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PROINFLAMMATORY EFFECTS OF INTERLEUKIN 18

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Thesis submitted to the
University of Glasgow for the degree of
Doctor of Philosophy

October 2002

Containing studies performed in the Department of Immunology, University of
Glasgow and the Centre for Rheumatic Diseases, Glasgow Royal Infirmary

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Abstract

Interleukin 18 (IL-18) was originally identified as an interferon γ inducing factor in the livers of mice treated with LPS and *Propionibacterium acnes*. Initial studies focused on IL-18 as a Th1 driving cytokine. Subsequently, myriad effects of IL-18 have emerged, indicating a broader role to include effects on innate immunity, via activation of macrophages and NK cells. This thesis sought to further investigate the role of IL-18 in innate immune responses. IL-18 neutralisation prior to LPS challenge reduces tissue myeloperoxidase levels, implicating IL-18 at some stage in neutrophil activation. This study defines direct biological effects of IL-18 on neutrophils. IL-18 promoted *in vivo* migration of neutrophils, concurrently, IL-18 enhanced neutrophil adhesion molecule expression *in vitro*. Furthermore, IL-18 induced neutrophil cytokine and chemokine release, including IL-8, which could further promote neutrophil accumulation. Additionally, IL-18 provoked neutrophil degranulation, and primed neutrophils for enhanced respiratory burst in response to fMLP. Finally, IL-18 stimulated leukotriene synthesis by neutrophils.

IL-18 promotes protective immune responses in parasitic, viral, fungal and bacterial infections. Aberrant immune responses are similarly associated with over expression of IL-18. Accordingly, elevated IL-18 is reported in rheumatoid arthritis (RA), inflammatory bowel disease (IBD), sarcoidosis, and sepsis. RA synovial fluid neutrophils are primed and activated *in vivo*, a process to which IL-18 may contribute. Neutrophils derived from the synovial fluid of patients with RA, exhibited enhanced cytokine release in response to IL-18, which included release of TNF α . Thus, IL-18 may promote joint damage via neutrophil activation. Neutralisation of IL-18 in a model

of acute inflammation significantly reduced swelling and neutrophil accumulation. Given its proinflammatory effects on T cells and macrophages, targeting IL-18 is suggested as an attractive therapeutic strategy. These data suggest that effects mediated by neutrophils may be reduced.

Effective neutrophil responses are paramount to clearance of gram-positive infections. IL-18-deficient mice exhibit enhanced arthritis but reduced systemic sepsis following infection with *Staphylococcus aureus*. Both features of this altered response may, in part, be explained by reduced neutrophil function. IL-18 promoted *in vitro* bactericidal activity of human neutrophils; cells derived from IL-18-deficient mice exhibited reduced killing ability *in vitro*. However, administration of IL-18 prior to infection with *S. aureus* resulted in significant mortality compared to controls, thereby suggesting that IL-18 promoted host toxicity rather than an early protective response. Further analysis revealed rapid, early IFN γ production, elevated Toll-like receptor expression and changes in *ex vivo* immune responses. In contrast, post-infection administration of IL-18 substantially reduced arthritis, although no direct evidence of enhanced neutrophil bacterial killing was observed. Supplementary IL-18 promotes vaccine efficacy and may therapeutically address Th2 diseases such as asthma. These data caution addition of exogenous IL-18. Additionally, this model highlights the complex evolution of immune responses, and demonstrates discrepant consequences of intervention at different time points.

IL-18 is involved in innate and adaptive responses. Although traditionally described as simple phagocytes, neutrophils have subsequently been shown to mediate adaptive responses, for example by release of cytokines and expression of MHC peptide

complexes. A novel system of investigating neutrophil – T cell interactions was established. Preliminary studies to investigate neutrophil antigen presentation were strongly suggestive that neutrophils could drive Class II restricted T cell responses. The role of soluble mediators in this interaction was investigated, however preliminary experiments elucidated no role for IL-18.

Data described here support a role of IL-18 in pathological inflammation, which can be perpetuated through neutrophil activation. Patients with elevated IL-18 *a priori* exhibit poor prognosis following gram-positive infection, similar to mice treated with IL-18 prior to *S. aureus* infection. Neutralising IL-18 in animal models of arthritis and inflammatory bowel disease has yielded promising results. Clearly, such manipulation of the immune system may leave the host vulnerable to infection. Therefore, the delineation of IL-18 mediated effects on neutrophils and *S. aureus* infection described herein requires close consideration in the context of therapeutic intervention.

Acknowledgements

I have been privileged to work with people whose talents have helped shape this work and my time in the Department of Immunology. Throughout the Department of Immunology, Western Infirmary and the Centre for Rheumatic Diseases, Royal Infirmary, numerous individuals have generously and patiently provided assistance. Some are described below, but thanks are due to the many others for whom space precludes individual mention.

The enthusiasm, unfailing optimism, and guidance of Prof. Iain McInnes have been invaluable. The support and encouragement provided by Prof. F.Y. ('Eddy') Licw is also greatly appreciated.

Dr. Bernard Leung expended immense effort and patience imparting his wealth of laboratory skills, and without his assistance with *in vivo* studies, the animals may have experimented on me! Drs. Carol Campbell and Duncan Thomson uncomplainingly tolerated my molecular biology mishaps and heroically rectified many of them, while Dr. Anne McKay skilfully bled 'normal' volunteers. I have been fortunate to enjoy their friendship, support and humour. The expertise of Dr. James Brewer allowed me to begin exploring a fascinating world of cells. Whilst working at the Royal Infirmary, Drs. Alistair Gracie, Susan Robertson and John Young, provided valuable contributions to this study.

Thanks to Mr. Eric Martindale, Dept. of Bacteriology, Western Infirmary, Glasgow, for assistance with bacterial culture and *Staphylococcus* identification. Also, Mr. P. Kerr,

Dept. of Pathology, University of Glasgow, for preparing tissue histology slides, and Mr P. Kerrigan, Dept of Pathology, University of Glasgow, for technical assistance with recording histology images. Susie's efforts to read this work and instil some punctuation are appreciated.

The essential contribution of normal donors, patients and the clinicians who obtained samples is acknowledged. So too is the compulsory participation of the animals involved. The present study was funded by an Arthritis Research Campaign Studentship.

My parents have offered me enormous opportunity and encouraged me to pursue my curiosity. Their acceptance of my unusual decisions is appreciated. The support, patience, tolerance and encouragement offered by Owain over the last 3 years deserves my special thanks.

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Abbreviations

APC	antigen presenting cell
bp	base pairs
cDNA	complementary deoxyribonucleic acid
CFU	colony forming unit
CRP	C-reactive protein
DMARD	disease modifying anti-rheumatic drug
DC	dendritic cell
EAE	experimental autoimmune encephalomyelitis
ELISA	enzyme linked immunosorbent assay
ESR	erythrocyte sedimentation rate
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
FIV	feline immunodeficiency virus
GM-CSF	granulocyte macrophage – colony stimulating factor
h	hours
ICAM	intercellular adhesion molecule
IDDM	insulin dependent diabetes mellitus
IFN	interferon
Ig	immunoglobulin
IL-	interleukin-
i.p.	intraperitoneal
IL-18BP	IL-18 binding protein
kD	kilo-Dalton
LPS	lipopolysaccharide
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
NOD	non obese diabetic
OD	optical density
PB	peripheral blood
PBL	peripheral blood leukocyte
PBS	phosphate buffered saline
PMN	polymorphonuclear neutrophil

PE	phycoerythrin
PFA	paraformaldehyde
PMA	phorbol 12-myristate 13-acetate
RA	rheumatoid arthritis
ROI	reactive oxygen intermediates
RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
SD	standard deviation
SEM	standard error of mean
SF	synovial fluid
TNF	tumour necrosis factor
TSST	toxic shock syndrome toxin

M	molar
mM	millimolar
mg	milligram
ml	millilitre
μ g	microgram
μ m	micrometer
μ M	micromolar
ng	nanogram
nM	nanomolar
pg	picogram
g	gram
x g	times gravity

Chapter 1 General Introduction

1.1 Cytokines

1.1.1 Cytokines and the inflammatory response

Communication between cells is obligatory for the orchestration of an inflammatory response. Cells may interact either via direct cell contact or the release of soluble factors. The term cytokine is applied to a rapidly growing number of small, non-structural proteins or glycoproteins that serve as protein messengers conveying information between and within cells through specific receptor molecules. In addition to their involvement in such processes as cell growth, differentiation, tissue repair, remodelling and development, cytokines play a central role in inflammatory and immune responses. Cytokines are instrumental in regulating the magnitude, nature and duration of the immune response, and as such are attractive therapeutic targets for modulating inflammatory diseases. The field of cytokine biology is rapidly expanding, promoted further by the near completion of the genome projects, which have allowed identification of new members of existing cytokine families and of novel cytokines.

1.1.2 Nomenclature and history

The generic term 'lymphokine' was introduced in 1969 to describe the origin and function of soluble lymphocyte derived factors (1). An increasing number of soluble factors were identified from cells of both lymphoid and non-lymphoid origin. Historically, individual soluble factors were identified on the basis of their biological function. However, it became apparent that proteins could share similar biological functions, and that many proteins had more than one function, therefore this

nomenclature became cumbersome. A consensus group formed in 1979 and coined the term 'interleukin'. As this project commenced, interleukin 18 (IL-18) was amongst the most recently characterised cytokines. At present, IL-27 is defined (2) and the number of cytokine entities purified is greater than 150 and increasing (3).

1.1.3 Cytokine action

The discovery of numerous cytokines with diverse functions makes generalising the mechanism of cytokine action difficult. Cytokines were originally thought to function like hormones. Indeed, IL-6 for example, operates like an endocrine factor, travelling via the circulation to reach a distant target, the hypothalamic-pituitary-adrenal axis, where it induces an acute phase response. Conversely, other cytokines act in a paracrine manner on adjacent cells. Some cytokines, which are associated with cell membranes, mediate effects via a cell contact dependent juxtacrine signalling. Cytokines may also function in an autocrine fashion, feeding back to the cell of origin. The mechanisms of cytokine actions are probably best described as a local intercellular communication network, providing a rapid and flexible modulation of immune responses. Cytokines also allow communication between immune cells and host tissues.

1.1.4 Cytokine receptors

Cytokines mediate effects by binding to their specific high affinity cell surface receptors. A relatively low concentration of cell surface receptors, approximately 100-1000 per cell, is required for biological effects (4) Depending on receptor affinity, usually between 10^{-12} and 10^{-9} M, the receptor requirement may be even lower; IL-1 can

induce effects in the presence of fewer than 10 receptors per cell. Cytokines can be divided into at least 6 different families depending on the receptors they bind. These include: interferon receptor family (IFN γ R, IL-10R), TNF family (Fas, TNF α), haematopoietic family (IL-3R, GM-CSFR), immunoglobulin family (IL-1R, IL-18R), type II – TGF β R, and the 7 transmembrane chemokine receptors. The extracellular domain of many receptors may be enzymatically cleaved and released, retaining ligand binding activity and thus acting as an inhibitor. Binding of cytokines to their specific receptors activates various signalling pathways, including MAP kinases, JAKS and STATS, JNKS, and NF κ B.

1.1.5 Cytokines activate the innate immune system

The innate immune system evolved in multicellular organisms to reduce the consequences of microbial invasion. This arm of the immune system functions regardless of the microbe's antigen specificity, serving not only as a first line of defence but potentially defining the nature of subsequent adaptive response, primarily through the release of cytokines. Activation of the innate immune system may be mediated by microbial components (e.g. cell wall polysaccharides) or endogenous signals of cellular damage (e.g. heat shock proteins). Cytokines are the primary means of communication for the activated innate immune system. TNF α , IL-1 and IL-6 are among the cytokines with key roles in innate immunity. Activation of the innate immune system leads to a hepatic acute phase response (release of acute phase proteins), a central nervous system response (fever, myalgia), a somatic tissue response (breakdown of muscle and fat), and an immunological response (release of neutrophils from the bone marrow, macrophage and neutrophil activation). If inappropriately activated, this 'hard-wired' response may

be lethal. Uncontrolled activation of the innate immune system can result in potentially fatal exaggerated IL-1 and TNF α release, as is evident in sepsis. Furthermore, there is evidence that persistent activation of the innate immune system occurs in chronic inflammatory conditions such as rheumatoid arthritis, where the effects of uncontrolled innate immunity manifest as metabolic abnormalities, tissue destruction and remodelling.

1.1.6 Cytokines influence adaptive immunity

Protective immunity to pathogens is the result of a complex interplay between the innate and adaptive immune systems. The innate immune system detects pathogens via a restricted range of pattern recognition receptors, providing a rapid response and mobilising the adaptive immune system. The adaptive response is almost infinitely flexible, capable of generating a broad immunological repertoire allowing specific recognition of pathogens. Cytokines play a key role in providing fine control required for evolution of the adaptive response; other regulators include co-stimulatory mediators vital to T cell – dendritic cell interactions. The dominant response to specific antigen may be cell mediated (Th1) or humoral (Th2) depending on factors driving the differentiation of naïve CD4 T cells. Most immune responses are likely to involve a balance of both Th1 and Th2 phenotypes. T cells initially stimulated in the presence of IL-12 and IFN γ develop into Th1 cells, whereas activation in the presence of IL-4 promotes Th2 development. The ability of IL-12 and IL-4 to drive Th1 and Th2 differentiation respectively is enhanced by their inhibitory effects on the opposing pathway (5, 6). In this context, IL-12 and IL-4 are prototype T cell driving factors,

however as more cytokines are identified, the cytokine networks influencing T cell differentiation become increasingly more complex.

1.2 Interleukin-18 (IL-18)

IL-18 was originally identified as an interferon γ inducing factor (IGIF), in sera and liver extracts of mice treated sequentially with *Propionibacterium acnes* and LPS (7). Sera from these mice induced copious IFN γ from anti-CD3 stimulated T cells. This IFN γ production was greater than that elicited by addition of excess IL-12 alone, indicating the presence of an additional IFN γ stimulating factor. Analysis of the amino acid sequence demonstrated that IL-18 was a new member of the IL-1 family (8).

1.2.1 Regulation of gene expression

IL-18 mRNA is constitutively and stably expressed in many tissues. The hIL-18 gene localises to chromosome 11 and mIL-18 maps to chromosome 9, linked to *Ildm2* locus. The gene order, ATM-IL-18-DRD2-THY1, is the same in mouse and human (9). The mIL-18 gene is composed of 7 exons, of which 1 and 2 are non-coding. Two TATA-less IL-18 promoters have been identified. Promoter activity upstream of exon 2 acts constitutively, whereas an area upstream of exon 1 can be LPS activated (10). The transcription factors, IFN consensus sequence binding protein (ICSBP) and PU.1 are critical for the activation of the IL-18 promoter upstream of exons 1 and 2, respectively (11). NF κ B recognition sequences have also been identified in the promoter region of IL-18. Unusually for a cytokine gene, the 3' untranslated region of human IL-18 mRNA lacks AUUUA destabilisation sequences, which may explain the constitutive expression

of IL-18 mRNA in freshly isolated human peripheral blood mononuclear cells, murine splenic macrophages and non-immune cell (12).

Three single nucleotide polymorphisms were detected in the IL-18 promoter. There were no significant differences in promoter activity between alleles without stimulation, but after stimulation with PMA/ionomycin one allele had distinctly lower activity than the other. Comparison of allele frequency in populations of multiple sclerosis patients and healthy controls demonstrated no significant differences (13). Whether IL-18, like IL-1, contains polymorphisms with strong disease associations remains to be elucidated.

1.2.2 IL-18 production and processing

IL-18 is transcribed as a 24 kD pro-molecule with no measurable biological activity. There is no signal peptide, which would be required for secretion, thus alternative processing occurs. Processing of pro to active forms may be mediated by several enzymes. Studies in caspase 1 (IL-1 β converting enzyme, ICE) deficient mice indicate that this is probably the principal IL-18 processing pathway (14). Caspase 1 cleaves IL-18 at Asp³⁵ residue, and although also a cleavage site for caspase 4, the processing predominantly occurs via caspase 1. Caspase 3 cleaves between Asp⁶⁹ and Ile⁷⁰ resulting in accumulation of inactive products, implying a role for caspase 3 as a scavenger for mature IL-18 (15). Proteinase-3, found in the azulophilic granules of neutrophils, also generates biologically active cleavage products of IL-18 (16). Nitric oxide, a destructive molecule in inflammatory reactions, inactivates caspase 1, thus regulating IL-18 processing (17). Other caspase regulatory factors remain largely unknown. Interestingly, both IL-18 and caspase 1 promoters contain consensus

sequences for IRF1 (interferon regulatory factor) and ICSBP, implicating interferons in the regulation of both IL-18 transcription and IL-18 processing.

In addition to pro and active forms, further species of IL-18 have been identified. Macrophages produce an inactive 48 kD dimer, which is recognised by pro-IL-18 specific antibodies (18). Another inactive form of IL-18 is found complexed with IgM in the serum. Termed 'Type II' IL-18, this is present in the serum, at relatively high concentrations, in approximately 30% of the normal population studied (19). The functional implications of these species of IL-18 are as yet unknown. Further regulation of IL-18 is presumed to occur at the level of release from the cell; however, like IL-1, the precise mechanisms are ill defined. Studies employing ATP to promote release of IL-18 demonstrated that caspase processing of IL-18 is not required for pro-IL-18 release; however, proteins recruited by caspase-1 are speculated to be involved in mature IL-18 release (20). IL-1 β release in response to ATP occurs through shedding of cytokine containing microvesicles; whether a similar mechanism mediates IL-18 release remains to be elucidated (21). Given the constitutive, widespread expression of IL-18 mRNA, extensive post-transcriptional regulation seems likely.

1.2.3 IL-18 receptors

The IL-18R α was originally identified on the Hodgkin's disease cell line, L428, on the basis of IL-18 binding assays. The receptor was subsequently identified as the previously orphan receptor IL-1R η 1 (IL-18R α /IL-1R5). Human IL-18R α maps to 2q12-21 near IL-1 α , IL-1RA and IL-1R1 and IL-R2 (22). A novel member of the IL-1R family, AcPL (IL-18R β /IL-1R7), was identified as a second receptor subunit. The IL-

IL-18R α alone binds with relatively low affinity (K_d of 20 – 50 nM) (23). IL-18R β does not itself bind IL-18, but the IL-18R $\alpha\beta$ heterodimer exhibits increased affinity for IL-18 (K_d of 0.2 nM), and is necessary for receptor signalling (24). Co-transfection studies demonstrate that the IL-18R complex is responsive to IL-18 in a highly specific (no response to other IL-1 ligands) and unique manner (no functional pairing with other IL-1 receptor family members, including IL-1R1, IL-1RII, IL-1RAcP, T1/ST2, IL-1Rrp2, SIGIRR and IL-1APL) (25). Extracellularly, the IL-18 receptors consist of three immunoglobulin domains, and intracellularly, both chains contain a TIR (Toll/IL-1 Receptor) domain, a motif that is conserved through evolution from *Drosophila*. Computer modelling and mutational analysis reveal that Glu⁴² and Lys⁸⁹ are critical amino acid residues for binding of IL-18 to cell surface receptors (26). Crystal structures of either IL-18 or the receptor complex have not yet been resolved.

There is no evidence of soluble forms of the IL-18 receptor, which is in contrast to the closely related cytokine homologue, IL-1R. Type II IL-1R acts as a non-signalling decoy receptor, which may be cleaved from the cell membrane to generate a soluble inhibitory receptor. Moreover, recombinant soluble IL-18 receptor α or β chains, generated by cloning the extracellular domains, do not bind IL-18 with sufficient affinity to mediate biological effects. A combination of both α and β chains mediate some inhibitory effects but only at large molar excesses (27). *In vivo* inhibition of IL-18 is mediated by the IL-18 Binding Proteins (IL-18BP, Section 1.2.5).

IL-18R α mRNA is widely distributed and in addition to being detected in lymphoid and myeloid cells, was identified in thymus, spleen, liver, lung, intestine, colon, placenta, prostate, heart, testis, ovary, and brain (22). Expression of IL-18R α does not confer

responsiveness to IL-18, as there is an absolute requirement for both chains (24). IL-12 enhances IL-18R α and β chain expression on T, B and NK cells, enhancing IL-18 responsiveness (28). This provides a Th1 response amplification loop, as IL-12R β is upregulated on T cells in response to IL-18. Conversely, IL-4 reduces IL-18R expression on T cells (29). IFN α also upregulates IL-18R expression on T cells (30). The receptor regulation on other cell types is ill defined.

1.2.4 Signalling via the IL-18R

The signalling pathways proposed to operate downstream of IL-18 are illustrated in Fig. 1.1. Binding of IL-18 to the IL-18R complex induces signalling pathways shared with other IL-1R family members. These involve recruitment of myeloid differentiation factor 88 (MyD88) and IL-1 receptor associated kinase (IRAK) (31). Activated, phosphorylated IRAK interacts with the adaptor protein, TNF receptor associated factor (TRAF6), which results in translocation of NF κ B to the nucleus (32). Dominant negative transfectants of I κ B α inhibit IL-18 dependent NF κ B activation and IFN γ expression by KG-1 cells (33). IL-18 may also activate the stress kinase pathways, and is documented to activate extracellular signal related kinases (ERK) and p38 (34). Furthermore, GADD45 α is induced by IL-18 in Th1 cells, which in turn binds MEKK4, amplifying the MAP kinase associated effects of IL-18 (35). IFN γ production in response to IL-18 is reduced in the absence of *tyk2*, a JAK family non-receptor tyrosine kinase (36). This raises the possibility of cross talk between JAK-STAT and MAP kinase pathways in cytokine signalling. Whether significant differences exist between IL-18 induced signalling pathways in different cell populations is not elucidated.

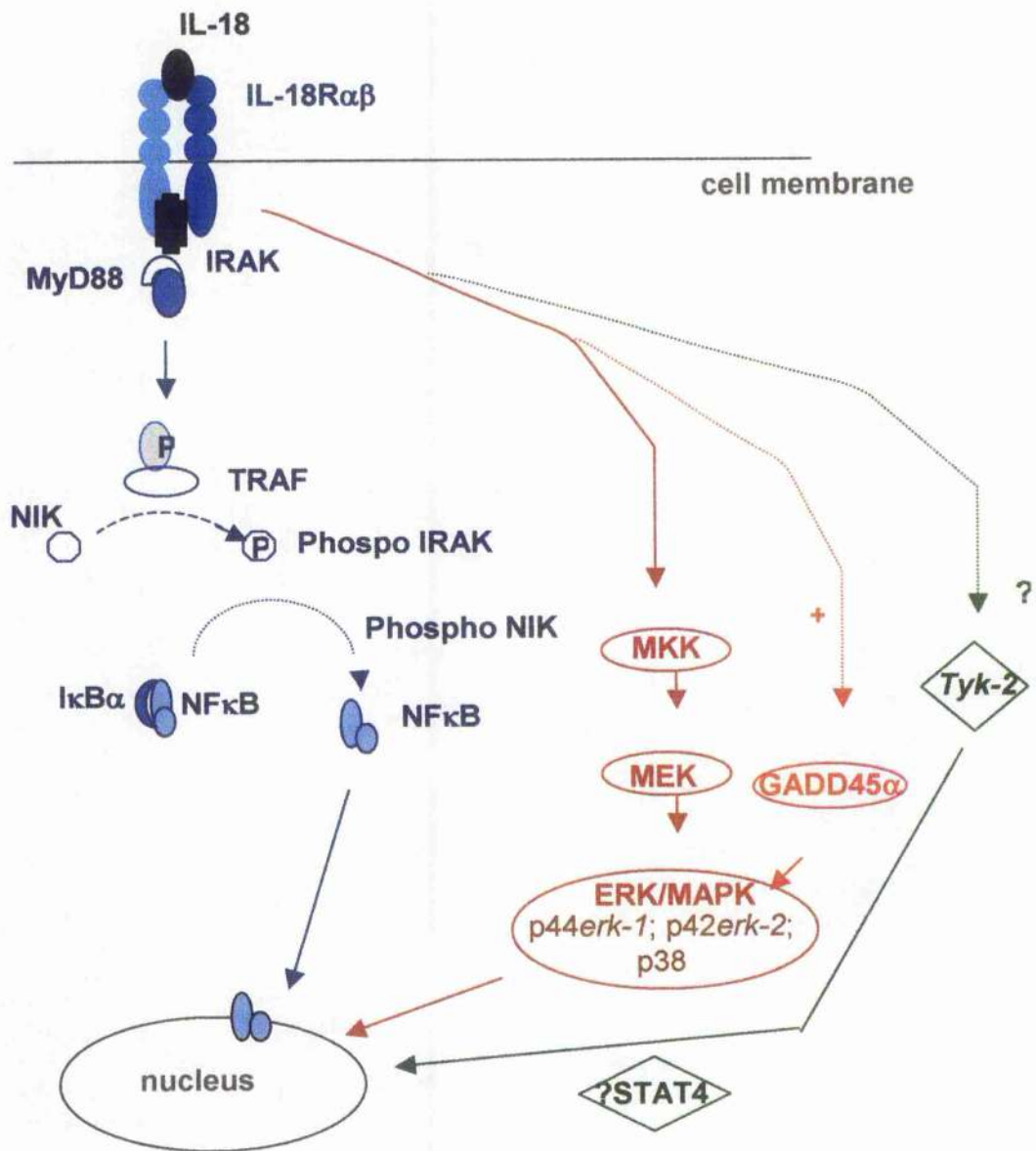


Figure 1.1 Signalling pathways activated by IL-18

1.2.5 IL-18 antagonists

IL-18 Binding Proteins (IL-18BP), like IL-18R, are Ig superfamily members. Notwithstanding the presence of a single Ig domain, IL-18BPs bear little homology to either chain of the IL-18R. The IL-18BP lacks a transmembrane domain, and is thus only found in soluble form. Four human and two mouse isoforms of IL-18BP exist, arising from alternative splicing. These binding proteins are widely expressed, and have been identified in various cDNA libraries (37). HIL-18BP_a exhibits highest affinity for hIL-18, which is similar to that of the IL-18R $\alpha\beta$ heterodimeric receptor. HIL-18BP_c also neutralises hIL-18, but differs from the 'a' isoform at its C terminus, resulting in lower affinity interaction. HIL-18BP_b and d lack complete Ig domains and do not bind hIL-18. Both murine IL-18BP isoforms (b and d) bind and neutralise mL-18, mL-18BP_d also cross reacts with hIL-18. The binding proteins effectively neutralise IL-18 at relatively low concentrations, neutralising approximately 95% of IL-18 activity at a molar excess of 2 (38). The function of the non-binding isoforms of the IL-18BP remains elusive. Although experimental evidence is awaited, preferential secretion of binding and non-binding isoforms seems likely to modulate IL-18 mediated responses.

Expression of IL-18BP is enhanced *in vitro* by proinflammatory cytokines including IFN γ , IL-12, IL-15, and IL-2, suggesting negative feedback regulation of IL-18 (39, 40). Similarly, in disease states associated with exaggerated IL-18 expression, such as sepsis and GvHD, IL-18BP levels are elevated (41, 42). Interestingly, IFN α , which exerts inhibitory effects on IL-1, TNF α and IL-18 release, also enhances IL-18BP expression (43). In healthy humans, the mean concentration of IL-18BP in the circulation is 2.15 ng/ml (range 0.5-7 ng/ml). The mean molar equivalent is 100 pM. Mean molar

concentration of IL-18 at 64 pg/ml is 2-3 pM, therefore, with a K_d of 400 pM, 85% of circulating IL-18 is free. In septic patients, IL-18BP concentrations increase approximately 10 fold, but IL-18 concentrations increase 20 fold (41). As such, relative levels of IL-18 and binding proteins are likely to determine overall IL-18 activity *in vivo*.

A recent addition to the IL-1 family, IL-1H4 (IL-1F7b), binds IL-18R α , but does not induce signalling. When expressed in mammalian cells, IL-1H is secreted as 2 polypeptides. IL-1H is cleaved by caspase 1 and exhibits some amino acid sequence homology with IL-1ra. Both pro- and mature IL-1H bind IL-18R α , although mature IL-1H binds with greater affinity (44, 45). Evidence for *in vivo* IL-18 inhibitory effects of IL-1H is awaited.

IL-18 antagonists are synthesised by viruses. Several poxvirus genes encode proteins with sequence similarity to IL-18BP. These viral IL-18BPs (vIL-18BP) are secreted from cells infected with *vaccinia*, *ectromelia* and *cowpox*, and inhibit NF κ B activation and IFN γ induction in response to either human or murine IL-18 (46). *Molluscium contagiosum* gene MC54L encodes a larger vIL-18BP, which exhibits functional similarity to other IL-18PBs. The extent to which these proteins contribute to virus virulence awaits deletion studies.

1.2.6 Functional effects of IL-18

IL-18 was originally defined as an IFN γ inducing factor that promoted the development of Th1 cells, however, since its discovery, the known effector roles of IL-18 have rapidly expanded.

IL-18 influences lymphocyte differentiation and function

IL-18 and IL-12 synergistically induce IFN γ production by T cells via reciprocal upregulation of cytokine receptors. Additionally, the IFN γ promoter includes consensus binding sites for AP-1 and NF κ B and STAT-4 (47, 48). Thus, in the presence of sub-optimal levels of IL-12, IL-18 induces significant IFN γ production by CD4 and CD8 T cells without TCR ligation (49, 50), although for cell commitment to Th/c1 lineage, TCR engagement is required (51). The requirement for both IL-12 and IL-18 for optimal IFN γ secretion is apparent *in vivo*; IL-18-deficient mice have normal IL-12 levels but produce limited IFN γ (52); similarly, administration of IL-12 to ICE-deficient mice resulted in reduced IFN γ production compared to wild type controls. IL-12 and IL-1 also induce IFN γ in human T cells, suggesting that IFN γ induction may be a feature of other IL-1 family members (53). IL-18R α has been proposed as a Th1 cell marker (54). IL-18 acts on differentiated Th1 cells to enhance FAS mediated cytotoxicity and co-stimulate IL-2, GM-CSF, and IL-2R α production and proliferation. Conversely, IL-18 has no effect on differentiated Th2 clones (55, 56).

In addition to promoting IFN γ production and Th1 development, IL-18 may influence the opposing Th2 arm of the immune response. Together with IL-2, IL-18 co-induces IL-13 in murine T and NK cells and, in the presence of TCR activation, induces T cell

IL-4, IL-10, IL-13 and IFN- γ production (57, 58). IL-18, in combination with IL-2, anti-CD3 and anti-CD28, markedly enhances IL-4 production by CD4 T cells. Naïve T cells express low levels of IL-18R α and respond to IL-18 by producing IL-13 and GM-CSF (59). When cultured alone or in combination with IL-4, IL-18 is known to induce murine T cell Th2 differentiation, although IL-18 responsiveness is lost in differentiated Th2 cells. These effects are dependent upon genetic influences, as spleen cells from BALB/c and C56BL/6 strains of mice stimulated with anti-CD3 and IL-18 exhibit enhanced Th2 and Th1 responses, respectively (60).

IL-18 synergises with IL-12 to induce IFN γ production by B cells (61); however, in isolation IL-18 induces high IgE expression by B cells (59). Daily administration of IL-18 augmented serum IgE, which was dependent of IL-4 but not IL-13 (58). Similarly, mice over-expressing caspase 1 in the skin, which results in enhanced expression of mature IL-18, exhibit elevated serum IgE and develop dermatitis. This dermatitis is partially abrogated by crossing these mice onto an IL-18-deficient background. Intriguingly, the skin pathology associated with IL-18 persists even in the absence of IgE in serum, demonstrated by crossing the animals which over-express caspase 1 onto a STAT-6-deficient background (59, 62). In a model of helminth infection, administration of IL-18 completely inhibited IgE production, which was dependent on IFN γ (61). Thus IL-18 can promote Th1 or Th2 lineage maturation dependent upon underlying genetic influences and the ambient cytokine milieu.

In addition to its effects on T cell cytokine production, IL-18 exerts effects on CD8 and NK T cell mediated cytotoxicity (63). IL-18 upregulates perforin and FAS dependent

cytotoxicity (55, 64), but has no effect on TRAIL (TNF related apoptosis inducing ligand) (65). These effects of IL-18 operate independently of IL-12.

In addition to modulating cytokine production, effects of IL-18 on lipid mediators have been investigated. Unlike IL-1, IL-18 does not induce cyclooxygenase (COX) or prostaglandin E₂ (PGE₂) expression in human PBMC. Furthermore, inhibition of endogenous IL-18 resulted in upregulation of spontaneous PGE₂ release (66). These effects may be cell type specific as IL-18 induces COX expression in chondrocytes (67).

IL-18 exerts effects on non T cell populations

Extensive effects of IL-18 have been documented on myeloid and tissue cell populations. In conjunction with IL-3, IL-18 induces IL-4 and IL-13 production by bone marrow derived basophils (68). IL-18 stimulates eosinophils to release IL-8 (69) and basophils and mast cells to release histamine. Histamine is reported to stimulate IL-18 and IFN γ production by PBMC and, as such, induction of this Th1 like response may be evidence of a negative feedback loop (70). Overexpression of IL-18 in the skin resulted in accumulation of neutrophils in the spleen (71). Moreover, repeated injection of IL-18 into mice resulted in eosinophilia and significant neutrophilia, suggesting that IL-18 has haematopoietic effects (72), although further studies in IL-18-deficient animals are awaited.

Direct effects of IL-18 on macrophages and dendritic cells (DC) have been observed. Stimulation of bone marrow derived macrophages or splenic DC with IL-12 and IL-18 can induce IFN γ production (73, 74). Langerhans cells (LC) also produce IL-18 (75), and IL-18-deficient animals exhibit defects in LC migration (76). IL-18 stimulation of

peritoneal macrophages induces IL-6 production, independent of the intermediate induction of endogenous cytokines such as TNF α or IL-1 β (77). Macrophages derived from rheumatoid arthritis (RA) synovial membrane (but not peripheral blood monocytes) respond directly to IL-18 with TNF α production (78).

Numerous cells not directly associated with the immune system respond to IL-18. IL-18 exerts effects on chondrocytes, promoting cartilage matrix degradation (67). Endothelial cells and synovial fibroblasts upregulate intracellular adhesion molecule-1 (ICAM-1) and VCAM-1 expression in response to IL-18 (79). In addition, IL-18 promotes migration and angiogenesis of endothelial cells both *in vitro* and *in vivo*. IL-18 has further been shown to inhibit osteoclast formation via T cell GM-CSF production (80). Keratinocytes, originally believed to produce but not process IL-18 (81), have been shown to secrete biologically active IL-18 when treated with dinitrochlorbenzene and proinflammatory mediators such as LPS (82).

Clearly IL-18 exerts extensive effects of IL-18 on numerous cell populations. Whilst some contradictory effects await resolution, IL-18 undoubtedly operates as part of an extensive cytokine network, thus its effects are critically dependent on ambient cytokine milieu and genetic background. Unsurprisingly, IL-18 modulation exerts significant effects not only in host protection against pathogens but also host autotoxicity.

The biological effects of IL-18 are summarised in Figure 1.2.

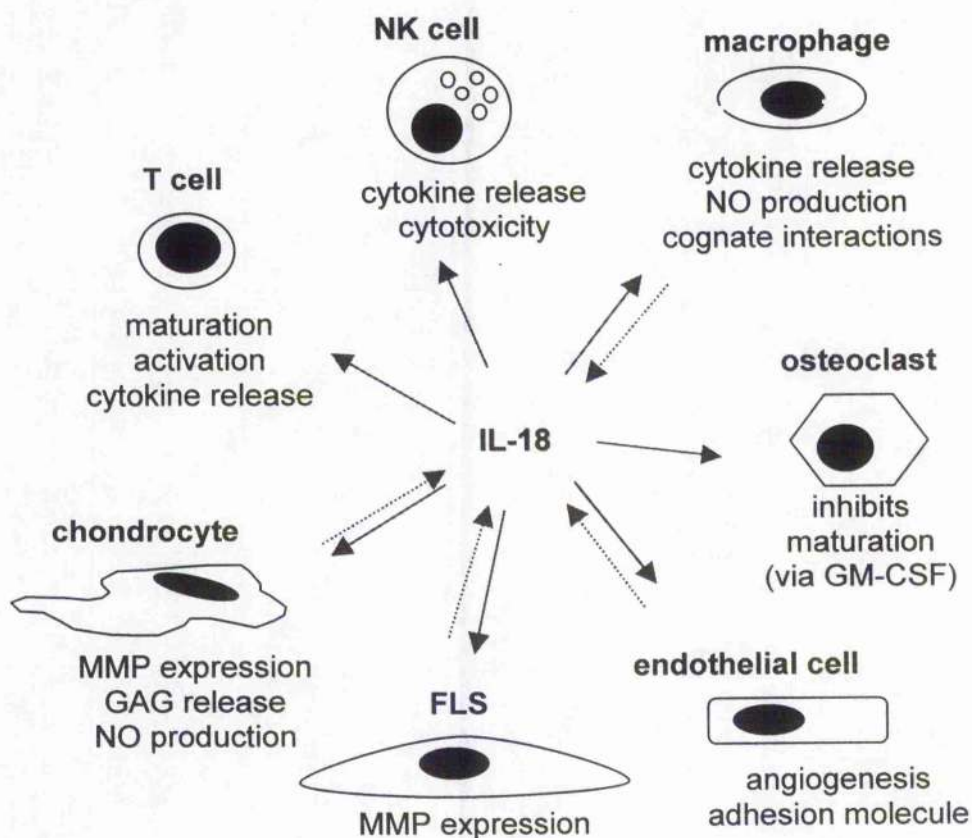


Figure 1.2 Biological effects of IL-18 adapted from McInnes *et al* (83)

1.2.7 Host defence

IL-18 possesses broad and potent immunomodulatory properties, thus IL-18 modulation dramatically alters the pathogenesis of a broad array of infectious diseases. The major effects of some such studies are summarised in Table 1.1. Interpreting the importance of endogenous IL-18, and establishing the potential benefits of supplementing with exogenous IL-18, is essential given the clinical potential of cytokine targeting therapies.

Table 1.1 IL-18 and infectious disease (adapted from (84))

Microorganism	rIL-18 administration	IL-18 neutralisation	IL-18 Knock Out
<i>Yersinia enterocolitica</i>	improved	exacerbated	*
<i>Staphylococcus aureus</i>	*	*	variable
<i>Mycobacterium tuberculosis</i>	*	*	exacerbated
<i>Salmonella typhimurium</i>	improved	exacerbated	*
<i>Legionella pneumophila</i>	*	not exacerbated	*
<i>Streptococcus pneumoniae</i>	*	exacerbated	exacerbated
<i>Cryptococcus neoformans</i>	improved	exacerbated	exacerbated
<i>Candida albicans</i>	*	exacerbated	exacerbated
Protozoa			
<i>Leishmania major</i>	*	*	exacerbated
	*	*	not exacerbated
	Improved (with IL-12)	*	exacerbated
<i>Toxoplasma gondii</i>	Improved	not exacerbated	*
<i>Plasmodium berghei</i>	Improved (with IL-12)	exacerbated	*
Herpes Simplex	improved	*	*
Murine cytomegalovirus	*	*	not exacerbated

Intracellular Bacterial infections

Intracellular bacteria require cell mediated immune responses for their eradication. The role of IL-12 in such protective Th1 type responses is well defined. The intracellular pathogen *Mycobacterium avium* has been widely studied using a variety of murine strains, including IL-18 and IL-18R α -deficient mice, demonstrating a critical role for IL-18 in expulsion of the pathogen (52, 85, 86). Healthy PPD responsive control donors display a greater ability to produce IL-18 and IFN γ in response to antigen, compared to patients with *M. tuberculosis* infection (87). However, patients with active disease exhibit raised plasma IL-18 levels (88) and *in vitro* stimulation of PBMC leads to increased IL-18 production (89). In leprosy the Th1/Th2 balance is key to disease outcome, but currently the role of IL-18 is conflicting. In resistant tuberculoid leprosy (TL), protective IFN γ production is associated with increased IL-18 mRNA expression within lesions. Following *in vitro* challenge with bacterial antigen, monocytes from TL patients show increased IL-18 mRNA expression and IFN γ production compared to cells from patients with susceptible lepromatous leprosy (LL) (90). However, one LL cohort studied exhibited elevated serum IL-18 levels (59), suggesting that IL-18 may promote the development of Th2 response, characteristic of LL. Further *in vivo* studies have shown the importance of IL-18 in the protective immune response to *Salmonella typhimurium* (91) and, although IL-18 expression is elevated in *Legionella pneumophila* infection, neutralisation of IL-18R α was of no consequence to the outcome of the infection (92). Treatment with anti-IL-18 receptor β chain antibody in a murine model of *Listeria monocytogenes* infection demonstrated the protective effects of IL-18 through modulation of early innate immune responses, Th1 development and memory responses (93).

Extracellular Bacterial Infections

Host responses to extracellular bacteria are at least partly mediated by neutrophil dependent killing mechanisms. IL-18 induces monocyte IL-8 production, which is chemotactic to neutrophils, and other effects of IL-18 on neutrophils are addressed throughout this study. Neutralisation of IL-18 in a murine model of *Yersinia enterocolitica* reduced resistance to infection, in an IFN γ independent manner (94). IL-18-deficient mice fail to clear *Streptococcus pneumoniae* as effectively as wild type controls (95). *Staphylococcus aureus* infection in IL-18-deficient mice results in reduced sepsis but enhanced arthritis, possibly indicative of a dual role for IL-18 in protective bactericidal responses, but a detrimental role in promoting sepsis (96).

Role of IL-18 in sepsis

Sepsis may be defined as a potentially fatal condition, which can occur as a sequel to severe illness or trauma and is caused by LPS from gram-negative bacteria; but similar symptoms may result from overwhelming gram-positive infection. The role of IL-18 in endotoxic shock is critically dependent on initiating factors. Administration of neutralising antibodies to IL-18 results in 100% survival following LPS administration that would otherwise be fatal (97). *Propionibacterium acnes* primed IL-18-deficient mice show a defect in negative regulation of TNF α , resulting in increased mortality following challenge with endotoxin (98). Septic patients have elevated levels of both IL-18 and its natural antagonist IL-18BP (41, 99). Interestingly, septic patients with gram-positive infections have significantly higher IL-18 plasma levels than patients with gram-negative infection (100).

Fungal infections

Stimulation of peritoneal exudate cells with IL-18 and IL-12 enhances the suppressive effects on growth of *Cryptococcus neoformans*. Similarly, protective effects of IL-18 administration on *in vivo* infection with *C. neoformans* were the result of increased IFN γ release from NK cells, downregulation of IL-4 production and enhanced NO generation by macrophages (101, 102). Furthermore, IL-18 appears effective even in the absence of IL-12 (103). In a chronic fungal asthma model, IL-18 promotes innate responses preventing the development of severe fungus-induced asthmatic disease (104). In caspase-1-deficient mice, exogenous IL-18 restores defective Th1 responses during *Candida albicans* infection (105).

