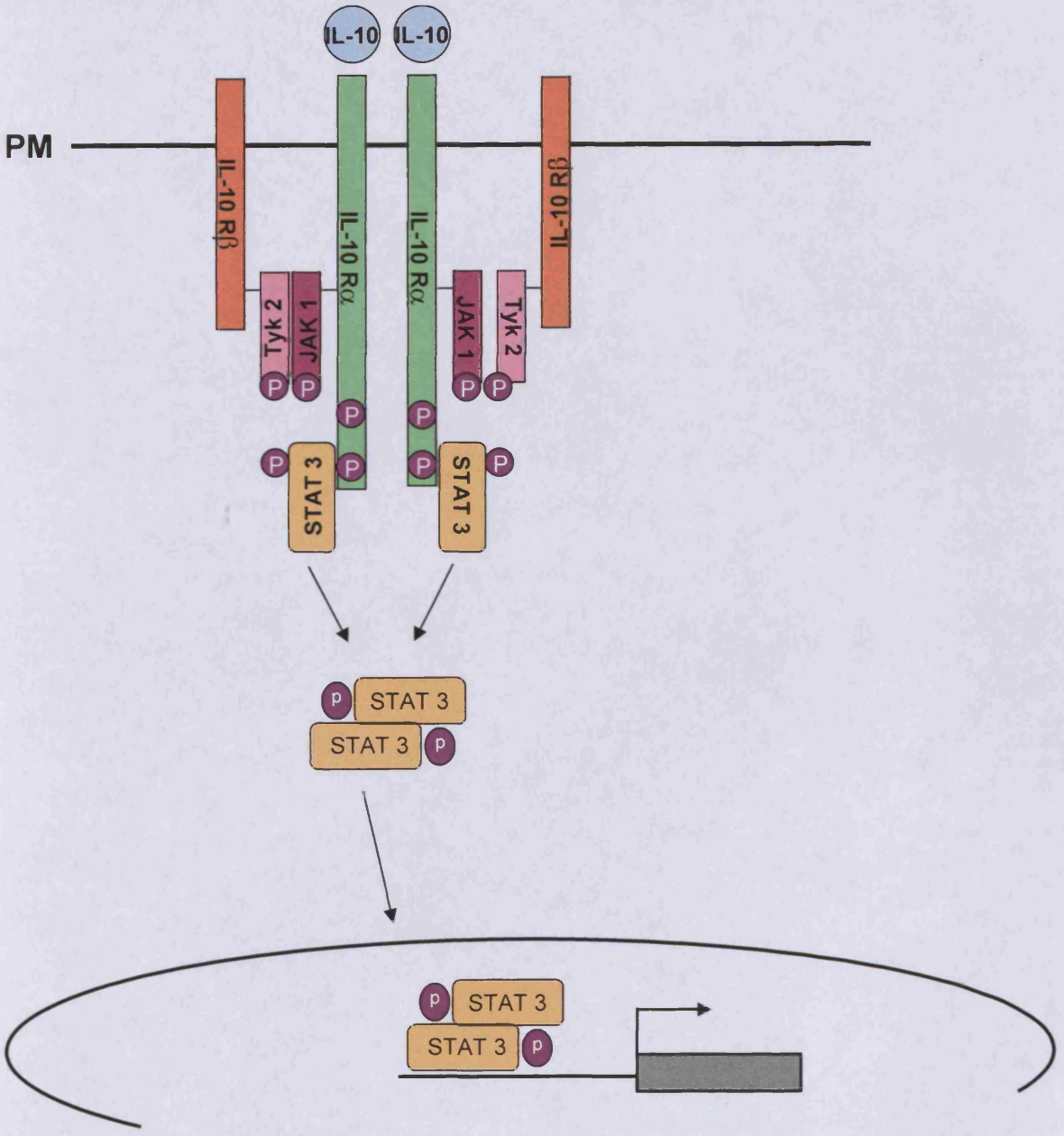


Figure 1.7: *IL-10 signalling pathway*. The receptor for IL-10 has two subunits: IL-10 Rα and IL-10 Rβ. As the ligand-binding chains interact with IL-10 they dimerise and become associated with two secondary chains. Receptor assembly leads to activation of the Janus kinases JAK1 and Tyk2 and phosphorylation of tyrosine residues on the IL-10 Rα . This leads to the recruitment and phosphorylation of STAT3, which forms homodimers and translocates to the nucleus to activate a wide range of IL-10 -responsive genes (Riley *et al* 1999, Ho *et al* 1993, 1994).

IFN-γ up-regulates many functions within the class I antigen presentation pathway to increase the number and diversity of peptides presented on the cell surface. Up-regulation of MHC I is important for the host response to intracellular pathogens and increases the likeliness of cytotoxic T cell recognition of foreign peptides thereby inducing cell mediated immunity (Schroder *et al* 2004). IFN-γ also up-regulates MHC II presentation pathways thereby promoting peptide specific activation of $CD4^+$ T cells (Mach *et al* 1996).

Finally, IFN-γ is able to co-ordinate the transition from innate to adaptive immunity by aiding the development of Th1 type responses, as well as directing B cell isotype switching, inducing immunoglobulin production, and regulating local leukocyte endothelial interactions (Schroder *et al* 2004). IFN-γ is involved in the development of Th1 responses and subsequent IFN-γ secretion by these cells further skews the immune response towards a Th1 phenotype. IFN-γ facilitates this response by promoting Th1 effector mechanisms such as innate cell mediated immunity by activation of NK cell effector functions, specific cytotoxic immunity by maximizing T cell:APC interactions to increase $CD4^+$ differentiation (a result of increased antigen processing, presentation, and expression of co-stimulatory molecules) and macrophage activation (Boehm *et al* 1997).

1.18.1 Interferon (IFN)-γ signalling

The receptor for IFN-γ has two subunits, the IFN-γ Rα ligand-binding chain and the IFN-γ Rβ signal-transducing chain. The receptor complex comes together and assembles before the ligand binds and IFN-γ binds the receptor complex as a non-covalent homodimer. Ligand binding induces JAK2 autophosphorylation and activation, which allows JAK1 trans-phosphorylation by JAK2. The activated JAK1 then phosphorylates functionally important tyrosine residue 440 of each alpha chain to form two adjacent docking sites for the SH2 domains of STAT1. The recruited STAT1 pair is then phosphorylated near the N terminus at Y701, most likely by JAK2, inducing their dissociation from the receptor complex as a homodimer. The STAT1 homodimer then translocates to the nucleus to activate a wide range of IFN-γ -responsive genes (Figure 1.8) (Schroder *et al* 2004).

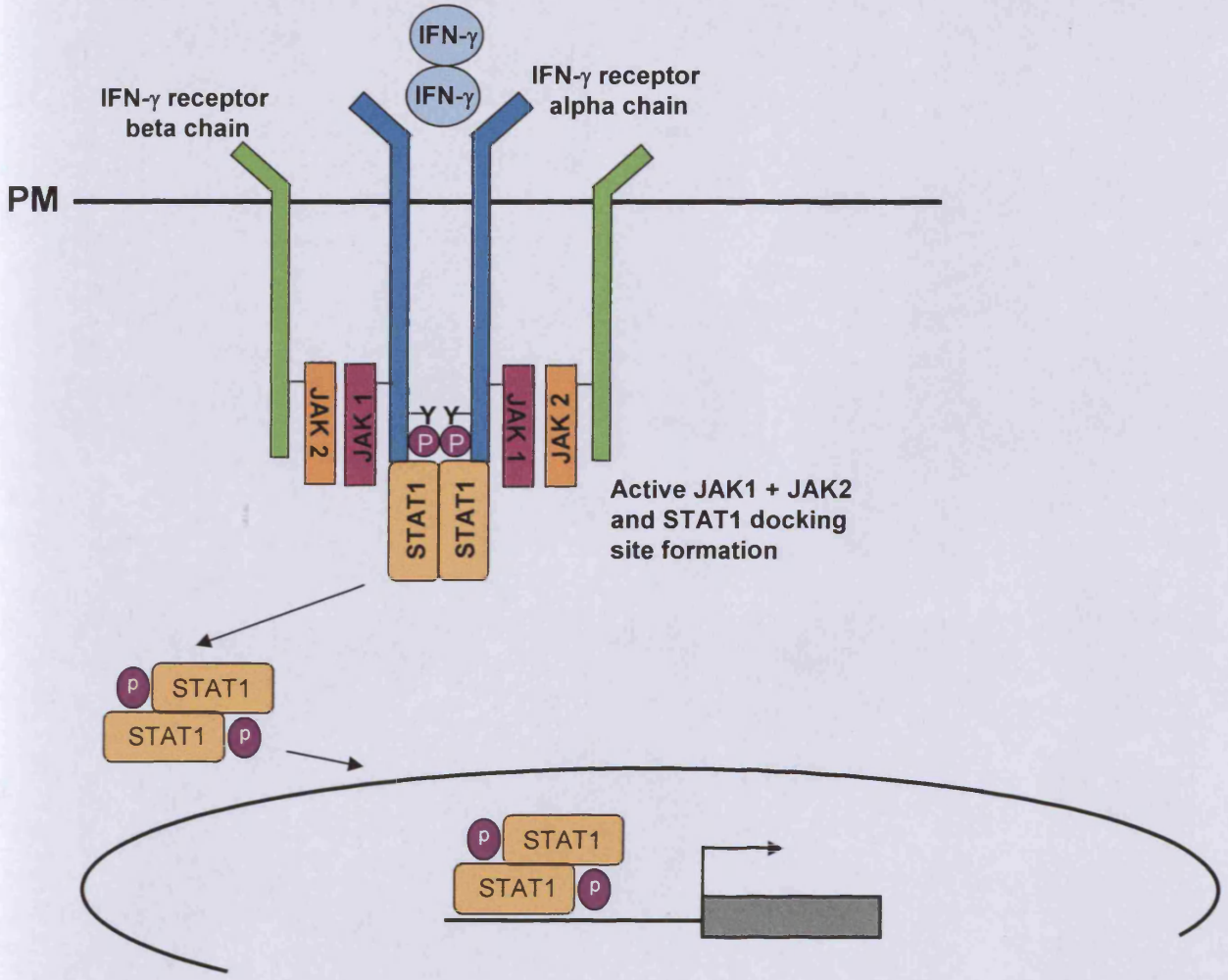


Figure 1.8: *IFN-γ signalling pathway*. The receptor for IFN-γ has two subunits, IFN-γ Rα, the ligand-binding chain and IFN-γ Rβ, the signal-transducing chain. The receptor complex comes together and assembles before the ligand binds and IFN-γ binds the receptor complex as a non covalent homodimer. Ligand binding induces JAK2 autophosphorylation and activation, which allows JAK1 trans-phosphorylation by JAK2. The activated JAK1 then phosphorylates tyrosine residue 440 of each R α chain to form two adjacent docking sites for the SH2 domains of STAT1. The recruited STAT1 pair is then phosphorylated near the N terminus at Y701, most likely by JAK2, which induces their dissociation from the receptor complex as a homodimer. The STAT1 homodimer then translocates to the nucleus to activate a wide range of IFN-γ -responsive genes (Schroder *et al* 2004).

1.19 Oncostatin M

Oncostatin M (OSM) is a pleiotropic cytokine of the interleukin-6 family and is produced by activated T cells and macrophages. OSM has been shown to drive the differentiation of megakaryocytes, inhibit tumour cell growth, induce neurotrophic peptides, and regulate cholesterol metabolism (Bruce 1992). In addition, OSM has also been implicated in wound healing and attenuation of the immune response, with evidence showing that OSM regulates the expression of IL-6 (Brown *et al* 1991), which has known potent pro-inflammatory capabilities. OSM can also regulate the expression of human acute phase proteins and protease inhibitors that have been implicated in modulating cytokine function and limiting tissue damage at sites of inflammation (Wallace *et al* 1995).

OSM is produced late in the cytokine response and protects from LPS-induced toxicities (shown by its inhibition of bacterial LPS-induced TNF-α and septic lethality), promoting the re-establishment of homeostasis by cooperating with pro-inflammatory cytokines and acute phase molecules to alter and attenuate the inflammatory response. Additionally, in many murine models of chronic inflammatory disease such as rheumatoid arthritis and multiple sclerosis, OSM potently suppresses inflammation and tissue destruction (Wallace *et al* 1999, Wahl *et al* 2001). In contrast to this data however, OSM has been shown to be involved in tissue injury. Langdon *et al* showed that OSM may be involved in inflamed joint pathology, due to its apparent ability to regulate synovial fibroblast function (growth, and expression of MCP-1, IL-6 and tissue inhibitor metalloproteinase-1) *in vitro*, and its ability to cause increases in the synovial cell proliferation, infiltration of mononuclear cells, and increases in extracellular matrix deposition in joints *in vivo* resulting in the induction of a proliferative invasive phenotype of synovium *in vivo* in mice on overexpression (Langdon *et al* 2000).

Finally, OSM has been shown to have a role in monocytic cell trafficking during acute inflammation. Hams *et al* showed that OSM limits monocytic cell recruitment, shown by using a murine model of peritoneal inflammation in OSM-receptor β knock out mice,

which exhibited enhanced monocytic cell trafficking. This was linked to the ability of OSM to inhibit IL-1β-mediated NF-κB activity and CCL5 expression (Hams *et al* 2008).

1.19.1 OSM signalling

OSM engages gp130 to form a heterodimer with the OSMRβ, upon which the intracellular signal is initiated. Gp130 dimerisation with OSMRβ leads to the activation of JAK (Janus activated kinases), specifically JAK1, JAK2 and Tyk2, by tyrosine phosphorylation. JAK then phosphorylates gp130 at the four C terminal tyrosine residues and this facilitates the recruitment of STAT (signal transducer and activation of transcription) 1,3 and 5, mediated by their SH2 domain. STAT1/3/5 bind to the phosphorylated YxxQ signalling motifs on gp130, where they then become phosphorylated by JAK. Phosphorylation occurs on a single tyrosine residue, which leads to the formation of active STAT dimers (STAT 1,3 and 5 homodimers and less commonly, heterodimers), also mediated by their SH2 domains. These STAT dimers are subsequently transported to the nucleus where they activate gene expression (Figure 1.9). OSM can also activate the mitogen-activated protein kinase (MAPK) pathway. This occurs when activated JAK tyrosine phosphorylate receptor bound SHP2 (a protein tyrosine phosphatase), which then binds the adaptor protein growth factor receptor binding protein 2 (Grb2), which is constitutively associated with the GDP/GTP Ras exchange factor Sos, and GTP bound Ras triggers ERK activation, which leads into the MAPK pathway (Figure 1.10) (Heinrich *et al* 2003).

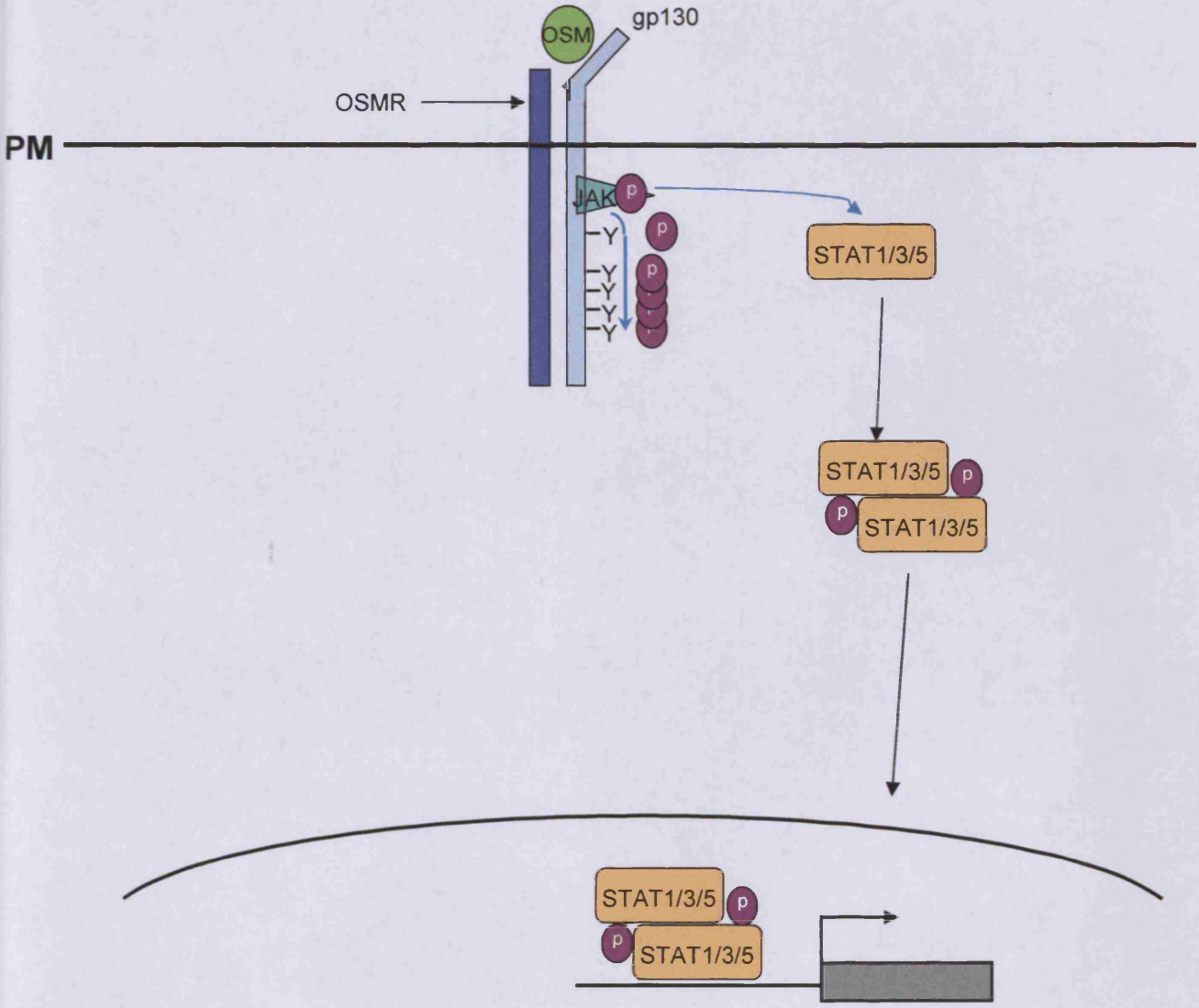


Figure 1.9: *OSM signalling via the STAT pathway*. Upon binding of OSM to its receptor, it becomes associated with gp130. This leads to the activation of JAKs (Janus activated kinases), by tyrosine phosphorylation, which then phosphorylate gp130, facilitating the recruitment of STATs (signal transducer and activation of transcription) 1,3 and 5. The STATs then become phosphorylated by JAKs, which leads to the formation of active STAT dimers, which are subsequently transported to the nucleus where they activate gene expression (Heinrich *et al* 2003).

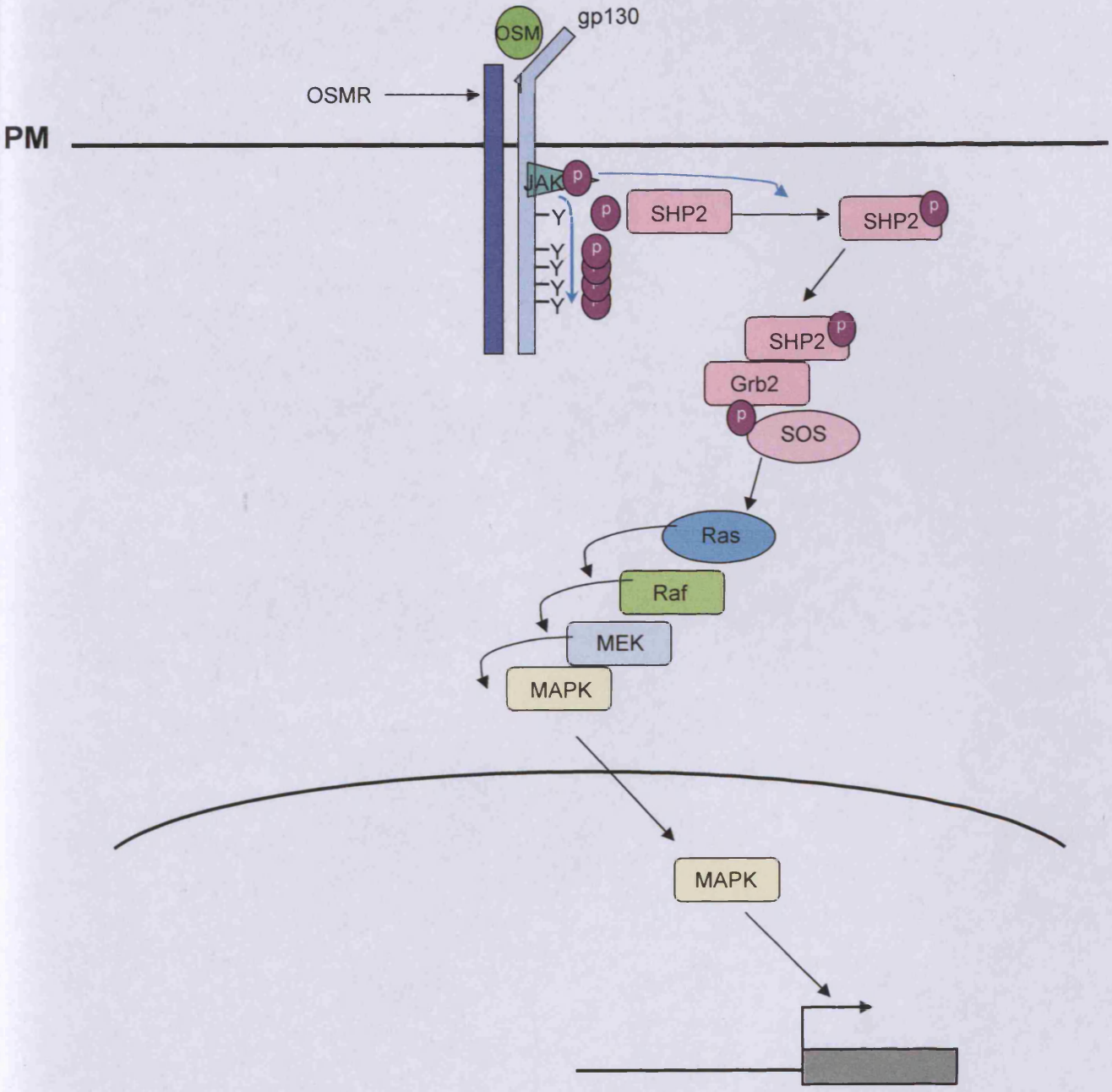


Figure 1.10: *OSM signalling via the SHP2 pathway.* Upon binding of OSM to its receptor, it becomes associated with gp130. This leads to the activation of JAKs (Janus activated kinases), by tyrosine phosphorylation, which then phosphorylate gp130. The JAKs then tyrosine phosphorylate receptor bound SHP2, which then binds the adaptor protein growth factor receptor binding protein 2 (Grb2), which is constitutively associated with the GDP/GTP Ras exchange factor Sos, and GTP bound Ras triggers ERK activation, which leads into the MAPK pathway. (Heinrich *et al* 2003, Ernst and Jenkins 2004).

1.20 Established Renal Failure

End stage renal failure arising as a consequence of chronic renal disease is characterised by a 90% reduction in renal function as determined by the glomerular filtration rate (GFR). Patients with chronic renal disease have an abnormally low GFR. Causes of renal failure can include hypertension and cystic and interstitial renal diseases, but the majority of cases occur as a direct result of diabetic nephropathy, following longstanding diabetes mellitus.

The most effective and preferred treatment for end stage renal failure patients is kidney transplantation. However a lack of donor kidneys and transplant failure substantially limits the effectiveness of this treatment. Alternative treatment strategies involve the use of haemodialysis, which uses hydrostatic pressure to achieve ultra-filtration of fluids and solutes from the blood, or peritoneal dialysis to remove solutes from the blood.

1.20.1 Peritoneal dialysis

Peritoneal dialysis (PD) is an alternative therapy to haemodialysis, and is a more widely used therapy for patients with end stage renal failure. Peritoneal dialysis involves the use of a highly concentrated sugar solution to remove solutes from the blood utilising the semi permeable and highly vascularised peritoneal membrane as a dialysing surface, which separates the peritoneal capillary blood from the dialysate that is instilled into the peritoneal cavity through an implanted catheter (Venkat *et al* 2006).

1.20.2 Peritoneal dialysis technique

Dialysis fluid, which contains a high concentration of glucose, is instilled into the peritoneal cavity *via* an implanted catheter, which enters the skin in the right or left lower abdominal quadrant. Dialysate is left in the peritoneal cavity for 6-8 hours to equilibrate and then drained to allow fresh dialysate to be instilled. Removal of solutes from the blood relies on the creation of an osmotic gradient between the dialysate and the capillary rich peritoneal membrane by using varying concentrations of glucose in the dialysis fluid. During this time, solute transport occurs across the peritoneal membrane (Venkat *et al* 2006). Water and small solutes including uremic metabolites move from the blood into

the dialysate through the combination of osmotic pressure and hydrostatic ultrafiltration. This dialysis fluid is then drained from the peritoneal cavity and the cycle repeated approximately four times daily.

1.20.3 Peritonitis

Peritonitis, an inflammatory condition caused by bacterial infection, is a common complication with peritoneal dialysis and the biggest reason for treatment failure. It is defined by a dialysate leukocyte count of 10^5 cells/ml or more, with or without accompanying symptoms such as abdominal pain and fever and cloudy dialysate (Davies *et al* 1996). Repeated episodes of peritonitis are directly related to increased peritoneal fibrosis and increased susceptibility to further peritoneal infection.

The most common pathogens associated with peritonitis are gram-positive staphylococci, including *Staphylococcus epidermidis* and *Staphylococcus aureus* (Woo *et al* 2004). Most episodes of peritonitis are associated with catheter site infections caused by touch contamination during dialysis exchange. Episodes of peritonitis have been associated with the onset of ultrafiltration failure and can cause morphological changes in the membrane, characterised by cellular necrosis and a loss of the mesothelium (Di Paolo *et al* 1986). A loss of the filtration barrier and the function of the mesothelium leads to a loss of host defence, resulting in increased incidence of further infection. This increased susceptibility to peritonitis further compromises the host immune response, and ultimately, leads to treatment failure (Davies *et al* 1996).

1.20.4 The peritoneal membrane

The peritoneal membrane is composed of two distinct layers: an epithelial layer known as the mesothelium; and its underlying connective tissue known as the interstitium or the compact zone (Krediet *et al* 2000). These layers are separated by a discontinuous basement membrane, composed of proteoglycans, type IV collagen and glycoproteins, which supports the mesothelium (Nagy and Jackman, 1998). The compact zone acts as a cellular barrier preventing fibroblasts in the connective tissue making contact with the

mesothelium. This zone is composed of extracellular matrix molecules including type I and III collagen, fibronectin and hyaluronic acid (Witz *et al* 2001).

The peritoneal membrane is highly vascularised to traffic molecules to and from tissues and organs of the abdominal cavity and a lymphatic system is also present, which is important for host defence within the peritoneum (Nagy and Jackman, 1998).

The mesothelium is composed of a monolayer of flattened mononuclear cells, which possess a luminal microvilli border to increase the surface area for exchange (Di Paolo *et al* 1986). The main function of the mesothelium is to maintain normal homeostasis within the peritoneal cavity, being involved in the transport of solutes and cells across serosal cavities, however they also play a role in regulating the inflammatory response after peritoneal infection or injury (Mutsaers *et al* 2004).

1.20.5 Peritoneal inflammation and the role of IL-6

Peritoneal inflammation can be characterised by an initial recruitment and subsequent clearance of neutrophils followed by the recruitment of a more sustained mononuclear cell infiltrate (Topley *et al* 1996) (Figure 1.11). Peritoneal inflammation, triggered by the presence of gram-positive bacteria, is driven by the early TLR-mediated activation and release of pro-inflammatory cytokines IL-1 and TNFα by "resident" peritoneal cells, usually monocytic cells such as macrophages. These pro-inflammatory cytokines signal the mesothelial cells, lining the peritoneal cavity, to produce an array of inflammatory mediators including CXCL1, CXCL2, CXCL5 and CXCL8, which are potent neutrophil activating CXC chemokines that promote neutrophil recruitment (Hurst *et al* 2001), and integrins such as CD62L, ICAM-1 and VCAM-1 in the mesothelium and endothelium of the surrounding blood vessels (Betjes *et al* 1993, Li *et al* 1998, Jonjic *et al* 1992). This initiates the strong adhesion of circulating neutrophils to the endothelium of blood vessels surrounding the site of inflammation and forms a chemotactic gradient down which neutrophils migrate into the peritoneal cavity where they form the initial response to the infection (Topley *et al* 1996). Upon entry into the peritoneal cavity, neutrophils are known to shed their surface bound IL-6R (McLoughlin *et al* 2004).

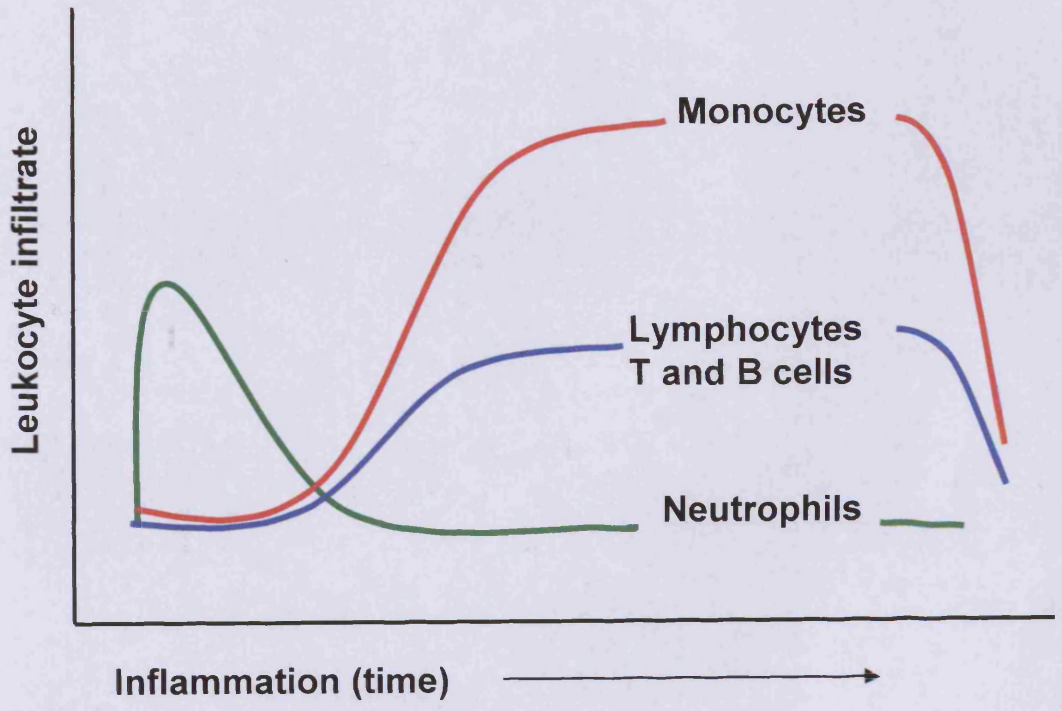


Figure 1.11: *Leukocyte infiltration into the murine peritoneal cavity following the induction of inflammation.* Upon induction of peritoneal inflammation, a characteristic infiltration of leukocytes can be observed. There is an initial influx of neutrophils which are cleared promptly, and followed by an infiltration of monocytic cells and lymphocytes.

This shedding is performed by the sheddase ADAM-17 and is thought to be initiated by: 1) C reactive protein (CRP) activation of neutrophils (produced in the liver in response to IL-6) (Jones *et al* 1999), 2) apoptosis of neutrophils (Chalaris *et al* 2007) and 3) by activation of neutrophils by CXCL1 and other chemoattractants (Marin *et al* 2001, McLoughlin *et al* 2004). This release of IL-6R facilitates the formation of the IL-6/sIL-6R complex, when the soluble receptor binds to exogenous IL-6 present in the peritoneal cavity having been secreted from the lining mesothelial cells (Topley *et al* 1993). The formation of this complex facilitates trans-signalling and subsequent STAT activation in the mesothelial cell lining of the peritoneal cavity, which has been shown to differentially regulate inflammatory chemokine expression (notably the down-regulation of CXCL1 and up-regulation of CCL2 and CCL5 by mesothelial cells) (Hurst *et al* 2001, McLoughlin *et al* 2005) and modulate leukocyte apoptosis (in particular IL-6 directs caspase-3-mediated neutrophil apoptosis, but prevents T cell apoptosis) (McLoughlin *et al* 2003, Teague *et al* 1997, Atreya *et al* 2000). Consequently, IL-6 is essential for directing the transition from innate to acquired immunity (Figure 1.12) by defining the successful resolution and clearance of neutrophils, and the subsequent recruitment of mononuclear cells (Jones, S.A. 2005, Hurst *et al* 2001, McLoughlin *et al* 2004, 2005). Regulation of these activities may be critical for resolving bacterial infections since IL-$6^{-/-}$ mice show an impaired ability to control infections (Kopf *et al* 1994, Dalrymple *et al* 1995).

When challenged, IL-6 deficient mice exhibit impaired control of leukocyte infiltration and apoptosis. These mice exhibit increased neutrophil recruitment to the peritoneal cavity following acute inflammatory challenge, due to increased secretion of neutrophil chemoattractants, and also an impaired clearance of these cells resulting from impaired induction of apoptosis. These mice also exhibit impaired T lymphocyte trafficking into the cavity as a result of impaired chemokine secretion and receptor expression (McLoughlin *et al* 2004, 2005). In WT mice, IL-6 selectively governs T cell infiltration by regulating chemokine secretion (CXCL10, CCL4, CCL5, CCL11, and CCL17) and chemokine receptor (CCR3, CCR4, CCR5, and CXCR3) expression on the CD3+ infiltrate (McLoughlin *et al* 2005).

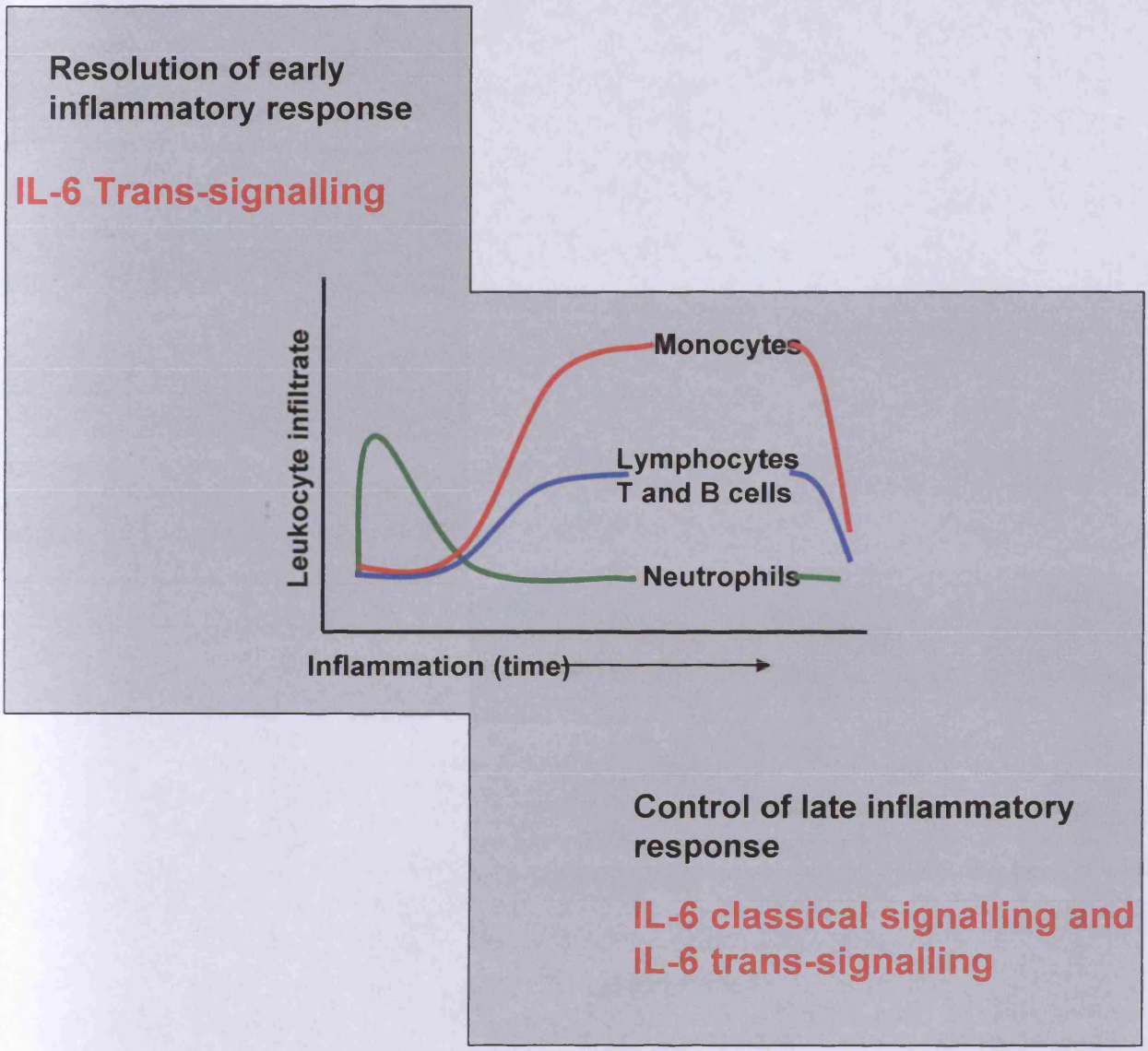


Figure 1.12: *Regulation by IL-6 signalling of the early innate and late/adaptive arms of the immune system.* The early innate immune response is regulated by IL-6 trans-signalling, while the late/adaptive immune response is regulated by both classical and trans-signalling.

Additionally, the IL-6/sIL-6R complex has been shown to be an inhibitor of apoptosis in T cells by inhibiting the down-regulation of anti-apoptotic regulator Bcl-2 (Teague *et al* 1997). IL-$6^{-/-}$ mice lacking this cytokine are more prone to T cell apoptosis, however this has not been proven in a model of peritonitis.

During initial cases of peritonitis, the influx of mononuclear cells effectively clears the pathogen and as a result, removes the stimulus for the production and release of pro-inflammatory cytokines. This then down-regulates the trafficking signals facilitating leukocyte infiltration into the cavity, and therefore leads to the resolution of the inflammatory response. In recurrent cases of peritonitis however, or in patients who have been undergoing PD for sustained periods of time, it is common to see a decreased clearance of pathogens during infection, and a constant up-regulation of pro-inflammatory cytokines, which lead to the onset of a chronic inflammatory state. This is characterised by an increased infiltration of neutrophils followed by a sustained influx and retention of mononuclear cells into the peritoneal cavity (Figure 1.13). The accumulation of leukocytes and the presence of elevated levels of cytokines in the peritoneal cavity have been shown to lead to loss of mesothelial integrity and thickening of the membrane. In cases of peritoneal membrane thickening, or 'fibrosis', the dialysing capacity of the membrane is compromised which can lead to treatment failure (Davies *et al* 1996, Selgas *et al* 2006).

1.20.6 Peritoneal dialysis associated fibrosis

Fibrosis is a pathological hallmark of many diseases, and is characterised by alterations in extracellular matrix deposition as a consequence of tissue damage or persistent inflammatory processes (Wynn *et al* 2007). Evidence has shown that exposure to bio-incompatible peritoneal dialysis fluids during prolonged PD treatment in combination with repeated peritonitis episodes leads to long term changes in both peritoneal membrane structure and function. These include partial or complete loss of the mesothelium, a thickening of the submesothelial tissue due to deposition of collagen (fibrosis or sclerosis), hypervascularization in the submesothelial zone and alterations in the mesothelial cells such as multinucleation and reduced regenerative capacities

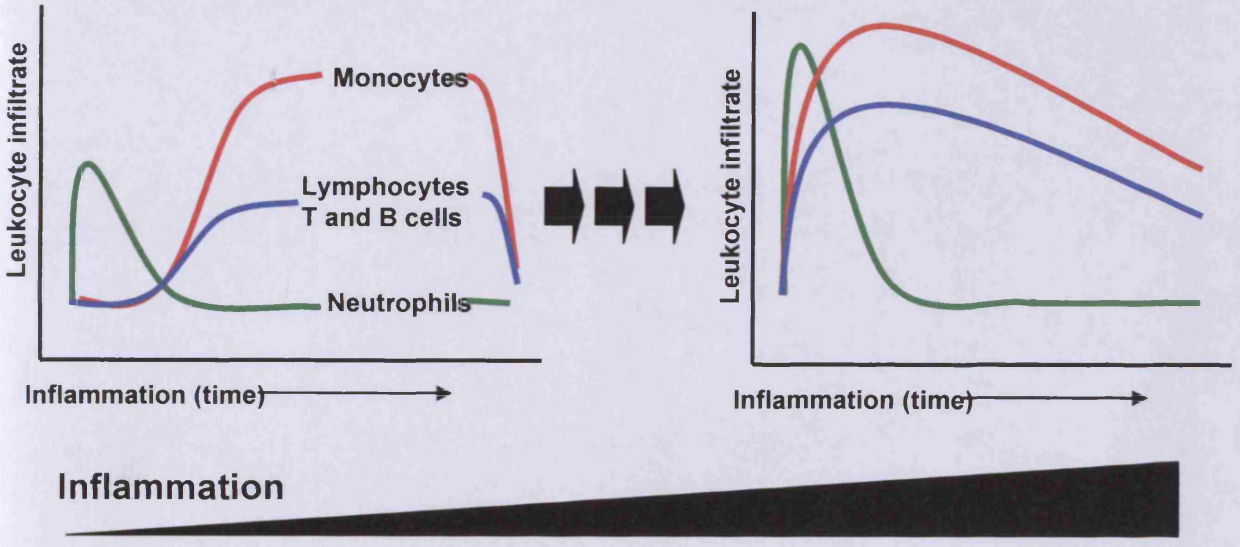


Figure 1.13: *Repeated rounds of infection lead to chronic inflammation characterised by enhanced neutrophil infiltration and retention of mononuclear cell populations.*

(Davies *et al* 1996, Williams *et al* 2002, Di Paolo *et al* 1986, Devuyst *et al* 2002). Clinical evaluation of peritoneal biopsies have shown that the normal thickness of the submesothelial zone is approximately 50 μm compared to a thickness of 270 μm in peritoneal dialysis patients with active peritoneal fibrosis (Williams *et al* 2002).

Glucose and glucose degradation products in the dialysis fluid are responsible for stimulating transforming growth factor-β (TGF-β) production by mesothelial cells. TGF-β is thought to be a key growth factor in the development of fibrosis in diabetic patients (Williams *et al* 2002), and increased peritoneal levels of TGF-β have been observed in PD patients (Williams *et al* 2002). TGF-β is a potent regulator of growth and differentiation of connective tissue cells and stimulates the conversion of fibroblasts to myofibroblasts during fibrogenesis (Roberts *et al* 1986). TGF-β also drives epithelial to mesenchymal transition (EMT) in the mesothelium. EMT is a process by which cells are diversified in complex tissues and by undergoing EMT, mesothelial cells are transformed into fibroblast like cells and acquire fibroblast capabilities with increased migratory, invasive and fibrogenic features (Selgas *et al* 2006). Epithelial to mesenchymal transition (EMT) is usually a normal physiologic process, during which local fibroblasts proliferate and secrete a non-rigid extracellular matrix rich in collagen type I and III, which is necessary for tissue repair under normal conditions. However if this process is uncontrolled, for example during PD when TGF-β levels are elevated, it can promote a fibrotic response and is well known to play a central role in the genesis of fibroblasts during organ fibrosis in adult tissues (Selgas *et al* 2006).

Glucose and glucose degradation products in the dialysis fluid are also responsible for stimulating vascular endothelial growth factor (VEGF) production by mesothelial cells. Local production of VEGF by transitional mesothelial cells in response to glucose products, appears to play a role in the processes leading to peritoneal angiogenesis (Selgas *et al* 2006). Peritoneal angiogenesis is one of the morphological membrane changes associated with fibrosis and is characterised by a progressive increase of

capillaries and vasculopathy, which leads to increased small solute transport across the membrane (Selgas *et al* 2006).

Peritonitis has been shown to exacerbate and accelerate the process of fibrosis. The inflammatory response that occurs during peritonitis provides an alternative source of the pro-fibrotic growth factor TGF-β, as well as IL-1 and other pro-inflammatory cytokines, and the accumulation of these factors that arises as a result of repeated infections can contribute to the onset of membrane fibrosis and treatment failure (Davies *et al* 1996, Selgas *et al* 2006).

The combination of fibrosis, increased vasculopathy and loss of the mesothelium leads to a decrease in ultrafiltration (Di Paolo *et al* 1986). The major cause of ultrafiltration failure is the increased vascular peritoneal surface due to neoangiogenesis in the peritoneum. This combined with the increased distance between endothelium and dialysate due to sub-endothelial fibrosis, can lead to the increased transport of low molecular weight solutes, which makes maintaining an osmotic gradient over long periods very difficult (Buemi *et al* 2004).

1.21 IL-6 regulation of monocytic cells

The role of IL-6 in directing the successful transition from innate to acquired immunity and ultimately the resolution of the acute inflammatory response has been extensively reviewed in this Chapter (Jones 2005). Using an *in vivo* model of peritoneal inflammation (induced by i.p administration of a cell free supernatant derived from *Staphylococcus epidermidis*), which enables a comparison of the inflammatory trafficking and production of inflammatory mediators between WT and IL-$6^{-/-}$ mice, it has been shown that IL-6 directs the successful resolution of neutrophil infiltration and subsequent recruitment of mononuclear cells *via* its control of chemokine production and apoptotic processes (Hurst *et al* 2001, McLoughlin *et al* 2005). However no observations have been made to suggest that IL-6 specifically regulates the recruitment and trafficking of monocytic cells (Hurst *et al* 2001, McLoughlin *et al* 2005). However these findings dealt with monocytic cells as a single population and did not discriminate

between the multiple monocytic cell subsets that are known to exist. Multiparameter flow cytometry can be used extensively to allow identification and discrimination of monocyte subpopulations with the use of antibodies against unique phenotypic markers. Using this technique, resident monocytic populations that reside in the peritoneal cavity have previously been characterised (Dioszeghy *et al* 2008). These studies have shown that the monocytic population in murine peritoneal cavity of wild type mice is comprised of approximately 50% macrophages and 2-5% dendritic cells. The remainder of the cells in the murine cavity are comprised of eosinophils, B and T lymphocytes. The resident macrophage and DC populations can be distinguished using flow cytometry by their distinct phenotypes of $F4/80^{hi}CD11b^{hi}$ and $F4/80^{int}CD11b^{hi}$ respectively. These subsets have been extensively characterised individually by Dioszeghy *et al* 2008, and the surface marker expression of these populations is outlined in Table 1.6.

The protective roles played by IL-6 (directing microbial clearance and inflammatory resolution) are balanced against the detrimental effects exerted by this cytokine during chronic inflammatory disease. In this respect, studies using the same *in vivo* model have shown that IL-6 deficient mice subjected to repeated peritoneal inflammatory episodes remained resistant to the onset of the fibrosis normally associated with this condition, while WT controls exhibited thickening of the peritoneal membrane (Fielding *et al* 2009, submitted). These WT mice exhibit dramatically enhanced leukocyte trafficking upon repeated inflammatory episodes, which is thought to contribute to the characteristic membrane fibrosis. While the neutrophil and lymphocyte populations have been characterised in this model, little is known about the composition of the monocytic cell infiltrate and how IL-6 may regulate their migration.

Surface marker	Resident macrophages	Resident DC
CD11b	+++	+++
F4/80	+++	+
CD14	+++	+++
7/4	-	-
Gr-1	-	-
CD11c	-	++
B220	-	-
CD3	-	-
CD4	+/-	+
CD8	-	-
CD83	-	-
M-CSF-R	+	++
GM-CSF-R	++	+++
Dectin-1	+/-	++
Dectin-2	-	-
MR	-	++
MARCO	+/-	+
SR-A	+	-
Siglec1	+/-	-
Syndecan1	-	+/-
TLR2	+	+
CCR2	-	-
CCR5	+/-	++
CCR6	-	-
CXCR4	+/-	+/-
CX3CR1	+/-	+
CD62L	+/-	+/-
ICAM-1	+++	+++
CD49b	-	-
CD40	+	+
CD80	+	+
CD86	+	+
MHC-II	+	+++
12/15-LOX	+	-

Table 1.6: *Murine resident peritoneal macrophage and dendritic cell surface marker expression.* Data have been assigned arbitrary symbols: + indicates low expression, ++ indicates intermediate expression, +++ indicates high expression, and – indicates no expression. Adapted from Dioszeghy *et al* 2008.

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 SV40 transformed human peritoneal mesothelial cells (HPMC)

SV40 transformed HPMC are derived from a primary HPMC cell line that has been transformed with a plasmid encoding a modified SV40 sequence which contains a deletion in the late region and a 1bp insertion that disrupts the origin of replication site within the SV40 genome. This cell line retains many of the phenotypic properties of primary HPMC including morphology and the expression of cytokeratins 8 and 18 and vimentin (Fischereder *et al*, 1997).

2.2.1.2 SV40 HPMC growth conditions

SV40 HPMC were cultured in DMEM (Dulbecco's modified medium) (Gibco, Invitrogen, Paisley, UK) with 10% (v/v) heat inactivated foetal calf serum (FCS) (Gibco, Invitrogen, Paisley, UK), supplemented with 2mM L-glutamine (Gibco, Invitrogen, Paisley, UK), 100U/ml penicillin, 100μg/ml streptomycin (Gibco, Invitrogen, Paisley, UK), 5μg/ml transferrin, 5μg/ml insulin and 0.4μg/ml hydrocortisone (Li *et al*, 1998). Cell monolayers were grown in T25 Falcon culture flasks (Beckton Dickinson, Oxford, UK) (incubated at 37°C, 5% CO_2) until confluent.

2.2.1.3 Monolayer sub-culture

On reaching confluence in the primary culture flask, cells were transferred to T75 flasks before passage into appropriate culture vessels for experimental purposes at a 1:3 ratio. Sub-culture involved the use of trypsin to remove cells from the bottom of the plate. Growth medium was removed by aspiration and cells were washed with 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) before trypsinisation with 0.1% trypsin/0.02% EDTA (ethylenediaminetetraacetic acid) (Gibco, Invitrogen, Paisley, UK) (w:v) diluted in sterile PBS. Cell detachment was monitored by light microscopy

following brief incubation at 37°C. Once all cells had detached, 10mls of appropriate growth medium supplemented with 10% (v/v) FCS was added to neutralise the trypsin. The cell suspension was then transferred to a 50ml universal tube for centrifugation. Cells were spun at 600xg for 6 minutes, the supernatant removed and the cell pellet resuspended in FCS supplemented medium and transferred to the appropriate culture vessel and incubated at 37°C, 5% CO_2.

2.2.1.4 Growth arrest and cell stimulation

At the beginning of each experiment, the growth phase of the cell population was synchronised by growth arresting the cells in serum free media containing appropriate culture supplements. In brief, the medium was removed, cells were washed with PBS to remove any residual FCS, and the media replaced with serum free medium. Cells were then incubated for 48 hours at 37°C, 5% CO_2. For cell stimulation, cells were washed twice with serum free medium, prior to treatment with the desired stimulant/cytokine.

2.2.2.1 Isolation and culture of mouse peritoneal leukocytes

To collect total peritoneal leukocytes, healthy animals were sacrificed by Schedule 1. The peritoneal cavity was lavaged with 2ml ice cold sterile PBS. Cell numbers were determined using a Coulter counter. Leukocytes were then enriched through a magnetic sorting column (MACS column) (Miltenyi Biotech, Surrey, UK) selecting for $CD11b^+$ cells (as described in section 2.2.2.2) prior to centrifugation (2000xg, 5 minutes) and resuspension in RPMI 1640 medium (Gibco, Invitrogen, Paisley, UK) supplemented with 10% FCS, 2mM L-Glutamine, 100U/ml penicillin, 100μg/ml streptomycin. Cells were subsequently stimulated in 12 well plates ($2x10^6$ cells/well in 2mls media) for 2 hours with appropriate inflammatory mediators and stimulants (as described in the relevant Figure Legends). Culture supernatant samples were rendered cell-free by centrifugation and stored at -70°C for further cytokine analysis. Cells were collected by scraping for flow cytometric analysis.

2.2.2.2 MACS purification of CD11b$^+$ murine peritoneal leukocytes

Murine peritoneal cells were pelleted from lavage fluid by centrifugation (300xg, 10 minutes). Cells were then resuspended in MACS buffer (PBS pH7.2, 0.5% BSA (Sigma, Poole, UK), 2mM EDTA) at a ratio of 90µl of buffer to every 10^7 total cells. Magnetic microbeads coated in anti-CD11b antibody (Miltenyi Biotech, Surry, UK) were then added at a ratio of 10µl beads to every 10^7 cells, and incubated at 4°C for 15 minutes. Cells were washed with 2 mls MACS buffer per 10^7 cells and centrifuged at 300xg for 10 minutes. Supernatant was discarded and the pellet resuspended in 500µl MACS buffer (up to a maximum of 10^8 cells) or 3mls (>10^8 cells). A MACS column (Miltenyi Biotech, Surry, UK) was prepared by placing in the magnet and rinsing with the appropriate volume of MACS buffer. For cell concentrations up to $2x10^8$, an MS column was used and rinsed with 500µl MACS buffer. For concentrations of up to $2x10^9$ labelled cells, an LS column was used and rinsed with 3mls of MACS buffer. The cell suspension was then applied to the column and allowed to run through, followed by three washes of either 500µl or 3 mls of MACS buffer as before. Finally bound cells were eluted from the column by removing from the magnetic field and applying either 1ml or 5mls of MACS buffer, depending on column size.

2.2.2.3 MoFlo purification and culture of murine peritoneal macrophages and dendritic cells

Murine cells were collected by peritoneal lavage using cold sterile PBS (pH 7.4) as described in section 2.2.2.1. Peritoneal cells from between 10 and 30 age-matched mice of mixed sex were pooled and leukocytes were collected by centrifugation at 400g for 10 minutes. Cells were resuspended in MACS buffer at a concentration of $20x10^6$ cells/ml and to this was added 4ug/ml Fc block (CD16/CD32 FcγR1) (BD Pharmingen, Oxford, UK). These cells were then incubated at 4°C for 30 minutes. Anti-mouse F4/80 FITC (AbD Serotec, Oxford, UK) and CD11b APC (BD Pharmingen, Oxford, UK) were then added directly to the cells at a 1/100 (2µg/ml) concentration before incubating for a further 1 hour at 4°C. Labelled cells were then sorted based on the expression of F4/80

and CD11b, with the $F4/80^{hi}/CD11b^{hi}$ population being resident macrophages and the $F4/80^{int}/CD11b^{hi}$ population being dendritic cells.

Cells were cultured in 24 well plates with or without SES (prepared as described in section 2.3.3) (1/50), human recombinant IL-6 (50ng/ml) (R&D systems, UK) and hyper IL-6 (30ng/ml) (Fusion protein comprising IL-6 bound to the soluble IL-6R. Donated by Stefan Rose-John, Christian-Albrechts, University of Kiel, Kiel, Germany) in RPMI + 10% (v:v) FBS, 2mM L-Glutamine, 100U/ml penicillin, 100μg/ml streptomycin (v:v) at a concentration of 400,000 cells per well for the resident macrophages in 800μl volume of media, and 200,000 cells per well for resident dendritic cells in 400μl volume of media. After 20 hours of culture, supernatants were rendered cell free by centrifugation at 2000rpm for 5 minutes, and stored at -80°C for cytokine determination by Luminex or protein array.

2.2.2.4 Isolation of murine peripheral blood leukocytes

Mice were sacrificed by schedule 1, and blood was extracted by cardiac puncture into a syringe primed with heparin (5,000U/ml) (LEO laboratories Ltd, Princes Risborough, Bucks, UK) as an anti-coagulant. All red blood cells were removed by hypertonic lysis (155mM NH_4Cl (BDH Chemicals, Poole, UK), 11.9mM $NaHCO_3$ (Fischer Scientific, Loughborough, UK), 1mM EDTA) at room temperature (RT) for 5 minutes. This process was repeated as necessary, and each time leukocytes were collected by centrifugation (2000rpm 5 minutes).

2.2.2.5 Isolation of murine bone marrow leukocytes

Murine femurs and tibias were taken, and stripped. The ends were trimmed with sterile scissors, to expose the marrow. The bone marrow was then flushed from the bones using sterile PBS in a syringe with a needle. Bones were flushed until all cells had been recovered. Red blood cells were lysed as described in Section 2.2.2.4.

2.3 Animal experiments

Experiments were performed in age matched 6-12 week C57/BL6 J wild type (WT), IL-$6^{-/-}$, IL-$10^{-/-}$, $OSMR^{-/-}$, IFN-$\gamma^{-/-}$, $gp130^{Y757F/Y757F}$ and $gp130^{Y757F/+}$ mice using a mixture of males and females. C57/BL6 J mice were bought from Charles River suppliers. Procedures were performed in accordance with Home Office approved project licence number PPL-30/2269.

2.3.1 Mice

WT C57BL6 mice were bought from Charles River Laboratories (Margate, UK).

IL-$6^{-/-}$ mice were generated as previously described (Kopf *et al*, 1994). Briefly, the IL-6 gene was disrupted in the second exon (first coding exon) by insertion of a neomycin resistance (*neo*^{r}) cassette, and loss of the WT alleles and IL-6 messenger RNA was confirmed by Southern blot analysis and reverse transcriptase PCR from lipopolysaccharide (LPS)-stimulated macrophages. $OSMR^{-/-}$ mice were generated as previously described (Tanaka *et al* 2003). $Gp130^{Y757F/Y757F}$ and $gp130^{Y757F/+}$ mice were generated as previously described (Tebbutt *et al* 2002). IL-$10^{-/-}$ mice were from an in house colony, originally purchased from Jackson Laboratories, and were generated as previously described (Kuhn *et al* 1993). IFN$\gamma^{-/-}$ mice were generated as previously described (Dalton *et al* 1993).

2.3.2 Determination of baseline leukocyte numbers

Differential cell counts and FACS analysis were performed on peripheral blood and peritoneal leukocytes from WT and IL-$6^{-/-}$ animals. Peripheral blood was collected by cardiac puncture into syringes flushed with heparin (5,000U/ml) as an anti-coagulant. All red blood cells were removed by hypertonic lysis at room temperature (RT) for 5 minutes. This process was repeated as necessary, each time leukocytes were collected by centrifugation (2000rpm 5 minutes). To collect peritoneal leukocytes, healthy animals were sacrificed by Schedule 1, and the peritoneal cavity was lavaged with 2 mls of ice cold sterile PBS pH 7.4. Cell numbers were determined using a Coulter counter. Slides for differential cell counting were prepared by cytospin (Thermo Fischer Scientific, Waltham, MA, USA) and stained with Wright GIEMSA (Sigma, Poole, UK) for 45

seconds followed by washing in deionised water. Leukocyte subsets were counted according to cell morphology as visualised by light microscopy. Leukocytes numbers were also determined by flow cytometry using leukocyte markers as described in section 2.4.

2.3.3 Preparation of a *Staphylococcus epidermidis* cell-free supernatant (SES)

A lyophilised cell free supernatant was prepared from a clinical isolate of *Staphylococcus epidermidis*. In brief, an inoculum of *S. epidermidis* was maintained on a dorset egg medium slope for cultivation and maintenance of stock culture (Oxoid Ltd, Basingstoke, Hampshire, UK). Using asceptic techniques, an agar plate (DST agar) (Oxoid Ltd, Basingstoke, Hampshire, UK) was inoculated with a loop of dormant *S. epidermidis* bacteria, and incubated at 37°C for 48 hours. A single colony was used to inoculate a volume of nutrient broth (Nutrient broth no.2) (Oxoid Ltd, Basingstoke, Hampshire, UK). The culture broth was incubated for 18-24 hours at 37°C with shaking (170rpm) until the cells reached the stationary phase of growth. Turbidity was checked by eye, and cultures were then centrifuged at 1800xg for 20 minutes at 20°C. Supernatants were discarded and pellets resuspended in 50mls of Tyrodes salts solution (1.8mM $CaCl_2 \cdot H_2O$, 1mM $MgCl_2 \cdot 6H_2O$, 2.6mM Kl, 137mM NaCl, 0.42mM NaH_2PO_4, 5.5mM D-glucose, with 12mM $NaHCO_3$ (Sigma, Poole, UK) and sterile filtered using a 0.2μm membrane). Bacteria were pelleted again at 1800xg for 20 minutes at 20°C. Supernatants were discarded and pellets resuspended in Tyrodes salts solution to an OD of 0.5 at 560nm, which corresponds to $5x10^8$ cfu/ml. The bacterial suspension was then incubated at 37°C for 18-24 hours. After this period, bacteria was pelleted at 5000xg for 30 minutes at 20°C, and following this the supernatant was collected and filtered through a 0.2μm filter to remove any remaining bacteria. The filtered supernatant was then dialysed against 5L deionised water at 4°C to remove excess salt (50-60mls of supernatant per dialysis tube, 2 tubes per 5L of water). Fresh water was exchanged 3 times (every 8-12 hours). The dialysed supernatant was pooled and 10ml aliquots were prepared for freeze-drying. Lyophilised SES batches were stored at -70°C. Batches of SES were reconstituted in PBS (750μl) directly before use and filtered. All SES preparations were routinely stored for no more than 6 months.

2.3.4 Determination of SES activity

A dose response bio-assay was used to validate each batch of SES prior to use *in vivo*. SV40 human peritoneal mesothelial cells (HPMC) were grown in 48 well plates to passage 2 (Sections 2.2.1.2-2.2.1.3), and then growth arrested once confluent for 48 hours in non-FCS-supplemented medium as described in Section 2.2.1.4. Cells were then stimulated for 24 hours with SES reconstituted in 2.5mls of appropriate serum free medium at the following dilutions: neat, 1/2, 1/10, 1/50, 1/100. Stimulation with 100 pg/ml IL-1β (R&D systems, Abingdon, UK) was used as a positive control, and un-stimulated cells as a negative control. All stimulations were in a volume of 300µl. Following 24 hours, supernatants were removed, and centrifuged at 2000rpm for 5 minutes to pellet and remove any cells. The activity of the SES was then quantified by CXCL8 ELISA (BD biosciences, Oxford, UK) on the supernatants diluted 1/20. SES was considered suitable for use if the IL-8 levels produced from neat SES cultures was within 80% of that produced in response to IL-1β stimulation (Figure 2.1).

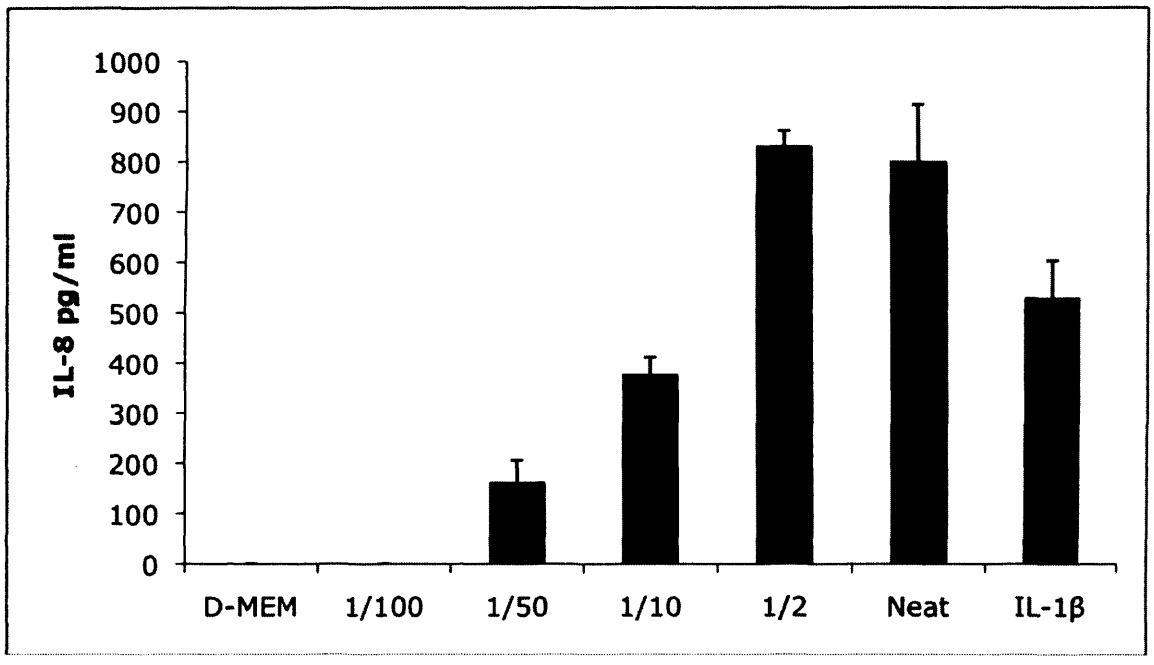


Figure 2.1: *Interleukin-8 production by SV40 cells following SES stimulation for 24 hours.*

2.3.5 Induction of peritoneal inflammation using *Staphylococcus epidermidis* cell-free supernatant

For each experiment groups of mixed sex, aged matched WT and certain genetically modified animals were used (IL-$6^{-/-}$, IFN-$\gamma^{-/-}$, IL-$10^{-/-}$, $gp130^{Y757F/Y757F}$, $gp130^{Y757F/+}$, $OSMR^{-/-}$). Inflammation was triggered in mice by intra-peritoneal (i.p) injection of SES (500µl). Dependent on the nature of the experiment, antibodies or cytokines were added to the SES prior to injection. These additions are stated in the Figure Legends in the appropriate Results Sections. At defined intervals (1 to 72 hours), mice were sacrificed by Schedule 1 and the peritoneal cavity lavaged with 2mls ice cold sterile PBS to collect the leukocytes. The leukocyte infiltrate was counted by direct counting (Coulter counter) (Section 2.3.2). Slides were prepared and stained for differential cell counts (Section 2.3.2). Remaining leukocytes were stained for flow cytometric analysis of macrophage and dendritic cell specific (F4/80, CD11b and CD11c) markers (Section 2.4.2). Peritoneal lavage supernatants were rendered cell free by centrifugation and stored at -70°C for future analysis by ELISA (Section 2.5).

Samples of peritoneal membrane were taken for histology from mice after resolution of four episodes of SES-induced inflammation. Groups of five IL-$6^{-/-}$ and five WT mice received four consecutive 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 (Sigma, Poole, UK). All histology was performed by Central Biotechnology Services, Cardiff University.

2.3.6 Preparation of a live *Staphylococcus epidermidis* inoculum for the live infection model

Bacteria ATCC *S.epidermidis* 12228 was maintained at room temperature on Agar slopes. An agar plate (prepared with Nutrient Broth # 2 (Oxoid Ltd, Baskingstoke, Hampshire, UK) was inoculated by aseptically transferring bacteria from a slope using a

flame-sterilised loop. The agar plate was then incubated for 48 hours at 37°C checking for growth at 24 hours. 10mls of Nutrient Broth # 2 was then inoculated with a single colony of bacteria aseptically using a flame sterilised loop. The culture was incubated overnight for 24 hours in a shaking incubator at 37°C. This overnight bacterial culture was then used to inoculate a second culture, in a suitable volume of Nutrient Broth # 2 (100 µl / 10 ml broth) to provide enough inoculum for the experiment planned. This culture was then incubated for 15 hrs in a shaking incubator and bacteria harvested from these log phase cultures. The bacterial broth suspension was pelleted at 4,000xg for 10 minutes at room temperature and the supernatant carefully decanted taking care not to dislodge the pellet. The pellet was resuspended in 40 ml sterile PBS as a wash and this step was repeated a total of 3 times. Finally the bacteria were centrifuged at 4,000xg for 10 minutes at room temperature and resuspended in sterile PBS to a cell density of $5x10^8$ colony forming units (cfu) per inoculum (500µl). The inoculum was stored at 4°C or on ice until 5 minutes prior to injection.

2.3.7 Induction of peritoneal inflammation using a live infection model with *Staphylococcus epidermidis*

For each experiment groups of mixed sex, aged-matched WT and IL-$6^{-/-}$ animals were used. Peritoneal inflammation was achieved in mice by intra-peritoneal (i.p) injection of $5x10^8$ cfu per mouse in a 500µl volume. At defined intervals (1-72 hours), mice were sacrificed by Schedule 1 and the peritoneal cavity lavaged with 2mls ice cold sterile PBS. The leukocyte infiltrate was assessed by direct counting (Coulter counter), differential staining and flow cytometric analysis with macrophage and dendritic cell specific markers (F4/80, CD11b and CD11c) (Sections 2.3.2 and 2.4.2). Lavage fluids were rendered cell free by centrifugation and stored at -70°C for analysis by ELISA as described in Section 2.5.

2.3.8 *In vitro* stimulation of inflammatory peritoneal leukocytes for intracellular cytokine staining

Inflammation was induced in WT, IL-$6^{-/-}$ and IL-$10^{-/-}$ mice by i.p injection SES (Section 2.3.5). Mice were sacrificed by schedule 1 at 24, 48 and 72 hours and peritoneal

leukocytes collected by lavage (Section 2.3.2). Cells were pelleted at 2000rpm for 5 minutes, and resuspended in RPMI medium containing 10% FCS, 2mM L-glutamine, 100U/ml Penicillin, 100μg/ml Streptomycin, 1mM sodium pyruvate (Gibco, Invitrogen, Paisley, UK) and 55μM β-mercaptoethanol (Gibco, Invitrogen, Paisley, UK). $1x10^6$ cells were cultured in 96 well plates stimulated with 50ng/ml PMA (Phorbol 12-myristate 13-acetate (Sigma, Poole, UK), 500ng/ml Ionomycin (Sigma, Poole, UK) and 3μM Monensin (Sigma, Poole, UK), for 4 hours at 37°C, 5% CO_2. Following this, cells were pelleted at 500xg for 3 minutes, and stained for flow cytometry (Section 2.4.3) to detect IL-17A, IFN-γ and IL-10 producing $CD4^+$ T cells.

2.4 Flow Cytometry

2.4.1 Use of Becton Dickinson FACS Calibur

Flow cytometric analysis allows information to be acquired on the relative size and granularity of cell samples, as well as enabling multi-parameter visualisation of defined cell marker expression using fluorochrome labelled antibodies.

2.4.2 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 2.1.

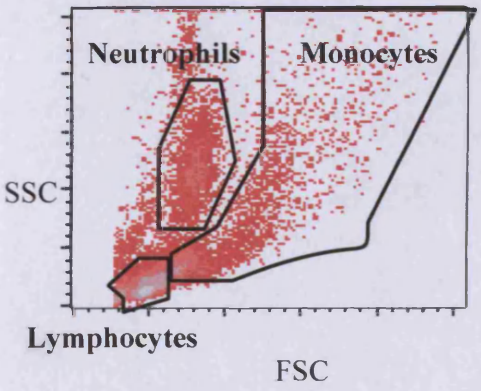


Figure 2.2: *Forward and side scatter profile of murine blood leukocytes indicating the separation of different subtypes of cells based on size and granularity.* Side scatter is an indication of granularity whereas forward scatter is an indication of size. Due to the characteristics of individual leukocyte 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.

2.4.3 General protocol for staining cells with fluorochrome labelled antibodies for visualisation by flow cytometry

Murine cells were collected by centrifugation at 2000rpm for 5 minutes and then resuspended to a concentration of $2x10^7$ cells/ml in Fc Block (4μg/ml rat anti-mouse CD16/CD32 FcγR1 monoclonal antibody (BD Pharmingen, Oxford, UK) in FACs buffer (5mM EDTA, 7.5mM NaH_3 (Sigma, Poole, UK), 0.5% BSA (USB Corporation, Cleveland, USA) in PBS pH 7.4). Cells were blocked for 30 minutes at 4°C before dilution to a concentration of between $2x10^5$ to $5x10^5$ cells/50μl in FACS buffer. Cells (50μl per well) were transferred to a 96 well plate and 50μl of a 2X concentrated antibody mix in FACS buffer was added to each well as appropriate. Cells were stained with primary fluorochrome-conjugated or biotin-conjugated antibodies at 2μg/ml for 30-60 minutes at 4°C. Where necessary, cells were incubated for a further 30 minutes with the appropriate fluorochrome-conjugated secondary antibody or streptavidin conjugated fluorochrome for biotinylated antibodies. Following staining, cells were pelleted by centrifugation at 500xg for 3 minutes, and washed in 200μl FACS buffer to remove unbound antibodies. This step was repeated twice.

For intracellular staining, cells were permeabilised for 20 minutes following surface staining in BD cytofix/cytoperm (BD Pharmingen, Oxford, UK). Cells were washed twice in 200μl BD cell perm/wash (BD Pharmingen, Oxford, UK) diluted 1/10 (v:v) in deionised water, and intracellular antibody (2μg/ml) staining performed for 45 minutes in BD cell perm/wash at 4°C. Cells were then washed twice in BD cell perm/wash and fixed in BD Cellfix (BD Pharmingen, Oxford, UK) diluted 1/10 (v:v) in deionised water.

Murine cells were analysed using a FACS Calibur flow cytometer. Data was acquired from 30,000-50,000 gated events and staining was compared to fluorochrome-conjugated isotype control antibodies. All antibody descriptions are shown in Table 2.1.

Antibody	Clone	Isotype	Fluorochrome	Working dilution	Source
F4/80	CI:A3-1	Rat IgG2b	FITC	2µg/ml	AbD Serotec
CD11b	M1/70	Rat IgG2b	APC	0.1µg/ml	BD Pharmingen
CD11b	M1/70	Rat IgG2b	PerCP-Cy5.5	0.5µg/ml	BD Pharmingen
CD11c	HL3	Hamster IgG1	PE	2µg/ml	BD Pharmingen
CD11c	HL3	Hamster IgG1	APC	4µg/ml	BD Pharmingen
CD14	Sa2-8	Rat IgG2a	PE	2µg/ml	eBioscience
CD4	RM4-5	Rat IgG2a	FITC APC PE	2µg/ml	Caltag
CD3	17A2	Rat IgG2b	PE	2µg/ml	BD Pharmingen
B220	RA3-6B2	Rat IgG2a	FITC/PE/PerCPCy5.5	2µg/ml	BD Pharmingen
CD19	1D3	Rat IgG2a	PE	2µg/ml	BD Pharmingen
Gr-1	RB6-8C5	Rat IgG2b	PE	2µg/ml	BD Pharmingen
CD40	3/23	Rat IgG2a	Biotin PE	2µg/ml	AbD Serotec BD Pharmingen
CD80	B7-1	Hamster IgG2	Biotin	2µg/ml	BD Pharmingen
CD83	Michel-17	Rat IgG1	PE	2µg/ml	eBiosciences
CD86	PO3	Rat IgG2b	Biotin	2µg/ml	BD Pharmingen
CD206	MR5D3	Rat IgG2a	Biotin	2µg/ml	R&D systems
MHCII	2G9	Rat IgG2a	Biotin	2µg/ml	BD Pharmingen
TLR2	6C2	Rat IgG2b	PE	2µg/ml	eBiosciences
CCR3	83101	Rat IgG2a	PE	2µg/ml	R&D systems
CCR5	C34-3448	Rat IgG2c	Biotin PE	2µg/ml	BD Pharmingen
CCR6	140706	Rat IgG2a	PE	2µg/ml	R&D systems

CCR7	4B12	Rat IgG2a	Biotin	2μg/ml	eBioscience
CXCR2	242216	Rat IgG2a	PE	2μg/ml	R&D systems
CXCR3	220803	Rat IgG2a	PE	2μg/ml	R&D systems
CXCR6	221002	Rat IgG2b	PE	2μg/ml	R&D systems
IL-6R	D7715A7	Rat IgG2b	PE	2μg/ml	BD Pharmingen
IL-6R	BAF1830	Goat IgG	Biotin	2μg/ml	R&D systems
IL-10	JES5-16E3	Rat IgG2b	APC	2μg/ml	eBioscience
CD62L	MEL-14	Rat IgG2a	PE	2μg/ml	BD Pharmingen
NK1.1	PK136	IgG2a	PE	2μg/ml	eBioscience
IFNγ	XMG1.2	Rat IgG1	FITC	2μg/ml	Caltag
IL-17A	TC11-18H10.1	Rat IgG1	PE	2μg/ml	BD Pharmingen
TNF-α	MP6-XT22	Rat IgG1	APC	2μg/ml	BD Pharmingen
IgG1	R3-34	Rat	FITC PE	2μg/ml	BD Pharmingen
IgG1	G235-5356	Hamster	PE	2μg/ml	BD Pharmingen
IgG1	G235-5356	Hamster	APC	4μg/ml	BD Pharmingen
IgG2a	R35-95	Rat	Biotin PE	2μg/ml	BD Pharmingen
IgG2b	A95-1	Rat	PE	2μg/ml	BD Pharmingen
IgG2c	A23-1	Rat	Biotin	2μg/ml	BD Pharmingen
IgG	polyclonal	Goat	Biotin	2μg/ml	Abcam
IgG2		Hamster	Biotin	2μg/ml	BD Pharmingen
CD16/CD32	2.4G2	Rat IgG2b	N/A	4μg/ml	BD Pharmingen

Table 2.1: *Anti-mouse antibodies used for flow cytometry staining*

2.4.4 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 fluorochrome 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. Any events to the right of the end of the negative isotype peak are considered to be positive. By utilising the statistics function on CellQuest Pro software, the geometric mean of the positive events and the negative events can be obtained, and the mean fluorescent intensity can then be calculated as the number given when the mean of the isotype control or negative events is subtracted from the mean of the cell marker or positive events. Calculation of the MFU enables the effect of cell stimulation on the relative receptor expression to be assessed.

2.4.5 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 or PerCPCy5.5 (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	Fluorochrome	Abbreviation
FL1	Fluoroscein / Fluoroscein isothiocyanate	FITC
FL2	Phycoerythrin	PE
FL3	peridinin–chlorophyll–protein complex–cyanine 5.5	PerCPCy5.5
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 and further quantification of specific cell subsets.

2.5 Enzyme-linked immunosorbent assays (ELISA)

Cell culture supernatants and murine peritoneal lavage fluids were assayed for cytokine and chemokine concentrations in accordance with the BD OptEIA™ (murine CCL2, CXCL1 (KC), IL-6, human CXCL8) or the R&D Systems Duoset (murine sIL-6R, CCL5) or the Antigenix America (murine CCL7) ELISA protocols. For specific antibody concentrations see Table 2.3.

Species	Chemokine/ Cytokine	Primary capture Ab concentration/ dilution	Secondary detection Ab concentrations	Top Standard concentration	Limit of detection
Human	CXCL8 *	1/250	1/250	200pg/ml	3.1pg/ml
Mouse	CXCL1 (KC) *	2μg/ml	200ng/ml	1000pg/ml	15.1pg/ml
	CCL2	1/250	1/250	1000pg/ml	15.1pg/ml
	CCL3 *	2μg/ml	400ng/ml	500pg/ml	7.8pg/ml
	CCL5 *	2μg/ml	400ng/ml	2000pg/ml	31.3pg/ml
	CCL7 •	0.5μg/ml	200ng/ml	1000pg/ml	15.1pg/ml
	IL-6	1/250	1/250	2000pg/ml	31.3pg/ml
	sIL-6R *	0.8μg/ml	100ng/ml	1000pg/ml	15.1pg/ml
	IL-10	4μg/ml	800ng/ml	2000pg/ml	31.3pg/ml

Table 2.3: *Antibody concentrations and standards for murine and human ELISA assays.* * correspond to R&D sourced kits. CCL7 ELISA was sourced from Antigenix America. All other kits were sourced from BD.

Each ELISA assay was run according to the manufacturers protocol and although the methods were similar among kits, some variations existed in the buffers and other components. In brief, 96 well plates were coated with specific monoclonal capture antibody (100μl) and incubated overnight at room temperature or at 4°C. For R&D ELISA kits, plates were coated in PBS, but for BD kits, plates were coated in 0.1M

sodium carbonate buffer (8.4g $NaHCO_3$, 3.56g Na_2CO_3 Sigma, Poole, UK). After each step the plate was washed in 0.05% (v:v) Tween20 (Sigma, Poole, UK) in PBS on a Jencons automatic plate washer (Jencons, West Sussex, UK). Plates were then blocked in PBS containing BSA (200µl per well), for one hour at room temperature. Composition of the blocking reagent varied between kits but was usually 3% (w/v) BSA in PBS for the BD OptEIA kits and 1% (w/v) BSA in PBS for the Duoset kits (R&D systems). Plates were washed once again and standards and test samples were added for a 2 hour incubation at room temperature. Samples and standards were diluted appropriately in reagent diluent (blocking reagents as described earlier). Each well was washed again prior to the addition of detection antibody (specific biotinylated monoclonal antibody and streptavidin-horseradish peroxidase (HRP) conjugate) (100µl per well), which was incubated in wells for 1 hour at room temperature, or two hours for R&D kits, with a separate streptavidin-HRP step for 20 minutes. Wells were once again washed prior to the final step, which was the addition of Tetramethyl Blue (Sureblue TMB (3,3',5,5'-tetramethylbenzidine) substrate) (Insight Biotechnology, Middlesex, UK) (100µl per well). Colour development was monitored by eye and once the required blue colour change was acquired, the reaction was stopped by addition of 2M H_2SO_4 (sulphuric acid) (Fischer Scientific, Loughborough, UK) 50µl per well. Absorbance changes were measured at 450nm (MRX Revelation, Dynex Technologies, Worthing, West Sussex, UK) and concentrations were deduced using the logarithmic relationship between the standards and their absorbances.

2.6 Luminex Bio-Plex Cytokine Assay

Luminex is a multisphere based multiplex system that allows detection of multiple cytokines/chemokines in a single well, and is ideal for multi-parameter analysis on precious and limited samples. The Bio-Plex cytokine assay was run according to the manufacturers protocol (Biorad, Hertfordshire, UK). In brief, fluorochrome labelled beads specific for 23 mouse cytokines (see Table 2.4) were diluted to the advised working concentration and aliquoted into a pre-wetted 96 well filter bottom plate (50µl/well). Beads were washed twice in 100µl of wash buffer provided with the kit. Wash buffer was removed between each step by vacuum filtration. Standards and samples were then added. Standards were diluted down the plate, with a 1/4 dilution between each well. Culture supernatants were added neat due to the top standard for each cytokine being at a very high concentration. The plate was then incubated on a shaker rotating at 1,100rpm for 30 seconds and then at 300rpm for 30 minutes at room temperature. The beads were then washed three times in wash buffer as previously described, and this was followed by the addition of a biotin conjugated detection antibody cocktail (25µl per well). The detection antibody was diluted in accordance with the manufacturers protocol in detection antibody diluent provided with the kit. The plate was then incubated for 30 minutes at room temperature with shaking as previously described. The beads were once again washed three times as before, and PE-conjugated streptavidin, (diluted as advised in Bio-Plex assay buffer) added (50µl per well). The plate was then incubated for 10 minutes at room temperature with shaking as previously described. Finally the beads were washed three times, and resuspended in 125µl of Bio-Plex assay buffer provided. Plates were then placed on a shaker for 30 seconds (1,100rpm) and immediately read on a pre-calibrated Bio-Plex system. This system works similarly to a FACS calibur in that it acquires a defined number of beads and then determines sample concentration of each cytokine relative to the standard based on the PE signal in each well.

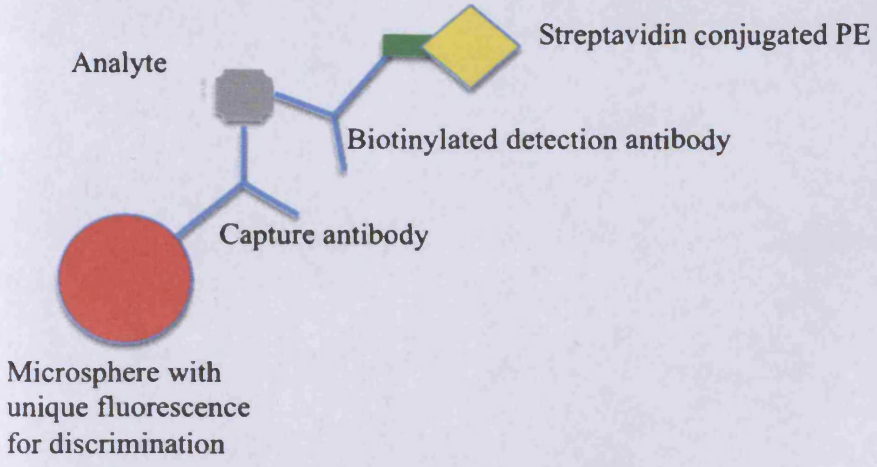


Figure 2.3: *A diagram to depict the mechanism of the Bioplex assay.* Capture antibodies specific for individual cytokines or chemokines are immobilised on the surface of microspheres, which possess a fluorescence unique to that soluble factor. Once the analyte has bound the capture antibody/microsphere complex, this is then incubated with a biotinylated detection antibody, which is subsequently conjugated to streptavidin PE. The plate reader can distinguish between different cytokines/chemokines according to their fluorescent microsphere, and quantify the levels of the analyte by measuring the PE fluorescence.

Cytokine/Chemokine	Top Standard concentration (pg/ml)
IL-1α	30998
IL-1β	20063
IL-2	74458
IL-3	30331
IL-4	66096
IL-5	24998
IL-6	17664
IL-9	23886
IL-10	108315
IL-12 (p40)	17376
IL-12 (p70)	15270
IL-13	53744
IL-17	50593
Eotaxin	49383
G-CSF	40736
GM-CSF	58435
IFN-γ	37387
KC	31953
MCP-1	35748
MIP-1α	31252
MIP-1β	27058
RANTES	16902
TNF-α	77425

Table 2.4: *Cytokines/chemokines detected in the Bioplex luminex assay, and their top standards used*

2.7 Proteome Profiler Arrays

Proteome profiler arrays (R&D Systems, Abingdon, UK) were used for parallel determination of the relative levels of selected cytokines and chemokines. Each kit contained four blots with capture antibodies for multiple cytokines and chemokines spotted in duplicate onto the surface. The membranes were blocked in array buffer provided for 1 hour on a rocking platform, and following this, cell free culture supernatants, that had previously been incubated for 1 hour with a detection antibody cocktail, were incubated with the membranes overnight at 4°C. Each membrane was then washed three times in 20mls of array wash buffer provided for 10 minutes with gentle rocking. The blots were then incubated with streptavidin-HRP for 30 minutes at room temperature, followed by three washes as described above. Finally a chemiluminescent substrate was incubated with the blots, in this case ECL western blotting detection reagents (GE Healthcare UK, Buckinghamshire, UK), for three minutes and blots were then developed for 10 minutes with X ray film.

2.8 Statistical analysis

In vivo experiments were performed on groups of five animals for each time point. *In vitro* experiments were performed to $n=\geq 2$. Statistical analyses were performed using the Student's *t*-test, with a p value of <0.05 considered significant. All data represent mean values +/- SEM.

CHAPTER 3

Phenotypic characterisation of resident peritoneal mononuclear subsets: Defining the involvement of STAT activating cytokines

3.1 Introduction

Resident macrophages and dendritic cells (DC) reside as homeostatic sentinels in the peritoneal cavity of both mice and humans where they are likely to undergo phagocytosis of apoptotic cells, antigen sampling, and the induction of peripheral tolerance to self molecules (Steinman *et al* 2000). However during infection, these cells act as the first line of defence, and act to co-ordinate the anti-microbial immune response (Dunn *et al* 1985). Resident macrophages and DC undergo a degree of turnover, particularly the DC, which are continually replenished. This is associated with their trafficking through the tissues into the lymphatic system and subsequent migration to the T cell area of the lymph nodes (Kamath *et al* 2000). This suggests that these populations are regularly replenished from the haematopoietic pool to maintain resident populations within the peripheral tissues.

It is well documented that gp130 mediated STAT3 signalling is critical for normal haematopoiesis, with evidence showing that mice exhibiting hyperactive STAT3 signalling and impaired SOCS3 regulation of gp130 signalling, display multiple hyperproliferative haemopoietic abnormalities (Jenkins *et al* 2002, 2005). Subsequent investigations showed that these mice also exhibit elevated myelopoiesis in the bone marrow, attributable to an increase by either IL-6 or IL-11 in the STAT3 driven impairment of TGF-β signalling (Jenkins *et al* 2007). Further studies have also illustrated that IL-6 is an important secondary cytokine in GM-CSF (Granulocyte macrophage colony stimulating growth factor) induced monocytopoiesis (Jansen *et al* 1992), and is required as an accessory cytokine to promote mono-DC haematopoiesis from normal progenitors (Santiago-Schwartz *et al* 1995). In contrast however, IL-6, Leukemia inhibitory factor (LIF) and ciliary neurotrophic factor have been reported to selectively inhibit the proliferation of committed macrophage-lineage progenitor cells indicating that gp130 activating cytokines may act as negative physiological regulators of mononuclear phagocyte differentiation or expansion (Clutterbuck *et al* 2000).

Since peripheral blood monocytes are a common precursor of macrophages and DC, there have been many contradictory studies demonstrating how IL-6 can regulate

macrophage or dendritic cell differentiation from monocytic cell precursors. In this respect, IL-6 has been shown to switch the differentiation of monocytes from dendritic cells to macrophages upon contact with fibroblasts, which occurs when monocytes cross the endothelium (Chomarat *et al* 2000). It is thought that IL-6 secretion by activated fibroblasts induces an up-regulation of M-CSF receptor expression on monocytes allowing an autocrinal activation of monocyte to macrophage differentiation by M-CSF (Chomarat *et al* 2000). In contrast however, Jenkins *et al* showed that the IL-6 family cytokines inhibited M-CSF-stimulated macrophage formation by the down-regulation of the M-CSF receptor gene *c-fms* (Jenkins *et al* 2004). The findings by Chomarat *et al* were supported by work from Mitani *et al*, who showed that IL-6 inhibited differentiation of monocytes to DC by promoting their differentiation towards macrophages, however this inhibition was reversed upon addition of TNF-α, LPS, IL-1β, CD40L or TGF-β (Mitani *et al* 2000). Similarly, Mentrier-Caux *et al* showed that IL-6 or M-CSF inhibited the differentiation of DC from $CD34^+$ progenitor cells, and redirected the differentiation of these cells towards a macrophage lineage (Mentrier-Caux *et al* 1998). Finally Bleier *et al* showed that the number of bone marrow derived DC from IL-6 deficient mice generated *in vitro* was significantly greater than that of WT mice, however, no difference was observed in the number and subtype composition of DC in the absence of IL-6 *in vivo* (Bleier *et al* 2004).

Whilst much of the work discussed so far infers that signalling by IL-6 promotes macrophage differentiation, there is work that supports a role for IL-6 in the promotion of dendritic cell differentiation. Brasel *et al* established that blocking IL-6 function reduced the number of DC generated in Flt-3 ligand-supplemented murine WT bone marrow cultures (Brasel *et al* 2000). Santiago-Schwartz *et al* showed that when $CD34^+$ progenitor cells were cultured with GM-CSF, TNF-α and stem cell factor (SCF), the proportion of DC generation was markedly enhanced by the presence of IL-6, and consequently reduced by the addition of blocking antibodies to IL-6 (Santiago-Schwartz *et al* 1995). Similarly, Bernhard *et al* showed that hyper IL-6 (an IL-6/sIL-6R fusion protein) in combination with SCF (Stem cell factor) and GM-CSF, promoted the

expansion and differentiation of DC from $CD34^+$ precursor cells from both bone marrow and peripheral blood stem cells (Bernhard *et al* 2000).

Given the contrasting data, Jenkins, B.J. suggests that differences in the transcriptional programming of immature and committed haematopoietic progenitors to respond to gp130 signalling, affect the ability of IL-6 family cytokines to determine the differentiation fate of these cells (Jenkins *et al* 2004). In light of this, the aim of this Chapter was to determine whether cytokine directed STAT signalling regulates the number of resident peritoneal macrophages and DC present in mice, and how it may potentially affect the phenotype of these cells.

3.2 Materials and methods

3.2.1 Isolation of basal resident peritoneal macrophages and dendritic cells

WT, IL-$6^{-/-}$, IL-$10^{-/-}$, $gp130^{Y757F/Y757F}$, $OSMR^{-/-}$ and IFN-$\gamma^{-/-}$ mice were sacrificed by schedule 1 and peritoneal leukocytes recovered by lavage as described in Section 2.2.2.1. Each of these strains are on a C57/BL6 background, except for the $gp130^{Y757F/Y757F}$ mice, which are on a mixed SV29/C57/BL6 background. For this reason, separate WT littermate controls, which had the same mixed background, were used for experimental comparisons against the $gp130^{Y757F/Y757F}$ mice.

3.2.2 Flow cytometric analysis of basal resident peritoneal macrophages and dendritic cells

Resident peritoneal macrophages and DC were labelled with anti-F4/80 FITC and anti-CD11b APC in combination with antibodies against a variety of macrophage and DC markers conjugated to either biotin or PE as described in Section 2.4.3. Details of the antibodies used are found in Table 2.1. Cell numbers were quantified by taking the flow cytometry gated population percentages and multiplying them by the total cell number.

3.2.3 MoFlo purification and culture of murine peritoneal macrophages and dendritic cells

Basal resident peritoneal leukocytes were collected by lavage from WT and IL-$6^{-/-}$ mice (Section 2.2.2.1). Cells were labelled with anti-F4/80 FITC and anti-CD11b APC conjugated antibodies as described in Section 2.2.2.3. Resident macrophage and DC populations were isolated on the MoFlo sorter based on their differential expression of these two antigens. Resident macrophages and DC were gated and sorted according to their $F4/80^{hi}$ $CD11b^{hi}$ and $F4/80^{int}$ $CD11b^{hi}$ phenotypes respectively. These populations were then individually cultured as described in Section 2.2.2.3, in the presence or absence of SES (as prepared in Section 2.3.3) at a 1/50 concentration or with 50ng/ml human recombinant IL-6 (R&D systems, UK). Cells were cultured for 20 hours and the supernatants removed and rendered cell free by centrifugation as described in Section 2.2.2.3. Supernatants were frozen at -70°C until required for cytokine quantification by either luminex or protein array.

3.2.4 Assays for cytokine/chemokine quantification of macrophage and DC culture supernatants

Culture supernatants were analysed on a Bioplex assay (BioRad), which could detect and quantify a panel of 23 murine cytokines/chemokines (see Section 2.6). The cytokines/chemokines measured are detailed in Table 2.4. Culture supernatants were also run on a protein profiler array (R&D Systems), which was used as an alternative method to the Bioplex assay for the semi-quantification of cytokines/chemokines. This was performed as described in Section 2.7 to verify results obtained from the bioplex assay.

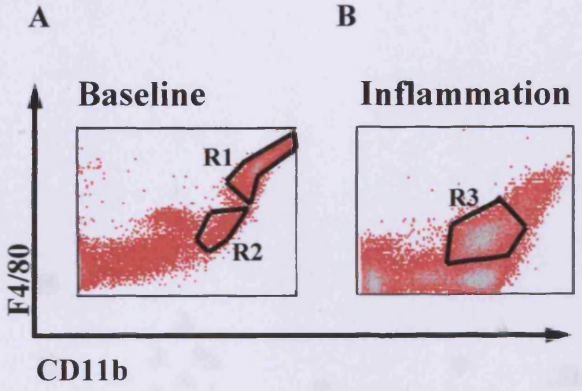
3.3 Results

3.3.1 Resident macrophages, DC and infiltrating monocytes have distinct phenotypes

Resident peritoneal macrophages and DC have been extensively characterised by Dioszeghy *et al* 2008, who showed that these subpopulations are easily identified by their differential expression of F4/80 and CD11b. In this respect, antibodies against these two markers were used to identify resident peritoneal macrophages and DC as well as infiltrating monocytes. Labelling of peritoneal leukocytes with anti-F4/80 and anti-CD11b revealed three distinct populations of monocytic cells (Figure 3.1). Resident peritoneal macrophages were defined as F4/80^{hi} CD11b^{hi}, and resident DC as F4/80^{int} CD11b^{hi}. Both these populations were present in the murine peritoneal cavity of unchallenged naive WT mice. Upon induction of peritoneal inflammation, a third infiltrating population of monocytes was observed. These infiltrating cells were defined as F4/80^{int} CD11b^{int} (Figure 3.1).

3.3.2 WT, IL-6$^{-/-}$, IL-10$^{-/-}$, IFNγ$^{-/-}$, OSMR$^{-/-}$ and gp130$^{Y757F/Y757}$ mouse total peritoneal leukocyte numbers

To determine differences in baseline leukocyte cell numbers in WT, IL-6$^{-/-}$, IL-10$^{-/-}$, IFNγ$^{-/-}$, OSMR$^{-/-}$ and gp130$^{Y757F/Y757}$ mice, peritoneal leukocytes were recovered by lavage, and the total leukocytes were counted using a coulter counter (Figure 3.2 and 3.5 A). WT, IL-6$^{-/-}$, IL-10$^{-/-}$, IFNγ$^{-/-}$, and OSMR$^{-/-}$ mice had equivalent numbers of total peritoneal leukocytes. Gp130$^{Y757F/Y757}$ mice, (which exhibit hyperactive STAT 1/3 signalling and impaired SOCS3 regulation of gp130 signalling as well as aberrant SHP2 signalling) had significantly more total peritoneal leukocytes compared to the WT mice.



C

R1	Resident peritoneal macrophages	F4/80^{hi}/CD11b^{hi}
R2	Resident peritoneal dendritic cells	F4/80^{int}/CD11b^{hi}
R3	Inflammatory/ infiltrating monocytes	F4/80^{int}/CD11b^{int}

Figure 3.1: *F4/80 and CD11b staining highlights three subpopulations of monocytic cells.* **(A)** Basal "resident" peritoneal leukocytes were labelled with anti F4/80 and anti CD11b highlighting the presence of two populations of monocytic cells. The F4/80^{hi}/CD11b^{hi} cells have been identified as resident macrophages, and the F4/80^{int}/CD11b^{hi} cells have been identified as resident dendritic cells (Dioszeghy *et al* 2008). **(B)** Staining of peritoneal leukocytes present during SES induced peritonitis (12 hours) highlighted the presence of F4/80^{int}/CD11b^{int}infiltrating monocytes. **(C)** Summary of the phenotypically distinct monocytic cell subsets.

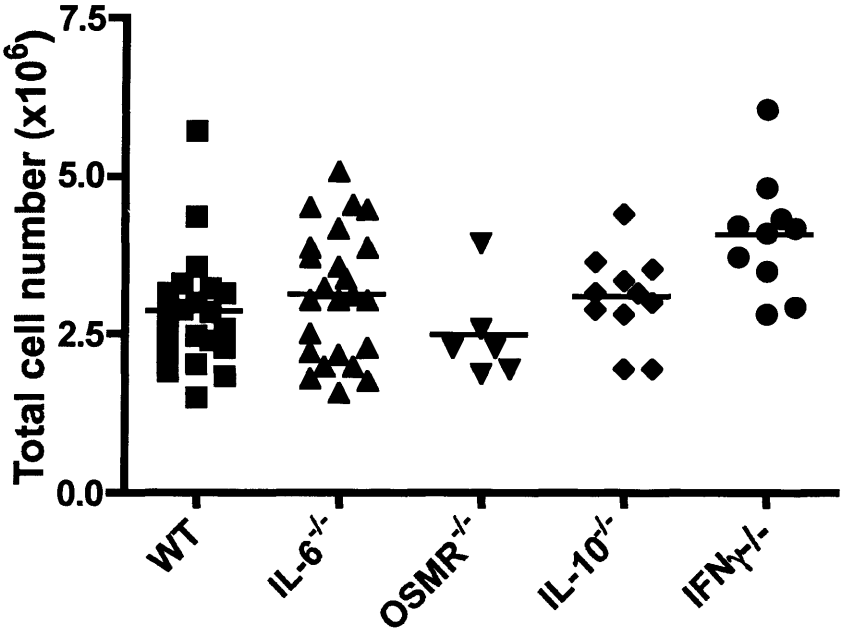


Figure 3.2: *Total peritoneal leukocyte counts for WT, IL-6$^{-/-}$, OSMR$^{-/-}$, IL-10$^{-/-}$, IFN-γ$^{-/-}$, and gp130$^{Y757F/Y757F}$ mice.* Peritoneal leukocytes were recovered by peritoneal lavage from WT, IL-6$^{-/-}$, OSMR$^{-/-}$, IL-10$^{-/-}$and IFN-γ$^{-/-}$ mice. Total cells were counted using a coulter counter.

3.3.3 Signalling by cytokines via the STAT pathway regulates resident peritoneal macrophage and dendritic cell numbers

To investigate whether STAT signalling regulates the proportion of resident monocyte populations, baseline levels of peritoneal macrophages and DC were recorded in a variety of mice deficient in cytokines that signal via the STAT pathway. The number of resident peritoneal macrophages present in the peritoneal cavity at baseline was plotted for WT, IL-$6^{-/-}$, OSM-$R^{-/-}$, IL-$10^{-/-}$ and IFN-$\gamma^{-/-}$ mice. No significant differences were observed between the WT and any of the genetic strains (Figure 3.3). However the number of resident peritoneal macrophages present in the $gp130^{Y757F/Y757F}$ mice was significantly greater than the number of macrophages present in the WT mice (Figure 3.5 B). The total cell number in $gp130^{Y757F/Y757F}$ mice is significantly higher than in WT mice (Figure 3.5 A), meaning that although the total number of resident macrophages was significantly greater in the $gp130^{Y757F/Y757F}$ mice, the proportion of these cells was equivalent in both strains.

Comparable analysis of the resident DC population in each of the 6 strains examined revealed that there were significantly fewer resident peritoneal DC present in the IL-$6^{-/-}$ mice, but significantly more of these cells in the IFN-$\gamma^{-/-}$ mice (Figure 3.4). The number of resident DC in $OSMR^{-/-}$ and IL-$10^{-/-}$ mice remained equivalent to the numbers observed in WT mice. $Gp130^{Y757F/Y757F}$ mice also showed no significant difference in the number of resident peritoneal DC compared to WT mice (Figure 3.5 C).

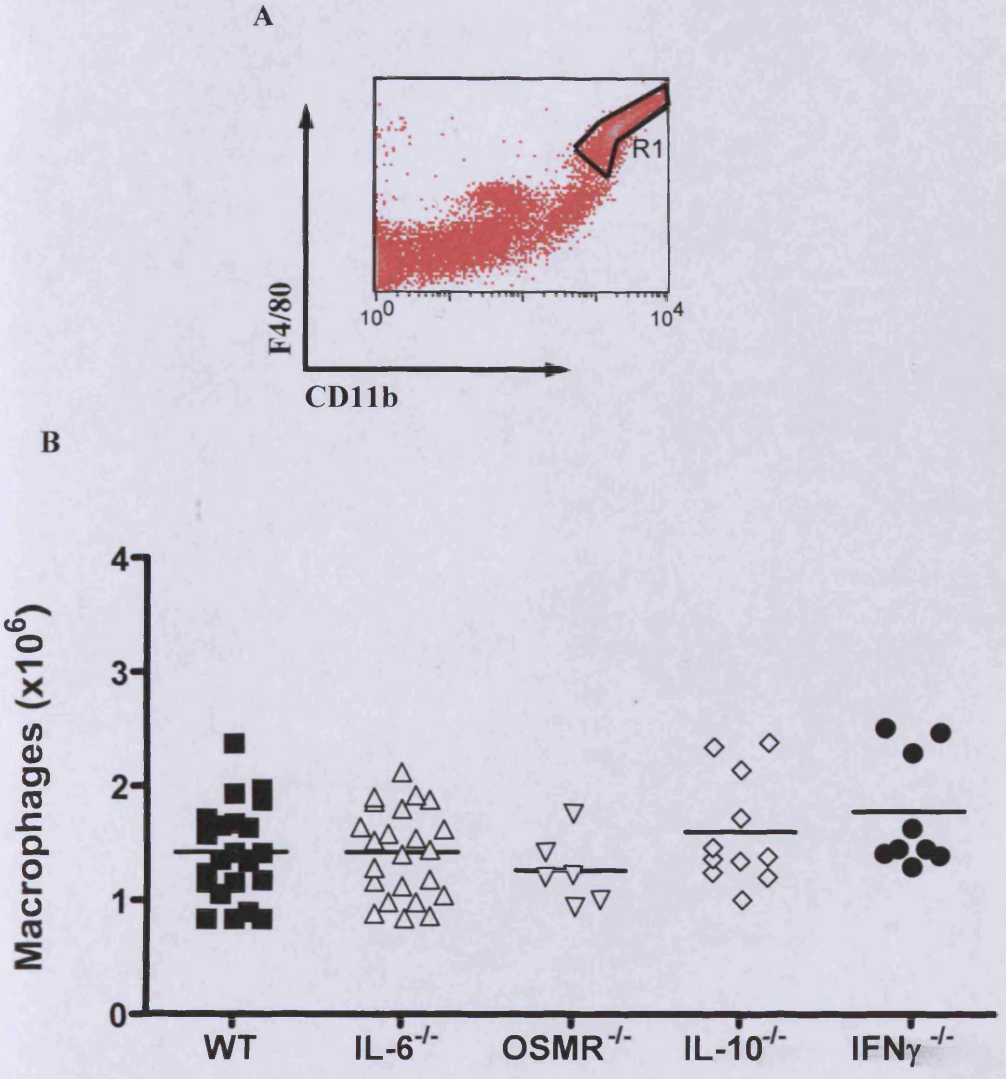


Figure 3.3: *IL-6, OSM, IL-10 and IFNγ driven STAT signaling does not determine the resident macrophage population size.* **(A)** Resident peritoneal macrophages were recovered by peritoneal lavage, and were identified by labelling with antibodies against F4/80 and CD11b. **(B)** This population size was determined in various knock out mice by taking into account the flow cytometry population gated percentage and applying this to the total cell number recovered from the peritoneal cavity.

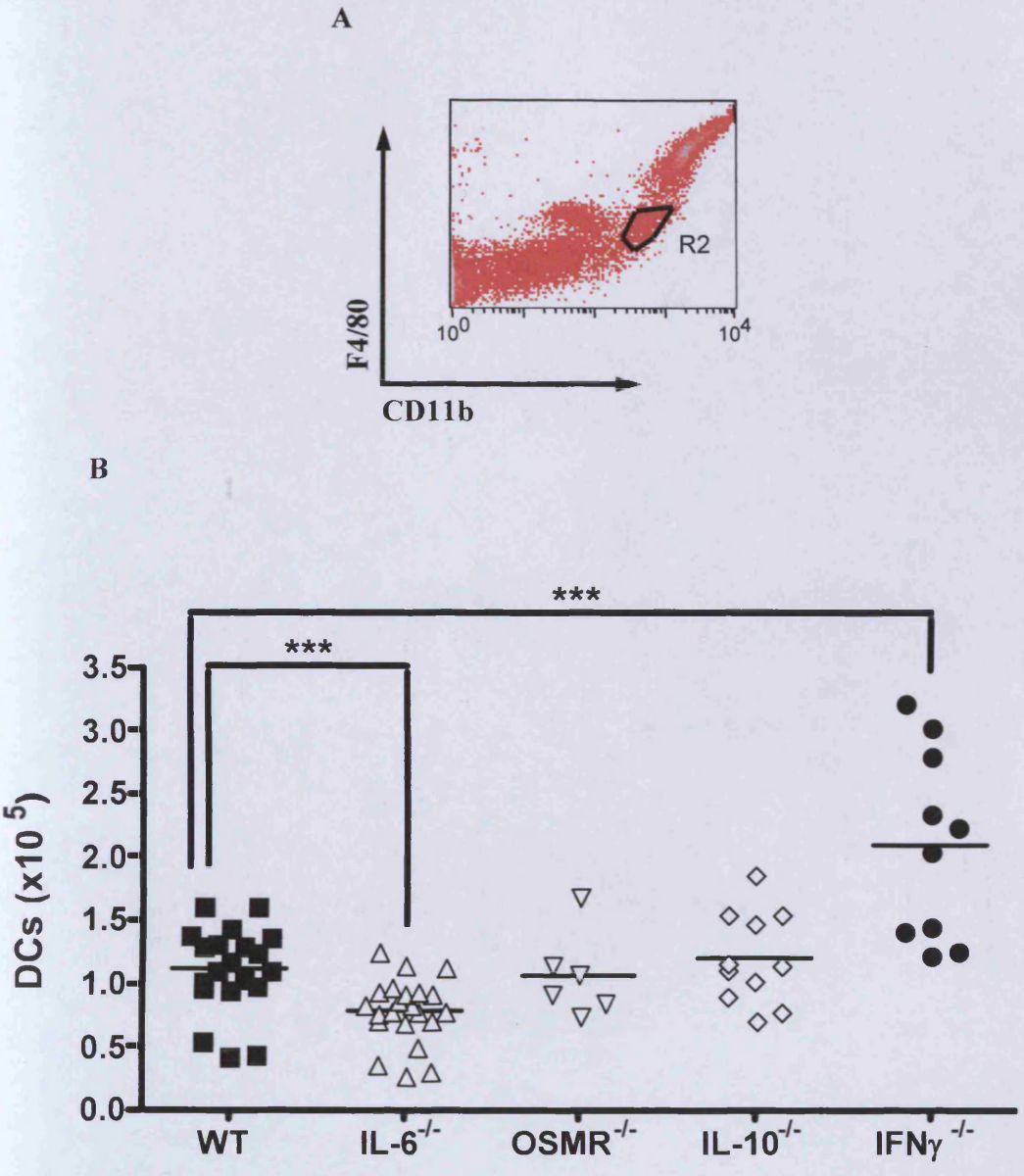


Figure 3.4: *IFN-γ mediated STAT1 signalling and IL-6 mediated Shp2 signalling plays a role in regulating the resident dendritic cell population size.* **(A)** Resident peritoneal DC were recovered by peritoneal lavage, and were identified by labelling with antibodies against F4/80 and CD11b. **(B)** This population size was determined in various knock out mice by taking into account the flow cytometry population gated percentage and applying this to the total cell number recovered from the peritoneal cavity. (*** $P < 0.001$)

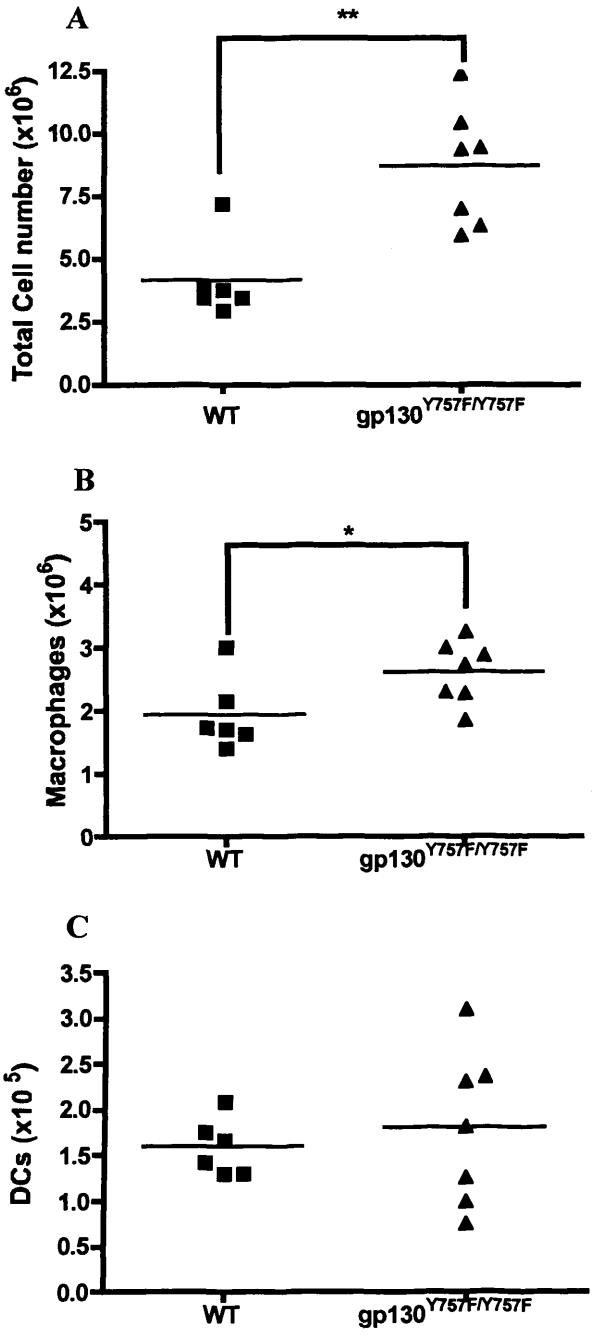


Figure 3.5: *Hyperactive gp130 mediated STAT1/3 signalling appears to play a role in regulating the resident macrophage population size, but does not plays a significant role in determining the resident dendritic cell population size.* Resident peritoneal leukocytes were recovered by peritoneal lavage, and counted **(A)** . Resident macrophages and DC were identified by labelling with antibodies against F4/80 and CD11b. The resident macrophage **(B)** and resident dendritic cell **(C)** population sizes were determined in WT and gp130$^{Y757F/Y757}$ mice (which display hyperactive STAT1/3 signaling) by taking into account the flow cytometry population gated percentage and applying this to the total cell number recovered from the peritoneal cavity. (* $P <$ 0.05, ** $P < 0.01$)

3.3.4 Phenotypic analysis of resident peritoneal macrophages and DC

Resident macrophages and DC were characterised using antibodies against pan markers associated with the phenotype of these cell types in the six murine strains, by flow cytometry (Figure 3.6). Wild type resident peritoneal macrophages expressed F4/80, CD11b, MHCII, CD80, CD86, ICAM-1, Scavenger RA, CD40, TLR2, IL-6R, CCR3 and CCR5. Wild type resident DC expressed F4/80, CD11b, MHCII, CD80, CD86, CD40, ICAM-1, TLR-2, Mannose receptor (MR), CCR3, CCR5 and IL-6R (Figure 3.6).

The expression of these characteristic macrophage and DC markers by resident peritoneal macrophages and DC was generally equivalent in the different genetic strains (Figures 3.7 & 3.8 respectively). The exceptions were resident macrophages from $gp130^{Y757F/Y757F}$ mice and IL-$10^{-/-}$ mice, which expressed more CCR5 and MHCII respectively than WT littermates (Figure 3.7 B&C). Finally, IL-$6^{-/-}$ mice expressed lower levels of CCR5 on their resident DC compared to WT mice (Figure 3.8 B).

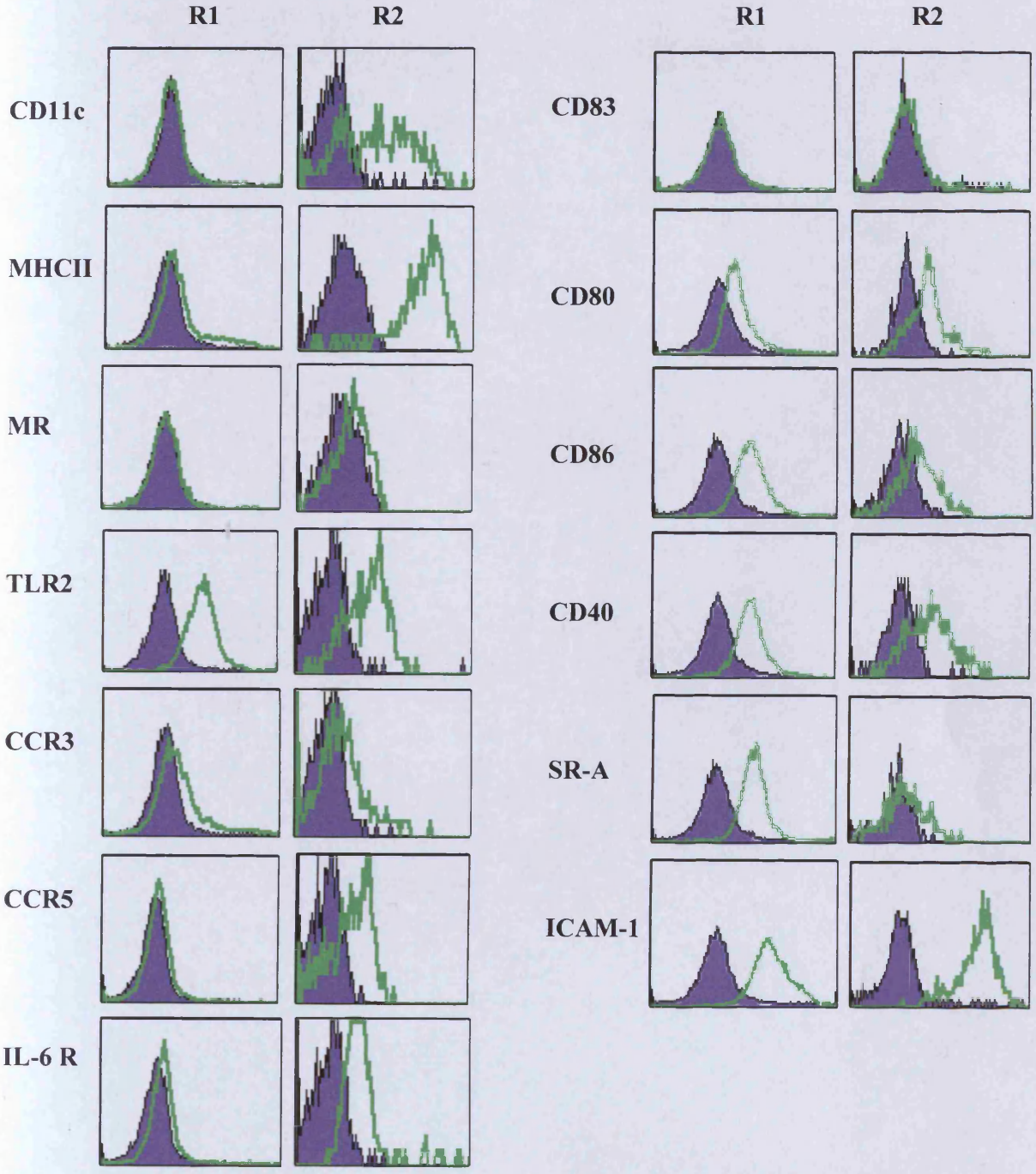


Figure 3.6: *Expression of macrophage and dendritic cell markers by WT resident peritoneal macrophages (R1) and dendritic cells (R2).* Resident macrophage and DC populations were identified by flow cytometry by labelling with anti F4/80 FITC and anti CD11b APC, and pan markers were measured using PE conjugated antibodies. Flow cytometry histogram plots are representative of four independent experiments.

A Resident peritoneal macrophage expression of pan markers.