Viral infections

Immunity to viral infection is mediated via early production of IFN α and IFN β , followed by NK cell activation and antigen specific CD8 cell responses. IFN γ production by CD8 and NK cells is crucial for controlling viral infections. The administration of IL-18 to animals prior to infection with *Herpes simplex virus* (HSV) results in upregulated IFN- γ dependent NO production, leading to improved survival; this effect was evident in the absence of T cells, implying IL-18 also augments innate immunity to viruses (106). In an *in vivo* model of *Vaccinia* infection, IL-18 administration reduces pock formation (107). Pirating of IL-18 binding proteins by poxviruses may contribute to their immunosuppressive abilities (Section 1.2.5), and is consistent with a critical role for IL-18 in defense against the virus. *In vitro*, IL-18 increased HIV-1 production in a chronically infected monocyte cell line (108). However, including IL-18 as an adjuvant enhanced the efficacy of a DNA vaccine against FIV and in models of HIV, apparently by promoting Th1 responses (109).

Protozoan Infections

The activation of macrophages by IFN γ is a critical component of the immune response against intracellular protozoan infection. In animal models, cutaneous inoculation of *Leishmania major* induces either self-limiting, localised lesions or fatal visceral lesions, depending on genetic background. Resistance is associated with CD4 cell IFN γ production. IL-18 deficiency renders mice susceptible to *Leishmania* infection (96, 110). Infection of IL-18-deficient mice on a resistant background results in severe local lesions, which eventually resolve, indicating a protective effect of IL-18, but also that immunity is not exclusively due to IL-18 (111). Studies of animal models of malaria indicate protective effects of IL-18. Treatment of mice with rIL-18 conferred protection to both *Plasmodium yoelii* and *P. berghei*, which was associated with elevated serum IFN γ . Similarly, IL-18-deficient mice or mice treated with neutralising anti-IL-18 antibodies were more susceptible to *P. berghei* than controls (112). Patients with uncomplicated *P. falciparum* malaria, who mount an effective Th1 response, exhibit elevated serum IL-18 (113).

Anti tumour activity

Anti tumour activity of IL-18 has been explored in the context of a wide range of tumours, including sarcoma (114), neuroblastoma (115), melanoma (116), glioma (117), colon carcinoma (118), and bladder carcinoma (119). Various mechanisms are proposed for the observed anti-tumour effects of IL-18, including activation of NK cells, CD4 and CD8 T cells as well as induction of FasL and augmentation of production of reactive oxygen species (120, 121). Early studies demonstrated inhibitory effects of IL-18 on tumour angiogenesis (122), although controversy arose following studies by Koch *et al* demonstrating pro-angiogenic properties of IL-18 (123).

1.2.8 Autoimmunity

Molecules involved in protection against pathogens are invariably involved in inflammatory reactions resulting in tissue injury. As such, multiple pathological roles of IL-18 in immune disturbances have been described.

Inflammatory arthritis

IL-18, in both pro and mature forms, is present in the synovial membrane of patients with RA (78, 124, 125) and with psoriatic arthritis (PsA; unpublished observations). Elevated expression of IL-18 is detected in the serum of patients with Adult Onset Still's disease (126). In the synovial membrane, IL-18 expression localises to CD14⁺ and CD68⁺ macrophages and in fibroblast-like synoviocytes (FLS) *in situ*. IL-18R (α and β) chains are detected *ex vivo* on up to 40% of synovial CD3 lymphocytes and on 20% of synovial CD14⁺ macrophages and *in vitro* on FLS (127). IL-18 may perpetuate inflammation by promoting cytokine release, particularly TNF α , GM-CSF and IFN γ , from both lymphocyte and macrophage populations. Marked synergy with IL-12 and IL-15 is observed in this respect (78). In turn, IL-18 expression is upregulated in FLS by IL-1 β and TNF- α , suggesting the existence of positive feedback loops. This feedback loop provides a link between monokine predominance in RA with innate cytokine production and Th/c 1 cell activation in synovial immune responses. IL-18 induces NO release by RA SM *in vitro* that, since NO inhibits caspase-1 activity, provides a further potential regulatory loop. IL-18 reduces chondrocyte proliferation, upregulates iNOS, stromelysin and cyclooxygenase 2 expression, and increases glycosaminoglycan (GAG) release *in vitro*, thus potentially promoting cartilage degradation (67). However, IL-18 may limit tissue destruction via inhibition of osteoclast maturation through GM-CSF

production by T cells (80). Furthermore, IFN- γ production suppresses COX expression with consequent effects upon prostanoid mediated local inflammation.

The predominantly proinflammatory function of IL-18 has been demonstrated by targeting IL-18 in several arthritis models *in vivo*. Upon challenge with type II collagen (CII) in CFA, IL-18-deficient mice on a DBA/1 background exhibit reduced incidence and severity of arthritis. *Ex vivo* analysis determined that both cellular and humoral responses to CII were suppressed (128). Moreover, administration of recombinant IL-18 abrogates erosive arthritis in DBA/1 immunised with CII in IFA (129). Neutralisation of IL-18 *in vivo*, using specific antibodies or IL-18BP, effectively reduces developing and established rodent arthritis in both streptococcal cell wall (130) and CIA models (131). These observations strongly suggest that the net effect of IL-18 expression is proinflammatory, at least in the context of antigen-driven articular inflammation. Clinical studies to test this hypothesis in RA are awaited.

Gastrointestinal inflammation

Elevated IL-18 mRNA and protein expression are reported in gut tissue from patients with Crohn's disease (CD) and ulcerative colitis (UC) (132, 133). Elevated IL-18 is present in serum of CD but not UC patients (134). IL-18BP isoforms are upregulated in CD mucosa in epithelial cells and macrophages. Correspondingly, IL-18/IL-18BP complexes are detected in tissues together with free mature IL-18 (135). *In vivo* model systems further implicate IL-18 in the pathology of IBD. IL-18 mRNA levels increase early in the colon during dextran sulphate sodium induced (DSS) colitis. Studies utilising several gene targeted murine strains suggest that IL-18 mediated effects on colonic inflammation are IFN γ dependent, but independent of NO, Fas-L and TNF α .

(136). ICE-deficient mice exhibit reduced severity of DSS colitis associated with reduced IL-18 expression (137). Moreover, DSS colitis is ameliorated either by anti-IL-18 antibody or by IL-18BP:Fc protein (138). IL-18BP:Fc treatment attenuated mRNA upregulation of multiple proinflammatory cytokine genes, chemokine genes, and matrix metalloprotease genes in the large intestine, which are commonly elevated during IBD. IL-12p40-deficient mice develop increased severity of TNBS induced colitis associated with enhanced IL-18 expression, suggesting interactions between IL-12 and IL-18 in colonic mucosa (139). Together these data strongly implicate IL-18 as an important mediator of gastrointestinal inflammation.

Insulin dependent diabetes mellitus

The NOD mouse is an accepted model of IDDM, and various studies in this model indicate a role for IL-18 in pathogenesis. Elevated IL-18 mRNA expression is detected in pancreata prior to onset of diabetes in the NOD mouse (140). However, addition of exogenous IL-18 appeared to bias islet infiltrating T cells to a Th2 phenotype, thus reducing disease severity (141). Thus, elevated IL-18 expression may be a preclinical feature of IDDM. Studies of diabetic patients demonstrated no significant elevation of serum IL-18 compared to normal controls; however, patients' first-degree relatives who fulfilled high risk of disease development criteria had significantly elevated serum IL-18 (142). IL-12-deficient NOD mice developed similar insulinitis and IDDM compared to normal controls; the development of IL-18-deficient NOD mice is awaited (143).

Pulmonary disease

Investigations into the role of IL-18 in pulmonary inflammation have yielded conflicting results. Antigen sensitised mice are nasally challenged with the same antigen, which induces lung eosinophilia and airway hyper-responsiveness (AHR), providing a murine model of asthma. In this model, adenoviral delivery of IL-18 in established AHR reduces AHR, IL-4 production, mucus expression and eosinophilia (144). Accordingly, IL-18-deficient mice, or mice treated with neutralising antibody against IL-18, exhibit marked eosinophilia together with exaggerated lung damage, compared with controls (145). However, administration of exogenous IL-18, intratracheally, at time of nasal challenge, enhanced eosinophilic infiltration. This transpired to be secondary to eotaxin production by bronchial epithelial cells in response to IL-18 (146).

In human studies, IL-18 has been implicated in allergic inflammation. Elevated serum IL-18 is detected in patients with acute asthma. Compared with normal control donors, the bronchoalveolar lavage (BAL) fluids obtained from asthmatics, have lower levels of IL-18 (147). These data suggest that the precise effect of IL-18 may depend upon the kinetics and nature of specific pulmonary antigen responses. Patients with sarcoidosis, exhibit elevated IL-18 in serum, epithelial lining fluid, BAL and BAL fluid cells compared to healthy controls (148). Whether neutralising IL-18 would successfully target the harmful effects of granuloma formation or simply permit the emergence of the underlying pathogen remains to be elucidated.

IL-18 exerts extensive biological effects, and over expression of this proinflammatory cytokine is associated with an array of diseases. The aims of this study included

exploration of the effects of IL-18 on neutrophils, therefore neutrophil biology is considered below.

1.3 Neutrophils

1.3.8 Background

Over 100 years ago, Ehrlich described the 'polynuclear' cell, which could spread secretory products to the environment as needed. Subsequently, myriad functions have been subscribed to neutrophils. The neutrophil is a terminally differentiated cell of 9-10 μm diameter. In humans, there are approximately $4 \times 10^6/\text{ml}$ in the circulation, constituting up to 75% of total circulating leukocytes. 5% of circulating neutrophils are immature 'band cells' (Fig.1.3). Neutrophils are derived from myeloblast stem cells in the bone marrow, which progressively lose cytoplasmic organelles and develop cytoplasmic granules, whilst simultaneously undergoing condensation and lobulation of the nucleus. The process of neutrophil maturation is outlined in Fig. 1.3. Progression from myeloblast to mature neutrophil takes about 2 weeks. The majority of neutrophils, rather than entering the blood, reside in the bone marrow for a period before being phagocytosed by bone marrow macrophages. The bone marrow contains up to 30 times more neutrophils than the blood, providing a buffer against neutrophil depletion and allowing a rapid outpouring of neutrophils in response to stimuli such as infection.

1.3.2 Role of neutrophils in the immune response

Neutrophils are the cellular hallmark of acute inflammation. On receiving signals from sites of infection and trauma, large numbers of neutrophils exit the blood stream, and migrate into tissue. As the first cells to arrive at the source of the insult, the neutrophils' principal role is believed to be ingestion and destruction of bacteria via the generation of reactive oxygen species and the release of lytic enzymes stored in granules. Neutrophils display a paucity of ribosomes and endoplasmic reticulum, yet retain some ability to synthesise proteins. Thus in addition to dealing directly with the offending stimulus, neutrophils may generate soluble mediators, allowing them to communicate with other cells. Furthermore, neutrophils display a dynamic and curious array of cell surface molecules, providing a further dimension to this communication network. Each of these aspects of neutrophil function is considered below.

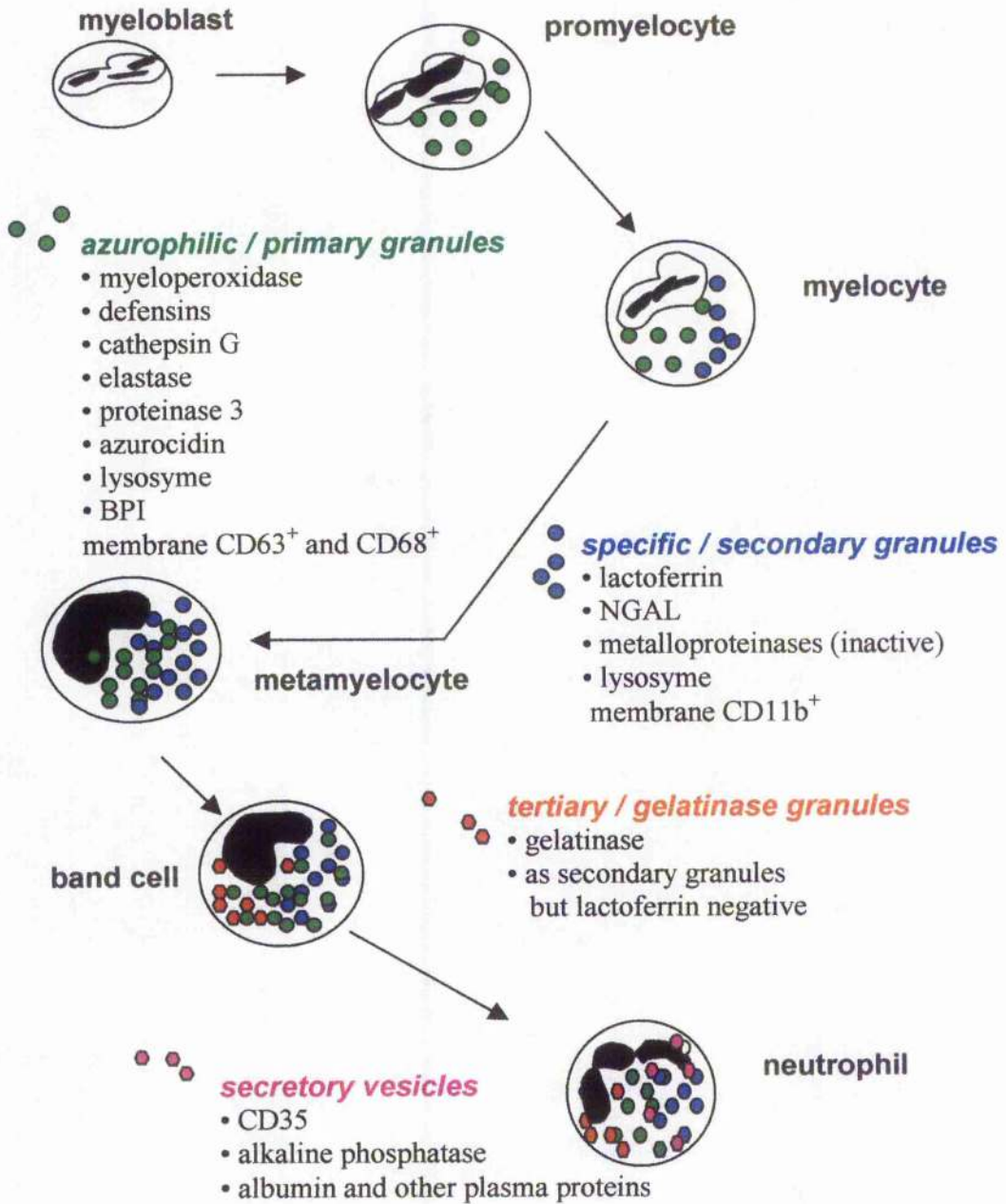


Figure 1.3 Neutrophil maturation in the bone marrow

1.3.3 Neutrophil migration

Neutrophils exit the blood, entering tissues, guided along gradients of molecules perceived by the cell. In the blood, approximately 50% of neutrophils flow with the circulating erythrocytes, while the remainders are 'marginated' and loosely adherent to the vascular endothelium. Neutrophil transmigration is illustrated in Fig. 1.4.

Neutrophils employ a multiplicity of mechanisms for locomotion as they encounter different substances on their march from blood to tissues. Selectins (L-, E- and P-selectin) exhibit rapid binding characteristics, sustaining dynamic interactions between neutrophils and endothelium. Neutrophils pause for milliseconds between each rolling step, and just 2 adhesive bonds may maintain this process (149, 150). Moreover, L-selectin binding can signal functional responses, such as upregulation of CD11b/CD18 (151). Arrest of the neutrophil is dependent on β_2 integrins, (CD11b/CD18 or CD11a/CD18 complexes). CD11a-deficient mice show reduced numbers of neutrophils at sites of inflammation, although this is less pronounced in CD11b-deficient animals (152). *In vitro*, cytokine activated endothelial cells induce shape change of neutrophils within 1-2 minutes of contact. Trans-endothelial migration follows and is complete within 1-2 minutes (153). Following shape change, L-selectin is shed and CD43 and CD44 are also partially shed. Other receptors traffic around the cell, for example, formyl peptide receptors and CR4 are sent to the forward regions (154-156). An anti-adhesive role is proposed for CD43, which would facilitate migration; CD43-deficient mice show reduced trans-endothelial migration even in the presence of increased adhesion (157). Once trans-endothelial migration is complete, neutrophils encounter extracellular matrix where β_1 integrins facilitate travel. Following transendothelial

migration, neutrophils now show increased $\alpha_4\beta_1$ dependent adhesion (158). $\alpha_9\beta_1$, which binds VCAM and extracellular matrix, is expressed by neutrophils. Additionally, $\alpha_9\beta_1$ contributes to neutrophil migration through fibroblast layers *in vitro* (159).

Neutrophil recruitment also influences subsequent cellular responses. In rodent models, depletion of neutrophils by specific monoclonal antibody (RB6-8C5 in mouse; RP-3 in rats) reduces both recruitment of macrophages and lymphocytes in autoimmune diseases, and immunity to tumours (160, 161). IL-8-induced CD4 T cell recruitment into subcutaneous tissues of rats is inhibited by RP-3 treatment (162). Furthermore, neutrophil infiltration of hapten challenge sites during contact hypersensitivity responses is essential for the subsequent recruitment of CD8 T cells (163). Accordingly, neutrophil migration into the tissues may affect the later accumulation of macrophages or T lymphocytes.

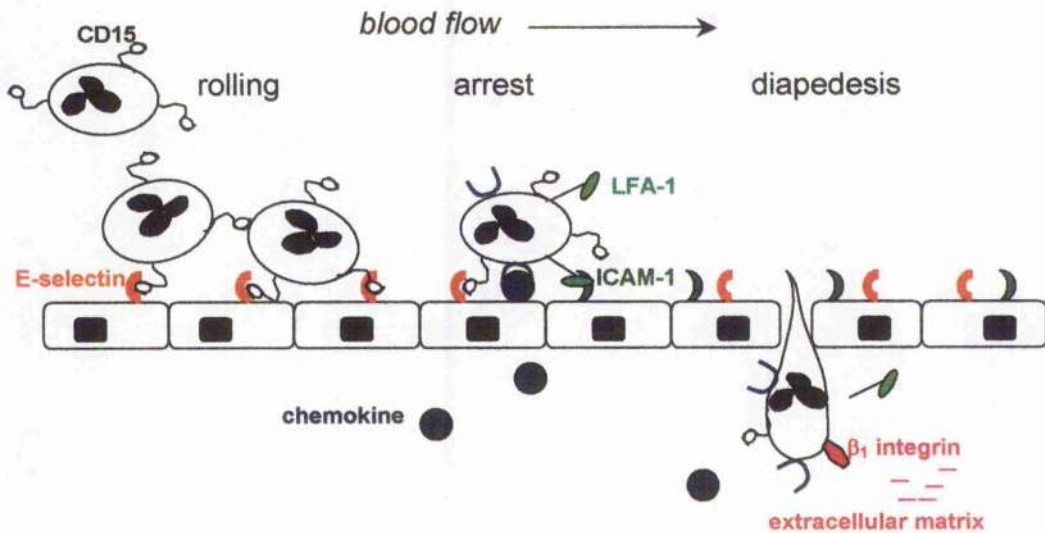


Figure 1.4 Neutrophil migration

1.3.4 Neutrophil anti-microbial defences

Patients with Chronic Granulomatous disease, who exhibit a deficiency of the NADPH oxidase system, are highly susceptible to bacterial and fungal infections, exemplifying the importance of the neutrophil respiratory burst. The oxidative burst depends on activation and assembly of the NADPH oxidase, a membrane associated electron transport chain. The primary products, superoxide anion and hydrogen peroxide, are relatively harmless, but give rise to potent anti-microbial compounds (Fig. 1.5). These reactive oxygen intermediates (ROI) also function as regulators of other immune cells and apoptosis. Toxic products of nitric oxide, such as peroxyntitrite (ONOO^-), may be generated by murine but not human neutrophils.

Neutrophil granules store myeloperoxidase, which catalyses numerous reactions of the respiratory burst, integral to neutrophil bactericidal activity. Details of proteins localised to granules, along with an overview of neutrophil granule accumulation through maturation is illustrated in Fig. 1.3. In addition to physically destroying the target, granule constituents may modify the ongoing immune response. For example, cathepsin G is chemotactic to mononuclear cells (164). Cathepsin G-deficient mice exhibit exaggerated neutrophil influx to wounds. The subsequent defects in wound repair are presumed to reflect the absence of macrophages, which promote wound healing and phagocytose neutrophils (165). Defensins are a large family of cysteine rich cationic proteins. Found in neutrophil granules and other cells (macrophages, keratinocytes), defensins display potent, non-specific anti-microbial activity. However, individual defensins exert other discrete effects such as degranulating mast cells (166), stimulating IL-8 production from epithelial cells (167), and acting as chemoattractants for distinct T cell subsets (168, 169). Patients with Chediak-Higashi syndrome have giant azurophil

granules, which do not secrete enzymes normally, and accordingly suffer from severe infections and an atypical lymphoproliferative syndrome (170). Neutrophils exert potent antibacterial effects through generation of reactive oxygen species and release of granule proteins, and these armaments may also be instructive to the ongoing immune response.

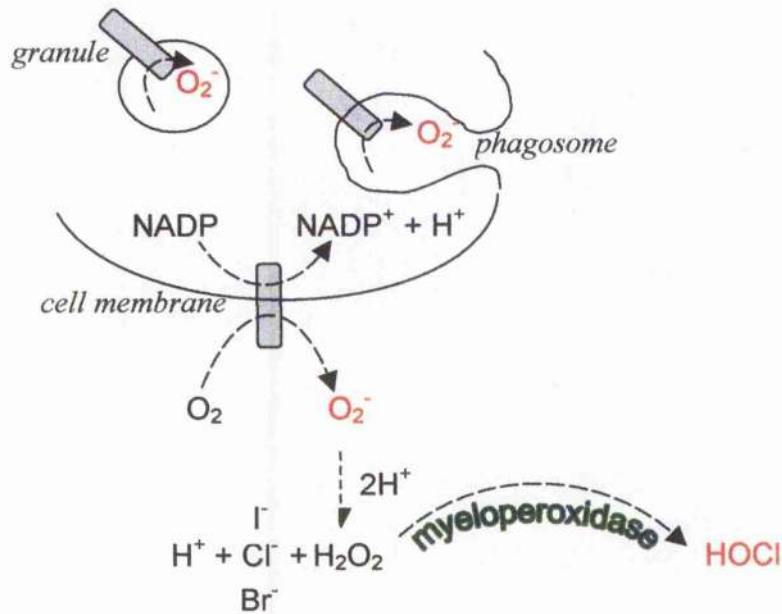


Figure 1.5 Possible sites for neutrophil oxidase assembly and reactions

1.3.5 Production of cytokines by neutrophils

Mature neutrophils are terminally differentiated cells containing meagre protein synthesis machinery. However, studies of highly purified (>99.5%) neutrophil populations, using molecular biology techniques and immunohistochemistry, convincingly demonstrate that neutrophils are capable of synthesising a variety of cytokines (Table 1.1).

Neutrophil cytokine release may contribute to orchestrating inflammatory responses, as indicated by depletion studies described previously (Section 1.3.3). Furthermore, neutrophil depletion, in mice otherwise susceptible to *Leishmania*, liberates sufficient pre-stored IL-12 to initiate a protective Th1 response (171). Mononuclear cells generally generate more cytokine than neutrophils on a per cell basis (Table 1.2). However, as neutrophils constitute the majority of infiltrating leukocytes in inflammation, their cytokine release is likely to provide a substantial contribution to the overall inflammatory response.

Cytokine release may be paracrine or autocrine in function. Many cytokines released by neutrophils exert an array of autocrine effects, and may prime and initiate respiratory burst and degranulation, delay apoptosis, and promote migration. As such, the cytokine network provides a potent amplification loop for neutrophil led responses. Conversely, cytokines may restrain neutrophils; IL-10 can suppress IL-8 production in response to LPS (172). A wide range of stimuli may induce cytokine production by PMN (Table 1.3). The magnitude, kinetics and nature of cytokines released vary substantially depending on the stimulus; for example, opsonised *Saccharomyces cerevisiae* (Y-IgG) induces only proinflammatory cytokines, while LPS induces both pro (e.g. IL-1) and anti inflammatory (e.g. IL-1ra) (173).

Table 1.2 Cytokines expressed by neutrophils (adapted from (173))

<i>in vitro</i>	<i>in vivo</i>
IL-1 α and β	IL-1 α and β
IL-1ra	IL-1ra
IL-8	IL-6
IL-12	IL-8
TNF α	IL-10
IFN α	IL-12
GRO α , GRO β	KC, GRO α
CINC-1,2 α ,3	CINC
IP-10	MIP-1 α /1 β
MIG	TNF α
MIP-1 α /1 β	TGF β
TGF α , TGF β	
IL-3, G-CSF, M-CSF	

Table 1.3 Examples of stimuli for neutrophil IL-8, IL-1 and TNF α production (adapted from (173))

	IL-8	IL-1	TNF α
LPS	+	+	+
fMLP	+	-	-
TNF α	+	+	+
IL-1 β	+	+	+
Leukotriene B ₄	+	-	-
IFN γ	+	+	+
Substance P	+	-	-
C5a	+	-	-
fibronectin	+	+	+
Monosodium ureate crystals	+	+	-
PMA	+	+	+
Respiratory Syncytial Virus	+	+	-
Epstein Barr Virus	+	+	-
<i>Candida albicans</i>	+	+	+
<i>Staphylococcus aureus</i>	+	+	+
<i>Cryptococcus neoformans</i>	+	+	+

Table 1.4 Relative cytokine production by neutrophils and monocytes (adapted from (174))

cytokine	cell type	stimulus	cell no./ml	concentration ng/ml/24 h
IL-8	PMN	LPS	10^7	2.8
	MNC		10^7	200
TNF α	PMN	Y-IgG	5×10^6	0.2
	MNC		5×10^5	3
IL-1 β	PMN	LPS	10^7	3.7
	MNC		10^5	0.4

1.3.6 Neutrophil antigen presentation

Quiescent circulating neutrophils express Class I MHC (MHC-I). Exogenous peptide antigen can bind neutrophil cell surface MHC-I and stimulate memory CD8 cells. Furthermore, neutrophils may process exogenous antigen via an alternate vacuolar pathway for presentation on MHC-I, and these processed peptides may be released for presentation by other cells (175). Stimulation with GM-CSF and IFN γ increases MHC-I expression and may induce Class II MHC (MHC-II) (176). Following treatment with GM-CSF, neutrophils respond to superantigen by producing IL-8. Moreover, mutant superantigens lacking MHC-II binding activity fail to induce IL-8 (177). MHC-II positive neutrophils mediate superantigen-induced T cell activation, but lack the ability to process antigen for Class II presentation (178). In addition to MHC-II, human PMN cultured with IFN γ express co-stimulatory molecules, CD80 and CD86 (179). Synovial fluid derived neutrophils constitutively express intracellular MHC-II, which is found on the cell surface following *ex vivo* culture (180). This suggests that neutrophils within the joint, but not in the synovial fluid, may express MHC-II, with the potential to present and process cryptic antigens. The *in vivo* relevance of neutrophil antigen presentation

awaits clarification, but provides another intriguing potential link between neutrophils and the adaptive response.

1.3.7 Role of neutrophils in sepsis and infection

Neutrophils are critical to the clearance of numerous microbes, a role best exemplified in gram-positive infections such as *Staphylococcus aureus*. However, in the event of overwhelming, severe infection, a systemic inflammatory response may develop. Whether neutrophils are friend or foe in sepsis is unclear. Neutrophilia arises following an outpouring of neutrophils from the bone marrow, which increases the proportion of immature 'band' cells in the circulation. Neutrophils derived from septic patients exhibit a complex altered functional phenotype. Although LPS may prime and activate neutrophils, a phenomenon of neutrophil endotoxin tolerance describes the refractory response of neutrophils to re-stimulation following appropriate encounter with endotoxin. Thus neutrophils obtained from patients with sepsis show decreased capacity to generate IL-1 upon stimulation with various agonists (181). This 'tolerance' is not restricted to initiation by LPS and is observed in patients with gram-positive sepsis. Following severe trauma, leukotrienes release by neutrophils diminishes, which correlates with the occurrence of immature neutrophils, mostly band cells, in the circulation. Similarly, expression of 5-lipoxygenase in neutrophil fractions consisting mainly of band cells is decreased (182). Neutrophils in sepsis display some features of activation including enhanced expression of CD11b. The resulting proclivity to sequester in microvessels in an activated state may perpetuate endothelial damage. Interestingly, immature neutrophils show greater tendency to accumulate in the microvasculature during gram-positive sepsis (183).

Migratory defects are reported in neutrophils during sepsis implying a scenario whereby activated neutrophils may deliver toxic oxygen species and proteolytic enzymes directly to the endothelium, promoting injury within microvascular beds. Interventions that target neutrophils or their products may offer protection from tissue injury and death in endotoxaemia, suggesting that these cells may be worthy of further clinical attention.

1.3.8 Role of neutrophils in arthritis

Normally acellular joints become heavily infiltrated with leukocytes in rheumatoid arthritis. A pannus of proliferating cells forms over the cartilage, consisting of synovial-like, fibroblast-like, macrophage-like cells, and lymphocytes. The cartilage-pannus junction (CPJ) is the site of actual cartilage destruction. This 'solid phase' is bathed in synovial fluid (SF), which becomes heavily infiltrated, primarily with neutrophils. In active disease, neutrophils may accumulate within the synovial fluid at concentrations exceeding 10^8 neutrophils/ml.

SF derived neutrophils display evidence of *in vivo* priming and activation. SF neutrophils contain less myeloperoxidase (MPO) than PB neutrophils; in SF, MPO is present in a form that suggests it has been co-secreted with oxidants (184). Compared to PB neutrophils, SF neutrophils exhibit increased CD11b, CD35 (CR1) (185) and reduced formyl peptide receptor expression. Functionally, SF neutrophils are refractory to priming; IFN γ stimulated PB, but not SF, neutrophils exhibit enhanced respiratory burst in response to fMLP (186).

Given the phenotype of SF neutrophils, predictably, the SF contains numerous neutrophil derived factors (including IL-1, IL-8, TNF α , LTB $_4$), which may exert autocrine priming and activation of neutrophils. Additionally, IgG containing immune complexes found within the joint are potent neutrophil agonists. Unlike PB neutrophils, SF neutrophils express FcRI; cell free SF induces FcRI expression by PB PMN (187). Whether FcRII and III expression is modulated on SF compared to PB neutrophil remains controversial (188).

Neutrophils are absent from synovial explant cultures, however these tissues are obtained at end stage disease and with culture methods that favour dividing or long-lived cells. However, histological examination of the cellular composition of the CPJ revealed the presence of neutrophils (189). An activated neutrophil adjacent to cartilage may release harmful concentrations of reactive oxygen and degradative enzymes, a process termed 'frustrated phagocytosis.' In the fluid phase, potential for neutrophil damage includes release of products that break down hyaluron (190), reducing its viscosity, and others which saturate the synovial fluid anti-proteinase and antioxidant activity (191).

Peripheral circulating neutrophils in RA patients exhibit characteristics predominantly equivalent to normal PB neutrophils. However, RA plasma levels of lactoferrin and elastase are greater than normal controls, possibly indicative of peripheral degranulation (192). Nevertheless, no difference in CD11b, CD16, CD32, CD35 and formyl peptide receptor expression is reported between RA and normal PB neutrophils (193). Other studies report elevated CD16 on RA PB neutrophils and such discrepancies may be attributable to drug effects (185). The majority of evidence indicates that, in rheumatoid

arthritis, rather than systemic deregulation, neutrophils undergo major changes on entering the joint.

Animal models of arthritis indicate that neutrophil depletion may ameliorate inflammation and joint damage. In the rat adjuvant arthritis model, administration of anti neutrophil antibody (RP3) reduced circulating neutrophils and neutrophil accumulation within the joint, concomitant with ameliorated arthritis. Interestingly, no parallel effects on T cell numbers within the joint were observed. Conversely, CD4 depletion, in the same model system, markedly reduced neutrophil accumulation within the joint (194). Circumstantial evidence indicates that targeting neutrophil activation in human disease may be beneficial. *In vitro*, leflunamide inhibits neutrophil migration, and patients receiving leflunamide display reduced neutrophil numbers in joints (195). Neutralising TNF α may modulate neutrophil function at many levels, as TNF α promotes neutrophil migration, priming, release of granules and ROI all of which may contribute to joint damage (reviewed (196)).

The SF is clearly a source of patho-physiologically altered neutrophils, which likely contribute to the disease process. This heterogeneous population of resting, primed and activated neutrophils indicates a dynamic population, which varies with prevailing conditions within the joint.

1.4 Cytokines as clinical targets

Cytokines are critically involved in the pathogenesis of several diseases such as arthritis, Crohn's disease and sepsis, providing a rationale for therapeutic targeting.

Cytokine targeting has been extensively investigated in arthritis. Targeting cells of the immune system, by depletion of CD3 cells, provides transient improvements but severe side effects. Other more common agents for the treatment of arthritis, such as corticosteroids, affect the transcription, release and activity of cytokines. Some patients do not respond to these non-specific agents and these drugs exert toxic side effects. Specific anticytokine targeting may be achieved with monoclonal antibodies (e.g. Infliximab), soluble receptors (e.g. Etanercept), recombinant natural antagonists (e.g. IL-1Ra) and anti-inflammatory cytokines (e.g. IL-10). The effects of targeting TNF α are summarised in Table 1.5. Treatment of arthritis with monoclonal antibody against TNF α results in a dramatic reduction in circulating IL-1, IL-6, ESR and CRP with associated clinical improvement; however, disease returns upon cessation of therapy (reviewed (4)).

Although animal models of sepsis provided promising results, treatment with soluble TNF α receptors actually increased mortality in one sepsis trial (the soluble TNF α R employed was an Fc fusion protein, and these results are speculated to relate to Fc effects) (197). There was no overall effect of TNF α neutralisation in another sepsis trial (198). A small subgroup of patients however, enjoyed clear benefit from this therapy.

Targeting IL-1 in arthritis revealed the importance of reagent choice. Use of soluble IL-1R1 was not effective in treating arthritis (199). Indeed in human trials of endotoxaemia, administration of soluble IL-1R1 resulted in decreased circulating IL-1Ra, and increased circulating TNF α and IL-8 (200). This recombinant soluble receptor binds the natural antagonist reducing its overall biological effectiveness. However,

treatment of arthritis patients with recombinant human IL-1Ra demonstrated clinical benefit, although arguably with less efficacy than TNF α targeting (201).

Table 1.5 Effect of targeting TNF α in experimental and human disease (adapted from (202))

animal models	efficacy	human clinical studies	efficacy
bacteraemic / endotoxic shock	+	rheumatoid arthritis	+
cerebral malaria	+	inflammatory bowel disease	+
experimentally induced arthritis	+	IL-2 toxicity	-
EAE	+	septic shock	-
inflammatory bowel disease	+	Jarisch-Herxheimer reaction	+
acute lung injury	+	cerebral malaria	-
acute pancreatitis	+	graft vs. host disease	-
graft vs. host disease	+		

Further potential benefits of cytokine directed therapies lie in combination therapy. This strategy provides better disease control, allows reduction in dose, reduces the toxicity of non-specific agents such as DMARDs, and may ease the impairment of host defences caused by specific cytokine targeting therapies. Clinical trials combining methotrexate and TNF α inhibitors demonstrate the efficacy of this rationale (203). Other combinations of therapies under consideration include targeting IL-1 and TNF α , and combining IL-1Ra with DMARDs.

Cytokine actions and interactions in the pathogenesis of disease continue to be explored. Promising results obtained so far indicate the potential of cytokine based therapies.

Furthermore, as novel cytokines are defined and characterised, these provide new avenues to explore the pathogenesis of disease and refine treatment strategies.

1.5 Objectives

The foregoing introduction demonstrates the importance of IL-18 in protective and pathogenic immune responses. This study aimed to address the role of IL-18 in early immune responses and neutrophil activation, specifically:

1. *In vitro* activation of human neutrophils by IL-18
2. *In vivo* effects of IL-18 on neutrophil function
3. The role of IL-18 in the host response to gram-positive infection, *in vitro* and *in vivo*
4. The role of cytokines and other factors that potentially allow neutrophils to interact with cells on the adaptive immune system

A series of *in vitro* and *in vivo* systems were employed to answer these questions.

Chapter 2 Material and Methods

2.1 Samples and ethical considerations

Samples were obtained from patients attending the Centre for Rheumatic Diseases, Glasgow Royal Infirmary and Gartnavel General Hospital. RA patients satisfied the 1987 American College of Rheumatology diagnostic criteria (204). Samples were collected in the presence of 10 U/ml sodium heparin (Leo Laboratories, Bucks, UK). Synovial fluid (SF) was surplus to clinical requirement following routine therapeutic or diagnostic procedures and would otherwise have been discarded. Blood and SF samples were collected only when clinically indicated and informed consent was obtained from patients prior to research use of samples. Peripheral blood was obtained from normal healthy donors with informed consent. This study (Dr. I.B. McInnes, A comparative study of synovial immune dysregulation in inflammatory arthritis; Project no. 00RH004) was reviewed and approved by the Glasgow Royal Infirmary Ethics Committee.

2.2 Reagents

The source of reagents (either purchased or donated) is given in the text and Tables 2.2-2.5. All chemicals were obtained from Sigma, Poole, UK, and all culture media and supplements were obtained from Invitrogen, Paisley, UK unless otherwise indicated.

2.3 Cell culture

Human cells were cultured in RPMI 1640 medium, supplemented with 2 mM L-Glutamine, 100 IU Penicillin, 100 µg/ml Streptomycin and 10% foetal calf serum ('complete RPMI'). Foetal calf serum (FCS, Harlan, Loughborough, UK) was certified mycoplasma free by the manufacturer and heat inactivated at 56°C for 30 minutes. Iscove's Modified Dulbecco's Medium (I-DMEM) was similarly supplemented and used for whole blood culture. Murine cell cultures were carried out in the presence of complete RPMI supplemented with 25mM HEPES. The DO11.10-GFP cell line, which contains a plasmid conferring antibiotic resistance, was cultured in complete medium supplemented with 0.5 mg/ml geneticin (Sigma). Cells were washed in medium containing antibiotics and L-Glutamine but not FCS. Cultures were incubated at 37°C in a humidified incubator with 5% CO₂. Cells were counted with a Neubauer haemocytometer (Weber Scientific International Ltd, UK) by diluting in cell counting fluid (0.01M acetic acid, 0.1% trypan blue in PBS), allowing viability to be assessed by trypan blue exclusion.

2.4 Human sample preparation

2.4.1 Peripheral blood neutrophil preparation

Venous blood collected in the presence of 10 U/ml sodium heparin was mixed with 1 part of 6% dextran (dextran from *Leuconstoc mesenteroides*, molecular weight approximately 160 000, Sigma; in 0.9% saline) to 4 parts blood, and incubated for 30 minutes at 37°C. The buffy coat was subsequently layered over 5 ml lymphoprep

(Nycomed, Oslo, Norway) and centrifuged at 400 x g in a swing bucket rotor for 25 minutes at room temperature. Mononuclear cells in the interface were removed, leaving neutrophils in the cell pellet. Remaining erythrocytes were lysed by 30 seconds exposure to sterile distilled water followed by the addition of an equal volume of 2 x PBS. Alternatively, neutrophils were obtained by layering 5 ml of blood over 5 ml of Polymorphoprep (Nycomed) and processed as for lymphoprep. The neutrophils, found in the lower interface, were of similar purity and viability to those obtained using lymphoprep. Neutrophils were washed 3 times in RPMI by centrifuging at 300 x g for 5 minutes at room temperature. Where indicated, autologous human serum was collected into a clot activator Vacutainer™ (Ref 367837, Becton Dickenson (BD), Oxford, UK).

2.4.2 Synovial fluid neutrophil preparation

Synovial fluids were diluted with an equal volume of RPMI followed by density centrifugation over lymphoprep, then washing of the neutrophils as in section 2.4.1.

2.5 Murine sample preparation

2.5.1 Murine blood preparation

Murine whole blood was obtained by cardiac puncture under terminal anesthesia (Schedule 1 procedure according to Home Office guidelines). The heart was exposed and blood withdrawn with a 23 G needle (all needles: BD Microlance™, BD). Anaesthesia was induced by intraperitoneal (i.p.) injection of 12.5 mg/animal avertin (2,2,2-tribromoethanol) in PBS, with a 25 G needle (stock anaesthetic was prepared by

dissolving 1 g avertin per 1 ml amyl alcohol and was stored at 4°C). Blood was collected in the presence of 20 U/ml heparin and processed within 20 minutes of collection. Murine buffy coats were obtained by addition of an equal volume of 6% dextran (as Section 2.4.1). Murine serum was obtained by allowing blood to clot overnight in Eppendorfs™ at 4°C. Clotted samples were centrifuged at 450 x g for 10 minutes and the serum removed and stored at -20°C. Murine peripheral blood leukocytes were obtained following cardiac puncture and erythrocyte lysis. One volume of blood was added to 4 volumes of freshly prepared, sterile filtered, red cell lysis buffer (155 mM KH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA, in dH₂O) and incubated for 5 minutes at 4°C. Remaining white cells were washed twice in RPMI at 400 x g for 5 minutes then resuspended at 2 x 10⁶ cells/ml.

2.5.2 Murine spleen and lymph node preparation

Single cell suspensions of spleens and lymph nodes were obtained by passing tissues through 100 µm Nitex membrane (Cadisch & Sons, London, UK) using a 5 ml syringe plunger, in the presence of RPMI. To enrich for lymphocytes in lymph node cell suspensions, cells were resuspended at 4 x 10⁶/ml in complete RPMI, and cultured in 150 cm³ polystyrene flasks (Iwaki, Japan), for 2 h after which non-adherent cells were removed. Cells were washed three times in RPMI and resuspended at the desired cell concentration in complete RPMI with 25mM HEPES.

2.5.3 Murine neutrophil preparation

Murine neutrophils were obtained from thioglycollate induced peritoneal exudate cells (PEC, Section 2.11.2). PEC obtained from 2 BALB/c mice were washed once then resuspended in 8 ml PBS, and layered over 3 ml Histopaque® 1083 (Sigma). The gradient was centrifuged at 700 x g for 30 minutes at 20°C. The enriched population of neutrophils contained within the cell pellet was washed twice in RPMI then resuspended at 2×10^6 /ml.