Marker	WT	IL-6$^{-/-}$	OSMR$^{-/-}$	IL-10$^{-/-}$	IFN-$\gamma^{-/-}$	WT	Gp130$^{Y757F/Y757F}$
F4/80	+++	+++	+++	+++	+++	+++	+++
CD11b	+++	+++	+++	+++	+++	+++	+++
CD11c	-	-	-	-	-	-	-
MHCII	+	+	+	++	+	+	+
MR	-	-	-	-	-	-	-
TLR2	++	++	++	++	++	++	++
CD80	+	+	+	+	+	+	+
CD83	-	-	-	-	-	-	-
CD86	+	+	+	+	+	+	+
ICAM-1	+++	+++	+++	+++	+++	+++	+++
CD40	++	++	++	++	++	++	++
SR-A	+	+	+	+	+	+	+
CCR3	+	+	+	+	+	+	+
CCR5	+/-	+/-	+/-	+/-	+/-	+/-	+
IL-6 R	+/-	+/-	+/-	+/-	+/-	+/-	+/-

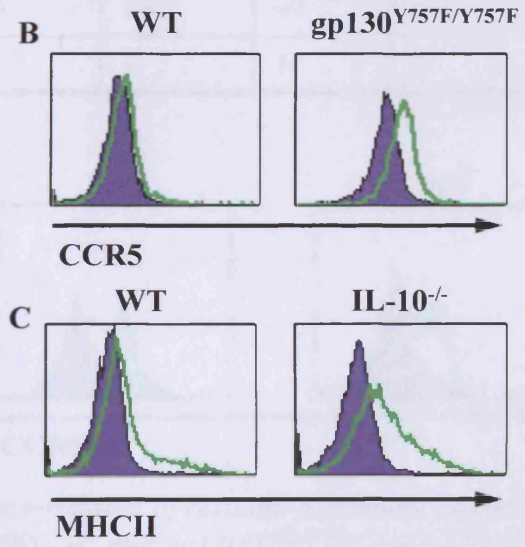


Figure 3.7: *Characterisation of resident peritoneal macrophages in WT, IL-6$^{-/-}$, OSMR$^{-/-}$, IL-10$^{-/-}$, IFN- $\gamma^{-/-}$ and gp130$^{Y757F/Y757F}$ mice.* **(A)** Peritoneal leukocytes were recovered by lavage, and labelled with antibodies against F4/80 and CD11b, to identify the macrophages, and an array of macrophage/DC markers. **(B)** CCR5 expression in WT and gp130$^{Y757F/Y757F}$ resident macrophages. **(C)** MHC II expression on WT and IL-10$^{-/-}$ resident macrophages. -, +, ++, +++ refer to no expression, low, medium and high expression respectively, as gauged by the relative intensity of staining defined from the histogram plot. Data is representative of four independent experiments.

A **Resident peritoneal dendritic cell expression of pan markers.**

Marker	WT	IL-6$^{-/-}$	OSMR$^{-/-}$	IL-10$^{-/-}$	IFN-$\gamma^{-/-}$	WT	Gp130$^{Y757F/Y757F}$
F4/80	+	++	++	++	++	++	++
CD11b	+++	+++	+++	+++	+++	+++	+++
CD11c	++	++	++	++	++	++	++
MHCII	+++	+++	+++	+++	+++	+++	+++
MR	+	+	+	+	+	+	+
TLR2	++	++	++	++	++	++	++
CD80	+	+	+	+	+	+	+
CD83	-	-	-	-	-	-	-
CD86	+	+	+	+	+	+	+
ICAM-1	+++	+++	+++	+++	+++	+++	+++
CD40	+	+	+	+	+	+	+
SR-A	-	-	-	-	-	-	-
CCR3	+	+	+	+	+	+	+
CCR5	++	+	++	++	++	++	++
IL-6 R	+	+	+	+	+	+	+

B

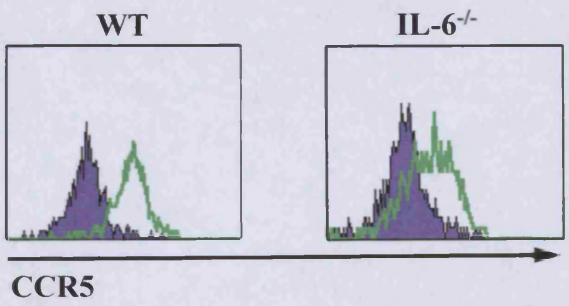


Figure 3.8: *Characterisation of resident peritoneal dendritic cells in WT, IL-6$^{-/-}$, OSMR$^{-/-}$, IL-10$^{-/-}$, IFN- $\gamma^{-/-}$ and gp130$^{Y757F/Y757F}$ mice.* **(A)** Peritoneal leukocytes were recovered by lavage, and labelled with F4/80 and CD11b, to identify the resident DC, and a number of macrophage/DC markers. **(B)** CCR5 expression in WT and IL-6$^{-/-}$ resident DC. -, +, ++, +++ refer to no expression, low, medium and high expression respectively, as gauged by the relative intensity of staining defined from the histogram plot. Data is representative of four independent experiments.

3.3.5 Cytokine/chemokine production by resident macrophages and DC, in response to SES and IL-6

To study the activity of resident monocytic cells upon inflammatory stimulation, cytokine/chemokine production by resident peritoneal macrophages and DC from WT and IL-6$^{-/-}$ mice was measured using a Bioplex assay and a proteome profiler array. Non stimulated resident macrophages and DC produced basal levels of cytokines/chemokines. Macrophages produced IL-1α, IL-1β, IL-6, IL-9, IL-10, IL-12 (p40 and p70), IL-13, G-CSF, GM-CSF, CXCL1, CCL2, CCL3, CCL4, CCL5 and TNF-α (Figure 3.9 & 3.10). The production of these cytokines/chemokines was however comparable between WT and IL-6$^{-/-}$ mice, with the exception of IL-6, which was absent in the IL-6$^{-/-}$ mice. In response to SES stimulation, production of all the cytokines/chemokines tested increased significantly, and also led to a low level of production of IL-2 and IL-3. SES stimulation induced equivalent changes in cytokine/chemokine production in macrophages from both WT and IL-6$^{-/-}$ mice. Interestingly, IL-6 stimulation of these resident macrophages did not induce any changes in cytokine or chemokine production in IL-6$^{-/-}$ mice. The same is true for WT macrophages, however since this population is likely to respond to autocrinal IL-6, no effect was expected by further IL-6 stimulation of these cells.

WT resident DC produced basal levels of IL-1α, IL-1β, IL-6, IL-9, IL-10, IL-12 (p40 and p70), IL-13, G-CSF, GM-CSF, CXCL1, CCL2, CCL3, CCL4, CCL5 and TNF-α in un-stimulated culture (Figure 3.11 & 3.12). Again, cytokine and chemokine production was significantly increased in the resident DC population challenged with SES. IL-6$^{-/-}$ mice lacked IL-6, GM-CSF and TNF-α, but were comparable to WT in their release of the other cytokines/chemokines tested. The production of GM-CSF and TNF-α by IL-6$^{-/-}$ resident DC was however rescued upon stimulation with IL-6 (Figure 3.11). However these values were below the limit of detection and so no real conclusions could be made from these results. No other regulation was observed in response to IL-6 stimulation in IL-6$^{-/-}$ resident DC, or in WT resident DC as was expected given their basal expression of IL-6 in un-stimulated culture.

This data was confirmed by protein array (data not shown).

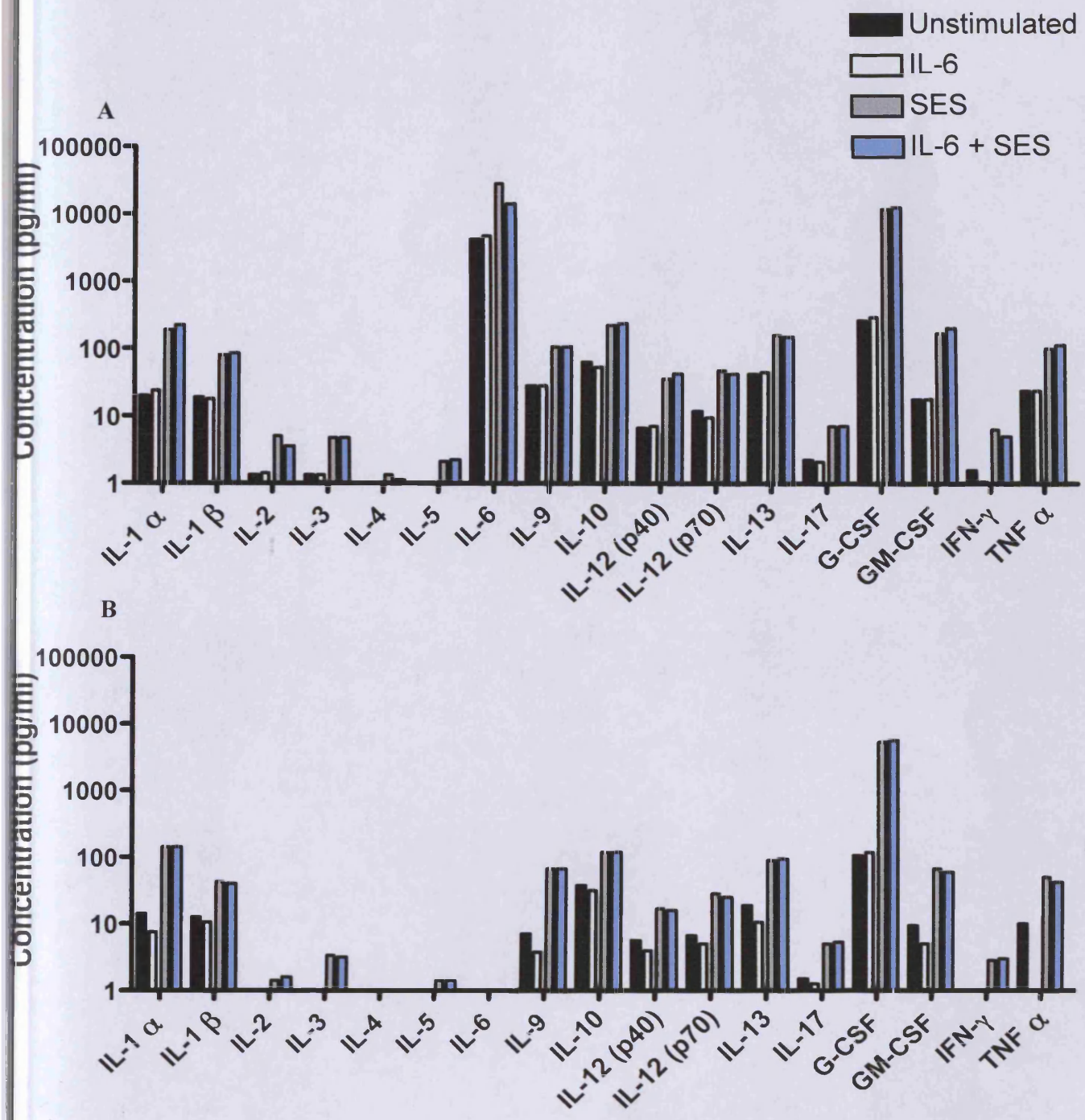


Figure 3.9: *Resident peritoneal macrophages produce basal levels of cytokines in culture which increases upon SES stimulation but not IL-6 stimulation.* Resident peritoneal macrophages were purified from pooled lavages based on their expression of F4/80 and CD11b. Cytokine production was measured by Bioplex assay. **(A)** Cytokine production by WT resident peritoneal macrophages in culture, un-stimulated, SES 1/50, recombinant (r)IL-6 50ng/ml, SES+hrIL-6. **(B)** Cytokine production by IL-6$^{-/-}$ resident peritoneal macrophages in culture, un-stimulated, SES 1/50, rIL-6 50ng/ml, SES+rIL-6. Data represents a single experiment following 20hrs of culture.

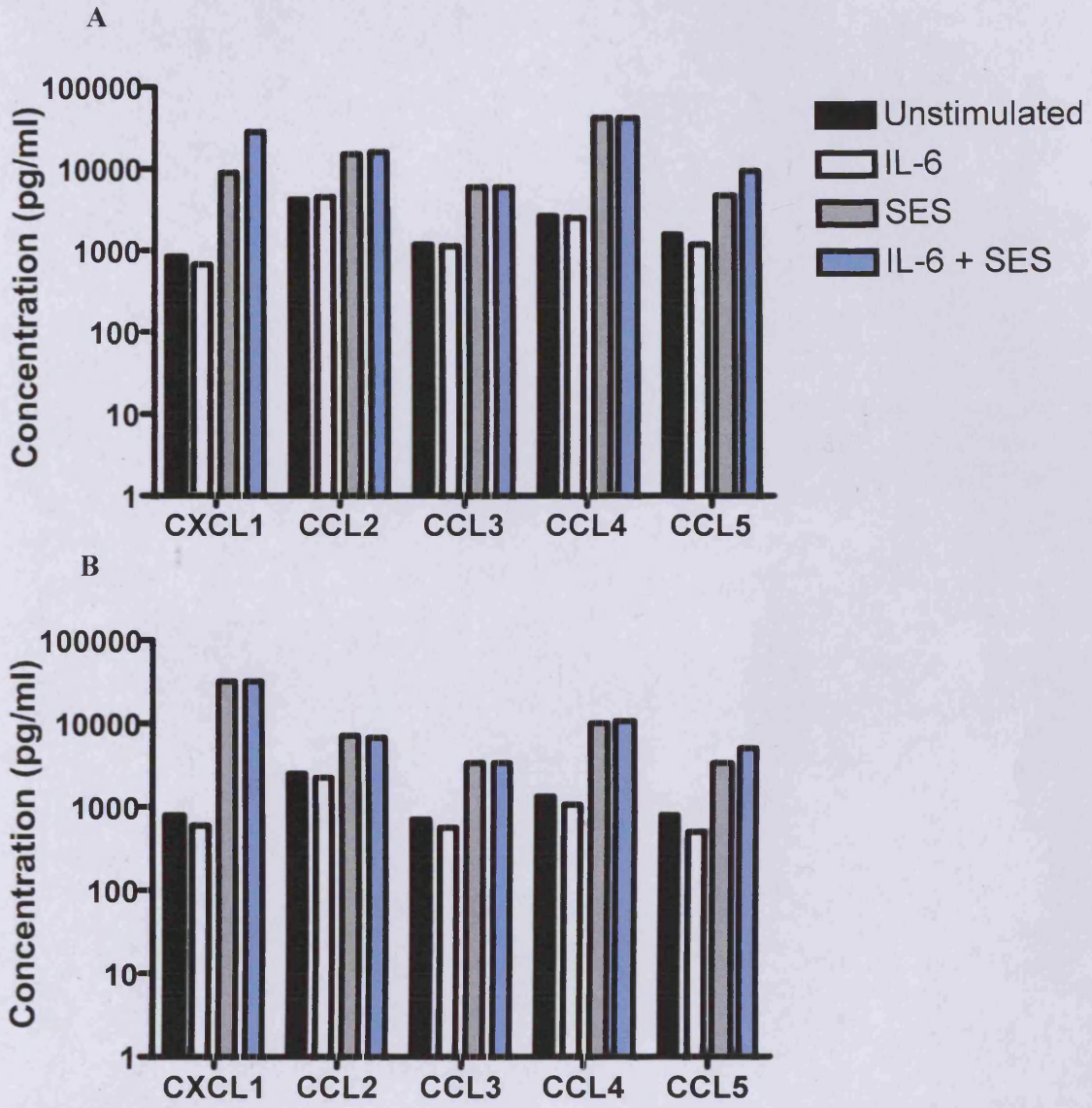


Figure 3.10: *Resident peritoneal macrophages produce basal levels of chemokines in culture which increases upon SES stimulation but not IL-6 stimulation.* Resident peritoneal macrophages were purified from pooled lavages based on their expression of F4/80 and CD11b. Chemokine production was measured by Bioplex assay. **(A)** Chemokine production by WT resident peritoneal macrophages in culture, un-stimulated, SES 1/50, recombinant (r)IL-6 50ng/ml, SES+rIL-6. **(B)** Chemokine production by IL-$6^{-/-}$ resident peritoneal macrophages in culture, un-stimulated, SES 1/50, rIL-6 50ng/ml, SES+rIL-6. Data represents a single experiment following 20hrs of culture.

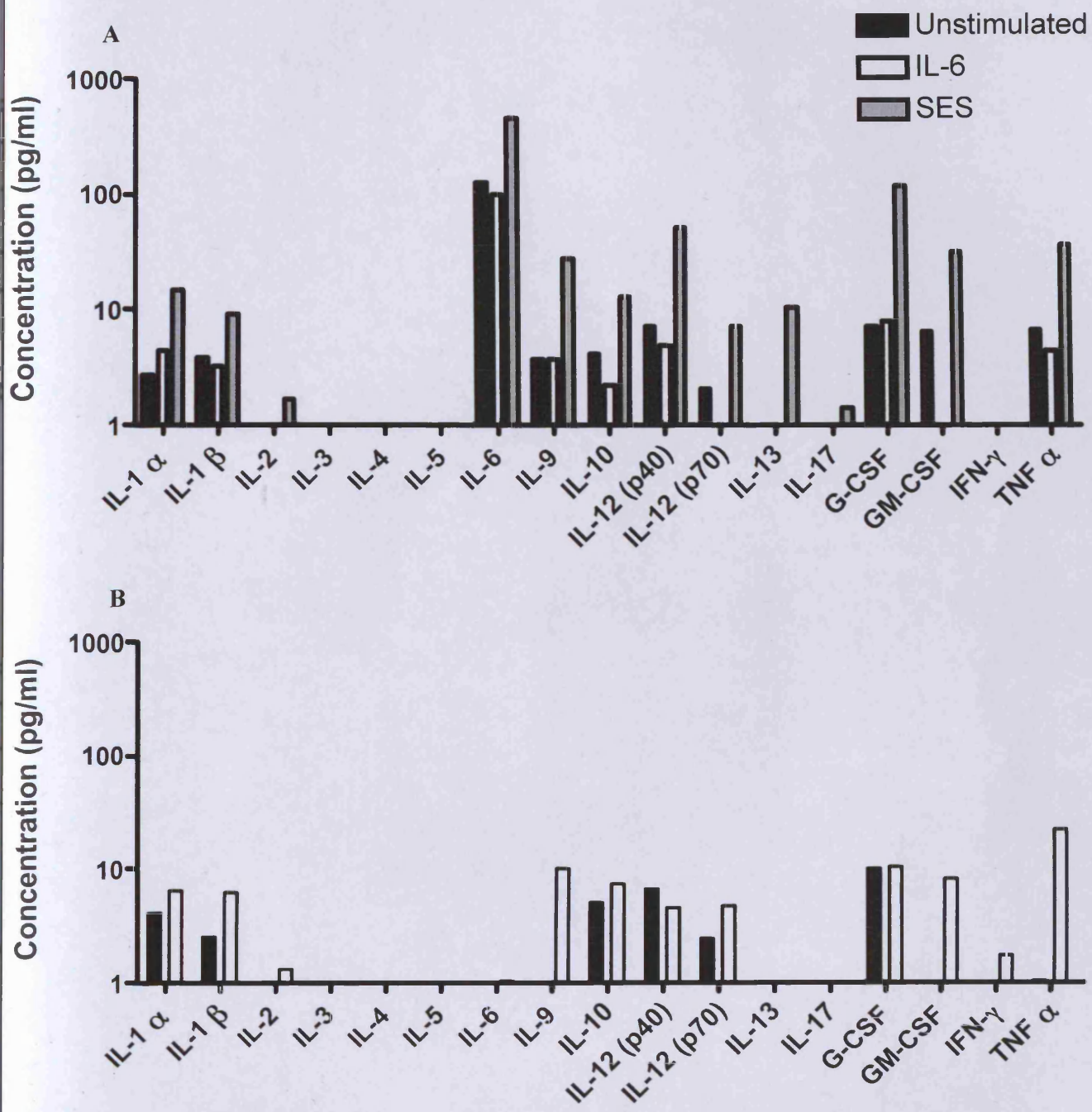


Figure 3.11: *Resident peritoneal DC produce basal levels of cytokines in culture which increases upon SES stimulation but not IL-6 stimulation.* Resident peritoneal DC were purified from pooled lavages based on their expression of F4/80 and CD11b. Cytokine production was measured by Bioplex assay. **(A)** Cytokine production by WT resident peritoneal DC in culture, un-stimulated, SES 1/50, recombinant (r)IL-6 50ng/ml. **(B)** Cytokine production by IL-$6^{-/-}$ resident peritoneal DC in culture, un-stimulated, rIL-6 50ng/ml. Data represents a single experiment following 20hrs of culture.

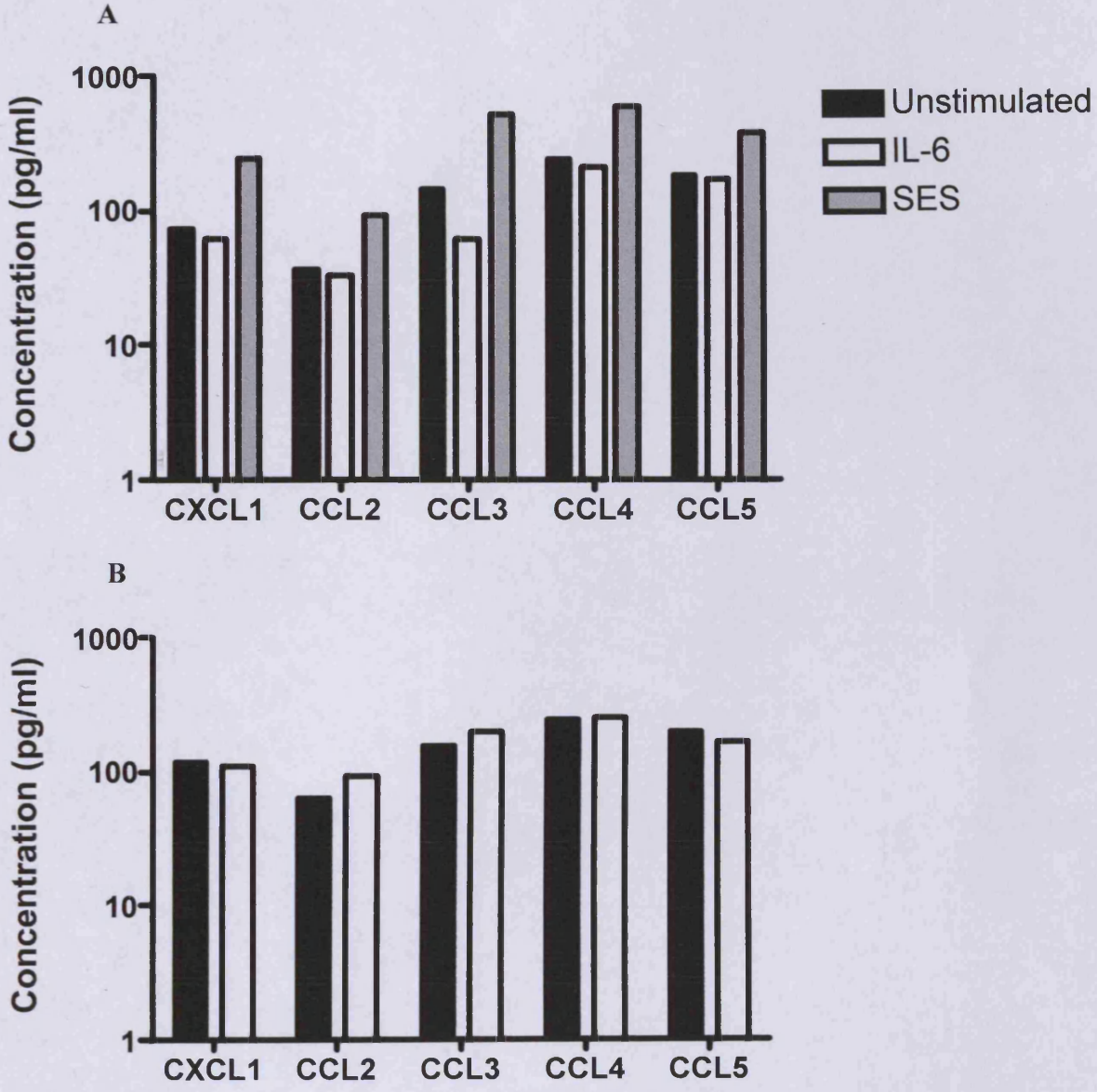


Figure 3.12: *Resident peritoneal DC produce basal levels of chemokines in culture which increases upon SES stimulation but not IL-6 stimulation.* Resident peritoneal DC were purified from pooled lavages based on their expression of F4/80 and CD11b. Chemokine production was measured by Bioplex assay. **(A)** Chemokine production by WT resident peritoneal DC in culture, un-stimulated, SES 1/50, recombinant (r)IL-6 50ng/ml. **(B)** Chemokine production by IL-$6^{-/-}$ resident peritoneal DC in culture, un-stimulated and rIL-6 50ng/ml. Data represents a single experiment following 20hrs of culture.

3.4 Discussion

Based on work published by a collaborating group (Dioszeghy *et al* 2008), we were able to use F4/80 and CD11b labelling of total resident peritoneal cells to identify distinct populations of resident peritoneal macrophages and dendritic cells. These resident macrophage and DC populations were defined by their phenotypes $F4/80^{hi}CD11b^{hi}$ and $F4/80^{int}CD11b^{hi}$ respectively. The data shown in this Chapter suggest that STAT signalling via IL-6, OSM, IFN-γ, and IL-10 does not regulate the number of resident macrophages found in the mouse peritoneal cavity under baseline conditions. It must be said however that it would be difficult to detect small variations in the resident macrophage population due to their large number (between $1.5x10^6$ and $6x10^6$ cells) present at any one time within the cavity. $Gp130^{Y757F/Y757F}$ mice however possessed significantly increased numbers of resident macrophages as compared to their littermate controls. These mice also exhibited a global increase in the number of peritoneal leukocytes indicating that hyperactive STAT signalling in these mice contributes to haematopoiesis in general, giving rise to the increased numbers of macrophages observed.

The studies described here have however shown that IL-6 directed STAT1/STAT3 or SHP-2 signalling and IFN-γ directed STAT1 signalling regulates the number of resident DC present in the peritoneal cavity of naive mice, in a positive and negative manner respectively. IL-6 is able to signal either via the STAT pathway or the SHP2 pathway (as detailed in Chapter 1, Section 1.16.2). To distinguish which IL-6-mediated signalling pathway may contribute to the regulation of DC numbers, this population was analysed in $gp130^{Y757F/Y757F}$ mice, which exhibit hyperactive STAT1/STAT3 signalling, and impaired SHP2 signalling (Tebbutt *et al* 2002). Given that the $gp130^{Y757F/Y757F}$ mice did not exhibit any increase in the number of resident DC present in the peritoneal cavity (as would be expected if the regulation was STAT1/STAT3 regulated), this positive regulation by IL-6 of the proportion of resident peritoneal DC is likely acting via the SHP2 mediated ERK MAP kinase pathway. Therefore, the evidence given in this chapter supports a role for the IL-6 mediated MAP kinase pathway in promoting resident DC numbers shown by the significantly reduced numbers of these cells in $IL\text{-}6^{-/-}$ mice. To

confirm these observations, it would have been elegant to test this notion further in $gp130^{\Delta STAT1/\Delta STAT3}$ mice, which retain SHP2 signalling capacity, but lack STAT1/STAT3 signalling capacity (Ernst *et al* 2001). However due to breeding difficulties within the colony we were unable to test this further. The apparent positive regulation of resident peritoneal DC numbers by IL-6 demonstrated by these findings, supports work by Santiago-Schwartz *et al* 1995, Bernhard *et al* 2000, and Brasel *et al* 2000, who all showed evidence to suggest that IL-6 drives DC differentiation. Conversely, it contrasts many reports of IL-6 skewing the differentiation of monocytes from macrophages to DC. The data shown in this Chapter, inferring a role for IL-6 in the regulation of the number of resident DC, is also supported by the fact that no decrease in resident peritoneal macrophages was observed in IL-$6^{-/-}$ mice suggesting that IL-6, in this case, is not regulating macrophage numbers, but does appear to be dictating DC numbers.

Studies in this Chapter also revealed a role for IFN-γ in the suppression of resident DC numbers. This negative regulation of resident peritoneal DC numbers by IFN-γ supports previous work by Delneste *et al* who showed that in humans, IFN-γ switches monocyte differentiation from dendritic cells to macrophages, by up-regulation of autocrine IL-6 and M-CSF production (Delneste *et al* 2003). In this respect, IL-6 has been shown to enhance M-CSF receptor expression and signalling (Chomorat *et al* 2000), which in turn drives macrophage differentiation (Becker *et al* 1987). It therefore follows that in the absence of IFN-γ, monocyte differentiation into DC would predominate, as was demonstrated in the findings discussed here.

Since WT, IL-$6^{-/-}$ and IFN-$\gamma^{-/-}$ mice possessed equivalent total peritoneal leukocyte numbers, the differences observed in DC numbers in these mice indicate that SHP2 and STAT1 responses driven by IL-6 and IFN-$\gamma^{-/-}$ respectively, contribute to the regulation of the DC population alone, and do not regulate the global haematopoiesis of all cell types. In this respect, while the data supports a clear role for IL-6 and IFN-γ in determining resident peritoneal DC numbers, it does not allow us to draw any conclusions on the mechanism by which they do this. These cytokines may either regulate the rate of homeostatic migration to the peritoneal cavity and local differentiation of these DC from

their precursors, or the rate of egress from the peritoneal cavity, and further experimental work would be required to determine the nature of this response.

No differences were observed in the number of resident DC in $OSMR^{-/-}$ and $IL\text{-}10^{-/-}$ mice indicating that the regulation of STAT signalling by these particular cytokines does not regulate the resident peritoneal DC population size. It has been previously reported that in humans, IL-10 prevents differentiation of monocytes to DC and instead promotes their maturation to macrophages (Allavena *et al* 1998), but no evidence of this was seen in the murine model in these studies.

To define a role for IL-6 in regulating resident monocytic cell activity, macrophages and DC were cultured in the presence of IL-6 as well as inflammatory stimulus SES, to determine how the activity of these cells are regulated upon inflammatory challenge. Cytokine and chemokine production in WT and $IL\text{-}6^{-/-}$ resident macrophages and DC was up-regulated upon SES stimulation and this is consistent with findings by Dioszeghy *et al* 2008, who showed similar up-regulation of cytokine and chemokine production in WT resident macrophages and DC using the same bioplex assay (Dioszeghy *et al* 2008). IL-6 stimulation of $IL\text{-}6^{-/-}$ resident macrophages showed no effect on cytokine and chemokine production. The level of IL-6R expression on these cells is very low (refer to Figure 3.6), which may explain why no response is seen from these cells. However, resident DC, which express higher levels of IL-6R, also did not appear to respond to IL-6 stimulation. Where differences were seen upon IL-6 stimulation, the measured levels were below the limit of detection of the assay, so no conclusions could be made from these results. These data clearly demonstrate that while the activity of resident macrophage and DC populations is potently initiated and up-regulated by an inflammatory stimulus (e.g. SES), the activity of these cells does not appear to be regulated in any way by the presence of IL-6. This is striking given the known impact of IL-6 on monocyte activity (Bleier *et al* 2004, Hedge *et al* 2004, Pasare *et al* 2003).

The absence of activity of resident peritoneal DC in response to IL-6 stimulation is further investigated in Chapter 4.

CHAPTER 4

The regulation of IL-6 receptor on murine resident peritoneal dendritic cells

4.1 Introduction

In addition to its role as a regulator of dendritic cell haematopoiesis and differentiation, IL-6 also controls dendritic cell activation, maturation and trafficking (Park 2004, Kitamura *et al* 2005, Hedge *et al* 2004). Following microbial invasion or tissue destruction, resting immature DC, which possess diverse recognition systems for monitoring danger signals, become activated and undergo maturation (Kaisho *et al* 2001). DC maturation is associated with the loss of antigen capturing ability and the acquisition of an antigen presenting ability. This maturation is coupled with the migration of DC from the peripheral tissue, to the lymph nodes where they are able to present antigen, and activate T cells (Novak and Beiber 2007, Ueno *et al* 2007, Wu and Dakie 2004, Foti *et al* 1999). Work by Park *et al* showed that IL-6 plays a major role in maintaining DC in an immature state. Specifically, IL-$6^{-/-}$ mice had increased numbers of mature DC indicating that IL-6 blocks DC maturation *in vivo*. In this respect, IL-6 activation of STAT3 was required for the suppression of LPS-induced DC maturation, with DC mediated T cell activation being enhanced in IL-$6^{-/-}$ mice and suppressed in gp130 knock in mice (Park *et al* 2004). Taken together these data indicate that IL-6 acts as an immunosuppressive cytokine for DC differentiation/activation/maturation and suggests that IL-6 continuously stimulates resting/immature DC in the steady state (Park *et al* 2004).

A key indicator of DC maturation is the induction of intracellular trafficking of MHCII vesicles and increased surface expression of MHCII. Work by Kitamura *et al* has shown that IL-6-STAT3 signalling suppresses MHCII expression on DC by suppressing both MHCII αβ dimer subunits and associated MHCII-adaptor molecules. Regulation of these processes is associated with an attenuation of T cell activation (Kitamura *et al* 2005). This work supports findings by Park *et al* who showed that IL-6 STAT-3 mediated signalling suppressed LPS-induced surface expression of MHCII (Park *et al* 2004).

Upon activation and maturation of DC, they migrate through the lymphatic system to the lymph nodes where they present antigen and activate T cells. This migration is chemokine mediated, and the predominant chemokine receptor for this process is CCR7 (Ohi *et al* 2004, Forster *et al* 1999, Sozzani *et al* 1998). DC maturation is accompanied

by a rapid inhibition of responsiveness to chemoattractants present at the sites of inflammation by down regulation of the relevant chemokine receptors (Sozzani *et al* 1998). This permits the maturing DC to leave the peripheral tissues, at which point an up-regulation of CCR7 allows a co-ordinated migration of DC towards the chemoattractants CCL19 and CCL21 expressed in the lymph nodes (Sozzani *et al* 1998, Ohi *et al* 2004, Forster *et al* 1999). The induction of CCR7 is regarded as a crucial integral part of the maturation process, as it confers responsiveness to CCL19, the chemoattractant produced in the T cell zone of the secondary lymphoid organs, and secondary lymphoid tissue chemokine CCL21. These chemokines are responsible for the homing of T cells and DC to secondary lymphoid tissue (Moser and Loetscher, 2001). IL-6 has been shown to inhibit CCR7 up-regulation on LPS matured DC, adding another immunosuppressive property to the growing list attributed to IL-6. Hedge *et al* showed that IL-6 does this by inhibiting nuclear factor-κB regulation of CCR7 transcription. This results in impaired chemotaxis of matured DC to CCR7-activating chemokines CCL19 and CCL21, required for recruiting DC to lymphoid tissues *in vivo* (Hedge *et al* 2004).

In contrast to these findings, IL-6 has been shown to be vital for the function of fully matured DC within the lymphoid organs. Bleier *et al* showed that IL-$6^{-/-}$ bone marrow derived DC (BMDC) and splenic DC induced significantly less allogenic T cell stimulation than their WT counterparts. Dendritic cells also produced high levels of IL-6 upon microbial stimulation, indicating that IL-6 is important for their function in activating T cells (Bleier *et al* 2004). Finally, IL-6 secretion by DC upon TLR stimulation has been shown to block the immunosuppressive activities of regulatory T cells (Pasare and Medzhitov 2003) thereby promoting pro-inflammatory T cell responses. Therefore IL-6 plays either an immunosuppressive role or a proinflammatory role on antigen presenting cells depending on their maturation state and tissue localisation.

The aim of this Chapter was to determine the effect of IL-6 on the activity of resident peritoneal dendritic cells, and to investigate possible activation induced changes in DC IL-6 receptor expression.

4.2 Materials and methods

4.2.1 Isolation of basal resident peritoneal macrophages and dendritic cells

WT, IL-$6^{-/-}$, IL-$10^{-/-}$, $gp130^{Y757F/Y757F}$, $OSMR^{-/-}$ and IFN-$\gamma^{-/-}$ mice were sacrificed by schedule 1 and peritoneal leukocytes were recovered by lavage as described in Section 2.2.2.1.

4.2.2 MACS purification of $CD11b^+$ murine peritoneal leukocytes

$CD11b^+$ murine peritoneal leukocytes were purified by MACS column separation using anti-CD11b antibodies conjugated to magnetisable particles (Section 2.2.2.2.). The $CD11b^+$ fraction of cells largely consists of macrophages and DC.

4.2.3 Culture of $CD11b^+$ murine peritoneal leukocytes

$2x10^6$ peritoneal leukocytes were incubated in 12 well plates for 2 hours at 37°C with SES (reconstituted in 750µl media and sterile filtered), PMA 50ng/ml, Ionomycin 500ng/ml, TAPI (TG-ase activating protease inhibitor) 100µM or PMA in combination with TAPI (Section 2.2.2.1). Cells were removed by scraping and centrifuged at 2000rpm for 5 minutes to collect the cells for flow cytometric analysis (see Section 4.2.6).

4.2.4 Induction of peritoneal inflammation with *Staphylococcus epidermidis* (SES)

Lyophilised SES was prepared as described in Section 2.3.3 and reconstituted in 750µl sterile PBS. Mice were injected i.p with a 500µl aliquot of SES (Section 2.3.5). At defined intervals, groups of mice (n=5, mixture of males and females) were sacrificed and peritoneal leukocytes recovered by lavage as described in Section 2.3.2.

4.2.5 Induction of bacterial peritonitis by administration of *Staphylococcus epidermidis*

Experiments were performed as outlined in Section 2.3.7. Mice were injected i.p with $5x10^8$ colony forming units (cfu) of live *Staphylococcus epidermidis* (prepared as described in section 2.3.6). At defined intervals, groups of mice (n=5, mixture of males

and females) were sacrificed by schedule 1 and peritoneal leukocytes recovered by lavage (Section 2.3.2).

4.2.6 Flow cytometric analysis of resident peritoneal dendritic cells

Freshly isolated and cultured resident peritoneal macrophages and DC were labelled with anti-F4/80 FITC and anti-CD11b APC, and PE or Biotin conjugated antibodies against either IL-6R, MHC II, CD83, CCR5, CCR3, CCR6 or CCR7 as described in Section 2.4.3. Resident peritoneal macrophages and DC from *in vivo* experiments were labelled with anti-F4/80 FITC, anti-CD11b PerCP Cy5.5, anti-CD11c APC, and anti-IL-6R PE as described in section 2.4.3. Details of the antibodies used are found in Table 2.1. Receptor expression was quantified by calculating the mean fluorescent intensity as described in Section 2.4.5.

4.3 Results

4.3.1 Changes in IL-6R levels on cultured resident dendritic cells

Due to the lack of IL-6 responsiveness of resident DC, observed in the previous Chapter (Section 3.3.5), it was decided to investigate the levels of the IL-6 receptor on this population during culture. Total $CD11b^+$ leukocytes, which included all resident macrophages and DC, were isolated by MACS separation, and established in culture. Cells were recovered two hours later and analysed by flow cytometry using antibodies against F4/80, CD11b and IL-6R. In contrast to resident peritoneal DC, resident peritoneal macrophages showed very low expression of IL-6R, and therefore it was not possible to determine any changes in IL-6R levels on this population. However prolonged culture of resident DC led to an almost complete loss of IL-6R expression on the cell surface (Figure 4.1B). This down regulation in IL-6R coincided with a significant increase in both CD83 and MHC II expression (Figure 4.1 C&D). This up-regulation of maturation markers was indicative of dendritic cell activation. The down regulation of IL-6R observed was not ligand dependent since a comparable loss in IL-6R was also noted in resident DC from $IL\text{-}6^{-/-}$ mice (Figure 4.2).

To determine if the loss of IL-6R on resident DC was a result of shedding, these cells were cultured in the presence of a variety of cell stimulators, and also the IL-6R sheddase inhibitor TAPI. Down-regulation of the receptor was quantified by calculating the mean fluorescent units (Figure 4.2). Down-regulation of IL-6R upon PMA, SES and ionomycin stimulation of resident DC was only marginally greater than that induced by culture alone, therefore culture induced activation alone was enough to mediate an almost complete loss of IL-6R on these cells. The addition of TAPI to these cultures only partially inhibited the down-regulation of IL-6R, returning the levels of IL-6R on these cells to that seen in cells in un-stimulated culture (Figure 4.2).

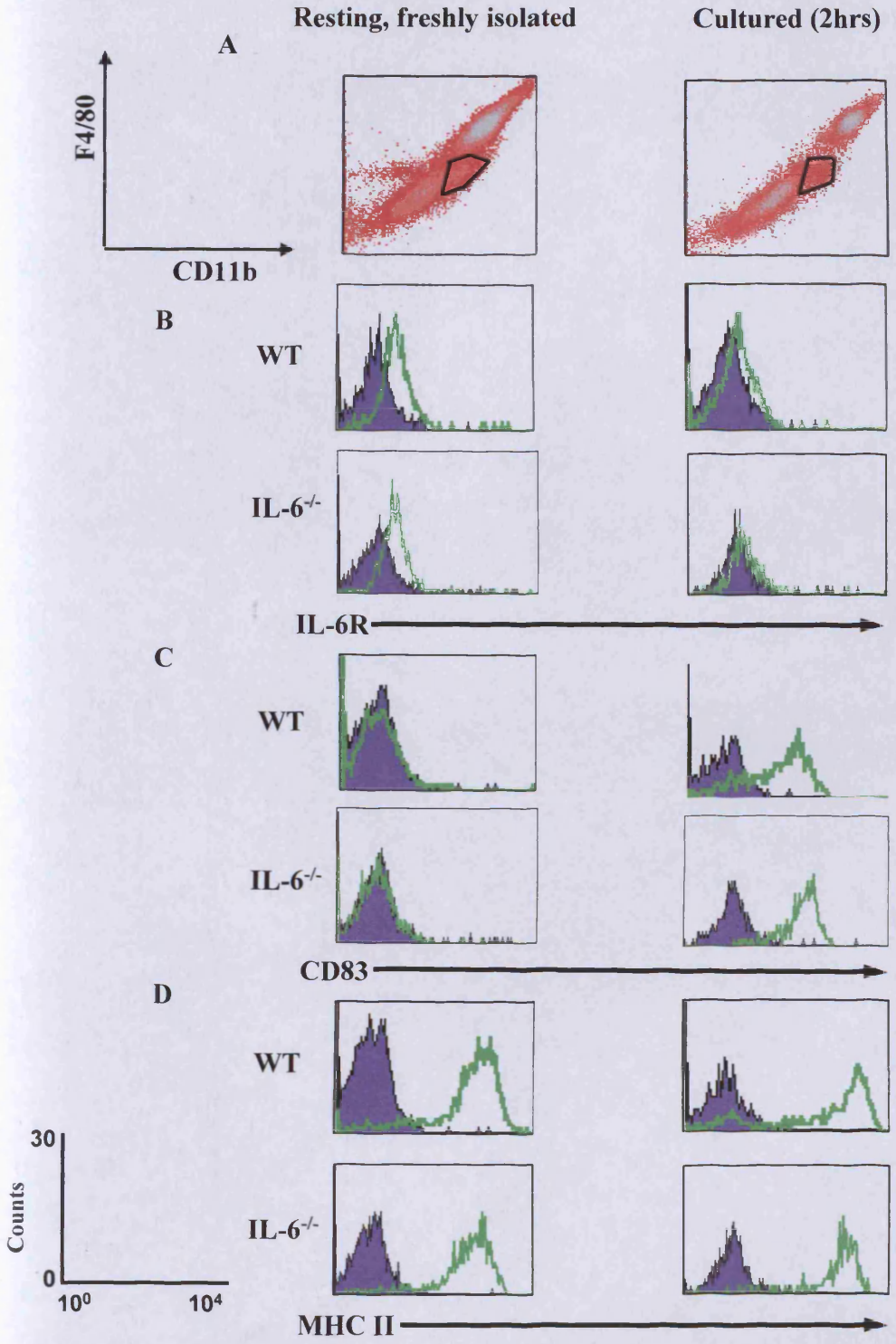


Figure 4.1: *WT resident dendritic cells down-regulate their IL-6R upon culture, coinciding with an up-regulation of CD83 and MHC II.* **(A)** Freshly isolated and cultured resident peritoneal DC were labelled with anti F4/80 and CD11b, and were subsequently identified by their phenotype F4/80^{int}CD11b^{hi}. **(B)** Flow cytometry histogram plots gated on resting and cultured resident dendritic cells (F4/80^{int}CD11b^{hi}) from 30,000 total events, measuring IL-6R levels. Representative histogram plots for **(C)** CD83 levels and **(D)** MHC II levels. Data is representative of 3 separate experiments.

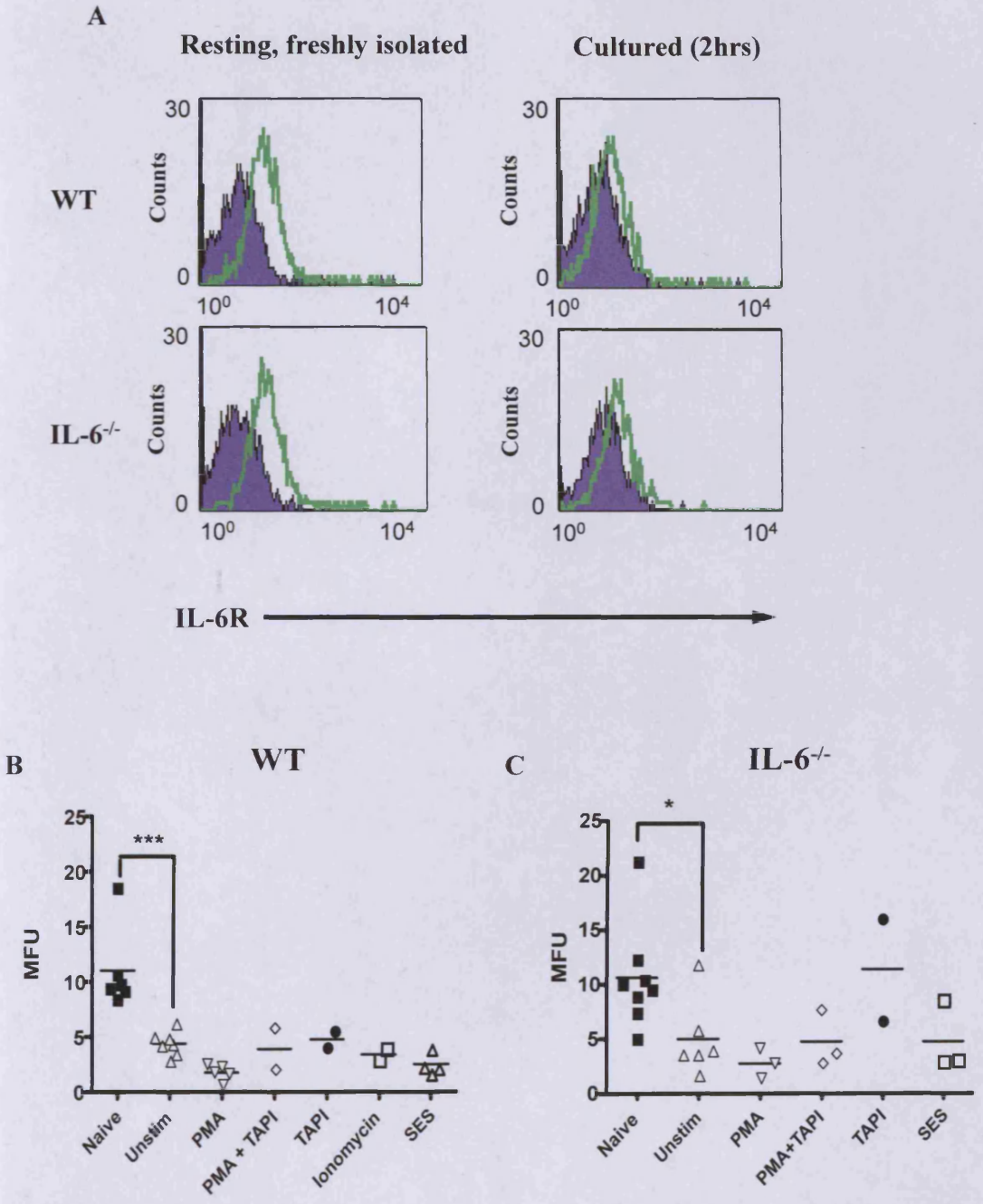


Figure 4.2: *WT and IL-6$^{-/-}$ mice exhibit a down regulation of IL-6 receptor on their resident DC in vitro both spontaneously in culture and upon stimulation with PMA, ionomycin and SES.* **A)** Representative flow cytometry histograms displaying the down regulation of IL-6R on WT and IL-6$^{-/-}$ murine resident peritoneal dendritic cells, based on 2-8 individual experiments. **(B)** Plots of the mean fluorescent units (MFU) taken from histogram plots, to represent down regulation of IL-6R from resident DC upon culture, and stimulation with PMA (50ng/ml), ionomycin (500ng/ml), SES (1/2) and with the addition of protease inhibitor TAPI (100μM) in WT and **(C)** IL-6$^{-/-}$ mice . (* $P < 0.05$, *** $P < 0.001$).

4.3.2 IL-6R level changes on resident dendritic cells *in vivo* upon activation of SES and *S.epidermidis* induced inflammation

To determine if resident DC down-regulated their IL-6R *in vivo*, peritoneal inflammation was induced either by i.p administration of SES, a cell free supernatant derived from a clinical isolate of *S.epidermidis,* or a live isolate of *S.epidermidis*. As a control, some mice were treated with PBS alone. Peritoneal cells were recovered by lavage after one and three hours and flow cytometry was performed using antibodies against F4/80, CD11b, CD11c and IL-6R to determine receptor levels in the resident DC population (Figure 4.3). Analysis of resident DC from both WT and IL-$6^{-/-}$ mice showed that inflammatory activation with either SES or live bacteria triggered a down-regulation of IL-6R on the resident DC population (Figure 4.3 A). No such changes were observed following PBS administration (Figure 4.3 B). Calculating the mean fluorescent units to quantify receptor level changes showed that IL-$6^{-/-}$ mice appeared to down-regulate their IL-6R at a slower rate than WT mice, which was particularly evident in mice administered with SES (Figure 4.4 C&D). Most WT mice exhibited a complete disappearance of the IL-6R from their resident DC by one hour post induction of inflammation. In IL-$6^{-/-}$ mice however, the receptor was not completely down-regulated on resident DC by 1 hour, but was completely absent from the cell surface by 3 hours (Figure 4.4).

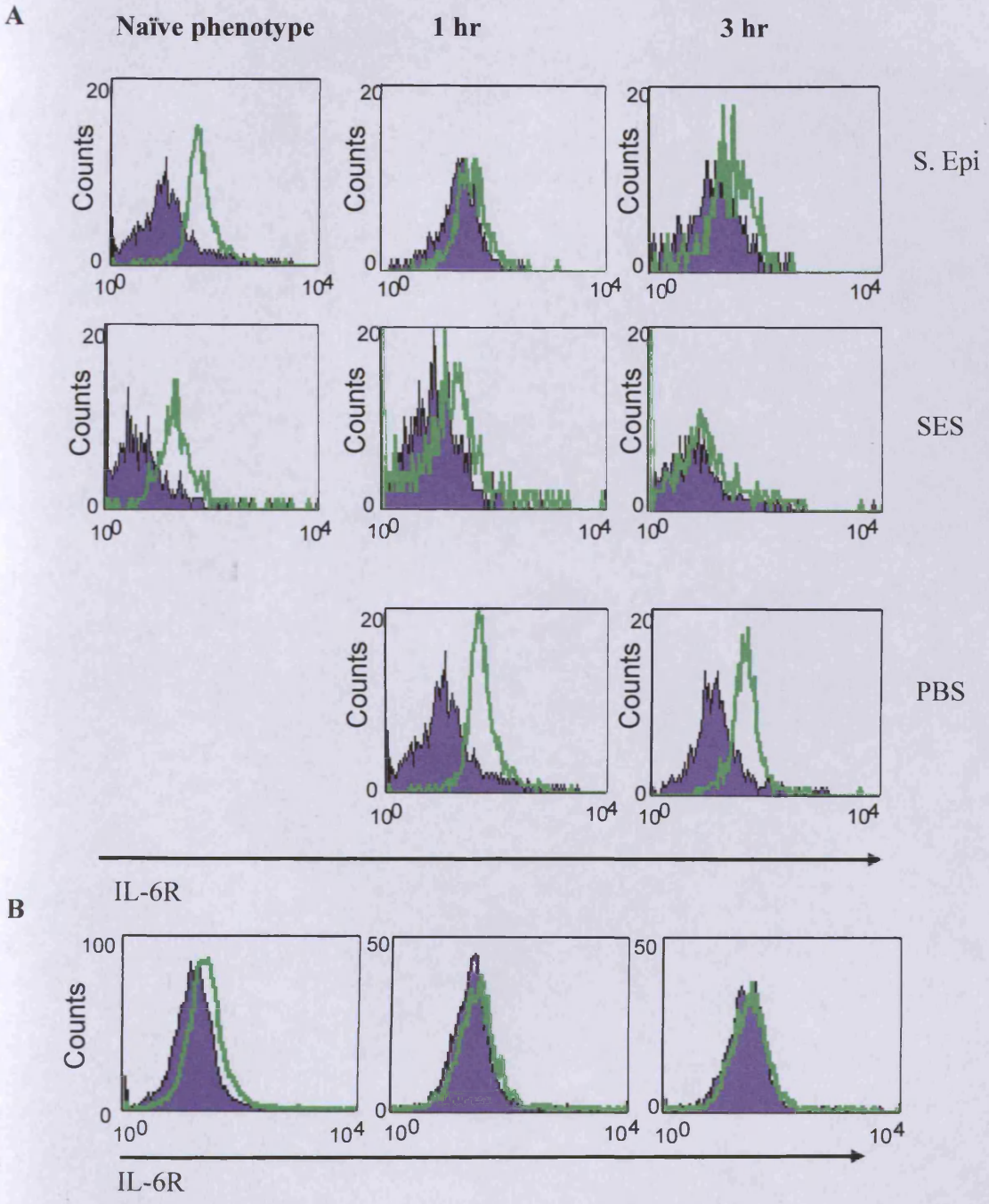


Figure 4.3: *IL-6R is down-regulated on resident peritoneal dendritic cells in vivo.* **(A)** Peritoneal inflammation was established in WT mice by i.p injection of either $5x10^8$ cfu *Staphylococcus epidermidis* or 500µl of SES. Some mice received 500µl of PBS as a control. IL-6R was measured on F4/80^{int}CD11b^{hi}CD11c^{+} resident DC. **(B)** IL-6R was measured on F4/80^{hi}CD11b^{hi} resident macrophages. Plots are representative from a total of 5 mice per time point.

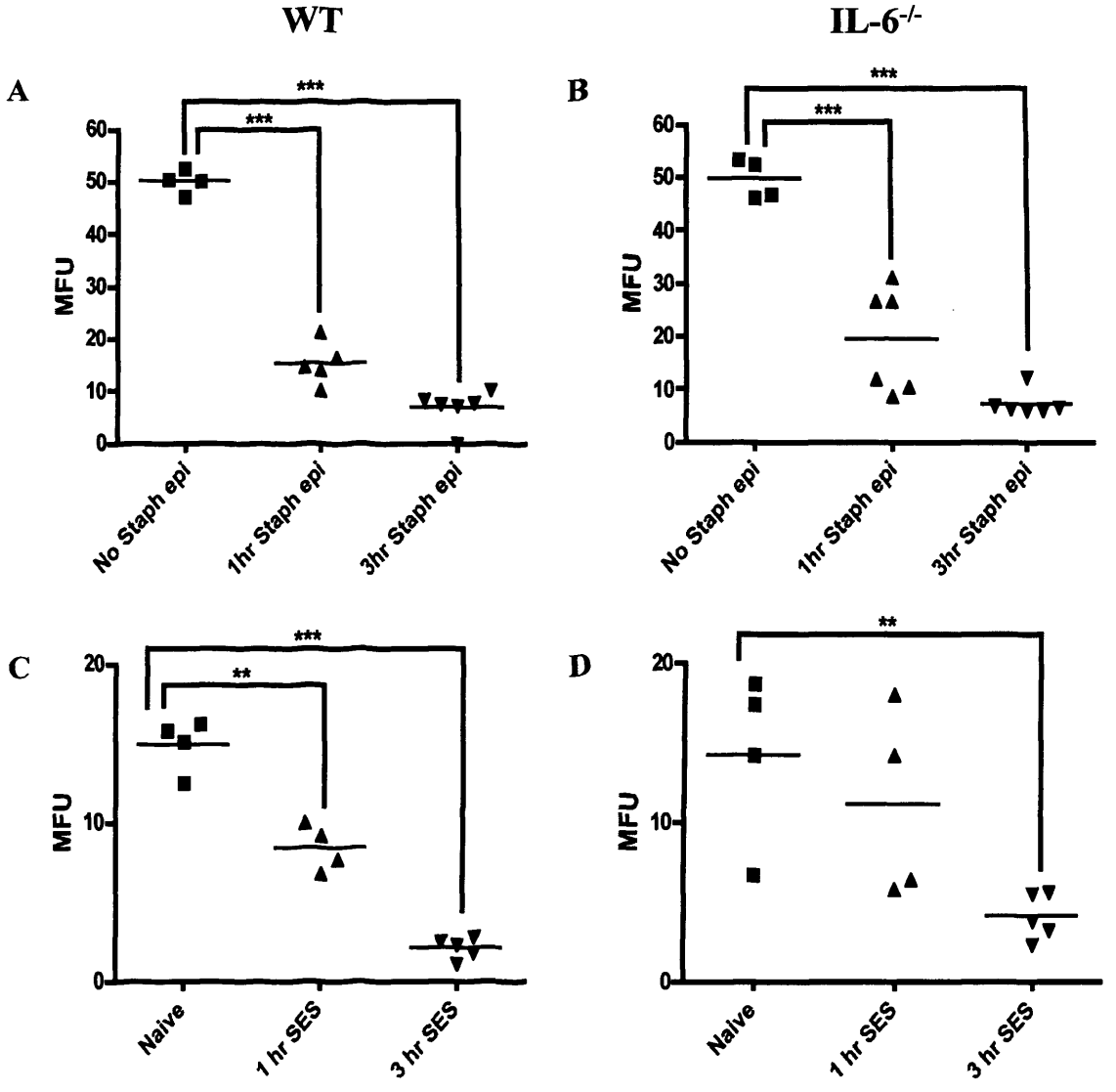


Figure 4.4: *WT and IL-6$^{-/-}$ mice exhibit a down regulation of IL-6 receptor on their resident DC in vivo upon induction of peritoneal inflammation.*
Peritoneal inflammation was established in WT and IL-6$^{-/-}$ mice by i.p injection of either 5x10^{8} cfu *Staphylococcus epidermidis* or 500µl of SES. Resident DC (F4/80^{int}CD11b^{hi}CD11c^{+}) were labelled with anti IL-6R, 1 and 3 hours following induction of peritoneal inflammation and changes in the mean fluorescent units were plotted for **(A)** WT mice that received live *Staphylococcus epidermidis* **(B)** IL-6$^{-/-}$ that received live *Staphylococcus epidermidis,* **(C)** WT mice that received SES. **(D)** IL-6$^{-/-}$ mice that received SES. (** $P < 0.01$, *** $P < 0.001$).

4.3.3 Regulation of CCR7 expression on resident dendritic cells

IL-6 has been shown to impair CCR7 up-regulation on maturing dendritic cells, and as a result impairs chemokine driven trafficking of DC to the draining lymph nodes following activation (Hedge *et al* 2004). To determine whether the down-regulation of IL-6R observed on resident peritoneal DC was associated with a concomitant increase in CCR7, expression of this receptor was measured on resident DC before and after activation. $CD11b^+$ peritoneal leukocytes, pooled from four mice, were cultured for two hours with or without SES stimulation, and flow cytometry performed using antibodies against F4/80, CD11b and CCR7 as well as IL-6R, CD83 and MHC II as controls. Resident DC from both WT (Figure 4.5) and IL-$6^{-/-}$ (data not shown) mice exhibited the previously observed down-regulation of IL-6R and corresponding up-regulation of CD83 and MHC II, but they did not show any expression of CCR7, both in the resting state, and following culture induced activation. This was irrespective of prior SES activation.

4.3.4 Regulation of monocyte chemoattractant receptors on resident dendritic cells

Since resident DC did not up-regulate CCR7 upon activation, alternative chemokine receptors were addressed with the aim of determining the mechanism by which resident DC migrate to the draining lymph node following activation. Resting, and culture activated (with and without SES) resident peritoneal DC from WT (Figure 4.6) and IL-$6^{-/-}$ (data not shown) mice were analysed by flow cytometry using antibodies against CCR3, CCR5 and CCR6. Resting resident DC did not express CCR6 basally, but did express CCR3 at a very low level, and CCR5 at a more moderate level. Upon activation in culture resident DC from both WT and IL-$6^{-/-}$ mice did not up-regulate any of these three chemokine receptors (Figure 4.6).

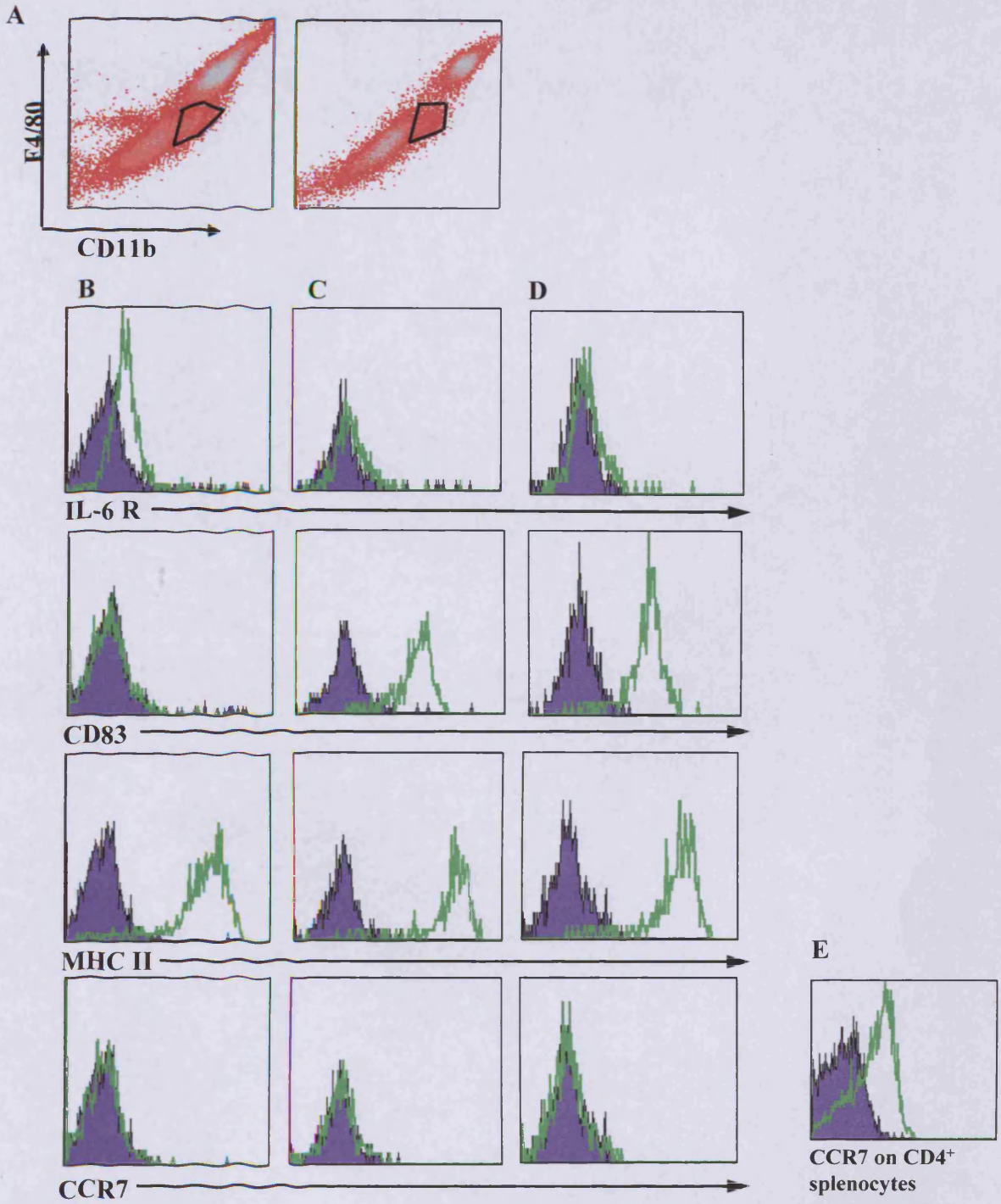


Figure 4.5: *Resident peritoneal DC do not exhibit an up-regulation of CCR7 upon culture.* **(A)** Wild type $CD11b^{+}$ MACS enriched peritoneal cells, pooled from 4 mice, were labelled with anti F4/80 and CD11b, and resident DC were identified and gated as $F4/80^{int}CD11b^{hi}$. This was followed by subsequent flow cytometry analysis. **(B)** Expression of IL-6R, CD83, MHCII and CCR7 on resting resident peritoneal DC from naïve WT mice. **(C)** Expression of IL-6R, CD83, MHC II and CCR7 on resident peritoneal DC following un-stimulated culture for two hours. **(D)** Expression of IL-6R, CD83, MHC II and CCR7 on resident peritoneal DC following stimulation with SES in culture for two hours. **(E)** Positive control for CCR7 antibody, staining of $CD4^{+}$ splenocytes. FACS plots are representative of two independent experiments.

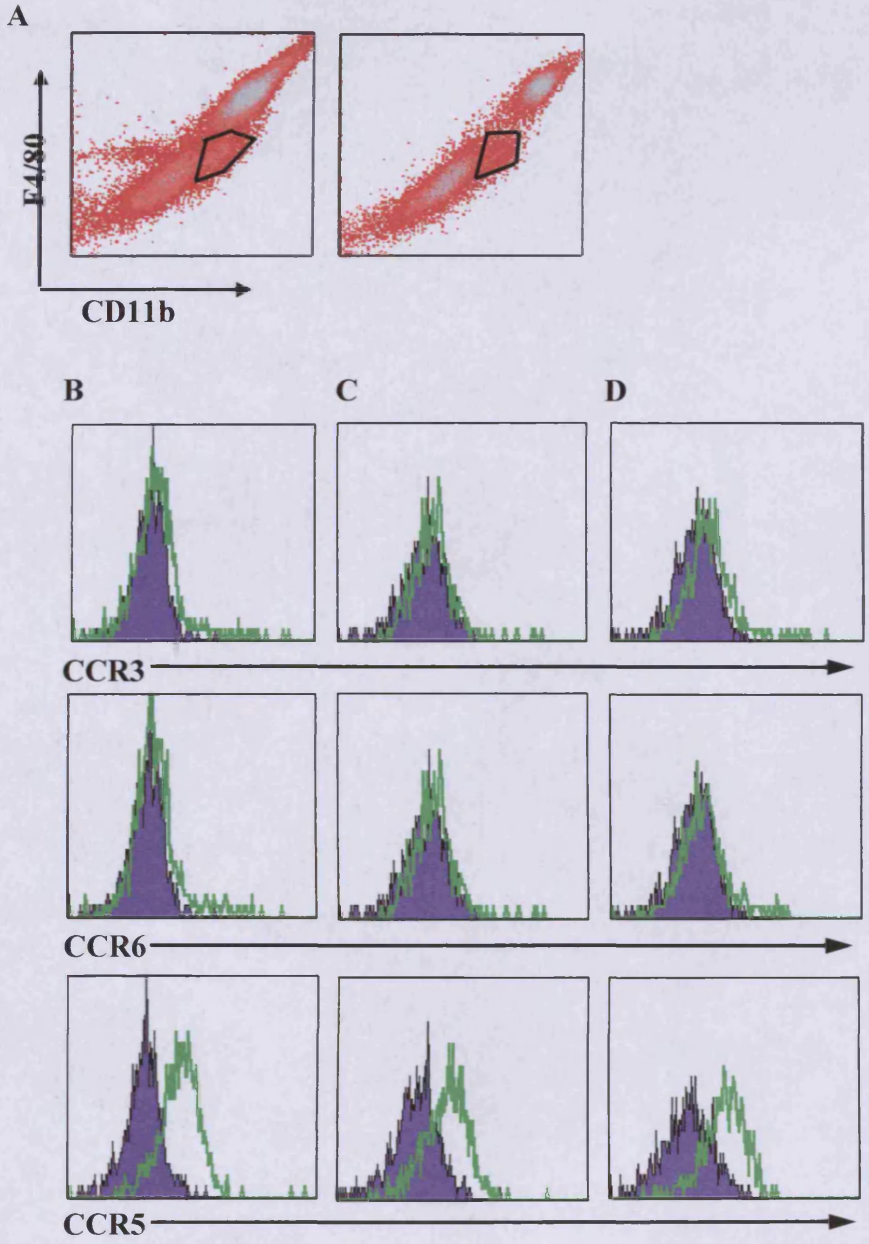


Figure 4.6: *Resident peritoneal DC do not up-regulate chemokine expression upon stimulation in culture.* **(A)** Wild type $CD11b^{+}$ MACS enriched peritoneal cells, pooled from four mice, were labeled with anti F4/80 and CD11b, and resident DC were identified and gated as $F4/80^{int}CD11b^{hi}$. This was followed by subsequent flow cytometry analysis. **(B)** Expression of CCR3, CCR6 and CCR5 on resting resident peritoneal DC from naïve WT mice. **(C)** Expression of CCR3, CCR6 and CCR5 on resident peritoneal DC following un-stimulated culture for two hours. **(D)** Expression of CCR3, CCR6 and CCR5 on resident peritoneal DC following stimulation with SES in culture for two hours. FACS plots are representative of two independent experiments

4.4 Discussion

Data presented in this Chapter supports a hypothesis that resident peritoneal DC down-regulate their IL-6 receptor upon activation, as part of the maturation process. The down-regulation of the IL-6R observed, coincided with the maturation of the resident DC population, and was linked with an increase in the DC activation markers MHCII and CD83. This activation-induced loss of IL-6R on resident DC goes some way to explain the lack of IL-6 responsiveness observed *in vitro,* where IL-6 failed to activate this cell population (data presented in Chapter 3). A number of studies have shown that the activation of T cells is associated with a reduced expression or complete loss of IL-6R resulting in IL-6 insensitivity (Teague *et al* 2000, Nowell *et al* 2009, Briso *et al* 2008). Similarly, an activation-induced loss of membrane bound IL-6R expression on DC is likely to abrogate IL-6 signalling, accounting for a down-regulation in IL-6 responsiveness by these cells. This would assume that these activated DC also lack responsiveness to IL-6 trans-signalling via the soluble receptor. The potential activation of resident DC by IL-6 trans-signalling is still unclear and due to a lack of an appropriate antibody we were unable to examine potential changes in the signalling receptor gp130.

The data shown in this Chapter fits nicely with what is already known about IL-6 and its immunosuppressive role on DC. IL-6 is currently believed to maintain resting DC in an immature state where they reside in the periphery (Park *et al* 2004). Once activated by a microbial stimulus, DC undergo maturation (Kaisho *et al* 2001). This process is an essential event that enables the activated DC to migrate to the lymph node where they can present antigen to T cells. The down-modulation of IL-6R on activated DC would therefore be predicted to remove the inflammatory constraint imposed upon DC by IL-6. This receptor down-regulation doesn't affect their ability to produce IL-6 however (as shown in Chapter 3, IL-6 production by resident DC increases upon SES stimulation), allowing the DC to effectively suppress T regulatory cells and activate T helper cells by the production of IL-6, without responding to IL-6 themselves (Pasare and Medzhitov 2003, Park *et al* 2004).

Support for this conclusion is derived from data relating to the IL-6 control of MHC II. As previously stated, the studies discussed here showed that resident peritoneal DC up-regulated MHCII upon activation *in vitro*. Based on data presented by Kitamura *et al,* IL-6 has been shown to act as a negative regulator of MHC II expression (Kitamura *et al* 2005), and the results presented in this Chapter would appear to support these findings. It could be speculated that the down regulation of IL-6R on these resident DC upon activation renders them unresponsive to IL-6R and therefore allows the up-regulation of MHCII on the cell surface. This would increase the antigen presenting capacity of these cells, and define their maturation from immature antigen capturing cells, to matured antigen presenting cells. However since an equivalent up-regulation of MHCII was observed in IL-$6^{-/-}$ DC, these studies would suggest that the increase in expression of this receptor was IL-6 independent. Further experiments would need to be performed to determine if the up-regulation of MHCII on these activated resident DC is attributed to the inability of IL-6 to stimulate these cells upon the loss of the receptor.

Previous studies have shown that IL-6 negatively regulates CCR7 expression, the chemokine receptor required for migration of matured DC to the lymph nodes (Hedge *et al* 2004). It was therefore hypothesised that upon down-regulation of the IL-6R on resident peritoneal DC, CCR7 expression would be increased due to the removal of the immunosuppressive effects of IL-6 on these cells. Unfortunately it was not possible to show an up-regulation of CCR7 or other potential chemokine receptors CCR3, CCR5 and CCR6 *in vitro*. It is conceivable that the initial migration of maturing resident peritoneal DC is attributed to an alternative chemokine, and the up regulation of CCR7 may occur at a later stage, perhaps during the transit into the lymphatic system at which point the cells become unrecoverable by peritoneal lavage. To determine this, CCR7 expression would need to be measured in resident DC upon induction of peritoneal inflammation *in vivo.* An alternative explanation could be that the resident DC in culture are being subjected to suppression by IL-6 trans-signalling, which allows IL-6 to signal via its soluble receptor even in the absence of IL-6R on the cells surface. This is unlikely however due to the absence of CCR7 observed on activated resident DC from IL-$6^{-/-}$ mice. Resident DC

from these mice do not secrete IL-6, and therefore are unable to produce the IL-6/sIL-6R complex required for trans-signalling in these cultures. Finally it is conceivable that the resident DC do not home to the lymph nodes upon activation in the same way that many other DC subtypes have been observed to do. The peritoneal cavity is known to be rich in B1 cells, which are involved in both the innate and the adaptive immune response (Itakura *et al* 2005). The resident peritoneal DC are established antigen presenting cells (Dioszeghy *et al* 2008), and it is conceivable that this subset may remain in the peritoneal cavity upon activation, to present antigen to and activate the resident B1 cells, triggering their roles in innate and adaptive immunity. This would provide an adequate explanation for the lack of expression of lymph homing chemokine receptors by the resident DC, and support a potential new role for these cells in triggering a local and more rapid adaptive immune response.

The next Chapter investigates the role of IL-6 in the trafficking of monocytic cell populations during acute inflammation.

CHAPTER 5

The role of IL-6 in the trafficking of monocytic cells during an *in vivo* model of peritoneal inflammation

5.1 Introduction

Bacterial peritonitis is an underlining complication of end-stage renal failure patients receiving peritoneal dialysis. This form of treatment uses the peritoneal membrane as a dialysing barrier, across which waste products can be removed from the blood into a dialysis fluid (Venkat *et al* 2006). Commensal bacteria are often introduced into the peritoneal cavity via the catheter port as a result of skin contact that occurs when attaching or detaching bags, resulting in an inflammatory episode. The most common bacterial infections associated with these episodes of peritonitis are either *Staphylococcus aureus* or *Staphylococcus epidermidis* (Woo *et al* 2004). Repeated episodes of peritonitis affect the integrity of the peritoneal membrane, causing thickening of the membrane and compromising its ability to act as a dialysis membrane (Davies *et al* 1996, Williams *et al* 2002, Di Paolo *et al* 1986, Devuyst *et al* 2002). Ultimately these inflammation-induced changes to the tissue architecture lead to treatment failure. IL-$6^{-/-}$ mice exhibit a remarkable protection from chronic inflammation, and are protected from a number of other immunological disorders such as rheumatoid arthritis and Crohn's disease (Grossman *et al* 1989, Swaak *et al* 1989, Houssiau *et al* 1988, Naugler and Karin 2008). This has lead to IL-6 being of huge interest as an important therapeutic target with anti-IL-6 based therapies currently being used to treat these chronic inflammatory disorders (Scheinecker *et al* 2009). Consequently, it is important to understand how IL-6 contributes to the control of the inflammatory process, and affects disease progression. A number of roles currently exist for IL-6 in controlling leukocyte recruitment and activation. For instance IL-6 is centrally required to direct the transition from innate to acquired immunity (Jones 2005). IL-6 does this by balancing both the suppression of neutrophil recruitment and concomitantly promoting the infiltration of mononuclear cells during acute inflammation. These IL-6 directed outcomes are mediated through a control of inflammatory chemokine production and apoptosis (Hurst *et al* 2001, McLoughlin *et al* 2003, 2004, 2005, Teague *et al* 1997, Atreya 2000). Although IL-6 has been well established as a key regulator of chemokine expression, neutrophil trafficking, apoptosis and T cell survival, the role it plays as a regulator of monocytic cells is less well defined.

During acute peritoneal inflammation, leukocyte recruitment is characterised by an initial infiltration of neutrophils, which are later replaced by a more sustained population of mononuclear cells (Topley *et al* 1996). Based on data obtained from cytospins of peritoneal leukocytes differentially stained for neutrophils, lymphocytes and monocytes, IL-6 does not appear to play a role in the inflammatory trafficking of monocytic cells (McLoughlin *et al* 2005). This method is however limited and does not allow a more phenotypic evaluation of specific monocytic subsets. It is well established that a number of monocytic cell subsets exist, distinguished by their unique phenotypic cell surface markers, that potentially perform very different functions in an inflammatory context. In the peritoneal cavity of naïve mice, two populations of resident monocytic cells have been characterised, and are defined as a resident macrophage population ($F4/80^{hi}/CD11c^{hi}$) which comprises around 50% of the total resting leukocyte population, and a resident dendritic cell population ($F4/80^{int}/CD11b^{hi}/CD11c^{+}$) which constitutes approximately 5% of the total resting leukocyte population (Dioszeghy *et al* 2008). These populations were extensively characterised in Chapters 3 and 4 of this thesis. Resident tissue macrophages and DC are regarded as sentinels of the innate immune system, and the front line of host defence (Dunn *et al* 1985). Recent work has shown that specific ablation of the resident peritoneal macrophages, followed by induction of peritoneal inflammation, results in an impaired neutrophil infiltration as a consequence of reduced neutrophil activating CXC chemokine expression (Specifically KC (CXCL1) and MIP-2 (CXCL2)). This indicates that the resident macrophages are essential for the initiation and orchestration of acute peritoneal inflammation (Cailhier *et al* 2005).

Upon induction of the inflammatory response, an infiltrating population of $F4/80^{+}/CD11b^{hi}$ monocytes are recruited to the peritoneal cavity (Dioszeghy *et al* 2008). Within the circulating blood, two subpopulations of monocytes exist. The "inflammatory" subset migrates to inflamed tissue where it can trigger immune responses, while the "resident" subset, with a longer life, migrates to non-inflamed tissues to replenish the resident populations (Geissman *et al* 2003). Upon infiltration to the site of infection, the 'inflammatory' monocytes are thought to differentiate into macrophages or DC to help combat the infection (Randolph *et al* 1998, Gordon and

Taylor 2005). The resident blood monocyte population have recently been assigned another function in that they also 'patrol' healthy tissues by crawling on the resting endothelium lining the blood vessels and can rapidly invade tissues upon infection. Once in the tissue, they help to trigger the immune response in combination with the resident macrophages and DC (Auffrey *et al* 2007).

In summary, the resident peritoneal macrophage and DC populations have been implicated in the initiation and orchestration of the acute inflammatory response (Cailhier *et al* 2005), while the recruited inflammatory monocytes are thought to be important for replenishing the macrophage and DC populations and maintenance of the immune response (Gordon and Taylor 2005, Geissman *et al* 2003, Randolph *et al* 1998). There is also evidence dictating that all these monocytic subsets described have antigen presenting abilities and are important in driving the adaptive immune response (Dioszeghy *et al* 2008, Randolph *et al* 2008).

Since little is known about the potential role of IL-6 in regulating the monocytic cell subsets described here, the studies outlined in this Chapter aimed to investigate the potential role of IL-6 in governing the inflammatory trafficking of monocytic cell subsets using an *in vivo* model of peritoneal inflammation.

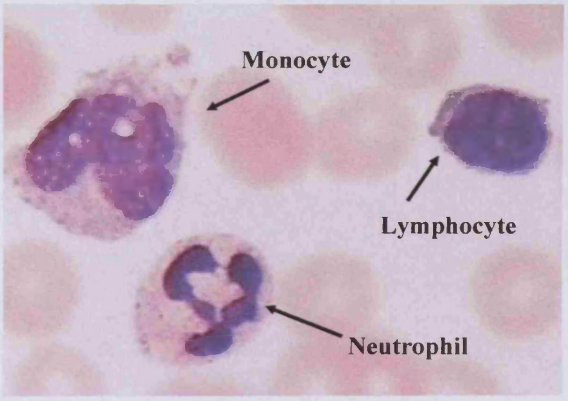
5.2 Materials and methods

5.2.1 Induction of peritoneal inflammation with *Staphylococcus epidermidis* (SES)

Lyophilised SES was prepared as described in Section 2.3.3 and reconstituted in 750µl sterile PBS. Inflammation was induced in groups of WT and IL-$6^{-/-}$ mice (n=5 per group, mixture of males and females) by i.p administration of SES (Section 2.3.5). At defined intervals during the inflammatory response (0, 1, 3, 6, 12, 15, 18, 24, 48 and 72 hours) mice were sacrificed and the leukocyte infiltrate collected by peritoneal lavage.

5.2.2 Cytospin analysis of the leukocyte infiltrate

Cytospin samples of peritoneal leukocytes were prepared and stained with Wright Giemsa stain and the composition of the inflammatory infiltrate was determined by differential cell counting (Section 2.3.2). The identification of each leukocyte subset was based on their morphology as visualised by light microscopy (Picture 5.1) .



Picture 5.1: *Murine blood leukocytes visualised by light microscopy following Giemsa staining.* Neutrophils are large and have multi-lobed nuclei. Monocytes are very large, and possess a kidney shaped nucleus. Lymphocytes are small, with very little cytoplasm and a large nucleus, which stains darker than that of the monocyte and neutrophil.

5.2.3 Flow cytometry

Peritoneal leukocytes were labelled with antibodies against the macrophage and dendritic cell markers F4/80, CD11b, and CD11c and analysed on a FACS calibur (Section 2.4.3). Details of the antibodies used are found in Table 2.1. Macrophages were defined as $F4/80^{hi}CD11b^{hi}$, dendritic cells as $F4/80^{int}/CD11b^{hi}$ and infiltrating monocytes as $F4/80^{int}CD11b^{int}$. Cell numbers were quantified by taking the flow cytometry population gated percentages and multiplying by the total cell number.

5.2.4 ELISA quantification of cytokine and chemokine levels

Peritoneal lavage fluids were rendered cell free by centrifugation and stored at -70°C. Supernatants were analysed by ELISA for IL-6, CCL2, CCL3, CCL5 and CCL7 and CXCL1 (KC) as described in Section 2.5.

5.3 Results

5.3.1 Comparison of leukocyte trafficking in WT and IL-6$^{-/-}$ mice following SES induced inflammation

To define the profile of leukocyte recruitment arising following SES challenge, differential cell counts were performed on cytospin preparations obtained at defined intervals following SES administration. Mice exhibited an initial transient infiltration of neutrophils which begun as early as one hour after SES challenge and peaked 12 hours post SES administration. This population was then cleared, and replaced with a more sustained population of monocytic cells and lymphocytes (Figure 5.1). As previously observed (Hurst *et al* 2001, McLoughlin *et al* 2004), IL-6$^{-/-}$ mice showed an enhanced peritoneal infiltration of neutrophils (approximately 2-fold higher than WT mice at 6 and 12 hours), which were retained for longer within the peritoneal cavity (Figure 5.1A).

Comparative analysis in WT and IL-6$^{-/-}$ mice did however emphasise that lymphocyte trafficking was unaffected by the absence of IL-6. Lymphocyte trafficking was characterised by an initial decrease in number during the first 3 hours after SES challenge, followed by a re-emergence of this population which was retained over the remainder of the time course (Figure 5.1B). Similarly, monocyte trafficking was also unaffected by the absence of IL-6. Monocyte trafficking was characterised by an initial decrease in number during the first 3 hours, followed by a substantial influx of these cells, which were retained throughout the remainder of the time course (Figure 5.1C).

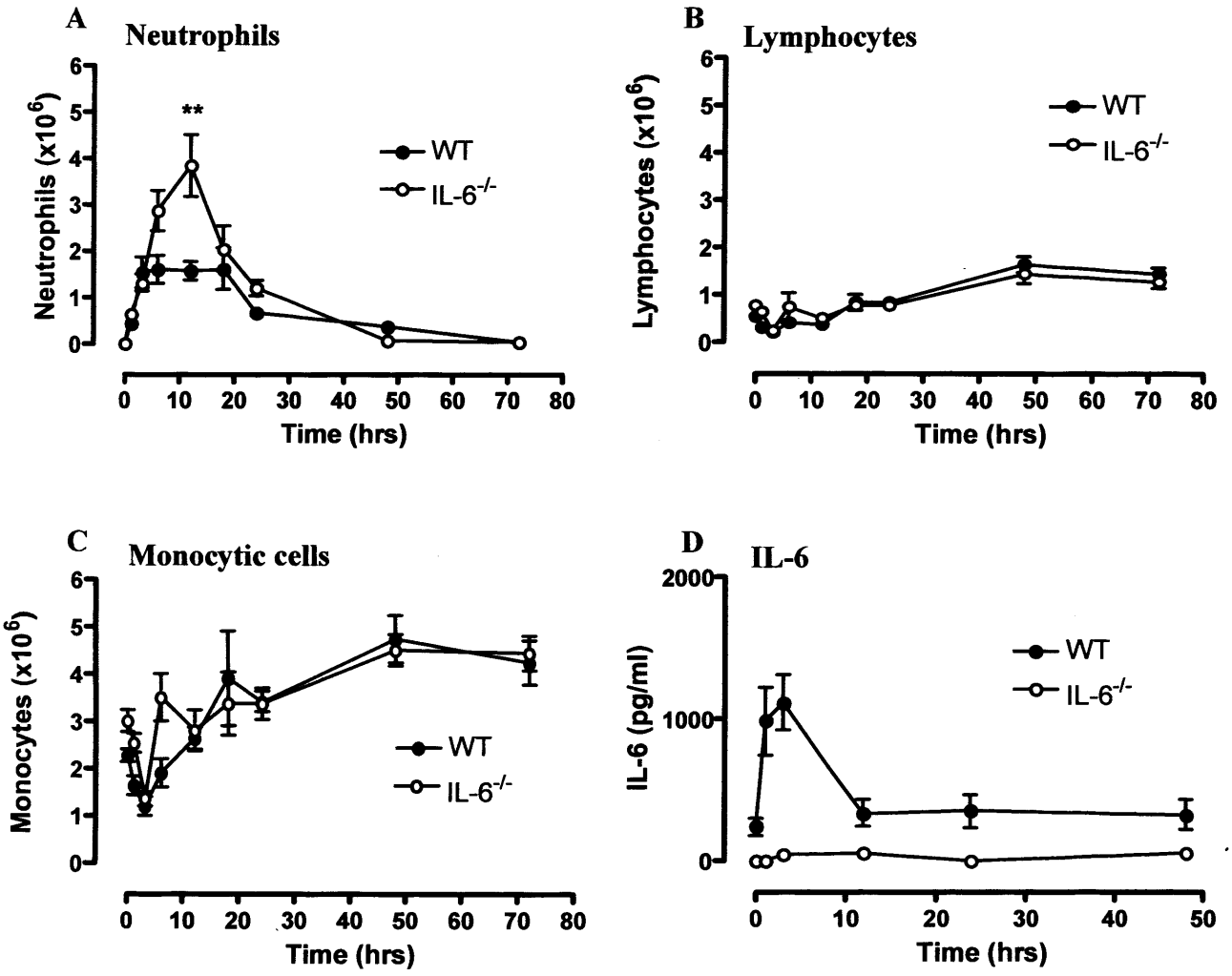


Figure 5.1: *Neutrophil, monocyte and lymphocyte trafficking following the induction of SES induced inflammation.* Peritoneal inflammation was induced by i.p administration of SES and peritoneal leukocytes were collected by lavage at defined intervals. **(A)** Differential cell counts were performed and the number of neutrophils calculated at each time point. **(B)** The number of lymphocytes calculated at each time point. **(C)** The number of monocytes calculated at each time point. **(D)** IL-6 levels were measured in lavage supernatants by ELISA as a control to confirm the absence of IL-6 in IL-$6^{-/-}$ mice. Values represent the mean ± SEM, n=5-15 mice per time point, ** $P < 0.01$.

5.3.2 The role of IL-6 in directing the inflammatory trafficking of resident-like macrophages

To tease apart potential differences in the inflammatory trafficking of defined monocytic populations, flow cytometry was used to monitor SES induced changes in $F4/80^{+}CD11b^{+}$ cell trafficking. Such analysis identified two distinctly defined populations characterised as resident-like $F4/80^{hi}CD11b^{hi}$ macrophages, and infiltrating $F4/80^{int}CD11b^{int}$ monocytes, which emerge following inflammatory activation (Figure 3.1). The aim of this experiment was to determine whether the trafficking of these populations during acute inflammation was regulated by IL-6. Prior to SES activation, the resident monocytic population was predominantly $F4/80^{hi}CD11b^{hi}$. SES stimulation however triggered an early exodus of the $F4/80^{hi}CD11b^{hi}$ resident macrophage population from the peritoneal cavity, decreasing dramatically during the first three hours of inflammation (Figure 5.2). This coincided with a marked influx of $F4/80^{int}CD11b^{int}$ monocytic cells (See Section 5.3.3). However as inflammation progressed, flow cytometry affirmed the re-emergence of a resident-like $F4/80^{hi}CD11b^{hi}$ macrophage population. This profile of cell emigration and recruitment was evident in both WT and IL-$6^{-/-}$ mice, with only minor differences observed between them. The emigration of the resident-like macrophage population from the peritoneal cavity did appear to be impaired in the IL-$6^{-/-}$ mice, demonstrated over the first 12 hours, and they also appeared to re-emerge at a faster rate in the IL-$6^{-/-}$ mice, demonstrated by the differences observed at 48 hours (Figure 5.2). However an analysis of the rate of clearance (WT: $3.1x10^5$ cells/hr, IL-$6^{-/-}$: $3x10^5$ cells/hr) and rate of re-emergence (WT: $1.7x10^4$ cells/hr, IL-$6^{-/-}$: $1.7x10^4$ cells/hr) showed that WT and IL-$6^{-/-}$ exhibited equivalent resident macrophage trafficking profiles.

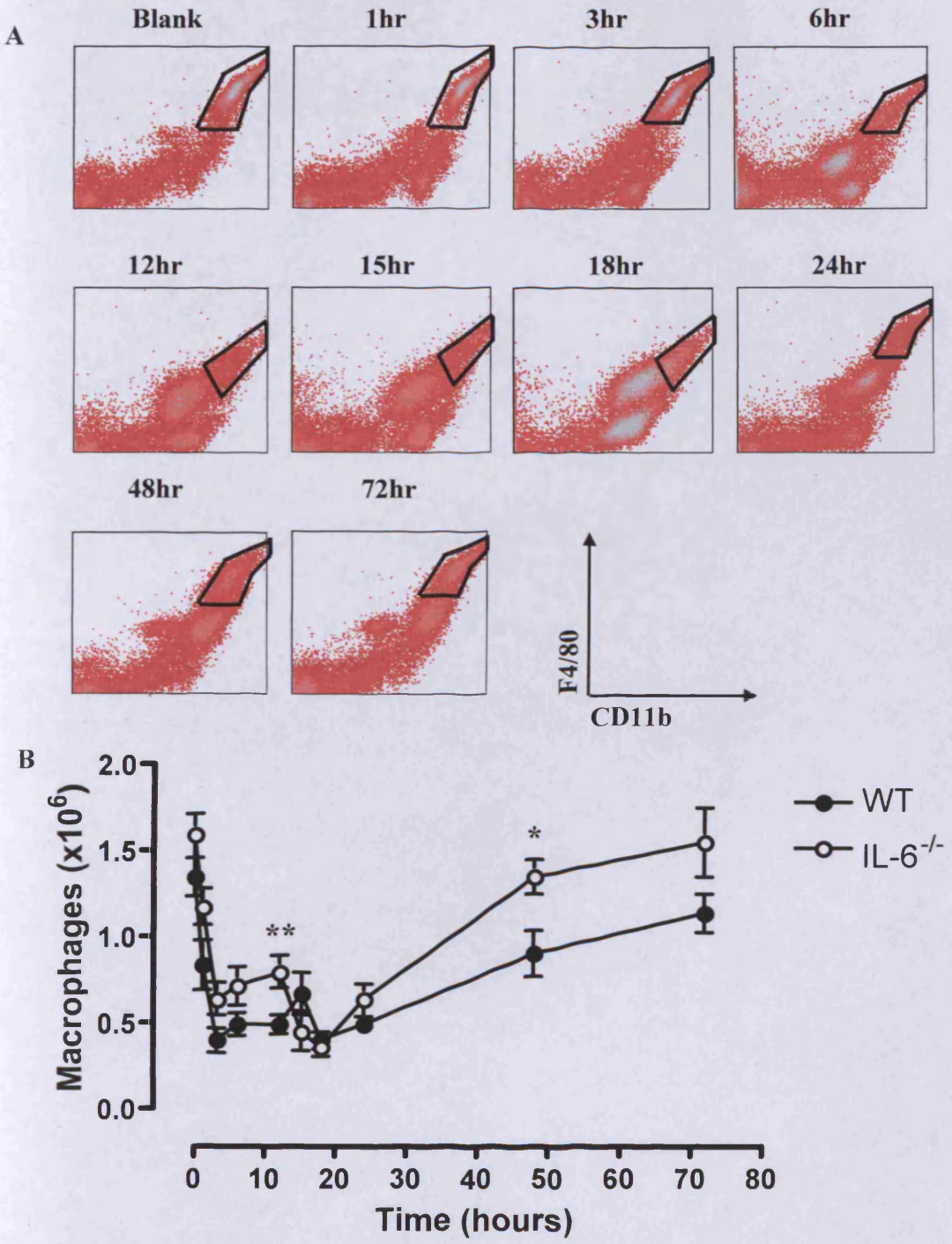


Figure 5.2: *IL-6 regulation of resident-like macrophage trafficking following SES induced peritonitis.* Peritoneal inflammation was induced by i.p administration of SES and peritoneal leukocytes were collected by lavage at defined intervals. **(A)** Peritoneal leukocytes were labelled with anti F4/80 and CD11b and the macrophages identified by their F4/80^{hi}CD11b^{hi} phenotype. **(B)** The number of resident-like macrophages, defined by the indicated gates (Panel A) were calculated at each time point by taking the flow cytometry gated percentage and multiplying by the total cell number. Values represent the mean ± SEM, n=5-15 mice per time point,* $P < 0.05$, ** $P < 0.01$.

5.3.3 The role of IL-6 in directing the inflammatory trafficking of infiltrating monocytes

Infiltrating monocytes were phenotypically identified as F4/80^{int}/CD11b^{int}. To extrapolate the infiltration of these cells, their number was calculated at each time point (Figure 5.3). Infiltrating monocytes began to appear in the peritoneal cavity at 3 hours, reaching a peak at 12 hours. This population was then retained in the peritoneal cavity, and were still present at 72 hours. No significant differences were observed in the trafficking of infiltrating monocytes in WT and IL-6$^{-/-}$ mice, apart from at 48 hours, when there was a slight elevation in the number of monocytes in WT mice.

5.3.4 IL-6$^{-/-}$ mice exhibit a temporal appearance of a CD11c^{+} monocytic like population during inflammation

To study the trafficking of resident F4/80^{int}/CD11b^{hi}/CD11c^{+} dendritic cells, the number of these cells was extrapolated from FACS data at each time point following SES administration (Figure 5.4). SES stimulation in both WT and IL-6$^{-/-}$ mice triggered an early clearance of the resident DC population during the first 3 hours, which was followed by a subsequent re-emergence of this population that was retained for the duration of the time course tested. The re-emergence of this DC like population was greatly enhanced in IL-6$^{-/-}$ animals, peaking at 12 hours (Figure 5.4). This enhanced re-emergence of the DC population was temporal, with their numbers becoming equivalent to WT mice towards the end of the time course. The DC population present in WT and IL-6$^{-/-}$ mice after 12 hours of SES was further analysed based on forward and side scatter properties. This mode of analysis gave information on the size and granularity of these cells (Figure 5.5). This identified two separate populations of DC-like cells present in IL-6$^{-/-}$ mice, which were distinguishable as being either relatively small, or larger and more granular. These were subsequently referred to as F4/80^{lo}CD11b^{hi}CD11c^{+} resident-like or monocyte-like DC respectively. WT mice possessed the smaller resident-like population of DC, but lacked the larger monocyte-like DC population (Figure 5.5). The data outlined in Figure 5.5 therefore supported the notion that IL-6 suppresses development of a novel CD11c^{+} monocyte-like DC population.

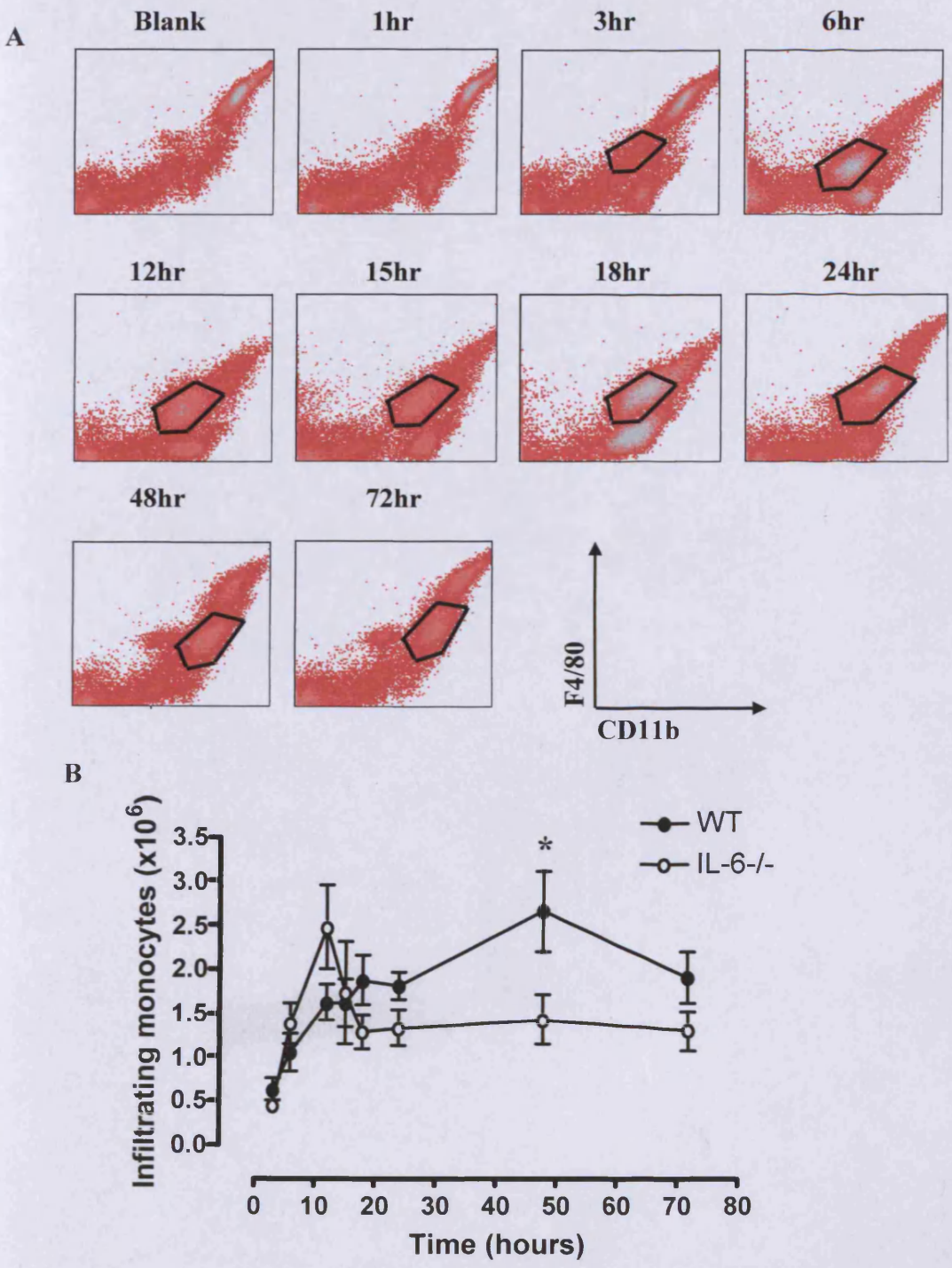


Figure 5.3: *IL-6 regulation of infiltrating monocytes following SES induced peritonitis.* Peritoneal inflammation was induced by i.p administration of SES. Peritoneal leukocytes were collected by lavage at defined intervals. **(A)** Peritoneal leukocytes were labelled with anti F4/80 and CD11b and monocytes were subsequently identified by their phenotype $F4/80^{int}CD11b^{int}$. **(B)** The number of infiltrating monocytes within the defined gates (Panel A) were calculated at each time point by taking the flow cytometry gated percentage and multiplying by the total cell number. Values represent the mean ± SEM, n=5-15 mice per time point, * $P < 0.05$.

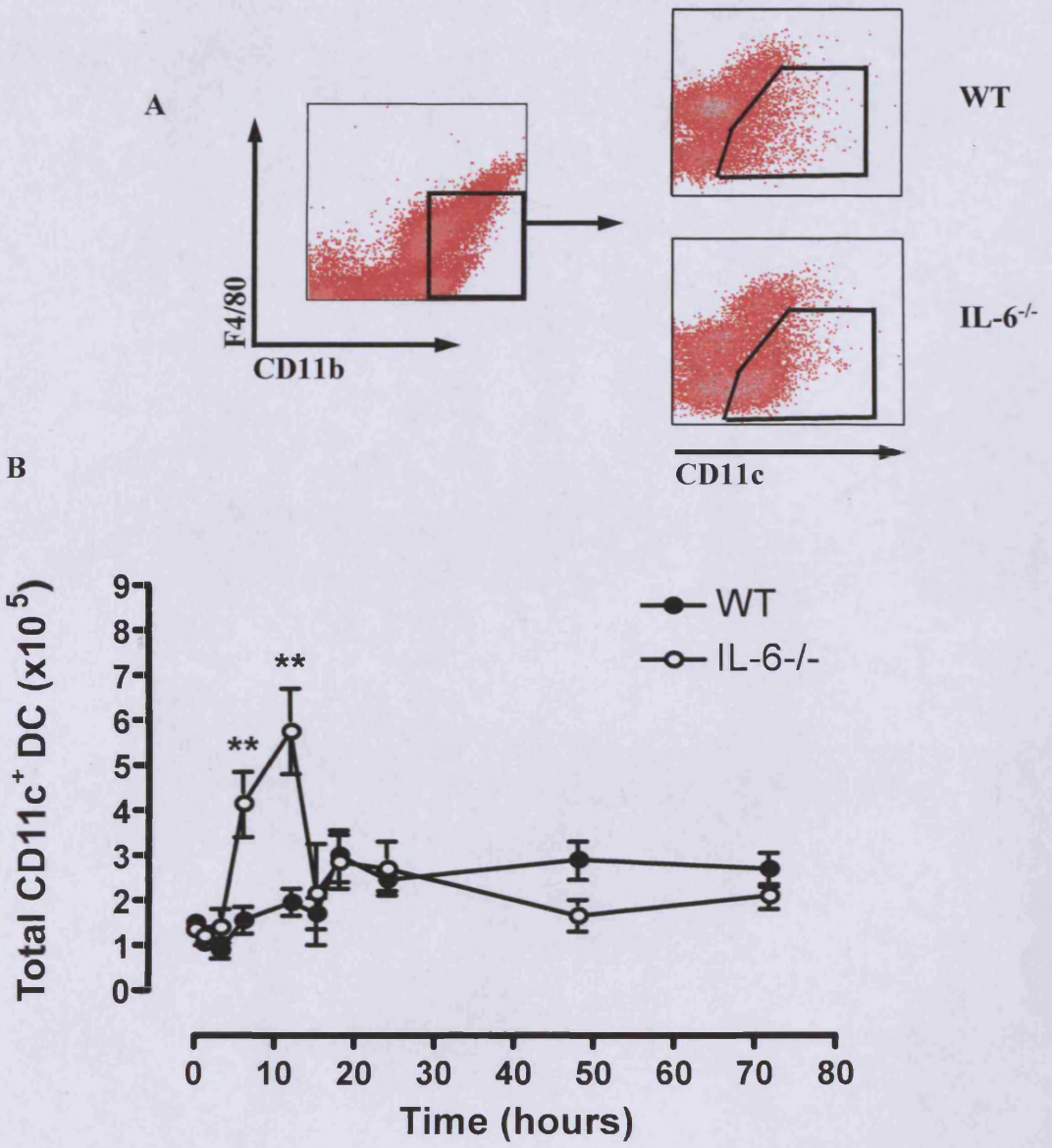


Figure 5.4: *IL-6 regulation of DC trafficking.* Peritoneal inflammation was induced by i.p administration of SES and peritoneal leukocytes were collected by lavage at defined intervals. **(A)** Peritoneal leukocytes were labelled with anti F4/80 and CD11b and cells that were positive for F4/80 and CD11b were labelled for CD11c. Cells that resided in this F4/80^{int}CD11b^{hi}CD11c+ gate were defined as DC. **(B)** The number of F4/80^{int}CD11b^{hi}CD11c+ DC within the defined gate (Panel A) was calculated at each time point by taking the flow cytometry gated percentage and multiplying by the total cell number. Values represent the mean ± SEM, n=5-15 mice per time point, ** $P < 0.01$.

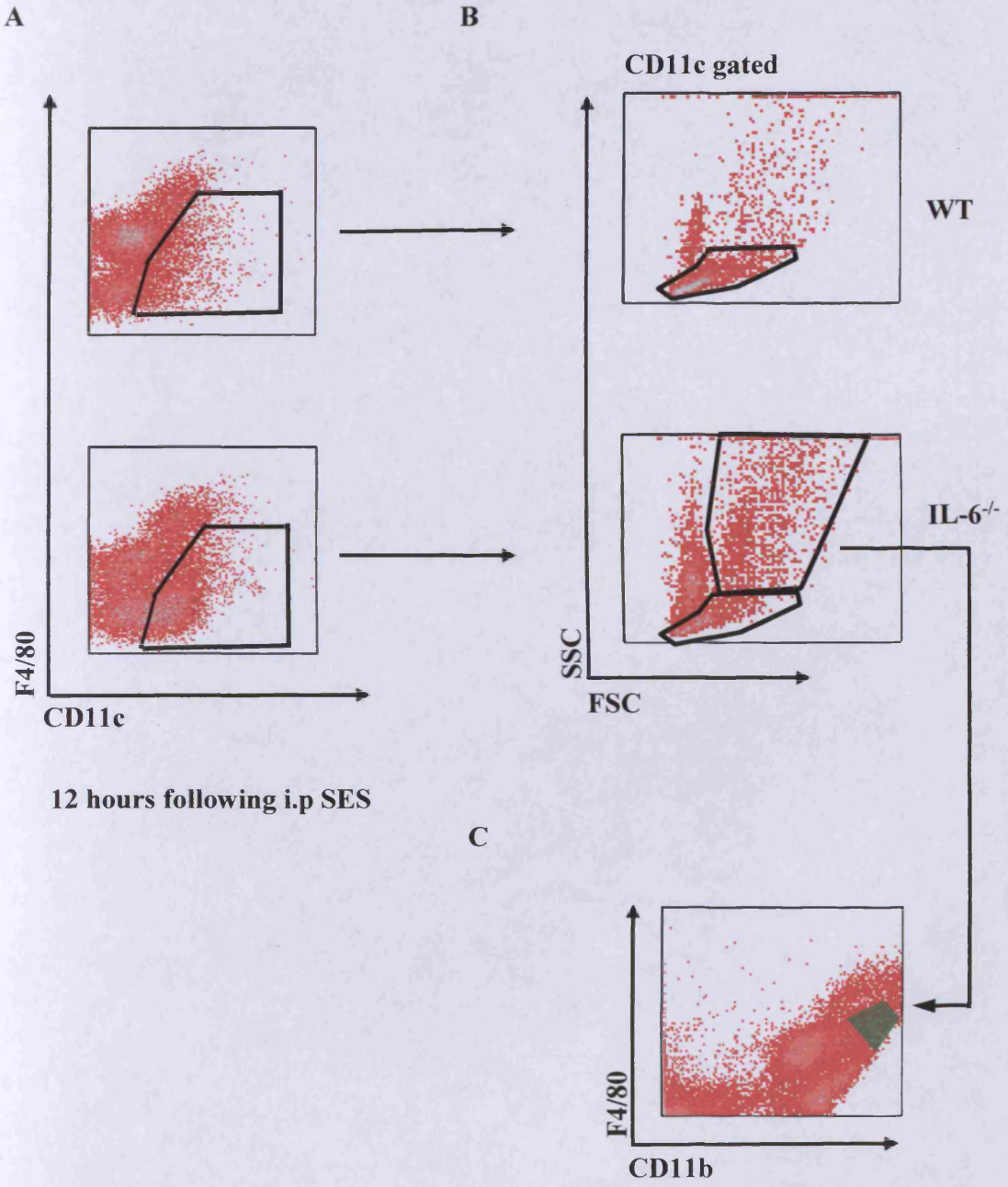


Figure 5.5: *IL-6$^{-/-}$ mice possess a novel CD11c^{+} monocytic population at 12 hours following induction of inflammation.* Peritoneal inflammation was induced by i.p administration of SES. Peritoneal leukocytes were collected by lavage after 12 hours and were labelled with anti F4/80, CD11b and CD11c. **(A)** Cells that were high for CD11b, low and medium for F4/80 and positive for CD11c were gated and analyzed by their forward and side scatter properties. **(B)** Forward and side scatter analysis of F4/80^{lo}CD11b^{hi}CD11c^{+} peritoneal DC. **(C)** Back gating of the high forward and side scatter population revealing information on exact F4/80 and CD11b expression by these cells.

To evaluate the trafficking properties of these two separate populations, the number of resident-like and monocyte-like DC were individually plotted for each time point (Figures 5.6 and 5.7). This showed that the trafficking of the resident-like DC population was equivalent in WT and IL-$6^{-/-}$ mice (Figure 5.6). In this respect, SES challenge led to an initial clearance of these cells over the first 3 hours, only to return later in the time course. The monocyte-like DC population however, exhibited a more temporal increase and showed a transient profile of detection (between 6-18 hours post SES challenge). This temporal appearance was more exaggerated in IL-$6^{-/-}$ mice, which possessed significantly more of these cells at 6 and 12 hours than WT mice (Figure 5.7).

5.3.5 The appearance of a $CD11c^{+}$ monocytic population in IL-$6^{-/-}$ mice does not appear to be chemokine driven

To determine whether the appearance of the monocyte-like DC population seen in SES challenged IL-$6^{-/-}$ mice was attributable to alterations in chemokine expression, CXCL1 (KC), CCL2, CCL3, CCL5 and CCL7 levels were measured in the peritoneal lavage supernatants at each time point by ELISA.

CXCL1 (KC) is a known neutrophil chemo-attractant. When measured, this chemokine could be temporally detected at the 1 hour time point, and was quickly cleared by 3 hours. The production of this chemokine was significantly enhanced in IL-$6^{-/-}$ mice as compared to the WT mice, with significantly more CXCL1 being produced in these mice at 1 hour (Figure 5.8).

Known monocyte chemo-attractants CCL2, CCL3, CCL5 and CCL7 all exhibited a temporal production in WT and IL-$6^{-/-}$ mice, with CCL2 and CCL5 peaking at 3 hours and CCL3 peaking at 6 hours. CCL7 peaked at 3 hours in WT mice, whilst IL-$6^{-/-}$ mice exhibited a delayed kinetic, with CCL7 peaking at 6 hours in these mice. This was followed by a complete clearance of the chemokine by 72 hours. The production of CCL2, CCL3 and CCL5 were all significantly impaired in IL-$6^{-/-}$ mice (Figure 5.9).

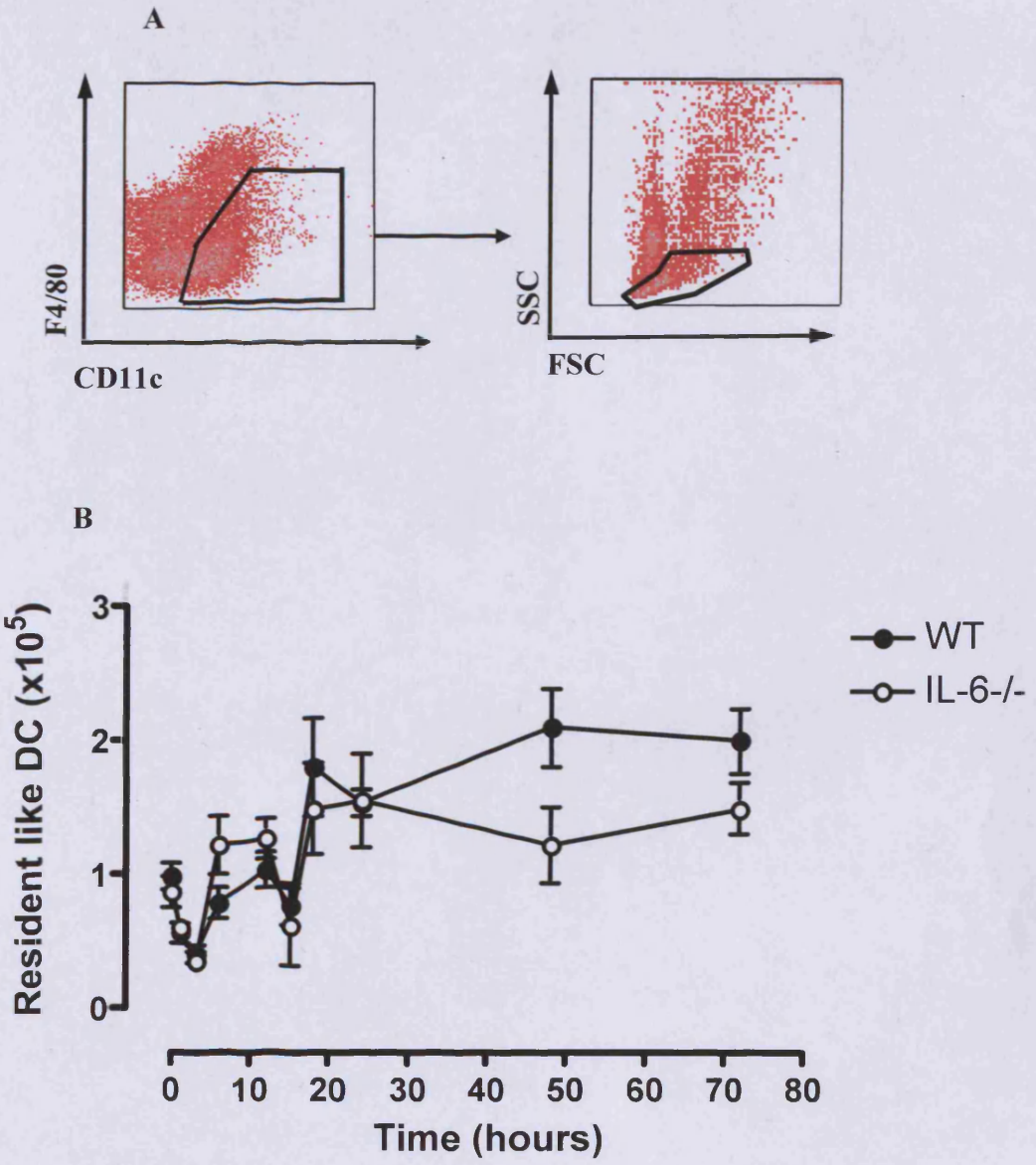


Figure 5.6: *Trafficking of resident-like DC following SES induced peritonitis.* Peritoneal inflammation was induced by i.p administration of SES. Peritoneal leukocytes collected by lavage at defined intervals. **(A)** Peritoneal leukocytes were labelled with anti F4/80 and CD11b and CD11c. Cells that were high for CD11b, low and medium for F4/80 and positive for CD11c were gated and analyzed by their forward and side scatter properties to identify the resident-like DC that display a $F4/80^{lo}CD11b^{hi}CD11c^{+}$ phenotype and reside in the low FSC/SSC gate. **(B)** The number of resident-like DC was calculated at each time point by taking the flow cytometry gated percentage and applying that to the total cell number. Values represent the mean ± SEM, n=5-15 mice.