2.5.4 Murine macrophage preparation

Peritoneal exudate cells were washed in PBS then resuspended in complete medium and adhered to a polystyrene flask for 3 – 4 h after which remaining adherent cells were incubated with ice cold PBS (without calcium or magnesium) at 4°C for 10 minutes. Cells were harvested with a cell scraper, then washed in RPMI and resuspended at 2×10^6 /ml.

2.5.5 Murine dendritic cell preparation

Mice were sacrificed by cervical dislocation prior to aseptic removal of tibias and fibias, which were transferred to ice cold RPMI. Muscle was removed and epiphyses cut off and bone marrow flushed out with 2 ml RPMI using a 2 ml syringe and a 23 G needle. The marrow plugs were broken up through a Nitex membrane and the resulting cell suspension washed twice in RPMI. Cells were resuspended at 2×10^5 /ml in complete RPMI supplemented with 10% GM-CSF (supernatant of x63 myeloma, kind gift of Dr.

Jim Brewer, Dept. of Immunology, University of Glasgow) and cultured in 60 mm petri dishes (Bibby Sterlin, Stone, UK). On day 3, a further 10 ml of complete RPMI supplemented with 10% GM-CSF was added. At days 6 and 8, 10 ml of cell culture medium was removed and centrifuged at $400 \times g$ for 5 minutes. The cell pellet was resuspended in 10 ml fresh medium containing GM-CSF and returned to the petri dish. Dendritic cells were harvested for assay on day 10.

2.5.6 Stimulation of murine cell cultures

Tissue culture plates were coated with $5 \mu\text{g/ml}$ anti-CD3 (BD, Clone 145-2C11) in PBS for 4 h at 37°C . Plates were washed with sterile PBS prior to addition of cells. In other experiments, cells were cultured with either $2 \mu\text{g/ml}$ concanavalin A (Con A, Sigma), toxic shock syndrome toxin-1 (TSST-1, Sigma), or formalin fixed *Staphylococcus aureus*. Concentration of stimuli is indicated in the figure legends. Fixed *S. aureus* were prepared by addition of 4% formaldehyde to an equal volume of 10^8 CFU/ml *S. aureus* in PBS (Section 2.9.8). Bacteria were incubated for 30 minutes at 4°C then washed 3 times with PBS at $700 \times g$ for 10 minutes. Cell contact dependency was investigated with tissue culture insert strips (Nunc, Roskilde, Denmark), consisting of a $0.2 \mu\text{m}$ anopore membrane, allowing separation of 2 cell populations within a 96 well format.

2.5.7 Proliferation assays

Proliferation assays were performed in triplicate in U bottom 96 well culture plates (Nunc) at 2×10^5 cells per well in a final volume of $200 \mu\text{l}$. $1 \mu\text{Ci}$ [^3H] thymidine (Amersham Life Science, Bucks, UK) in $25 \mu\text{l}$ complete medium was added during the

final 6 or 18 h of culture (as indicated in results) before harvesting onto filter mats (Wallac, Milton Keynes, UK) using a 1295-004 Betaplate™ 96 well harvester (Wallac). [³H] thymidine incorporation was measured using a 1205 Betaplate™ liquid scintillation counter (Wallac). In some experiments parallel cultures were performed in flat bottom 96 well plates and supernatants were stored at -20°C until estimation of cytokine content by ELISA (Section 2.6).

2.6 Cytokine assays

Concentration of cytokines in supernatants and serum samples were estimated by ELISA. Paired antibodies and ELISA kits were used in accordance with the manufacturer's recommendations (Table 2.2). All assays were optimised and validated prior to use. Maxisorp 96 well plates (Nunc) were coated with capture antibody in 0.1 M NaHCO₃ overnight at 4°C. All subsequent steps were carried out at room temperature with reagents diluted in blocking buffer (PBS, 10% FCS). Plates were blocked for 2 h; samples and standards added for 2 h, followed by biotinylated secondary antibody for 1 h, then ExtrAvidin peroxidase (Sigma) conjugate for 45 minutes. Plates were washed 4-8 times between each step with wash buffer (PBS 0.05% Tween 20). For each wash, wells were filled with wash buffer and allowed to stand for at least 1 minute prior to removal of wash buffer. For the final wash, remaining wash buffer was removed by pounding the plate on paper towels. Plates were developed with TMB (3,3',5,5'-tetra-methylbenzidine, KPL, Gaithersburg, USA), colour allowed to develop, and then read at 630 nm with a Dynex MRX II plate reader and analysed using Dynex Revelation 3.2 software (Dynex, Middlesex, UK).

2.7 Flow cytometry

2.7.1 Cell surface antigen staining

Human or murine cells were resuspended in FACS buffer (PBS, 0.2% sodium azide, 2% FCS) at approximately 4×10^6 cells/ml. 100 μ l of cell suspension, or 200 μ l anticoagulated whole blood were added for each test in a 12 x 75 mm polystyrene tube ('FACS tubes' Falcon 2052, BD). Antibody binding non-specifically to murine Fc receptors was reduced in murine samples by incubation at room temperature for 15 minutes with 0.5 μ g Fc block (anti-mouse CD16/32, BD). Cells were stained with various antibodies or appropriate isotype controls (Table 2.3), for 30 minutes, at 4°C in the dark. Where secondary detection reagents were necessary, cells were washed in FACS buffer, centrifuging at 400 x g for 5 minutes, then resuspended in 100 μ l of FACS buffer containing the appropriate secondary reagent and incubated at 4°C in the dark for 30 minutes. At this stage, whole bloods were lysed with 2 ml BD FACSLysing™ solution for 10 minutes at room temperature. Cells were washed twice in FACS buffer, then resuspended in 200 μ l FACS Flow, passed through Nitex and analysed on a FACSCaliber using CellQuest Software (all BD).

2.7.2 Intracellular cytokine staining

For intracellular staining, cells were resuspended at approximately 2×10^7 cells/ml and cell surface antigens were stained as above. Cells were fixed by addition of 2 ml of 2% formaldehyde in PBS for 30 minutes at 4°C, washed twice in 'perm' buffer (FACS buffer supplemented with 0.5% saponin) and then incubated with antibody in perm

buffer in the dark for 30 minutes at 4°C. Finally, cells were washed twice in perm buffer, then in FACS buffer and analysed as above. Details of antibodies are contained in Table 2.3.

2.7.3 FACS acquisition and analysis

Cells were acquired and analysed on a Becton Dickinson FACSCalibur. The instrument was calibrated monthly with CaliBRITE™ beads (BD), which are designed for use with the FACSCalibur™. The beads were used to adjust instrument settings, set fluorescence compensation, and check instrument sensitivity.

Prior to running labelled cells, FSC (forward scatter) and SSC (side scatter) were adjusted so cells of interested could be gated on the screen. Unstained samples were used as auto-fluorescence controls allowing FL1 (FITC) and FL2 (PE/PI) detector settings to be adjusted so that auto-fluorescence background was roughly within the first decade of the log scale of the fluorescence intensity histogram. Unstained samples were compared with isotype and positively stained controls to confirm the correct scale. Compensation settings were set such that on a FL1 vs. FL2 dot plot, an FL1 positive population is vertically aligned with negative population, and an FL2 positive population is horizontally aligned with the FL2 negative population. Results are expressed either as % positive cells or mean fluorescence intensity (MFI).

2.8 Histology

2.8.1 Cytospins and staining

Cells were resuspended at approximately 4×10^5 cells/ml in PBS and 200-500 μ l spun onto a SuperFrost Plus glass slide (Shandon, Pittsburgh, PA) at 300 rpm, for 3 minutes, using a Shandon cytopsin system. Cytospins were stained with DiffQuick™ (TBS, Skelmersdale, UK). Cells were fixed onto slides by immersion in methanol for 5 minutes, and then allowed to air dry. Slides were immersed in Solution B (Eosin Y in phosphate buffer) for 2-3 minutes then immediately transferred to Solution C (Methylene blue in phosphate buffer) for 2-3 minutes. Staining was checked for intensity before cells were allowed to air dry then mounted with DPX mountant (BDH, Poole, UK). DiffQuick™ stained nuclear chromatin and neutrophil granules blue/purple, and eosinophil granules red/pink.

2.8.2 Tissue histology

Murine hind limbs were removed above the knee and immediately fixed in 10% neutral buffered formalin for at least 1 week. Paws were decalcified by soaking in 5% HNO₃ for a further 3 weeks, then 6 μ m sections were cut using a bone microtome. Sections were stained with haematoxylin and eosin using the standard method of the Dept. of Pathology, Western Infirmary, University of Glasgow.

2.8.3 Image recording

A photographic system consisting off a Leica DM RB microscope connected to a digital camera, allowed recording of images via ImageAccess Software. In figure legends, low power magnification refers to an original magnification x 100; magnification x 630 is defined as high power

2.8.4 Fluorescence microscopy

3×10^6 cells per test were incubated for 15 minutes with 0.5 μ g Fc block then incubated with biotin-conjugated antibody for 30 minutes at 4°C in the dark. Cells were washed with sterile PBS containing 2% FCS, prior to addition of streptavidin conjugate. To distinguish green fluorescence protein (GFP) from a second fluorescent label, biotin conjugated antibodies were detected with streptavidin Texas Red (Vector Laboratories Inc, CA, USA), which has a narrow emission spectrum, reducing overlap between 'red' and 'green' signals. Cells were washed then fixed by the addition of 1 ml 1% paraformaldehyde (PFA) in PBS for 1 minute. Cells were washed in PBS and remaining PFA quenched by 30 seconds exposure to 1 ml 0.06% Gly-Gly in PBS. Cells were washed in PBS then in phenol free RPMI, and finally resuspended in phenol free complete RPMI with 25 mM HEPES. Cultures and analysis were performed in 8-chambered coverglass (borosilicate) slides (Nunc). Up to 10^6 cells in 1 ml were added to each chamber. Fluorescence was assessed by with an Axiovert S100 fluorescence microscope. Images were recorded via a digital camera, acquired and analysed with Openlab software (Improvision, Coventry, UK).

2.9 Neutrophil function and phenotype assays

2.9.1 Neutrophil purity

Cytospins of neutrophil preparations were stained (Section 2.8.1) and cells were identified as neutrophils by the presence of a multilobed nucleus and granular cytoplasm. Sample purity was also assessed by flow cytometry, identifying neutrophils both by forward/side scatter (Fig. 3.1) and staining contaminating cells with either FITC conjugated anti-CD3, anti-CD19, anti-CD14, or anti-CD15 (staining as Section 2.7, antibodies detailed Table 2.3).

2.9.2 Neutrophil cytokine production in response to IL-18

Neutrophils were added to 96 well flat bottom plates (Nunc), at 2×10^6 cells/ml in 100 μ l complete RPMI 1640. Cells were stimulated, in triplicate, with recombinant human IL-18 (kind gift of Dr. J.A. Gracie, Centre for Rheumatic Diseases, Glasgow Royal Infirmary) at 1, 10, or 100 ng/ml for 1-24 h at 37°C. To determine whether IL-18 induces *de novo* protein synthesis, cyclohexamide or actinomycin D (both at 2 μ g/ml) was added to cultures for 30 minutes prior to IL-18 stimulation. Dexamthasone and methotrexate (both at 1 μ M) were used in further inhibition studies. IL-18 signalling through the MAP Kinase pathways was investigated with the p38 inhibitor, SB 203580 (5 μ M), and the ERK inhibitor, PD 98059 (20 μ M, both Alexis Biochemicals, Nottingham, UK). Stocks of inhibitors were prepared at ≥ 1000 fold working concentration in DMSO and diluted in complete RPMI. Inhibitors and DMSO control

were included throughout the culture and toxicity assessed by MTT. Culture supernatants were stored at -20°C until cytokine levels were estimated by ELISA.

2.9.3 MTT assay

To assess cell viability in culture, 2×10^5 neutrophils in 200 μ l were plated in 96 well plates and incubated for up to 24 h. 100 μ l of supernatant was removed and 10 μ l of 5 mg/ml MTT (3-[4,5-Dimethylthiazol -2-yl] - 2,-5 diphenyltetrazolium bromide) in dH₂O, was added for the final 4 h of culture. Cells were lysed with an equal volume of isopropanol and the coloured product quantified using a plate reader to detect OD₅₇₀ nm.

2.9.4 CD11b expression

Whole blood was diluted in I-DMEM and cultured for 1 h in the presence or absence of 100 ng/ml rhIL-18. The cells were stained for CD11b, erythrocytes lysed then samples analysed by flow cytometry (Section 2.7).

2.9.5 Apoptosis

Neutrophils were incubated in complete medium, in the presence or absence of IL-18 for up to 24 h. Cells were washed twice with FACS buffer and resuspended in binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂). 5 μ l of Annexin V FITC and 10 μ l 50 μ g/ml PI was added to approximately 2×10^5 cells and

incubated in the dark, at room temperature for 15 minutes before adding 400 μ l binding buffer to each tube. Samples were analysed by flow cytometry (Section 2.7).

2.9.6 Effect of IL-18 on neutrophil respiratory burst

Neutrophils were placed in 24 well flat bottom plates (Nunc) at 10^7 cells/ml in complete medium and stimulated with 100 ng/ml rhIL-18 for 1 h at 37°C. Cells were transferred to triplicate wells of a luminometer microtitre plate, in the presence of 10 μ M Luminol. 1 μ M N-formyl-methionyl-leucyl-phenylalanine (fMLP) was added and the plate read immediately in a MLX microtitre plate luminometer (Dynex, Middlesex, UK). Magnitude of respiratory burst was estimated by Revelation software. This program recorded a V_{max} , which is the rate of increase in emitted light to the maximum level recorded (A/B, Fig. 2.1).

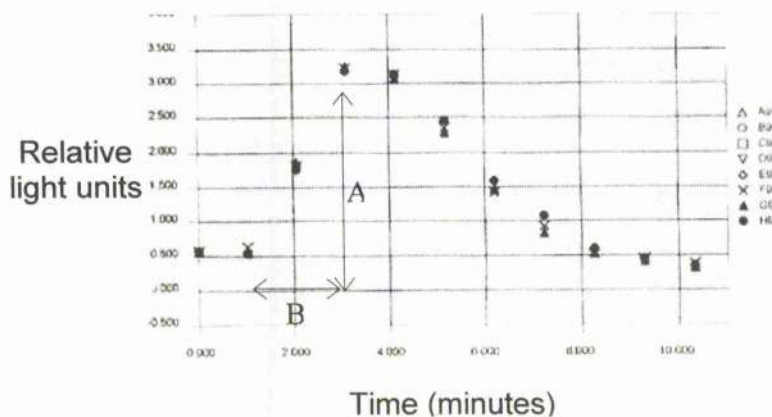


Figure 2.1 Assessment of neutrophil respiratory burst

2.9.7 Neutrophil myeloperoxidase assay

This was carried out as previously described (205). Neutrophil preparations or murine footpads were homogenised with a pellet pestle motor homogeniser (Anachem, Luton, UK) in 2 ml of 20 mM potassium phosphate (pH 7.4) then centrifuged in a J2-MC centrifuge (BD) at 35 000 x g for 30 minutes, at 4°C. The pellet was resuspended in 2 ml of 50 mM potassium phosphate (pH 6), containing 0.5 g/dl cetrimonium bromide. Resuspended pellets were frozen at -70°C until assay. Samples were thawed and sonicated for 1 minute at 50% power, 50% pulse with a Branson sonifier 250, then incubated for 2 h in a 60°C water bath, and centrifuged for 10 minutes at 12 000 x g in a Jouan BR4 microfuge. 25 µl of the supernatant were added to 750 µl 50 mM potassium phosphate containing 0.167 mg/ml o-dianisidine and 5 x 10⁻⁴% H₂O₂. Absorbance at 460 nm was recorded from 1-3 minutes following initiation of reaction.

MPO activity per gram of tissue was calculated by:

$$\text{MPO (U/gwt)} = \frac{(A_{460})(13.5)}{\text{Weight (g)}}$$

A₄₆₀ is the change in absorbance of 460 nm light from 1-3 minutes after initiation of the reaction. 13.5 is an empirically derived coefficient such that 1 unit MPO activity is the amount of enzyme that will reduce 1 µM peroxide per minute.

2.9.8 *In vitro* bacterial killing

Murine buffy coat or human neutrophil preparations were resuspended at 10⁷/ml, in Media 199 containing 10% autologous serum. The cells were stimulated with 100 ng/ml

rhIL-18 (Peprotech, EC Ltd, UK) or rhIL-18 for 1 h. *S. aureus* Cowan strain (SAC, kind gift of Prof. Curtis Gemmell, Dept. of Bacteriology, Glasgow Royal Infirmary) were stored in Microbanks (Pro-Lab Diagnostics, Neston Wirrel, UK) at -70°C and were cultured on 5% blood agar (kind gift of Dept. of Bacteriology, Western Infirmary, Glasgow) for 18 h at 37°C , followed by overnight growth in Mueller Hinton Broth (Sigma). The broth culture was washed 3 times in sterile PBS by centrifuging at $600 \times g$ for 10 minutes at room temperature. Bacteria were resuspended in sterile PBS and standardised spectrophotometrically to contain 10^8 CFU/ml. This suspension was diluted in Media 199 containing 10% autologous serum, to 10^7 CFU/ml, and allowed to opsonise for 20 minutes, at 37°C with gentle shaking. Bacteria were washed and resuspended at 10^7 per ml. Equal volumes of stimulated cells and bacteria were mixed and incubated with gentle shaking for 20 minutes at 37°C . After incubation, 3 volumes of cold water were added to lyse the neutrophils and release bacteria which had been phagocytosed but not killed. Finally, remaining live bacteria were counted by plating dilutions in triplicate. Percentage killing was estimated by calculating $(\text{Bacteria only} - \text{Test})/(\text{Bacteria only}) \times 100$. 'Bacteria only' is a control from which addition of cells were omitted.

2.10 Gene expression analysis

2.10.1 RNA extraction

Cell pellets of $10^6 - 10^7$ PMN were resuspended in 0.2 ml RNAzol B (Biogenesis Ltd, Poole, U.K.) per 10^6 cells and stored at -70°C prior to processing. Samples were thawed on ice, 50 μl chloroform added and samples shaken for 15 seconds before incubation on

ice for 5 minutes. The samples were centrifuged at 11,000 x g, at 4°C for 15 minutes. The upper aqueous phase was removed to a clean tube and an equal volume of isopropanol added, mixed and incubated for 45 minutes on ice before centrifuging as above. The RNA containing pellet was washed in 70% ethanol then resuspended in sterile distilled water (Injection grade, Steripak Ltd, Cheshire, UK).

RNA was extracted from 400 µl murine heparinised blood by RNAqueous™ Blood module and kit (Ambion, Cambridge, UK) according to the manufacturer's instructions. Erythrocytes were lysed by 10 minutes incubation with 1.2 ml of the manufacturer's buffer, at 4°C. Samples were centrifuged at 10,000 x g for 30 seconds in a microfuge and the supernatant removed. Remaining erythrocytes were lysed by mixing with a further 1 ml of lysis buffer and centrifuging as before. The leukocytes were disrupted in a high concentration guanidinium salt solution that simultaneously lysed the cells and inactivated endogenous RNAses (206). The lysate was diluted with ethanol allowing RNA to bind to a glass fibre filter, while most other cellular components flowed through. The filter was washed 3 times then the RNA eluted the manufacturer's buffer (a very low ionic strength solution). Sections of murine spleen and footpads were stored in RNAlater™ (Ambion) at 4°C until RNA extractions were performed with 10 mg of spleen, or total footpad, using Nucleospin® RNA II columns (Macherey-Nagel, Duren, Germany) according to the manufacturer's protocol. Tissues, immersed in the manufacturer's lysis buffer, were homogenised with a pellet pestle motor homogeniser (Anachem). This system worked on an identical principal to the RNAqueous Kit™. Concentration of RNA was determined by its optical density by the following formula (207):

$$[(62 \times OD_{260}) - (36 \times OD_{280})] \times \text{dilution of sample} = \text{'RNA' } \mu\text{g/ml}$$

Contaminating genomic DNA was removed with DNA-free™ (Ambion) according to the manufacturer's instructions. Typically, 2-4 units of DNase I was added to 20 µl of RNA in the presence of the manufacturer's buffer and incubated at 37°C for 30 minutes. DNase inactivation reagent was added for 2 minutes then removed by centrifugation leaving RNA in the supernatant.

2.10.2 Reverse transcription

RNA (1-5 µg) was incubated with 0.5 µg Oligo(dT)₁₂₋₁₈ (Invitrogen) at 70°C for 10 minutes, quick chilled, then reverse transcribed using 100 U Superscript II RT (Invitrogen) at 42°C for 50 minutes in the presence of the manufacturer's buffer (50 mM Tris.HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂) with 0.01 M DTT and 0.5 mM dNTPs in a total volume of 20 µl. Finally, the Superscript was inactivated by heating to 70°C for 10 minutes. Where appropriate 'no RT' controls were performed in the absence of Superscript.

2.10.3 Primer design

Oligonucleotide primers were designed to span regions of interest using Primer 0.5 software (Whitehead Institute for Biomedical Research, Cambridge, MA). This program screens a given sequence for pairs of oligonucleotide primers that fit given parameters, including an optimal primer length of 20 bases, melting temperatures of between 50 and 60°C, a GC content of between 40 and 65% and self complementarity of less than 3 bp overall. Selected primer pairs were tested using Amplify software (v1.2, University of Wisconsin, WI, USA) to predict the size of PCR product, and ensure the primers did not

bind other regions of the sequence. Where possible, primers were designed to span two intron-exon boundaries, allowing discrimination between products resulting from amplification of cDNA and contaminating genomic DNA. A predicted annealing temperature for the PCR was obtained using HyperCard 2.2 software (Apple Computer Inc. CA). Primer specificity was checked by analysing the sequences using the basic local alignment search tool (BLASTn), within the National Center for Biotechnology Information's (NCBI) World Wide Web site.

2.10.4 Polymerase chain reaction

Amplification reactions were performed in a final volume of 25 μ l (unless otherwise stated) in a 0.2 ml Thermo-Tube (ABgene, Surrey, UK). Reactions were carried out using *Thermus aquaticus* DNA Polymerase (Invitrogen) in the manufacturer's buffer (1x; 20 mM Tris.HCl (pH 8.4), 50 mM KCl). The standard conditions in each PCR were:

1 μ l template cDNA (approx. 100 ng)

0.5 μ l 100 μ M each primer

0.5 μ l 10 mM dNTP mix

0.25 units *Taq* DNA polymerase

0.5 – 1 μ l 50 mM MgCl₂ (independently optimised for each PCR)

Reaction conditions are shown in Table 2.1. Primers are listed in Table 2.4.

Table 2.1 PCR reaction conditions

		Temperature (°C)	Time
Denaturing		94	2 minutes
Amplification (35 cycles)	denature	94	30 seconds
	anneal	56	30 seconds
	extend	72	30 seconds
Extend		72	10 minutes

The product of the hIL-18R α PCR was confirmed by cloning into TA vector (Invitrogen), and sequencing with T7 DNase polymerase kit (Amersham, Buckinghamshire, U.K.) according to the manufacturer's protocol. PCR products were analysed on gels containing 1% agarose (w/v) (Molecular Biology Grade, Promega Inc.) in 1 x TBE buffer with 0.5 μ g/ml ethidium bromide. The samples were loaded beside 0.5 μ g 1 Kb Ladder (Invitrogen) with 25% (v/v) loading buffer (10 mM Tris.HCl (pH 8.0), 1 mM EDTA, 33% (w/v) glycerol and 600 μ g/ml bromophenol blue) and the gel was run at a constant 5 V/cm in 1 x TBE until the bromophenol blue had migrated approximately 5 cm through the gel. The product was visualised under ultraviolet illumination (320 nm).

2.10.5 Real time PCR

Taqman real-time PCR (208) was performed using an ABI prism 7700 sequence detector according to the manufacturer's instructions (PerkinElmer Applied Biosystems, Foster City, CA). The principal of the method is outlined below (Fig 2.2).

Primers and fluorescent probes (Table 2.5) were designed using the PrimerExpress™ v1.0 program and purchased from Biosource UK. The fluorescent probes contained a reporter dye (FAM or VIC) covalently attached at the 5' end and a quencher dye

(TAMRA) attached at the 3' end. Probes and primers were HPLC purified. Extension from the 3' end of the probe was blocked by the attachment of a 3' phosphate group.

Each reaction contained:

200 μ M each dATP, dCTP, dGTP

400 μ M dUTP

5 mM MgCl₂

0.625 U AmpliTaqGold™

0.25 U AmpErase Uracil N-Glycosylase

50 mM KCl, 10 mM Tris.HCl (pH 8.3), 10 mM EDTA

The above were purchased as qPCR™ Core Kit, (Eurogentec, Belgium).

300 nM each primer

200 nM detection probe

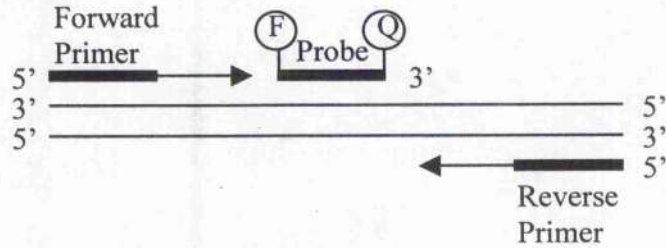
1 μ l cDNA template (approx. 100 ng)

total volume = 25 μ l.

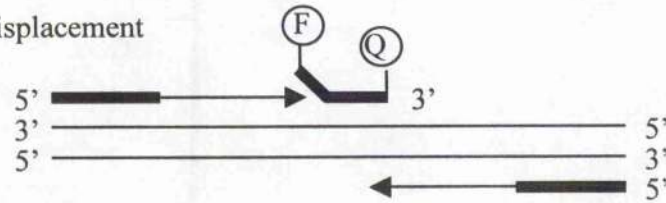
Amplifications were performed in triplicate using an initial cycle of 50°C for 2 minutes and 95°C for 10 minutes followed by 45 cycles of 95°C for 15 seconds and 50°C for 1 minute. Data analysis was performed using Sequence Detector software (PerkinElmer Applied Biosystems), which calculates the threshold cycle (Ct). This represents the PCR cycle at which an increase in fluorescence above a threshold can first be detected. The cDNA levels during the linear phase of amplification were normalised against hypoxanthine phosphoribosyltransferase (HPRT) controls, by subtracting the mean Ct value of HPRT from that of the gene of interest (Δ Ct). To obtain a value for fold increase relative to HPRT the formula $2^{-\Delta$ Ct} was used. Multiplication of this value was used to estimate mRNA expression as % HPRT. The error is the standard deviation of the difference: $s = \sqrt{(S_1^2 + S_2^2)}$ where S₁ and S₂ are the standard deviations of the mean Ct

of HPRT and the gene of interest respectively. Results are expressed as mean \pm percentage error.

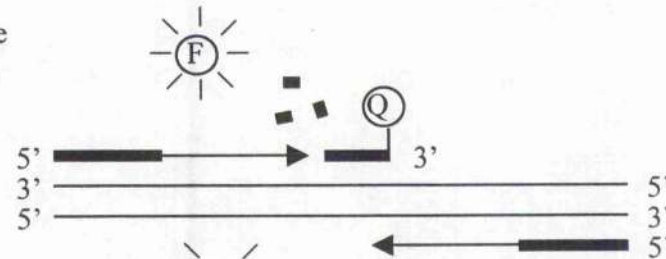
1. Polymerisation



2. Strand displacement



3. Cleavage



4. Polymerisation complete

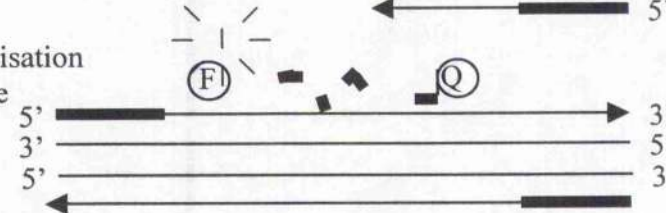


Figure 2.2 Schematic representation of real time PCR

A reporter dye, FAM or VIC was covalently attached to the 5' end of the probe (F). A quencher (Q), TAMRA was attached to the 3' end. PCR product amplification resulted in cleavage of the probe, thus removing the inhibitory action of the quencher from the reporter.

2.11 Animal models

2.11.1 Animals

All animal experiments were performed according to UK Home Office regulations under Project Licence 60/2402, procedure 8, and 60/2217, procedures 1 and 7.

Outbred 6-8 week old male Swiss mice (original breeding stock NIH) were obtained from Harlan (Orlac, Bicester, UK). IL-18-deficient and wild type control mice were obtained as previously described (96). 6-8 week old BALB/c, C57Bl/6 and C3H/HcJ mice were obtained from Harlan. TNFR p55 knock out mice (209) were a kind gift of Prof. Allan Mowat, Dept of Immunology, University of Glasgow. The DO11.10 T cell receptor transgenic mice, on a BALB/c background, were a kind gift of Prof. Paul Garside, Dept of Immunology, University of Glasgow. Mice were fed standard mouse chow and water *ad libitum* under standard conditions of temperature and light in accordance with UK Home Office regulations.

2.11.2 Carrageenan induced acute inflammation

Carrageenan induced inflammation was initiated in 6-8 week old female BALB/c mice as previously described (210). Neutralising monoclonal anti-IL-18 Ab or isotype control Ab (both 25 µg in 50 µl PBS per animal, equivalent to 1.5 mg/kg, R&D Systems) was administered i.p. 30 min before and 24 h after sub cutaneous injection of 300 µg λ carrageenan, in 50 µl PBS, into the right hind footpad; a 28 G needle was used for both injections. The change in footpad thickness between the right and left hind limbs was

measured with a dial caliper (Kroeplin, Munich, Germany). Differences are recorded as change in footpad thickness expressed in millimeters.

2.11.3 Cell recruitment to the peritoneal cavity

6-8 week old BALB/c, C3H/HeJ or C57Bl/6 TNFR p55-deficient mice or wild type controls, received rmIL-18 in PBS with 0.05% BSA, or carrier alone, i.p. 4-48 h following injection, mice were sacrificed by exposure to rising concentrations of carbon dioxide and the peritoneal cavity cells were harvested by making a ventral midline incision with scissors and retracting the abdominal skin, exposing the intact peritoneal wall. 5 ml PBS containing 10 U/ml sodium heparin (Leo Laboratories) was injected through the peritoneal wall with a 23 G needle. The abdomen was gently massaged then the fluid withdrawn. A similar volume of lavage was recovered from each animal. Total cell counts were performed in a cell counter (COULTER ACT; Coulter, Miami, FL) and differential cell counts enumerated on cytocentrifuge slides stained with DiffQuick™. Differential counts (200 cells) were performed under a light microscope and the results presented as number of neutrophils per cavity. Alternatively, cells were stained and analysed by flow cytometry. Cell recruitment into the peritoneal cavity was also induced with thioglycollate. Brewers thioglycollate medium (Difco, East Molesey, UK) was prepared according to the manufacturer's instructions; 40.5 g medium was dissolved by boiling in 1 liter of sterile de-ionised water, aliquoted, then autoclaved and stored in the dark. 1.5 ml was injected i.p. into BALB/c mice and 4 h later cells were harvested by peritoneal lavage.

2.11.4 Induction and assessment of septic arthritis

This was performed as previously described (211). *S. aureus* LS-1, a TSST-1 producing strain, was originally isolated from a spontaneous outbreak of murine septic arthritis (kind gift of Dr. T. Bremell, University of Lund, Lund, Sweden). Bacteria were stored and prepared as for SAC (Section 2.9.8). Mice received 5×10^7 CFU *S. aureus* LS-1 in 150 μ l PBS injected i.v. Mice were treated with 200 ng rmIL-18 or carrier alone (PBS with 0.05 % BSA) administered i.p either 2 h prior to, or 18 h post infection. LPS contamination of rmIL-18 was assessed by E-Toxate (Limulus Amebocyte Lysate, Sigma) according to manufacturer's instructions and found to be <25 pg LPS per 1 μ g IL-18. The maximum contaminating dose of LPS was administered in some control experiments. Mice were observed daily for up to 14 days. Mice were weighed and weight loss recorded as % starting weight. Incidences of arthritis and footpad diameter (calliper measurement) were measured. An arthritic index was derived by observation of each limb, scoring each footpad as below, giving a maximum score of 12 per animal.

- 1 erythema alone
- 2 swelling and erythema
- 3 erythema, swelling and loss of function (ability to grip cage)

The severity of septicaemia was judged clinically by characteristic changes, scoring 1 per each of the following, maximum septic index score of 3 per mouse:

- i) coat appearance ('staring' coat)
- ii) posture (hunched posture)
- iii) lack of spontaneous movement

McInnes *et al* (224) previously validated the above clinical indicators. Animals were scored by two observers, BPL and author. To assess *in vivo* bacterial distribution,

animals were sacrificed by avertin-induced terminal anaesthesia (Section 2.5.1), and heparinised bloods, sections of spleens (weighed), kidneys and footpads were obtained. Tissues were homogenised, serially diluted in PBS and 10 μ l of each dilution was plated in triplicate on 5% blood agar and cultured at 37°C for 18 h to determine the CFU present. Serum was collected and stored at -20°C until assay for cytokine concentration by ELISA. Heparinised blood and spleen were collected for RNA extraction (Section 2.10.1), or for *in vitro* culture (Section 2.5.1 and 2).

2.11.5 Confirmation of *S. aureus*

The presence of *S. aureus* LS-1 in the tissues of infected mice was confirmed by comparing the original stock of LS-1 with bacteria isolated from infected animals. The presence of *S. aureus* was confirmed by positive coagulase and staphylase tests, and the stock and test bacteria showed an identical antibiogram result. An ID 32 STAPH (bioMerieux, Lyon, France) system of standardised miniaturised biochemical tests, used in conjunction with a specific database, also confirmed the presence of *S. aureus* and indicated the test and stock bacteria were highly likely to be of the same strain.

2.12 Statistical analysis

Data were obtained from observations and measurements made on individuals (mice, humans, cells etc). Statistical tests were applied to evaluate how far the observed evidence differed from what would be expected if the null hypothesis were true. From these statistics, *p* values were calculated and if $p < 0.05$ the null hypothesis was rejected and the difference considered significant. 2-sample (Student's) *t* tests were applied to

test whether 2 normally distributed populations had the same mean. When populations exhibited skewed distribution, a Mann Whitney *U* test was applied to test the difference between the means. Chi-squared tests were used with data sets in which individuals fell into mutually exclusive categories. Kaplan Meier analysis was applied to survival data allowing withdrawals from the study to be compensated for. Statistical analysis was performed using Minitab software (State College, PA).

Table 2.2 ELISA reagents

ELISA	Supplier	Capture $\mu\text{g/ml}$	Detection $\mu\text{g/ml}$	Sensitivity range pg/ml
Murine				
IFN γ	Pharmingen	4	2	10-10 000
IL-6	Pharmingen	2	2	10-10 000
IL-5	Pharmingen	2	2	10-10 000
IL-4	Pharmingen	2	2	10-10 000
TNF α	Pharmingen	8	2	20-20 000
IL-18	Peprrotech	2.5	0.3	40-40 000
human				
IL-1 α	Genzyme	2	1	10-10 000
IL-8	R&D	4	2	40-40 000
TNF α	Pharmingen	1	1	10-10 000
IFN γ	Biosource*	4	0.4	10-10 000
IL-6	Biosouce*	1	0.4	10-10 000
LTB $_4$	R&D	Kit	Kit	20-12 000

Murine ELISA antibodies were purified rat anti-mouse antibodies; human ELISA antibodies were mouse anti-human antibodies. Detection antibodies were biotin conjugated. Peroxidase conjugate was used at 1 $\mu\text{g/ml}$ (ExtrAvidin®, Sigma).

* Biosource Cytosets (Fleurus, Belgium).

Table 2.3 Antibodies for flow cytometry

Antigen	Isotype	Supplier (Clone)	Label
Murine			
CD3	Rat IgG2b	BD (17A2)	PE
CD4	Rat IgG2a	BD (RM4-5)	PE
CD19	Rat IgG2a	BD (1D3)	FITC
Pan NK	Rat IgM	BD (DX5)	FITC
OVA -TcR	Mouse IgG2a	(KJ1.26)	Bio
GR-1 (Ly-6G)	Rat IgG2b	Caltag (RB6-8C5)	FITC, PE and Bio
F4/80 (Ly-71)	Rat IgG2b	Caltag (C1:A3-1)	FITC and PE
Human			
CD3	Mouse IgG1	Sigma (UCHT-1)	FITC
CD4	Mouse IgG1	Sigma (H129.19)	PE
CD11b	Mouse IgG1	Sigma (44)	FITC
CD14	Mouse IgG2a	BD (M5E2)	FITC
CD15	Mouse IgM	Sigma (DU-HL60-3)	FITC
CD19	Mouse IgG1	Sigma (SJ25-C1)	FITC
IL-18R	Mouse IgG1	R&D (70625)	PE
IL-18	Mouse IgG2a	R&D (MAB 318)	none

Antibodies were used according to the suppliers' recommendations. Biotin conjugated antibodies were detected with 0.2 μ g test streptavidin-FITC (BD). Anti hIL-18 was detected with FITC-anti mouse conjugate (Serotec).

Table 2.4 PCR Primers

Gene	Species	Forward primer 5' → 3'	Reverse primer 5' → 3'
β actin	Human	AGGGCAGTGATCTCCTTCTGCATCCT	CCACACTGTGCCCACTACGAGGGGT
IL-18Rα	Human	ACTTGTCAATTAGGTTGGCG	ACTCAGTCACCCCACTGGTCC
IL-18Rβ	Human	CCGCATCACATAAGCAAGAC	ACCACCTCCCTCTTTCTTTCA

PCR primers were obtained from Invitrogen.

Table 2.5 Taqman real time PCR probes and primers

Gene	Forward primer 5' → 3'	Probe 5' → 3'	Reverse primer 5' → 3'
mIFN γ	TCAAAGTGGCATAGATGTGGAAGAA	TCACCATCCTTTTGGCAGTTCCTCCAG	TGGCTCTGCAGGATTTTTCATG
mIL-18	CAAAATCACTTCCTCTTGGGCC	CTGCCATGTCAGAAAGACTCTTGGGTCAACT	GTGAAGTCGGCCCAAAAGTTGTC
mHPRT	GCAGTACAGCCCCAAAATGG	TAAAGTGTGCAAGCTTGCTGGTGA AAAAGGA	AACA AAGTCTGGCCTGTATCCAA
mTNF α	PE*	PE	PE
mIL-12p40	GGAATTTGGTCCACTGAAATTTTAAA	AACAAGACTTTCCTGAAGTGTGAAGCACCCAAAAT	CACGTGAACCCGTCCGGAGTA
mIL-18R α	TCCAGGAGATCCATTCACCTGATAG	AAAAGCCGGAGGCTAATCATCGTTCTCA	CTCGGCTCCGTTAGTCAGGTAA
mILR2	AAGATGGGCTTCCTGAAATTTG	CGTTTTTACCACCCGGATCCCTGTACTG	TCCAGCGTCTGAGGAAATGC
mILR9	AGGCTGTCAATGGCTCTCAGTT	TGCCCGCTGACTAATCTGCAGGTGCT	TGAACGATTTCCAGTGGTACAAAGT
mIL-18	GGCCTCTATTTGAAAGATATGACTGATT	TGACITGTAGAGATAATGCACCCCGGACC	CCTCTAGGCTGGCTATCTTTATACATACT

*PE – supplied by PerkinElmer as pre-developed reagents. All other primers and probes were obtained from Biosource, UK.

Chapter 3 A role for IL-18 in neutrophil activation

Introduction

IL-18 is a pleiotropic cytokine which influences both adaptive and innate immune responses. In addition to driving Th1 responses, a role for IL-18 in innate immunity is increasingly recognised. IL-18 enhances NK cell cytotoxicity and directly induces IFN γ production (52) and directly induces monokine production by macrophages that constitutively express IL-18R (78). Furthermore, neutralisation of IL-18 before LPS challenge reduces tissue myeloperoxidase (MPO) levels, suggesting that IL-18 is implicated at some stage in neutrophil activation (97). Commensurate with a putative early role in immune responses, IL-18 mRNA is widely distributed, facilitating rapid generation of cytokine if required.

Neutrophils are the most abundant circulating leukocyte in humans, with up to 2×10^{10} cells in circulation. The neutrophil population undergoes rapid turnover, the bone marrow generating up to 10^{10} per day. Such cell turnover is presumably justified by the protective value of such cells, which react rapidly and destroy pathogens. The neutrophil armoury may promote not only host defense but also tissue damage. Neutrophil infiltration is a feature of many autoimmune lesions including psoriasis, inflammatory bowel disease, and RA, although their qualitative and quantitative contribution therein is unclear. In RA, neutrophils constitute up to 90% of synovial fluid (SF) cells and their presence has been documented at the cartilage-pannus junction (189). Activated neutrophils secrete many of the cytokines and extracellular matrix degrading enzymes implicated in RA pathogenesis (reviewed (188, 196)). IL-18 is involved in the pathogenesis of several inflammatory disease states and is present at significant levels in the RA synovium (78), where it induces and sustains articular Th1

cell responses and independently promotes TNF α production. In the present study, I have explored the hypothesis that the functional role of IL-18 in innate immune responses may extend to neutrophil activation, and that this property of IL-18 may be of importance in chronic inflammatory diseases. Effective neutrophil regulation is crucial to protective, controlled immune responses, thus understanding factors that influence neutrophil function reveals further details of the orchestration of the immune response. The present report documents for the first time direct biological effects of IL-18 upon neutrophils.

Results

3.1 Assessment of neutrophil sample purity

Neutrophil sample purity was assessed by flow cytometry, allowing observation of size (forward scatter), granularity (side scatter) and expression of cell specific surface markers. Human peripheral blood leukocytes formed 3 populations when analysed by forward and side scatter (Fig. 3.1a). Following purification by polymorphoprep (Fig. 3.1b) or lymphoprep (Fig. 3.1c), monocyte and lymphocyte populations were largely removed. Contaminating cells present in neutrophil samples were specifically identified by labelling with monoclonal antibodies, demonstrating that neutrophil preparations routinely contained <1% CD3 positive T cells (Fig. 3.2b), <1% CD19 positive B cells (Fig. 3.2c), and importantly, <1% CD14 positive monocytes (Fig. 3.2d). CD15, a neutrophil marker was expressed by >96% cells in purified neutrophil samples (Fig. 3.2e). Finally, to confirm these observations, cytopreps of neutrophil preparations were stained with DiffQuick™ and examined microscopically. Nuclear chromatin and neutrophil granules were stained purple, and eosinophilic granules stained pink. This allowed neutrophils to be identified by their characteristic multilobed nuclei, and granular cytoplasm. The majority of cells were easily identified as neutrophils, rare (<0.5%) eosinophils were also identified by this method (Fig. 3.3a and b).