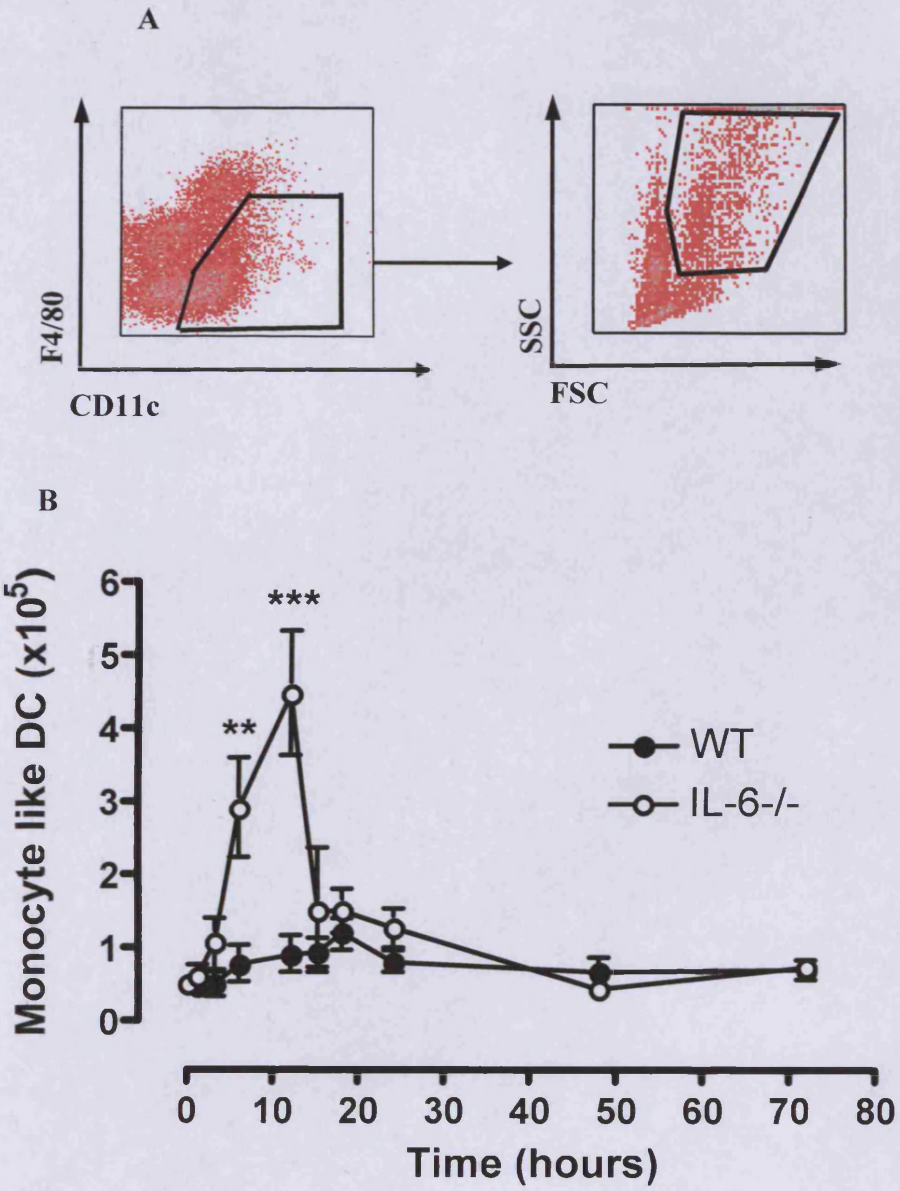


Figure 5.7: *Trafficking of monocyte-like DC following SES induced peritonitis.* Peritoneal inflammation was induced by i.p administration of SES. Peritoneal leukocytes were collected by lavage at defined intervals. **(A)** Peritoneal leukocytes were labelled with anti F4/80 and CD11b and CD11c. Cells that were high for CD11b, low and medium for F4/80 and positive for CD11c were gated and analysed by their forward and side scatter properties to identify the monocyte-like DC that display a $F4/80^{lo}CD11b^{hi}CD11c^{+}$ phenotype and reside in the high FSC/SSC gate. **(B)** The number of monocyte-like DC was calculated at each time point by taking the flow cytometry gated percentage and applying that to the total cell number. Values represent the mean ± SEM, n=5-15 mice per time point, ** $P < 0.01$, *** $P < 0.001$.

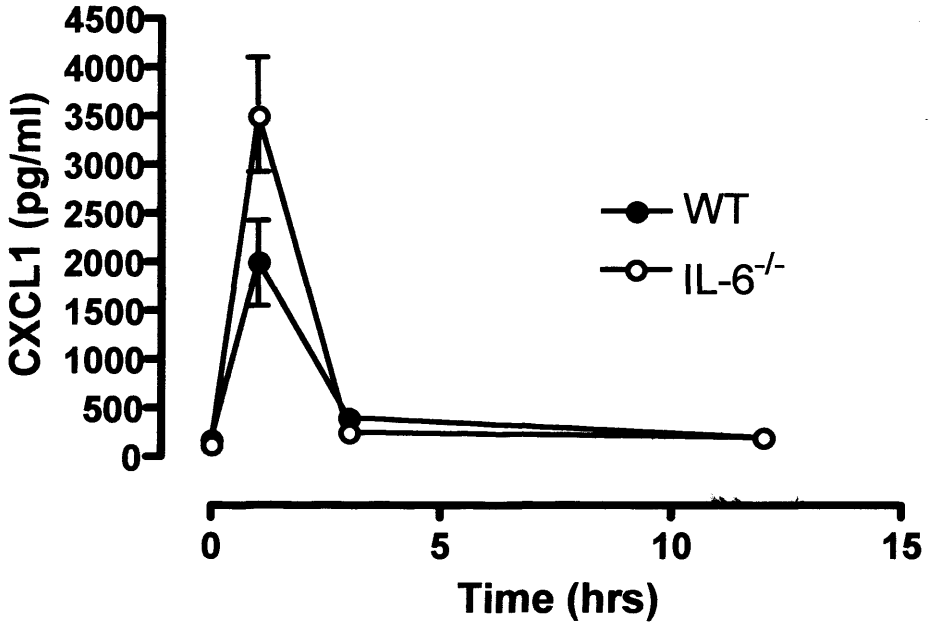


Figure 5.8: *CXCL1 (KC) production in the peritoneal cavity of WT and IL-6$^{-/-}$ mice following induction of SES induced peritoneal inflammation.* Peritoneal inflammation was induced by i.p administration of SES. CXCL1 (KC) levels were quantified by ELISA in cell free lavage fluids. Values represent the mean ± SEM, n=5-15 mice per time point.

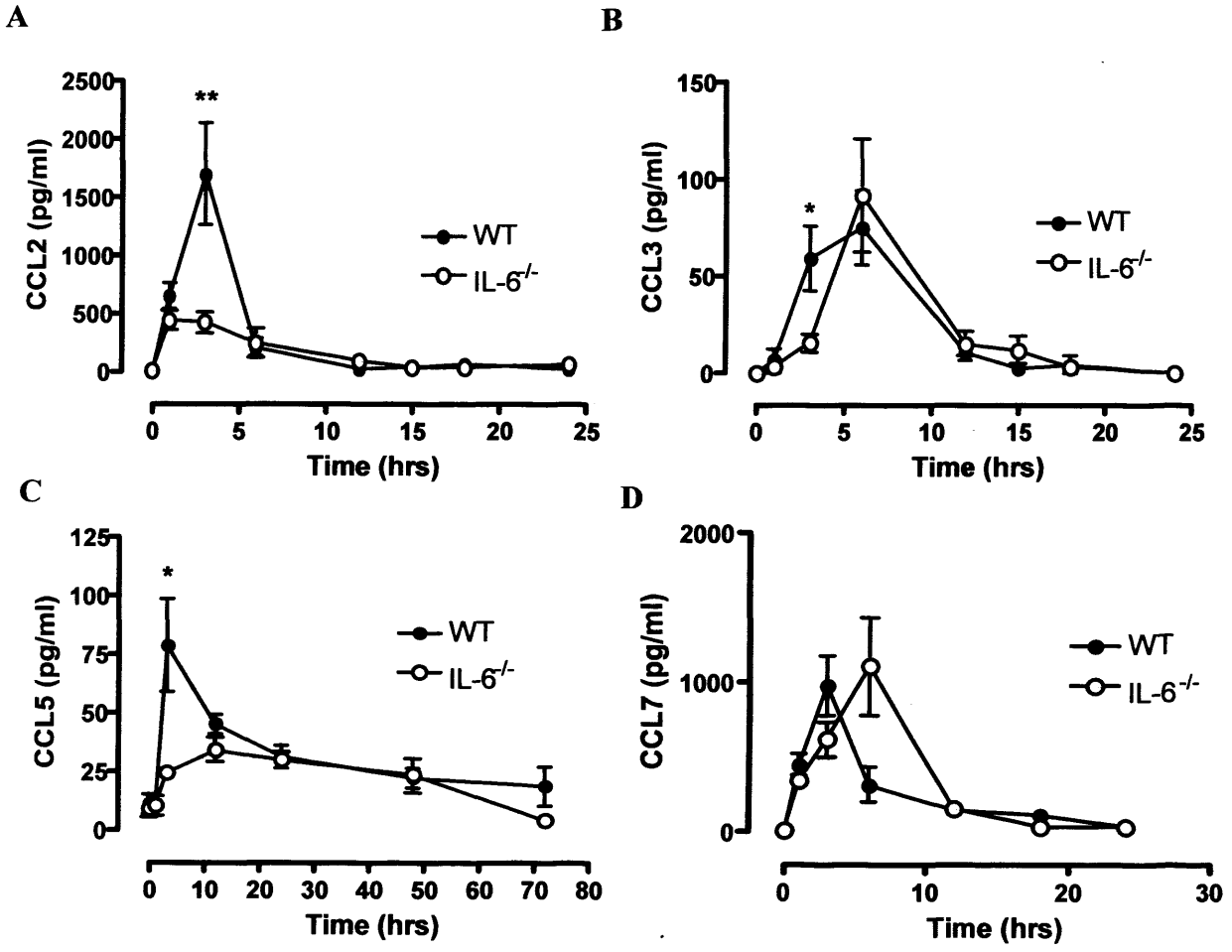


Figure 5.9: *Mononuclear chemo-attractant production in the peritoneal cavity following SES induced inflammation.* Peritoneal inflammation was induced by i.p administration of SES. **(A)** CCL2 **(B)** CCL3, **(C)** CCL5, **(D)** and CCL7 levels were quantified by ELISA in cell-free lavage supernatants. Values represent the mean ± SEM, n=5-15 mice per time point, * $P < 0.05$, ** $P < 0.01$.

5.4 Discussion

As has previously been reported by the group, data presented in this Chapter highlights a role for IL-6 in the regulation of neutrophil trafficking (Hurst *et al* 2001). This conclusion is based on differential cell count data, which showed an enhanced influx of neutrophils in IL-$6^{-/-}$ mice. IL-6 is essential for directing the transition from innate to acquired immunity by directing the successful resolution of neutrophil infiltration during inflammation, and the subsequent recruitment of mononuclear cells, which is critical for the successful resolution of acute inflammation (Jones, S.A. 2005). Upon entry to the peritoneal cavity, neutrophils shed their surface IL-6 receptor (McLoughlin *et al* 2004). The release of IL-6R from neutrophils facilitates IL-6 trans-signalling in the mesothelium, which differentially regulates inflammatory chemokine expression (notably the down-regulation of CXCL1 and up-regulation of CCL2 and CCL5 by mesothelial cells), and modulates leukocyte apoptosis (in particular IL-6 directs caspase-3-mediated neutrophil apoptosis), thereby driving a switch from a predominantly neutrophil rich environment to a more sustained mononuclear cell recruitment (Hurst *et al* 2001, McLoughlin *et al* 2003 McLoughlin *et al* 2004, McLoughlin *et al* 2005). In the absence of IL-6, CXCL1 production by the mesothelial cells is prolonged and the onset of neutrophil apoptosis is delayed resulting in increased recruitment and retention of these cells (Hurst *et al* 2001, McLoughlin *et al* 2003 and 2004).

It is also documented, but not seen in this study, that IL-$6^{-/-}$ mice exhibit impaired lymphocyte infiltration during inflammation, specifically T lymphocytes (McLoughlin *et al* 2005, Jones *et al* 2009, J.Immunol, under revision). This is attributed to IL-6 which selectively governs T cell infiltration by regulating chemokine secretion (CXCL10, CCL4, CCL5, CCL11, and CCL17) and chemokine receptor (CCR3, CCR4, CCR5, and CXCR3) expression on the CD3+ infiltrate (McLoughlin *et al* 2005). Additionally, the IL-6/sIL-6R complex has been shown to be an inhibitor of apoptosis in T cells by inhibiting the down-regulation of anti-apoptotic regulator Bcl-2 (Teague *et al* 1997). IL-$6^{-/-}$ mice lacking this cytokine are therefore likely to be more prone to T cell apoptosis. Regulation of these activities may be critical for resolving bacterial infections since

IL-$6^{-/-}$ mice show an impaired ability to control infections (Kopf *et al* 1994, Dalrymple *et al* 1995, Coles *et al* unpublished). This regulation of T lymphocyte trafficking by IL-6 was not supported by the data presented in this Chapter, however the differential cell counting method utilised did not allow a discrimination between T and B lymphocytes, therefore the data represents the trafficking profile of the total lymphocyte population. Multi-parameter flow cytometry, which was used by both McLoughlin *et al* and Jones *et al,* allows the trafficking of B and T lymphocyte trafficking to be analysed individually providing an explanation as to why differences were not seen in these studies described here. In this respect, a flow cytometric analysis of $CD4^{+}$ T lymphocytes during SES induced peritonitis is outlined in Chapter 8.

The data presented in this Chapter showed that IL-6 does not regulate the inflammatory trafficking of resident-like macrophages. Although an impaired clearance of these macrophages was observed in IL-$6^{-/-}$ mice during the initial stages, naive mice (0h time point) began with a larger number of resident macrophages, and these cells were cleared and re-emerged at an equivalent rate to that of WT mice. It is currently unknown what causes the initial exodus of this population, but a number of explanations are possible. Firstly cells could adhere to the peritoneal membrane as a result of activation, meaning that they would not be recovered in the peritoneal lavage. A review of adhesion molecules present on the surface of these cells would shed light on this. Secondly, these cells may undergo apoptosis, whilst their homing to the lymphatic system or omental milky spots may represent an alternative explanation (Rangal-Moreno *et al* 2009). Further work would be needed to confirm this. The reappearance of this population is likely a result of replenishment from the infiltrating monocytes, and there is currently no evidence to show that the original population might be returning to the peritoneal cavity upon resolution of the inflammatory episode. To address this, experiments were performed utilising a fluorescent marker to label resident leukocytes prior to induction of inflammation in an attempt to distinguish between resident macrophages and newly recruited cells. Whilst the resident like macrophage population remained labelled throughout the time course following the induction of inflammation, this could not be attributed to their retention in the peritoneal cavity since newly recruited cells may

become labelled by the phagocytic uptake of labelled apoptotic resident cells. Therefore alternative experiments would be required to try to conclusively answer this question.

The initial egress of the resident macrophage population, which was observed following the induction of inflammation, coincided with a recruitment of infiltrating monocytes from the circulation. These cells are known to enter the peritoneal cavity during inflammation where they differentiate into either macrophages or DC to replenish the resident populations and also contribute to the immune response against the pathogen (Geissman *et al* 2003, Randolph *et al* 1998, Auffrey *et al* 2007). In this respect they form an important support to the resident DC and macrophages, which initiate the immune response, but are unable to clear the infection alone (Ajuebor *et al* 1999, Cailhier *et al* 2005). Although IL-6 did not drive the trafficking of these infiltrating monocytes, shown by the data in this Chapter, the absence of IL-6 enabled the appearance of a monocyte-like $CD11c^+$ DC population of cells. This population was characterised by an $F4/80^{lo}CD11b^{hi}CD11c^+$ phenotype and are large and granular cells. This DC population, which is not observed in WT mice, may conceivably represent a novel inflammatory DC subset. Inflammatory DC appear as a consequence of inflammation or microbial stimuli. An example of an inflammatory DC is the tumour-necrosis factor and inducible nitric-oxide sythase-producing DC, also termed Tip DC, which were first discovered in mice infected with *Listeria monocytogenes* (Serbina *et al* 2003, Wu and Lui 2007 and Wu and Dakie 2004). Another example of inflammatory DC are the monocyte-derived DC, which are derived from $Gr1^+$ monocytes, and are thought to be essential for antimicrobial immunity (Lopez-Bravo and Ardavin 2008). It is possible that the $CD11c^+$ monocyte-like DC observed in these current studies may have differentiated from infiltrating monocytes. It is documented that IL-6 switches the phenotype of monocytes from DC to macrophages by facilitating the M-CSF (Macrophage colony stimulating factor) regulation of macrophage differentiation (Chomarat *et al* 2000). Although a base line appraisal of resident peritoneal DC numbers supports the notion that IL-6 favours DC formation, (Chapter 3), it is possible that the reverse is true under inflammatory conditions where the absence of IL-6 may enable an increased differentiation into dendritic cells. The functional significance of this population is of interest given that

IL-6$^{-/-}$ mice exhibit protection to various autoimmune diseases and this novel population of cells could contribute to that protection. It has been published that IL-6$^{-/-}$ mice have increased numbers of mature DC in the spleen and lymph nodes indicating that IL-6 blocks DC maturation *in vivo* (Park *et al* 2004). In this respect, it could be speculated that the emerging population of CD11c^{+} monocyte-like DC seen in IL-6$^{-/-}$ mice, may undergo increased maturation and a subsequent trafficking to secondary lymph nodes where they can mediate antigen presentation. Alternatively, it has also been published that IL-6$^{-/-}$ mice have increased numbers of bone marrow derived DC with reduced function (Bleier *et al* 2004). An attenuated function by the CD11c^{+} monocyte-like DC may be important for the protective phenotype exhibited by this strain as this may act to dampen down the immune response and thereby prevent the development of chronic inflammatory conditions. This concept will be discussed in Chapter 6.

The appearance of the CD11c^{+} monocyte-like DC population in IL-6$^{-/-}$ mice does not appear to be chemokine driven, given that the monocytic chemokines measured were either equivalent or significantly lower in IL-6$^{-/-}$ mice compared to WT. While it is possible that an alternative chemokine is responsible for the trafficking of these cells, it may also be possible that the trafficking of these cells is chemokine independent and regulated in another way. This has been seen previously in work by Dioszeghy *et al* who also showed an increased inflammatory trafficking of monocyte/macrophages in mice that coincided with a significantly impaired monocyte chemokine production in these mice (Dioszeghy *et al* 2008). The studies described in this Chapter did however show significantly elevated levels of the neutrophil activating chemokine KC in IL-6$^{-/-}$ mice. Interestingly, work by Huo *et al* has shown that KC is able to trigger monocyte arrest on the early atherosclerotic endothelium thereby promoting their recruitment *in vivo* (Huo *et al* 2001). This is an important finding and could provide an explanation as to the emergence of the monocyte like DC subset in IL-6$^{-/-}$ mice described here.

This novel monocyte-like DC population is studied in a repeat inflammatory model in Chapter 6, and its regulation is studied in more detail in Chapter 7.

CHAPTER 6

The role of IL-6 in the trafficking of monocytic cells in an *in vivo* repeat model of inflammation

6.1 Introduction

During an acute infectious episode, leukocyte infiltration is tightly regulated to ensure both clearance of the invading microbe and successful resolution of the inflammatory response to prevent damage to the host tissue. IL-6 is essential for defining leukocyte recruitment and clearance during acute peritoneal inflammation (Hurst *et al* 2001, Jones 2005, Fielding *et al* 2008) *via* its control of signalling events that occur through the transcription factors STAT1 and STAT3, which appear to be critical for inflammatory resolution (McLoughlin *et al* 2005, Fielding *et al* 2008). In this respect, IL-6 deficient mice have an impaired ability to clear bacterial infections, suggesting a central role for IL-6 in cellular immunity against infection (Kopf *et al* 1994, Coles *et al* unpublished). These protective properties of IL-6 however are balanced against the detrimental effects of IL-6 biology seen in chronic inflammatory disease (Jones 2005). Chronic inflammation arises through an inappropriate control of the inflammatory response and is exacerbated by the prolonged retention of activated leukocytes within the affected tissue (Jones 2005). IL-6 plays a significant role in the emergence of a chronic inflammatory state, given its heightened levels in a number of chronic inflammatory diseases, and a capacity to direct mononuclear cell trafficking and prevent normal apoptotic processes in effector T cells, including Th17 and Th1 cells (Jones 2005, McLoughlin *et al* 2005, Atreya *et al* 2000, Yamamoto *et al* 2000, Kikly *et al* 2006). In this respect, IL-$6^{-/-}$ mice exhibit a marked protection from chronic inflammation, and are resistant to experimental models of rheumatoid arthritis, systemic lupus erythematous, psoriasis and Crohn's disease (Grossman *et al* 1989, Swaak *et al* 1989, Houssiau *et al* 1988, Naugler and Karin 2008).

Persistent or recurrent episodes of bacterial peritonitis are a common complication in renal failure patients receiving dialysis treatment, and dramatically affect the integrity of the peritoneal membrane, giving rise to fibrotic thickening and scarring of the membrane (Davies *et al* 1996, Williams *et al* 2002). Fibrosis is characterised by alterations in extracellular matrix deposition as a consequence of tissue damage or persistent inflammatory processes (Wynn *et al* 2007). This compromises the dialysing capacity of

the membrane and ultimately these inflammation-induced changes to the tissue architecture lead to treatment failure (Davies *et al* 1996, Williams *et al* 2002).

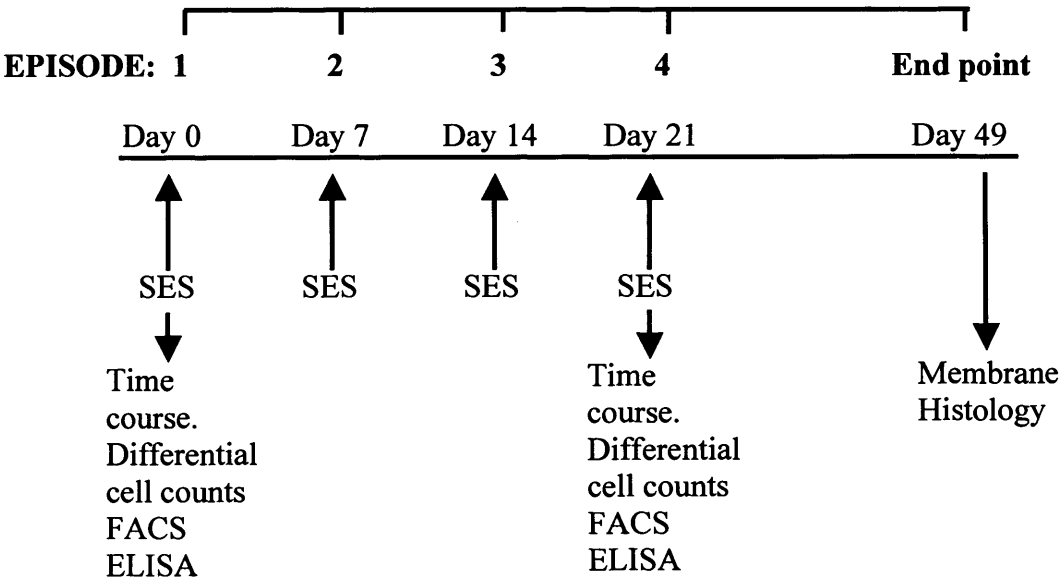
The induction of tissue damage is a key marker of chronic disease pathology. The underlining tissue damage that can lead to fibrosis is associated with the damage or apoptosis of cells within the inflamed tissue such as resident stromal and parenchymal cells (Shimizu *et al* 1995). Inflammatory macrophages have been well documented to play a major role in the induction of tissue damage, and this is indicated by a strong correlation between macrophage influx and apoptosis of resident tissues (Tesch *et al* 1999). Macrophages induce tissue damage via a number of mechanisms, which include their ability to release a variety of cytokines and free radicals such as nitric oxide, and oxygen radicals such as hydrogen peroxide, all of which have been shown to induce apoptosis and oxidative damage within target tissues (Duffield *et al* 2000, Duffield *et al* 2001, Kipari *et al* 2002, Duffield *et al* 2001). Macrophages also release TGF-β (Khalil *et al* 1996, Bissell *et al* 1995, Letterio and Roberts 1998), which is known to drive epithelial to mesenchymal transition (EMT), transforming mesothelial cells to fibroblasts, which, when uncontrolled, can lead to overproduction of extracellular matrix and tissue fibrosis (Selgas *et al* 2006). Recent work has shown that dendritic cells also express high levels of inducible nitric oxide synthase (Serbina *et al* 2003), and can induce cell death (Stary *et al* 2007). It therefore follows that dendritic cells may also contribute to tissue injury during chronic disease.

Given that both macrophages and dendritic cells have the potential to induce tissue damage, the aim of this Chapter was to determine whether recurrent or persistent inflammation caused changes in the recruitment of the defined peritoneal mononuclear subsets that have been characterised in Chapters 3-5, and if IL-6 modulated any of these outcomes.

6.2 Materials and methods

6.2.1 Recurrent induction of SES induced peritoneal inflammation

SES induced peritoneal inflammation was established in groups of WT and IL-$6^{-/-}$ mice (n=5 per group, mixture of males and females) as previously described in Section 2.3.5. However to mimic the incidence of recurrent peritonitis in humans, mice were sequentially activated with a maximum of four rounds of SES induced inflammation, which were triggered at seven day intervals. At defined intervals (0, 1, 3, 6, 12, 15, 18, 24, 48 and 72 hours) during the first and fourth episode, groups of mice were sacrificed and the peritoneal lavage sample collected. A further group of mice, that had received four sequential injections of SES as described above, were maintained until day 49 to allow complete resolution of the inflammatory response, and their peritoneal membranes were collected for histological analysis. These were compared to age matched controls, which had received no SES (see Diagram 6.1 for an overview).



Diag. 6.1: *Schematic depicting the recurrent model of peritoneal inflammation.*

6.2.2 Cytospin analysis of leukocyte infiltrate

Cytospins of peritoneal leukocytes from lavage samples were stained with Wright Giemsa stain and the composition of the inflammatory infiltrate examined by differential cell counting (Section 2.3.2).

6.2.3 Flow cytometric analysis of leukocyte infiltrate

Multi-parameter flow cytometry (Section 2.4.3) using defined surface markers (F4/80, CD11b and CD11c) was used to assess changes in $F4/80^{hi}CD11b^{hi}$ resident macrophages, $F4/80^{int}CD11b^{int}$ monocytes and $F4/80^{int}CD11b^{hi}CD11c^{+}$ DC. Cell numbers were quantified by taking the FACS gated population percentages and multiplying by the total cell number.

6.2.4 ELISA quantification of cytokine and chemokine levels in lavage supernatants

Peritoneal lavage supernatants were analysed by ELISA for CCL2, CCL5 and CCL7 as described in Section 2.5.

6.2.5 Histological analysis of peritoneal membranes

Histological evaluation of peritoneal membranes was performed on fixed and paraffin embedded sections (Section 2.3.5). Tissue sections (5-6μm) were cut and stained with haematoxylin and eosin. The thickness of the mesothelium was measured by light microscopy and QWin software. Measurements were taken in μm and an average was taken from a number of measurements across the membrane.

6.3 Results

6.3.1 Peritoneal membrane thickening as a result of repeated inflammatory episodes

Repeated peritoneal inflammatory episodes is associated with increased tissue damage and peritoneal fibrosis (Davies *et al* 1996, Williams *et al* 2002, Fielding *et al* 2009, submitted). To mimic these processes, WT and IL-$6^{-/-}$ mice were challenged with four consecutive rounds of SES induced inflammation. At day 49, the peritoneal membrane was collected and histologically analysed (Figure 6.1). Evaluation of the membrane structure revealed two distinct cellular regions consisting of an underlining area of muscle and a thin mesothelial layer. Analysis of the membrane revealed that WT mice exhibited increased thickening of the mesothelium following four episodes of SES induced peritoneal inflammation. IL-$6^{-/-}$ mice however exhibited no histological changes in the mesothelium integrity and remained comparable with non-treated WT controls.

6.3.2 Recurrent peritoneal inflammation results in increased retention of leukocytes

A chronic inflammatory state is often associated with a retention of activated mononuclear cells, which are known to affect fibrosis and the underlining tissue damage (Jones 2005, Duffield *et al* 2000, Duffield *et al* 2001, Kipari *et al* 2002, Duffield *et al* 2001, Stary *et al* 2007, Gordon 2003, Deodhar *et al* 1997). Therefore the retention of leukocytes was analysed in a recurrent inflammatory model to determine whether repeated inflammation controlled leukocyte recruitment and how IL-6 may affect the retention of leukocytes in the peritoneal cavity once the inflammatory episodes have resolved. To analyse the peritoneal leukocyte composition during a recurrent inflammatory model, WT and IL-$6^{-/-}$ mice were administered with SES and the inflammatory infiltrate profiled prior to the induction of the first and fourth round of peritoneal inflammation. As seen in Figure 6.2, these baseline measurements showed that prior inflammatory episodes gave rise to a retention of inflammatory leukocytes in WT and IL-$6^{-/-}$ mice (Figure 6.2 A). Specifically, increased numbers of monocytes, lymphocytes and neutrophils were found in the peritoneal cavity prior to the fourth episode of inflammation (Figure 6.2 B). Wild type mice exhibited a greater retention of monocytes and lymphocytes prior to the fourth inflammatory episode, compared to IL-$6^{-/-}$ mice (Figure 6.2B).

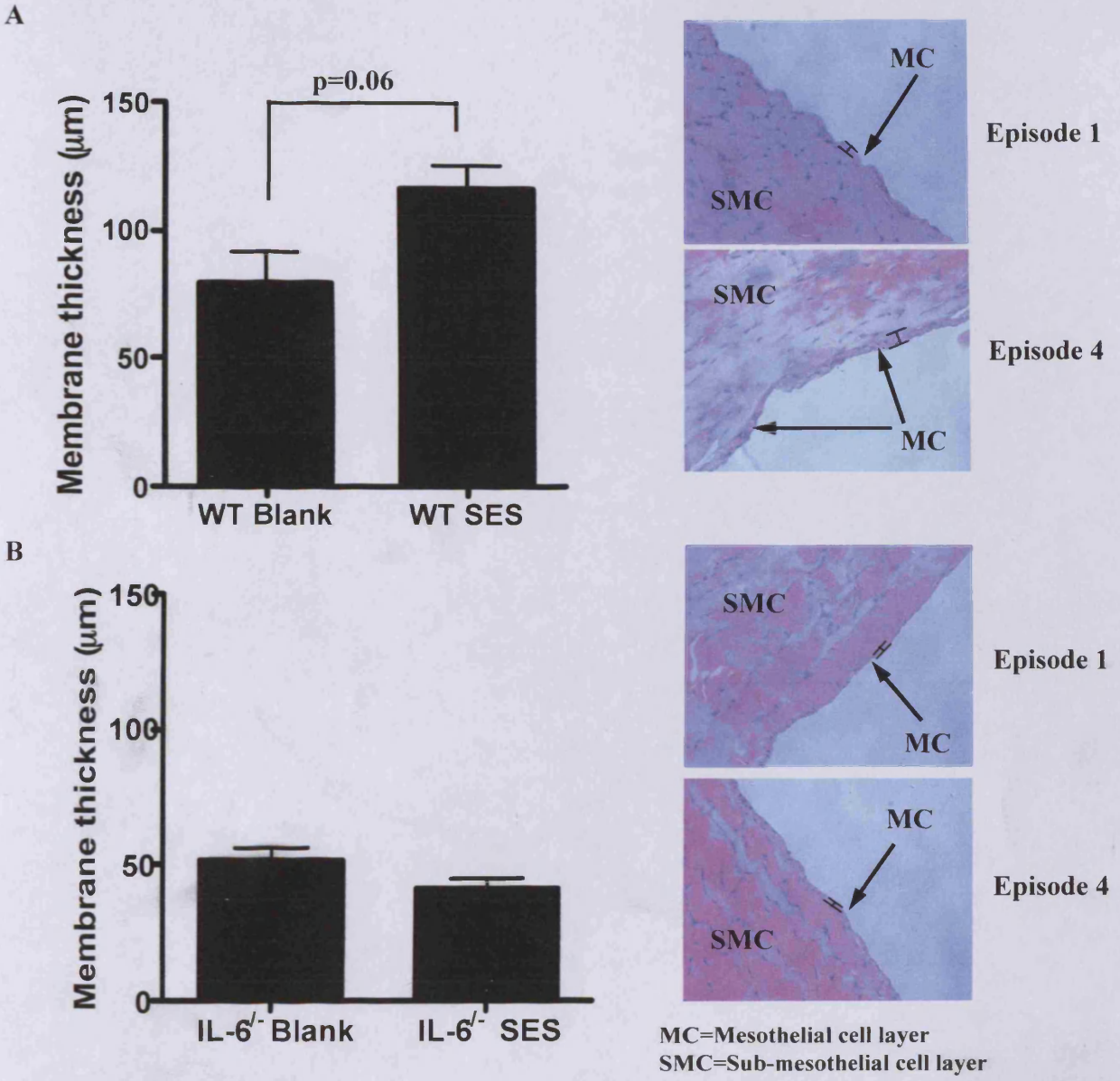


Figure 6.1: *WT but not IL-6$^{-/-}$ mice exhibit peritoneal membrane thickening following repeated inflammatory episodes.* Peritoneal inflammation was induced by i.p administration of SES. WT and IL-6$^{-/-}$ mice received four consecutive rounds of inflammation, at 7 day intervals, and were then maintained to day 49 to allow complete resolution. Peritoneal membranes were then recovered, sectioned and stained for histological analysis. Membrane thickness was observed and measured at multiple points along a 5µm membrane section by light microscopy in **(A)** WT and **(B)** IL-6$^{-/-}$ mice. Representative pictures of membranes are shown. n=5.

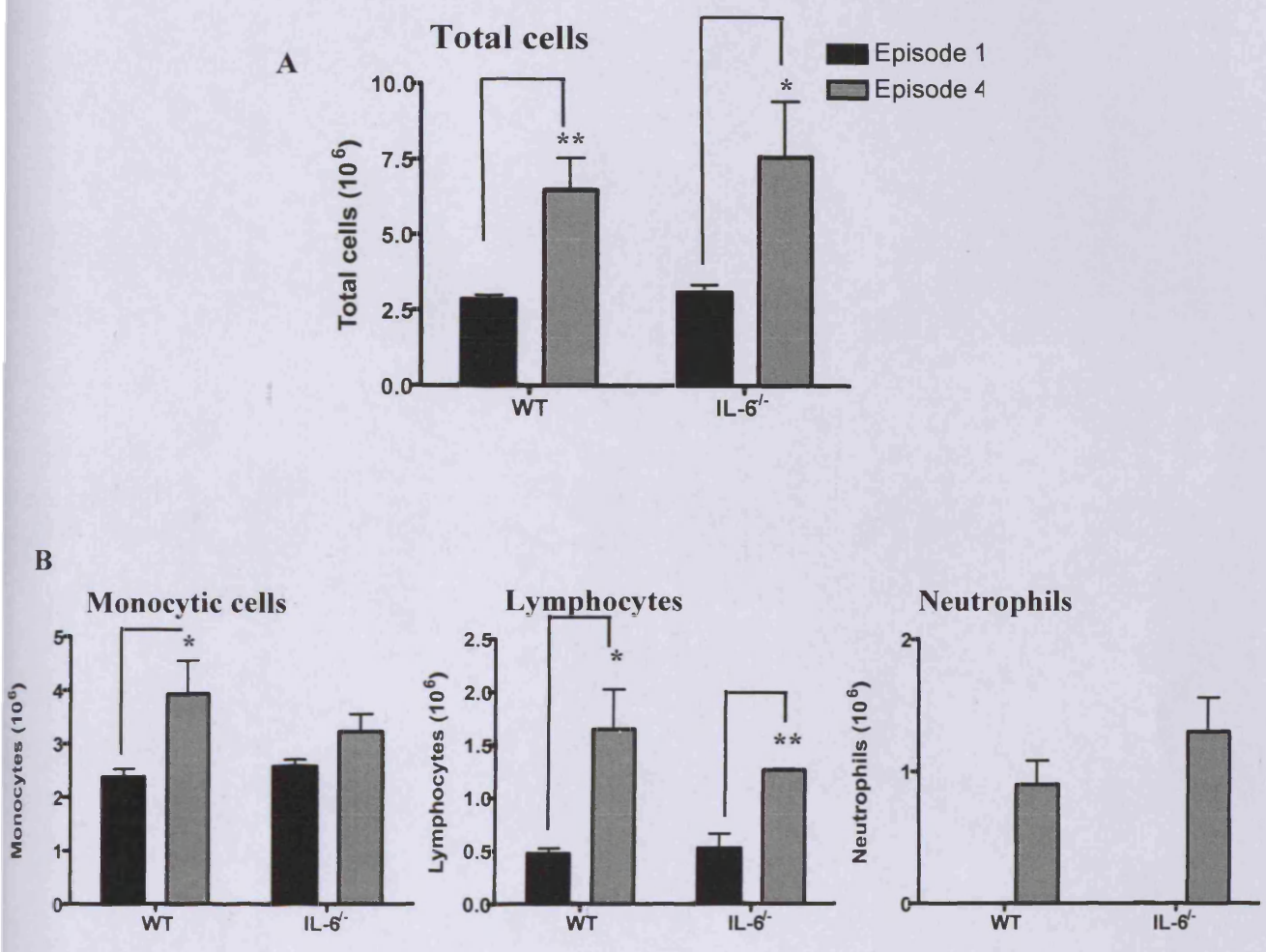


Figure 6.2: *WT and IL-6$^{-/-}$ mice displayed an increased retention of peritoneal leukocytes following repeated inflammatory episodes.*
(A) Baseline leukocyte numbers were calculated in WT and IL-6$^{-/-}$ mice prior to receiving the first and fourth SES induced inflammatory episodes. Peritoneal leukocytes were collected by lavage and counted. **(B)** Differential cell counts were performed to determine monocyte, lymphocyte and neutrophil numbers. Values represent the mean ± SEM, n=4-5 mice per time point, * $P < 0.05$, ** $P < 0.01$.

6.3.3 Recurrent inflammation results in increased leukocyte infiltration in WT mice

To determine whether IL-6 regulates the inflammatory trafficking of leukocytes in this recurrent inflammatory model, trafficking of neutrophils and mononuclear cells were monitored by differential cell counting in WT and IL-$6^{-/-}$ mice during the first and fourth inflammatory episodes (Figure 6.3).

During the fourth episode of inflammation WT mice exhibited a significantly increased recruitment of neutrophils compared to that observed during the first episode (Figure 6.3 A). This was coupled with an enhanced retention or delayed clearance of neutrophils in these mice compared to the first episode. As shown in Figure 6.3 B, WT mice also exhibited a significant increase in the recruitment of monocytic cells into the peritoneal cavity during the fourth inflammatory episode as compared to the first. This was particularly evident at 48 hours, at which point there was a 2 fold increase in the number of monocytes present compared to the first inflammatory episode. Similarly, WT mice also exhibited a significant increase (2.5 fold) in lymphocyte trafficking at 48 hours upon repeated SES challenge (Figure 6.3 C).

The leukocyte trafficking observed in IL-$6^{-/-}$ mice following repeated SES challenge showed few changes from that observed in a single inflammatory episode. Neutrophil trafficking in IL-$6^{-/-}$ mice remained unchanged from the first episode (Figure 6.3 A). This resulted in the neutrophil trafficking profiles for WT and IL-$6^{-/-}$ mice being equivalent during the fourth episode, contrasting that seen during the first episode.
Similarly, IL-$6^{-/-}$ mice did not display an enhanced trafficking of monocytic cells, and their migratory patterns remained equivalent to that seen during a single inflammatory episode (Figure 6.3 B). Finally IL-$6^{-/-}$ mice exhibited only a small increase (1.5 fold) in lymphocyte trafficking at 48 hours during the fourth inflammatory episode (Figure 6.3 C).

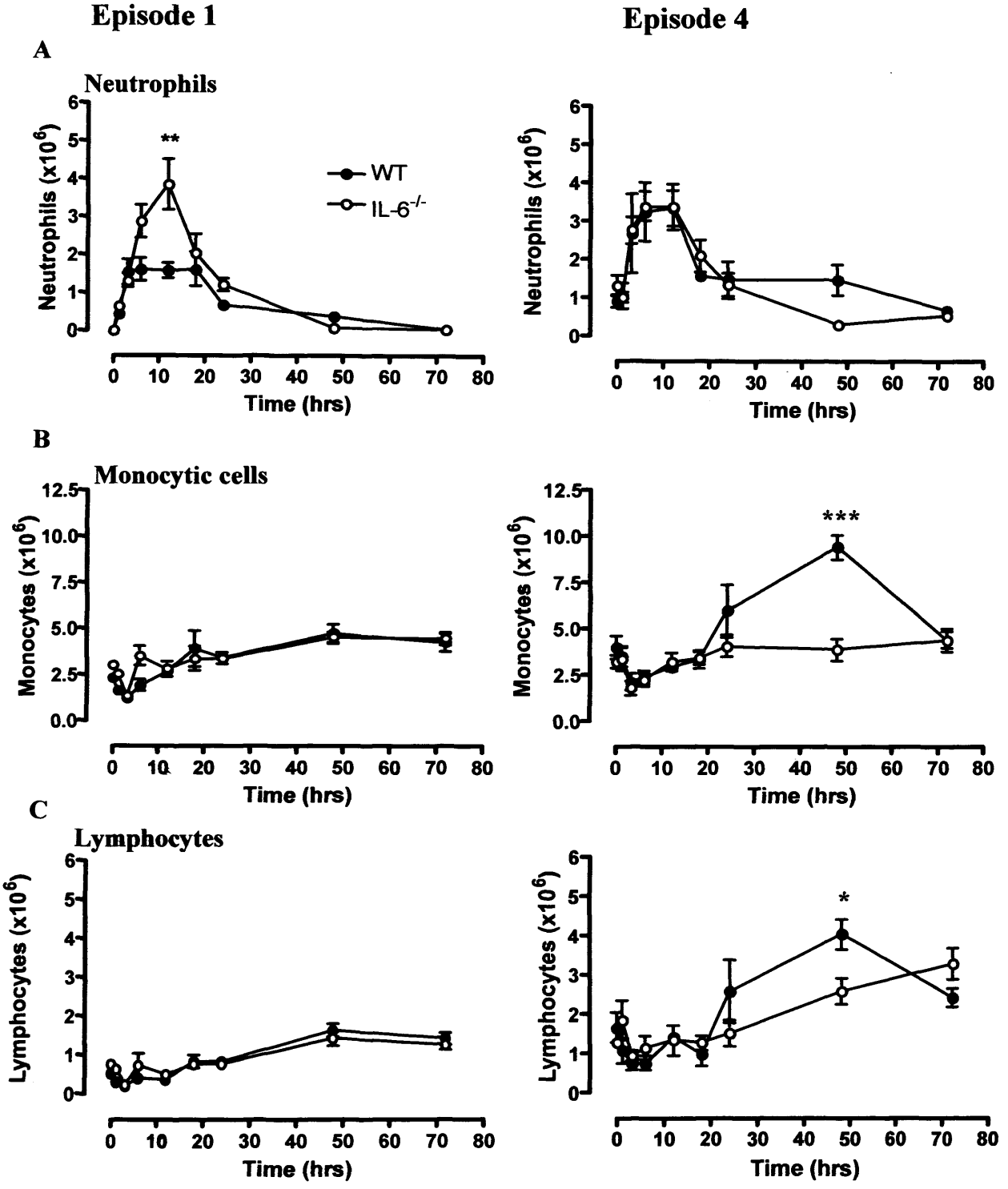


Figure 6.3: *Repeated episodes of peritoneal inflammation resulted in increased infiltration of all leukocyte types in WT mice.* Peritoneal inflammation was established by i.p injections of SES. Mice received either one or four injections (7 days apart). Peritoneal leukocytes were recovered by lavage and cytospins prepared for differential cell counts. **(A)** Neutrophil numbers determined by differential cell counting. **(B)** Monocyte numbers determined by differential cell counting. **(C)** Lymphocytes numbers determined by differential cell counting. Values represent the mean ± SEM, n=5-15 mice per time point, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

6.3.4 The effect of repeated inflammatory episodes on chemokine production

To determine if increased levels of inflammatory chemokines resulted in the increased and retained inflammatory infiltrate observed in WT mice, ELISAs were performed to measure chemokine levels in the lavage fluids (Figure 6.4).

As seen in Chapter 5, CCL2 levels were significantly impaired in IL-$6^{-/-}$ mice during the first episode of SES induced inflammation. This was not observed during the fourth episode, with WT and IL-$6^{-/-}$ mice exhibiting equivalent levels of CCL2 in the peritoneal cavity (Figure 6.4 A). CCL5 and CCL7 production in WT and IL-$6^{-/-}$ mice during the fourth inflammatory episode remained equivalent to that seen during the first episode (Figure 6.4 B&C).

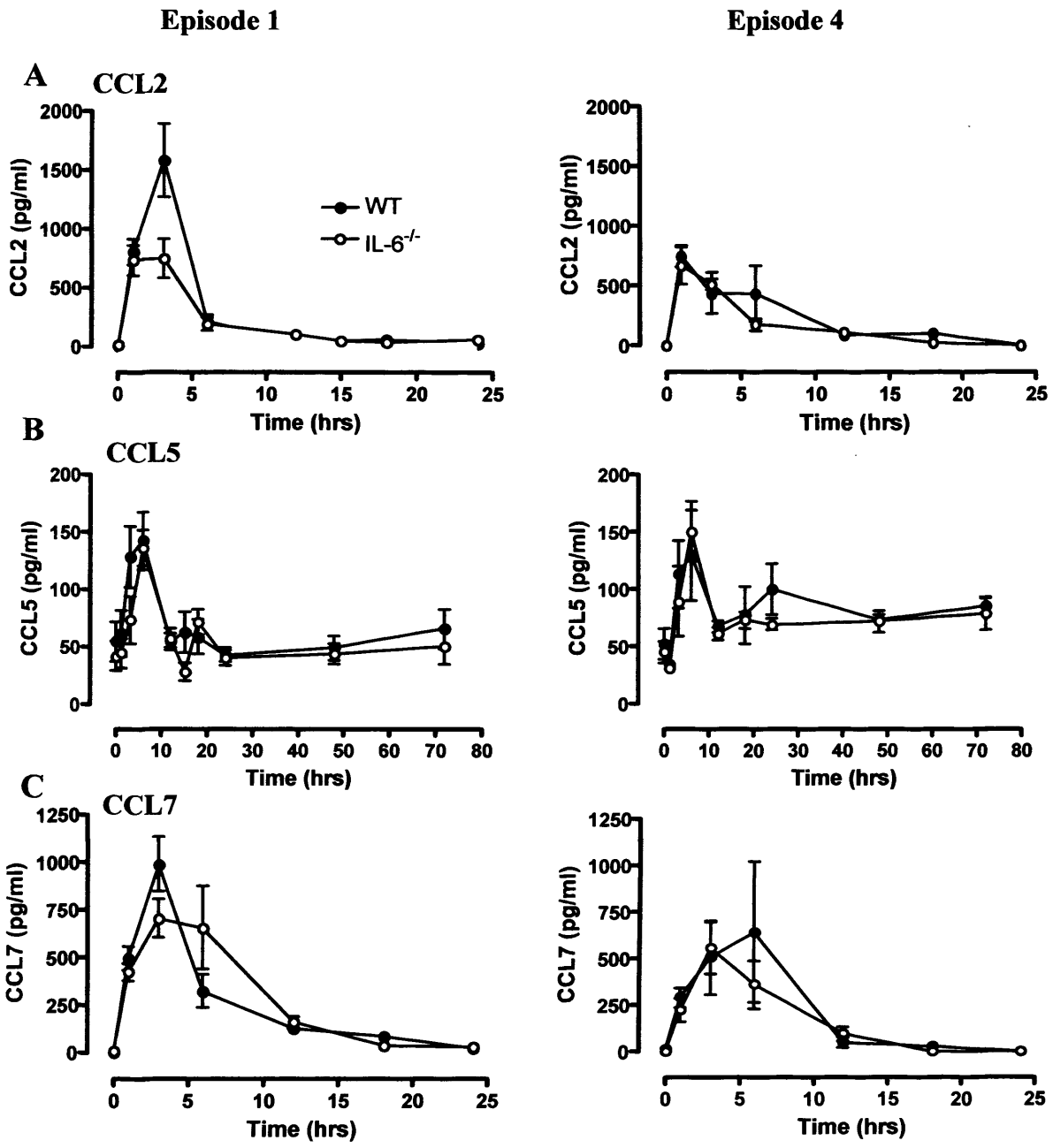


Figure 6.4: *Mononuclear chemo-attractant production in the peritoneal cavity lavage fluids in WT and IL-6$^{-/-}$ mice during the first and fourth inflammatory episodes.*
Peritoneal inflammation was established by i.p injections of SES. Mice received either one or four injections (7 days apart). Peritoneal fluids were recovered by lavage and centrifuged to remove leukocytes. Chemokines were then measured by ELISA **(A)** CCL2 production in WT and IL-6$^{-/-}$ mice during episodes 1 and 4. **(B)** CCL5 production in WT and IL-6$^{-/-}$ mice during episodes 1 and 4. **(C)** CCL7 production in WT and IL-6$^{-/-}$ mice episodes 1 and 4. Values represent the mean ± SEM, n=5-15 mice per time point.

6.3.5 Regulation of resident like macrophage trafficking during a recurrent inflammatory model

As previously discussed, macrophages have the potential to contribute to tissue injury during chronic inflammation (Duffield *et al* 2000, Duffield *et al* 2001, Kipari *et al* 2002, Duffield *et al* 2001), as well as their potential to drive EMT by their production of TGFβ (Khalil *et al* 1996, Bissell *et al* 1995, Letterio and Roberts 1998, Selgas *et al* 2006). Since WT mice exhibit membrane fibrosis upon repeated episodes of inflammation, while the IL-$6^{-/-}$ mice remain protected, resident macrophage trafficking was analysed in these mice to assess whether IL-6 plays a role in the trafficking/retention of this subset during a recurrent inflammatory model. To do this, the leukocyte infiltrate was further analysed at each time point by multi-parameter flow cytometry to identify changes in the trafficking of specific monocytic cell subsets as a consequence of repeated inflammatory episodes. The trafficking of resident like macrophages, identified as $F4/80^{hi}$ $CD11b^{hi}$, was monitored and the number of these cells present in the peritoneal cavity of WT and IL-$6^{-/-}$ mice during the first and fourth inflammatory episodes was plotted at each time point (Figure 6.5 A&B). Resident-like macrophages were retained in the peritoneal cavity of WT mice prior to induction of the fourth inflammatory episode, exhibited by a 2.3 fold increase in the number of peritoneal resident-macrophages in the base line (0 hr) group. During both the first and fourth inflammatory episodes, the resident like macrophages exhibited a defined trafficking pattern characterised by an initial clearance followed by a subsequent re-emergence of this population at the later time points within the inflammatory response. The rate of clearance (WT: $9x10^5$ cells/hr, IL-$6^{-/-}$:$1.4x10^6$ cells/hr) and re-emergence (WT: $1.9x10^4$ cells/hr, IL-$6^{-/-}$: $2.9x10^4$ cells/hr) of the macrophage population was significantly accelerated during the fourth inflammatory episode as compared to the first (clearance WT: $3.1x10^5$ cells/hr, IL-$6^{-/-}$: $3x10^5$ cells/hr, re-emergence WT: $1.7x10^4$ cells/hr, IL-$6^{-/-}$: $1.7x10^4$ cells/hr). Interestingly the emerging macrophage population re-established itself to numbers equivalent to those seen at the beginning of the inflammatory episode, following both single and repeated SES challenge. As seen during the first episode, there were no differences observed between WT and IL-$6^{-/-}$ mice (Figure 6.5).

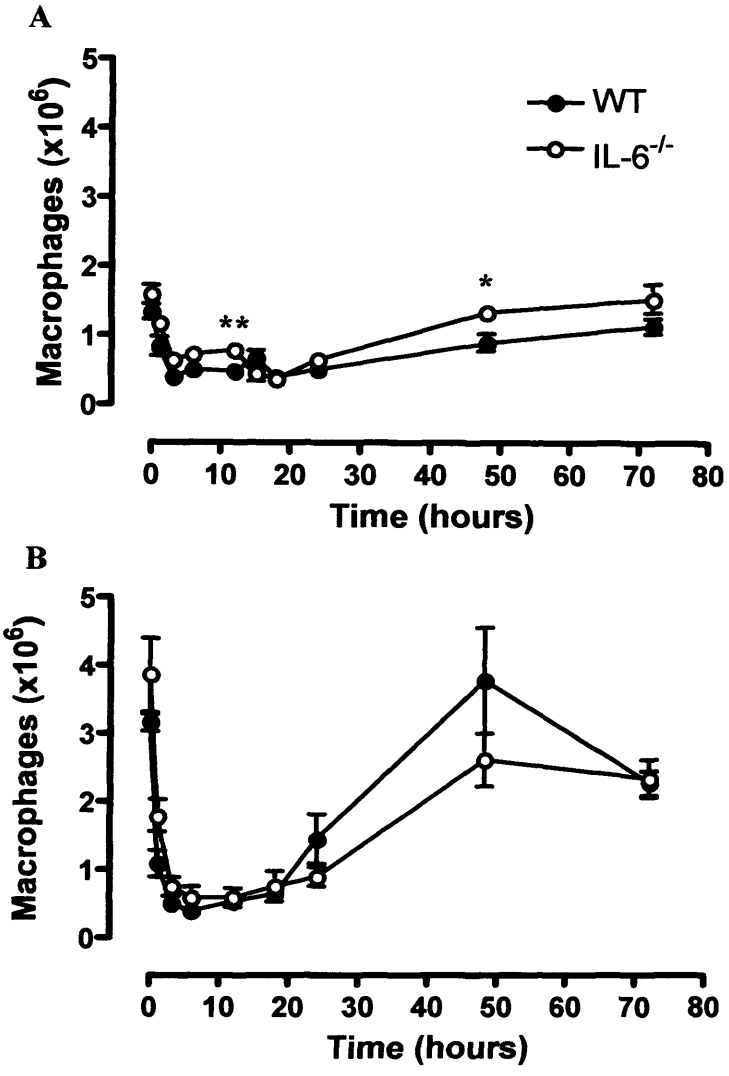


Figure 6.5: *IL-6 does not regulate resident like macrophage trafficking in a single inflammatory episode or a recurrent inflammatory state.* Peritoneal inflammation was established by i.p injection of SES. Groups of mice received either one **(A)** or four **(B)** sequential inflammatory episodes. Peritoneal leukocytes were collected by lavage and labelled with F4/80 and CD11b. Resident macrophage were identified based on their phenotype F4/80^{hi}CD11b ^{hi}, and their number plotted at each time point by taking the flow cytometry gated percentage and multiplying by the total cell number. Values represent the mean ± SEM, n=5-15 mice per time point, * $P < 0.05$, ** $P < 0.01$.

6.3.6 Regulation of infiltrating monocyte cell trafficking during a recurrent inflammatory model

Peritoneal infiltration of F4/80^{int} CD11b^{int} monocytes was monitored in WT and IL-6$^{-/-}$ by flow cytometry following a single and repeated SES activation (Figure 6.6 A&B).

Monocyte recruitment into the peritoneal cavity began one hour after the induction of the fourth inflammatory episode, compared to 3 hours as was observed during the first episode. Monocytes continued to infiltrate the cavity, reaching a peak at 18 hours, followed by a subsequent clearance of this population. WT mice exhibited an enhanced magnitude of infiltration during the fourth inflammatory episode, which was particularly evident at 48 hours when the number of monocytes present was 2.8 fold higher than that observed during the first episode. Subsequently the clearance of these cells was faster ($1.6x10^5$ cell/hr in the fourth episode compared to $3.3x10^4$ cells/hr in the first episode).

This confirmed data obtained from differential cell counting for this group, which highlighted an enhanced monocyte infiltrate in WT mice.

The absence of IL-6 did not alter the recruitment or infiltration of monocytes, with IL-6$^{-/-}$ mice exhibiting an equivalent pattern and magnitude of infiltrating monocyte trafficking during the fourth episode to that seen in the first episode.

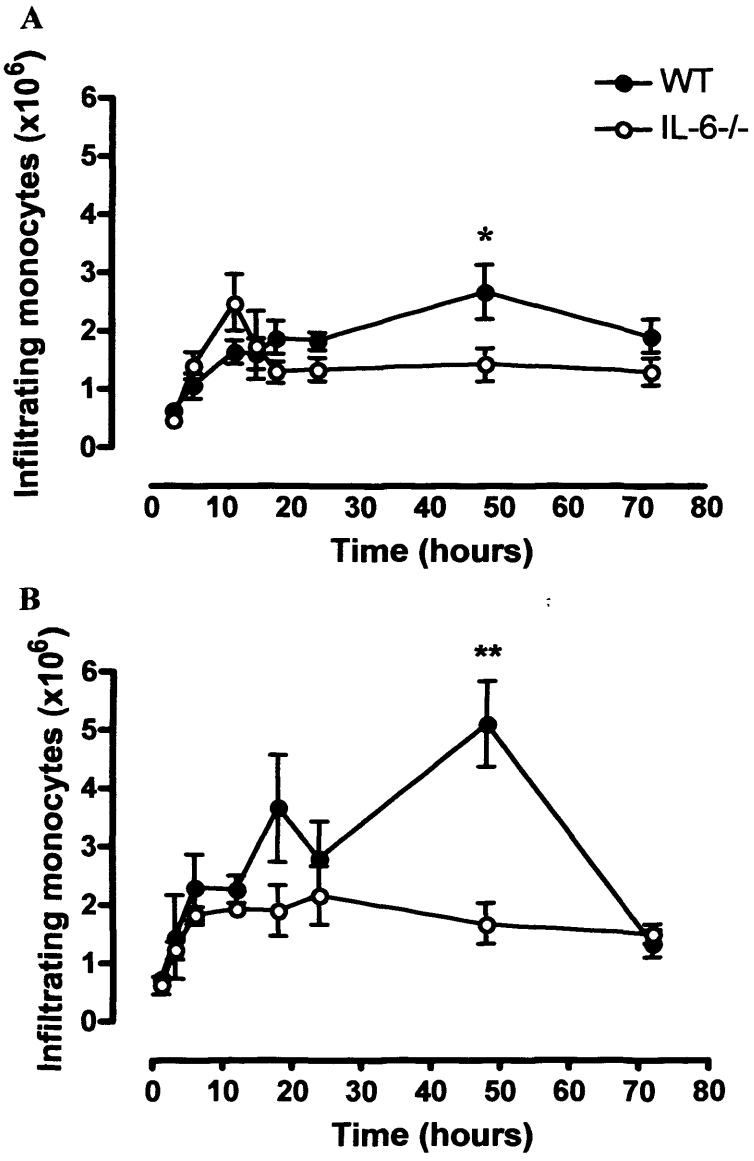


Figure 6.6: *IL-6 regulates infiltrating monocyte trafficking in a single inflammatory episode and a recurrent inflammatory state.* Peritoneal inflammation was established by i.p injection of SES. Groups of mice received either one **(A)** or four **(B)** sequential inflammatory episodes. Peritoneal leukocytes were collected by lavage and labelled with anti F4/80 and anti CD11b. Infiltrating monocytes were identified based on their phenotype F4/80^{int}CD11b^{int} and their number plotted at each time point by taking the flow cytometry gated percentage and multiplying by the total cell number. Values represent the mean ± SEM, n=5-15 mice per time point, * $P < 0.05$, ** $P < 0.01$.

6.3.7.1 Regulation of dendritic-like cell trafficking during a recurrent inflammatory model

Reports have suggested that dendritic cells have the capacity to contribute to tissue damage based on their ability to express high levels of inducible nitric oxide synthase (Serbina *et al* 2003), and to induce cell death (Stary *et al* 2007). In this respect, the inflammatory trafficking of DC was monitored in a single and recurrent inflammatory model to determine if regulation by IL-6 contributed to the retention and recruitment of these cells, and subsequent tissue damage observed in this model. The trafficking of dendritic cells, defined as $F4/80^{int}CD11b^{hi}CD11c^{+}$, were monitored by flow cytometry and the number of these cells present in the peritoneal cavity of WT and IL-$6^{-/-}$ mice was plotted at each time point during the first and fourth inflammatory episodes (Figure 6.7 A&B).

Dendritic-like cells were retained in the peritoneal cavity of WT mice prior to induction of the fourth inflammatory episode, exhibited by a 2.3 fold increase in the number of peritoneal resident DC in the base line (0 hr) group (Figure 6.7 B). Following the induction of the fourth inflammatory episode, the DC population exhibited a brief clearance, followed by a recruitment of cells, which were retained for the remainder of the time course. The magnitude of infiltrating DC in the fourth episode was significantly enhanced in WT mice, with 2.5 fold more DC-like cells being present in the peritoneal cavity at 48 hours.

As was observed during the first inflammatory episode (Figure 6.7 A), IL-$6^{-/-}$ mice exhibited an enhanced infiltration of DC-like cells compared to the WT mice during the fourth episode, and this was particularly evident at 6 and 12 hours. The number of DC-like cells present in the peritoneal cavity of IL-$6^{-/-}$ mice at these time points however, was equivalent to the number present at the same time points during the first inflammatory episode indicating that their infiltration was not enhanced as a result of repeated SES activation. In this respect, these IL-$6^{-/-}$ mice also did not exhibit the enhanced infiltration of DC at 48 hours that was observed in WT mice.