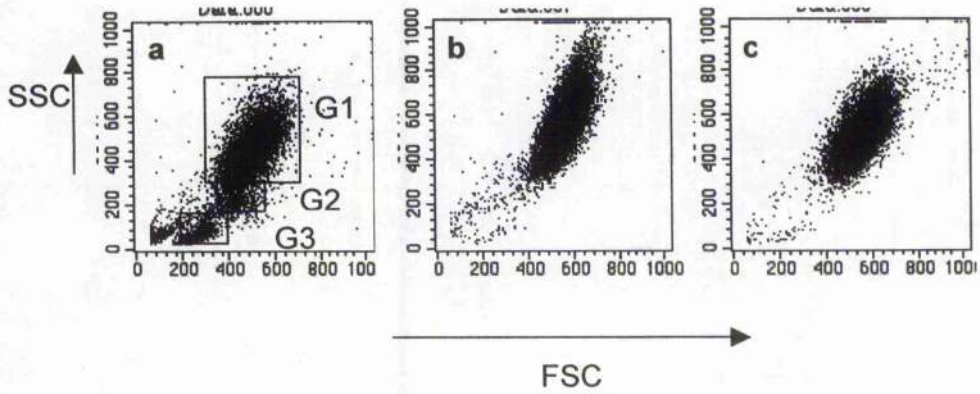


Figure 3.1 Assessment of neutrophil sample purity

Neutrophil sample purity was routinely assessed by flow cytometry. Cells were characterised by cell size (forward scatter, FSC) and granularity (side scatter, SSC). (a) Human peripheral blood leukocytes prior to separation; G1 containing neutrophils, G2 monocytes and G3 lymphocytes. (b) Cells following purification by polymorphoprep or (c) dextran sedimentation and lymphoprep. Cells shown were derived from a normal donor and representative of > 10 similar experiments.

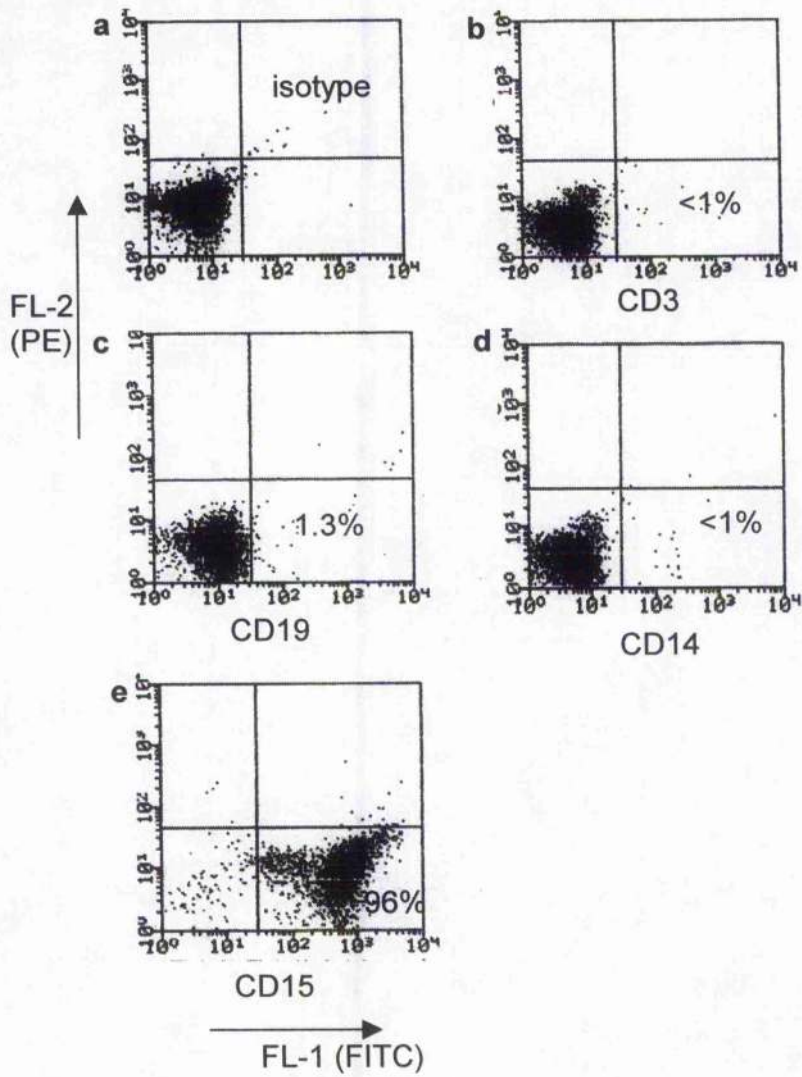


Figure 3.2 Assessment of neutrophil sample purity (2)

FACS analysis of neutrophils purified from a normal donor and labelled with the antibodies indicated on the axes; representative of 3 similar experiments.

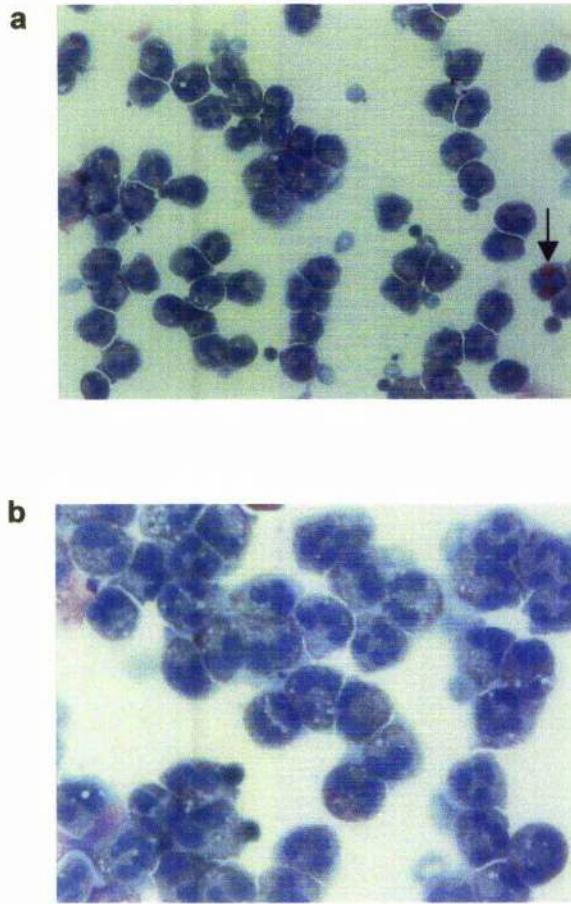


Figure 3.3 Cytoprep of neutrophil samples

Cytopreps of neutrophils purified with lymphoprep were stained with DiffQuick™ then examined microscopically. (a) Low power magnification, arrow highlights a contaminating eosinophil, which were found to be <0.5% of total cells, (b) high power magnification. Cells shown are derived from a normal donor and representative of 3 similar experiments.

3.2 Neutrophil expression of IL-18 receptor

To investigate whether IL-18 may modulate neutrophil function, evidence of IL-18R expression was sought, initially at the mRNA level. Neutrophils derived from both normal donors and RA patients constitutively expressed IL-18R α and IL-18R β chain mRNA (Fig. 3.4a). Surface expression of IL-18R α protein was confirmed by FACS in PB samples from both normal donors and RA patients. No significant difference in the level of expression was observed in PB derived from RA or normal donors (Fig. 3.4b). Similar data were obtained using a whole blood FACS method in which IL-18R α expression was assessed by gating the neutrophil population on the basis of forward and side scatter (Fig. 1a) and assessing the fluorescence of cells within the gate. This suggests that IL-18R α expression on peripheral blood PMN was constitutive and unlikely to reflect partial activation during purification. To determine whether IL-18R α expression is sustained after tissue entry, IL-18R α levels on neutrophils derived from RA SF were examined. Significantly higher levels of IL-18R α were observed (Fig. 3.4b), indicating that IL-18 responsiveness could be maintained, or even enhanced, within a chronic inflammatory lesion. Detection of IL-18R β at the protein level awaits development of appropriate reagents at this time.

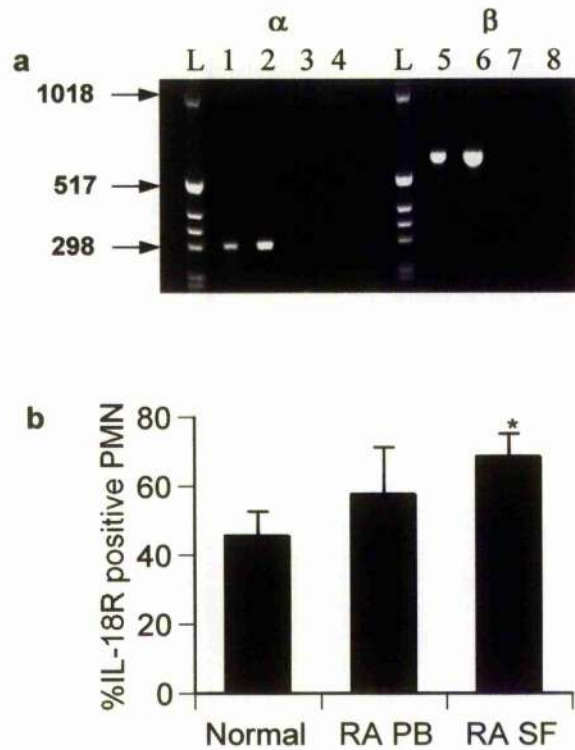


Figure 3.4 Neutrophils express IL-18R mRNA and protein.

(a) RT-PCR was used to detect IL-18R α chain and β chain mRNA expression. Lanes 1–4, IL-18R α expression; lanes 5–8, IL-18R β expression; L, ladder. Lanes 1 and 5 are representative of normal PB derived neutrophils, 2 and 6 of positive control (cDNA derived from driven human Th1 cells, kind gift of Duncan Thomson), 3 and 7 of no RT control, and lanes 4 and 8 negative (water) control. (b) Cell surface expression of IL-18R α on neutrophils was assessed by FACS analysis of normal PB neutrophils ($n = 6$), RA PB ($n = 7$), and RA SF ($n = 12$). To ensure sample processing did not affect IL-18R α expression, analysis was performed on either gated whole blood (Fig. 3.1a) or purified neutrophils. Neutrophil purification did not alter IL-18R α expression and data in (b) are pooled from both methods. Data are mean \pm SEM of each group. * $p < 0.05$, vs. normal PB, by Student's t test.

3.3 IL-18 modulated neutrophil adhesion molecule expression

Constitutive expression of IL-18R α indicated that IL-18 could exert early effects on neutrophil function. In the blood, approximately 50% of neutrophils flow with the circulating erythrocytes while the remainder are 'marginated' and loosely adherent to the vascular endothelium. Neutrophils roll along the endothelium by reversible interactions between E-selectin on the endothelium and the sialyl-Lewis^x moiety of CD15 on the neutrophil. Activation of the neutrophil induces expression of the integrin CD11b (CR3) allowing high affinity binding to endothelial ICAM-1, facilitating diapedesis and allowing the neutrophil entry to the tissue. Since neutrophils constitutively expressed IL-18R, and IL-18 could be involved in early innate responses, whether IL-18 mediated regulation of CD11b on neutrophils was investigated. IL-18 enhanced neutrophil CD11b expression; a representative histogram is illustrated in Fig. 3.5. PB neutrophils from RA patients expressed significantly higher basal levels of CD11b than normal donors, indicating partial prior activation *in vivo*. Incubation of PB neutrophils from either normal donors or RA patients with rhIL-18 resulted in a significant increase in CD11b expression compared with control cells (Table 3.1). Maximal CD11b expression was similar in RA and normal donor groups. Regulation of CD11b on SF derived neutrophils was not assessed as these neutrophils are highly activated *in vivo* and express elevated CD11b (185). These observations suggest a possible central role for IL-18 in leukocyte migration. Commensurate with this, IL-18 has been shown to be released from stressed endothelial cells (212) and induce ICAM-1 expression on endothelial cells (213).

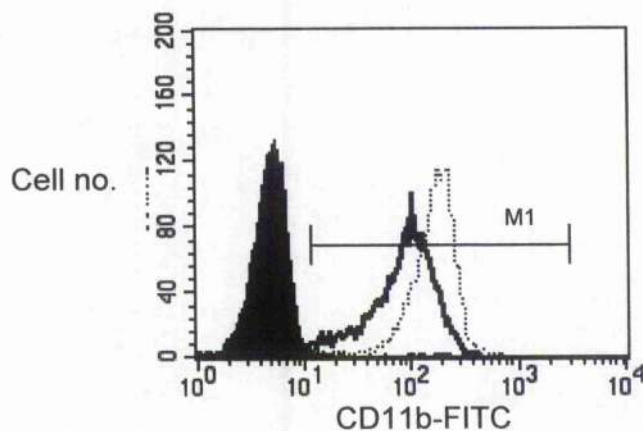


Figure 3.5 Representative histogram of CD11b expression by neutrophils

Whole blood was cultured with medium alone or rmIL-18 for 1 h then neutrophils were stained for CD11b expression and analysed by flow cytometry. Solid histogram – isotype control, solid line - medium control stained with CD11b, dotted line - cells cultured with IL-18 then stained for CD11b. Mean fluorescence intensity in MI was assessed (Table 3.1).

Table 3.1 IL-18 up-regulated CD11b expression in normal and RA PB neutrophils

PB Neutrophils	CD11b Expression (MFI)	
	Medium	IL-18
Normal (n = 4)	38.7 ± 15.9	312.7 ± 39.5**
RA (n = 8)	148 ± 51.7*	300 ± 27**

Whole blood cultures were prepared either with medium alone or in the presence of rhIL-18 (100 ng/ml) for 1 hour, after which CD11b expression was assessed by flow cytometry. Neutrophils were gated by forward/side scatter and the MFI of CD11b expression within the neutrophil gate was assessed. Data are expressed as mean fluorescence intensity ± SD. IL-18 induced CD11b up regulation in both normal and RA derived PB neutrophils, although significant increase in CD11b was observed *a priori* in RA PB neutrophils when compared with neutrophils derived from normal donors. ** $p < 0.05$ vs. respective medium control. * $p < 0.05$ vs. normal PB, by Mann-Whitney *U* test.

3.4 IL-18 induced chemokine production by neutrophils

Neutrophils are abundant in inflammatory lesions, and their ability to produce both C-C and C-X-C chemokines is extensively documented. Neutrophils derived from normal, RA-PB or RA SF produced high levels of IL-8 in a dose-dependant manner in response to IL-18. Of interest, neutrophils derived from different cohorts exhibited different responsiveness to IL-18. RA peripheral blood derived neutrophils were hyporesponsive

to IL-18 compared to neutrophils obtained from normal donors (Fig. 3.6a, also addressed in Section 3.7). RA SF neutrophils exhibited significantly enhanced responses to IL-18 compared to blood derived neutrophils. This is likely to be a reflection of events mediated within the joint, rather than patient variation; when neutrophils obtained from different compartments within the same RA patient were compared, the SF derived neutrophils exhibited a clearly enhanced response to IL-18 compared to PB derived neutrophils (Fig. 3.6b). Chemokine release in response to IL-18 was rapid; IL-8 was detectable 4 h following exposure to IL-18, although this response was maximal after 24 h (Fig. 3.7). These data provide a further mechanism whereby IL-18 may promote neutrophil recruitment into inflammatory lesions.

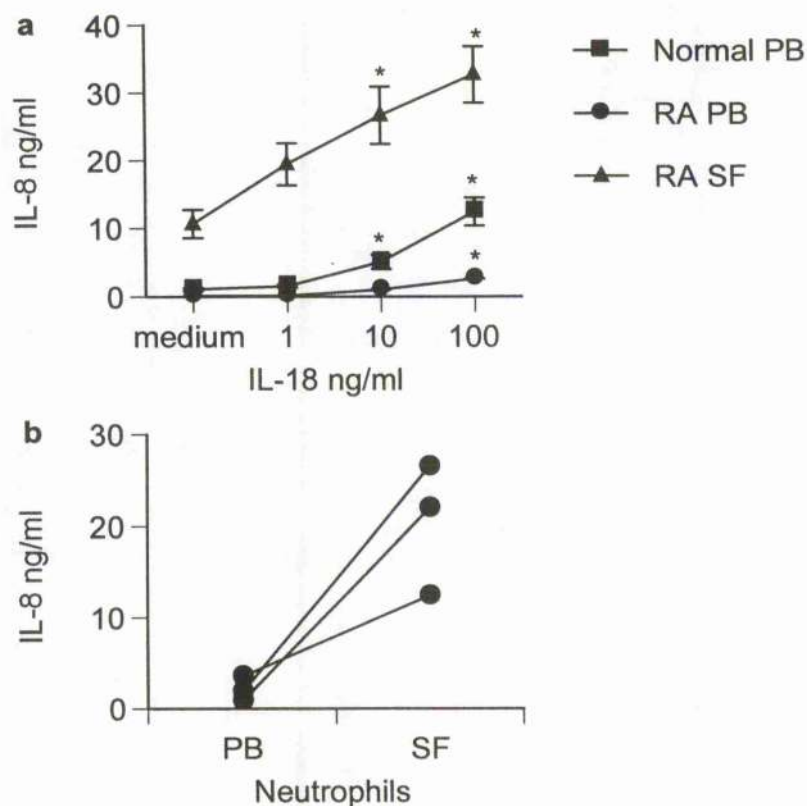


Figure 3.6 IL-18 induced chemokine release by neutrophils.

(a) IL-18 induced IL-8 production by PB neutrophils from normal donors (11 of 11 tested), RA donors (12 of 12 tested) and from RA SF (14 of 14 tested). Data are mean \pm SEM of cytokine production for all donors in each cohort. (b) IL-8 production from matched PB and SF neutrophils obtained from individual patients and stimulated with 100 ng/ml IL-18. * $p < 0.05$ vs. medium alone, by Student's t test.

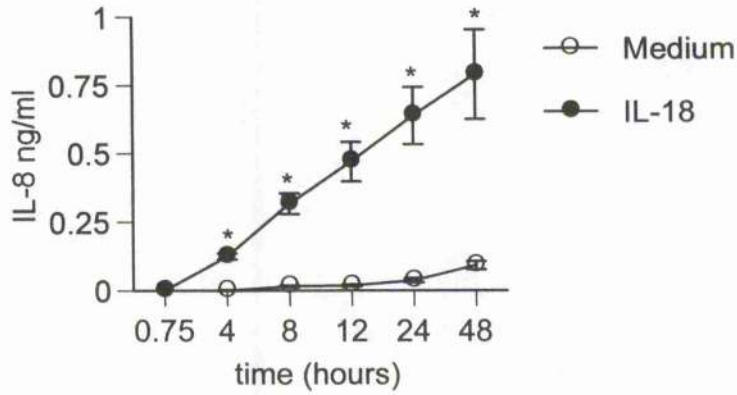


Figure 3.7 Time course of IL-18 stimulated IL-8 release

Normal PB neutrophils were stimulated with 100 ng/ml IL-18 and supernatants harvested at the times indicated. IL-8 release was assessed by ELISA. Data are mean \pm SEM of 3 normal donors. * $p < 0.05$ vs. medium, by Student's t test.

3.5 IL-18 induced cytokine production by neutrophils

In addition to releasing chemokines, neutrophils produce a wide variety of cytokines including IL-1, 3, 10, 12, and TNF α . Neutrophils were therefore stimulated with rhIL-18 for 24 h and cytokine concentration in supernatants was assessed by ELISA. Only low levels of IL-1 α production were consistently observed from PB neutrophils derived from normal donors (Fig. 3.8a). No IL-1 α was produced by RA PB neutrophils. Neither patient nor normal PB donor neutrophils produced TNF α . In contrast, RA SF neutrophils released high levels of IL-1 α and TNF α in response to IL-18 in a dose-dependent manner (Fig. 3.8a and b). Thus, the potential for *ex vivo* cytokine and chemokine release appeared to be qualitatively and quantitatively altered by prior *in vivo* activation. As IL-18 is known to be a potent IFN γ inducing factor, and neutrophils

are reported to be capable of IFN γ production, neutrophil culture supernatants were assayed for IFN γ by ELISA. Neutrophils derived from normal donors did not release IFN γ in response to any dose of IL-18.

3.6 IL-18 induced *de novo* cytokine and chemokine production

To determine whether *de novo* protein synthesis was required for the observed chemokine and cytokine production, neutrophils were cultured in the presence of protein synthesis inhibitors. Actinomycin D (AD) is an intercalating agent, which inhibits transcription, and cyclohexamide (CH) binds ribosomes, inhibiting translation. Addition of AD or CH led to highly significant suppression of all chemokine (Fig. 3.9) and cytokine (Fig. 3.10) production, in all donor cohorts. To exclude the possibility of cell death accounting for reduced chemokine production, inhibitor toxicity was estimated by MTT, demonstrating no effect on cell viability (Fig. 3.11). Accordingly, *de novo* cytokine synthesis, rather than granule release alone, most likely explains these observations.

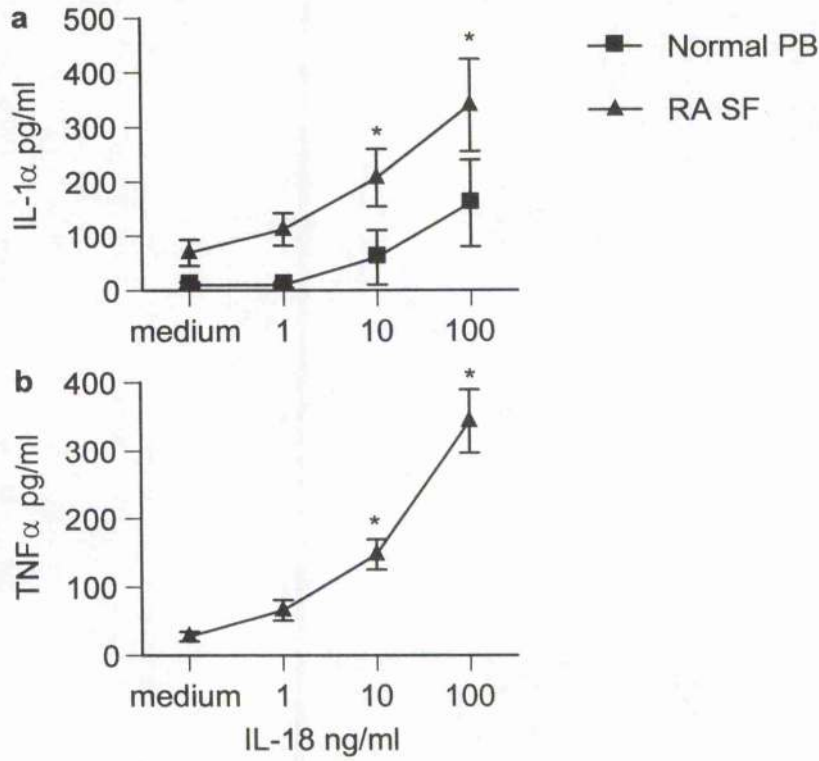


Figure 3.8 IL-18 induced cytokine release from human neutrophils

(a) IL-18 induced IL-1 α release by PB neutrophils from 5 of 11 normal donors and 14 of 14 RA SF samples. Only 2 out of 12 RA-derived PB neutrophil samples produced low levels of IL-1 α (data not shown). (b) IL-18 induced TNF α release in 14 of 14 RA SF neutrophil samples. No TNF α release was observed from either normal or RA-derived PB neutrophils. Data are mean \pm SEM of cytokine production for all donors in each cohort. * $p < 0.05$ vs. medium control by Student's t test.

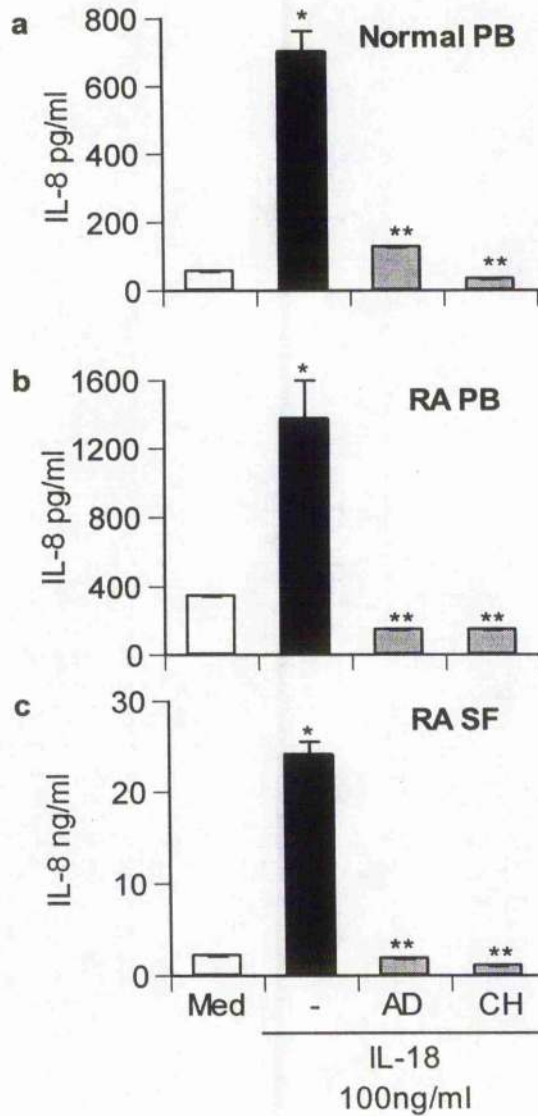


Figure 3.9 IL-18 induced *de novo* chemokine transcription and translation.

Neutrophils were incubated with either 2 $\mu\text{g/ml}$ actinomycin D (AD) or 2 $\mu\text{g/ml}$ cyclohexamide (CH) for 30 min before activation with 100 ng/ml IL-18. IL-8 production by (a) normal PB neutrophils ($n = 5$), (b) PB neutrophils from RA patients ($n = 5$) and (c) SF neutrophils from RA patients ($n = 2$) is shown. Data are mean \pm SEM of each group. * $p < 0.05$ vs. medium control, ** $p < 0.01$ vs. IL-18 by Student's *t* test.

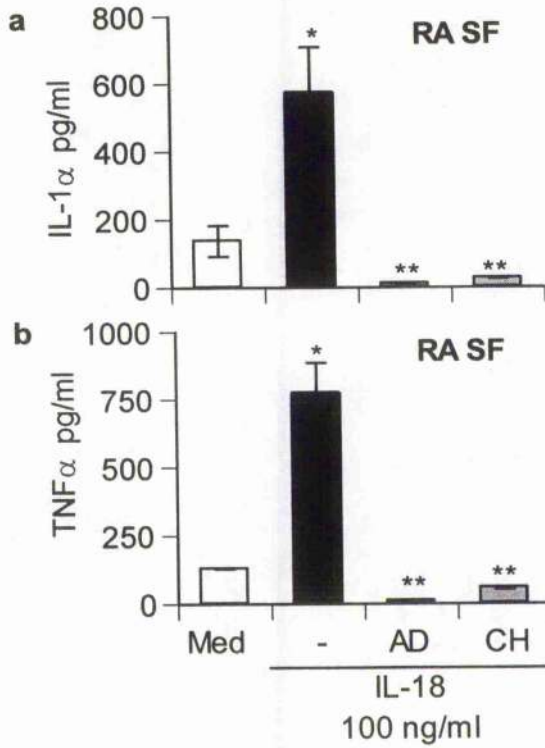


Figure 3.10 IL-18 induced *de novo* cytokine transcription and translation.

Neutrophils from RA SF ($n = 2$) were cultured as described in Fig. 3.9. (a) IL-1 α and (b) TNF α production by RA SF neutrophils were measured by ELISA. Data are mean \pm SEM of each group. * $p < 0.05$ vs. medium control, ** $p < 0.01$ vs. IL-18 by Student's *t* test.

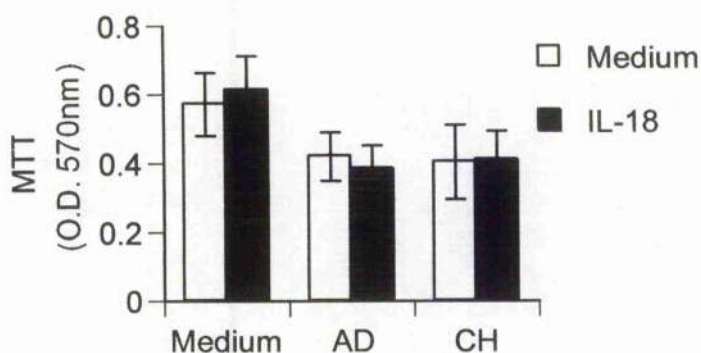


Figure 3.11 Toxicity of Actinomycin D and Cyclohexamide

Neutrophils from normal donors ($n = 3$) were cultured as described in Fig. 3.9 and cell viability after 18 h was assessed by MTT assay. Data are mean \pm SEM.

3.7 Drug effects on IL-18 activation of neutrophils

The reduced IL-8 production in response to IL-18 in RA donors (Fig. 3.5) was of interest. To determine effects of concomitant drug therapy, neutrophils were preincubated with the synthetic corticosteroid, dexamethasone (DEX), or an anti-metabolite, methotrexate (MTX). The dose of drug used *in vitro* was determined by previous reports and a titration of 100 nM to 10 μ M. The optimum dose of 1 μ M reproducibly reduced IL-18 stimulated cytokine release. Dexamethasone exhibited more potent inhibitory effects although methotrexate also significantly reduced IL-8 release (Fig. 3.12). Several other factors are likely to influence neutrophil function including age, disease duration and current disease activity. Furthermore, *in vitro* culture with such drugs is at best a surrogate for prolonged *in vivo* exposure.

3.8 IL-18 signalling through MAP kinase pathways in neutrophils

Dexamethasone is reported to exert effects via inhibition of MAP kinase pathways (214). Furthermore, emerging evidence suggests that IL-18 may activate MAP kinases, which are involved in neutrophil activation (215). Studies in which p38 MAP kinase and ERK were specifically inhibited indicated that IL-18 stimulated IL-8 release from neutrophils was primarily p38 dependent, and may also involve ERK (Fig. 3.13a). The dose of inhibitors used in these studies was previously determined (216). Toxicity of these inhibitors was assessed by MTT assay and found to be insignificant (Fig. 3.13b).

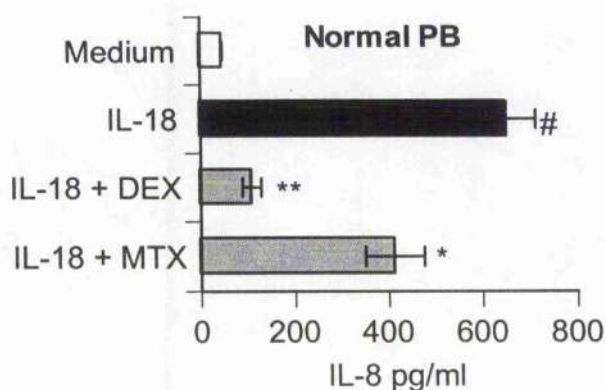


Figure 3.12 DMARDS inhibited IL-18 induced IL-8 release

Normal PB neutrophils ($n = 5$) were pretreated with $1 \mu\text{M}$ dexamethasone (DEX) or $1 \mu\text{M}$ methotrexate (MTX) for 30 min before activation with 100 ng/ml IL-18. Dexamethasone and methotrexate significantly reduced IL-18 induced IL-8 release. Data are mean \pm SEM. # $p < 0.01$ vs. medium, * $p < 0.05$ and ** $p < 0.01$ vs. IL-18, by Student's t test.

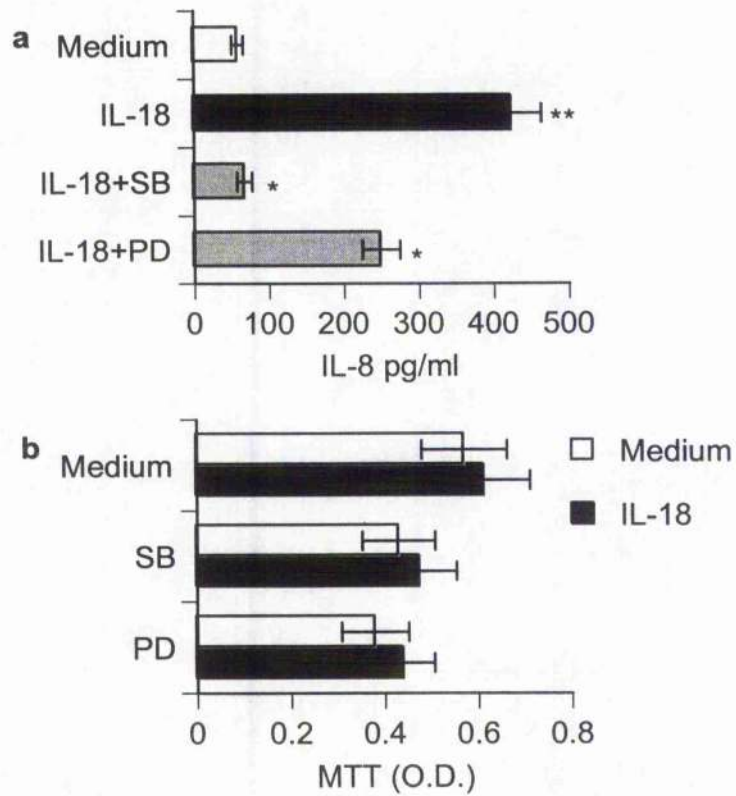


Figure 3.13 IL-18 activated neutrophils through MAP kinase dependent pathways

Normal human neutrophils were cultured in the presence of 5 μ M SB203580 (p38 inhibitor) or 20 μ M PD98059 (ERK inhibitor) for 18 h with or without stimulation with 100 ng/ml IL-18. (a) IL-8 release was assessed by ELISA ($n = 5$). (b) Toxicity of inhibitors was assessed by MTT ($n = 3$). Data are mean \pm SEM. * $p < 0.01$ vs. IL-18, ** $p < 0.05$ vs. medium, by Student's t test.

3.9 Effects of IL-18 on neutrophil degranulation

Neutrophil granules store potent anti-bacterial proteins which contribute to degradation of ingested material, or may be released extracellularly in response to stimuli such as IL-8 or immune complexes binding Fc γ R. To determine whether IL-18 promoted neutrophil granule release, neutrophil culture supernatants were assayed for a secondary granule constituent, lactoferrin, which serves as a surrogate marker for neutrophil degranulation. Lactoferrin release increased dose dependently in response to IL-18 (Fig. 3.14a). These data were extended using the maximum stimulating dose of IL-18 (100 ng/ml). Lactoferrin release in response to IL-18 was significantly higher from SF neutrophils than from RA or normal PB neutrophils, commensurate with *in situ* activation in the synovial compartment (Fig. 3.14b). This further confirmed that neutrophils maintained an IL-18 responsive state in an inflamed compartment.

3.10 Effects of IL-18 on respiratory burst

The key role of the neutrophil oxidase system in host defense against bacteria is clearly illustrated in patients with chronic granulomatous disease. With respect to oxidative activity, neutrophils may exist in quiescent, primed or activated states. In the primed state there is no increase in oxidative activity yet subsequent stimulation provokes a response that is larger than in non-primed cells. Inflammatory mediators such as formyl peptides and C5a prime at low concentrations but initiate a respiratory burst at high concentrations. Other mediators including TNF α and GM-CSF may be described as 'dedicated primers' and as such do not activate respiratory burst but prime for subsequent activation. IL-18 alone did not induce respiratory burst in human neutrophils ($n = 5$, data not shown). However, prior incubation of neutrophils with IL-18 led to

significant enhancement of the response to fMLP that was evident in normal and in RA-derived PB neutrophils (Fig. 3.15). SF neutrophils did not exhibit an enhanced response to fMLP following preincubation with IL-18. This observation that may be related to the previously described phenomenon of neutrophil "exhaustion" relating to respiratory burst (186). Synovial fluid neutrophils are highly activated *in vivo* and a greater proportion of this population is likely to have been exposed to priming stimuli or stimulation of respiratory burst. Thus, the SF derived neutrophils are less susceptible to priming effects of IL-18 than PB derived neutrophils.

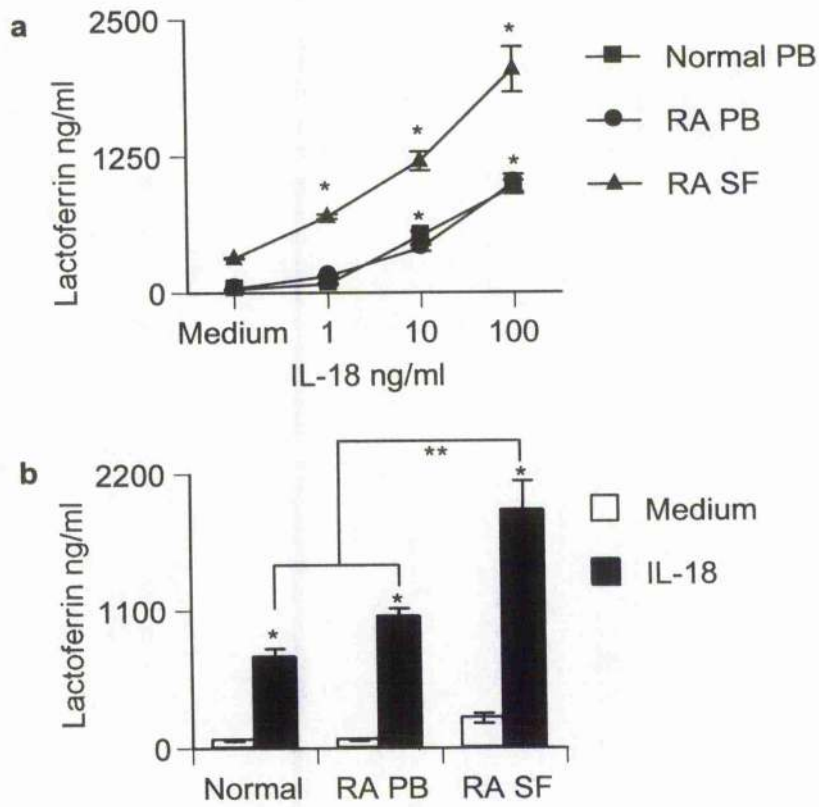


Figure 3.14 IL-18 induced granule release by neutrophils

(a) Neutrophils from normal PB, RA PB, or RA SF ($n = 4$) were cultured as described in Fig. 3.6 with increasing concentrations of IL-18, and granule release was estimated using lactoferrin release as a surrogate. (b) These data were extended using 100 ng/ml IL-18 ($n = 8$). Data are mean \pm SEM of each group. * $p < 0.05$ vs. medium control, ** $p < 0.01$ SF vs. normal and RA PB neutrophils, by Student's t test.

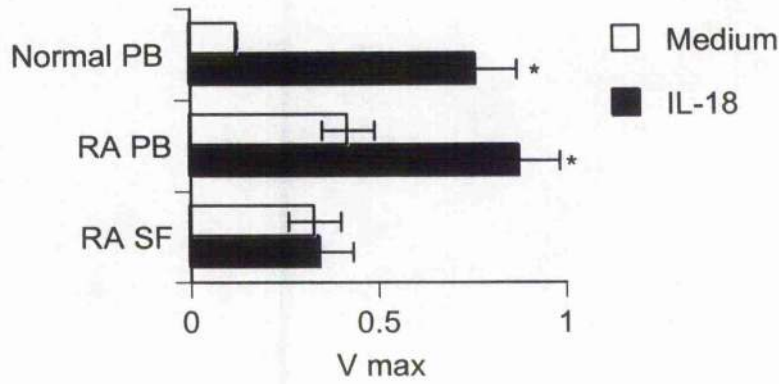


Figure 3.15 IL-18 enhanced neutrophil respiratory burst

Neutrophils from normal PB ($n = 5$), RA PB ($n = 5$), or RA SF ($n = 7$) were incubated with 100 ng/ml IL-18 or medium alone for 1 h before stimulation with 1 μ M fMLP. Reactive oxygen intermediate release was assessed immediately as in methods. V_{\max} is the rate of increase in emitted light to the maximum level recorded (see Fig. 2.1). Data are mean \pm SEM of each group. * $p < 0.01$ vs. medium control by Student's t test.

3.11 Effects of IL-18 on leukotriene release by neutrophils

There are limited studies of the relationship of IL-18 to fatty acid metabolism. Unlike its relative IL-1, IL-18 has no effect on cyclooxygenase activity. IL-18 exerts inhibition of prostaglandin E_2 synthesis by PBMC, mediated via $IFN\gamma$ (217). However, the role of IL-18 in metabolism of arachidonic acid (AA) by neutrophils, which do not produce $IFN\gamma$ in response to IL-18, has not been investigated. Cytokines are known to activate neutrophil phospholipase A_2 , releasing AA. Neutrophils contain AA, which they are capable of metabolising to produce LTB_4 . LTB_4 is a potent proinflammatory mediator,

promoting neutrophil chemotaxis, adhesion, superoxide production and degranulation. IL-18 treatment for 30 minutes promoted significant LTB₄ release from human neutrophils (Fig. 3.16a and b). LTB₄ activates neutrophils, for example by enhancing CD11b expression. To investigate if this rapid production of LTB₄ mediated IL-18 induced neutrophil cytokine production, parallel cultures were set up for 18 h and IL-8 release into the supernatant was assessed by ELISA. MK-886 (3-[1-(*p*-Chlorobenzyl)-5-(isopropyl)-3-*t*-butylthionindol-2-yl]-2,2-dimethylpropanoic acid, Sodium) is a leukotriene synthesis inhibitor that binds the 5-lipoxygenase-activating protein, preventing activation of 5-lipoxygenase. Addition of this inhibitor demonstrated that IL-8 release in response to IL-18 was independent of LTB₄ synthesis (Fig. 3.16c). This does not however conclusively demonstrate that other neutrophil functions induced by IL-18 are independent of LTB₄. However, IL-18 promoting release of LTB₄, provides an amplification loop for whereby IL-18 may perpetuate neutrophil activation.

3.12 Effects of IL-18 on neutrophil apoptosis

Anti-apoptotic effects of innate cytokines including IL-15 and GM-CSF have been previously observed for neutrophils. Therefore, we investigated whether IL-18 can promote neutrophil survival at sites of inflammation. After up to 24 h of incubation with IL-18 (100 ng/ml), apoptosis was determined by propidium iodide and annexin V staining. No difference was observed in IL-18-treated cells compared with medium controls at any time point (B. Leung, personal communication).