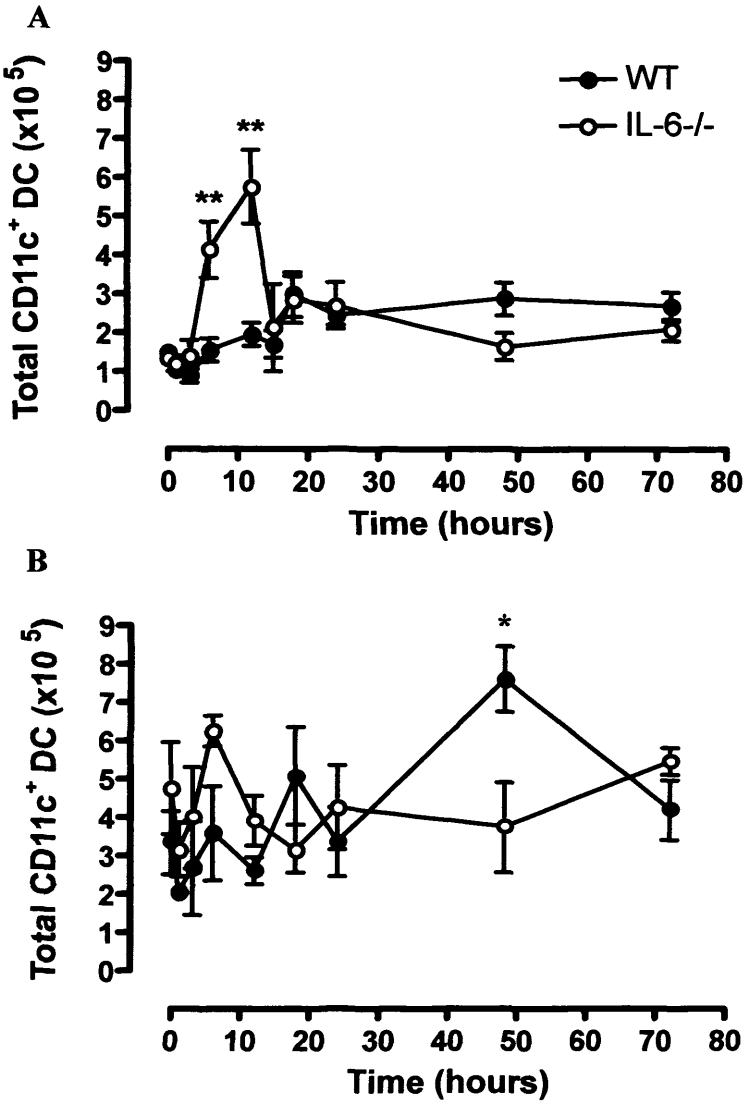


Figure 6.7: *IL-6 regulates DC trafficking in a single inflammatory episode and a recurrent inflammatory state.* Peritoneal inflammation was established by i.p injection of SES. Groups of mice received either one **(A)** or four **(B)** sequential inflammatory episodes. Peritoneal leukocytes were collected by lavage and labelled with anti F4/80, CD11b and CD11c. Total DC were identified based on their phenotype F4/80^{int}CD11b^{hi}CD11c^{+} and their number plotted at each time point by taking the flow cytometry gated percentage and multiplying by the total cell number. Values represent the mean ± SEM, n=5-15 mice per time point,
** $P < 0.01$.

6.3.7.2 Regulation of resident- and monocyte-like DC trafficking during a recurrent inflammatory model

Prior investigation outlined in Section 5.3.4 revealed that the inflammatory DC population was comprised of two phenotypically distinct $F4/80^{lo}CD11b^{hi}CD11c^{+}$ subsets, being distinguishable based on low (resident-like) and high (monocyte-like) forward and side scatter properties. The number of these cells present in the peritoneal cavity of WT and IL-$6^{-/-}$ mice was therefore plotted at each time point following induction of a single and recurrent inflammatory episodes (Figure 6.8 and 6.9).

Resident-like DC were retained in the peritoneal cavity of mice prior to induction of the fourth inflammatory episode, exhibited by a 1.8 fold increase in the number of peritoneal resident-like DC in the base line (0 hr) group (Figure 6.8 B). During the fourth inflammatory episode, the trafficking of these cells followed a similar pattern to that exhibited during the first episode, characterised by an initial clearance followed by a recruitment of this population at the later time points. The infiltration of this subset in WT mice was significantly enhanced when compared to that observed during a single inflammatory episode, with a 2.5 fold increase in the number of these cells present at 48 hours.

Monocyte-like DC were also retained in the peritoneal cavity of mice prior to induction of the fourth inflammatory episode, exhibited by a 4 fold increase in the number of peritoneal monocyte-like DC in the base line (0 hr) group (Figure 6.9 B). During the fourth inflammatory episode, the trafficking of these cells was equivalent to that observed during the first episode, characterised by a temporal increase in the number of these cells, followed by their clearance during the later time points. The infiltration of the monocyte-like DC population peaked at 6 hours during the fourth episode, 6 hours earlier than that seen during the first episode. As was observed during the first episode, IL-$6^{-/-}$ mice exhibited an increased recruitment of monocyte-like DC compared to the WT mice. Interestingly however, the number of recruited monocyte-like DC during the fourth episode was no higher than the number recruited during one inflammatory episode indicating that their infiltration was not enhanced as a result of repeated SES activation.

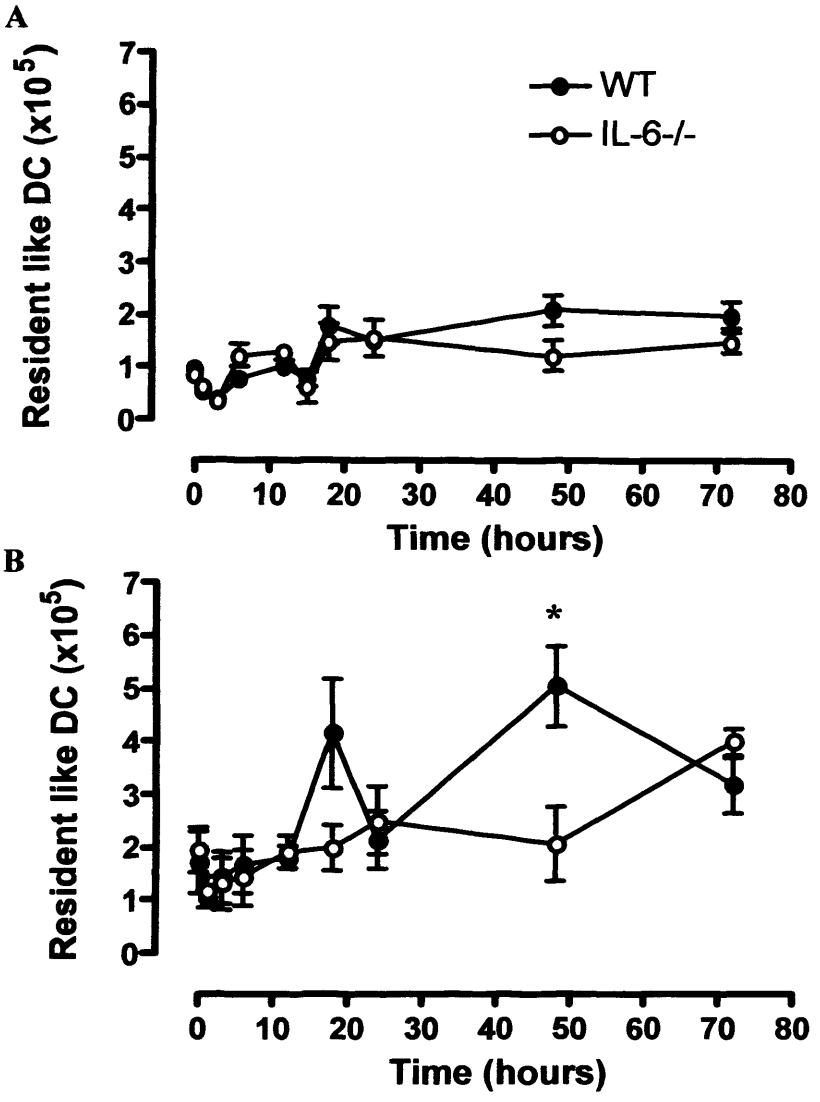


Figure 6.8: *IL-6 does not regulate resident like DC trafficking in a single inflammatory episode but does during a recurrent inflammatory state.* Peritoneal inflammation was established by i.p injection of SES. Groups of mice received either one **(A)** or four **(B)** sequential inflammatory episodes. Peritoneal leukocytes were collected by lavage and labelled with F4/80, CD11b and CD11c. Resident like DC were identified based on their phenotype F4/80^{lo}CD11b^{hi}CD11c^{+} and their low forward and side scatter properties, and their number plotted at each time point by taking the flow cytometry gated percentage and multiplying by the total cell number. Values represent the mean ± SEM, n=5-15 mice per time point, * $P < 0.05$.

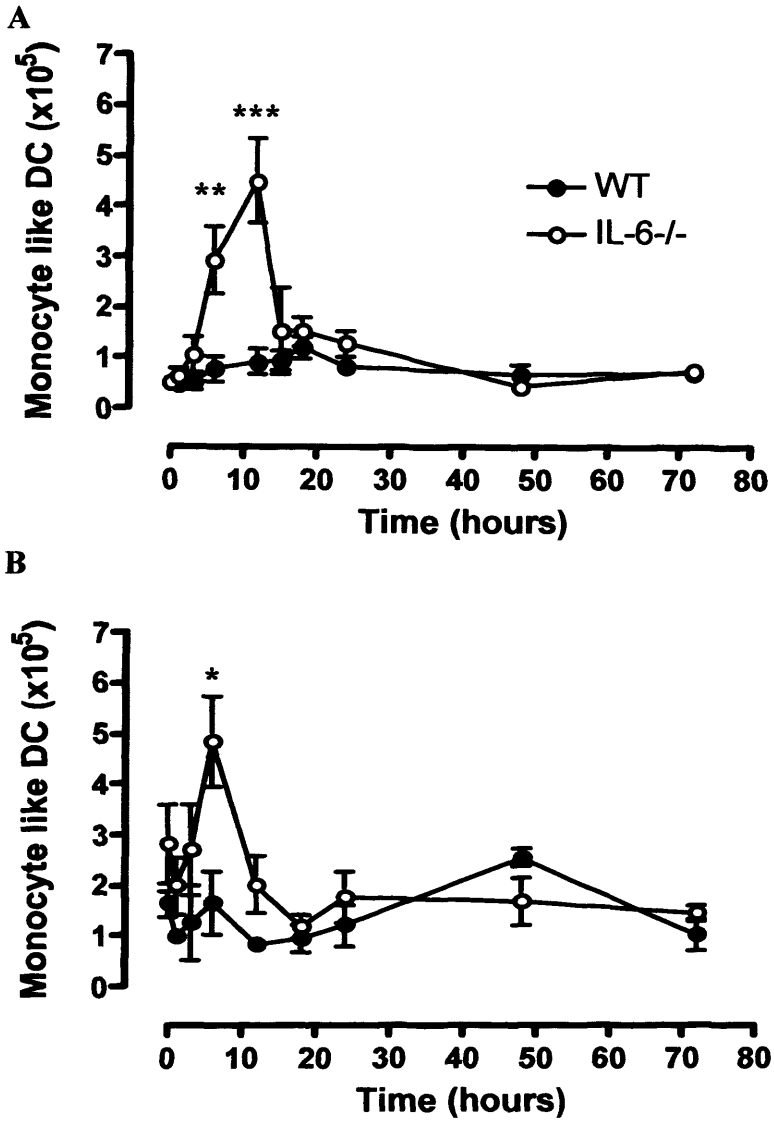


Figure 6.9: *IL-6 regulates monocyte like DC trafficking in a single inflammatory episode and a recurrent inflammatory state.* Peritoneal inflammation was established by i.p injection of SES. Groups of mice received either one **(A)** or four **(B)** sequential inflammatory episodes. Peritoneal leukocytes were collected by lavage and labelled with F4/80, CD11b and CD11c. Monocyte like DC were identified based on their phenotype $F4/80^{lo}CD11b^{hi}CD11c^{+}$ and their high forward and side scatter properties, and their number plotted at each time point by taking the flow cytometry gated percentage and multiplying by the total cell number. Values represent the mean ± SEM, n=5-15 mice per time point, * $P < 0.05$ ** $P < 0.01$, *** $P < 0.001$.

6.4 Discussion

Studies described here were performed to determine whether repeated activation altered mononuclear cell trafficking and recruitment during peritoneal inflammation, and whether IL-6 modulated these outcomes. The rationale for doing this was taken from previous experiments that showed IL-$6^{-/-}$ mice to be protected against peritonitis associated membrane thickening or fibrosis (Fielding *et al* 2009, submitted). In these studies, IL-$6^{-/-}$ mice displayed resistance to membrane fibrosis upon recurrent inflammation, and exhibited no increase in inflammatory leukocyte trafficking characteristic of a chronic inflammatory state (Fielding *et al* 2009, submitted). This was consistent with findings by other groups showing that IL-6 deficient mice are resistant to the induction of other chronic inflammatory diseases including experimental encephalitis, Crohn's disease, colitis, lupus nephritis and rheumatoid arthritis (Galle *et al* 2007, Atreya, R. *et al* 2000, Nowell, M.A. *et al* 2003, Mihara, M. *et al* 1998). It has been documented that monocytic cell subsets such as macrophages and dendritic cells are involved in facilitating inflammation induced tissue injury through their release of cytokines and the generation of free radicals such as nitric oxide, and reactive oxygen species (Duffield *et al* 2000, Duffield *et al* 2001, Kipari *et al* 2002, Duffield *et al* 2001). Macrophages are also involved in tissue repair and are able to promote fibrosis and the deposition of extracellular matrix (Deodhar *et al* 1997, Gordon 2003), which could implicate them in contributing to the induction of uncontrolled fibrotic processes when present at large numbers. Therefore the aim of this Chapter was to determine a potential role for IL-6 in directing monocytic cell trafficking, and to correlate this to the onset of peritoneal fibrosis. These studies were performed using a recurrent model of SES induced inflammation, and the leukocyte infiltrates were examined both by differential cell counting and multiparameter flow cytometry.

Upon repeat inflammatory challenge, both WT and IL-$6^{-/-}$ mice exhibited a retention of the resident like macrophage and DC populations within the peritoneal cavity. This is potentially important, given that the retention of monocytic cells has previously been associated with a chronic disease state (Jones 2005). Wild type mice also exhibited an increased trafficking of inflammatory leukocytes into the peritoneal cavity upon recurrent

SES challenge, with the infiltrate being comprised of monocytes, lymphocytes and neutrophils. Most noticeable was the increased infiltration of the mononuclear cells seen in WT mice at the later time points, which consisted of infiltrating monocytes and lymphocytes. This increased and retained inflammatory infiltrate is highly characteristic of a chronic inflammatory state (Nowell *et al* 2003, 2009, Liote *et al* 1996). The IL-$6^{-/-}$ mice however, did not exhibit quite the same increased inflammatory leukocyte trafficking following SES induced peritonitis. This phenomenon is in part attributed to IL-6 signalling which is known to prevent T cell apoptosis (Atreya *et al* 2000, Teague *et al* 1997) and therefore may contribute to their retention within the peritoneal cavity. IL-6 also directs mononuclear cell trafficking via the induction of CCL2 and CCL5, and this is likely to direct the increased leukocyte recruitment and retention during chronic inflammatory conditions (Hurst *et al* 2001, Nowell 2003, McLoughlin *et al* 2005). The absence of these IL-6 directed events may explain why the altered trafficking profile was not so significant in IL-$6^{-/-}$ mice following repeated SES activation. Interestingly, the studies described here showed that repeated rounds of peritoneal inflammation did not alter the magnitude of chemokine production in either WT or IL-$6^{-/-}$ mice. The mechanism by which increased leukocyte trafficking into the peritoneal cavity occurs in a repeat inflammatory model is therefore unknown.

The profile of resident-like macrophage trafficking during the fourth inflammatory episode was unchanged from the first, in that there was an initial clearance of the population, followed by a re-emergence later on in the time course. As previously discussed, this may be a result of trafficking of these cells to the lymphatic system following activation, and being replenished from the infiltrating monocyte pool, or due to increased adherence to the peritoneal membrane. Despite there being no change to the trafficking profile of these cells, there was a noticeable retention of the resident macrophage population, in both WT and IL-$6^{-/-}$ mice indicated by an increased presence of these cells at the beginning and end of the fourth inflammatory episode. This retention of macrophages in the peritoneal cavity could potentially contribute to the associated tissue damage observed in the recurrent inflammatory model, however this is unlikely given that IL-$6^{-/-}$ mice exhibited the same retention of these cells. Alternatively, the

absence of IL-6 may alter or attenuate the activity of these resident macrophages, resulting in reduced tissue injury inflicted by these cells.

The studies outlined in Section 6.3.6 showed that the recruitment of infiltrating monocytes was significantly increased in WT but not IL-$6^{-/-}$ mice, which exhibited normal monocyte trafficking into the peritoneal cavity. An enhanced monocytic cell recruitment is commonly associated with inflammatory disorders, an example being the accumulation of monocytes/macrophages in lungs during influenza infection, which has been associated with the development of lung injury (Lin *et al* 2007), and in other chronic inflammatory models such as rheumatoid arthritis where blood monocytes exhibit increased adhesion and trafficking into the synovium (Liote *et al* 1996). Infiltrating monocytes are considered to differentiate into inflammatory macrophages or DC (Randolph *et al* 1998, Gordon and Taylor 2005, Geissman *et al* 2003), and the increased presence of these cell types could potentially contribute to tissue injury and/or the promotion of tissue fibrosis in the peritoneal cavity. Given that approximately 50% of resident peritoneal cells are macrophages, and this population is rapidly cleared upon induction of the inflammatory response, it is reasonable to assume that infiltrating monocytes are recruited to provide a pool of cells from which the macrophage population can be replenished. This can however induce detrimental side effects in WT mice, with the increased monocyte infiltrate having the potential to contribute to tissue injury, especially since recruited macrophages are ascribed a greater destructive potential than resident macrophages (Gordon 1995). Monocyte/macrophages induce tissue damage via a number of mechanisms, which include their release of cytokines and generation of nitric oxide, and reactive oxygen species, which have been shown to induce apoptosis (Duffield *et al* 2000, Duffield *et al* 2001, Kipari *et al* 2002, Duffield *et al* 2001). Macrophages are also involved in tissue repair, being able to promote fibrosis and the deposition of extracellular matrix (Deodhar *et al* 1997, Gordon 2003), which could implicate them in contributing to the induction of uncontrolled fibrotic processes if large populations of these cells are retained for long periods. In this regard an increased presence of macrophages may result in an up-regulation of extracellular matrix deposition and fibrosis, resulting in the membrane thickening observed in WT mice. The

absence of an enhanced recruitment of these inflammatory cells in IL-$6^{-/-}$ mice provides one potential mechanism for their protection against chronic disease.

Dendritic cells are also thought to contribute to tissue damage during chronic inflammation due to their ability to express high levels of inducible nitric oxide synthase (Serbina *et al* 2003), and induce cell death (Stary *et al* 2007). Analysis of the peritoneal DC subsets by flow cytometry confirmed the retention and heightened trafficking of these cells, demonstrated by the increased numbers of DC present in the peritoneal cavity of WT and IL-$6^{-/-}$ mice during the fourth inflammatory episode compared to the first. It would be reasonable to speculate that these may contribute to tissue damage in these animals as a result of repeated inflammation. However, it was observed that IL-$6^{-/-}$ mice exhibited an enhanced increase in the number of DC in the peritoneal cavity following both a single and repeated rounds of inflammation, in particular the monocyte like DC. This indicates that the presence of DC does not significantly contribute to chronic inflammation associated tissue damage, since IL-$6^{-/-}$ mice have widely been reported to have a protected phenotype and do not incur the characteristic fibrosis that is seen in WT mice in this model. This leads onto further explanations that could be considered in the forms of IL-6 and its regulation of T helper cell differentiation and T regulatory (T-reg) cell activity. It has been documented that IL-6 is critical for $CD4^+$ T cell memory responses to influenza virus, and the suppression of virus specific $CD4^+$ $CD25^+$ T-regs (Longhi *et al* 2008). It has also been shown that IL-6 production by DC in response to TLR ligation during infection is critical for T cell activation as it allows pathogen specific T cells to overcome suppression by $CD4^+$ $CD25^+$ T-regs (Pasare *et al* 2003). Finally, IL-6 is required for the commitment of IL-17 secreting T helper cells (Bettelli *et al* 2006, Veldhoen *et al* 2006). This would provide a mechanism for the resistance of IL-$6^{-/-}$ mice to autoimmunity and their susceptibility to infection, in that the failure to overcome T-reg mediated suppression due to the absence of IL-6 may contribute to their protected phenotype, by dampening the immune response and inhibiting effector T cell responses and memory recall. This would directly apply to the studies described in this Chapter where IL-$6^{-/-}$ mice show protection from fibrosis. The inability of DC to overcome T-reg mediated suppression in these mice would result in the inflammatory response being

much more tightly controlled, and potentially result in limiting the recruitment of tissue damage causing immune cells to the peritoneal cavity.

The significance of the monocyte like DC present in IL-$6^{-/-}$ mice during inflammation has yet to be determined, and do not appear to contribute to chronic disease pathology given that these mice are protected. One consideration is that they may be contributing to the protected phenotype of these animals, possibly through their regulation of T-reg activity, and the next chapter focused to investigate their phenotype and function, to further determine their significance.

CHAPTER 7

An *in vivo* characterisation of the F4/80^{lo}CD11b^{hi}CD11c^{+} monocyte-like dendritic cell population unique to IL-6 deficient mice, and a study of the regulation of its inflammatory trafficking

7.1 Introduction

Dendritic cells (DC) within peripheral tissues are functionally involved in antigen capture, processing and presentation to T cells (Novak et al 2008). In this respect, activated DC are involved in the initiation and modulation of an inflammatory response and direct both the innate and adaptive immune response to infection. DC are also important for the maintenance of immune tolerance to self antigens (immune homeostasis) (Wu and Liu 2007).

DC are heterogeneous and although they are all capable of antigen uptake, processing and presentation to naïve T cells, DC subtypes differ in location, migratory capacity, immunological function, and dependence on infectious/inflammatory stimuli for their generation (Shortman and Naik 2007, Wu and Liu 2007). There are thought to be three subclasses of DC, which include the Pre-DC, Conventional DC and Inflammatory DC (Shortman and Naik 2007). Pre-DC develop into functional DC, sometimes upon inflammatory stimuli and an example includes the interferon producing plasmacytoid DC. Conventional DC (cDC) can be divided into two categories: (1) Migratory DC act as sentinels in peripheral tissue and migrate to the lymph nodes through the lymphatics bearing antigens from the periphery where they present to T cells. Examples include Langerhans cells and dermal DC; (2) Lymphoid tissue resident DC life and function are restricted to one lymphoid organ where they collect and present foreign and self antigens. Examples include thymic cDC and splenic cDC (Shortman and Naik 2007). The final subclass of DC is the Inflammatory DC, which are not normally present in the steady state but appear as a consequence of inflammation or microbial stimuli. An example is the tumour necrosis factor and inducible nitric-oxide synthase producing DC (Tip DC) (Serbina *et al* 2003). Inflammatory monocytes also have the capacity to generate Inflammatory DC (Geissman *et al* 2003, Sunderkotter *et al* 2004, Gordon and Taylor 2005, Shortman and Naik 2007).

Monocyte-derived inflammatory DC originate from the "inflammatory" $CX_3CR1^{lo}CCR2^{+}Gr1^{+}$ mouse monocyte subset, which are recruited as a consequence of inflammation. They make no contribution to the proportion of homeostatic/steady state

"resident" DC in naive mice (Geissman *et al* 2003, Sunderkotter *et al* 2004, Gordon and Taylor 2005). When $Gr1^+$ "inflammatory" monocytes are transferred into mice subjected to a vigorous inflammatory response, they differentiate into DC in the peritoneum and enter the spleen to mature into a distinctive DC population (Geissman *et al* 2003). This process is dependent on GM-CSF, with GM-CSF receptor deficiency resulting in migration of this cell type to the spleen but failure to develop MHC class II^+ DC (Shortman and Naik 2007). Monocyte-derived DC can be distinguished from steady state splenic DC by their intermediate rather than high expression of CD11c, and by their expression of CD11b and MAC3 (a glycoprotein found on activated macrophages). These cells also characteristically lack expression of CD4 and CD8. Monocyte-derived DC generated in culture also express low MHCII levels (Agger *et al* 2000, Leon *et al* 2004). These inflammatory DC resemble Tip DC previously found in mice, which are functionally observed during infection (e.g. *L. monocytogenes*) and autoimmunity (Serbina *et al* 2003).

The generation of monocyte-derived DC was demonstrated *in vitro* using a reverse trans-endothelial migration model, designed to mimic the process of differentiation of monocytes into DC following extravasation into the target tissue. This showed that the differentiation of DC from monocytes is associated with the trans-migration of this inflammatory population (Randolph *et al* 1998). Following trans-migration, monocyte-derived DC are formed in the immature state, and their maturation is induced by immuno-modulators such as LPS, TNFα, IFN-γ or CD40L (Ardavin *et al* 2001). These induce the up-regulation of MHCII, and co-stimulatory molecules as well as causing dramatic changes in antigen processing, presentation ability, migration behaviour and capacity to induce and modulate T cell, NK cell and B cell responses (Leon *et al* 2005). Interestingly, it has been shown that the immuno-modulators that induce the maturation of immature monocyte-derived DC, actually block their initial differentiation from monocytes into DC, and promote macrophage differentiation instead (Rotta *et al* 2003, Palucka *et al* 1999). Consequently, inflammatory processes may impact not only on the recruitment of monocytic cells, but also affect their subsequent activation, maturation and local differentiation.

Having identified a novel population of monocyte-like dendritic cells that are unique to IL-$6^{-/-}$ mice during inflammation, more experiments were needed to characterise their phenotype and biological significance in an inflammatory setting. The aim of this Chapter was therefore to further characterise this novel population, and determine how IL-6 may contribute to its functional capacity.

7.2 Materials and methods

7.2.1 Generation of SES induced peritoneal inflammation

Lyophilised SES was prepared as described in Section 2.3.3 and reconstituted in 750μl sterile PBS. Peritoneal inflammation was induced by the injection of 500μl of SES, and peritoneal leukocytes were collected by lavage at 12 hours following injection, as described in Section 2.3.2. To study the regulation of the $CD11b^{hi}CD11c^{+}F4/80^{lo}$ monocyte-like DC, recombinant mouse IL-6 (R&D systems, UK), human hyper-IL-6 (From Stefan Rose-John, Christian-Albrechts, University of Kiel, Kiel, Germany) or anti IL-6R blocking antibodies 2B10 and 25F10 which block global IL-6 signalling, or IL-6 trans-signalling respectively (from Walter Ferlin, Novimmune, Switzerland) were administered in combination with the SES. After 12 hours, peritoneal leukocytes were recovered as described in Section 2.3.2, and flow cytometric analysis was used to examine the presence of the monocyte-like DC.

7.2.2 Phenotypic characterisation of $F4/80^{lo}$ $CD11b^{hi}$ $CD11c^{+}$ monocyte-like dendritic cells

$F4/80^{lo}$ $CD11b^{hi}$ $CD11c^{+}$ monocyte-like DC were phenotyped for various cell surface markers by flow cytometry. The population was identified by labelling with anti-F4/80 FITC, anti-CD11c PE and anti-CD11b APC, and a range of markers were measured using biotinylated antibodies, conjugated to PE-Cy5. All flow cytometry staining and acquisition was performed as described in Section 2.4.3.

7.2.3 Labelling of resident peritoneal leukocytes

Resident peritoneal leukocytes were labelled by i.p injection of 500μl of PKH26 red fluorescent dye (Sigma, Poole, UK) at a 1μM concentration. 3 hours following the labelling of peritoneal cells, SES-induced inflammation was established and peritoneal cells recovered after 12 hours to analyse both resident (dye labelled) and elicited (dye free) cell populations.

7.2.4 Isolation of bone marrow and blood total leukocytes

Bone marrow leukocytes were isolated by flushing out the marrow from mouse tibia and fibulas with sterile PBS as described in Section 2.2.2.5. Peripheral blood leukocytes were collected by cardiac puncture as described in Section 2.2.2.4. In both cases red blood cell contamination was removed by lysis (Section 2.2.2.4).

7.3 Results

7.3.1 Characterisation of the F4/80^{lo} CD11b^{hi} CD11c^{+} monocyte-like dendritic cell population

Previous experiments detailed in Chapter 5, Section 5.3.4, showed that IL-6$^{-/-}$ mice exhibited a temporal appearance of a novel DC-like population during SES induced inflammation, which was characterised as CD11b^{hi}CD11c^{+} and F4/80^{lo} and subsequently referred to as a monocyte-like DC population. To fully characterise the phenotype of this subset, flow cytometry was used to examine defined pan DC markers and chemokine receptor expression (Figure 7.1). Analysis of the monocyte like DC showed that they expressed the DC markers CD40, CD80, CD86, and low levels of MHCII. They also expressed the chemokine receptors CCR3, CCR5, CCR6 and CXCR4, and displayed low IL-6R expression. These cells did not express CD83, the DC maturation marker, and were negative for CD3, CD4, B220, and CD19. Expression was however high for both the monocyte marker CD14, and monocyte/neutrophil marker Gr1 (Figure 7.1).

7.3.2 F4/80^{lo} CD11b^{hi} CD11c^{+} monocyte-like dendritic cells are elicited from the circulation during inflammation

To determine whether the monocyte-like DC population arose from a localised differentiation or as a result of inflammatory recruitment, resident cells were globally labelled with a red fluorescent dye prior to the induction of SES-mediated inflammation in order to differentiate between resident and recruited leukocytes. Analysis of the F4/80^{lo} CD11b^{hi} CD11c^{+} monocyte-like DC showed that they did not possess the dye and were therefore elicited cells recruited from the circulation in response to SES activation (Figure 7.2).

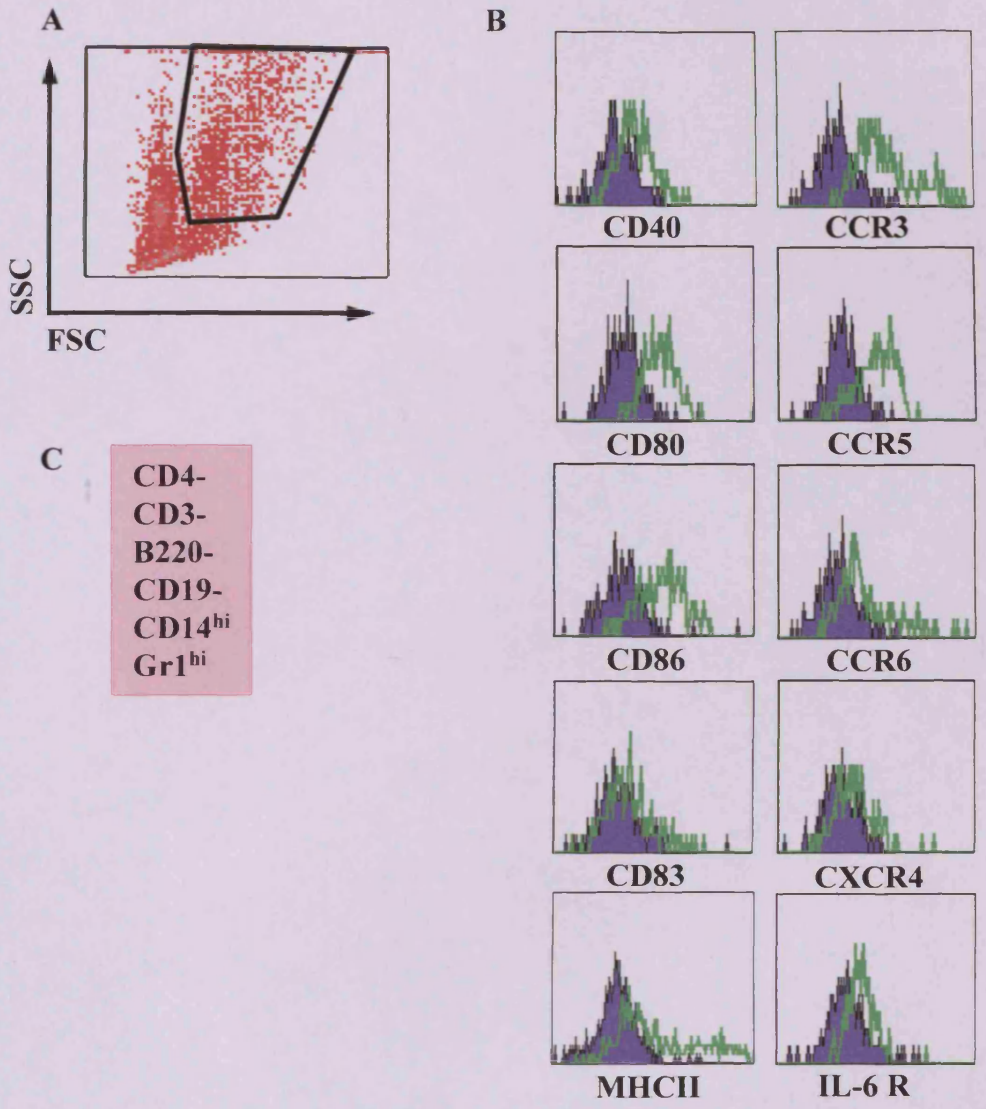


Figure 7.1: *Phenotypic analysis of the CD11c$^+$ monocyte like DC.*
IL-6$^{-/-}$ mice were challenged with SES for 12 hours, and the peritoneal leukocytes were recovered by lavage. The CD11c$^+$ monocyte like DC were analysed by flow cytometry for pan DC and monocytic markers. **(A)** The cell gate was set to identify the CD11c$^+$ monocyte like DC population with high forward and side scatter as previously described (Figure 5.5). **(B)** This population was labelled with antibodies against a variety of DC markers. **(C)** This population was also labelled with antibodies against lymphocyte markers CD3, CD4, B220 and CD19, and myeloid markers CD14 and Gr1. Data is representative of two independent experiments.

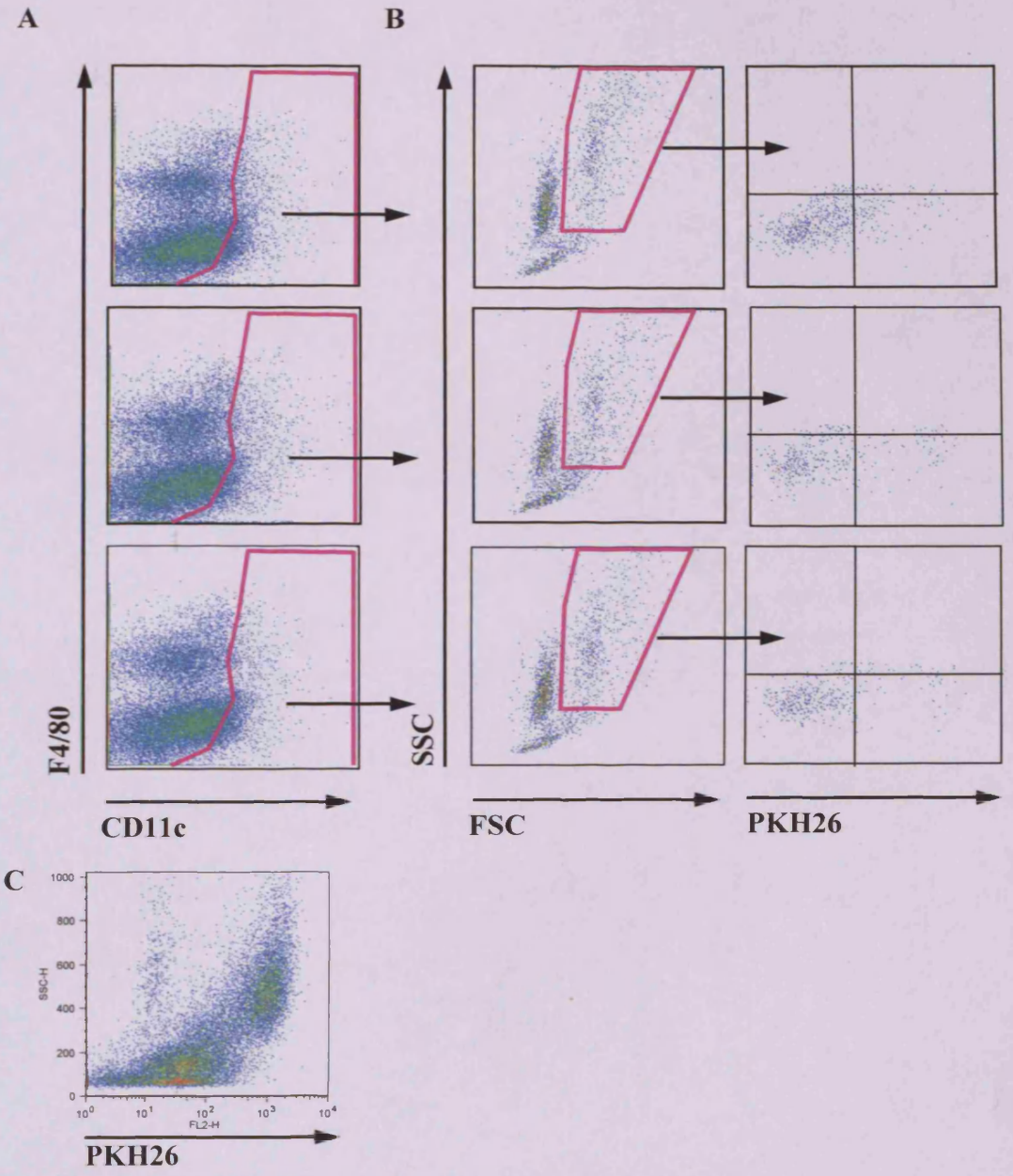


Figure 7.2: *The monocyte-like DC present in IL-6$^{-/-}$ mice at 12 hours are elicited from the circulation.* To determine if the monocyte like DC population was elicited from the circulation, resident peritoneal leukocytes were labelled by i.p injection of 500µl of PKH26, a dye that permanently labels cells and fluoresces in the FL2. This allows infiltrating leukocytes to be separated visually from resident cells. Inflammation was then induced in these mice by i.p injection of SES, and after 12 hours the leukocyte infiltrate was recovered by lavage and labelled with anti F4/80, CD11b and CD11c for analysis by flow cytometry. **(A)** CD11c^{+} cells were gated and the monocyte like DC gated based on their forward and side scatter. **(B)** The high forward and side scatter gated monocyte like-DC were analysed for fluorescence in the FL2 indicating dye labelling. Each plot represents an independent experiment from one mouse. **(C)** Whole peritoneal cells labelled with PKH26 dye following i.p injection.

7.3.3 $CD11c^+$ bone marrow and blood monocytic cell numbers are equivalent in WT and IL-$6^{-/-}$ mice

To assess whether the monocyte-like DC arose as a result of the inflammatory response or were an inherent part of the peripheral circulation, the number of $CD11c^+$ bone marrow and blood monocytes in WT and IL-$6^{-/-}$ mice were analysed (Figure 7.3).
Bone marrow monocytes were identified by their low forward and side scatter properties characteristic of monocytic cells. The number of these cells expressing CD11c was quantified and no significant differences were observed between WT and IL-$6^{-/-}$ mice. Similarly blood monocytes were identified by their phenotype $CD11b^{int}$ $F4/80^{int}$, and the number of these monocytes that expressed CD11c were equivalent between WT and IL-$6^{-/-}$ mice.

7.3.4 The $F4/80^{lo}$ $CD11b^{hi}$ $CD11c^+$ monocyte-like dendritic cell population is unique to IL-$6^{-/-}$ mice

To determine whether the appearance of the $F4/80^{lo}$ $CD11b^{hi}$ $CD11c^+$ monocyte-like DC population was also regulated by alternative cytokines that signal via the STAT pathway, peritoneal inflammation was induced in WT, IL-$6^{-/-}$, IFN-$\gamma^{-/-}$, IL-$10^{-/-}$, $OSMR^{-/-}$ and $gp130^{Y757F/Y757F}$ mice to analyse their infiltrating dendritic cell composition. The number of $F4/80^{lo}$ $CD11b^{hi}$ $CD11c^+$ monocyte-like dendritic cells was quantified in each of these groups. These data indicated that this population was unique to IL-$6^{-/-}$ mice (Figure 7.4).

Subsequent experiments were designed to verify the role of IL-6 in coordinating the development of this inflammatory $F4/80^{lo}$ $CD11b^{hi}$ $CD11c^+$ monocyte-like dendritic cell population. Specifically, IL-$6^{-/-}$ mice were administered with SES (i.p) in combination with either 100ng of hyper IL-6 or 500ng of recombinant IL-6, to reconstitute signalling by this cytokine in these mice, while WT mice received SES alone. After twelve hours, the peritoneal leukocytes were recovered by lavage and the presence of the monocyte like DC population was then determined by flow cytometry (Figure 7.5 A&B). IL-$6^{-/-}$ mice receiving SES alone exhibited the appearance of the $F4/80^{lo}$ $CD11b^{hi}$ $CD11c^+$ monocyte-like DC population, as was previously observed (Chapter 5, section 5.3.4), while WT mice lacked this population.

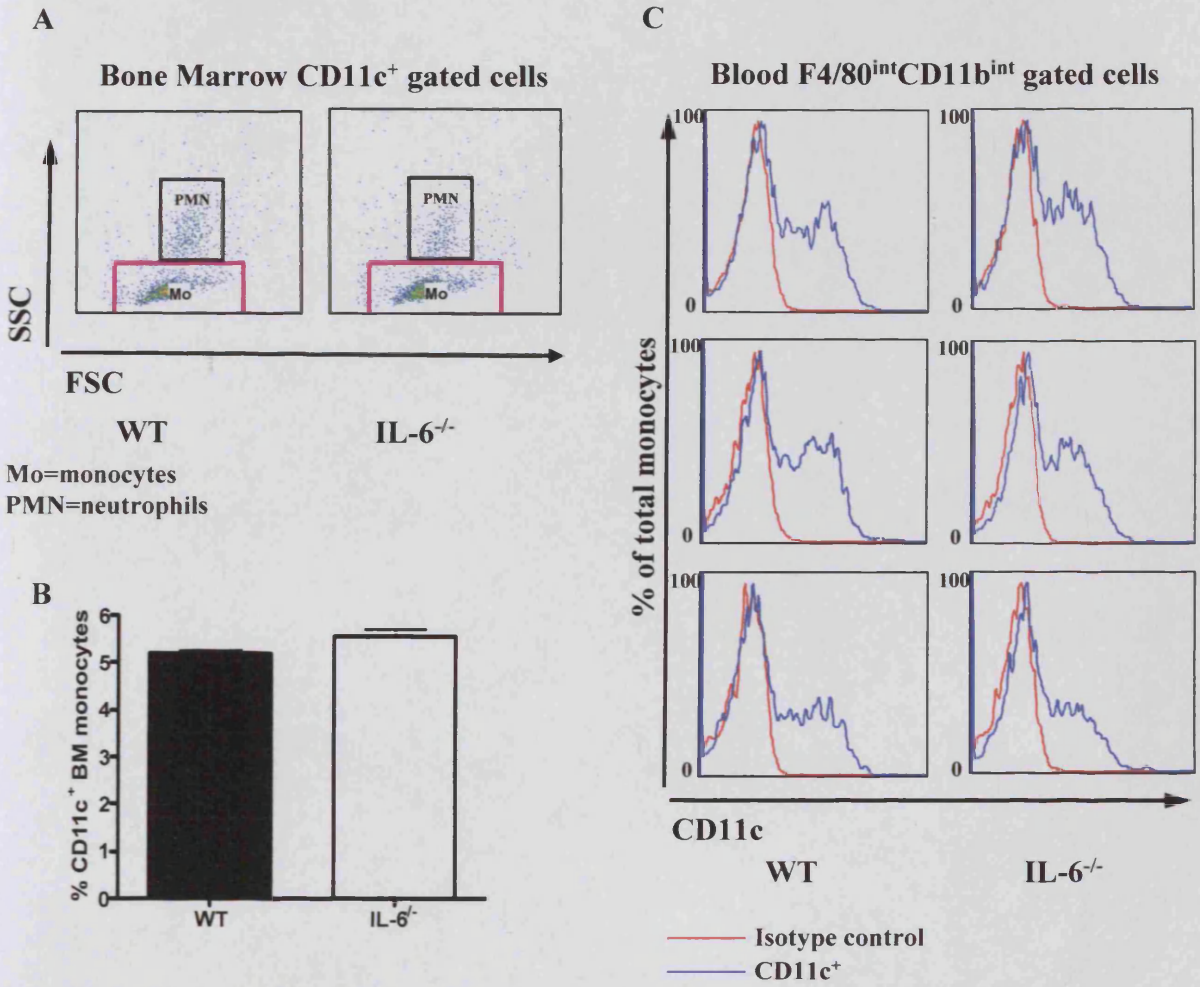


Figure 7.3: *IL-6$^{-/-}$ mice do not exhibit increased numbers of CD11c^{+} bone marrow and blood monocytic cells.* **(A)** Total bone marrow cells were recovered from WT and IL-6$^{-/-}$ mice by flushing the leg bones with PBS. These leukocytes were then labelled with anti CD11c and the CD11c $^{+}$ monocytic cells were identified by their low forward and side scatter properties (Plots are representative of three independent experiments). **(B)** Total percentage of CD11c^{+} bone marrow monocytes in WT and IL-6$^{-/-}$ mice (data represents three independent experiments). **(C)** Murine peripheral blood cells were labelled with anti F4/80, CD11b and CD11c. Monocytes were identified by their phenotype F4/80^{int} CD11b^{int}, and the number of CD11c expressing monocytes in this population was analysed (each plot represents an independent experiment from a single mouse).

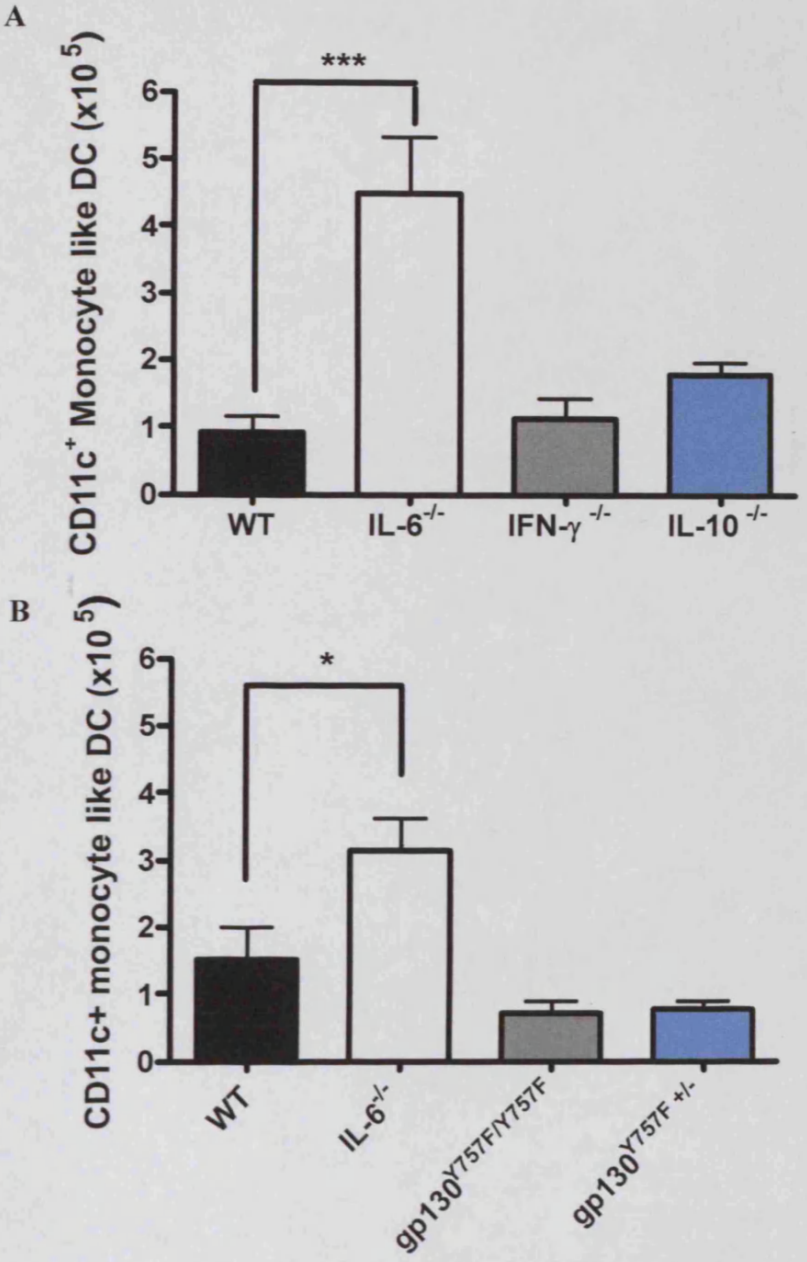


Figure 7.4: *The appearance of CD11c$^+$ monocyte-like DC is restricted to IL-6$^{-/-}$ mice.* WT, IL-6$^{-/-}$, IFN-$\gamma^{-/-}$, IL-10$^{-/-}$, gp130$^{Y757F/Y757F}$ and gp130$^{Y757F/+}$ mice were injected i.p with 500µl SES. After 12 hours, peritoneal leukocytes were recovered by lavage and labelled for FACS with anti-F4/80, CD11b and CD11c. The CD11b^{hi}CD11c$^+$F4/80^{lo} monocyte like DC were identified and quantified. **(A)** The number of CD11c$^+$ monocyte-like DC present in the peritoneal cavity of WT, IL-6$^{-/-}$, IFN-$\gamma^{-/-}$ and IL-10$^{-/-}$ mice at 12 hours was quantified by taking the flow cytometry gated percentage and multiplying by the total cell number. **(B)** The number of CD11c$^+$ monocyte like DC present in the peritoneal cavity at 12 hours was measured by flow cytometry for gp130$^{Y757F/Y757F}$ and gp130$^{Y757R\ +/-}$ mice. Values represent the mean ± SEM, n=5-10 mice, * $P < 0.05$,
*** $P < 0.001$.

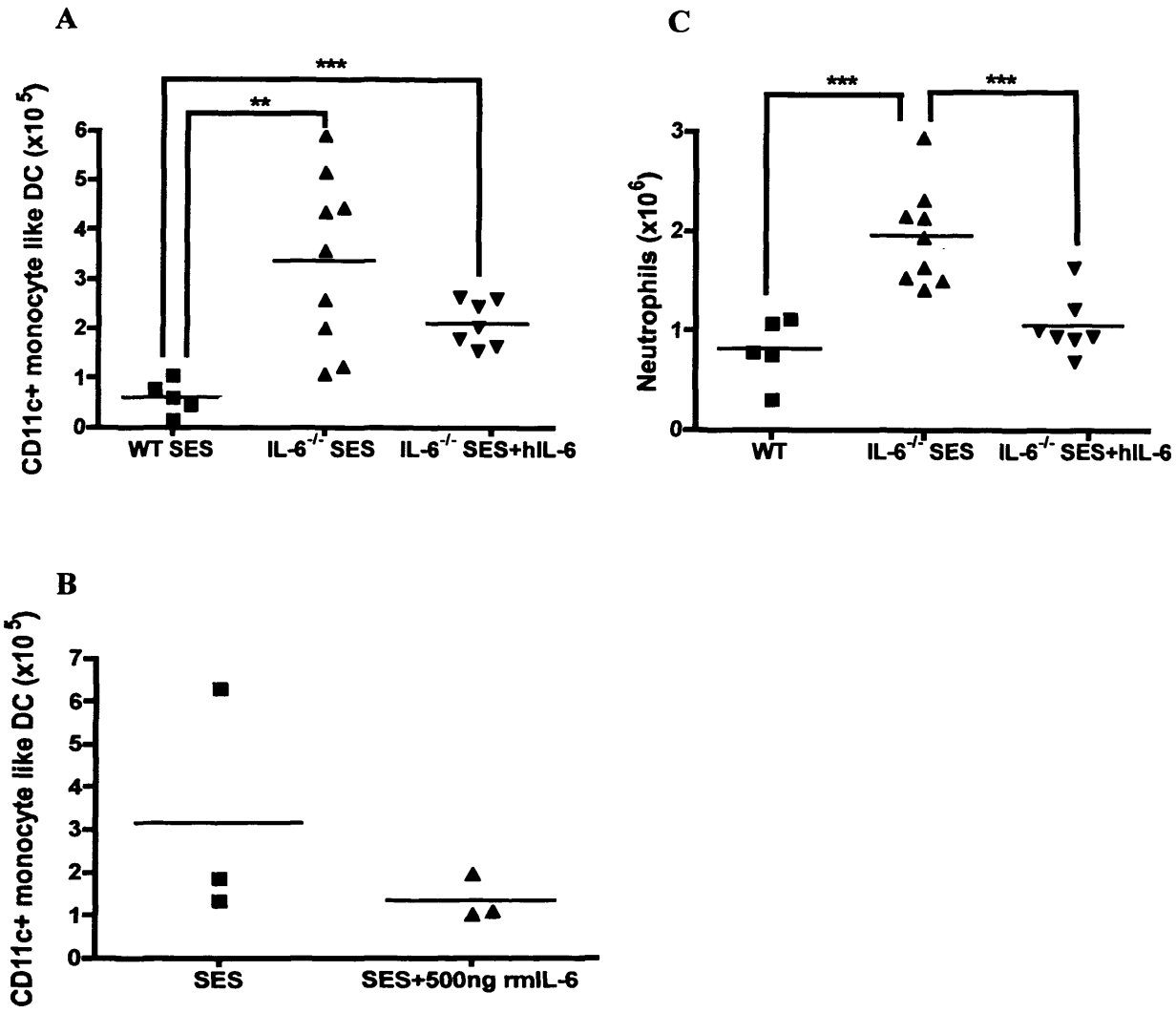


Figure 7.5: *The appearance of the monocyte-like DC population can be regulated by IL-6 signalling.* To induce peritoneal inflammation, WT mice were injected i.p with SES. IL-6 $^{-/-}$ mice were injected i.p with SES alone, or SES in combination with 100ng hyper IL-6 **(A)** or 500ng of recombinant human IL-6 **(B)**. These mice were then sacrificed at 12 hours, and the leukocyte infiltrate analyzed for the presence of CD11c^{+} monocyte like DC. The monocyte like DC were identified based on their phenotype F4/80^{lo}CD11b^{hi}CD11c^{+} and their high forward and side scatter properties, and the number of these cells present in the peritoneal cavity was quantified by taking the flow cytometry gated percentage and multiplying by the total cell number. **(C)** Neutrophil numbers were calculated in these mice by differential cell counting. (** P <0.01, *** $P < 0.001$) .

IL-6$^{-/-}$ mice that received SES in combination with hyper-IL-6 displayed a decrease in the number of F4/80^{lo}CD11b^{hi} CD11c^{+} monocyte-like dendritic cells as compared to IL-6$^{-/-}$ mice challenged with SES alone. IL-6$^{-/-}$ mice that received SES in combination with recombinant human IL-6 also exhibited a decrease in the number of these cells as compared to mice that received SES alone (Figure 7.5 A&B). As a control, the number of neutrophils in these animals was quantified, as IL-6 trans-signalling is well known to regulate neutrophil trafficking (Hurst *et al* 2001, McLoughlin *et al* 2003 and 2004). As expected, i.p administration of hyper-IL-6 in combination with SES reduced the number of infiltrating neutrophils in the IL-6$^{-/-}$ mice (Figure 7.5 C).

To support these findings, IL-6 signalling was also blocked in WT mice by administering two doses of an anti-IL-6R antibody (2B10) in combination with the SES required to induce peritoneal inflammation. These mice exhibited increased numbers of F4/80^{lo} CD11b^{hi} CD11c^{+} monocyte-like DC in the peritoneal cavity at 6 hours following induction of inflammation. Mice that received SES challenge in combination with the 25F10 antibody, which is designed to block only trans-signalling exhibited no change in their DC inflammatory infiltrate (Figure 7.6 B). Again, neutrophil numbers were quantified in these mice as a control, and the number of these cells increased with the blockade of IL-6 signalling (Figure 7.6 C).

7.3.5 MoFlo purification of CD11b^{hi} CD11c^{+} F4/80^{lo} dendritic like cells

Once the role of IL-6 in regulating the monocyte-like DC population had been verified, subsequent cell sorting approaches were adopted to more closely examine the morphology and functional characteristics exhibited by this F4/80^{lo}CD11b^{hi}CD11c^{+} population. Various cell sorting techniques were used in an attempt to achieve this. When using live cells for sorting, problems arose because the population was not easily visualised and located on the FACS calibur. In addition to this, the DC-like cells were also not detectable on the MoFlo cell sorter or the FACS Aria cell sorter (data not shown). It was only then discovered that fixation of these cells with BD cellfix made the population more defined and easily located by forward and side scatter as visualised by flow cytometry. It was therefore not possible to sort this population of cells for further analysis.