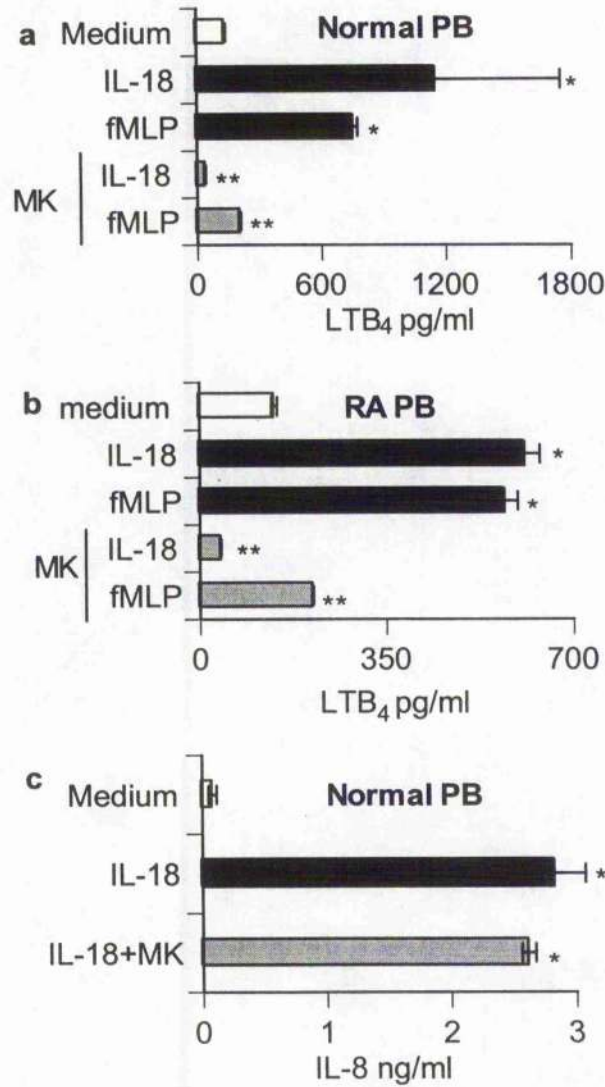


Figure 3.16 IL-18 induced LTB₄ from neutrophils

Purified neutrophils were cultured for 30 minutes in the presence of 100 ng/ml IL-18 or 1 μ M fMLP. Release of LTB₄ was inhibited by 1 μ M MK-866. Supernatants were harvested and assessed for LTB₄ by ELISA. (a) Normal PB neutrophils ($n = 2$) and (b) RA PB neutrophils ($n = 2$). (c) Normal PB neutrophils cultured for a further 18 h and IL-8 release assessed by ELISA. IL-18 induced IL-8 was not LTB₄ dependent. Data are mean \pm SEM. * $p < 0.05$ vs. medium, ** $p < 0.05$, significant inhibition of LTB₄ synthesis in response to IL-18 or fMLP.

Discussion

The present report clearly demonstrates that IL-18 can play an important role in neutrophil activation. Data described define a novel pathway whereby IL-18 can amplify acute inflammation through promoting neutrophil adhesion, cytokine and chemokine production, granule release, respiratory burst and arachidonic acid metabolism. This strongly indicates that the biological effects of IL-18 extend beyond promoting Th/c1 type responses, to include a role in developing innate immune responses. By implication, regulation of IL-18 may be an important determinant in the evolution of immune responses from acute to acquired or chronic phases. Polymorphonuclear cell activation by IL-18 may also be important in host pathogen interactions, especially during gram-positive bacterial infection, in which neutrophil function is paramount.

Constitutive IL-18R α chain expression indicated that IL-18 dependent responses might be rapidly instigated. Higher levels of IL-18R expression were detected in neutrophils derived from RA synovial fluids than from blood. Wide variability was observed in the magnitude of neutrophil IL-18R expression between individuals and in cells from different tissue compartments. Since samples were collected and treated in a similar manner, it is unlikely that processing artefacts can explain all of the observed variation. This presumably therefore reflects altered neutrophil activation status *in vivo*. The relative importance of IL-18 in regulating acquired and innate responses implies that IL-18R α and β chain expression will be tightly regulated. Factors regulating IL-18R expression on neutrophils are as yet unknown. IL-18R is a member of the Toll/IL-1R family, suggesting that the IL-18/IL-18R system has evolved as a component of the innate response (39). Pathways that modulate IL-18R will likely modulate neutrophil

function and may in due course have implications for Toll/IL-1R family regulation on neutrophils and other cell types.

Commensurate with a role in early immune responses, IL-18 induced CD11b expression by peripheral blood neutrophils. Subsequent work by Koch *et al* demonstrates that IL-18 upregulates ICAM-1 on endothelial cells and promotes migration of endothelial cells through a gel (123) Further work in our laboratory has shown that IL-18 also induces lymphocyte migration (Komai *et al*, submitted, *J. Immunol.*). Direct evidence for IL-18 promoting neutrophil migration has been discussed in chapter 4. Moreover, the effects of IL-18 on CD11b expression by neutrophils have recently been confirmed by Silliman and colleagues, who showed upregulation of CD11b within 15 minutes of exposure to IL-18 (218).

IL-18 induced IL-8, granule release, enhanced respiratory burst and LTB₄ release by peripheral blood neutrophils. Additionally, recent work has shown that IL-18 also induces IL-1 β , and soluble IL-1RII by neutrophils (219). The observed IL-18 induced neutrophil degranulation has been confirmed in an independent study, which assessed elastase release, instead of lactoferrin, as a surrogate marker for granule release (218). Whether IL-18 stimulates neutrophil production of auto-regulatory inhibitors such as IL-18BP or IL-1H awaits investigation. Although these data suggest that neutrophils could contribute directly to the cytokine milieu, the extent to which neutrophils augment overall inflammatory mediator production *in vivo* is difficult to evaluate. On a per cell basis, neutrophil cytokine production is less than a macrophage, but the relative abundance of neutrophils at sites of inflammation may readdress this balance. This has

important implications in considering critical cell targets in chronic diseases such as RA or Crohn's disease, in which cytokine production is crucial.

The pathways involved in the observed responses to IL-18 were only superficially investigated. IL-8 release in response to IL-18 required *de novo* protein synthesis, was sensitive to steroids, but did not require LTB₄ production. Signalling inhibition studies demonstrated that p38 MAP kinase activation was necessary for IL-18 stimulated IL-8 release. Pathways involved in other neutrophil responses to IL-18 (e.g. respiratory burst and granule release) were not delineated to the same extent. However, recent work demonstrated p38 dependence of IL-18 induced upregulation of CD11b and enhanced respiratory burst in response to fMLP (218), suggesting that this may be a predominant signalling pathway activated by exposure of neutrophils to IL-18.

The pathophysiological importance of IL-18 induced neutrophil activation is intriguing. Sustained IL-18 expression has been reported in a number of chronic inflammatory disease states including RA, inflammatory bowel disease and sarcoidosis (78, 132, 220). The mechanisms whereby IL-18 could contribute to disease pathogenesis remain poorly defined. In RA, proposed mechanisms whereby IL-18 can drive synovitis include regulation of local Th1 responses, effects on proinflammatory cytokine production, enhanced nitric oxide production, and direct effects on chondrocytes and matrix degradation (67, 78). Commensurate with high levels of IL-18R expression, synovial neutrophils retained a high degree of responsiveness to IL-18, leading to significant cytokine and chemokine production. The quantitative contribution of neutrophils to cytokine expression in RA is unclear but could be considerable given their abundance within the synovial compartment. That IL-18 also induced degranulation is of

importance since neutrophil granule contents include collagenase, cathepsin G, gelatinase, elastase and phospholipase A₂, all of which may be of functional significance in promoting articular destruction. Although neutrophils are detected in low numbers at the cartilage pannus junction, the majority are trafficked to synovial fluid. Thus, IL-18 activated neutrophils could contribute to altered cartilage turnover, since synovial fluid directly bathes the cartilage matrix, or could modulate synovial lining layer cells through adjacent release of cytokines or chemokines. Work presented in this chapter are relevant to other disease states in which IL-18 is over expressed and in which neutrophils are present in high numbers, such as psoriasis and Crohn's disease.

Taken together, the data suggest a role for IL-18 in innate neutrophil led responses in addition to its clearly defined role in T cell maturation. These data have two-edged consequences for therapeutic targeting of IL-18. IL-18 blockade may simultaneously abrogate host toxic effects of neutrophils, but could potentiate infections in which neutrophils are essential in host responses. I therefore sought to explore in more detail the *in vivo* effects of IL-18 in inflammatory (Chapter 4) and infectious (Chapter 5) models of acute inflammation.

Chapter 4 *In vivo* effects of IL-18 on neutrophil function

Introduction

IL-18 effectively activates neutrophils *in vitro* (Chapter 3). Such *in vitro* observations have significant limitations, which may be addressed by manipulation of IL-18 *in vivo*, for example with IL-18-deficient mice or neutralising antibodies. Effects of IL-18 modulation have been extensively studied *in vivo* demonstrating important effects during host responses to infection. Thus, protective responses during murine *Cryptococcus neoformans*, *Yersinia* and *Streptococcus pneumoniae* infections may be abrogated or enhanced by manipulation of IL-18 expression (13, 14), clearly indicating a role in microbial host defense. Neutralisation of IL-18 before LPS challenge reduces tissue myeloperoxidase (MPO) levels, suggesting that IL-18 is implicated at some stage in neutrophil activation (16). Moreover, we recently demonstrated that IL-18-deficient mice exhibit reduced capacity to kill *Staphylococcus aureus in vivo* associated with increased severity of septic arthritis (15). These studies imply a role for IL-18 in neutrophil activation *in vivo* although none specifically address direct effects on neutrophil function. Evaluation of human neutrophil activation *in vivo* is limited to studies of exudated neutrophils using a skin blister/chamber technique. This model of aseptic inflammation allows study of *in vivo* activated neutrophils and factors that may influence their migration. However, this system was not ethically appropriate for studying the effects of modulating IL-18 expression. Therefore murine models were investigated. Murine neutrophil migration into the peritoneal cavity, and a model of acute footpad inflammation were studied to assess the role of IL-18 in neutrophil activation *in vivo*.

Results

4.1 IL-18 promoted migration of neutrophils to the peritoneal cavity

Constitutive expression of IL-18R, together with the observation that IL-18 up-regulated CD11b expression by human neutrophils implied that IL-18 exerts early effects on neutrophil migration. *In vivo* evidence of this was sought by injecting rmIL-18 into the peritoneal cavity of BALB/c mice. Doses of both 20 ng and 60 ng of rmIL-18 effectively induced neutrophil recruitment to the peritoneal cavity after 4 h (Fig. 4.1a). This response was maximal at 4 h and reduced by 48 h (Fig. 4.1b), commensurate with IL-18 exerting early, rapid effects.

4.2 Effects of contaminating LPS present in rmIL-18

RmIL-18 was commercially produced in *E. coli* (Peprotech), and certified to contain <0.1 ng endotoxin per 1 µg IL-18. To confirm that significant LPS was not introduced by the handling procedure, reconstituted IL-18 was subjected to an E-toxate assay, which demonstrated that the rmIL-18 used in these experiments contained < 25 ng LPS per 1 µg IL-18 (the limit of sensitivity of the assay). To ensure this dose of LPS did not contribute to the neutrophil recruitment observed in Fig. 4.1, identical experiments were carried out in which up to 60 ng of LPS were injected i.p., and shown to induce minimal cell recruitment (personal communication, Dr. Fernando Cunha, Department of Pharmacology, School of Medicine Ribeirao Preto, University of Sao Paulo, Sao Paulo, Brazil). C3H/HeJ mice have a spontaneous mutation at the LPS response locus (*Tlr4^{LPS}*^d), which renders the animals endotoxin resistant. IL-18 effectively induced neutrophil

migration into the peritoneal cavity C3H/HeJ mice, confirming that LPS contamination is unlikely to explain our observations (Fig. 4.2).

4.3 Mechanisms of IL-18 induced cell recruitment

To investigate the mechanism of IL-18 induced cell recruitment I next sought to identify downstream pathways. $\text{TNF}\alpha$ is involved in leukocyte migration, and IL-18 induces $\text{TNF}\alpha$ from a variety of cells. This led us to examine whether IL-18 operated via a $\text{TNF}\alpha$ dependent pathway to promote cell migration. The majority of biologic responses classically attributed to $\text{TNF}\alpha$ are mediated by the p55 receptor subunit (209). IL-18 was administered i.p. to TNFR p55-deficient or wild-type control C57Bl/6 mice. Compared to PBS control, IL-18 induced significant migration of leukocytes into the peritoneal cavity of wild type C57Bl/6 mice. However, IL-18 administration had no effect in TNFR p55-deficient mice, demonstrating that $\text{TNF}\alpha$ signalling was critical for the response to IL-18 (Fig. 4.3). A 'background' response to PBS was observed in each of the neutrophil migration studies, presumably caused by physical stimulation and stretch responses following the i.p. injection. Interestingly, TNFR p55 $-/-$ mice exhibited a reduced background response to PBS alone, implying that $\text{TNF}\alpha$ is required for all cell migration, independent of stimuli, and that administration of IL-18 did not compensate for this defect.

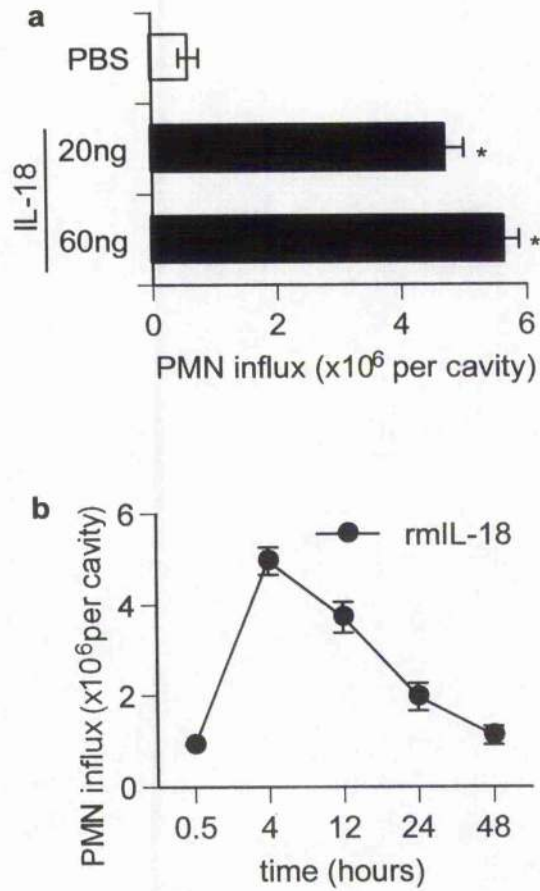


Figure 4.1 IL-18 promoted migration of PMN to the peritoneal cavity

IL-18 administration induced accumulation of neutrophils in the peritoneal cavity of 4 to 5 week old BALB/c mice. (a) Mice received 0, 20, or 60 ng rmIL-18 in a total volume of 0.5 ml PBS. Cell counts and cytopins of peritoneal lavage were performed after 4 h, and neutrophil accumulation was assessed by histology. (b) Thereafter, to establish a time course, 20 ng IL-18 was injected, and neutrophil accumulation was estimated at the time points shown. Data are mean \pm SEM, representative of five separate experiments with five mice per group. * $p < 0.05$ vs. PBS control group by Student's t test. These experiments were performed in collaboration with Dr. Fernando Cunha.

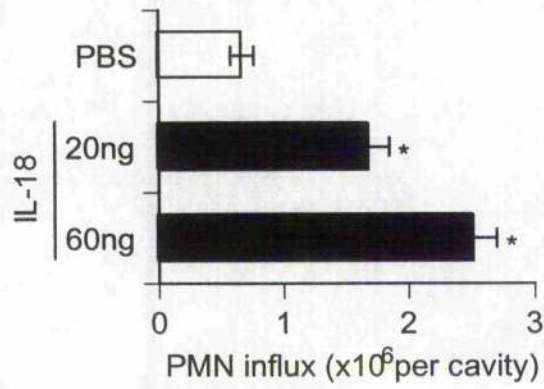


Figure 4.2 LPS contamination did not contribute to IL-18 induced neutrophil migration

LPS resistant C3H/HeJ mice received 0, 20 or 60 ng of IL-18 (derived from same batch as the rmIL-18 administered in Fig. 4.1) in PBS in a total volume of 0.5 ml. Cell counts and cytopins of peritoneal lavage were performed after 4 h, and neutrophil accumulation was assessed by flow cytometry of lavage cells and confirmed by histology. Data are mean \pm SEM of 5 mice per group.

* $p < 0.05$ vs. PBS by Student's t test.

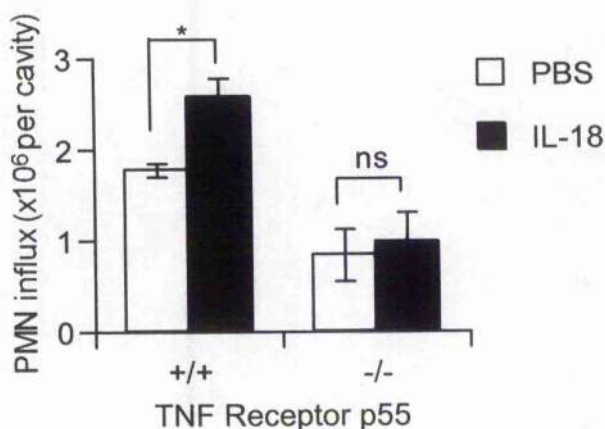


Figure 4.3 IL-18 induced neutrophil recruitment is TNF α dependent

C57Bl/6 wild type (+/+) and TNFR p55 knock out (-/-) mice received an i.p. injection of 60 ng rmIL-18 or carrier alone. 4 h post injection, cell counts and cytopspins of peritoneal lavage were performed, neutrophil accumulation was assessed by flow cytometry of lavage cells, and confirmed by histology. Data are mean \pm SEM of 5 mice per group, representative of 2 similar experiments. * $p < 0.05$ vs. PBS by Student's t test; ns, not significantly different to PBS group.

4.4 Role of IL-18 in acute inflammation

The murine model of carrageenan induced footpad inflammation is associated with rapid neutrophil recruitment, and was therefore appropriate for further investigating the role of IL-18 in acute, neutrophil led inflammation. IL-18 mRNA was upregulated in the lymph node draining the carrageenan injected footpad compared to contralateral control (Fig. 4.4). To investigate the role of extracellular IL-18 in this system, a

neutralising antibody was administered i.p. 1 h prior to injection of carrageenan. The antibody manufacturer (R&D Systems) demonstrated neutralising activity of this antibody (0.3-1 μ g of the anti-IL-18 antibody neutralised 50% IFN γ from activated T cells stimulated with 15 ng/ml rIL-18). Prior data indicate cytokine inhibitory antibodies operate in the range of 1-10 mg/kg (221). Given antibody availability, a dose of 1.5 mg/kg was selected empirically. Following carrageenan administration, footpad swelling was assessed by caliper measurement and the difference between injected and contralateral control paws was calculated (Δ footpad thickness). Administration of anti-IL-18 significantly reduced early (6 h post injection) signs of inflammation and this effect was consistently observed up to 48 h (Fig 4.5 and Table 4.1).

4.5 Mechanisms of anti-IL-18 action

To investigate the molecular and cellular effects of anti-IL-18 antibody treatment, changes in cytokine mRNA expression, locally at the site of inflammation (the footpad) and in the draining lymph node, were assessed 24 h after footpad challenge. RNA was extracted and cytokine mRNA expression was assessed by real time PCR. IL-18 drives TNF α and IFN γ production at sites of inflammation; therefore changes in TNF α and IFN γ mRNA expression were sought. In addition, to investigate autocrine mechanisms of IL-18 regulation, changes in IL-18 mRNA expression was investigated. Neutralisation of IL-18 *in vivo* reduced TNF α mRNA expression in the footpad (Fig. 4.6a). No difference was observed in TNF α mRNA expression in the draining lymph nodes when mice receiving anti-IL-18 were compared with those receiving isotype control (Fig 4.6b). Similarly, IFN γ mRNA expression was reduced in footpads from anti-IL-18 treated animals (Fig. 4.6c), although there was some elevation of IFN γ

mRNA in the draining lymph node (Fig.4.6d). Neutralisation of IL-18 had no consistent effect on IL-18 mRNA expression in either footpads or lymph nodes. These results suggested the effects of IL-18 neutralisation were predominantly manifest locally.

To confirm that the effects of neutralising extracellular IL-18 operated locally at the site of inflammation rather than influencing responses in the draining lymph nodes, single cell suspensions of lymph nodes, pooled from 10 mice per group, were stimulated with immobilised anti-CD3 for 48 h. [³H] Thymidine was added for the final 6 h of culture and proliferation assessed. Lymph node cultures exhibited a strong proliferative response to anti-CD3, which was not modulated by prior *in vivo* treatment with anti-IL-18 antibody (Fig. 4.7). Un-stimulated cells showed no significant background proliferation. Cytokine release into the supernatant of anti-CD3 stimulated cultures was assessed by ELISA. Anti-CD3 stimulated significant monokine (IL-6 and TNF α) release into the supernatant which was unaffected by prior treatment with anti-IL-18 antibody *in vivo* (Fig 4.8a). Cytokine production (IL-5 and IFN γ) by lymph nodes cultures derived from anti-IL-18 or sham treated mice was similar (Fig. 4.8b).

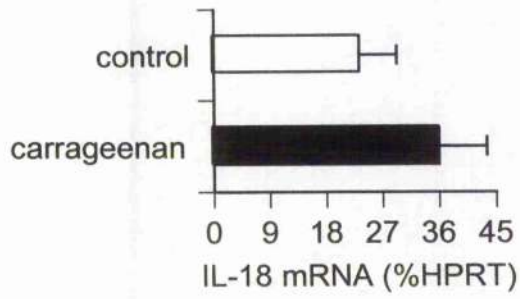


Figure 4.4 Elevation of IL-18 mRNA in draining lymph node following carrageenan administration

BALB/c mice received 300 μ g carrageenan in 50 μ l PBS s.c. into one footpad. Lymph nodes draining the carrageenan injected footpad or contralateral control footpad were removed, RNA extracted and IL-18 mRNA expression assessed by real time PCR. Data are mean \pm percentage error, lymph nodes were pooled from 5 mice per group.

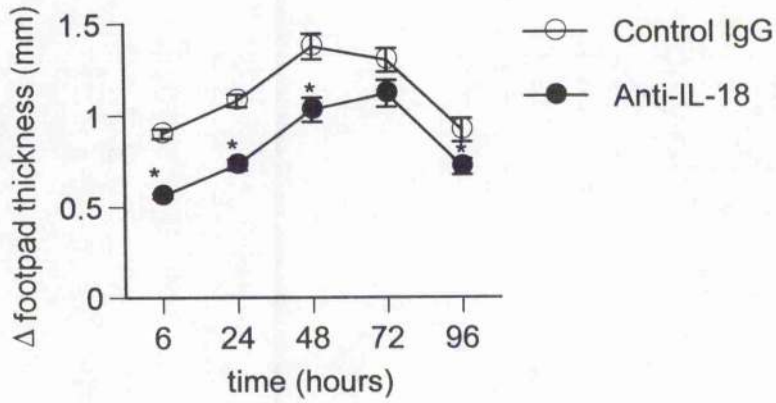


Figure 4.5 Effect of neutralising IL-18 on acute inflammation

BALB/c mice received 1.5 mg/kg rat anti-mouse IL-18 mAb or isotype control by i.p. injection 1 h before injection of 300 μ g carrageenan in 50 μ l PBS into the right hind footpad. Footpad thickness was assessed in injected and contralateral control paw by caliper measurement and the difference expressed in millimeters. Data are mean \pm SEM of at least 20 mice/group pooled from 4 independent experiments (See Table 4.1). * $p < 0.01$ by Mann-Whitney U test.

Table 4.1 Effect of neutralising IL-18 on carrageenan induced inflammation

Time (h)	No. mice/group	Δ Footpad thickness (mm) mean \pm SEM		<i>p</i> (PBS vs. Anti-IL-18, Mann Whitney)
		PBS	Anti-IL-18	
6	40	0.86 \pm 0.022	0.56 \pm 0.028	0.0001
24	40	1.02 \pm 0.025	0.67 \pm 0.027	0.0001
48	20	1.38 \pm 0.07	1.03 \pm 0.067	0.0027
72	17	1.3 \pm 0.065	1.12 \pm 0.07	ns
96	17	0.92 \pm 0.064	0.72 \pm 0.044	0.0138

Experimental conditions are as in Fig. 4.4. The reduction in number of mice over time is due to sacrifice of animals for *in vitro* analysis.

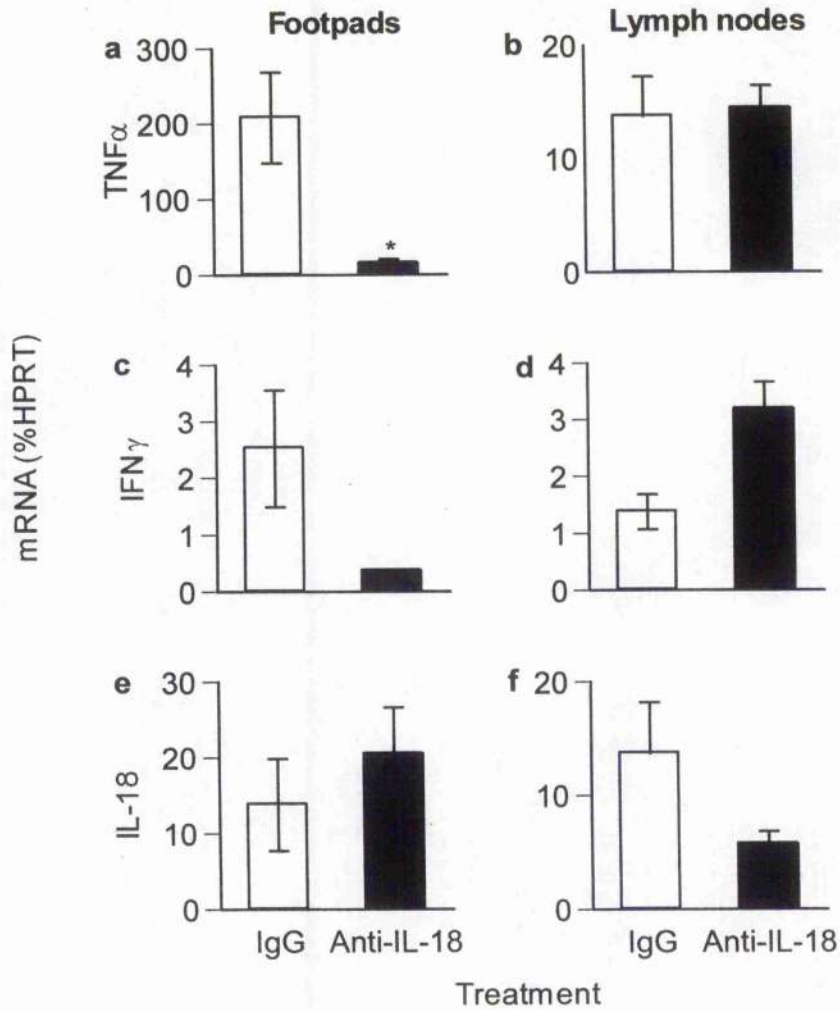


Figure 4.6 Cytokine mRNA expression

Pretreatment with anti-IL-18 reduced TNF α (a) in footpad but had no effect on TNF α expression in (b) lymph nodes. Similarly, neutralisation of IL-18 reduced IFN γ mRNA expression in (c) footpads; however, expression was elevated in (d) lymph nodes of treated mice. IL-18 mRNA expression in footpads (e) was unaffected by treatment with neutralising antibody. The observed reduction in IL-18 mRNA expression in draining lymph (f) nodes was not consistent in the 2nd experiment. TNF α and IL-18 footpad data are mean \pm SEM of 5 individual footpads per group; lymph node data are

representative of 2 similar experiments in which lymph nodes were pooled from 5 mice per group per experiment. The IFN γ data are derived from 2 footpads per group and lymph nodes from one experiment only. * $p < 0.05$ vs. IgG control by Mann Whitney U test.

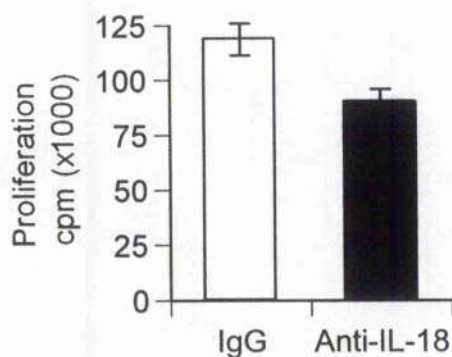


Figure 4.7 Effect of neutralising IL-18 on draining lymph node cell proliferation

24 h after injection of carrageenan, draining lymph nodes were removed from 10 animals per group, single cell suspensions prepared, and stimulated with 5 $\mu\text{g}/\text{ml}$ immobilised anti-CD3 for 48 h. Proliferation was assessed by addition of [^3H] thymidine for the final 6 h of culture. No significant differences were found in proliferation when lymph nodes from anti-IL-18 or sham treated mice were compared. Data shown are mean \pm SEM of triplicate cultures and are representative of two similar experiments.

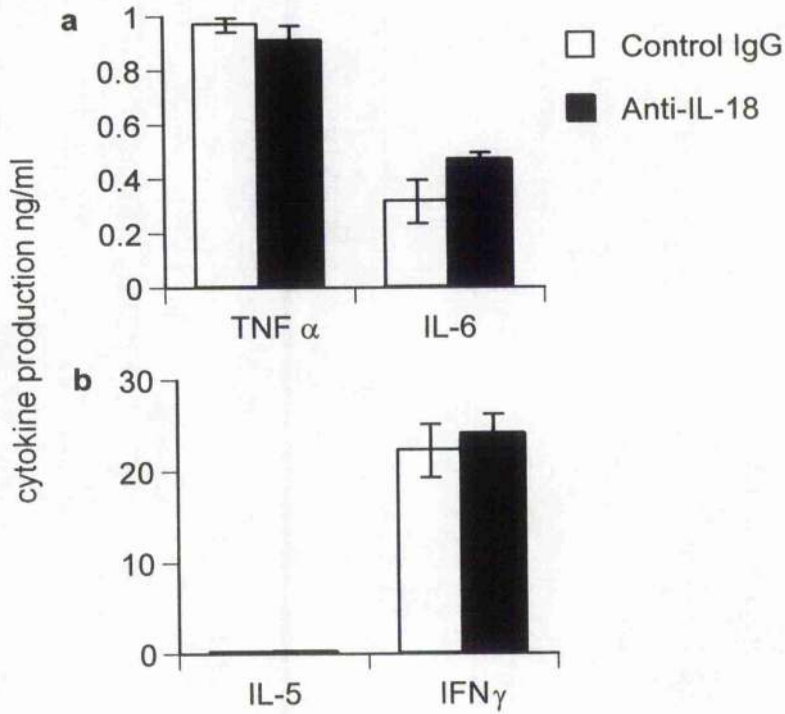


Figure 4.8 Effect of neutralising IL-18 on draining lymph node cell cytokine production

Single cell suspensions were prepared as described in Fig. 4.7. (a) TNF α and IL-6 and (b) IFN γ and IL-5 release into culture supernatants was assessed by ELISA. No significant differences were found in cytokine production when lymph node cultures from anti-IL-18 or sham treated mice were compared. Data are mean \pm SEM of triplicate cultures, representative of 2 similar experiments.

4.6 Local effects of anti-IL-18 treatment

The observed changes in footpad mRNA expression and absence of measurable changes in draining lymph node responses suggested that anti-IL-18 antibody administration mediated local effects. To investigate neutrophil infiltration in the footpad, myeloperoxidase (MPO) content was assessed as a marker of neutrophil accumulation. MPO is stored in neutrophil primary granules and catalyses the production of bactericidal hypochlorite. This enzyme activity may be quantified colorimetrically by oxidation of *o*-dianisidine in the presence of hydrogen peroxide. Footpads were removed 24 h after carrageenan challenge, weighed and MPO activity assayed and expressed as units of MPO activity per gram of tissue. Treatment with anti-IL-18 antibody compared to IgG control significantly reduced MPO content in footpads in 2 independent experiments (Fig. 4.9). This suggested that neutraliation of IL-18 reduced neutrophil accumulation in the inflamed tissue. To confirm this observation, sections of footpads were examined histologically. Normal, unchallenged footpad (Fig. 4.10a) demonstrated a defined tissue architecture, which was clearly disrupted by the inflammatory infiltrate induced 24 h following carrageenan challenge (Fig. 4.10b). Under high power, this infiltrate was identifiable as predominantly polymorphonuclear cells (Fig. 4.10c). Prior treatment with anti-IL-18 markedly reduced inflammatory infiltrate and (Fig. 4.10d), examination under high power demonstrates that fewer neutrophils were present in the tissue (Fig. 4.10e).

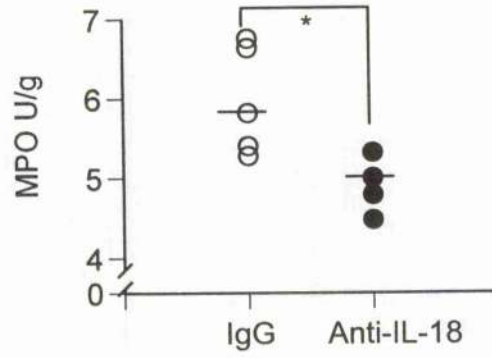


Figure 4.9 Myeloperoxidase activity in footpads

24 h following carrageenan injection, footpads were removed from 5 mice per group. Footpads were weighed, homogenised and MPO activity estimated. Data are measurements from individual paws. $*p < 0.05$ vs. control IgG by Student's *t* test, representative of 2 similar experiments.

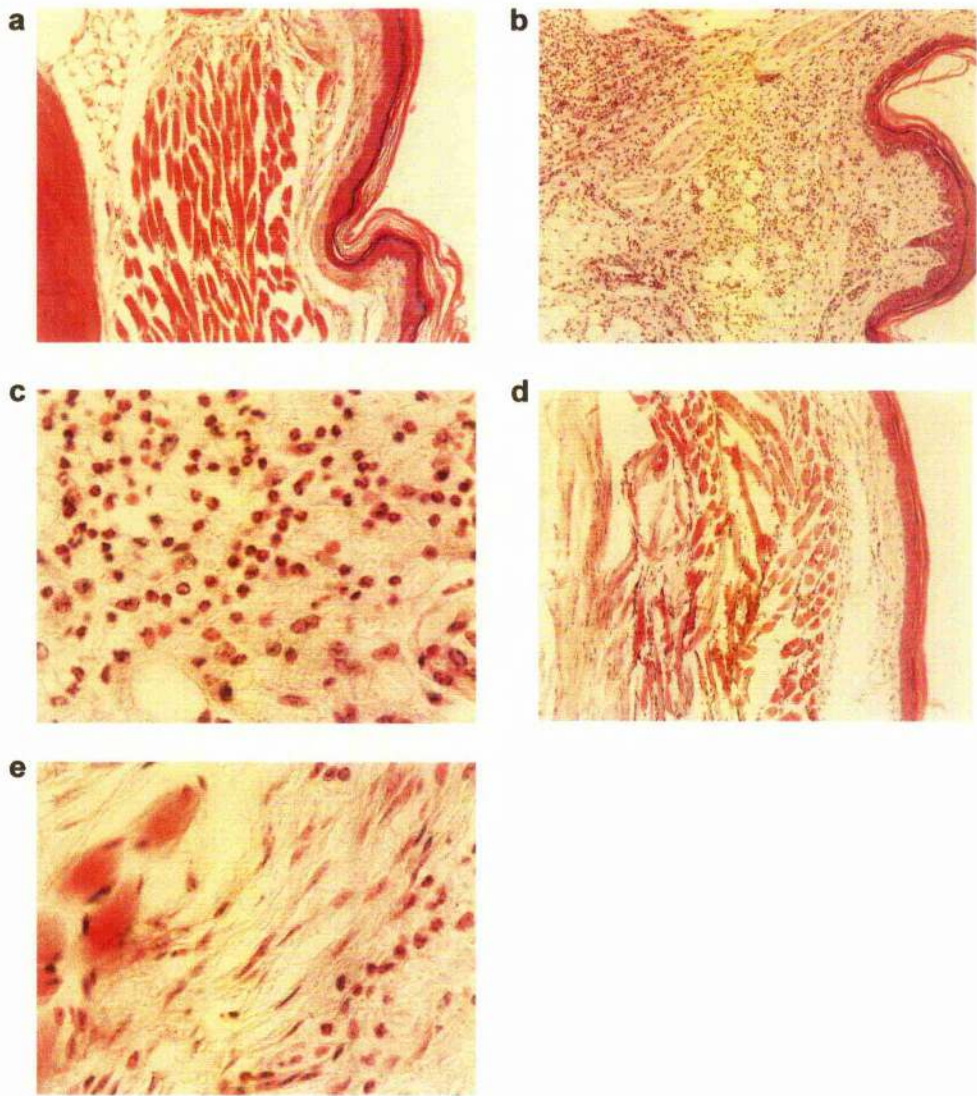


Figure 4.10 Histology of carrageenan induced inflammation

Footpads were removed 24 h after administration of carrageenan, fixed in formaldehyde, decalcified, then cut and stained with H&E. (a) Normal footpad, low power magnification, (b) carrageenan injected footpad from sham treated animal, low power and (c) high power. (d) Carrageenan injected footpad from anti-IL-18 treated animal, low power and (e) high power magnification.

Discussion

These data are the first to explore a role for IL-18 in neutrophil function *in vivo*. Injection of rIL-18 into the peritoneal cavity induced a rapid influx of neutrophils, which was dependent on intact TNF α signalling. Furthermore, in a model of carrageenan induced acute inflammation, prior neutralisation of IL-18 reduced inflammation and neutrophil accumulation, demonstrated histologically and by tissue MPO accumulation. There was also evidence of a TNF α -IL-18 axis in this model, as local TNF α mRNA expression was reduced by anti-IL-18 treatment. Although changes were observed in the footpad, systemic neutralisation of IL-18 had no measurable effects on draining lymph node responses. Together these data suggest that the *in vitro* observations reported in chapter 3 have *in vivo* relevance.

Administration of anti-IL-18 antibody reduced carrageenan induced footpad MPO content, histological evidence of neutrophil infiltration, and local TNF α mRNA expression, suggesting effects on the local inflammatory processes. The relationship and functional hierarchy between TNF α and IL-18 in the early phases of inflammation is intriguing. Evidence from these models suggests that IL-18 activities may lie upstream of TNF α . Neutralization of IL-18 reduced local TNF α mRNA expression in carrageenan induced inflammation. Furthermore, IL-18 induced neutrophil migration required intact TNF α signaling, demonstrated by the absence of IL-18 induced neutrophil migration in TNFR p55-deficient mice. Further investigation of the reduced 'background' neutrophil recruitment (i.e. PBS control) in the TNFR p55-deficient mice could be addressed, for example, by administration of thioglycolate. It is of interest that IL-18 did not compensate for this defect. Blocking free TNF α with soluble receptors or

neutralising antibody would also be appropriate to further investigate the IL-18-TNF α axis in these models, however reagents were not available to perform this study.

The use of LPS controls in this study was important, as BALB/c mice are particularly sensitive to LPS. Titration of low doses of LPS demonstrated that the maximum contaminating dose of LPS in the rmIL-18 could not induce significant neutrophil infiltration. Combined with the studies in endotoxin resistant mice, this provided sound evidence that contaminating LPS was not masquerading the effects of rmIL-18. The analysis of draining lymph node function could be further investigated using an antigen specific system and phenotypic analysis of cell populations. Furthermore, the cytokine mRNA expression data in footpad and lymph node were obtained from a limited number of animals. These studies were not extended due to limited antibody availability and in accordance with the refinement and reduction of animal experiments. It would, however, be of interest to determine if subtle effects were exerted within draining lymph nodes, and to extend the molecular approach of investigating the effects of administering a neutralising antibody. Detailed information could be provided by gene array technology, and protein analysis would also be desirable. Moreover, the expression of other mediators could be explored in detail, perhaps providing insights into networks of cytokine function. Delineating whether these *in vivo* observations were the consequence of direct effects on neutrophil responses, or secondary to effects on other cells would also be of interest. These experiments were beyond the scope of this project, but are worthy of consideration given the therapeutic potential of neutralising IL-18 in human disease.

Other approaches to neutralising IL-18 include targeting the IL-18R. Administration of anti-IL-18R antibody in this carrageenan model reduced footpad swelling but inhibited the Th1 response in the draining lymph, presumably by targeting IL-18R expressing T cells (54). The natural IL-18 antagonist, IL-18BP, has been administered in models of CIA and IBD with promising results (131, 138). This protein mediates disparate effects dependent on the dose given. Furthermore, IL-18BP is elevated along with IL-18 in several pathologies including sepsis and adult Still's disease (ASD) (99), indicating that relative levels of agonist and antagonist activity are determining overall effect. Application of IL-18BP to the carrageenan model awaits availability of reagents. Several studies have demonstrated that soluble IL-18R α or β chains are of insufficient binding affinity to mediate useful neutralising effects. Recombinant heterodimers also failed to bind as effectively as a binding protein (25, 27).

Neutralising IL-18 is an attractive therapeutic strategy, and whilst targeting pathological inflammatory effects, may also lead to immune compromise. These effects are especially difficult to evaluate in short term, murine experiments performed in carefully controlled environments with inbred animals. To partially address this issue, it would be of interest to neutralise IL-18 in the carrageenan and other models, in different strains of mice. Reagent limitations precluded this approach from the current study.

These data provide further evidence that IL-18 is involved in neutrophil migration, and recruitment to inflammatory lesions. As such, neutralising IL-18 may be beneficial, alone and in combination with targeting other cytokines. However, the full implications of neutralising IL-18, and the ideal vehicle for this purpose are not clear. Clearly, manipulation of cytokines in inflammation may have dramatic effects. To explore the

consequences of such manipulation in host defense and inflammation initiated by a pathogen, *S. aureus* infection was investigated.

Chapter 5 The role of enhanced IL-18 expression in gram-positive infection

Introduction

Data in previous chapters show that IL-18 activates neutrophils *in vitro* (Chapter 3) and *in vivo* (Chapter 4). Bacterial killing is a key function of neutrophils. To extend the physiological relevance of the previously described effects of IL-18 on neutrophil activation a model of gram-positive infection was investigated. IL-18 is a critical regulatory cytokine linking innate and acquired responses. Manipulation of IL-18 expression *in vivo* in models of parasitic (112), viral (107), fungal (222), and mycobacterial (52) infection indicates protective roles for IL-18. In murine models of gram-negative infection, IL-18 contributes to host resistance (94), but also promotes shock associated with administration of LPS (97). This role in endotoxic shock is critically dependent on initiating factors. Thus, *Propionibacterium acnes* primed IL-18-deficient mice show a defect in negative regulation of TNF α resulting in increased mortality following challenge with endotoxin (98).