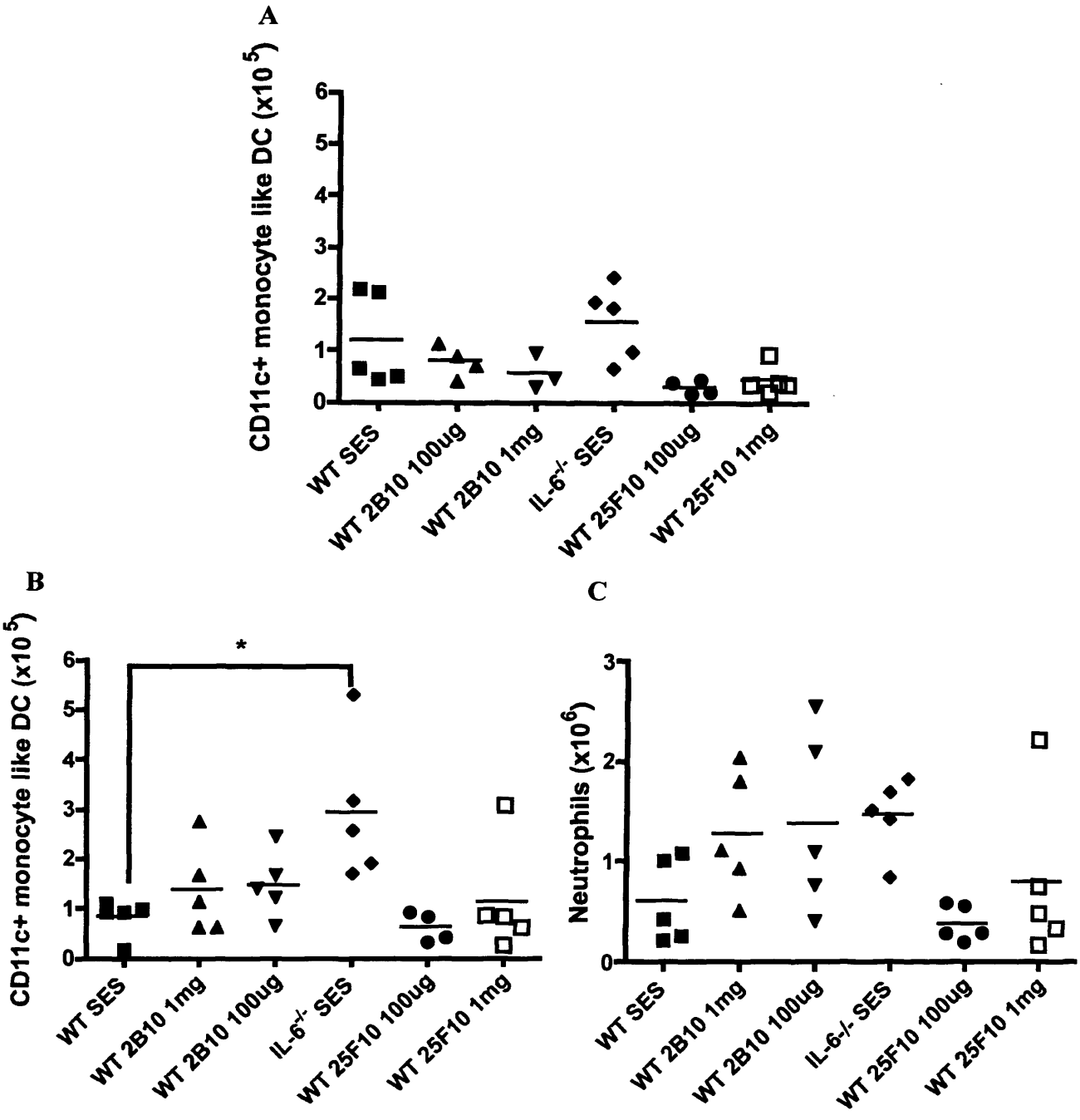


Figure 7.6: *Blocking IL-6 signalling in WT mice induced the appearance of the monocyte-like DC population during inflammation.* WT mice were injected with either SES alone or in combination with IL-6 cis and trans-signalling blocker 2B10 or IL-6 trans-signaling blocker 25F10. IL-$6^{-/-}$ mice were injected with SES alone. Mice were sacrificed at **(A)** 3 and **(B)** 6 hours following induction of inflammation and the peritoneal leukocytes were recovered by lavage and labelled with anti F4/80, CD11b and CD11c. The monocyte-like DC were identified based on their phenotype F4/80^{lo}CD$11b^{hi}$CD$11c^{+}$ and their high forward and side scatter properties, and the number of these cells present in the peritoneal cavity was quantified by taking the flow cytometry gated percentage and multiplying by the total cell number. **(C)** As a control, neutrophils were quantified at 6 hours in each group by differential cell counting. (* $P < 0.05$).

7.3.7 Doublet exclusion from acquired FACS samples ruled out the F4/80^{lo} CD11b^{hi} CD11c^{+} monocyte-like DC as a real population

Following the sorting difficulties outlined in Section 7.3.6, steps were taken to verify the true identity of the F4/80^{lo}CD11b^{hi}CD11c^{+} monocyte-like DC population. It was speculated that the population observed in IL-6$^{-/-}$ mice may have arisen as a result of the formation of neutrophil aggregates which were acquired on the FACS calibur. These suspicions arose due to the fact that the migratory pattern observed in the monocyte-like DC population mirrored that seen by the neutrophils (Figures 5.1A, 5.7, 7.5 and 7.6). In addition, IL-6$^{-/-}$ mice exhibited increased trafficking of neutrophils upon SES challenge, making the proportion of neutrophils recovered from these mice much greater (Figure 5.1A). Adding to these concerns was the fact that the regulation of the monocyte-like DC population by IL-6 signalling, as outlined in Figures 7.5 and 7.6, was identical to the regulation of the neutrophil population seen concurrently in these mice. To determine if aggregation was occurring, peritoneal leukocytes from IL-6$^{-/-}$ mice challenged with SES for 12 hours, were recovered by lavage and labelled with the required antibodies to detect the F4/80^{lo}CD11b^{hi}CD11c^{+} monocyte-like DC population. These labelled cells were then acquired on both the BD FACS calibur, and the Becton Coulter CyAn ADP analyser for comparison (Figure 7.7). The population could be identified as usual using the FACS calibur, however the CyAn ADP analyser detected a much smaller number of these cells within the gate. The CyAn ADP analyser allows doublet exclusion from acquired samples, and upon application of this gate, the F4/80^{lo}CD11b^{hi}CD11c^{+} monocyte-like DC population was completely excluded.

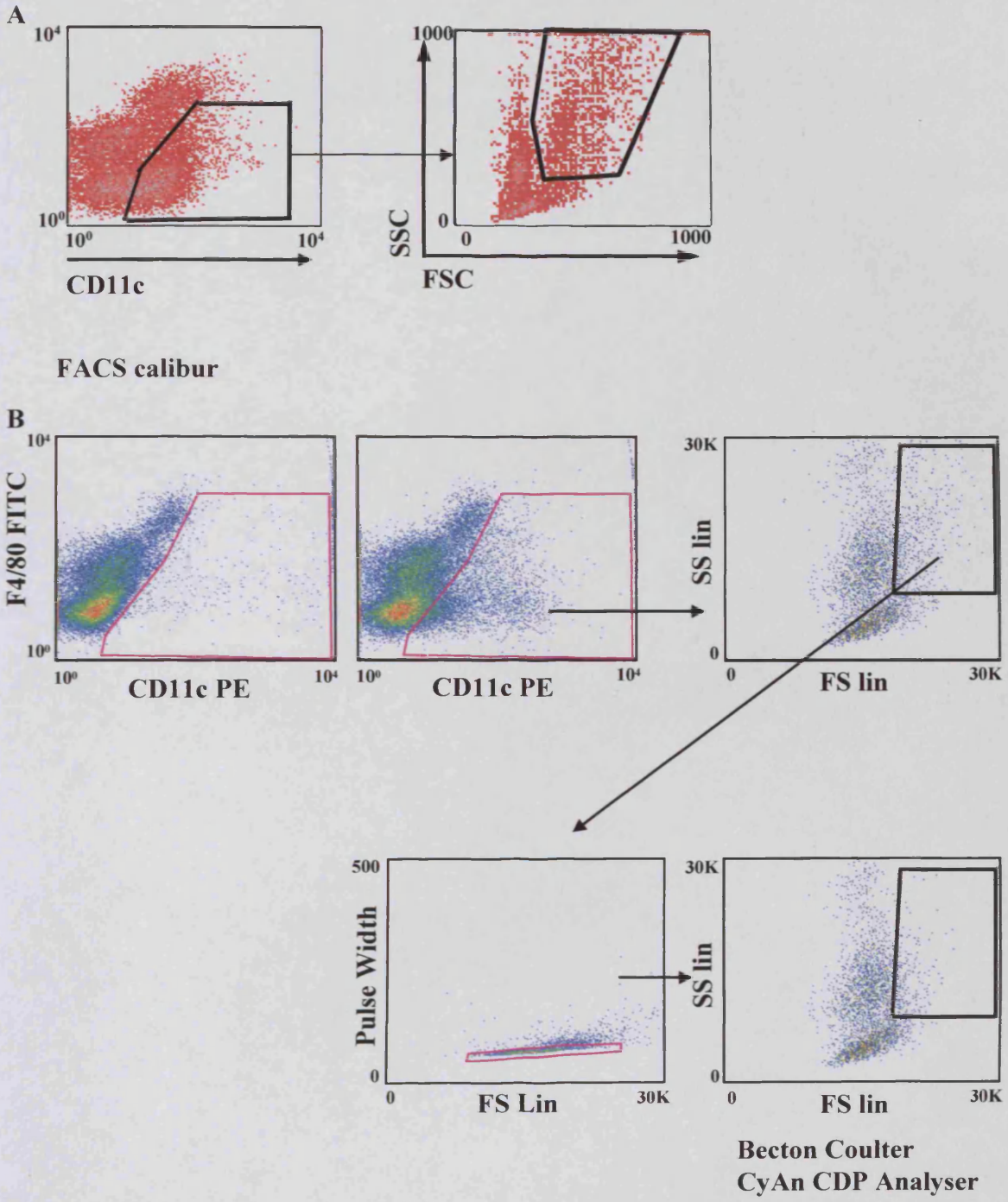


Figure 7.7: *Doublet exclusion from acquired flow cytometry data eliminated the monocyte-like DC population.* IL-6$^{-/-}$ mice were injected i.p with SES to induce inflammation. After 12 hours, peritoneal leukocytes were collected and labelled with anti F4/80, CD11b and CD11c for flow cytometry, and samples were acquired on **(A)** the FACS calibur and **(B)** the CyAn CDP analyser respectively. CD11c^{+} cells were gated and analysed for forward and side scatter to isolate the monocyte like DC population. A doublet exclusion gate was applied to data acquired on the CyAn CDP analyser.

7.3.8 CCR5 staining of the anomalous population revealed a mixed population of neutrophil aggregates and infiltrating monocytes

Upon reviewing the characterisation data shown in Figure 7.1, it was noted that the CCR5 staining of the anomalous/monocyte-like DC population revealed two populations of cells within this gate, one being CCR5 positive, the other CCR5 negative (Figure 7.8). This highlights the presence of two separate leukocyte populations within the anomalous gate. Back gating of these populations indicated the $CCR5^+$ cells were likely to be part of the infiltrating monocyte population, while the $CCR5^-$ cells were probably aggregated neutrophils, making them appear in the higher forward and side scatter region of the plot.

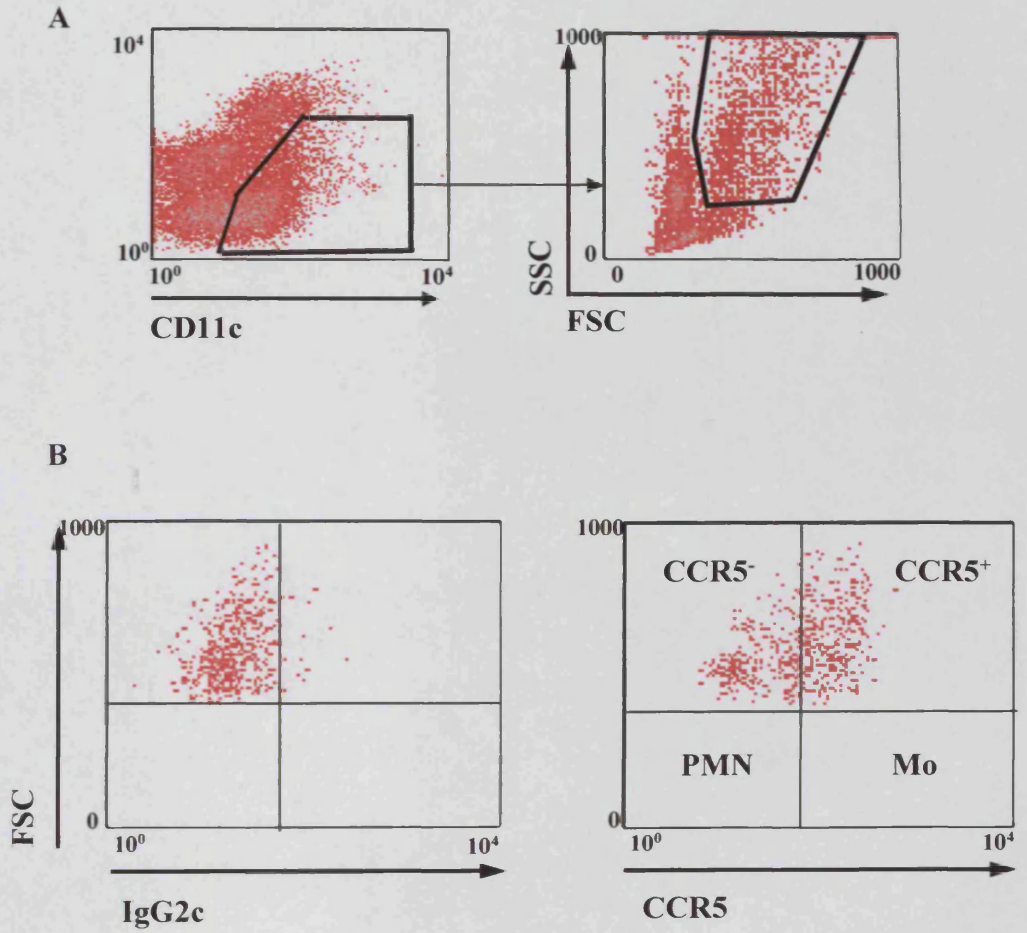


Figure 7.8: *CCR5 staining of the anomalous population reveals a mixed population of neutrophil aggregates and infiltrating monocytes.* IL-$6^{-/-}$ mice were injected i.p with SES to induce inflammation. After 12 hours peritoneal leukocytes were collected and labelled with anti F4/80, CD11b and CD11c for flow cytometry. **(A)** $CD11b^{hi}$ $CD11c^{+}$ $F4/80^{lo}$ cells with high forward and side scatter (monocyte-like DC) were gated and **(B)** labelled for CCR5. PMN-neutrophils, Mo-infiltrating monocytes.

7.4 Discussion

As these studies progressed, it became increasingly evident that the population of monocyte-like DC identified in the IL-$6^{-/-}$ mice were appearing as an anomaly. A number of observations confirmed this: First it was observed that fixation of the FACS labelled cells was necessary for the identification of the monocyte like DC population by flow cytometric forward and side scatter analysis. Fixation makes the cells smaller and more granular, and this "tightens" the population into a defined area, making it more distinguishable from other populations. Secondly, problems arose in attempts to purify this population for further phenotypic and functional analysis. It was not possible to detect this population on either the FACS Aria cell sorter or the MoFlo, both when the cells were live and fixed, making it impossible to purify this population. Upon revisiting the *in vivo* data in Chapter 5, it was noted that the trafficking pattern exhibited by the monocyte-like DC was identical to that observed for neutrophils. This arose suspicions that the monocyte-like DC population observed in IL-$6^{-/-}$ mice may have arisen as a result of neutrophil aggregates acquired by the FACS calibur flow cytometer, particularly since these mice have a significantly increased number and proportion of infiltrating neutrophils compared to WT mice at 6 and 12 hours following SES challenge. These suspicions were compounded by the identical regulation observed in the trafficking of the monocyte-like DC and neutrophil populations concurrently by IL-6 (Figure 7.5 and 7.6). This hypothesis was tested utilising data acquired on the Becton Coulter CyAn ADP analyser, which allows doublet exclusion. The application of a pulse width gate, which removes aggregates from the flow cytometry plot, lead to the complete exclusion of the monocyte-like DC confirming that this population arose as a result of an anomaly caused by aggregation of cells being detected by the flow cytometer. It is most likely that the aggregates seen during the flow cytometric acquisition were a result of neutrophil doublets. This can be concluded from a number of facts. First, the IL-$6^{-/-}$ mice exhibit increased infiltration of neutrophils into the peritoneal cavity during inflammation, peaking at the 12 hour time point. This increased recruitment, which occurs as a result of enhanced CXCL1 expression in the peritoneal cavity (McLoughlin *et al* 2004), coincides with the appearance of the monocyte-like DC population. The higher concentration of neutrophils in theses animals would result in a higher concentration of neutrophils being passed through the flow cytometer, which would consequently increase the chances of

aggregate formation. Forward and side scatter analysis would therefore give rise to a larger and more granular cell population. This is likely since the anomalous monocyte-like DC population displayed almost identical granularity to the known neutrophil population, differing only in their size, being indicative of aggregate formation. Finally the regulation of the monocyte like DC trafficking by IL-6 was identical to that seen in neutrophils, indicating that they were in fact the same population, one being represented by an aggregated form of neutrophils. The reduction in neutrophil infiltration observed in IL-$6^{-/-}$ mice that were reconstituted with IL-6 most likely led to a reduction in doublet formation during the flow cytometric acquisition. Similarly, WT mice that received blocking antibodies for IL-6 signalling exhibited increased neutrophil infiltration, and as a result, the appearance of doublets was increased during the flow cytometric acquisition for these mice. This would have accounted for the appearance of the anomalous population observed, which were originally described as monocyte like DC. CCR5 labelling of this population further indicated that this population was not uniquely made up of $CCR5^-$ neutrophil aggregates, but likely included a small number of $CCR5^+$ infiltrating monocytes too.

Because the FACS calibur was used exclusively for the majority of work done for this thesis, and this machine does not have the capabilities for doublet exclusion, this anomaly did not come to light until the attempts to purify the so called monocyte-like DC population were made.

CHAPTER 8

The role of IL-10 in the peritoneal inflammatory response

8.1 Introduction

The principle role of IL-10 is to suppress the inflammatory response. In this respect, IL-10 contributes to the destruction of the pathogen, whilst simultaneously dampening the immune response to limit collateral tissue damage. In addition to this, IL-10 regulates the growth and differentiation of various immune cells such as dendritic cells, macrophages, cytotoxic and helper T cells, and plays a key role in the differentiation and function of T regulatory cells (Moore *et al* 2001).

During inflammation, IL-10 plays a regulatory role and suppresses the pro-inflammatory functions of APC by suppressing their expression of co-stimulatory molecules, their release of inflammatory cytokines and chemokines, and their maturation, therefore rendering them unable to stimulate adaptive immune effector cells (Moore *et al* 2001). In this respect, IL-10 inhibits the release of the inflammatory chemokines monocyte inhibitory protein-1α (MIP-1α), MIP-1β, MCP-1 and IL-8 (Olszyna *et al* 2000, Berkman *et al* 1995), as well as cytokines including TNF-α, IL-1β and IL-6 (Fiorentino *et al* 1991, Chernoff *et al* 1995). As a result, IL-10 inhibits cytokine production and proliferation of $CD4^+$ T cells via immunosuppression of APC function (Fiorentino *et al* 1991, de Waal Malefyt *et al* 1991, Chernoff *et al* 1995). Recently these observations have been elaborated to show that IL-10 treatment of DC can induce or contribute to a state of anergy in allo-antigen or peptide-antigen-activated T cells (Groux *et al* 1996, Takayama *et al* 1998).

IL-10 has been shown to inhibit the differentiation of monocytes into DC and instead promote their maturation to macrophages. Studies with human monocytes show that when cultured with GM-CSF and IL-4 for 6 days, monocytes differentiate into immature DC that can be activated by LPS, CD40 ligand or TNF to mature into efficient IL-12 secreting APC, capable of inducing Th1 polarisation. Addition of IL-10 inhibits the generation and maturation of monocyte-derived DC, and instead induces the differentiation of these immature DC into macrophage like cells. These macrophages are characterised by a lower expression of co-stimulatory molecules including MHC II, and display enhanced phagocytic capabilities (Allavena *et al* 1998, Fortsch *et al* 2000).

Many animal studies have been performed to dissect the role of IL-10 in the inflammatory response to pathogens. These have determined that both the innate and acquired immune responses are enhanced or impaired by experimental depletion or elevation of IL-10 respectively. For example, increased IL-10 activity compromised resistance to *Listeria monocytogenes* in *scid* mice, through suppression of the innate immune response (Kelly and Bancroft 1996, Tripp *et al* 1995). Similarly, the enhanced innate response to *Listeria* in IL-$10^{-/-}$ mice or anti-IL-10 treated mice lead to a rapid control of *Listeria* within the first few days of infection. This was accompanied by enhanced acquired immunity during primary and secondary infection (Dai *et al* 1997). Therefore IL-10 facilitates the outgrowth and dissemination of bacteria, with IL-$10^{-/-}$ mice showing improved clearance of *Escherichia coil* and *Staphylococcus aureus* during infection (Sewnath *et al* 2001, Coles *et al* unpublished).

Studies have shown that pre-treatment of macrophages with IL-10 inhibits subsequent STAT3 activation by IL-6, however this does not work in reverse, and IL-10 induced STAT3 activation is not counteracted by pre-treatment with IL-6. This cross inhibition has been demonstrated to be dependent on active transcription and is likely explained by different sensitivities of IL-10 and IL-6 signalling towards the negative feedback inhibitor Suppressor of cytokine signalling 3 (SOCS3), which is induced by both cytokines (Niemand *et al* 2003). The activation of SOCS3 acts as a negative regulator of gp130-mediated signalling (Nicholson *et al* 2000, Schmitz *et al* 2000). However the induction of SOCS3 by IL-10 does not suppress STAT3 signalling *via* the IL-10R (Niemand *et al* 2003). This difference in sensitivity of IL-10 and IL-6 to SOCS3 regulation of the JAK/STAT pathway defines an important mechanistic distinction between these two STAT3-activating cytokines (Niemand *et al* 2003). Contributing to this interplay is the ability of IL-10 to inhibit IL-6 production by activated monocytes (Fiorentino *et al* 1991), and the ability of IL-6 to regulate IL-10 expression *in vivo* (Fielding *et al* unpublished).

The aim of this Chapter was to investigate the role of IL-10 in cytokine and chemokine production and leukocyte trafficking in an *in vivo* model of acute peritonitis, drawing conclusions on its interplay with IL-6.

8.2 Materials and methods

8.2.1 Determination of IL-10 production by murine peritoneal leukocytes *in vitro*

Peritoneal lavage fluids from 5 WT mice were pooled. Cells were cultured for three hours in 6 well microtitre plates in RPMI 1640 containing 10% FCS, 2mM L-glutamine, 100U/ml Penicillin, 100μg/ml Streptomycin, 1mM sodium pyruvate and 55μM β-mercaptoethanol in the presence or absence of SES (1/10) and 3 μM monensin. Cells were scraped and surface labelled with antibodies against F4/80, CD11b, CDllc, B220, NK1.1 and CD4 as described in Section 2.3.4. Following permeabilisation, cells were labelled intracellularly for IL-10, and analysed by flow cytometry (Section 2.3.4).

8.2.2 Induction of SES induced peritoneal inflammation

SES induced inflammation was established in groups of WT, IL-$10^{-/-}$ and IL-$6^{-/-}$ mice (n=5 per group, mixture of males and females) by i.p administration of SES (Section 2.3.5). At defined intervals during the inflammatory response (0, 1, 3, 6, 12, 15, 18, 24, 48 and 72 hours) mice were sacrificed and the leukocyte infiltrate collected by peritoneal lavage.

8.2.3 Cytospin analysis of leukocyte infiltrate

At each time point, animals were sacrificed by schedule 1 and peritoneal leukocytes collected by lavage as described in Section 2.3.2. Cytospins were prepared, stained with Wright Giemsa stain and differential cell counts performed as described in Section 2.3.2.

8.2.4 Flow cytometric analysis of leukocyte infiltrate

Peritoneal leukocytes were labelled for flow cytometric analysis with antibodies against macrophage and dendritic cell markers F4/80 FITC, CD11b APC, and CD11c PE (Section 2.4.3). Details of the antibodies used are found in Table 2.1. Defined monocytic subsets were gated as described in Chapters 4 and 5. Cell numbers were quantified by taking the flow cytometry population gated percentages and multiplying by the total cell number.

8.2.5 *In vitro* stimulation of inflammatory peritoneal leukocytes and intracellular cytokine staining to identify $CD4^+$ T helper cell subsets

Peritoneal leukocytes were cultured ($1x10^6$ cells/well) in 96 well plates for four hours in the presence of 50ng/ml PMA, 500ng/ml Ionomycin and 3μM monensin, as described in Section 2.3.8. Following this, cells were labelled with antibodies against the T helper cell marker CD4, and subsequently labelled with anti-IL-17A, IFN-γ and IL-10 intracellularly to determine cytokine production by these cells (Section 2.4.3).

8.2.6 ELISA quantification of cytokine and chemokine levels in lavage supernatants

Peritoneal lavage supernatants were rendered cell free by centrifugation and stored at -70°C. Supernatants were analysed by ELISA for IL-6, sIL-6R, IL-10, CCL2 and CXCL1 as described in Section 2.5.

8.3 Results

8.3.1 Resident macrophages are the predominant IL-10 producing cells in the peritoneal cavity

To determine the peritoneal source of IL-10 following SES activation, resident peritoneal leukocytes were isolated and cultured in the presence or absence of SES and intracellular staining performed to identify IL-10 production by defined cell types (Figure 8.1). In this respect, the predominant cell type to produce IL-10 upon SES stimulation was the $F4/80^{hi}CD11b^{hi}$ resident macrophages (Figure 8.1 B). Resident $F4/80^{int}CD11b^{hi}CD11c^{+}$ dendritic cells exhibited a small shift indicating a low level of heterogeneous IL-10 expression by these cells (Figure 8.1 A). $CD11b^{int}B220^{int}$ B1 cells also produced IL-10 but not to the same magnitude as resident macrophages, whilst $B220^{hi}CD11b^{-}$ B2 cells produced low levels of IL-10 (Figure 8.1 C). NK1.1 Natural Killer cells produced no IL-10, while $CD4^{+}$ T cells exhibited only a negligible shift for IL-10 staining (data not shown).

8.3.2 IL-10 limits leukocyte infiltration during acute peritoneal inflammation

To study the potential role of IL-10 in governing leukocyte recruitment, WT and IL-$10^{-/-}$ mice were challenged with SES and the leukocyte infiltrate quantified at defined time points (Figure 8.2). Since the inflammatory trafficking of leukocytes in IL-$6^{-/-}$ mice has been so extensively characterised in Chapter 5, and there is a known interplay between IL-6 and IL-10, it was decided to include this group for comparison. The data revealed that while each group had equivalent numbers of resident peritoneal leukocytes prior to SES challenge, IL-$10^{-/-}$ mice exhibited significantly enhanced leukocyte recruitment following induction of peritonitis, as compared to age matched WT and IL-$6^{-/-}$ mice.

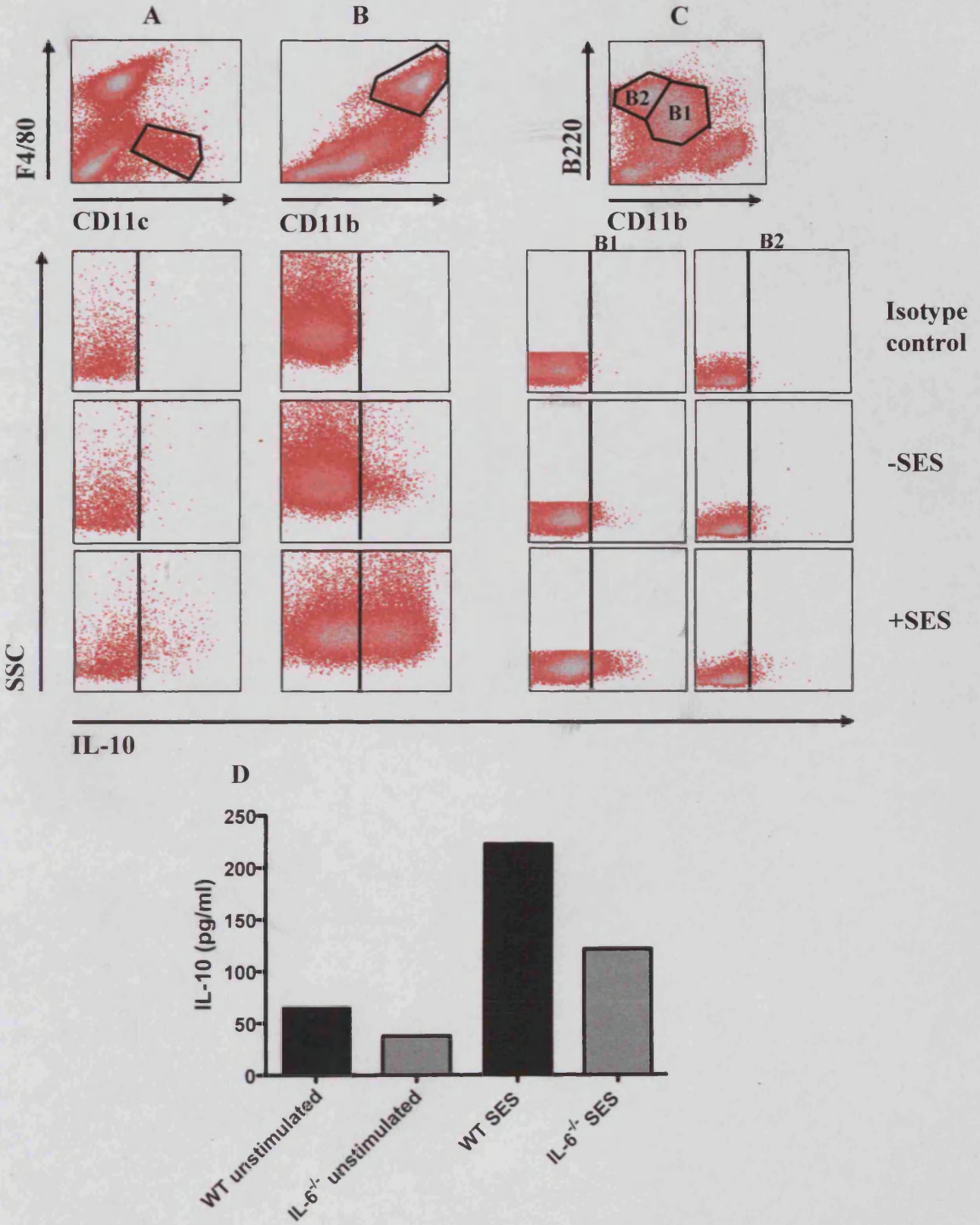


Figure 8.1: *IL-10 expression by resident peritoneal cells.* Peritoneal leukocytes were recovered by lavage from 5 WT mice and pooled. Cells were cultured for three hours in the presence or absence of SES (1/10) and 3μM monensin. Cells were then labelled with antibodies against markers for macrophages, DC, and B cells followed by intracellular staining for IL-10. **(A)** IL-10 expression by F4/80^{int}CD11b^{hi} resident DC, **(B)** F4/80^{hi}CD11b^{hi} resident macrophages, **(C)** B220^{int}CD11b^{int} B1 cells and B220^{hi}CD11b^{-} B2 cells . **(D)** IL-10 production by WT and IL-6$^{-/-}$ resident macrophages, as measured by luminex following 20 hours of culture in the presence or absence of SES (1/50).

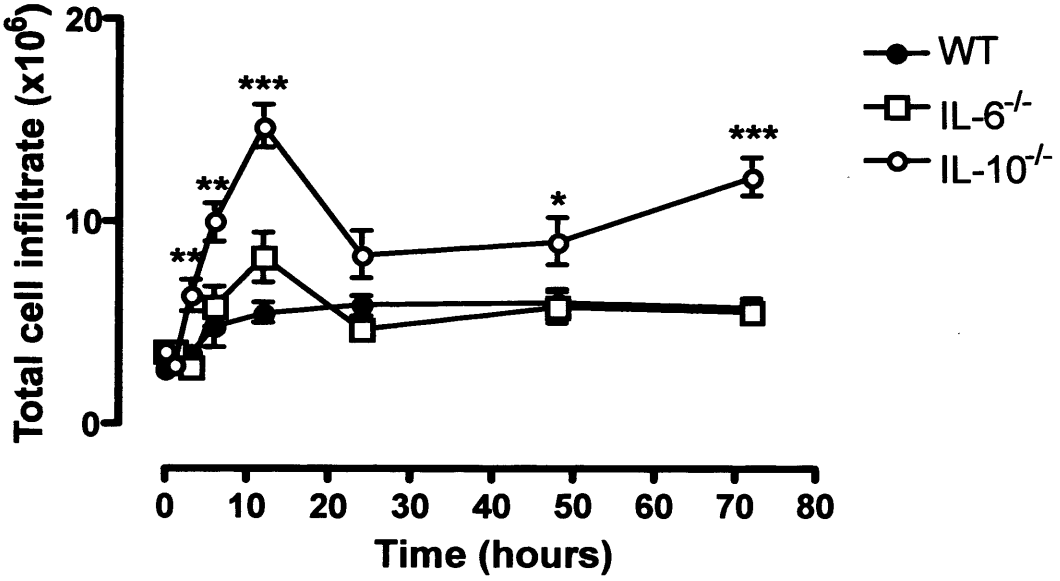


Figure 8.2: *Total leukocyte trafficking during SES-induced peritoneal inflammation.* Peritoneal inflammation was induced by i.p administration of SES and peritoneal leukocytes collected by lavage at defined intervals. Total cell counts were determined using a Coulter counter. Values represent the mean ± SEM, n=5-15 mice per time point, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

8.3.3 IL-10 regulates the inflammatory recruitment of neutrophils and mononuclear cells

To better understand the role of IL-10 in regulating the inflammatory recruitment of leukocytes, the inflammatory infiltrate was analysed by differential cell counting to determine the proportion of each individual cell type (Figure 8.3). The data revealed that IL-$10^{-/-}$ mice exhibited an increased recruitment of neutrophils, monocytes and lymphocytes in comparison to both WT and IL-$6^{-/-}$ mice. Neutrophil recruitment in IL-$10^{-/-}$ mice was 2-3 fold higher than in IL-$6^{-/-}$ mice and 3-4 fold higher than WT mice (Figure 8.3 A). Monocyte recruitment in IL-$10^{-/-}$ mice was approximately 2.5 fold higher than in both WT and IL-$6^{-/-}$ mice (Figure 8.3 B). Lymphocyte recruitment in IL-$10^{-/-}$ mice was between 1 and 3 fold higher than in WT and IL-$6^{-/-}$ mice depending on the time point (Figure 8.3 C).

8.3.4 IL-10 regulation of monocytic cell trafficking during SES induced peritonitis

To define a role for IL-10 in the regulation of F4/80^{hi}/$CD11b^{hi}$ resident macrophage, F4/80^{int}/$CD11b^{hi}$/$CD11c^{+}$ resident DC trafficking, and F4/80^{int}/$CD11b^{int}$ infiltrating monocytes, these populations were monitored by flow cytometry following the induction of SES induced peritoneal inflammation in WT and IL-$10^{-/-}$ mice (Figure 8.4). IL-10 did not alter the trafficking profile of the F4/80^{hi}/$CD11b^{hi}$ resident-like macrophages, which remained equivalent in WT and IL-$10^{-/-}$ mice (Figure 8.4 B). In this respect, these cells exhibited an initial clearance, followed by a re-emergence of this population at later time points. The F4/80^{int}/$CD11b^{hi}$/$CD11c^{+}$ resident DC population also exhibited an initial clearance followed by a recruitment at the later time points (Figure 8.4 D). However IL-$10^{-/-}$ mice exhibited an enhanced recruitment of this population as compared to the WT controls. Finally, IL-$10^{-/-}$ mice exhibited an enhanced recruitment of F4/80^{int}/$CD11b^{int}$ infiltrating monocytes compared to the WT mice (Figure 8.4 F).

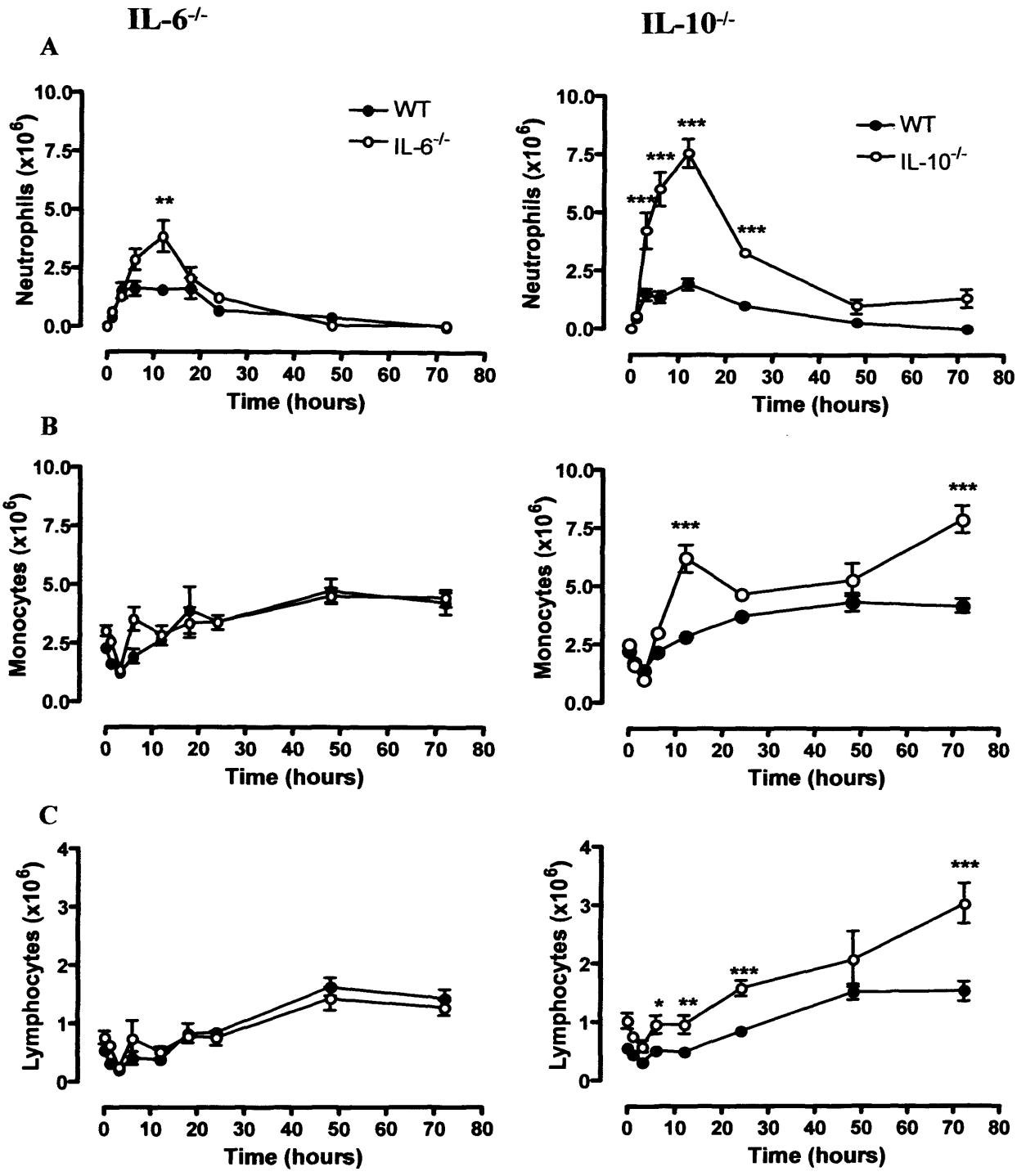


Figure 8.3: *Neutrophil, monocyte and lymphocyte trafficking following the induction of SES induced inflammation in WT, IL-6$^{-/-}$ and IL-10$^{-/-}$ mice.* Peritoneal inflammation was induced in WT, IL-6$^{-/-}$and IL-10$^{-/-}$ mice by i.p administration of SES and peritoneal leukocytes were collected by lavage at defined intervals. **(A)** Differential cell counts were performed and the number of neutrophils calculated at each time point. **(B)** The number of lymphocytes calculated at each time point. **(C)** The number of monocytes at each time point. (values represent the mean ± SEM, n=5-15 mice per time point, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

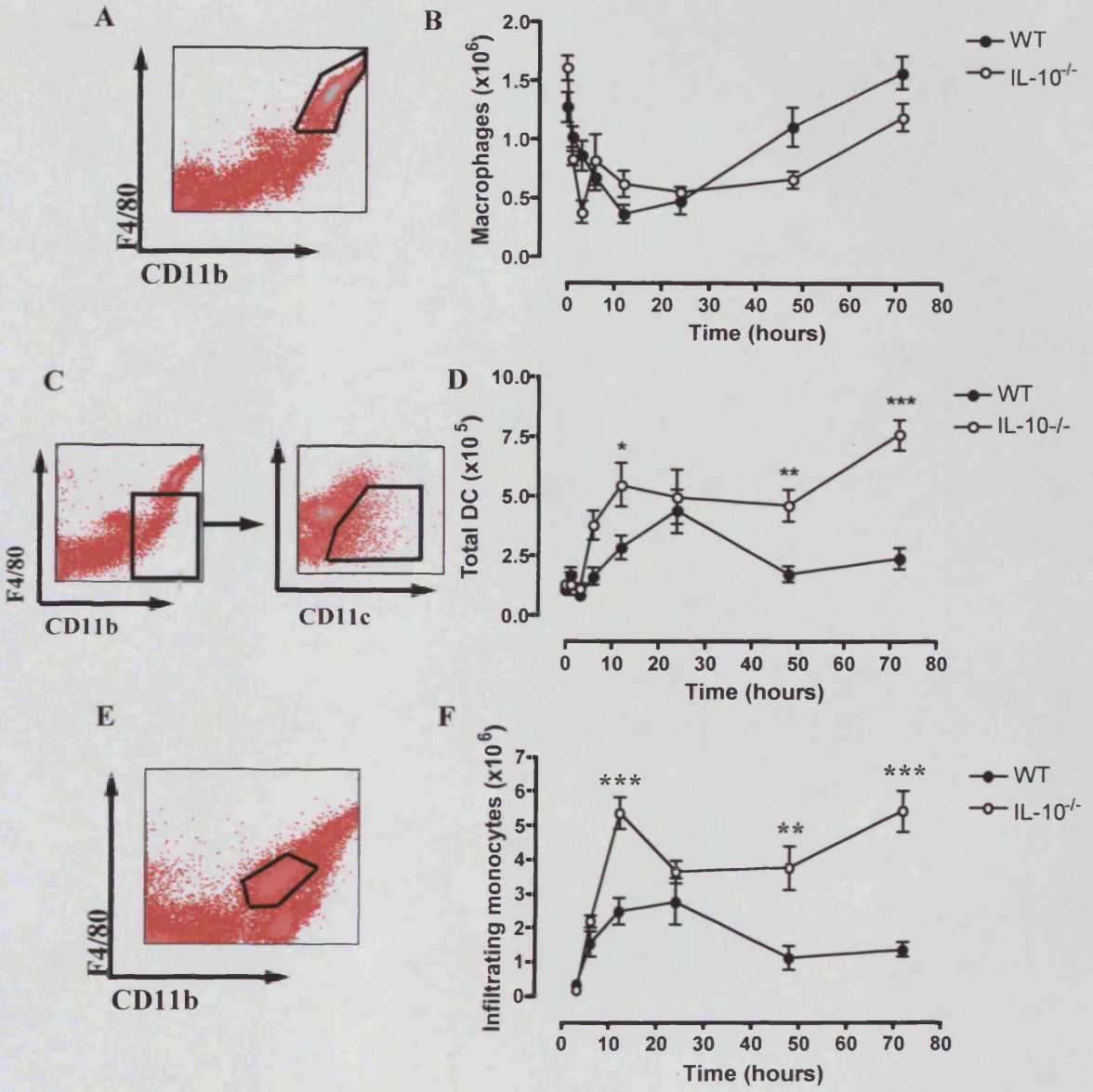


Figure 8.4: *IL-10 regulation of monocytic subset trafficking following SES induced peritonitis.* Peritoneal inflammation was induced by i.p administration of SES and peritoneal leukocytes were collected by lavage at defined intervals. **(A)** Peritoneal leukocytes were labelled with anti F4/80 and CD11b and the macrophages identified by their F4/80^{hi}CD11b^{hi} phenotype. **(B)** The number of resident-like macrophages, defined by the indicated gates (Panel A) were calculated at each time point by taking the flow cytometry gated percentage and multiplying by the total cell number. **(C)** Peritoneal leukocytes were labelled with anti F4/80 and CD11b and cells that were positive for F4/80 and CD11b were labelled for CD11c. Cells that resided in this F4/80^{int}CD11b^{hi}CD11c+ gate were defined as DC. **(D)** The number of F4/80^{int}CD11b^{hi}CD11c+ DC within the defined gate (Panel C) was calculated at each time point. **(E)** Peritoneal leukocytes were labelled with anti F4/80 and CD11b and infiltrating monocytes identified by their F4/80^{int}/CD11b^{int} phenotype. **(F)** The number of infiltrating monocytes, defined by the indicated gates (Panel E) were calculated at each time point. Values represent the mean ± SEM, n=5 mice per time point, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

8.3.5 IL-10 regulates the production of soluble mediators upon SES activation

To determine the mechanism of the increased leukocyte recruitment observed in Section 8.3.3, inflammatory chemokines were measured in lavage supernatants by ELISA (Figure 8.5). IL-$10^{-/-}$ mice exhibited an enhanced production of CCL2 as compared to the WT mice, while IL-$6^{-/-}$ mice exhibited impaired levels of CCL2 production as seen previously in Chapter 5 (Figure 8.5 A). CCL2 production in IL-$10^{-/-}$ mice was 1.5-2.5 fold higher than in WT mice, while IL-$6^{-/-}$ mice exhibited 2-3 fold lower levels of CCL2 than WT.

WT and IL-$6^{-/-}$ mice exhibited a transient expression of the neutrophil activating chemokine CXCL1 (KC), that occurred one hour after SES activation, and disappeared by three hours (Figure 8.5 B). This expression was elevated in IL-$6^{-/-}$ mice as was seen previously (Chapter 5). The absence of IL-10 led to a prolonged production of CXCL1 as compared to WT and IL-$6^{-/-}$ mice. In this respect, while IL-$10^{-/-}$ mice expressed similar levels of CXCL1 to the WT at one hour, this chemokine was still detectable in the peritoneal cavity of the IL-$10^{-/-}$ mice three hours post SES activation.

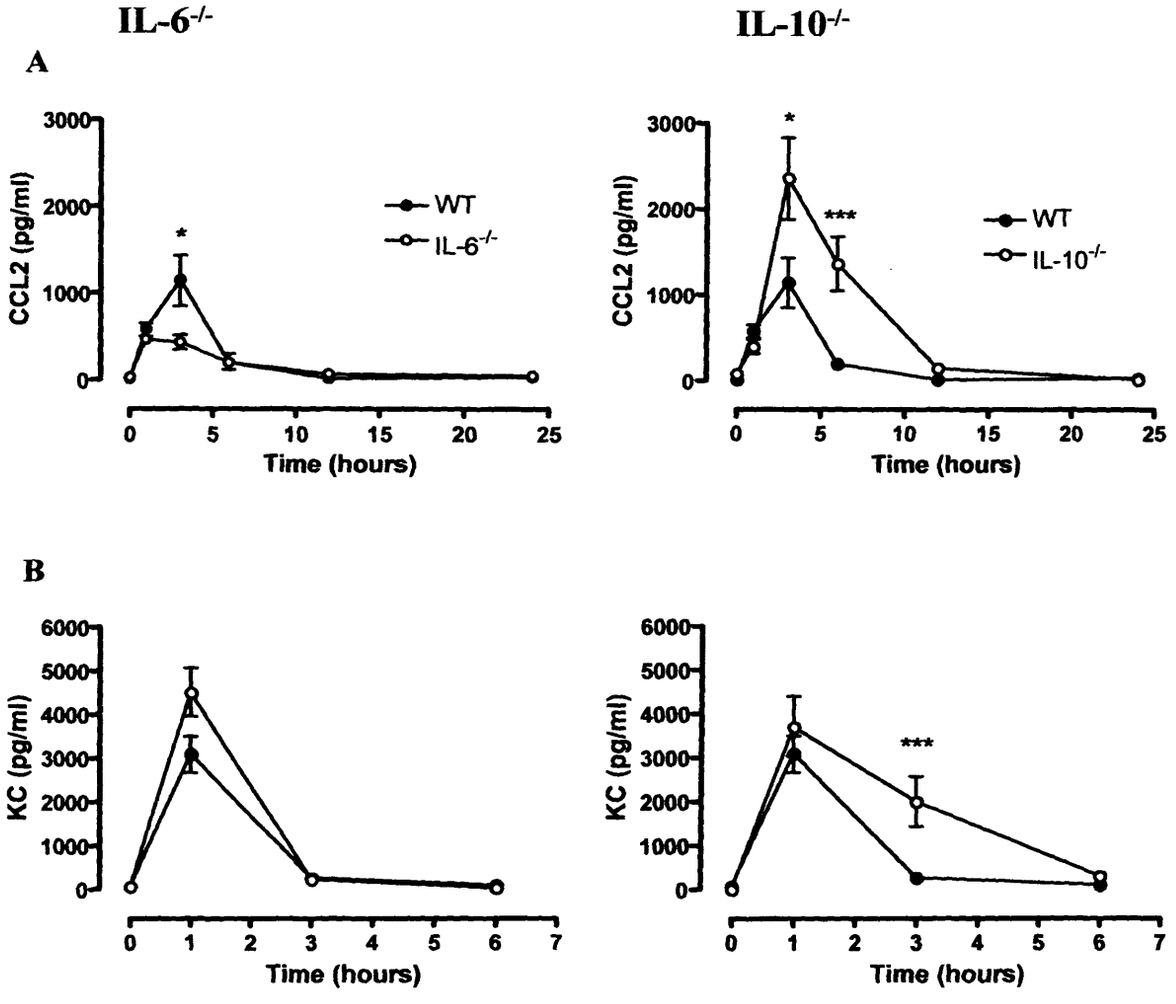


Figure 8.5: *Regulation of inflammatory chemokine expression by IL-6 and IL-10.* Peritoneal inflammation was induced in WT, IL-$6^{-/-}$and IL-$10^{-/-}$ mice by i.p administration of SES. Peritoneal fluids were recovered by lavage. Chemokines were then measured by ELISA . **(A)** CCL2 and **(B)** CXCL1 (KC) expression. Values represent the mean ± SEM, n=5-15 mice per time point, * $P < 0.05$, *** $P < 0.001$.

8.3.6 IL-10 impairs IL-6 production, and regulates sIL-6R levels

It has previously been published that IL-10 impairs the release of IL-6 by APC. To confirm this, IL-6 levels were measured in lavage fluids by ELISA (Figure 8.6 A). These results confirmed previous findings, with IL-$10^{-/-}$ mice exhibiting a large and prolonged increase in IL-6 production upon SES activation. In this respect, IL-6 levels were 10 fold higher than those observed in WT mice three hours after SES activation. Soluble IL-6R levels were also measured since the control of chemokine directed leukocyte trafficking and apoptosis by IL-6 trans-signalling is reliant on the formation of the IL-6/sIL-6R complex (Figure 8.6 B). Results revealed that sIL-6R levels were equivalent in WT and IL-$6^{-/-}$ mice, whilst IL-$10^{-/-}$ mice exhibited an enhanced production, suggesting that IL-10 influences the inflammatory shedding of IL-6R (Jones *et al* 1998). Finally IL-10 levels were measured to determine if IL-6 regulates the production of IL-10, as has previously been indicated by Fielding *et al* who showed an IL-6 dependent regulation of IL-10 production *in vivo* (Fielding *et al* unpublished). IL-10 levels peaked at one hour, with no significant differences existing between WT and IL-$6^{-/-}$ mice.

8.3.7 IL-10 regulates T cell recruitment

To investigate how IL-10 directed the recruitment of $CD4^+$ T cells and potentially their commitment, leukocytes were recovered from WT, IL-$10^{-/-}$ and IL-$6^{-/-}$ mice 24, 48 and 72 hours after the induction of peritoneal inflammation, and cultured *in vitro* (Section 2.3.8). Cells were then labelled with anti-CD4, IFN-γ and IL-17A to determine the phenotype of the activated T helper cell infiltrate. Cell numbers were calculated by multiplying the FACS gated population percentages by the total cell number (Figure 8.7). The data revealed that the absence of IL-10 led to an enhanced recruitment of $CD4^+$ T cells (Figure 8.7 A). In this respect, IL-$10^{-/-}$ mice exhibited a significantly increased recruitment of Th17 cells (Figure 8.7 B), while the recruitment of Th1 cells remained equivalent to WT mice (Figure 8.7 C). In comparison, IL-$6^{-/-}$ mice exhibited an impaired recruitment of total $CD4^+$ T helper cells (Figure 8.7 A), specifically Th1 (Figure 8.7 B) and Th17 cells (Figure 8.7 C). Whilst this was not observed from differential cell count data in Chapter 5, flow cytometry allows a specific analysis of $CD4^+$ T helper cells without interference from other lymphocyte subsets.

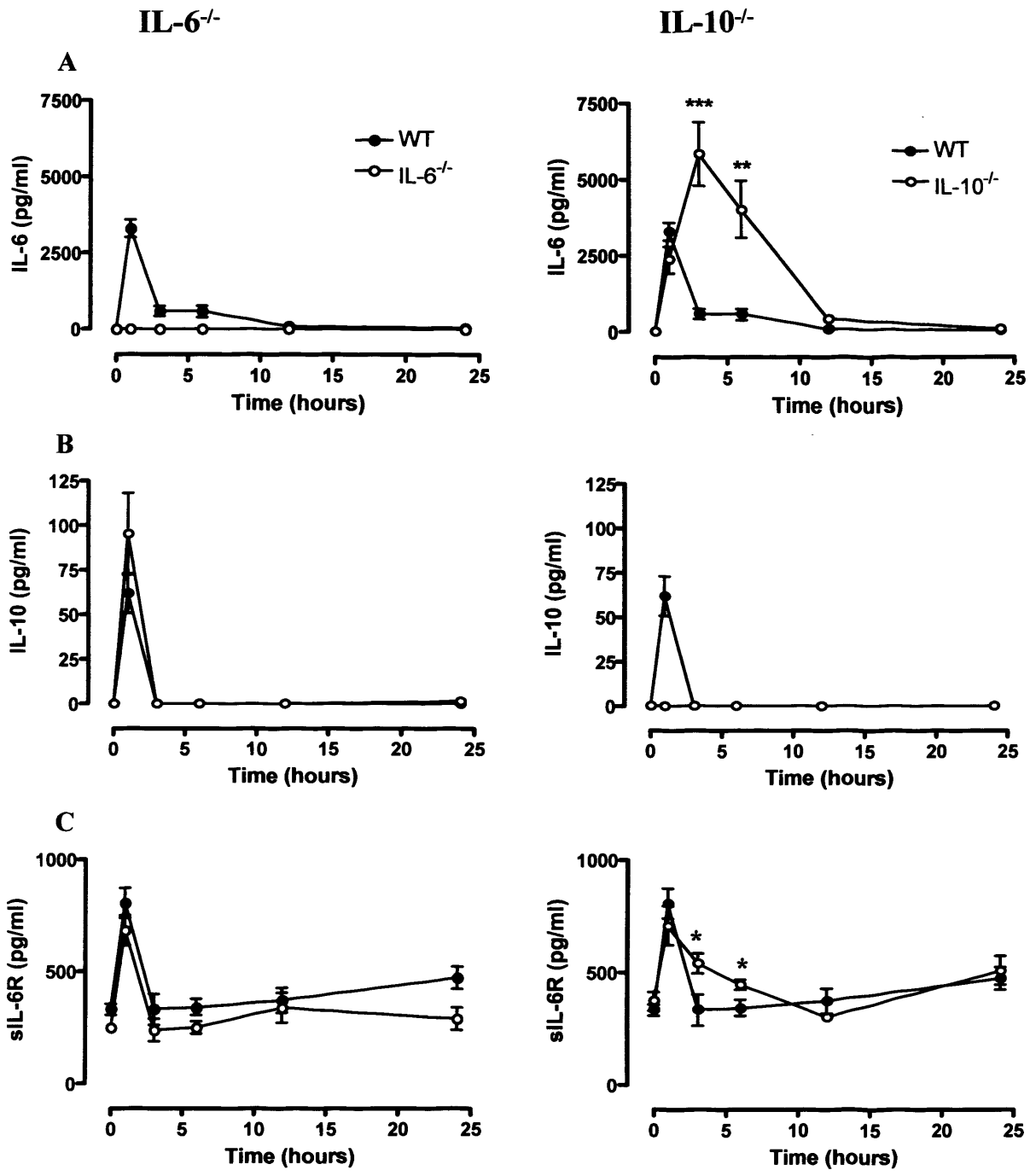


Figure 8.6: *Regulation of inflammatory cytokine expression by IL-6 and IL-10.* Peritoneal inflammation was induced in WT, IL-$6^{-/-}$and IL-$10^{-/-}$ mice by i.p administration of SES. Peritoneal fluids were recovered by lavage and cytokines quantified by ELISA . **(A)** IL-6, **(B)** sIL-6R and **(C)** IL-10 expression . Values represent the mean ± SEM, n=5-15 mice per time point, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

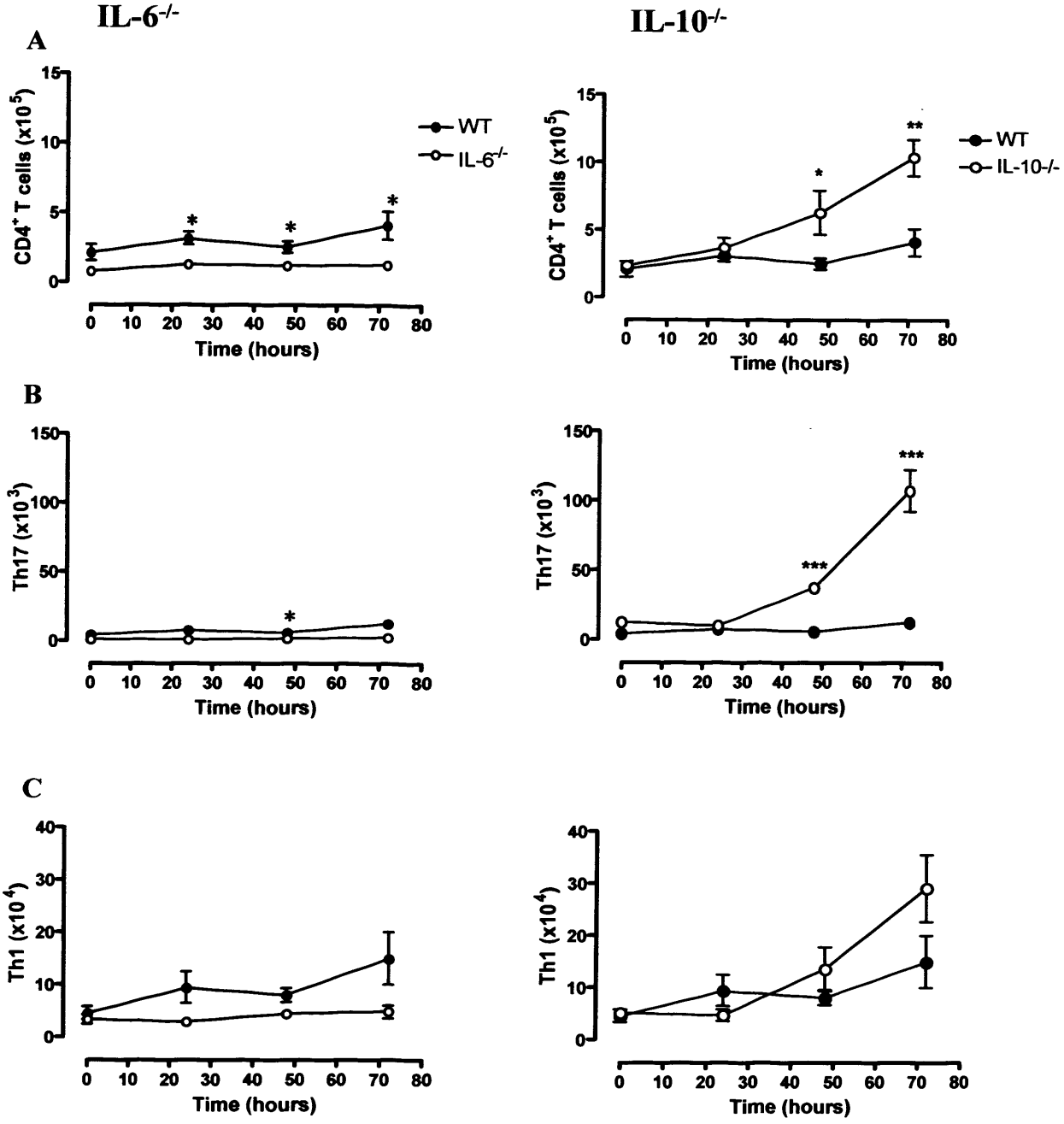


Figure 8.7: *T helper cell commitment and recruitment is regulated by IL-6 and IL-10.* Peritoneal inflammation was induced by i.p administration of SES and peritoneal leukocytes collected by lavage at defined intervals. $1x10^6$ Cells were cultured for 4 hours in 200µl of R10 medium supplemented with 50ng/ml PMA, 500ng/ml ionomycin and 3µM monensin. Cells were surface labelled with anti CD4, and intracellularly stained for IL-17A and IFN-γ. **(A)**The total number of $CD4^+$ T cells was calculated by multiplying the flow cytometry gated population percentage by the total cell number. **(B)** The number of IL-17A secreting Th17 cells at each time point. **(C)** The number of IFN-γ secreting Th1 cells at each time point. Values represent the mean ± SEM, n=5 mice per time point, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

8.3.8 IL-6 regulates the recruitment but not the commitment of IL-10 secreting T cells

To investigate how IL-6 directed the recruitment of IL-10 secreting T cells, leukocytes were recovered from WT, IL-$10^{-/-}$ and IL-$6^{-/-}$ mice 24, 48 and 72 hours after the induction of peritoneal inflammation, and cultured *in vitro* as described in Section 2.3.8. Cells were then labelled with anti-CD4 and IL-10 to determine the phenotype of the activated T helper cell infiltrate (Figure 8.8). The data revealed that IL-$6^{-/-}$ mice exhibited impaired recruitment of IL-10-secreting T cells. This was due to a global impairment of $CD4^{+}$ T helper cell recruitment as opposed to an impaired commitment. This was indicated by the equivalent proportion of IL-10 secreting T cells in both WT and IL-$6^{-/-}$ mice, suggesting that the differences in number of these cells arise as a result of an impaired recruitment of T cells in IL-$6^{-/-}$ mice.