Emerging evidence implicates IL-18 in gram-positive infections *in vivo*. Treatment with anti IL-18 receptor β chain antibody in a murine model of *Listeria monocytogenes* infection demonstrated protective effects of IL-18 through modulation of early innate immune responses, Th1 development and memory responses (93). Furthermore, IL-18-deficient mice fail to clear *Streptococcus pneumoniae* as effectively as wild type controls (95). Finally, septic patients have elevated levels of both IL-18 and its natural antagonist IL-18BP (41, 99). Interestingly, septic patients with gram-positive infections have significantly higher IL-18 plasma levels than patients with gram-negative infection (100). Following staphylococcal infection, IL-18-deficient mice exhibit reduced

septicaemia but increased end organ infection, namely inflammatory arthritis (96). This increased bacterial load may be indicative of a deficiency in neutrophil function.

Septic arthritis is a common complication of RA, affecting up to 3% of RA patients. Infection is associated with high mortality (20%), joint destruction and irreversible loss of joint function. In 30-60% of cases, the causative organism is *Staphylococcus aureus*. Furthermore, 1-5% of prosthetic joints become infected with *S. aureus*. Patients with RA, who are known to exhibit elevated IL-18 levels in tissues and serum, are more susceptible to gram-positive infection, particularly *S. aureus*, which is often complicated by septicaemia with significant associated mortality (223). That patients with *a priori* elevated IL-18 expression show a poor prognosis following gram-positive infection led us to investigate the protective versus pathogenic effects of IL-18 in a murine model of gram-positive sepsis. Cytokine modulation is an attractive therapeutic strategy for many diseases; therefore determining whether IL-18 plays a predominantly protective or pathogenic role in the pathogenesis of *S. aureus* infection is crucial.

Results

5.1 Human neutrophil expression of IL-18

Neutrophils are capable of synthesising a broad array of cytokines. Neutrophil IL-18 expression was investigated by intracellular FACS analysis. This facilitated identification of neutrophils by size and granularity, reducing the potential for false positive results. Contaminating cells within purified neutrophil populations (Fig. 3.2), which express IL-18, could give false positives in sensitive assays such as PCR or Western blot. Neutrophils were cultured with an inhibitor of golgi function, Brefeldin A, to promote accumulation of intracellular protein. A modest increase in the fluorescence intensity of staining with anti-IL-18 antibody was observed compared to isotype control (Fig. 5.1a and b). This suggested that either neutrophils express low levels of IL-18 on a per cell basis or that only a subpopulation of neutrophils are IL-18 positive. The latter was implied by dot plot analysis (Fig. 5.1c and d). The anti-IL-18 antibody used in this study does not differentiate pro and mature forms of IL-18. However, control of neutrophil production, processing and release of IL-18 is currently under investigation (Dr. S. Roberstons, Centre for Rheumatic Diseases, Glasgow Royal Infirmary). Nevertheless, these data suggest that endogenous IL-18 may potentially auto-activate neutrophils, which may contribute to their bactericidal role.

5.2 IL-18 promoted bacterial killing

To determine if IL-18 may promote bacterial killing by human neutrophils *in vitro*, neutrophils were incubated with 100 ng/ml IL-18 for 1 h before exposure to opsonised *S. aureus*. This period of incubation with IL-18 was previously determined as optimal

for enhancing subsequent respiratory burst in response to fMLP (Section 3.9). Prior incubation of human neutrophils with IL-18 modestly enhanced bacterial killing (Fig. 5.2). Such a modest effect was unsurprising given that neutrophils were derived from normal healthy donors, and bactericidal activity of such neutrophils should be highly efficient in any case.

To further investigate if the IL-18 identified in neutrophils may play a role in bacterial killing, leukocytes were obtained from IL-18-deficient DBA1 mice, or wild type controls. Thioglycolate induced peritoneal neutrophils are highly activated *in vivo* (CD11b^{hi}, CD62L^{lo}), and thus less susceptible to priming than human blood derived neutrophils. Therefore murine buffy coat leukocytes were assayed. Accordingly, whole blood was collected via cardiac puncture under terminal anaesthetic and buffy coats containing peripheral leukocytes separated by dextran sedimentation. Neutrophils were not purified from murine peripheral blood due to the limited blood volume available and the relatively low frequency of neutrophils (Chapter 6, Fig. 6.1). Leukocytes derived from IL-18-deficient mice exhibited significantly reduced ability to kill bacteria *in vitro*, compared to wild type controls. This deficiency in bactericidal activity was partially restored by addition of exogenous IL-18 (Fig. 5.3). Addition of exogenous IL-18 to leukocytes derived from wild type mice did not enhance bacterial killing, as was evident in human samples. This may reflect prior *in vivo* activation caused by the traumatic route of blood collection, or the different constitution of blood cell subsets in mice compared with humans. The compromised bactericidal capacity observed in IL-18-deficient mice *in vitro* may contribute to their increased susceptibility to bacterial infections.

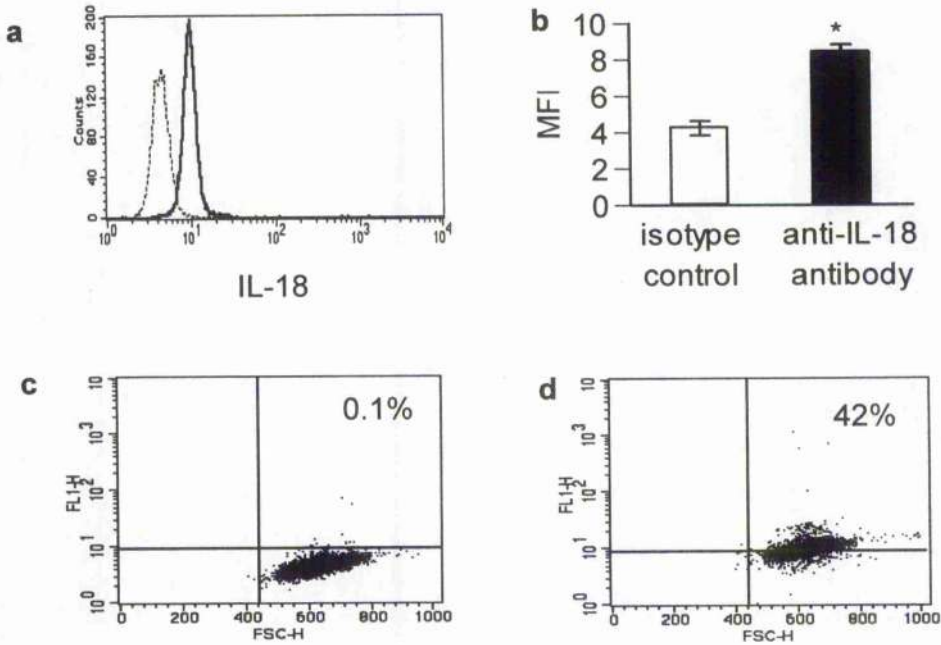


Figure 5.1 Human neutrophils from healthy donors expressed IL-18 protein

Neutrophils were purified from normal donors ($n = 4$), cultured with $10 \mu\text{g/ml}$ brefeldin A for 2 h, stained for intracellular cytokine expression, and analysed by flow cytometry. (a) Representative histogram analysis of neutrophils stained with anti-human IL-18 (solid line) or isotype control (dotted line). (b) Pooled data of mean fluorescent intensity of 4 donors. Data are mean \pm SEM. $*p < 0.01$ vs. control. (c) Representative dot plot of isotype control and (d) dot plot of IL-18 staining.

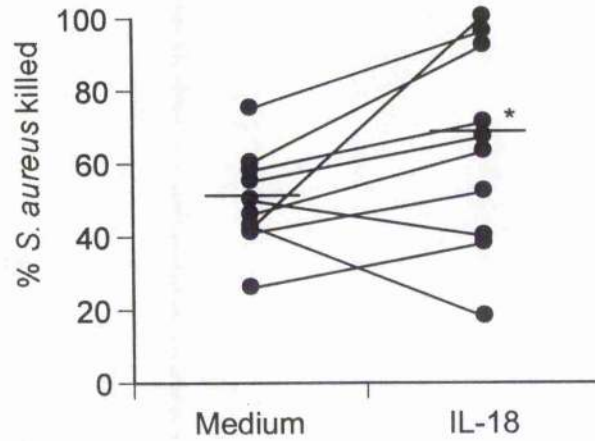


Figure 5.2 IL-18 promoted bacterial killing by human neutrophils

Peripheral blood neutrophils were purified from normal human volunteers ($n = 10$) and incubated at 10^7 /ml for 1 h with medium alone or 100 ng/ml IL-18. Neutrophils were added to an equal volume of opsonised *S. aureus* (10^7 CFU/ml, assessed spectrophotometrically, confirmed by plating serial dilutions on blood agar). After 20 minutes, remaining viable bacteria were counted by plating serial dilutions on blood agar. Mean bacterial killing of neutrophils pretreated with IL-18 was 63.7% compared with 49.6% bacterial killing by neutrophils incubated in medium alone. * $p < 0.05$ vs. medium by 1 sample t test.

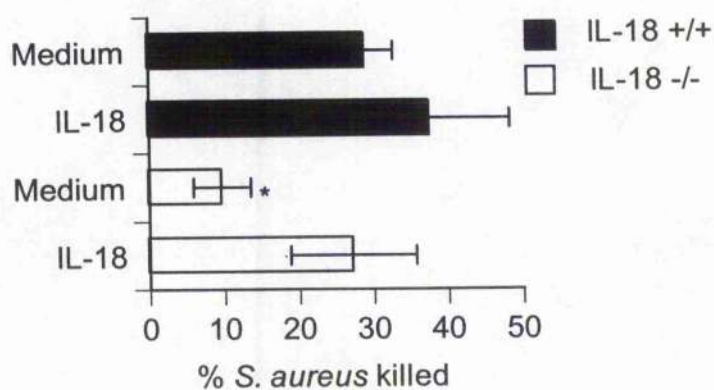
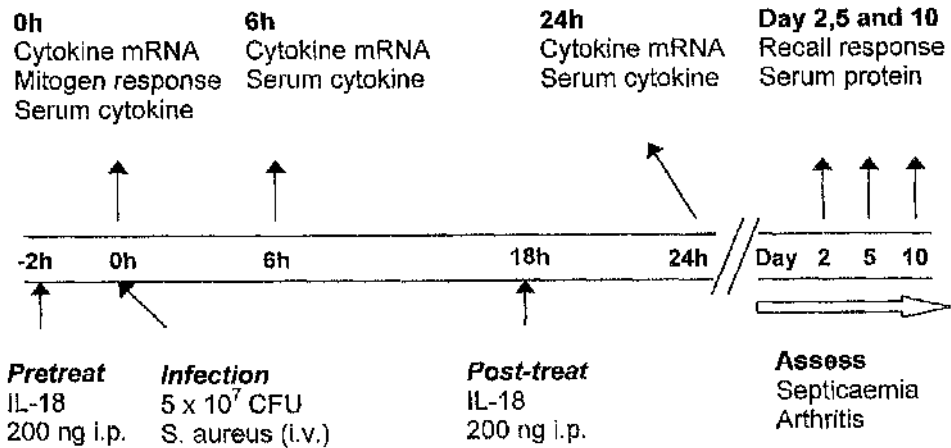


Figure 5.3 Endogenous IL-18 promoted bacterial killing

IL-18 wild type (+/+) or deficient (-/-) DBA-1 mice were terminally anaesthetised by i.p. administration of 12.5 mg avertin, blood was collected via cardiac puncture into heparinised syringes and leukocytes separated by dextran sedimentation. Murine cells were incubated with 100 ng/ml rmIL-18 or medium alone for 1 hour prior to addition of bacteria as in Fig. 5.2. Data are mean \pm SEM of 4 experiments with 3 mice per group in each experiment. * $p < 0.05$ vs. wild type medium by Student's t test.



3 groups, n = 30 per group

- PBS (i.p. PBS at either -2h or 18h)
- Pretreat (i.p. IL-18 at -2h)
- Posttreat (i.p. IL-18 at +18h)

Figure 5.4 Outline of experimental design to investigate the role of elevation of IL-18 in bacterial infection

5.3 Incidence and severity of septicaemia following IL-18 administration

Previous investigation determined that IL-18 deficiency modified *S. aureus* infection in mice (96). A more common scenario is *a priori* elevated IL-18 levels, such as observed in autoimmune diseases. The incidence and severity of sepsis following rmIL-18 administration was therefore investigated as shown in Fig. 5.4. Swiss mice were infected with 5×10^7 CFU/animal *S. aureus* by intravenous inoculation. Tarkowski *et al* previously established that low dose infection ($\cong 10^7$ CFU per animal) promotes predominantly arthritis whereas high dose infection ($\cong 10^8$ CFU per animal) induces predominantly septicaemia (211). An intermediate dose was selected in the following experiments that caused severe arthritis and evidence of sepsis in a small-scale pilot experiment. The dose of IL-18 employed in these studies was estimated on the basis of studies in CIA models, in which a similar bolus effectively modulated immune responses (129). In compliance with Home Office recommendations, which stipulate a reduction and refinement in the use of animals, further dose response experiments were not performed to minimise animal usage.

In 2 experiments, mice received 200 ng of rmIL-18, 2 h prior to infection, (n = 30, 'pretreat'), 18 h post infection (n = 30, 'posttreat'), or received carrier alone (n = 30 'PBS'). Significantly increased mortality was observed in pre-treated mice compared with controls. Survival at day 5 in the pre-treated group was 58%, compared with 93% in the PBS group (Fig. 5.5). Post infection administration of IL-18 had no effect on mortality. Onset of clinical evidence of septicaemia was accelerated in IL-18 pre-treated mice compared with the control group (Fig. 5.6a). The severity of systemic disease, quantified by determining septic index (only in involved mice), was greater at early time

points (day 2 and 4) in pre-treated mice compared to controls (Fig. 5.6b). From day 4 through day 10 there was no significant difference in clinical signs of sepsis between groups suggesting that the predominant effects of prior IL-18 elevation were manifest at an early stage. Post infection administration of IL-18 had no effect on incidence or severity of sepsis. Weight loss was monitored daily, revealing significant weight loss in all groups, which was not affected by administration of IL-18 at any time point (Fig. 5.6c).

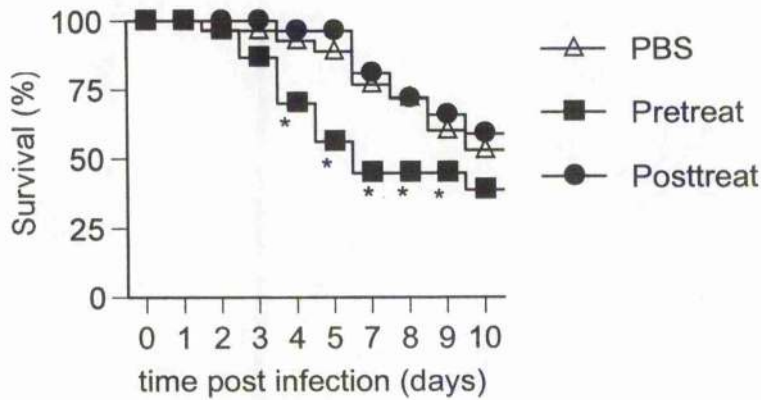


Figure 5.5 Administration of IL-18 modulated mortality following *S. aureus* infection

Mice received 200 ng rmIL-18, 2 h prior to ('pretreat') or 18 h post ('posttreat') infection. The 'PBS' group received carrier alone either prior to or post infection (Fig. 5.4). Mortality was assessed by Kaplan Mayer analysis, which accounts for animals sacrificed for *in vitro* analysis. $n = 30$ mice per group, from 2 independent experiments. $*p < 0.05$ vs. PBS by Chi-squared test.

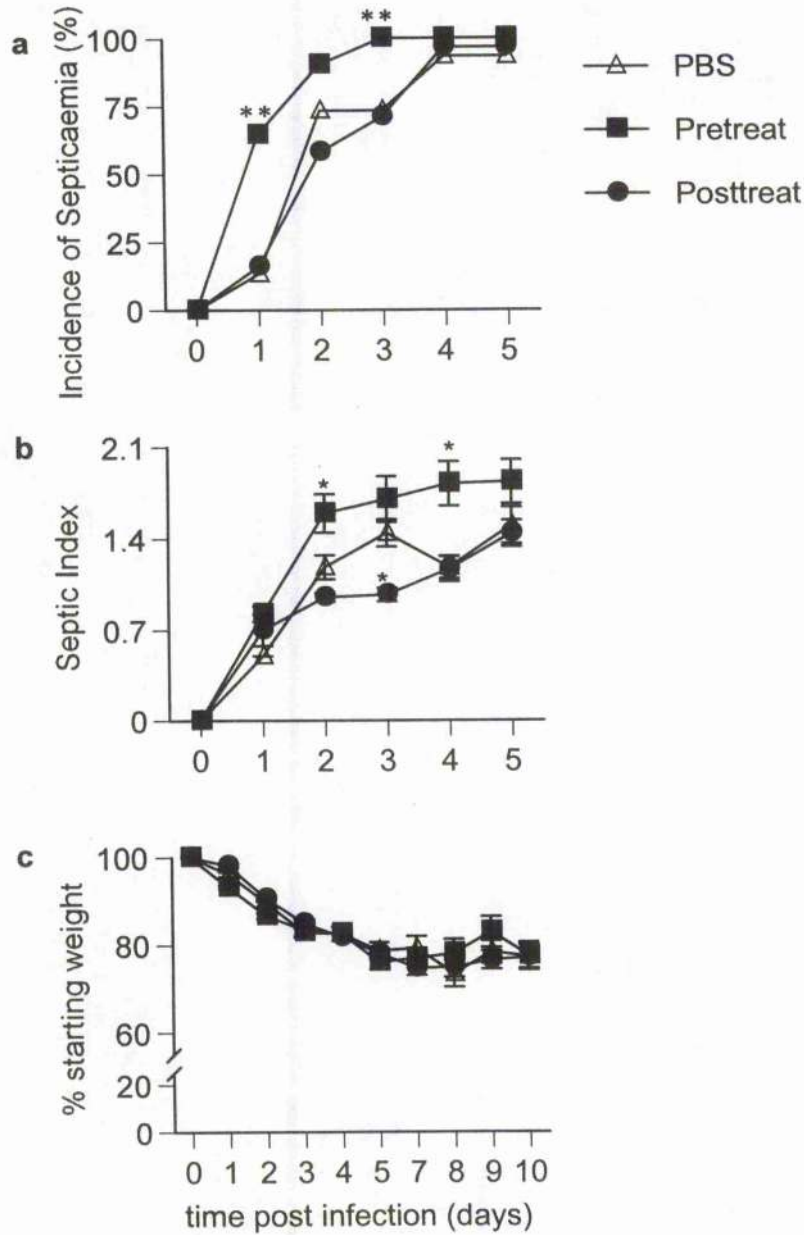


Figure 5.6 Clinical assessment of sepsis following *S. aureus* infection

Mice were treated as in Fig. 5.4. (a) Incidence of septicaemia, representing the proportion of animals exhibiting signs of sepsis. (b) Severity of sepsis, judged by septic index (Section 2.11.3), data are from involved animals only. (c) Weight loss, recorded as % starting weight. Data are mean \pm SEM, $n = 30$ mice

per group, representative of 2 similar experiments. * $p < 0.01$ vs. PBS by Mann Whitney U test; ** $p < 0.005$ vs. PBS by Chi-squared test.

5.4 Incidence and severity of septic arthritis

Within 2 days of infection with *S. aureus*, clinical signs of arthritis were observed in the majority of mice. 100% of the animals exhibited involved joints from day 4 through day 10. Incidence of arthritis was similar in all groups (Fig. 5.7a). Control mice developed severe arthritis, determined by articular index, and pre-treatment with IL-18 did not further increase the magnitude of such already severe articular disease. However, post infection administration of IL-18 significantly reduced the severity of arthritis (Fig. 5.7b). To further investigate this observation, hind paws were subjected to histological analysis at day 5. Footpads were formalin fixed, then decalcified and 4 μm sections stained with heamatoxylin and eosin. Sections were examined for signs of synovial hyperplasia, joint space invasion and cartilage and bone destruction. There was evidence of all these features of joint destruction (Fig. 5.8). Surprisingly, no differences were observed between the groups, suggesting either that the reduced articular index was due to altered oedema in the footpads, or that the animals obtained were not representative. It is likely that larger studies would be required to accurately determine mechanisms of altered pathogenesis.

5.5 Distribution of bacteria

Distribution of bacteria was assessed by sequentially diluting cultures of blood and homogenised tissues at 6 h, 24 h, and at days 2, 5 and 10, post infection. 24 h post

infection, IL-18 pretreated mice showed elevated levels of bacteria in peripheral blood and kidney compared to controls (Table 5.1). This suggested some inefficiency in killing at early time points in pretreated mice. Similar numbers of viable *S. aureus* were detected in the blood, kidneys, spleens and footpads of mice at later time points, in all groups.

In post infection IL-18 treated mice, there was no evidence of consistently reduced bacterial load in the footpads. Thus increased bacterial clearance did not account for the observed reduction in articular index, or the footpads obtained were not representative.

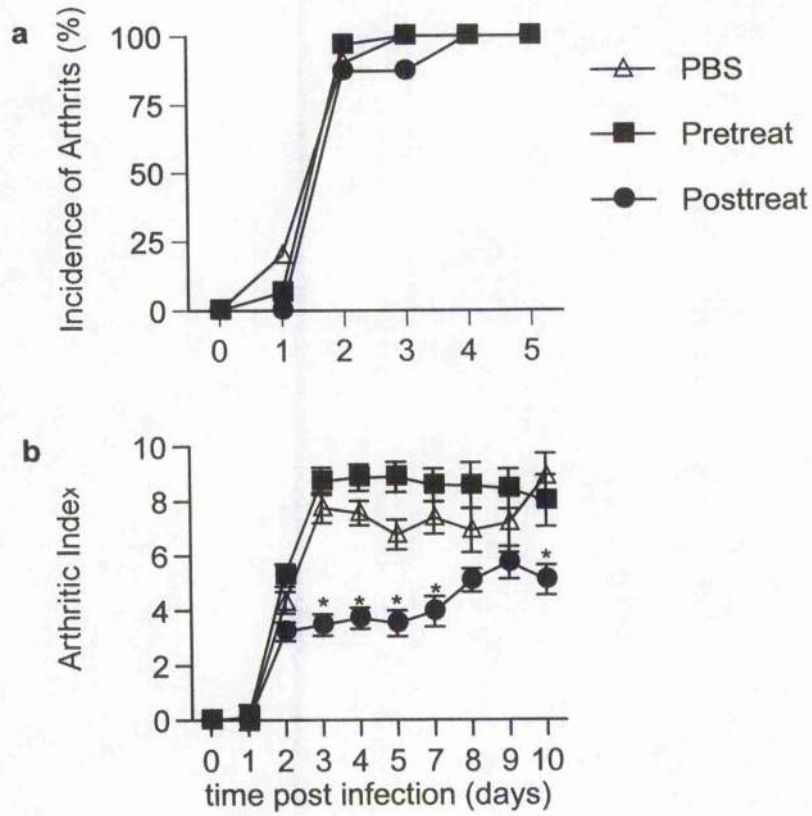


Figure 5.7 Pathogenesis of septic arthritis following infection with *S. aureus*

Experimental protocol is described in Fig. 5.4. (a) Incidence of arthritis representing the proportion of animals exhibiting signs of arthritis. (b) Severity of arthritis was judged by arthritic index (Section 2.11.3), data are mean \pm SEM, of involved animals only. From day 4 through day 10, all animals exhibited clinical signs of arthritis. $n = 30$ mice per group, from 2 independent experiments. * $p < 0.01$ vs. PBS by Mann Whitney U test.

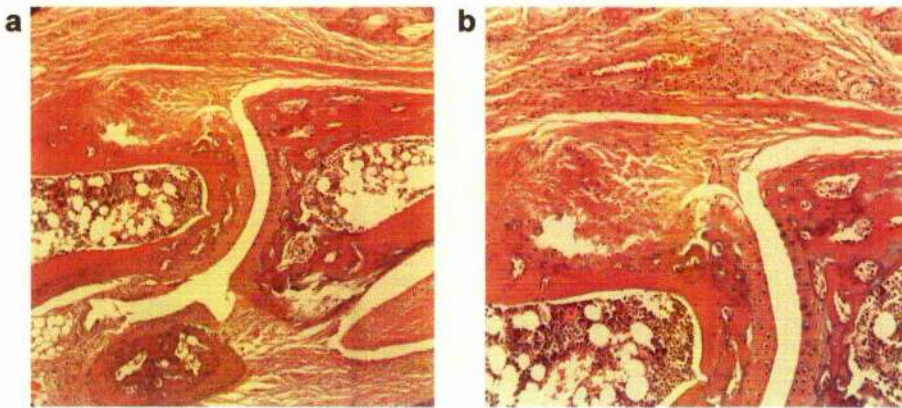


Figure 5.8 Articular histology subsequent to *S. aureus* infection

Left and right hind paws were removed at day 5 post *S. aureus* infection, fixed in formaldehyde then decalcified. 4 μm Sections were prepared from 5 mice per group, and stained with H&E. Degree of erosion, infiltration and synovial hyperplasia were assessed as previously described (224). No histological differences were observed at this time point between the 3 groups. (a) Representative section, low power magnification, and (b) same section, high power magnification.

Table 5.1 Peripheral distribution of bacteria following infection with *S. aureus*¹.

Time	Group	n per group	Blood CFU/ml $\times 10^3$	Kidney CFU/kidney $\times 10^5$	Spleen CFU/g $\times 10^4$	Paws CFU/paw $\times 10^4$
6h	PBS	5	21701±4365	0.19±0.05	2170±436	not
	Pretreat	5	18063±6054	0.58±0.39	1806±605	done
24h	PBS	5	0.27±0.20	0.08±0.03	167±58	22.4±8.0
	Pretreat	5	2.15±0.69*	1.54±0.59*	151±49	128±98.2
	Posttreat	5	0.41±0.20	0.07±0.03	186±37	25.8±8.3
48h	PBS	5	0.28±0.19	9.90±8.08	4.36±0.82	94.0±29.0
	Pretreat	5	0.21±0.08	263±181	2.85±1.20	183.0±68.5
	Posttreat	5	0.32±0.13	0.25±0.16	2.01±1.15	196.0±93.6
d5	PBS	3	226.33±188.06	440.04±302.63	0.46±0.40	622.0±190.0
	Pretreat	3	466.68±466.66	770.65±367.85	0.02±0.01	659.8±275.9
	Posttreat	3	0.00±0.00	818.20±437.92	0.06±0.04	335.1±166.1
d10	PBS	3	0.00	910.69	0	131.1
	Pretreat	3	0.00	1124.67	0	55.6
	Posttreat	3	0.00	799.33	0.05	82.0

¹Experimental design outlined in Fig. 5.4. Values are mean \pm SEM of number of mice per group as indicated in the table. The data obtained at 24 h are representative of 2 similar experiments. * $p < 0.05$ vs. PBS control by Mann Whitney U test.

5.6 Pharmo-kinetics of IL-18 administration

As a surrogate for pharmacokinetics of IL-18 administration, IL-18 levels in the sera were assessed by ELISA. IL-18 was detected in the serum 2 and 6 h following i.p. administration. IL-18 levels declined rapidly over the next 48 h (Fig. 5.9). Comparison of IL-18 levels 6 to 8 h post administration (i.e. 6 h post infection for pretreated mice, and 24 h post infection for post-treated mice) indicated similar levels of circulating IL-18. This suggests that any up regulation of IL-18 binding proteins or other negative regulators by *S. aureus* was not sufficient to interfere with detection of circulating IL-18 levels at this time point. Measurement of such inhibitors was hampered by absence of reagents. Tissue IL-18 protein levels could not be easily measured. Of particular importance, the 1-3 ng/ml of IL-18 detected in these murine sera are comparable to IL-18 concentrations detected in sera of patients with severe diseases such as sepsis and Adult Still's Disease. Septic patients exhibit elevated serum IL-18 at various stages of disease. It was therefore surprising that only very low levels of IL-18 were detected in infected mice which been treated with PBS instead of IL-18.

5.7 Altered cytokine mRNA and protein expression 2 h after administration of IL-18

To investigate changes induced by IL-18 prior to infection, mice were sacrificed 2 h after IL-18 administration and cytokine mRNA expression in peripheral blood leukocytes (PBL) was determined. Significant increase in expression of IFN γ mRNA in IL-18 treated mice was observed compared to PBS controls (Fig. 5.10a). Since monokine over-expression can be induced by IL-18 *in vitro* and is associated with septicaemia (225) TNF α mRNA expression was also analysed, and found to be

significantly elevated in PBL following IL-18 administration (Fig. 5.10b). In parallel, we investigated whether exogenous IL-18 administration altered the cytokine mRNA profile in spleen. IL-18 enhanced IFN γ mRNA (Fig. 5.10c), but did not alter TNF α (Fig. 5.10d). To provide an indication of modulation of Th1 response induction, IL-12p40 mRNA expression was assessed, and to assess whether IL-18 exerted auto-regulatory effects at the mRNA level, expression of IL-18 mRNA was also investigated. No effect on IL-12p40 or IL-18 mRNA expression in PBL or spleen was observed 2 h post IL-18 administration.

Serum IFN γ protein was detected 2 h post IL-18 administration in 80% of recipients at low levels up to 300 pg/ml (Fig. 5.11). TNF α protein was not detected in the serum of any group.

5.8 IL-18 modulated TLR expression

To determine if in addition to altering cytokine expression, IL-18 administration affected other pathways of immune response to bacterial infection, Toll-like receptor (TLR) expression was investigated. TLRs are a large family of pattern recognition molecules that mediate responses to various pathogen components. In this study, modulation of TLR2 and TLR9 were investigated. TLR2 binds bacterial peptidoglycan and is involved in immunity to gram-positive bacteria; TLR9 mediates host responses to bacterial CpG motifs. To determine whether IL-18 could affect TLR mRNA expression, experiments were performed as in section 5.5. Two hours post IL-18 administration, TLR2 mRNA expression in PBL was significantly elevated in IL-18 treated mice compared to PBS controls (Fig. 5.12a). TLR9 mRNA expression in peripheral blood

followed a similar trend of elevation, although the difference between the groups was not statistically significant (Fig. 5.12b). Administration of IL-18 had no effect on TLR2 or TLR9 mRNA expression in the spleen (Fig. 5.12c and d). Protein detection reagents for murine TLR2 and TLR9 were not available at the time of these studies.

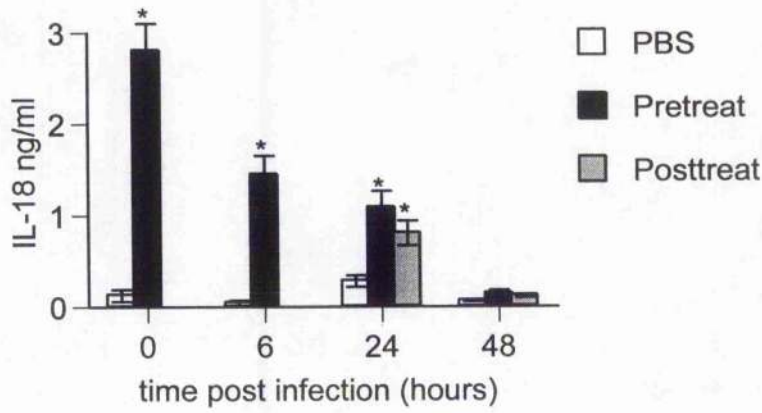


Figure 5.9 Serum IL-18 following i.p. administration of IL-18

Blood was obtained at times indicated following inoculation with *S. aureus*. Blood was allowed to clot overnight at 4°C and centrifuged to obtain serum, which was assayed for cytokines by ELISA. Data are mean \pm SEM of 5 mice per group per time point. * $p < 0.05$ vs. PBS group by Student's *t* test. Serum IL-18 levels at days 5 and 10 were similar to those observed at 48 h.

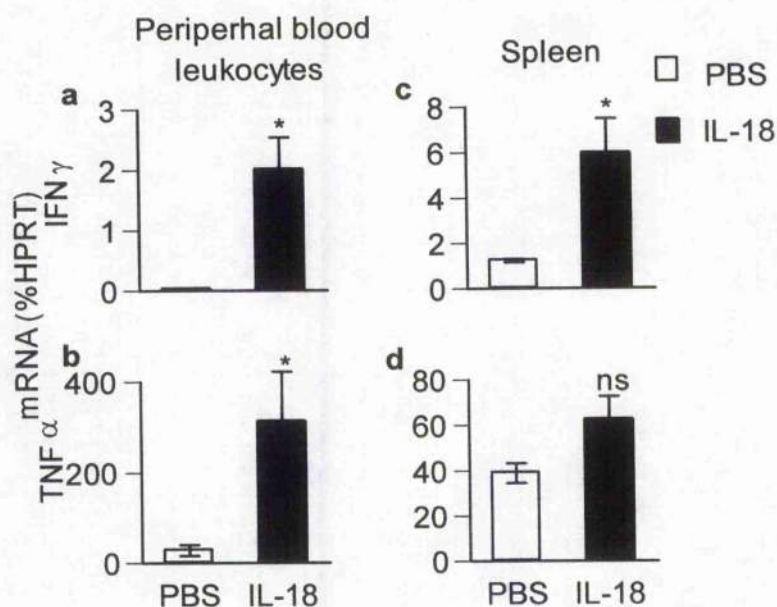


Figure 5.10 Cytokine mRNA expression 2 h post IL-18 administration

To investigate the immediate effects of IL-18 administration, mRNA was extracted from peripheral blood leukocytes (PBL) or sections of spleen. Changes in cytokine mRNA expression were assessed by real time PCR. (a) IFN γ and (b) TNF α mRNA expression in PBL was elevated 2 h post i.p. administration of IL-18. (c) Splenic expression of IFN γ mRNA was similarly elevated but administration of IL-18 had no effect on TNF α mRNA expression in spleen (d). Data are mean \pm SEM of 5 mice per group. * $p < 0.05$ vs. PBS by Mann Whitney U test. These data are also described in Tables 5.2 and 5.3.

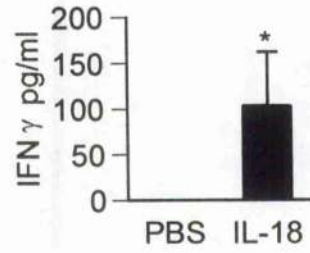


Figure 5.11 Serum IFN γ 2 h post i.p. administration of IL-18

Whole blood was obtained via cardiac puncture 2 h post i.p. administration of 200 ng of IL-18. Serum cytokine concentration was assayed by ELISA. Data are mean \pm SEM of 5 mice per group. * $p < 0.05$ vs. PBS by Mann Whitney U test.

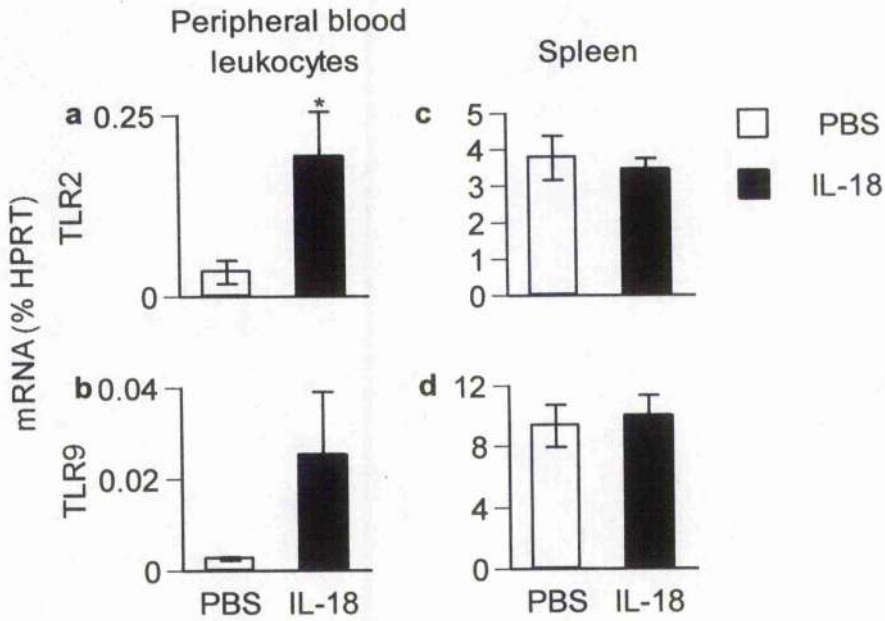


Figure 5.12 Changes in TLR mRNA expression following IL-18 administration

Experiments were performed as in Fig. 5.10. (a) TLR2 and (b) TLR9 mRNA expression in PBL was elevated 2 h post i.p. injection of IL-18. Splenic expression of (c) TLR2 mRNA or (d) TLR9 mRNA was unaffected by administration of IL-18. Data are mean \pm SEM of 5 mice per group. $*p < 0.05$ vs. PBS by Mann Whitney *U* test.

5.9 Effects of IL-18 administration on *ex vivo* immune responses

To investigate whether this rapid induction of cytokine mRNA (Section 5.5) indicated 'priming' of the immune response to subsequent stimulus, spleen and heparinised blood were obtained 2 h after i.p. injection of IL-18. Single cell suspensions were cultured for 48 h with Con A or formalin fixed bacteria.

i) *Ex vivo* response to Con A

Proliferative responses to Con A in peripheral blood or spleen cultures were similar irrespective of prior IL-18 administration (Fig. 5.13a and b). However, in leukocyte cultures, Con A induced production of IFN γ was significantly increased by prior treatment with IL-18 *in vivo* (Fig. 5.13c). IFN γ production to Con A in spleen cultures was similar in both groups (Fig. 5.13d), suggesting that the primary early effects of IL-18 administration had been in the periphery. Con A stimulated IL-6 production by both leukocyte and spleen cultures. IL-6 production by PBL in response to Con A was moderately depressed by prior *in vivo* administration of IL-18. (Fig. 5.13e and f) suggesting some selectivity in the modulation of cytokine release. Residual free recombinant IL-18 was unlikely to influence subsequent leukocyte cultures since erythrocytes were lysed and remaining leukocytes thoroughly washed prior to addition of stimulus.

ii) *Ex vivo* response to *S. aureus*

In vitro stimulation with formalin-fixed *S. aureus* did not induce significant proliferation (Fig. 5.14a and b) or IFN γ release in leukocyte cultures (Fig. 5.14c). Spleen cell cultures produced only modest levels of IFN γ in response to fixed *S. aureus*, regardless of prior exposure to IL-18 (Fig. 5.14d). *S. aureus* stimulated significant IL-6 production by both PBL and splenocyte cultures. Furthermore, the PBL response was

enhanced by prior *in vivo* exposure to IL-18 (Fig. 5.14e and f). Together these data suggest that IL-18 administration alters subsequent immune responsiveness, primarily in the periphery, and this in turn could alter early responses upon subsequent *S. aureus* infection.

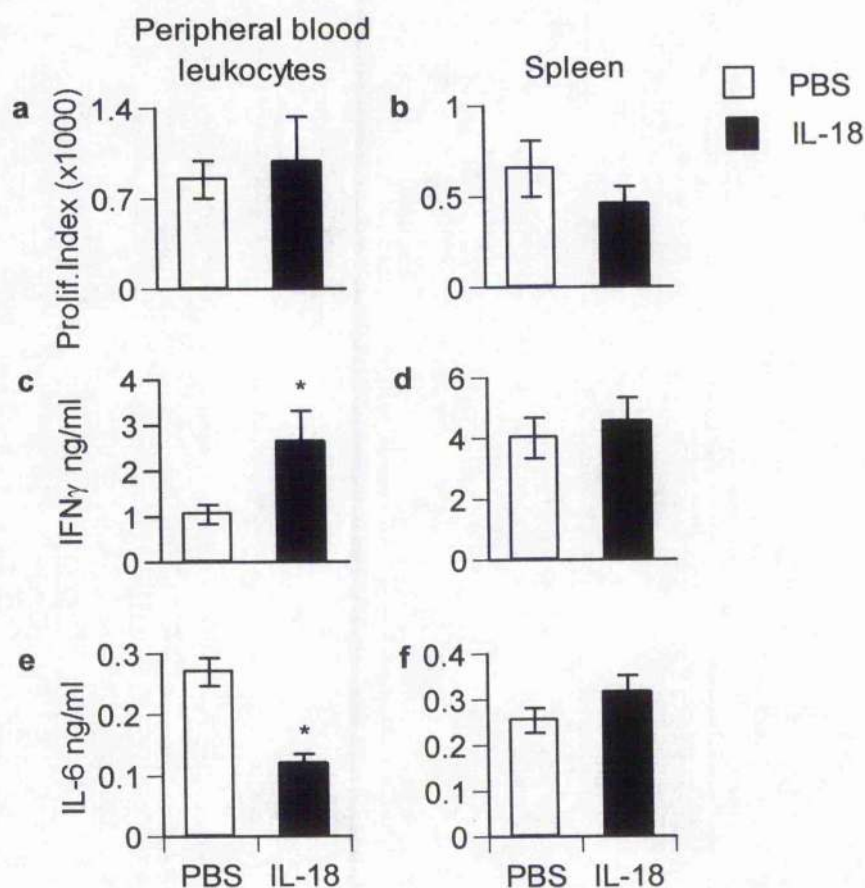


Figure 5.13 Administration of IL-18 altered *ex vivo* immune responses to Con A.

Mice were sacrificed 2 h after receiving 200 ng rIL-18 or PBS i.p. Single cell suspensions of spleen and peripheral blood leukocytes were stimulated with Con A. Proliferation of (a) PBL and (b) spleen cell cultures, was assessed by [3 H] thymidine incorporation. Data are expressed as a proliferative index which normalises the response to medium control. Con A induced IFN γ release in (c) PBL and (d) spleen cultures. IL-6 release in response to Con A was detected in PBL (e) and spleen (f) cultures. Data are mean \pm SEM of 5 mice per group. * p < 0.05 vs. PBS by Student's t test.

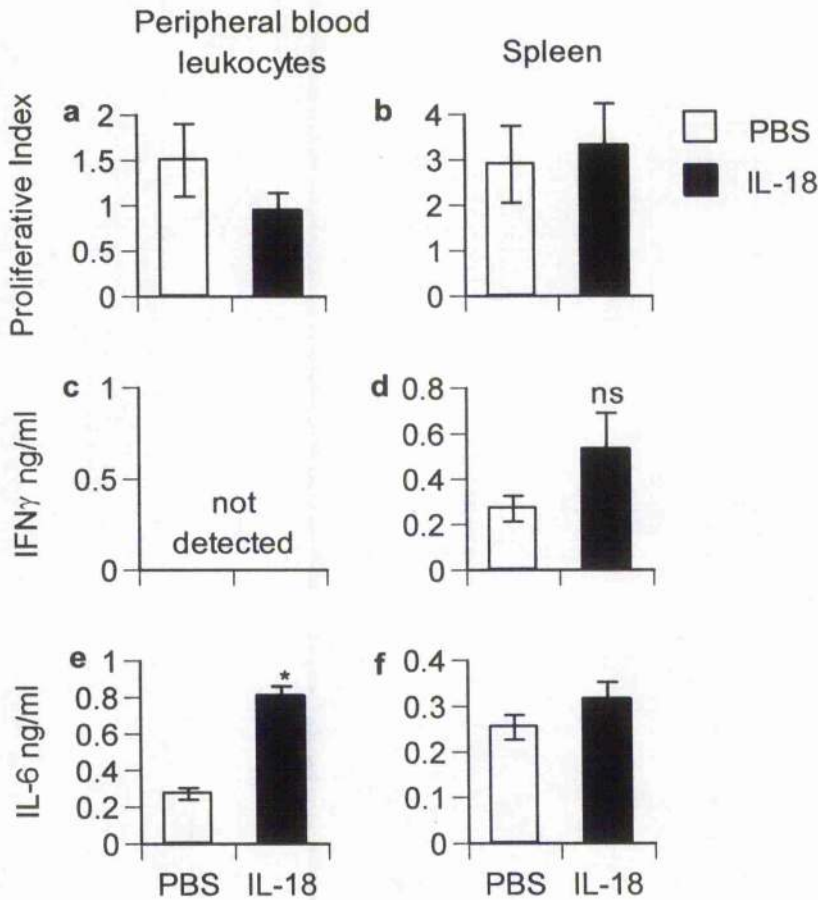


Figure 5.14 Administration of IL-18 altered *ex vivo* immune responses to *S. aureus*

Experiments were performed as in Fig. 5.14, except cell cultures were stimulated with 10^7 CFU/ml formalin fixed *S. aureus*. Proliferation in response to fixed *S. aureus* was minimal in both (a) PBL cultures and (b) spleen cell cultures. *S. aureus* failed to induce IFN γ release in (c) peripheral blood cultures, but (d) stimulated IFN γ release by spleen cultures. Prior *in vivo* administration of IL-18 enhanced PBL IL-6 release in response to *S. aureus* (e), although IL-6 production by spleen cultures (f) was unaffected. Data are mean \pm SEM of 5 mice per group. * $p < 0.05$ vs. PBS, by Student's *t* test, ns, not significantly different to PBS control.

5.10 Immunomodulatory effects of prior IL-18 administration on subsequent *S. aureus* infection

Potential immune mechanisms whereby prior IL-18 administration modified subsequent responses to *S. aureus* were investigated by measuring serum cytokine concentration and cytokine mRNA expression in tissue compartments 6 h and 24 h after infection.

i) Peripheral responses

Cytokine mRNA expression in peripheral blood leukocytes was assessed prior to infection and 6 h and 24 h post infection. In PBS treated, control mice, *S. aureus* infection resulted in increased TNF α , IFN γ , and IL-12p40 mRNA expression, but no change in expression of IL-18 mRNA. Changes in mRNA expression were maximal 6 h post infection and largely returned to prior infection levels 24 h post infection ('PBS' group, Table 5.2). Administration of IL-18 prior to infection with *S. aureus* resulted in exaggerated IFN γ mRNA expression at 6 h and 24 h post infection. Pretreated mice also exhibited elevated IL-18 mRNA expression 6 h post infection ('Pretreat' group, Table 5.2 and Fig. 5.15). Prior treatment with IL-18 had no effect on TNF α or IL-12p40 mRNA expression.

Changes in protein expression in the periphery were assessed by ELISA of serum. Infection with *S. aureus* resulted in little measurable change in serum IL-4, IL-5, IFN γ or TNF α at any time point. However, serum IL-6 was modestly elevated 24 h and 48 h post infection. Significant changes in serum cytokine expression were observed in mice receiving IL-18 prior to infection. Pretreated animals exhibited elevated serum IL-6 and IFN γ 6 h post infection. Serum IFN γ remained modestly elevated at 24 h post infection in pretreated mice, but was undetectable in any group after that time (Fig. 5.16).

ii) Spleen responses

Changes in splenic cytokine mRNA expression were assessed in parallel with peripheral blood leukocytes (Section (i), above). Similar to PBL, infection with *S. aureus* resulted in elevated TNF α , IFN γ , and IL-12p40 mRNA expression; changes were also maximal 6 h post infection. Like PBL, no change in splenic IL-18 mRNA expression was observed following infection ('PBS' group, Table 5.3). Prior administration of IL-18 predominantly enhanced expression of IFN γ mRNA (illustrated in Fig. 5.15). Interestingly, in contrast to PBL mRNA expression, prior IL-18 administration also resulted in elevated IL-12p40 mRNA expression, 6 h post infection ('Pretreat' group, Table 5.3).

Together these data clearly indicate that exposure to IL-18 predisposed to accelerated and exaggerated IFN γ transcription and protein expression upon subsequent *S. aureus* infection.

5.11 Immunomodulatory effects of post infection administration of IL-18

Post infection treatment with IL-18 resulted in increased IFN γ mRNA expression in both PBL ('Posttreat' group, Table 5.2) and spleen ('Posttreat' group, Table 5.3), although this effect was less pronounced than that caused by prior infection administration of IL-18. Similarly, post infection treated mice exhibited elevated circulating IFN γ (Fig. 5.18).

Table 5.2 Cytokine mRNA expression in peripheral blood leukocytes following IL-18 administration and infection with *S. aureus*¹.

Time (Post infection)	Treatment Group	IFN γ	TNF α	IL-12 p40	IL-18
0 h	PBS	0.03 \pm 0.02	28.1 \pm 11.9	0.08 \pm 0.07	15.61 \pm 3.69
	Pretreat	1.88 \pm 0.53*	311 \pm 111*	0.00 \pm 0.00	16.7 \pm 0.65
6 h	PBS	22.8 \pm 6.20	362 \pm 96.0	0.40 \pm 0.20	36.9 \pm 9.80
	Pretreat	149 \pm 40.1*	380 \pm 200	0.30 \pm 0.10	124 \pm 27.5*
24 h	PBS	1.36 \pm 0.44	6.12 \pm 6.79	0.02 \pm 0.01	33.4 \pm 6.90
	Pretreat	16.9 \pm 5.21*	20.8 \pm 1.16	0.08 \pm 0.04	27.4 \pm 4.70
	Posttreat	15.2 \pm 4.40*	6.30 \pm 1.00	0.01 \pm 0.00	30.0 \pm 3+6

¹Values are mean \pm SEM of 5 mice per group. * p < 0.05 vs. PBS by Mann Whitney U test. See also Fig. 5.15.

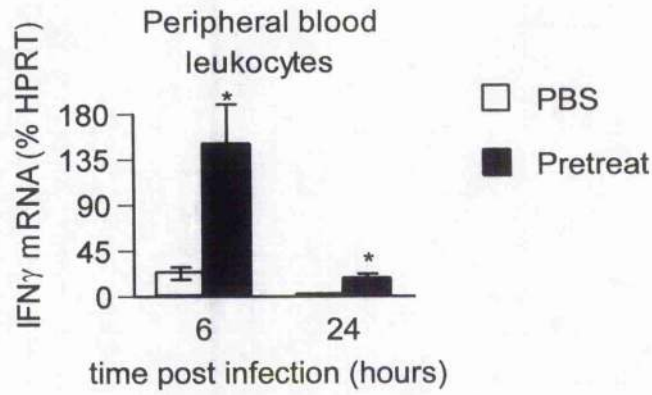


Figure 5.15 Effects of administering IL-18 on early *S. aureus* infection, in peripheral blood leukocytes

Mice received either PBS or IL-18 (Pretreat) 2 h prior to infection with *S. aureus*, and were sacrificed 6 or 24 h post infection. RNA was extracted from peripheral blood leukocytes, reverse transcribed and mRNA levels quantified by real time PCR. IFN γ mRNA expression was assessed in PBL. Data are mean \pm SEM of 5 mice per group. * $p < 0.05$ vs. PBS by Mann Whitney *U* test.

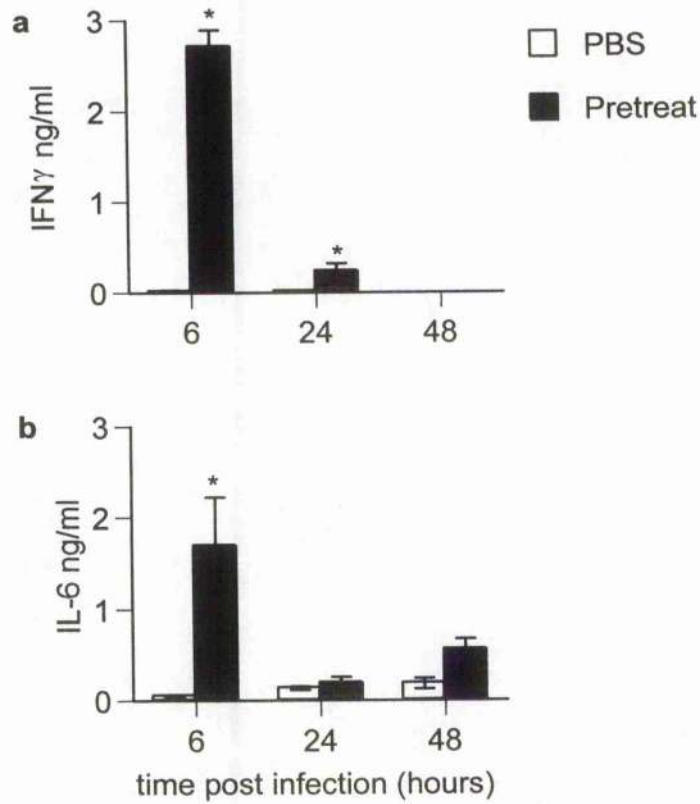


Figure 5.16 Effects of prior treatment with IL-18 on serum cytokines following *S. aureus* infection

Bloods were obtained at the time points indicated after inoculation with *S. aureus*. Serum was separated from clotted blood and assayed for cytokines by ELISA. Data are mean \pm SEM of 5 mice per group per time point, representative of 2 similar experiments. * $p < 0.05$ vs. PBS group by Student's *t* test. Serum cytokines were also measured at days 5 and 10; no cytokines were consistently detected in any group.

Table 5.3 Changes in cytokine mRNA expression in spleen following IL-18 administration and infection with *S. aureus*¹.

Time (Post infection)	Treatment				
	Group	IFN γ	TNF α	IL-12 p40	IL-18
0 h	PBS	1.24 \pm 0.08	38.9 \pm 4.39	0.142 \pm 0.005	48.4 \pm 1.3
	Pretreat	5.94 \pm 1.54*	62.1 \pm 10.5	0.09 \pm 0.009	40.2 \pm 2.01
6 h	PBS	18.1 \pm 6.45	426 \pm 90.6	1.79 \pm 0.47	50.7 \pm 7.94
	Pretreat	177 \pm 28.2*	604 \pm 102	8.79 \pm 0.73*	52.1 \pm 11.6
24 h	PBS	4.89 \pm 0.96	40.4 \pm 7.30	0.87 \pm 0.18	26.1 \pm 3.36
	Pretreat	20.9 \pm 4.49*	69.1 \pm 28.7	1.79 \pm 0.61	24.6 \pm 3.57
	Posttreat	10.74 \pm 2.87	22.2 \pm 1.63	0.36 \pm 0.06*	11.2 \pm 1.37*

¹Values are mean \pm SEM of 5 mice per group. * $p < 0.05$ vs. PBS by Mann Whitney U test.

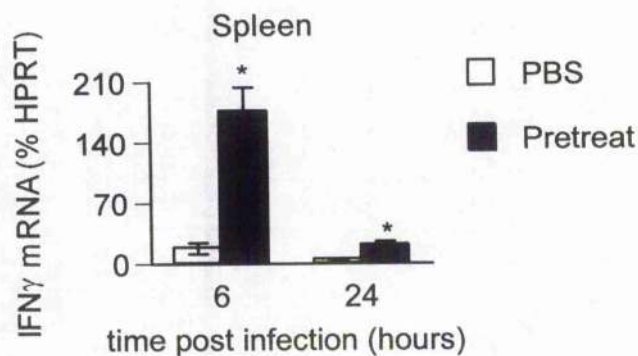


Figure 5.17 Effects of administering IL-18 on early *S. aureus* infection in spleen

Mice received either PBS or IL-18 (Pretreat) 2 h prior to infection with *S. aureus*, and were sacrificed 6 or 24 h post infection. RNA was extracted from whole spleen, reverse transcribed and IFN γ mRNA expression quantified by real time PCR. Data are mean \pm SEM of 5 mice per group. * $p < 0.05$ vs. PBS by Mann Whitney *U* test.

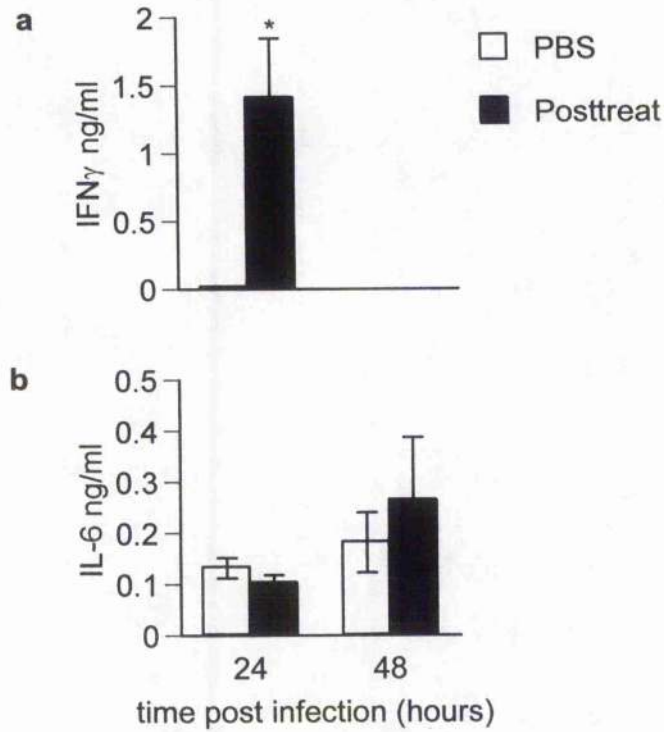


Figure 5.18 Effects of post infection administration of IL-18 on serum cytokines following *S. aureus* infection

Bloods were obtained at the time points indicated after inoculation with *S. aureus*. Serum was separated from clotted blood and assayed for cytokines by ELISA. Data are mean \pm SEM of 5 mice per group per time point, representative of 2 similar experiments. * $p < 0.05$ vs. PBS group by Student's *t* test. Serum cytokines were also measured at days 5 and 10 and no cytokine expression was consistently detected in any group.

5.12 Low dose LPS does not prime for enhanced IFN γ release

To determine if LPS contamination of the IL-18 contributed to the exaggerated responses observed, mice received either the maximum contaminating dose of LPS (5 μ g, determined by E-Toxate assay), 200 ng rmIL-18, or PBS carrier, 2 h prior to infection with 5×10^7 CFU/animal *S. aureus*. Animals were sacrificed 6 h post infection, and mRNA extracted from peripheral blood leukocytes and spleen, and serum cytokines analysed by ELISA. Prior treatment with IL-18, but not LPS, significantly enhanced IFN γ mRNA expression in the PBL (Fig. 5.19a) and spleen (Fig. 5.19b), and serum IFN γ (Fig. 5.19c) and IL-6 (Fig. 5.19d), confirming the previous observations and indicating that low dose contaminating LPS did not account for these changes.

5.13 Longevity of altered responsiveness

Finally, to determine the duration of the foregoing effects, serum was obtained at days 2, 5 and 10 following infection (Fig. 5.4). No cytokine protein levels (TNF α , IFN γ , IL-4 and IL-5) were consistently detected in serum at these time points. Variable levels of serum IL-6 were measured at each time point, with no difference between the groups. Accordingly, spleens were obtained at days 2, 5 and 10, and single cell suspensions stimulated with formalin-fixed *S. aureus*, to which the mice were now likely to be responsive. At day 2, proliferation of spleen cell cultures in response to fixed *S. aureus* was modest but equivalent in all groups (Fig. 5.20a), however, production of IFN γ in response to fixed *S. aureus* remained higher in IL-18 pre-treated mice than in PBS or post treated groups (Fig. 5.20b), whereas IL-6 (Fig. 5.20c) and TNF α (Fig. 5.20d) production and proliferation were similar between groups. Only low levels of IL-4 and

IL-5 were detected in all cultures commensurate with a dominant Th1 response. Similar cultures on day 5 and day 10 yielded IL-6, TNF and IFN γ production that was similar between groups (data not shown), suggesting that the early effects of IL-18 administration had been lost.

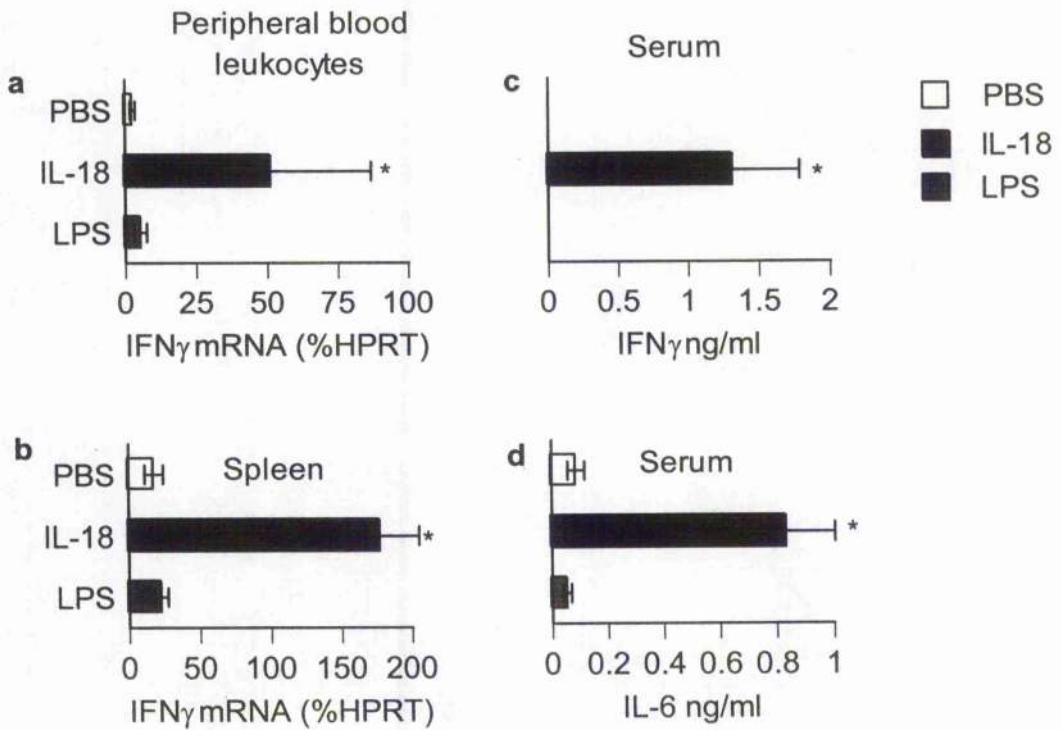


Figure 5.19 Low dose LPS did not prime for subsequent enhanced IFN γ expression

Mice received 5 ng LPS, 200ng IL-18, or PBS alone 2 h prior to infection with *S. aureus*. 6 h post infection animals were sacrificed, tissues harvested and IFN γ mRNA expression assessed in (a) peripheral blood leukocytes and (b) spleen. ELISA of serum demonstrated changes in circulating (c) IFN γ and (d) IL-6. Data are mean \pm SEM of 5 mice per group. * $p < 0.05$ vs. PBS by Mann Whitney *U* test.

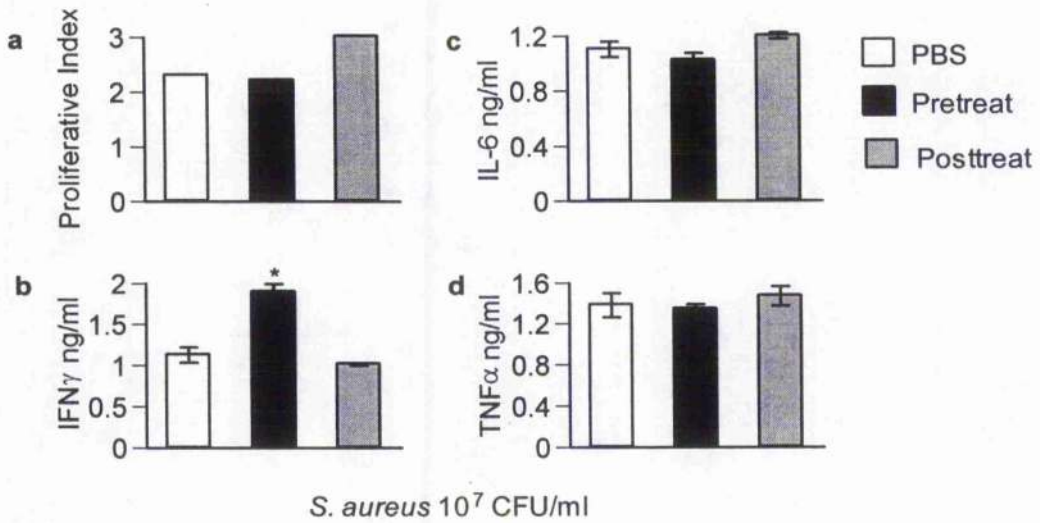


Figure 5.20 Spleen cell responses following *staphylococcal* infection

Spleens were removed 2 days post infection and cultured for 48 h in the presence of 10⁷ formalin fixed *S. aureus* per ml. A dose response of between 10⁵ and 10⁷ CFU/ml demonstrated that 10⁷ CFU/ml elicited maximum response. (a) Proliferation was assessed by [³H] thymidine incorporation. Cytokine and monokine release into the supernatant was assessed by ELISA. (b) IFN γ release was enhanced by prior *in vivo* exposure to IL-18. Cultures produced significant IL-6 (c) and TNF α (d) in response to formalin fixed *S. aureus* which was not affected by exposure to IL-18 *in vivo*. Data are mean \pm SEM of 3 mice per group per time point. * p < 0.05 vs. PBS group by Student's *t* test. Similar experiments were performed on days 5 and 10 post infection, although no differences were observed between the groups.

5.14 Cytokine detection in serum of patients with septic arthritis

Patients attending the Glasgow Royal Infirmary were diagnosed with septic arthritis on the basis of isolation of bacteria in synovial fluids, blood cultures or both. Mean patient age and underlying arthropathies, where known, are indicated in table 5.4. Serum samples were obtained from sample stores retained at -70°C , at the Royal Infirmary.

Serum $\text{IFN}\gamma$ has not been previously measured in patients with septic arthritis. Such patients may have *a priori* elevated IL-18 levels related to an underlying autoimmune disease, such as RA. In light of the exaggerated $\text{IFN}\gamma$ response seen in animals receiving IL-18 prior to infection, it was of interest to determine the presence of $\text{IFN}\gamma$ in patient sera. IL-6 levels in serum were assessed as a measure of acute phase response. Normal control sera were obtained from laboratory colleagues, thus the average age is lower than in the patient cohort (average age of normal cohort was 36.1 years, patient cohort 72.25 years). However, neither $\text{IFN}\gamma$ nor IL-6 were detected in any normal sera. IL-6 was detected at variable levels in 21 of 33 patients (Fig. 5.19a). 7 of 33 septic patients had measurable $\text{IFN}\gamma$ present in the sera (5.19b). Interestingly, within the $\text{IFN}\gamma$ positive cohort of septic patients, there was a strong correlation between IL-6 levels and $\text{IFN}\gamma$ levels in the sera (Fig. 5.19c). No correlation between serum cytokine levels and underlying disease was observed. However, these are at best preliminary observations given the insufficient power of this study. A larger, prospective patient cohort and age matched normal donors would be appropriate to further investigate these observations.

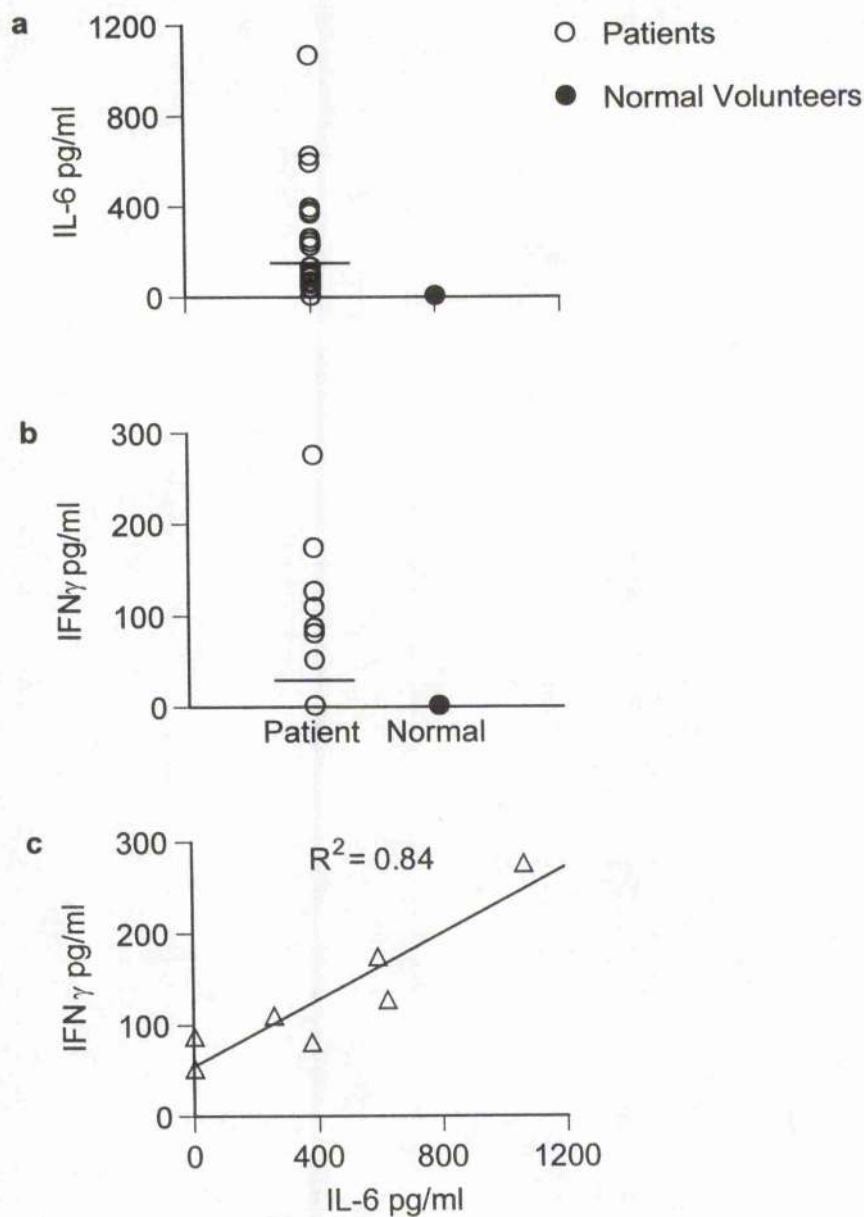


Figure 5.21 Serum from patients with septic arthritis

Serum was obtained from 33 septic patients and 11 normals. IFN γ and IL-6 were assayed by ELISA. Neither IFN γ nor IL-6 was detected in normal sera. (a) IL-6 was detected in the majority of septic patients, (b) 7 of 33 patients exhibited detectable IFN γ in the sera. (c) Within the IFN γ positive patient

population there was a strong correlation of IFN γ with levels of IL-6. *Pearson* correlation = 0.916, $p = 0.004$.

Table 5.4 Details of patients diagnosed with septic arthritis.

Disease	n	n (known age)	Average Age (years)	Min	Max	n positive for cytokine	
						IL-6	IFN γ
RA	11	5	68	43	86	7	1
OA	11	10	74	74	74	6	3
NonRA	3	1	80	67	92	2	2
ND	8	4	75	67	88	6	1
TOTAL	33	20	72.25	43	92	21	7

RA – rheumatoid arthritis, OA – osteoarthritis, ND – no diagnosis

Discussion

The pleiotropic effects of IL-18 render it a candidate for promoting not only host defence but also deleterious auto-toxicity during gram-positive infection. *In vitro*, IL-18 promoted bacterial killing; exogenous rhIL-18 enhanced normal human neutrophil bactericidal activity, and IL-18-deficient cells failed to kill bacteria as effectively as wild type controls. Discrepancies were manifest *in vivo*; in the presence of *a priori* elevation of IL-18, infection with *S. aureus* led to increased mortality, however, administration of IL-18 post infection reduced the severity of arthritis. This pattern reflects previous work in which IL-18-deficient animals exhibited reduced systemic sepsis but more severe arthritis following *S. aureus* infection.

Alterations in bacterial distribution following IL-18 administration either pre or post infection may have predisposed to the clinical changes observed. Administration of IL-18 induced IFN γ , which is associated with increased phagocytosis and bacterial killing, thus the early elevation of bacteria in kidney and blood were surprising. The cause of these changes in early bacterial distribution is not clear but may reflect impaired neutrophil function associated with sepsis or early haemodynamic change. *A priori* elevation of IL-18 caused immune deregulation, which may have resulted in 'immune paralysis,' and this in turn led to inefficient bacterial clearance. No changes were observed in bacterial loads of the footpads, however, the use of labeled bacteria would be appropriate to further investigate this observation.

To further investigate mechanisms underlying the clinical observations, the effects of IL-18 administration alone were examined. Given the haematogenous route of infection,

in addition to obtaining spleens, peripheral blood leukocyte responses were investigated. Furthermore, these are routinely studied in humans, but seldom explored in mice. Enhanced IFN γ , at both mRNA and protein level, was evident in PBL and spleen 2 h post IL-18 administration. Elevated TNF α mRNA was observed in PBL only. Identification of the IFN γ producing cells, hypothesised to be NK cells, by intracellular FACS analysis was unsuccessful; the reagents available detected IFN γ only in cells producing the cytokine in high concentrations, such as driven Th1 cells. *Ex vivo* responses to mitogen and superantigen were also enhanced by prior exposure to IL-18, but only in the peripheral compartment. An alternative explanation for the modified response to *S. aureus* following administration of IL-18 was speculated to lie in modification of Toll-like receptors. In peripheral blood leukocytes, IL-18 administration enhanced TLR2, and to a lesser extent TLR9, mRNA expression. This is consistent with studies in a model of murine fungal induced asthmatic disease, in which neutralisation of IL-18 was associated with reduced TLR2 expression (104). These changes in TLR expression await verification at protein level, but may contribute to subsequently altered immune responses. That the peripheral blood leukocytes showed several discrepant responses, compared to whole spleen, following administration of IL-18 may have important implications for studying *S. aureus* and other infections.

Analysis of cytokine profiles post infection demonstrated that IL-18 predominantly exaggerated IFN γ production following infection. It was assumed that TNF α deregulation may account for some of our observations. Administration of IL-18 alone induced elevated TNF α mRNA expression in PBL. However, although TNF α mRNA expression was elevated following infection, this was not further modulated by *a priori* addition of IL-18. This is surprising given the potential ability of IL-18 to drive TNF α

release directly from NK cells and macrophages (77). However, our methodology did not allow detection of membrane bound $\text{TNF}\alpha$, or soluble $\text{TNF}\alpha$ receptors. To determine if this enhanced $\text{IFN}\gamma$ was directly responsible for the increased mortality of pretreated mice, $\text{IFN}\gamma$ neutralising antibodies could be administered. Previous studies have shown that administration of $\text{IFN}\gamma$ before or after infection reduces mortality and increases septic arthritis. Neutralising anti- $\text{IFN}\gamma$ antibody has no effect on survival but reduces arthritis (226). $\text{IFN}\gamma$ receptor deficient mice exhibit greater arthritis and display altered mortality dependant on stage of disease (227). The discrepant results of these studies highlight the complex role of $\text{IFN}\gamma$ throughout the disease. As an upstream modulator of $\text{IFN}\gamma$, IL-18 is likely to be important in understanding these observations. Remarkably, post infection IL-18 treatment had no apparent effect on Th1 responses, as assessed by recall responses of spleen cells. Prior administration of IL-18 enhanced Th1 responses at 2 days post infection. No significant effects on Th1 responses were measured from day 5 onwards arising from early enhanced IL-18 expression. This is unsurprising since IL-12 and IL-18 signalling were intact in the present model and we did not anticipate that a single IL-18 bolus would significantly alter these processes.

These studies were extended to include a preliminary investigation of septic patients. Some of the patient cohort expressed $\text{IFN}\gamma$ protein in the sera. It is tempting to speculate that, as this correlated with IL-6 level, the appearance of $\text{IFN}\gamma$ in the serum may be linked to a severe, systemic inflammatory response. However, the cohorts are too small for conclusions to be drawn and preexisting and current levels of IL-18 in these patients are unknown.

Incidence of *staphylococcal* infection is likely to increase further with wider therapeutic use of immunosuppressive drugs and invasive investigative techniques. Moreover, new treatment strategies are essential in the face of emerging antibiotic resistant bacterial strains. Therefore, whether IL-18 plays a primarily protective or aggressive pathogenic role in gram-positive sepsis is important to elucidate. Elevated IL-18 prior to infection may be found in individuals with autoimmune disease. Furthermore, IL-18 has been employed as a vaccine adjuvant (228, 229), shows a protective role in tumour immunity (230), and therapeutically reverses established murine allergic asthma (144, 231). Conversely, neutralising IL-18 is an attractive therapeutic option for patients with IBD (138) and RA (78, 131). Our data suggest that redressing immune system imbalances, for example by targeting IL-18, may reduce pathogenic immunity to gram-positive infection. Moreover, these data caution the use of IL-18 as a vaccine adjuvant or to augment anti-tumour immunity.

Chapter 6 Neutrophil interactions with the adaptive immune response

Introduction

Neutrophils clearly respond to IL-18 *in vitro* and *in vivo*. Moreover, IL-18 promotes neutrophil bactericidal activity, and may alter complex immune responses to pathogens. IL-18 was originally defined with a role in the adaptive immune response, promoting Th1 development. A role for IL-18 in innate immunity has only subsequently emerged. Intricate links between innate and adaptive responses are becoming apparent. Neutrophils were traditionally described as terminally differentiated, short-lived phagocytes that mediate only innate immune responses, however, these cells are increasingly believed to influence the ultimate outcome of adaptive immunity. Emerging evidence suggests that neutrophils and T cells communicate. This could occur via direct mechanisms, for example antigen processing and co-stimulation; or via cytokine release, which may for example influence the polarisation of the immune response.

In vivo depletion of neutrophils potently alters adaptive immune responses. Depletion of neutrophils prior to *Leishmania* infection of BALB/c mice results in a switch to a protective Th1 response. This effect was IL-12 dependent, perhaps indicating that neutrophil lysis released pre-stored IL-12, sufficient to mediate a predominantly Th1 response (171). The role of neutrophil derived cytokines has been documented in rodent models of *Legionella pneumophila* (232), *Toxoplasma gondii* (233), *Mycobacterium tuberculosis* (234) and *Candida albicans* (235). Human neutrophils are also reported to express IL-12 (236).

There are limited studies of cross talk between neutrophils and T cells. Fixed PHA/PMA stimulated T cells stimulate neutrophils to release IL-1 β and TNF α , and prime for enhanced respiratory burst in response to fMLP. This cell contact is speculated to localise neutrophil activation to sites of activated T cells, thus limiting neutrophil mediated tissue damage (237). The hypothesis that neutrophils may actively modulate T cells has recently received further attention. Murine neutrophils process phagocytosed bacteria via an alternate MHC Class I antigen-processing pathway, which allows MHC Class I presentation of peptides derived from the bacteria. Such neutrophils may release processed peptide into the extracellular space and this peptide may then bind MHC-I on neighbouring cells (e.g. macrophages) for presentation to CD8 cells (175).

MHC class II expression can be induced on a number of cells including eosinophils, keratinocytes, synovial fibroblasts, tubular epithelial cells and others, by culturing in the presence of cytokines, notably IFN γ , GM-CSF, and IL-3. These cytokines also induce *in vitro* synthesis of MHC Class II by PMN derived from healthy donor. Of note is the variable response when cells of different donors are compared (176). Class II expression is observed on PMN of patients with active Wegener's Granulomatosis, and correlates with disease activity (238). Class II is upregulated in patients receiving GM-CSF (239), and enhanced expression has been reported on RA synovial fluid derived neutrophils (240). Interestingly, no elevation of Class II expression is found in patients with acute bacterial infections. In addition to MHC Class II expression, culturing normal human PMN with IFN γ induces B7.1 and B7.2 expression (179). As such, these neutrophils may present tetanus toxoid peptide antigen on MHC Class II to T cell clones and induce proliferation and cytokine production (241).

To estimate the relative contribution of neutrophil antigen presentation and cytokine production to an adaptive response, preliminary studies were undertaken, using both novel and more established experimental systems. These studies first addressed whether murine neutrophils present or process a Class II restricted antigen. Secondly, experiments were designed to determine whether soluble mediator release by neutrophils could influence their interactions with T cells. In particular, I was interested to explore a role for IL-18 in this process, given previous data implicating IL-18 in the progression from innate to adaptive immunity.

Results

6.1 Purification of murine neutrophils

Neutrophils were purified from thioglycollate induced peritoneal exudate cells (PEC), as there is a relative paucity of neutrophils in murine peripheral blood. To confirm the latter, whole blood obtained via cardiac puncture was stained with anti-GR1 antibody, lysed, and analysed by flow cytometry. Less than 20% of circulating leukocytes in the mouse were GR1 positive neutrophils (Fig. 6.1a and b). Published data suggest that neutrophils constitute 17.9% (range 6.7-37.2%) of circulating leukocytes. Intraperitoneal injection of 1.5 ml thioglycollate induced a rapid influx of cells into the peritoneal cavity. PEC were harvested 4 h post thioglycollate administration as it was previously shown that the number of neutrophils present peaks at this time, after which an increasing proportion of macrophages are recruited (242). Approximately 65% of thioglycollate induced PEC were identified as neutrophils on the basis of GR1 staining (Fig. 6.1c and d). PEC were further enhanced for neutrophils with a Ficoll gradient purification as previously described (242). Following purification, samples were routinely 85% GR1 positive (Fig. 6.2a and b). Importantly, samples contained few macrophages (<4%) as determined by F4/80 staining (Fig. 6.2c). Cytospins of neutrophil preparations were also prepared and stained (Section 2.8.2), allowing identification of neutrophils by their characteristic multilobed nuclei (Fig. 6.3). Percoll gradient purifications are reported to yield up to 97% pure neutrophil populations, however this method provided less consistent samples than the Ficoll purifications.

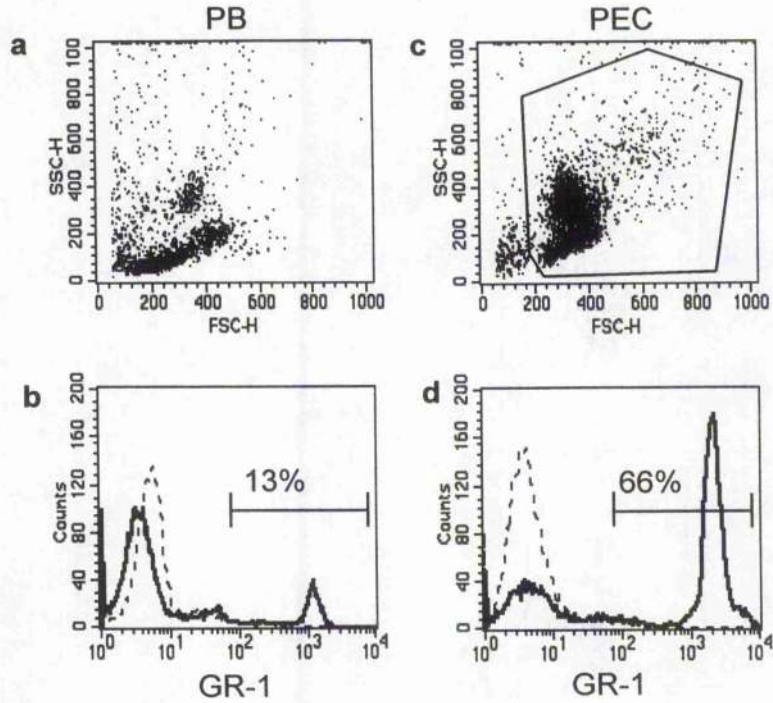


Figure 6.1 Neutrophil populations in murine peripheral blood and thioglycollate induced peritoneal exudate cells

Heparinised peripheral blood (PB) was collected from untreated BALB/c mice via cardiac puncture, and analysed by flow cytometry demonstrating (a) cell size and granularity and (b) proportion of GR-1 positive neutrophils as % total cells. Peritoneal exudate cells were obtained from BALB/c mice 4 h after receiving 1.5 ml thioglycollate i.p. (c) Cells size and granularity was assessed by flow cytometry, cells outside the gate stained positive for propidium iodide, and (d) cells within the PI negative gate, labelled with FITC anti-GR-1 antibody or an isotype control to identify neutrophils. Data are representative of 2 similar experiments, with 3 mice per group in each.