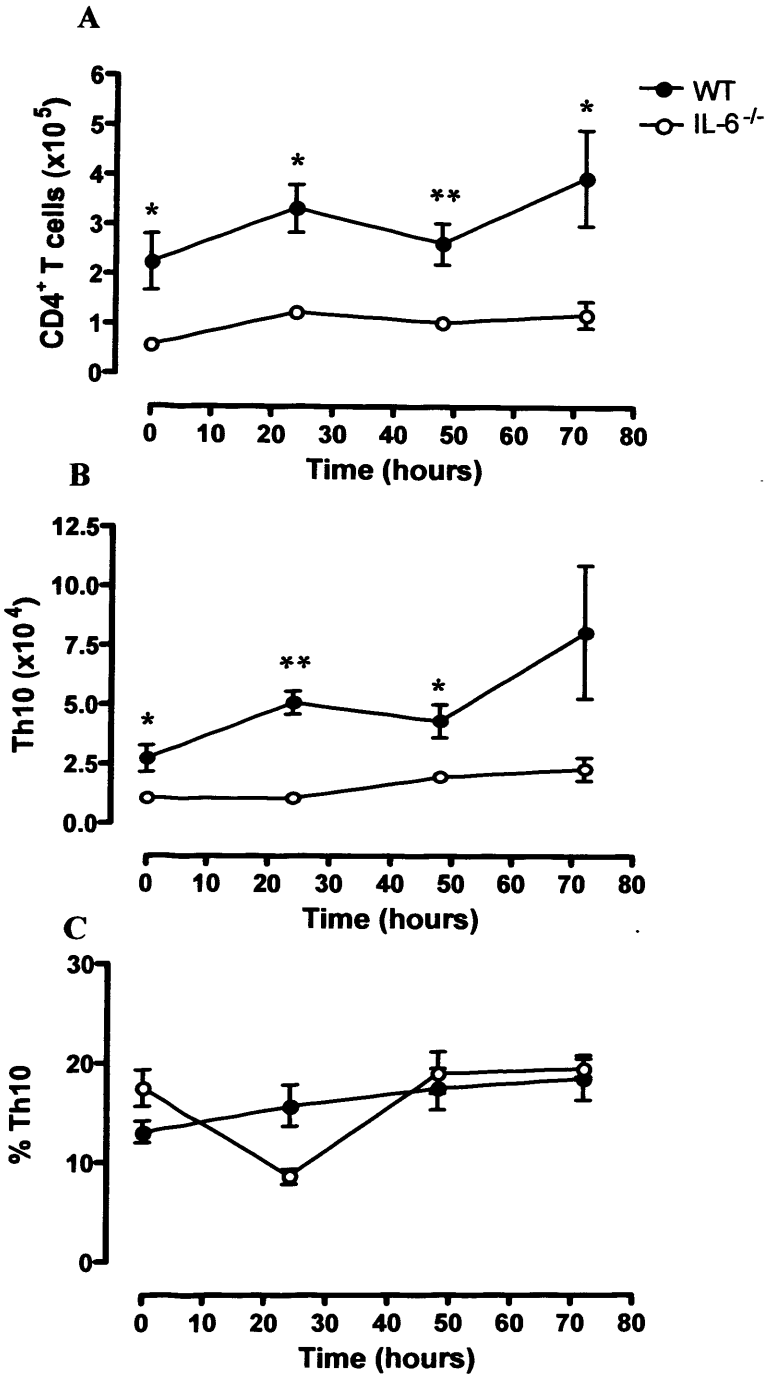


Figure 8.8: *IL-6 regulates the recruitment of IL-10 secreting T cells.* Peritoneal inflammation was induced by i.p administration of SES and peritoneal leukocytes collected by lavage at defined intervals. $1x10^6$ Cells were cultured for 4 hours in 200µl of R10 medium supplemented with 50ng/ml PMA, 500ng/ml ionomycin and 3µM monensin. Cells were surface labelled with anti-CD4, and intracellularly stained for IL-10 **(A)** The total number of $CD4^+$ T cells was calculated by multiplying the flow cytometry gated population percentage by the total cell number. **(B)** The number of IL-10 secreting Th10 cells at each time point. **(C)** The percentage of Th10 cells at each time point. Values represent the mean ± SEM, n=5 mice per time point, * $P < 0.05$, ** $P \leq 0.01$.

8.4 Discussion

Previous literature has reported an interplay between IL-6 and IL-10 (Niemand *et al* 2003). The data presented in this Chapter support a role for IL-10 in regulating both the innate and adaptive arms of the inflammatory response. These studies show that IL-10 appears to limit neutrophil recruitment by its control of CXCL1 (KC) production in response to SES activation. IL-10 is known to inhibit IL-1, TNFα and IL-17 expression in macrophages, all of which are important for driving the expression of CXCL1 (KC) by mesothelial cells and the subsequent recruitment of neutrophils (Gu *et al* 2008, Witowski *et al* 2000, Fiorentino *et al* 1991, Chernoff *et al* 1995, Hurst *et al* 2001). This therefore provides a potential mechanism by which IL-10 limits neutrophil recruitment. Neutrophils are important for microbial killing, an integral part of the innate immune response, but the anti-microbial agents and reactive oxygen species released by these leukocytes are well known to have the potential to cause collateral tissue damage (Mahmudi-Azer and Eeden 2003). In this respect, IL-10 potentially regulates neutrophil recruitment to ensure that there is an appropriate level of infiltrate to clear the pathogen, whilst avoiding tissue damage that may be caused by the presence of excessive numbers of these cells. Interestingly, current studies in the group, using a model of live bacterial infection, have shown that bacterial clearance is significantly improved in IL-$10^{-/-}$ (Coles *et al* unpublished). This supports other studies that have shown improved clearance in IL-10 deficient mice, indicating that IL-10 facilitates the outgrowth and dissemination of bacteria during infections (Sewnath *et al* 2001). IL-10 therefore can be shown to control the rate of bacterial clearance by regulating the recruitment of neutrophils to the peritoneal cavity upon induction of inflammation. IL-10 may also control the rate of bacterial clearance *via* the regulation of neutrophil activation, activity or their apoptotic clearance following the phagocytic uptake of bacteria. The increased recruitment of neutrophils and prolonged production of CXCL1 (KC) observed in IL-$10^{-/-}$ mice (Figures 8.3A and 8.5B respectively), correlated with a prolonged production of sIL-6R in these mice (Figure 8.6C). Since neutrophils are the main source of sIL-6R during peritoneal inflammation, and shedding is usually triggered by activation or apoptosis, these results imply that IL-10 may also negatively regulate the chemokine (KC)-directed activation or apoptotic clearance of these cells, accounting for the increased sIL-6R levels in the

peritoneal cavity and the improved microbial clearance capacity in the IL-$10^{-/-}$ mice. In this respect, IL-10 is known to inhibit bacterial phagocytosis and attenuate neutrophil microbicidal activity towards internalised bacteria (Laichalk *et al* 1996). Further studies would need to be performed to properly demonstrate this, which could investigate the activation, killing capacity and apoptotic clearance of neutrophils from a STAT3 conditional knock out mouse.

Data presented in Figure 8.1 showed that the predominant IL-10 secreting cell types in the peritoneal cavity upon SES activation were the resident macrophages, and this appears to be IL-6 regulated since IL-$6^{-/-}$ macrophages produced lower levels of IL-10. This supports previous data showing that IL-10 levels were elevated in the gp130 knock in mice, that exhibit constitutive STAT1/3 signalling, and this was attenuated when these mice were crossed to an IL-$6^{-/-}$ background, implying an IL-6 dependent release of IL-10 in these mice *in vivo* (Fielding *et al* unpublished). However in this regard a detectable difference in the overall IL-10 levels in the peritoneal cavity was not observed (Figure 8.5).

IL-10 production by the resident macrophages implicates these resident cells in a role for the regulation of the recruitment of neutrophils and other cell types during acute peritonitis through their production of IL-10. Previous work has shown that depletion of resident peritoneal macrophages resulted in increased neutrophil recruitment upon zymosan-induced peritonitis, which was attributed to an inhibitory action of macrophage derived IL-10 on chemokine production and neutrophil recruitment (Ajuebor *et al* 1999). This supports the general belief that resident macrophages are primarily involved in immune modulation and resolution, and infers a role for these cells in immunoregulation *via* the release of IL-10. However this contrasts with data presented by Callhier *et al* who showed that macrophage depletion resulted in impaired neutrophil recruitment following thioglycollate-induced peritonitis (Callhier *et al* 2005). This could be explained by the use of different inflammatory models.

The results discussed in this Chapter have also highlighted a role for IL-10 in the regulation of monocyte recruitment during peritoneal inflammation. In this respect, IL-10 limits the recruitment of monocytic cells during the peritoneal inflammatory response, which would appear to be due to its negative regulation of the monocyte chemoattractant CCL2. Evidence has shown that upon migration across the endothelium, inflammatory monocytes are able to differentiate into both macrophages and DC (Randolph *et al* 1998, Gordon and Taylor 2005). As discussed in Chapter 6, macrophages and DC are both known to augment inflammation-induced tissue damage (Duffield *et al* 2000, Duffield *et al* 2001, Kipari *et al* 2002, Duffield *et al* 2001, Stary *et al* 2007, Serbina *et al* 2003) whilst macrophages are also involved in tissue repair, promoting fibrosis and the deposition of extracellular matrix (Deodhar *et al* 1997, Gordon 2003). These activities therefore potentially implicate monocytic cells in contributing to the induction of uncontrolled fibrotic processes when present in large numbers. Consequently, it is proposed, that IL-10 functions to limit monocytic cell recruitment in order to contain tissue injury or fibrosis afflicted by these cells. Previous studies by the group have shown that IL-6 differentially effects mesothelial expression of neutrophil activating chemokines and chemokines associated with mononuclear cell recruitment. In this respect, IL-6 is important in inducing a switch from neutrophil activating chemokine production, to mononuclear chemokine production thereby promoting the transition from innate to acquired immunity (Jones 2005, Hurst *et al* 2001, McLoughlin *et al* 2005). In the current study, data has shown that IL-$10^{-/-}$ mice have significantly elevated levels of peritoneal IL-6, which is potentially a result of increased IL-6 production by monocytic cells, which would confirm previous reports that IL-10 inhibits IL-6 release from activated monocytes (Fiorentino *et al* 1991). Since IL-6 drives the molecular switch to turn on CCL2 expression by mesothelial cells, it is proposed that the mechanism by which IL-10 limits monocyte recruitment is *via* both its negative control of IL-6 mediated signalling and its ability to inhibit IL-6 production. Further experiments would need to be performed to confirm this, and could potentially be demonstrated by reconstituting IL-10 in IL-$10^{-/-}$ mice by administering a defined dose of recombinant IL-10 or by introducing blocking antibodies for IL-10 in WT mice, and monitoring the levels of IL-6 and CCL2 upon induction of inflammation.

Multi-parameter flow cytometry revealed an increased recruitment of both infiltrating monocytes and DC in IL-$10^{-/-}$ mice. Based on previous literature stating that IL-10 inhibits IL-6 production by APC (Fiorentino *et al* 1991, Chernoff *et al* 1995), it is proposed that the recruited DC observed in IL-$10^{-/-}$ mice (Figure 8.4) may produce elevated levels of IL-6. This would implicate them in facilitating an enhanced adaptive immune response *via* an increased ability to suppress T-reg activity, promote T cell commitment and activation and promote the T cell memory response (Pasare and Medzhitov 2003, Longhi *et al* 2008, Park *et al* 2004, Veldhoen *et al* 2006, Betelli *et al* 2006). Evidence for this was shown following investigations into the trafficking of T cells in IL-$10^{-/-}$ mice, which is discussed below. Overall, this provides a potential role for IL-10 in limiting the recruitment of inflammatory DC during inflammation, and their secretion of IL-6, thereby limiting the potential for these APC to suppress T-regs and activate T helper cells. This would support evidence that has shown that IL-10 attenuates the adaptive immune response (Dai *et al* 1997).

The studies described in this Chapter finally showed that IL-10 also limits lymphocyte recruitment during peritoneal inflammation. Flow cytometric analysis of the lymphocyte population showed that IL-10 regulates the recruitment of $CD4^+$ T cells, and may also regulate their commitment. In this respect, IL-10 appears to suppress a Th-17 phenotype, but does not appear to regulate Th-1 cell commitment. This could be explained by the increased expression of IL-6 in IL-$10^{-/-}$ mice, which is known to drive Th-17 cell commitment (Bettelli *et al* 2007, Veldhoen *et al* 2006) and the fact that IL-10 has been shown to block Th17 commitment *ex vivo* (Gu *et al* 2008). Th-17 cells have been widely associated with autoimmune disease pathology (Langrish *et al* 2005), revealing another mechanism by which IL-10 is likely to dampen the immune response. Alternatively, the increased numbers of Th17 cells in IL-$10^{-/-}$ mice could simply be a result of increased expansion and recruitment of these cells, potentially facilitated by the IL-6 mediated inhibition of T-regs, and further work would be required to determine this. The opposite effect is true for IL-$6^{-/-}$ mice, which exhibit impaired recruitment of $CD4^+$ T cells (Figure 8.7), which is expected since IL-6 trans-signalling drives the recruitment of chemokine

mediated T cell recruitment (McLoughlin *et al* 2005). In this respect IL-$6^{-/-}$ mice show an impaired commitment and recruitment of Th-17, Th1 and IL-10 secreting T cells. IL-6 has previously been shown to drive the commitment of IL-17 secreting T cells (Bettelli *et al* 2006, Veldhoen *et al* 2006) and also induce IL-10 secretion by Th1, Th2 and Th17 cells (Stumhofer *et al* 2007), thereby presenting an alternative mechanism by which IL-$6^{-/-}$ mice suppress the T cell content within the peritoneal cavity.

When the profile of lymphocyte recruitment is overlaid with the profile of bacterial clearance obtained from studies using a model of live bacterial peritonitis, it can be seen that the majority of bacteria is cleared between 18 and 24 hours of infection whilst the recruitment of T helper cells occurs between 36-72 hours post infection (Coles *et al* unpublished, data not shown). This implies that the recruited T cell population does not contribute to the bacterial clearance, which brings into question their role in the inflammatory response. One possible explanation could be that they direct the re-establishment of the resident monocytic populations by the production of soluble mediators which have the potential to drive macrophage or DC differentiation from recruited monocytes. Alternatively they may control memory responses by the formation of a memory T cell pool within the cavity, providing a faster immunological response during recurrent infection. Finally, they may contribute quite substantially to tissue injury or the induction of membrane fibrosis, which has previously been associated with increased recruitment of IFN-γ secreting T cells and the activation of a STAT1 driven response (Fielding *et al* 2009, submitted).

In summary the findings outlined in this Chapter have effectively demonstrated that IL-10 plays an immunoregulatory role by limiting leukocyte recruitment during the peritoneal inflammatory response. This supports its known role as an anti-inflammatory cytokine that serves to limit the damage triggered by the pathogen by contributing to its destruction, while simultaneously limiting collateral damage by dampening the immune response (Moore *et al* 2001). These anti-inflammatory activities appear to be mediated through the interplay between IL-10 and IL-6, with the regulation of IL-6 by IL-10 being implicated as a mechanism for the control of IL-6 directed production of specific

chemokines and the subsequent chemokine directed recruitment of inflammatory leukocytes.

It would be interesting to see how the integrity of the peritoneal membrane of IL-$10^{-/-}$ mice is affected during a repeat model of inflammation. Since the data presented in this Chapter support a role for IL-10 in limiting the inflammatory response by suppressing the excessive recruitment of activated leukocytes, one would expect that in the absence of this cytokine, tissue damage to the peritoneal membrane would be significantly enhanced. This may arise due to an increased presence of neutrophils and mononuclear cells, which are known to contribute to tissue injury and fibrosis by their production of pro-fibrotic agents including reactive oxygen species, nitric oxide and TGF-β, but may also be driven by increased numbers of Th-17 cells (Duffield *et al* 2000, Duffield *et al* 2001, Kipari *et al* 2002, Duffield *et al* 2001, Stary *et al* 2007, Serbina *et al* 2003, Deodhar *et al* 1997, Gordon 2003). Importantly, IL-17 has recently been shown to induce MMP and TIMP activity, which, if unbalanced, could contribute to aberrant tissue metabolism or collagen deposition (Koshy *et al* 2002, Jovanovic *et al* 2001). This could be confirmed using the model of repeat inflammation described in Chapter 6, and may implicate IL-10 as a potential therapeutic molecule, which could be administered to prevent the onset of chronic inflammatory or autoimmune diseases, which are strongly associated with high levels of detrimental tissue damage.

CHAPTER 9

General Discussion

9. Discussion

Prior experiments have illustrated that IL-6 plays an important role in controlling both the chemokine directed recruitment and apoptotic removal of neutrophils and lymphocytes (Hurst *et al* 2001, McLoughlin *et al* 2003, McLoughlin *et al* 2005). However little is currently known about the role of IL-6 in governing the phenotype, activation and inflammatory trafficking of monocytic cells. The studies described in this thesis have provided a vast array of answers to these questions, by focusing on the IL-6 regulation of both resident and infiltrating monocytic cells within the peritoneal cavity. These studies also introduce the role of IL-10 in regulating the peritoneal inflammatory response, with a focus on leukocyte recruitment and chemokine production. These are discussed herein.

9.1 IL-6 exerts an immunosuppressive role in resident peritoneal DC

The findings presented in Chapter 3 demonstrated that IL-6 promotes resident DC numbers in the peritoneal cavity. This supports previous findings stating that IL-6 promotes the differentiation of DC from their precursors (Brasel *et al* 2000, Santiago-Schwartz *et al* 1995, Bernhard *et al* 2000). Findings outlined in Chapters 3 and 4 showed that these resident cells demonstrated an activation-induced loss of the IL-6R, coinciding with an up-regulation of maturation markers MHC II and CD83, thereby rendering them unresponsive to IL-6 stimulation. This data would appear to provide a potential mechanism by which DC liberate themselves from the immunosuppressive constraint exerted by IL-6, which is known to maintain resident DC in an immature state (Park *et al* 2004). In this respect, IL-6 mediated STAT3 activation was shown to be required for the suppression of LPS-induced bone marrow derived DC maturation, thereby implicating IL-6 as a potent regulator of DC maturation thought to continually stimulate and maintain immature DC in the steady state (Park *et al* 2004). *Ex vivo* activation of resident DC, contributed to an induction of a wide range of inflammatory mediators, including IL-6 (Chapter 3). IL-6 is important for the function of mature DC, the activation of naive T helper cells (Bleier *et al* 2004), and is known to attenuate the activity of antigen specific T-regulatory cells (Longhi *et al* 2009, Pasare and Medzhitov 2003). In this respect, initial *in vitro* studies using isolated peritoneal DC and T cells, showed that these cells can promote generation of both the IFN-γ and IL-17A-secreting T cells (data not shown).

Therefore, these studies have provided support to previous work, thereby shaping a potential two-sided immunoregulatory model for IL-6 in the control of DC activity (Figure 9.1): 1) IL-6 can initially exert an immunosuppressive constraint on resident DC by maintaining them in the immature state (Park *et al* 2004). Upon activation, DC down-modulate their surface IL-6 as part of the maturation process thereby becoming unresponsive to IL-6 stimulation and liberating themselves from the suppressive effects of IL-6 (Chapters 3&4). This coincides with an up-regulation of MHCII (Chapter 4), potentially ameliorating their antigen presenting capacity, but not CCR7. The expression of both MHC II and CCR7 have previously been shown to be negatively regulated by IL-6 (Kitamura *et al* 2005, Hedge *et al* 2004). 2) Following this, inflammatory release of IL-6 by resident DC following SES-mediated TLR2 activation (Chapter 3) would be predicted to take on a pro-inflammatory role, and is important for DC mediated T cell activation and the suppression of $Foxp3^+$ T-reg activity (Longhi *et al* 2009, Pasare and Medzhitov 2003, Bleier *et al* 2004).

Further work would be required to rule out an IL-6 trans-signalling responsiveness by the resident DC following activation, and to determine their ability to drive effector T helper cell expansion and suppression of T regulatory cell activity.

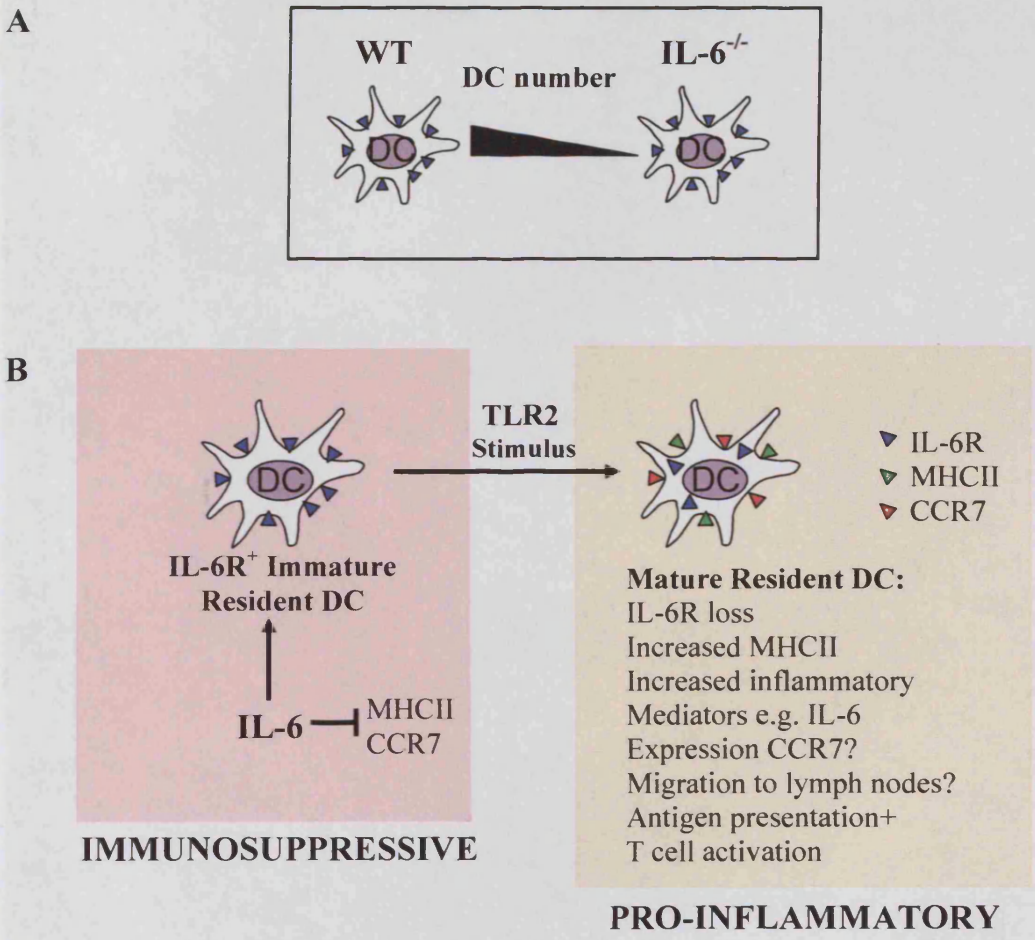


Figure 9.1 *Immunosuppressive and pro-inflammatory IL-6* **(A)** IL-6 positively regulates resident DC numbers in the murine peritoneal cavity (Chapter 3). This supports previous findings stating that IL-6 promotes the differentiation of DC from their precursors (Brasel *et al* 2000, Santiago-Schwartz *et al* 1995, Bernhard *et al* 2000) **(B)** Removal of IL-6 responsiveness is predicted to direct the transition from an immature resident DC to mature DC capable of T cell activation. It is hypothesised that resident DC may down-regulate their surface IL-6R upon activation (Chapter 4) in order to allow maturation, since previous work has defined a role for IL-6 in maintaining DC in an immature state (Kitamura *et al* 2004, Hedge *et al* 2005). SES mediated TLR2 stimulation induces an up-regulation in IL-6 production (Chapter 3), thereby potentially promoting DC activity.

9.2 Regulation of leukocyte trafficking by IL-6

Over the past 5-10 years, our group has defined a role for IL-6 in regulating the inflammatory response during bacterial peritonitis. Findings have characterised the IL-6 control of chemokine directed recruitment and apoptotic clearance of neutrophils and lymphocytes (Hurst *et al* 2001, McLoughlin *et al* 2003, 2004, 2005) and the important role for IL-6 in driving microbial clearance (Coles *et al* unpublished). These protective qualities of IL-6 biology are however tempered by the detrimental outcomes associated with IL-6 activity in driving the induction of chronic inflammatory damage and the associated fibrosis (Nowell *et al* 2003, 2009, Fielding *et al* 2009, submitted). In this respect, many members of the group have contributed to work that has shown that IL-$6^{-/-}$ mice are protected from inflammatory induced peritoneal fibrosis as a consequence of recurrent inflammatory challenge. Work in this thesis has contributed to the group effort by identifying the role of IL-6 in directing the trafficking of defined subsets of monocytic cells. Monocytic cell subsets, including monocytes, macrophages and DC, are important in the immune response both for the removal of the pathogen, clearance of apoptotic cells and activation of T helper cells. Studies outlined in this thesis have shown that while IL-6 does not regulate the trafficking of these cells during a single episode of acute inflammation (Chapter 5), it does contribute to their dysregulation during a repeat inflammatory model (Chapter 6), resulting in retention of resident populations, and increased recruitment of infiltrating monocytes (Figure 9.2). This phenomenon appears to be chemokine independent since no changes in KC, CCL2 or CCL5 levels were observed following multiple rounds of inflammation, although other chemokines would need to explored to be conclusive (Chapter 6). Consequently this increased retention and recruitment could be explained by an increased number of circulating monocytes, a local expansion of monocytic cells, or reduced adhesion of monocytic subsets to the peritoneal membrane. Future experiments are required to define this.

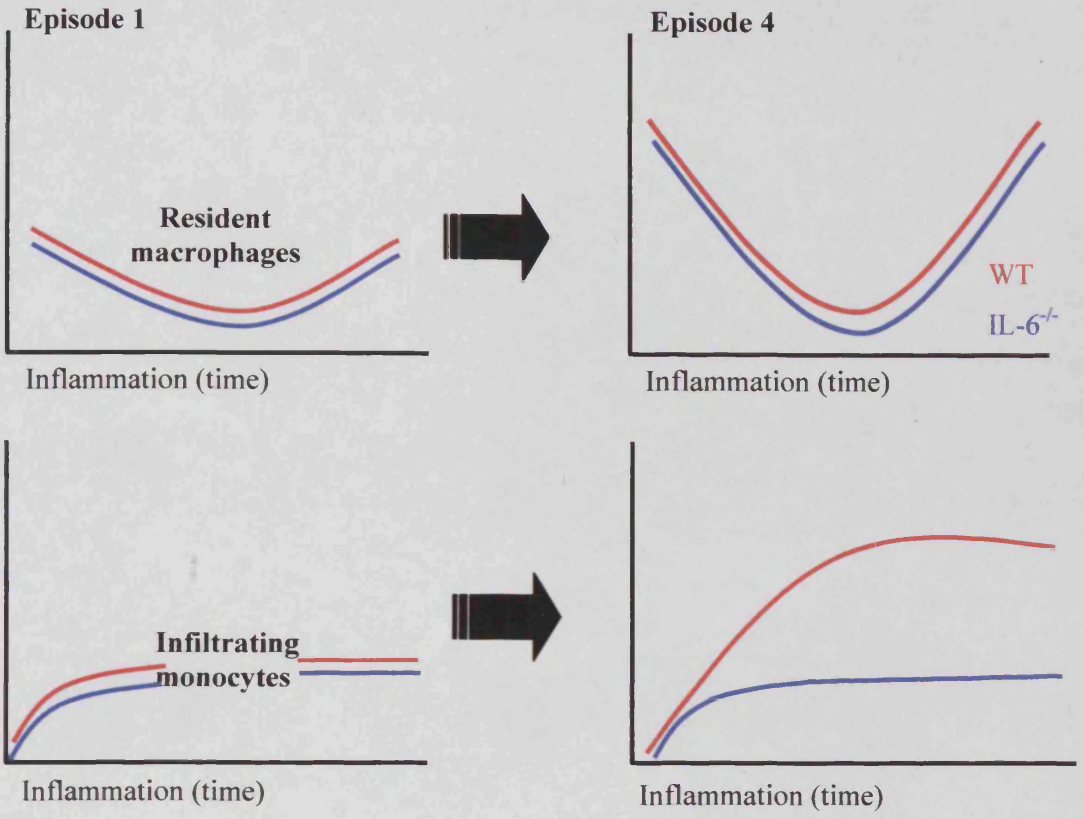


Figure 9.2 *The distortion of monocytic cell trafficking following repeated rounds of inflammation.* Red lines represent WT leukocyte trafficking, blue lines represent IL-$6^{-/-}$ mice. Repeated inflammatory episodes in WT mice result in an enhanced recruitment of infiltrating monocytes and increased retention of resident macrophages (Chapter 6). The increased and retained inflammatory monocytic infiltrate may contribute to the tissue damage associated with recurrent inflammation. IL-6 appears to drive the increased recruitment of infiltrating monocytes, but not the retention of resident populations, implicating the recruited monocytes as the predominant monocytic cell subset contributing to fibrosis. This correlates with the widely accepted opinion that recruited monocytes macrophages are ascribed a much greater destructive potential than their resident family members (Gordon 1995).

Monocytic cells are known contributors to tissue damage and degradation, due to their production of reactive oxygen species, nitric oxide and degradative enzymes such as metalloproteinases (MMPs) (Duffield *et al* 2000, Duffield *et al* 2001, Kipari *et al* 2002, Duffield *et al* 2001, Serbina *et al* 2003, Stary *et al* 2007, Gueders *et al* 2006). Additionally some macrophages have the ability to facilitate tissue repair and promote fibrosis and the deposition of extracellular matrix (Deodhar *et al* 1997, Gordon 2003). Impaired regulation of either of these processes could lead to uncontrolled injury and overcompensated repair mechanisms. Consequently monocytic cells have the potential to contribute substantially to tissue injury and the induction of peritoneal membrane scarring and fibrosis.

Given the significance of monocytic cells in mediating fibrotic processes, the trafficking of these cells was analysed in a recurrent inflammatory model, utilising flow cytometry to enable an independent analysis of resident macrophage and DC populations and recruited monocytes. Analysis of the resident monocytic populations (macrophage and DC) highlighted a retention of these cells following repeated SES activation (Chapter 6). However it was concluded that this retention of resident populations was unlikely to contribute substantially to tissue injury or fibrosis since the IL-$6^{-/-}$ mice, which have a protective phenotype, exhibited an equivalent retention of these resident populations.
Adding to this, infiltrating inflammatory monocytes are considered to differentiate into inflammatory macrophages or DC upon recruitment to the inflammatory site (Geissman *et al* 2003), and elicited inflammatory macrophages have been ascribed a greater destructive potential than resident macrophages (Gordon 1995). Consequently, the enhanced infiltration of monocytes, observed in the WT mice following repeated inflammatory episodes, are likely to be the predominant monocytic cell contributors to fibrosis in this model. The differentiation of these recruited cells into macrophages and DC would lead to their potential contribution to peritoneal membrane thickening either *via* the damage of surrounding tissues by the release of nitric oxide and reactive oxygen species, or by the induction of fibrosis. Macrophages are known to promote fibrosis either by the regulation of fibroblast recruitment, or by contributing to the production of extracellular matrix (Deodhar *et al* 1997, Gordon 2003). Furthermore, macrophages have

been shown previously to release TGF-β (Khalil *et al* 1996, Bissell *et al* 1995, Letterio and Roberts 1998), and this growth factor is known to drive epithelial to mesenchymal transition (EMT), the dysregulation of which is key for the induction of membrane thickening or fibrosis (Selgas *et al* 2006). This would not normally occur in an acute resolving inflammatory episode, however following recurrent inflammatory events, tissue injury may become more severe, and potential increases in local TGF-β levels may increase monocytic cell recruitment or activation status to promote EMT and subsequent fibrotic changes.

In addition to its control of monocytic cell recruitment during repeated inflammatory episodes, IL-6 similarly induced an increased recruitment of other inflammatory cell types, including neutrophils (Chapter 6), and effector Th1 and Th17 cells (Jones *et al*- under revision, J.Immunol. 2009). Neutrophils can contribute to tissue injury by their production of anti-microbial peptides and reactive oxygen species (Mahmudi-Azer and Eeden 2003), whilst the IL-6 control of effector T cell migration, activation-induced T cell clearance, and suppression of DC and regulatory T cell activity may lead to the retention of activated T cell subsets within the affected or damaged tissue (Mahmudi-Azer and Eeden 2003, Teague *et al* 2000, Longhi *et al* 2008). In this respect, Fielding *et al* have shown that the development of peritoneal membrane fibrosis as a consequence of repeated inflammatory episodes, correlates with enhanced STAT3 activity and the emergence of STAT1 signalling within the peritoneal membrane of WT but not IL-$6^{-/-}$ mice. This shift in the STAT1/STAT3 balance was attributable to the IL-6 dependent regulation of IFN-γ secreting T cells. In this respect IL-6 is proposed to drive the development of an IFN-γ secreting T cell population, potentially *via* the attenuation of $CD4^{+}CD25^{+}$ T regulatory cell activity, thereby resulting in an increased recruitment of IFN-γ secreting T cells and a subsequent initiation of STAT1-mediated fibrotic events (e.g. MMP regulation) within the peritoneal membrane (Fielding *et al* 2009, submitted). Whilst the IL-6 dependent induction of IFN-γ responsiveness further underlines the role of IL-6 in T cell memory expansion, IL-6 suppression of T-reg activity has been linked to the positive control of type I IFN responses and the development of adaptive immunity by DC (Detournay *et al* 2005, Longhi *et al* 2008).

A combined regulation of cellular events by IL-6, as detailed above, including the potential for uncontrolled activity of regulatory T cells, resulting in an attenuated expansion of pro-inflammatory effector and memory T cells, may help to explain why IL-$6^{-/-}$ animals display a highly protected phenotype in experimental models of chronic peritonitis. Contributing to this is their protection against dysregulated leukocyte recruitment, including infiltrating monocytes, neutrophils and lymphocytes.

It is therefore conceivable that the enhanced recruitment of monocytes, T helper cells and neutrophils following repeated SES activation (Chapter 6) are collectively responsible for driving fibrosis. This may also be coupled with the ability of IL-6 to suppress T regulatory cell activity, thereby promoting pro-inflammatory T helper cell differentiation and activation. Therefore during a persistent inflammatory condition, IL-6 evolves from providing a tight control of the inflammatory response, driving the transition from innate to acquired immunity, and promoting the successful resolution of the inflammatory response, to facilitating the increased recruitment of pro-inflammatory leukocytes, which contribute to membrane fibrosis. The cause of this switch is still unclear, and future experiments are required to unravel this phenomenon. However a specific balance in the control of STAT1 and STAT3 activity may contribute to these inflammatory changes (Figure 9.3) (Fielding *et al* 2009, submitted).

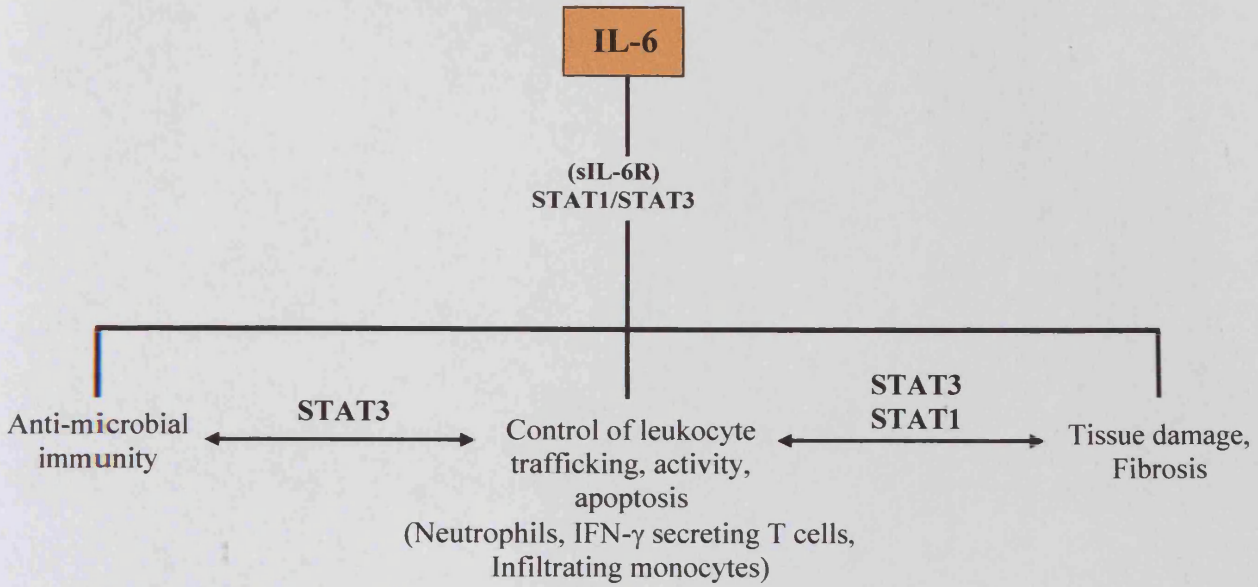


Figure 9.3: *The appropriate balance of STAT activity is required for the successful orchestration of the inflammatory response leading to the competent cell mediated immunity against infection whilst limiting collateral tissue injury.* IL-6 mediated STAT3 activation is vital for the resolution of acute neutrophil infiltration and the promotion of chemokine directed T cell trafficking (Hurst *et al* 2001, McLoughlin *et al* 2005, Fielding *et al* 2008, Nowell *et al* 2009). IL-6 is also required for the generation of IFN-γ secreting T cells (Fielding *et al* 2009, submitted), potentially through a suppression of T regulatory cell activity (Longhi *et al* 2008, Pasare and Medhitzov 2003), and their subsequent recruitment (Fielding *et al* 2009, submitted). Increased recruitment of these cells during recurrent inflammation results in an IFN-γ induced STAT1 activation in the peritoneal membrane, which is associated with, and thought to facilitate tissue injury and fibrosis during a persistent inflammatory condition (Fielding *et al* 2009, submitted). This detrimental biology exhibited by IL-6 must be balanced by the importance of IL-6 in regulating anti-microbial immunity. Mice deficient in IL-6 exhibit impaired clearance and increased dissemination (Coles *et al* unpublished). Since IL-6/STAT3 activity is important for neutrophil resolution and T cell recruitment, it is proposed that the ability of IL-6 to control STAT3 mediated processes is required for successful anti-microbial immunity.

9.3 IL-10: Anti-inflammatory roles in peritonitis and the interplay with IL-6.

The protective qualities afforded by IL-6 during infection are balanced by the deleterious outcomes associated with IL-6 activity in chronic disease. This is mirrored by the activity of IL-10, which hinders anti-microbial immunity (Coles *et al* unpublished, Sewnath *et al* 2001) but provides protection in chronic inflammation (Chmiel *et al* 1999, Mu *et al* 2005) (Figure 9.4). An interplay between IL-6 and IL-10 has been known to exist for some time, with studies showing that IL-10 inhibits IL-6 production by monocytes (Fiorentino *et al* 1991), and also inhibits IL-6 mediated STAT3 activation by the induction of the negative regulator SOCS3 (Niemand *et al* 2003). Studies detailed in Chapter 8 of this thesis have added further evidence for the known interplay between IL-6 and IL-10. The predominant cell type to secrete IL-10 upon SES/TLR2 mediated activation was the resident macrophage population (Chapter 8). Mice deficient in IL-10 produced very enhanced levels of IL-6 during acute peritoneal inflammation, which is potentially attributable to the IL-10 inhibition of IL-6 production by monocytic cells (Fiorentino *et al* 1991). In addition, other work by the group has shown that IL-6 mediated STAT activity induces an up-regulation of IL-10 in the peritoneal cavity following SES activation, indicating a control of IL-10 expression by IL-6 (Fielding *et al* unpublished). This is suggestive of a negative feedback regulation of IL-6 production by resident macrophages, mediated by their production of IL-10. In combination these studies show an important interplay between the pro-inflammatory IL-6 and anti-inflammatory IL-10, demonstrated by the IL-6 induction of IL-10 expression, and the IL-10 inhibition of IL-6 expression and activation of STAT3 (Fielding *et al* 2009 submitted, Fiorentino *et al* 1991, Niemand *et al* 2003). The balance of this interplay is important for the tight regulation of the inflammatory response.

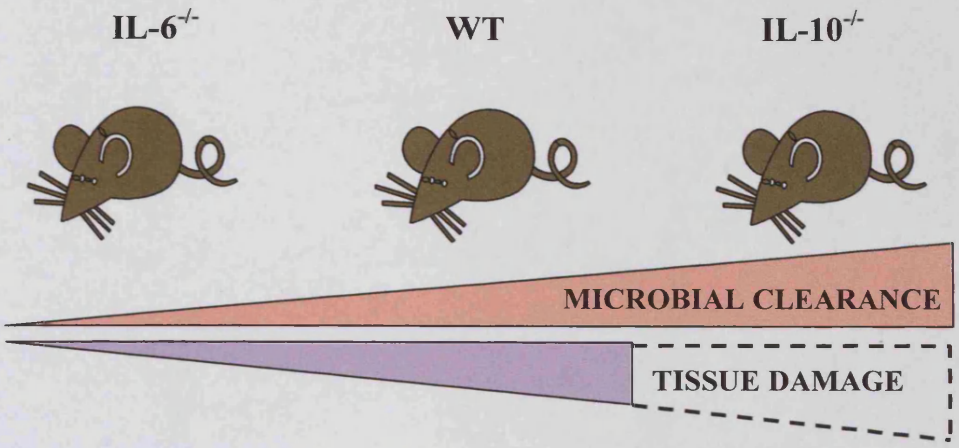


Figure 9.4: *IL-10 and IL-6 must be finely balanced to promote microbial killing and clearance, whilst preventing tissue injury.* WT mice exhibit tissue fibrosis of the peritoneal membrane upon repeated inflammatory insult, whilst IL-$6^{-/-}$ mice remain protected (Chapter 6). WT mice exhibit increased recruitment of inflammatory leukocytes, including infiltrating monocytes that are likely to contribute to the tissue damage seen in these mice (Chapter 6). Since IL-$10^{-/-}$ mice exhibit enhanced recruitment of inflammatory leukocytes during acute peritonitis (Chapter 8), it is hypothesised that this would be heightened upon repeated inflammatory insult, which would exacerbate chronic disease and tissue damage, resulting in a more severe fibrotic score. Microbial clearance on the other hand is enhanced in IL-$10^{-/-}$ mice (Coles *et al* unpublished) but impaired in IL-$6^{-/-}$ mice (Kopf *et al* 1994). This would define opposing roles for IL-6 and IL-10, with the former being pro-inflammatory and vital for microbial killing, but also pro-fibrotic and the latter being anti-fibrotic, and anti-inflammatory, thereby attenuating microbial clearance but protecting against fibrosis. As a result, these two cytokines, being interrelated and regulated by one another must be tightly regulated on order to maintain a competent cell mediated immunity to infection whilst preventing collateral damage. How this balance is altered during repeated inflammatory challenge remains to be determined. Dotted lines refer to hypothesised results that are yet to be determined experimentally.

9.3.1 Potential mechanism of IL-6 regulation by IL-10

Upon induction of inflammation, IL-6 is produced predominantly by the mesothelial cells lining the peritoneal cavity (Topley *et al* 1993), but is also secreted by the resident macrophage population (Chapter 3). Since stromal cells do not respond to IL-10, it is likely that this cytokine inhibits IL-6 production by the resident macrophages, as has previously been shown (Fiorentino *et al* 1991). Of course, IL-10 may also regulate IL-6 expression in the mesothelial cells by the regulation of other pro-inflammatory cytokines including IL-1 and TNF-α, which are known inducers of IL-6 production by mesothelial cells (Topley *et al* 1993). Previous work has in fact shown that IL-10 inhibits IL-1 and TNF-α production by activated macrophages (Fiorentino *et al* 1991), which would support this notion. This provides an alternative mechanism for the regulation of IL-6 production by IL-10.

9.3.2 Anti-inflammatory activities of IL-10

Studies outlined in Chapter 8 demonstrate that IL-10 exhibits potent anti-inflammatory activities during peritoneal inflammation, by limiting the recruitment of leukocytes, specifically neutrophils, lymphocytes (Th17 cells) and monocytes, to the peritoneal cavity (Chapter 8). This was facilitated by control of inflammatory chemokines, with IL-$10^{-/-}$ mice showing a prolonged production of the neutrophil activating chemokine KC, and enhanced levels of the mononuclear chemokine CCL2. The production of CCL2 by mesothelial cells is regulated by IL-6 trans-signalling (Hurst *et al* 2001), compounded by data from IL-$6^{-/-}$ mice, which exhibit an impaired production of this chemokine (Chapter 5). Therefore the enhanced production of CCL2 in IL-$10^{-/-}$ mice is likely to be a result of the increased levels of IL-6 in these mice, which is known to drive CCL2 expression and consequently the recruitment of mononuclear cells (Hurst *et al* 2001). The IL-10 mediated limitation of chemokine expression and leukocyte recruitment is important to ensure that the magnitude of the inflammatory response is sufficient to successfully clear the pathogen, whilst preventing collateral tissue damage that may arise from the presence of large numbers of infiltrating leukocytes (Moore *et al* 2001). In this respect, IL-10 plays an important role in maintaining and controlling both the innate and acquired arms of the immune response (Kelly and Bancroft 1996, Tripp *et al* 1995, Dai *et al* 1997).

When comparing the roles of IL-6 and IL-10 in directing the inflammatory response, it becomes clear that IL-6 exhibits a strong pro-inflammatory role and directs the transition from innate to acquired immunity (Jones 2005, Hurst *et al* 2001, McLoughlin *et al* 2003, 2004, 2005), while IL-10 functions to limit the inflammatory response to prevent large-scale leukocyte infiltration and potential tissue damage (Chapter 8). Further work could be done to determine how IL-10 co-ordinates its anti-inflammatory roles in a repeat model of peritonitis, and how this impacts on the potential fibrosis in these animals. One might expect that IL-$10^{-/-}$ mice would exhibit more severe tissue damage following repeat episodes of inflammation, given that the infiltration of leukocytes is so enhanced in these mice during acute inflammation, and their levels of pro-inflammatory cytokines such as IL-6 are elevated. This would contrast with what is known in the IL-$6^{-/-}$ mice, which exhibit protection from tissue fibrosis and have normal leukocyte trafficking (Chapter 6, Fielding *et al* 2009, submitted). If IL-$10^{-/-}$ mice proved to exhibit more severe tissue damage, this potentially would provide a central concept for the role of STAT3 activating cytokines in the regulation of the immune response, with opposing roles defined for IL-6 and IL-10 in the regulation of the inflammatory response and their exacerbation or protection from the onset of chronic disease associated fibrosis respectively.

While the role of IL-10 in fibrosis is likely to be protective, the suppressive properties of IL-10 may hinder anti-microbial immunity. Due to its role in containing the inflammatory response in order to prevent tissue damage and fibrosis, IL-10 also selectively impairs microbial clearance upon infection, and facilitates bacterial outgrowth and dissemination (Sewnath *et al* 2001). This is highlighted in IL-$10^{-/-}$ mice, which exhibit significantly improved clearance of *Staphylococcus epidermidis* and *Escherichia coli* when compared to WT littermate controls (Coles *et al* unpublished, Sewnath *et al* 2001). In WT mice, inflammatory challenge induces a rapid and transient STAT3 activation in the peritoneal membrane. This STAT3 activation in the mesothelial cells is driven uniquely by IL-6, and coincides with the initial infiltration of neutrophils (Fielding *et al* 2008). The removal of inflammatory neutrophils is also reliant on IL-6 activity (McLoughlin *et al* 2003, Fielding *et al* 2008), and the profile of bacterial killing correlates with the clearance of this neutrophil population (Coles *et al* unpublished). In

this respect, IL-6/STAT3 signalling is vital for the resolution of acute neutrophil infiltration by the regulation of their apoptotic clearance, and the promotion of chemokine directed T cell trafficking (Hurst *et al* 2001, McLoughlin *et al* 2005, Fielding *et al* 2008). However T cell recruitment is only observed once the bacterial infection has been resolved. Therefore it is proposed that successful anti-microbial immunity relies upon the ability of IL-6 to control STAT3-mediated processes, and the communication between peritoneal stromal cells and innate effector cells. This regulation may potentially occur at two levels, either by regulating innate anti-microbial effector functions, or by the clearance of activated neutrophils that are laden with phagocytosed bacteria. This brings a potential indirect role for IL-10 in the regulation of microbial clearance *via* its regulation of IL-6 and subsequent IL-6 driven STAT activity. The regulation of IL-6 by IL-10 is important to allow competent host defence against infection, whilst preventing dysregulation of cellular immunity (by limiting neutrophil recruitment and their apoptotic clearance), which could result in tissue injury. Indeed, IL-10 has previously been shown to impair the recruitment and subsequent microbicidal activity of neutrophils (Laichalk *et al* 1996, Ajuebor *et al* 1999).

This concept is however complicated by the fact that IL-$6^{-/-}$ mice also exhibit enhanced neutrophil recruitment (Chapter 5) and yet their capacity to kill and clear away bacteria is inhibited (Coles *et al* unpublished). The question therefore remains, how do two STAT3 activating cytokines differ so dramatically in their ability to regulate microbial immunity? Neutrophil recruitment is significantly greater in IL-$10^{-/-}$ mice than in IL-$6^{-/-}$ mice (Chapter 8), therefore this may play a significant role in their improved ability to clear bacterial infections. In addition, IL-$6^{-/-}$ mice may potentially exhibit an impaired activation of recruited neutrophils, thereby inhibiting their ability to mediate microbial killing. In this respect, STAT3 conditional mutants have been shown to exhibit impaired mature neutrophil function and microbicidal activity (Panopoulos *et al* 2006, Matsukawa *et al* 2003). This provides a potential explanation as to why these mice exhibit impaired microbial immunity, even in the presence of increased neutrophils. In addition to this, IL-6 trans-signalling is regulated by the availability of the soluble IL-6R *in vivo*. This presents a level of regulation for IL-6 that is absent for IL-10, which could potentially

contribute to the differences seen between these two STAT3 activating cytokines. An alternative explanation could also come in the form of the regulation of antimicrobial peptide production by the mesothelial cell layer. There have been reports that human peritoneal mesothelial cells produce β-defensin-2, which is regulated by TNF-α (Zarrinkalam *et al* 2001). IL-10 could potentially inhibit or limit the production of these defensins by mesothelial cells *via* its known negative regulation of TNF-α production by monocytes (Fiorentino *et al* 1991, de Waal Malefyt *et al* 1991), thereby impairing the antimicrobial properties exhibited by these cells. Finally, the results presented in Chapter 8 implied that IL-10 negatively regulates the chemokine (KC)-directed neutrophil activation or apoptotic clearance, indicated by an increased level of sIL-6R in the peritoneal cavity of IL-$10^{-/-}$ mice. This would provide yet another alternative mechanism by which these mice exhibit an improved microbial clearance capacity. To support this, IL-10 is known to inhibit bacterial phagocytosis and attenuate neutrophil microbicidal activity towards internalised bacteria (Laichalk *et al* 1996). Further experiments are therefore required to determine the actual mechanisms by which IL-10 inhibits microbial clearance, whilst the potential inflammatory interplay between IL-6 and IL-10 requires further mechanistic clarification.

In summary, the IL-10 regulation of IL-6-STAT3 activation therefore provides a potential mediation of microbial clearance, regulated and balanced by an interplay between IL-6 and IL-10. This interplay is in place to allow competent host defence against infection whilst limiting tissue injury that arises due to the dysregulation of inflammatory infiltrate recruitment. The mechanisms by which this is facilitated must be investigated further, with potential investigations into how the balance between these two cytokines is maintained or altered during the onset of chronic disease.

9.4 Lessons learned in Flow Cytometry

The ability to use flow cytometry to characterise the trafficking patterns of dendritic cells was complicated by the emergence of aggregate formation following neutrophil recruitment (Chapter 6&7). The phenomenon of aggregate formation appears to be a common occurrence in the practise of flow cytometry. This can arise when the flow rate is set too high during data acquisition, or when the concentration of cells is too high. The formation of neutrophil aggregates was observed exclusively in IL-$6^{-/-}$ mice, and were absent in $gp130^{Y757F/Y757F}$ or IL-$10^{-/-}$ mice, despite these animals exhibiting a significantly greater recruitment of neutrophils compared to WT (Chapter 7). This can be attributed to the fact that these mice had higher total cell counts in general, meaning that the proportion or concentration of neutrophils in these mice remained equivalent to WT. In contrast, IL-$6^{-/-}$ mice possessed equivalent overall peritoneal cell numbers to the WT, but with a higher abundance of neutrophils, resulting in the increased incidence of aggregate formation upon their acquisition on the FACS calibur.

Aggregates or doublets can be visualised and excluded from flow cytometry data files using a pulse width size exclusion gate. This option is only available on more modern models of flow cytometers and was unfortunately not compatible with the BD FACS Calibur used for the studies presented in this thesis. The FACS Calibur does have an area width setting which is used for cell cycle analysis and could potentially be used for doublet exclusion however a linear scale is required and was not possible in this case. This teaches a valuable lesson and emphasises the importance of exercising vigilance when it comes to data analysis and exploring the alternative explanations for a result. It also highlights the importance of routinely using size exclusion to remove aggregates from flow cytometry plots, in order to exclude any erroneous events that may distort the result.

9.5 Future directions

The work presented in this thesis has defined a role for IL-6 in the homeostatic regulation of monocytic cell numbers and their dysregulated recruitment following repeated inflammatory challenge. Subsequent studies began to introduce IL-10 and define its role in the regulation of the inflammatory response during peritonitis, highlighting important anti-inflammatory functions by this cytokine and a potential interplay with IL-6. Further experiments are required to develop these studies and are listed below:

- Resident DC are important for homeostatic antigen sampling, and the induction of the adaptive immune response during infection. As a result, it is important to maintain a considerable pool of DC resident in the peripheral tissues. Studies in Chapter 3 showed that the number of resident DC was regulated by IL-6 and IFN-γ in a positive and negative manner respectively. Future work is required to determine whether these cytokines differentially regulate haematopoiesis, their inward migration of DC, their differentiation from monocyte precursors, or their turnover/egress.
- Studies in Chapter 3 showed that resident macrophage and DC do not respond to signalling by IL-6 through the classical route of engagement with the surface bound IL-6R. IL-6 is also known to signal *via* a soluble form of the receptor and therefore studies should be done to determine if activated resident DC are able to respond to IL-6 trans-signalling following loss of the IL-6R. This would be achieved by measuring their release of inflammatory mediators following stimulation of these cells by Hyper IL-6. To reduce the number of mice required to sort sufficient numbers of cells for culture, work could be done to derive an immortalised cell line from these cells, which would better enable one to perform such studies. This could be performed using the Hox oncoprotein, which enforces self renewal of factor-dependent myeloid progenitors (Wang *et al* 2006).
- It has long been characterised that resident macrophages and DC exhibit a clearance upon inflammatory activation, followed by a subsequent re-emergence

later in the inflammatory response. However it is currently unknown what is responsible for this pattern of trafficking. Therefore it would be important to investigate the migratory fate of these resident populations following SES activation. Is the initial clearance of these cells a result of migration to the lymph nodes or increased adherence to the peritoneal membrane? Further investigation of chemokine receptor expression, adhesion molecules and integrins could provide potential explanations. Also, recovery of the draining lymph nodes for analysis of cell content following induction of peritoneal inflammation would allow us to determine if resident DC are in fact trafficking away from the peritoneal cavity.

- Studies so far have defined a role for IFN-γ secreting T cells in mediating STAT1 activation in the peritoneal membrane following repeated inflammatory challenge, which is associated with fibrosis (Fielding *et al* 2009, submitted). Studies outlined in Chapter 6 have shown that increased recruitment of neutrophils and monocytes are also evident following repeat inflammatory episodes. The contribution of this process by monocytic cells and neutrophils has not been determined, therefore further studies are required to dissect the role played by individual cell types in the onset of membrane fibrosis.
- Studies performed to characterise the inflammatory trafficking of leukocytes in IL-10 deficient mice showed that these mice have dramatically enhanced leukocyte recruitment (Chapter 8). The next step in these studies would be to perform the repeat inflammatory model in IL-$10^{-/-}$ mice to study changes in leukocyte trafficking, inflammatory mediator production and peritoneal tissue injury. IL-6 is extremely detrimental in chronic disease, and drives tissue injury and fibrosis through the regulation of T cell effector function, trafficking and through the prevention of activation-induced cell death. Given the known interplay between IL-6 and IL-10, and the ability of IL-10 to inhibit IL-6 production by monocytic cells, it is important to determine how this balance is regulated to provide immunity against infection whilst preventing collateral tissue damage. It is also important to understand how this balance is altered upon repeat inflammatory challenge, resulting in peritoneal fibrosis. It is

predicted that IL-10$^{-/-}$ mice, which have elevated IL-6 production during peritoneal inflammation, would exhibit a more severe fibrotic score compared to WT, given that the absence of IL-6 protects mice from peritoneal fibrosis (Chapter 6). Experiments would determine the fibrotic score in these mice and correlate this to potential changes in T cell (IFN-γ secreting), monocytic cell and neutrophil recruitment and retention.

- Finally, the group has seen from recent studies that the complete clearance of bacteria in mice subjected to the live peritoneal inflammation model (Section 2.3.7) precedes the recruitment or recall of inflammatory T helper cell subsets (Th1, Th17) (Coles *et al* unpublished, Jones *et al* 2009-under revision). This brings into question the role of the recruited T lymphocytes, since they are unlikely to be contributing to the antimicrobial response. Experimental work would therefore try to determine the contribution of T helper cells to the inflammatory response, addressing questions as to whether they are important for directing the re-establishment of resident leukocyte populations following inflammatory challenge, the formation of memory cells, and how they potentially contribute to tissue fibrosis.

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10 References

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