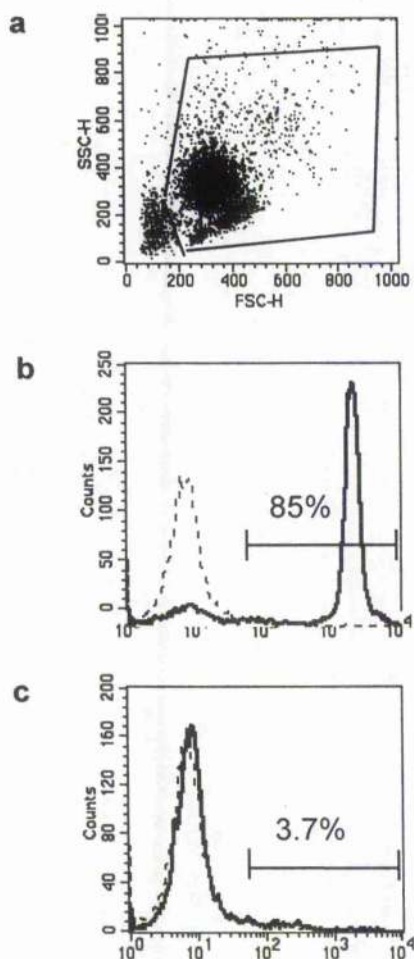


Figure 6.2 Purification of murine neutrophils

BALB/c mice received 1.5 ml i.p. 4 h prior to harvest of PEC by lavage. PEC from 2 mice were washed, resuspended in 8 ml PBS and separated by density centrifugation over Ficoll. Cell pellets were resuspended and analysed by flow cytometry. (a) Forward/side scatter indicating cells size and granularity; cells out with the gate were propidium iodide positive. (b) Neutrophils stained by GR-1 and (c) staining of macrophages by F4/80. Sample purity was routinely assessed and data are representative of > 6 similar experiments.

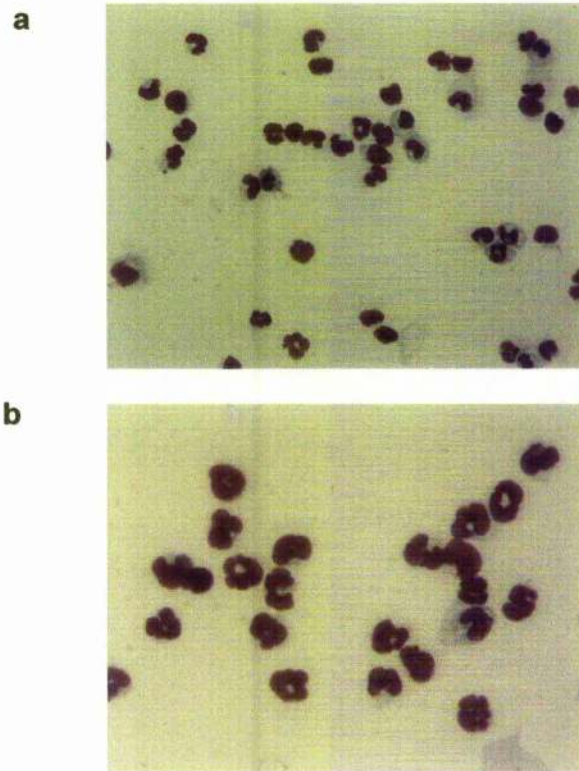


Figure 6.3 Cytospin preparations of murine neutrophils

Neutrophils were purified as in Fig. 6.2 and cytospins prepared. Cytospins were stained with DiffQuick™ and examined microscopically, (a) low power magnification, (b) high power magnification.

6.2 Murine neutrophils presented peptide antigen

To investigate whether murine PEC derived neutrophils were capable of antigen presentation on MHC Class II, a system for detecting antigen presentation was sought. The murine T cell hybridoma, DO11.10, expresses a T cell receptor (TcR) that recognises the ovalbumin peptide, OVA₃₂₃₋₃₃₉ in the context of the MHC class II, I-A^d.

The DO11.10 hybridoma was transfected with a plasmid allowing expression of green fluorescent protein (GFP) under the NFAT promoter. When the transfected hybridoma, DO11.10-GFP, recognises OVA₃₂₃₋₃₃₉ presented by I-A^d, GFP expression is induced within 4-5 h, and declines after 18 h. Expression of GFP is co-stimulation independent, providing a quantitative measure of antigen presentation via Class II. The DO11.10-GFP cells were a kind gift of David Underhill, Dept. of Immunology, University of Washington, Seattle, Washington. This system is schematically outlined in Fig.6.4.

DO11.10-GFP cells were grown in medium containing a 0.5 mg/ml geneticin. This enhanced the proportion of plasmid containing cells prior to the start of the experiments. 12 h prior to use, DO11.10-GFP cells were washed and grown in complete medium without antibiotic, reducing carry-over toxicity to other cells. Initially, antigen presentation to the DO11.10-GFP cells was investigated by flow cytometry. Co-cultures were established in which neutrophils or macrophages were incubated with 10 µg/ml OVA₃₂₃₋₃₃₉ peptide and DO11.10-GFP hybridoma cells, in the ratio indicated in the figures. Cells were cultured for 14 h, washed, and then analysed by flow cytometry. The hybridoma does not respond to peptide alone (Fig. 6.5, 'background'), and plate bound anti-CD3 provided a positive control in these experiments. Neutrophils or macrophages alone had no effect on GFP expression by DO11.10-GFP. However, neutrophils or macrophages in the presence of OVA peptide stimulated significant GFP expression when cells were cultured at a 1:1 ratio. A dilution of macrophages was performed to provide some indication of the contribution of contaminating macrophages to the results observed with neutrophil populations. This demonstrated that at a ratio of 1 macrophage to 10 DO11.10-GFP cells, there was significantly less expression of GFP protein compared to that induced by neutrophils at a 1:1 ratio (Fig. 6.5). At a ratio of 1:100,

there was no consistent GFP expression detected (data not shown). As there were <5% F4/80 positive macrophages contaminating the neutrophil samples (Fig. 6.2c), these titration experiments indicated that only a small proportion of the T cell hybridoma activation observed in the 'neutrophil presentation' assays could be attributable to macrophage contamination.

To visualise the cell-cell interactions, similar co-cultures were established for 4-6 h and then viewed microscopically. As the microscopy was less sensitive to the green fluorescent protein than flow cytometry, dendritic cells were used as a positive control to provide optimum *in vitro* antigen presentation. Co-culture of DO11.10-GFP with DC+OVA₃₂₃₋₃₃₉ resulted in a distinct clustering of GFP positive cells, presumably around the APC (Fig. 6.6a). Of interest, discrete GFP positive cells were also observed when purified neutrophils were co-cultured with DO11.10-GFP (Fig. 6.6b). Cells in co-cultures were identified as neutrophils by biotinylated anti-GR1 antibodies and detected with streptavidin Texas red. Control cells were stained with biotin labelled isotype control antibody and secondary detection reagents. These experiments demonstrated that neutrophils could be specifically stained for GR-1 and, in some fields, were seen in contact with individual green cells (Fig. 6.6b). In further control experiments, no GFP positive cells were observed in co-cultures in the absence of peptide (data not shown).

Preliminary studies were undertaken to follow these neutrophil-DO11.10-GFP cell interactions in real time, which showed promising results but time constraints prohibited fully exploring this avenue. Nevertheless, these experiments, using a novel cellular technology, strongly support the notion that neutrophils can indeed present peptide to T cells in a Class II restricted manner.

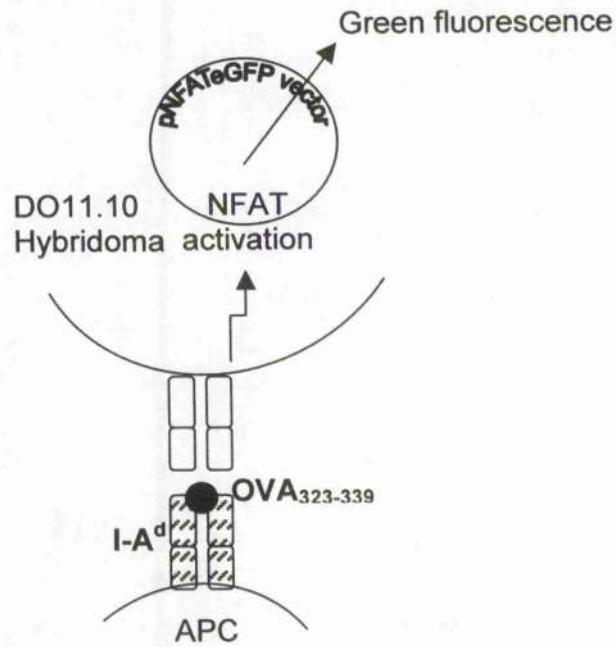


Figure 6.4 T cell hybridoma, DO11.10, transfected with pNFATeGFP

A T cell hybridoma, DO11.10, was stably transfected with a construct in which green fluorescent protein (GFP) expression is controlled by a NFAT responsive promoter. Thus, upon productive interaction with an APC the T cell becomes fluorescent. This stably transfected cells line was a kind gift of David Underhill, Dept. of Immunology, University of Washington, Seattle.

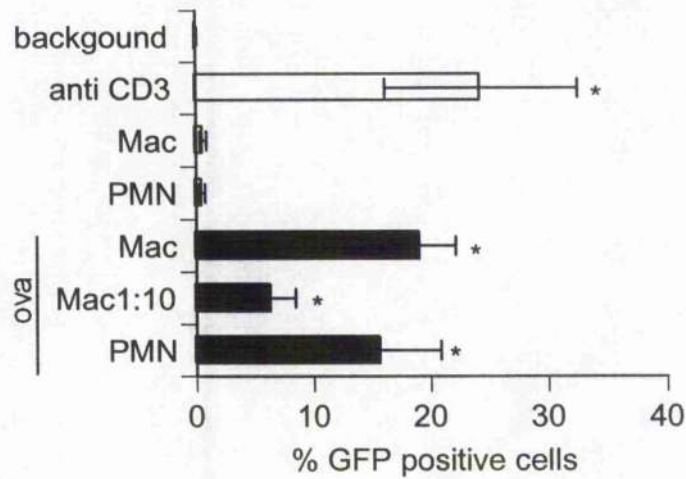


Figure 6.5 GFP expression by the DO11.10-GFP hybridoma following co-culture with APCs

Purified murine neutrophils (PMN) or macrophages (Mac) were cultured in the presence of 10 $\mu\text{g/ml}$ OVA₃₂₃₋₃₃₉ peptide with DO11.10-GFP cells for 14 h. Cells were washed and GFP expression was assessed by flow cytometry. Background is a measure of GFP expression of DO11.10-GFP cells cultured with ova peptide alone; anti-CD3 is a positive control. Data are mean \pm SEM of 3 experiments. In each experiment, neutrophils were purified from thioglycollate induced PEC pooled from 5 BALB/c mice. Macrophages were obtained from 2 similarly treated BALB/c mice. * $p < 0.05$ vs. background by Mann Whitney U test.

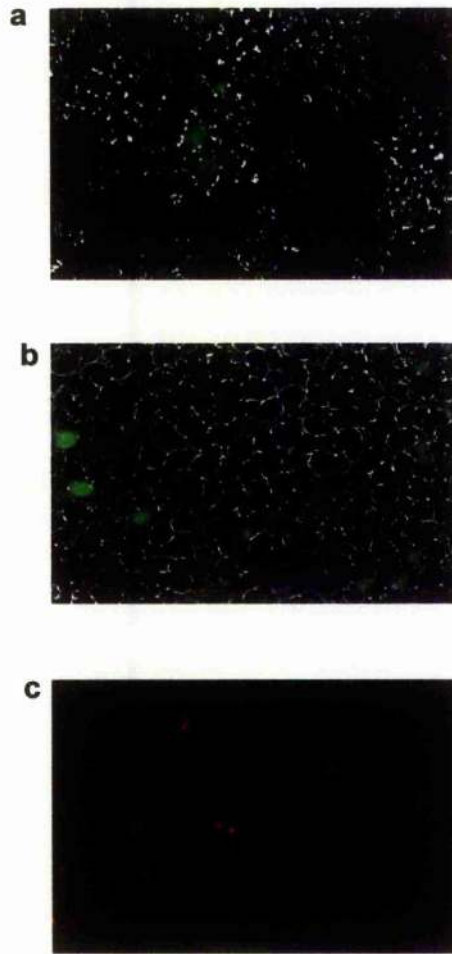


Figure 6.6 Analysis of DO11.10-GFP by fluorescence microscopy

DO11.10-GFP cells were co-cultured with (a) dendritic cells and (b) purified neutrophils in the presence of OVA₃₂₃₋₃₃₉ for 12 h then examined microscopically. (c) Neutrophils were stained, in the presence of OVA, with GR-1-Texas red, and co-cultured with DO11.10-GFP for 6 h. No GFP expression was observed in the absence of peptide. The GR-1 isotype control antibody staining of neutrophils provided no red signal (data not shown). (a) Representative of a single experiment, (b) of 3 and (c) of 2 similar experiments.

6.3 Neutrophil interactions with lymph node derived T cells

To investigate a more physiological system than T cell hybridomas, neutrophils were co-cultured with primary T cells. Lymph nodes were obtained from DO11.10 transgenic BALB/c mice. These mice express the OVA specific TcR on a proportion of their T cells, which is recognised by a clonotypic antibody, KJ 1.26. Fig. 6.7a shows a representative plot of KJ 1.26 positive cells derived from lymph nodes of DO11.10 mice. Single cell suspensions of lymph nodes were adhered to plastic for 3 h to remove the majority of adherent antigen presenting cells, after which the population was routinely 70% CD3 positive (data not shown). The presence of endogenous APCs in the lymph node facilitated a strong response to OVA peptide alone (Fig. 6.8a). Therefore, neutrophils and macrophages were pulsed with peptide for 2 h then washed 3 times to remove unbound peptide. In these experiments, Con A was used as a positive control mediating antigen independent stimulation. Cells were co-cultured for 72 h at the ratios indicated in the figures. Proliferation was assessed by [³H] thymidine incorporation for the final 18 h of culture. Cytokine release into the supernatants was assessed by ELISA.

Neutrophils pulsed with OVA induced proliferation (Fig. 6.8a), IL-2 (Fig. 6.8b) and IFN γ (Fig. 6.8c) production. Low levels of IL-5 were detected in some experiments (data not shown). The neutrophil induced responses were less than those evoked by macrophages at the same cell ratio. A titration of macrophages was performed to give a more physiologically relevant cell ratio and an estimate of the contribution of contaminating cells. This result suggested that not all the observed neutrophil induced activity could be attributable to contaminating macrophages.

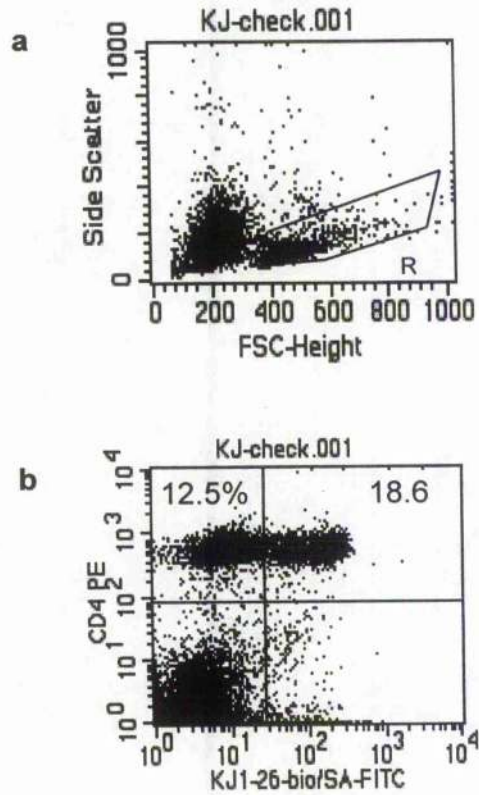


Figure 6.7 Lymph node derived DO11.10 transgenic T cells

Single cell suspensions were prepared from lymph nodes of DO11.10 BALB/c mice and stained for analysis by flow cytometry. The lymphocyte population was gated according to forward/side scatter (a) and this was analysed on the basis of CD4-PE expression and KJ 1.26-FITC staining (b), identifying the OVA-specific TcR on CD4 cells. Data shown are representative of > 5 similar experiments.

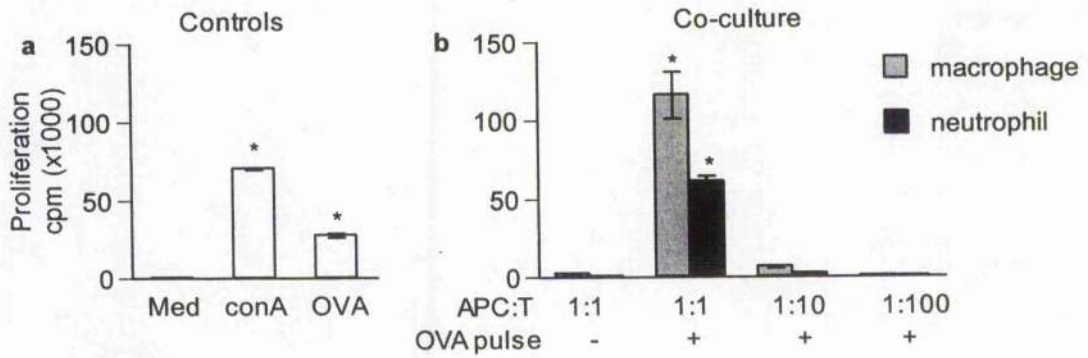


Figure 6.8 *In vitro* proliferation of DO11.10 lymph node cells

Single cell suspensions of lymph nodes were obtained from a DO11.10 transgenic mouse and cultured for 72 h and proliferation was assessed by [^3H] thymidine incorporation over the final 18 h of culture. (a) Control experiments in which DO11.10 lymph node cells alone were cultured with the stimuli indicated. (b) DO11.10 lymph nodes were cultured with either macrophages or neutrophils derived from thioglycollate induced PEC. The ratio of APC to T cell in the culture, and whether the APC was pulsed with OVA prior to addition to the co-culture, is indicated on the x-axis. Data are mean \pm SEM of triplicate cultures. * $p < 0.05$ vs. medium by Student's t test, representative of 3 similar experiments.

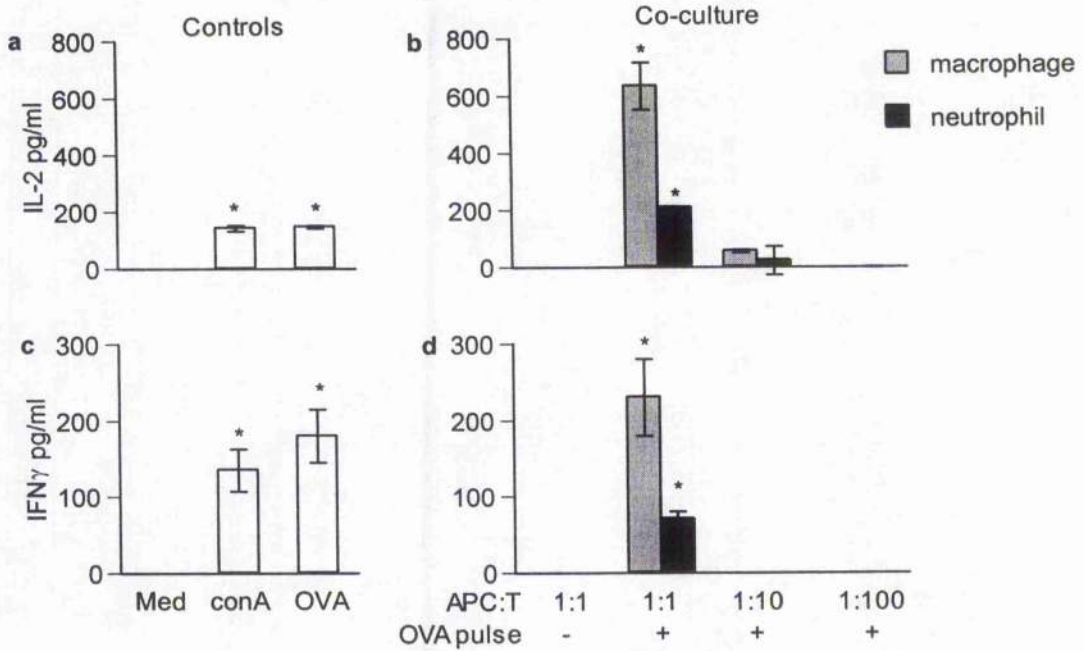


Figure 6.9 *In vitro* cytokine production by DO11.10 lymph node cells

Single cell suspensions of lymph nodes were obtained from a DO11.10 transgenic mouse and cultured for 72 h, after which IL-2 and IFN- γ levels in the supernatant were assessed by ELISA. (a) and (c) Control experiments in which DO11.10 lymph node cells alone were cultured with the stimuli indicated. (b) and (d) DO11.10 lymph nodes were cultured with either macrophages or neutrophils derived from thioglycollate induced PEC. The ratio of APC to T cell in the culture, and whether the APC was pulsed with OVA prior to addition to the co-culture, is indicated on the x-axis. Data are mean \pm SEM of triplicate cultures. * $p < 0.05$ vs. medium by Student's t test, representative of 3 similar experiments.

6.4 Effect of IL-18 on neutrophil T cell interactions

Neutrophil enzymes process IL-18. Furthermore, APCs (e.g. macrophages and DCs) produce IL-18; hence I next investigated whether soluble factors, such as IL-18, had any impact on the responses observed in the previous experiments (Fig. 6.8 and 6.9). Neutrophils were obtained from IL-18-deficient BALB/c mice. Co-cultures of APCs and DO11.10 lymph node cells were performed as in section 6.3. Again, neutrophils pulsed with OVA stimulated proliferation (Fig. 6.9a) and IFN γ production (Fig. 6.9b) by DO11.10 derived lymph node cultures. Neutrophils derived from IL-18 wild type or IL-18-deficient mice behaved similarly.

6.5 Cell contact dependence of neutrophil T cell interactions

To determine the relative contribution of cell contact dependent mechanisms and soluble factors in neutrophil-T cell interactions, transwells were placed between the cell populations. These consist of 0.2 μ m membranes, allowing movement of soluble proteins but not cells. The responding OVA specific T cells were not a 100% pure T cell population and contained some APCs, demonstrated by the lymph node response to OVA alone. Thus, the transwells permitted preliminary investigation of whether lymph node derived APCs were presenting soluble proteins derived from dead or degraded neutrophils. Bone marrow derived dendritic cells (DC) were used as a positive control. Free OVA peptide or Con A crossed the transwell membrane and stimulated cell proliferation and cytokine production (Fig. 6.11). When separated from the lymph node cells by the transwell, pulsed cells, either neutrophils or DCs did not release sufficient OVA to cross the membrane and stimulate a response from the OVA specific T cells

below (Fig. 6.11). These experiments demonstrated that cell contact was primarily responsible for the proliferation and cytokine production observed in the previous experiments (Fig. 6.8 and 6.9).

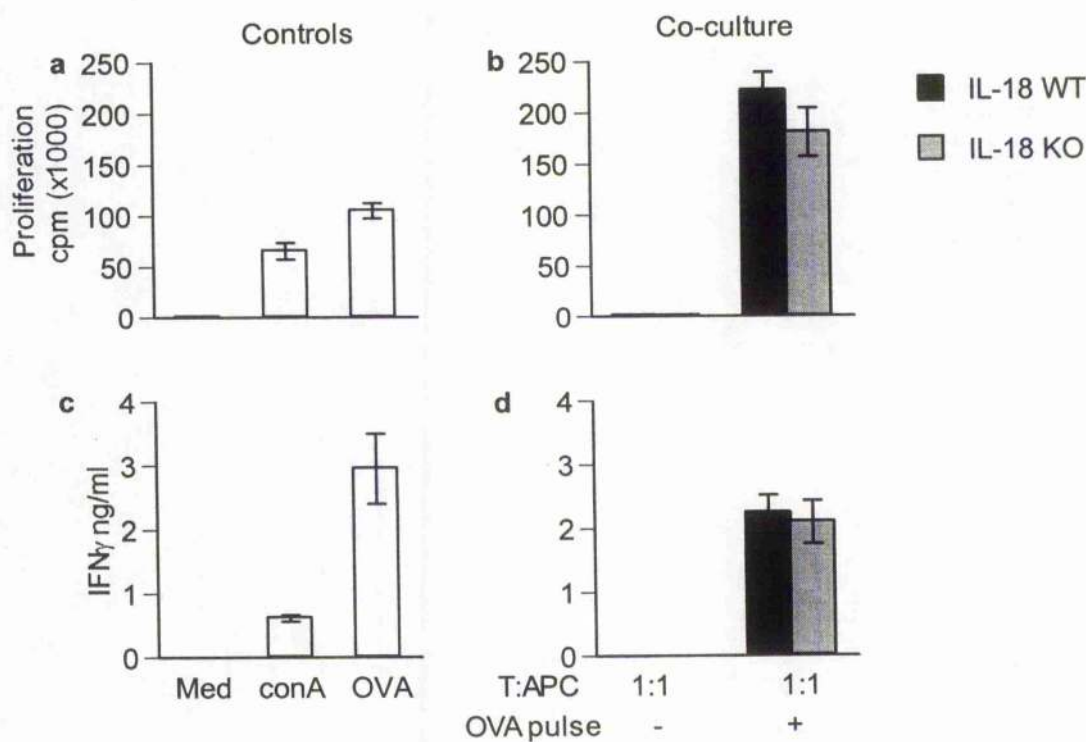


Figure 6.10 APCs from IL-18-deficient mice

Experiments were performed as in Fig. 6.8, except neutrophils were derived from IL-18-deficient mice (-/-) or wild type littermate controls (+/+). (a) Proliferation was assessed by [3 H] thymidine incorporation and (b) IFN γ release was assessed by ELISA. Thioglycollate induced PEC were pooled from 4 mice per group per experiment. Data are mean \pm SEM of triplicate cultures.

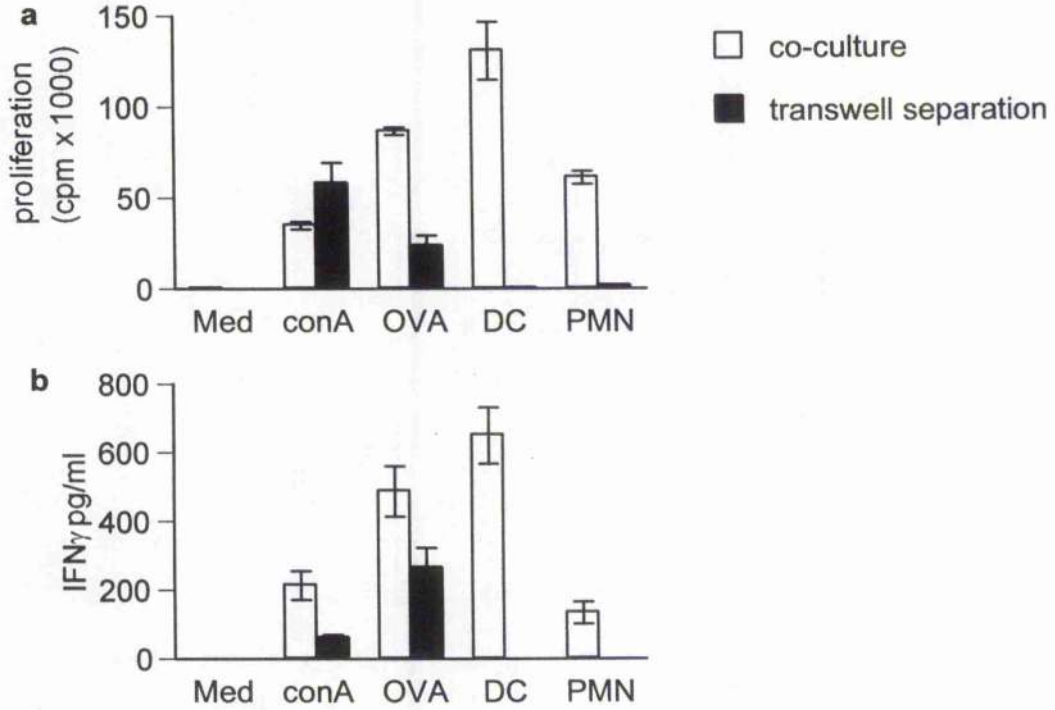


Figure 6.11 Antigen specific proliferation and cytokine production was cell contact dependent

Single cell suspensions derived from lymph nodes of DO11.10 mice, were stimulated with Con A, OVA peptide, or APCs (DCs or neutrophils), which had been pulsed with OVA peptide. In parallel cultures, the stimulus was separated from the responding T cells by a transwell membrane. The stimulus was placed in the upper compartment, and proliferation (a) and IFN γ production (b) of the OVA specific T cells in the lower chamber was assessed. T cells did not respond when cultured with APCs alone (data not shown). Similarly, culturing T cells in the upper chambers resulted in no measurable response in the lower chambers. Data are mean \pm SEM of triplicate cultures and representative of 2 similar experiments.

Discussion

Data presented here demonstrate a novel system for investigating whether neutrophils present antigen. The use of the DO11.10-GFP hybridoma cells allowed both preliminary quantitative analysis of antigen presentation and visual evaluation of cell/cell interactions. These data suggest that neutrophils are indeed capable of presenting antigen on Class II MHC.

The DO11.10-GFP hybridoma cells respond in a Class II restricted manner, and the data presented demonstrate that it is highly likely that neutrophils, rather than contaminating cells, were mediating this response. Furthermore, the interactions of neutrophils with lymph node derived cells were clearly cell contact dependent. Future use of MHC class II neutralising antibodies should confirm the specificity of these interactions. Additionally, the C4H3 antibody identifies hen egg lysosyme peptide complexed to I-A^k, and as such could be used to further address the question of neutrophil Class II expression and peptide presentation. The expression of Class II by neutrophils seems critically dependent on activation status. For example, SF derived neutrophils require *ex vivo* culture, but not stimulation, before MHC Class II can be detected on their surface (240). Preliminary investigations of MHC-II expression on murine neutrophils were inconclusive.

The impact of neutrophil derived cytokines on adaptive immune responses was of interest. Neutrophils both secrete and process cytokines, including IL-18. Neutrophil elastase, cathepsin G, and proteinase 3 process IL-18; both from pro to mature forms and also to generate other IL-18 fragments, the functions of which remain unknown. The relative importance of IL-18 on T cell mediated responses suggested that this

neutrophil processing was worthy of further investigation. However, studies using IL-18-deficient mice demonstrated no role for endogenous IL-18 in neutrophil interactions with T cells in this system. Murine neutrophils express IL-12, which may additionally influence Th1 type responses. This awaits further investigation and availability of IL-12/18 double knock out mice. Use of the transwell system confirmed the lack of involvement of soluble factors in this system, although the proliferation and cytokine production assays may not be sufficiently sensitive to detect fine-tuning of T cell responses. Analysis of cell surface markers or intracellular signalling events may better characterise subtle changes.

Data described here demonstrate neutrophil presentation of a peptide antigen. Using the DO11.10-GFP technology, preliminary investigations into antigen processing capacity of neutrophils were performed, using whole ovalbumin compared to OVA peptide. These studies were inconclusive although the neutrophils did not appear to process the ovalbumin effectively, demonstrated by a lack of response by DO11.10-GFP T cells (data not shown). An MHC Class I processing pathway within neutrophils was elegantly defined with *E. coli* transfected to express a fusion protein containing a MHC Class I restricted epitope of OVA. This demonstrated that neutrophils are capable of non-classical antigen processing for presentation on MHC Class I (175). However, there is no current evidence of any Class II processing pathway. These studies could be addressed by use of bacteria transfected with the MHC Class II restricted epitope, OVA₃₂₃₋₃₃₉. In addition to Class II, there is an array of molecules, associated with T cell activation, reportedly expressed on neutrophils (e.g. CD80 and CD86). It will be important to elucidate if this reflects variable responsiveness of different sub-

populations of neutrophils, or different susceptibility of neutrophils to activation dependent on maturation status.

I investigated neutrophil interactions with primary, naïve T cells, from a population containing an artificially high proportion of antigen specific cells. Physiologically, the location of such neutrophil-T cell interactions is likely to be restricted to sites of chronic or established inflammation. Therefore, investigation of the interaction of neutrophils with memory type T cells may be more relevant. However, these antigen experienced cells are likely have acquired a cytokine producing phenotype, which neutrophil derived factors are less likely to influence. The aberrant expression of MHC on neutrophils may be of particular significance in chronic inflammatory diseases such as RA. Due to the neutrophils' unique proteolytic activities, this may represent a mechanism of presentation of novel or cryptic antigens.

There are discrepancies between human and murine neutrophils especially in terms of documented MHC Class II expression. Given that only 20% of circulating leukocytes in the mouse are neutrophils, compared to 70% in humans, this also raises questions as to whether murine and human neutrophils perform parallel functions as is assumed.

Chapter 7 General Discussion

Cytokine networks operating in host defence and a variety of disease states provide a highly evolved, complex communication system for the immune system. The character of these messages critically influences the outcome of insults to host integrity. Understanding the role played by each messenger, both individually and in context of a cytokine network, provides vital insight into protective and pathogenic immune responses, in turn providing opportunities for therapeutic intervention.

The most important observation in this study is that documenting IL-18 induced neutrophil activation. IL-18 enhanced neutrophil adhesion molecule expression, cytokine release, degranulation, respiratory burst and arachidonic acid metabolism. These observations support a proinflammatory role for IL-18 in the context of neutrophil activation. This work has been extended and confirmed by other investigators (218, 219). Interestingly, the latter studies have shown that IL-18 stimulates IL-1 β and sIL-1RII. Thus, like LPS, IL-18 initiates both pro- and anti-inflammatory activities, the balance of which may determine the net effect of IL-18. This could alter in distinct tissues, or as immune response kinetics change. Dinarello *et al* have shown that the effects of IL-18 on neutrophils are mediated largely through p38 MAP kinase activation (218), which is of interest given the reported predominance of IRAK/NF κ B signalling pathways in other cell types. Clarifying whether IL-18 stimulates a secondary wave of cytokines, and how important these are for each neutrophil response, would allow more accurate charting of the position of IL-18 within the cytokine network.

In vivo relevance of IL-18 neutrophil activation was demonstrated by neutralising IL-18 in a model of acute inflammation. The subsequent reduction in tissue myeloperoxidase,

and histological evidence of reduced neutrophil accumulation implicates IL-18 as a modulator of *in vivo* neutrophil function. Moreover, addition of exogenous IL-18 prior to infection, promoted an exaggerated early immune response to *S. aureus*, with devastating consequences in this model. A system of investigating neutrophil – T cell interactions was established. However, in preliminary studies, no role for neutrophil derived IL-18 was observed in mediating neutrophil interactions with T cells. Nevertheless, IL-18 effects in DC function have been clearly documented and likely constitute the primary mode of action of IL-18 in T cell activation (74, 76).

IL-18 represents part of a complex intracellular communication network. Previously, IL-18 was described as a T cell differentiation factor, working primarily in synergy with IL-12, to regulate the phenotype of subsequent adaptive responses. However, data in the present studies advocate an independent inflammatory role for IL-18 early in the innate response. Figure 7.1 schematically outlines how the role of hypothetical cytokine may be considered, emphasising that this may alter throughout an immune response.

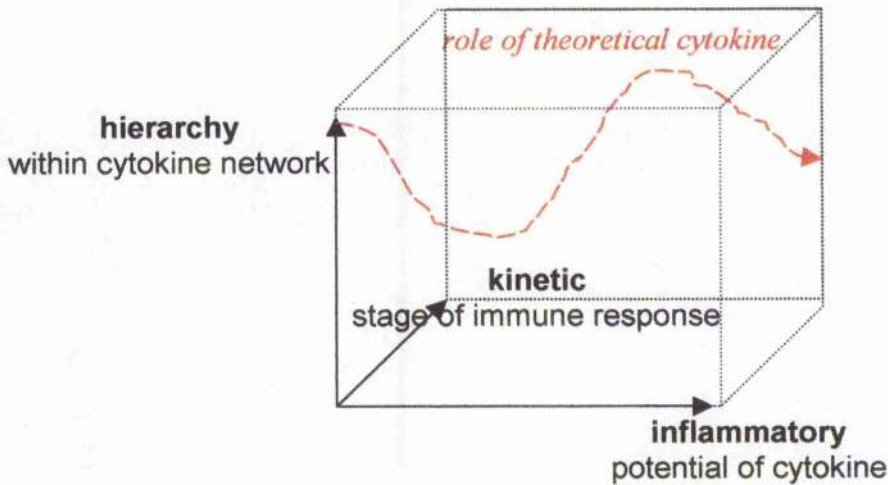


Figure 7.1 A hypothetical cytokine may mediate discrete functions as an immune response develops (adapted from (243))

The above figure illustrates, in 3 dimensions, how the qualitative and quantitative contribution of a cytokine may vary during different stages of an immune response.

The hierarchical relationship between IL-18 and TNF α remains intriguing. In the acute inflammatory model described in Chapter 4, neutralising IL-18 abrogated TNF α mRNA expression. Furthermore, addition of IL-18BP in a murine model of colitis reduces expression of numerous inflammatory genes, including TNF α (138). However, RA patients receiving anti-TNF α therapy exhibit persistent decrease in serum IL-18 levels (244). Thus, either in chronic disease, such as arthritis, TNF α regulates IL-18, or these two cytokines cooperatively promote inflammation. Such observations may be interpreted as rational for combined cytokine targeting therapy, or are an indication of deficiencies in evidence provided by animal models.

Further evidence suggesting that IL-18 operates very early in the immune response, and may form part of the first response to insult, is documented below.

- *Widespread expression of IL-18 mRNA and pro-IL-18 protein*

IL-18 has been identified in a diverse array of cells and tissues, from kidney mesangial cells to platelets (JA Gracie, personal communication). Thus this cytokine seems ideally positioned for rapid mobilisation.

- *Constitutively high levels IL-18 mRNA expression*

Very few, if any, investigators demonstrate up regulation of IL-18 mRNA in response to proinflammatory stimulation. In the *S. aureus* infection model (Chapter 5), IL-18 mRNA expression was unaltered, in any group, 6 h post infection; which contrasted sharply with TNF α , IFN γ or IL-12 p40 mRNA expression, all of which were markedly increased following infection. Thus, IL-18 mRNA appears constitutively, highly expressed, a further provision for rapid deployment.

- *IL-18 homologues*

Structurally, IL-18 is a member of the IL-1 family. Matzinger classifies IL-1 β as “an endogenous, non-foreign alarm signal” to which APCs respond by upregulating co-stimulatory molecules and initiating immune response. Furthermore, the IL-18 receptor chains contain Toll/IL-1 Receptor domains (TIRS), which links IL-18 to the Toll-like receptor (TLR) family, which are conserved through evolution from *Drosophila*. Similarly, IL-18R and TLRs share common molecules, such as MyD88 and IRAK, within their signalling pathways.

The TLR family comprises 10 currently defined receptors (TLR1-10), which recognise conserved pathogen motifs, and host molecules, such as heat shock proteins (reviewed (245, 246)). These receptors are fundamental to immune response initiation and may be considered either as conveyors of 'danger signals,' or for recognition of 'infectious, non-self.' According to either hypothesis, the TLR family constitute essential first line host defence. However, whether the host evolved to recognise and respond to pathogens via TLRs, or pathogens evolved to bind TLRs, remains controversial. Accordingly, the homology between the IL-1 receptor family and TLRs raises the possibility that this cytokine receptor family is an evolutionary progression from the TLR family. In this context, IL-1 family members, such as IL-18, may be considered to function at one level more like heat shock proteins, i.e. as endogenous alarm signals. Perhaps, pathogens are currently evolving to further 'hijack' this cytokine receptor system, which may already occur, unbeknown to investigators.

The precise role of neutrophils in early immune system activation is as yet poorly understood. Although the first cells to migrate to sites of insult, neutrophils *respond* to insults; sounding the 'alarm bell' is the task of the tissues and cells within them, such as resident dendritic cells. As the earliest cells to enter tissues, neutrophil responses are likely to be crucial to the subsequent immune response. Various data describe a role for TLRs in neutrophil function (247, 248). Interplay between IL-18 and TLRs is alluded to in this and other studies; IL-18 modulates TLR2 expression (104). The extent to which IL-18 may regulate other TLRs awaits examination. Similarly, whether IL-18 primes for enhanced neutrophil responses to TLR ligands awaits investigation.

The addition of exogenous IL-18 prior to onset of *S. aureus* infection resulted in increased mortality. This scenario mirrors human disease, such as RA, in which IL-18 is elevated *a priori*, which is similarly associated with increased mortality. Thus supplementing with IL-18 to promote protective immunity, as has been proposed for vaccine adjuvants, should be undertaken with caution. The corollary of these observations is that neutralising IL-18 in inflammatory disease may rebalance the immune system and under certain circumstances actually promote protective rather than pathogenic reactions. These studies, performed on genetically similar animals in a constant environment, should be cautiously interpreted. In general, targeting IL-18 is an attractive therapeutic goal for the assorted diseases in which elevated expression of IL-18 has been documented. Indeed, neutralising IL-18 in animal models of arthritis and IBD provide promising results. Predicting the long-term outcome of disturbing the cytokine networks in human populations is difficult. Dose, timing, route of administration, and choice of inhibitor (e.g. recombinant soluble receptors or humanised antibodies) will likely prove important.

Whether strong disease association polymorphisms exist in IL-18, IL-18R, or IL-18BP loci, as described for IL-1 family, has not been determined. Allelic differences in IL-18 mediate enhanced IL-18 expression, only apparent following mitogenic stimulation. No differences in allele frequency were observed when a cohort of MS patients was compared with a normal population (13). Preliminary data (McInnes, Gracie, unpublished) suggest the 'low production' phenotype predominates in RA patients. Such apparent contradiction may be resolved by hypothesising that a deficiency in IL-18 production may predispose to infection, which in turn could promote autoimmunity in a genetically otherwise predisposed individual. Once inflammation associated with

autoimmune disease is established, there is an aberrant over expression of IL-18. However, further studies of IL-18 polymorphisms and their functional implications are awaited.

Conclusions

Work carried out in this project provided the following novel information which advanced our understanding of the biology and potential therapeutic role of IL-18.

1. IL-18 promoted human neutrophil activation *in vitro*.
2. IL-18 induced *in vivo* neutrophil recruitment and neutralising IL-18 abrogated acute, neutrophil led, inflammation.
3. Addition of exogenous IL-18 promoted *in vitro* bactericidal activity of neutrophils. However, *in vivo*, IL-18 promoted lethal sepsis rather than bacterial clearance. Thus, IL-18 is a two edged sword in the immune system.
4. Murine neutrophils were capable of Class II restricted presentation of peptide to specific T cells. This was cell contact dependent.

Future Work

Reference has been made in the text to further investigations at specific points of interest. Key areas include the following:

- IL-18 expression is confirmed in human neutrophils. Neutrophils are capable of protein processing and generate discrete fragments of IL-18. The functional implications of this are under investigation.
- The hierarchy of IL-18 with respect to IFN γ and TNF α is interesting. For example, neutralising IL-18 abrogated TNF α mRNA expression in a model of acute inflammation. However, in a model of *S. aureus* infection, the predominant response was IFN γ mediated. Studies including neutralising antibodies would adequately address these issues.
- Further exploration of IL-18, IL-18R, or IL-18BP polymorphisms and their functional consequences.

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