

**IL-6/sIL-6R in Rheumatoid Arthritis:  
Interplay with TNF $\alpha$  and implications  
in anti-TNF $\alpha$  therapy**

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A thesis submitted to Cardiff University  
for the Degree of Doctor of Philosophy

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## **ABSTRACT**

Recent advances in the therapeutic management of rheumatoid arthritis have identified that selective targeting of inflammatory cytokines represents a valid approach to the treatment of rheumatoid arthritis (RA). Although blockade of the inflammatory response at its inception by anti-TNF $\alpha$  agents has shown considerable clinical promise, this approach is not without its drawbacks. Consequently, identification of novel therapeutic strategies is essential. In recent clinical trials, favourable results were found with modalities that block interleukin-6 signalling. However, it is unclear whether blockade of IL-6 bioactivity offers a true advantage over anti-TNF $\alpha$  therapies, and raises the possibility of combination therapies for selected patient cohorts. The ultimate objective of this thesis was to provide proof of concept that combination TNF $\alpha$  and IL-6 blockade may offer true advantages in selected RA patient cohorts.

To elicit a response from a target cell, IL-6 must first form a complex with its receptor. It was found that neutrophils and macrophages within the RA joint had lower levels of IL-6R expression than neutrophils and macrophages obtained from matched blood samples in the same patients. Stimulation of neutrophils with TNF $\alpha$  led to rapid shedding of cell surface IL-6R. TNF $\alpha$  and IL-6 trans-signalling interacted to modulate chemokine production (particularly CCL2 macrophage chemo-attractant) and adhesion molecule expression (ICAM-1) on RA synovial fibroblasts. This could lead to significant effects on leucocyte recruitment in the RA joint.

A murine model of arthritis (collagen induced arthritis) was used to investigate the efficacy of combination therapy with sgp130:Fc and etanercept on clinical and pathological outcomes of disease. Combination therapy for mCIA resulted in reduction in clinical disease severity. Macrophage recruitment was reduced and all parameters of histological damage. It appears that there is now proof of concept for the theory that combination therapy with etanercept and sgp130:FC may be effective in selected patients with rheumatoid arthritis.

## **ABBREVIATIONS**

Ab	Antibody
ACR	American College of Rheumatology
ACR 50	American College of Rheumatology 50% response
ADAM	A disintegrin and metalloprotease
AIA	Antigen induced arthritis
BSA	Bovine serum albumin
BSR	British Society for Rheumatology
CIA	Collagen induced arthritis
COMP	Cartilage oligomeric protein
CRP	C reactive protein
DAS	Disease activity score
dH <sub>2</sub> O	Distilled water
DMARD	Disease modifying anti-rheumatic drug
DMEM	Dulbecco's modified eagle's medium
DS	Differentially spliced
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunoabsorbent assay
EMSA	Electrophoretic mobility shift assay
ESR	Erythrocyte sedimentation rate
FITC	Fluorescein isothiocyanate
FCS	Fetal calf serum
gp130	glycoprotein 130
H&E	Haematoxylin and eosin
HLA	Human leucocyte antigen
ICAM	Intracellular adhesion molecule
IFA	Incomplete Freund's adjuvant

IP	Intra-peritoneal
IL-	Interleukin
IL-6	Interleukin 6
IL-6R	Interleukin 6 receptor
MMP	Matrix metalloproteinase
NBFS	Neutral buffered formalin saline
NFκB	Nuclear factor-kappa B
NS	Not significant
OA	Osteoarthritis
OA-SF	Osteoarthritis synovial fibroblasts
PBS	Phosphate buffered saline
PC	Proteolytically cleaved
PE	Phycoerythrin
PMN	Polymorphonuclear leucocyte
RA	Rheumatoid arthritis
RA-SF	Rheumatoid arthritis synovial fibroblast
SD	Standard deviation
SEM	Standard error of mean
SF	Synovial fluid
sIL-6R	Soluble Interleukin 6 receptor
STAT	Signal transducer and activator of transcription
TBS	Tris buffered saline
TNFα	tumour necrosis factor alpha
TACE	Tumour necrosis factor cleavage enzyme
TMB	Tetramethylbenzidine
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor

## CHEMOKINE NOMENCLATURE

<b>Current nomenclature</b>	<b>Previous name</b>
<b>CXCL8</b>	IL-8 (Interleukin 8)
<b>CCL5</b>	Rantes (Regulated on activation normal T Cell expressed and secreted)
<b>CCL2</b>	MCP- 1 (Macrophage chemo-attractant protein)

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# 1 INTRODUCTION

## 1.1 Background

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease affecting approximately 1% of the population (Silman 1998). It is characterised by widespread symmetrical arthritis together with tendonitis, serositis and systemic malaise. The primary site of pathology is within the synovial lining of the joint. Within the joint, the synovial lining proliferates and becomes inflamed forming pannus which erodes into cartilage, bone and surrounding ligaments leading to joint damage and subsequent deformities.

The clinical manifestations can be very broad and there is some overlap between RA and other inflammatory conditions. Symptoms of RA may begin abruptly or evolve over weeks, months or years (Jacoby *et al* 1973). Common patterns of disease are:

- a) Disease of small or medium joints particularly metacarpophalangeal (MCP) and proximal interphalangeal (PIP) joints of the hands, metatarso-phalangeal joints of the feet, wrists and ankles. There may also be variable large joint disease;
- b) Predominantly large joint disease;
- c) Disease involving only a few joints, or sometimes only one joint;
- d) Less common presentations include pain and stiffness affecting the shoulder and hip girdles (polymyalgic presentation); systemic symptoms such as weight loss and joint pain without a true arthritis; intermittent short-lived attacks of arthritis ('palindromic arthritis').

The course of RA is heterogeneous and variable. Within 2 years of diagnosis, patients usually experience moderate disability and a meta-analysis of functional activity suggested that over time 42% of patients with rheumatoid arthritis are in Steinbrocker functional class III or IV. Functional

class III is classified as limited and IV as incapacitated largely or wholly confined to bed or wheelchair and with little or no ability to self care (Steinbrocker *et al* 1949). This does not appear to have changed in recent years despite the advent of more effective therapies for RA (Scott and Steer 2007). Approximately a third of patients cease work because of disease, and life expectancy in RA is reduced (Fex *et al* 1998).

## **1.2 Rheumatoid Arthritis Historical Perspective**

Skeletal remains from North America suggest that RA was present over 4000 years ago (Rothschild and Woods 1990). In Europe paleo-pathology has failed to identify characteristic rheumatoid changes although other forms of arthritis are commonly seen.

In Europe the first pictorial representation of RA began to appear in Dutch art in the early 17<sup>th</sup> century (Dequeker 1977).

The first recognised description of RA came from Garrod in 1859. He described arthritis and used the phrase rheumatoid to distinguish it from the well-known forms of arthritis gout and rheumatic fever (Garrod, 1892).

In 1957 RA was described definitively and separated from other inflammatory conditions by Charles Short. The American Rheumatology Association devised criteria for the diagnosis of RA which were revised in 1987 to the following:

- a) Morning stiffness in and around joints lasting at least 1 hour before maximal improvement\*
- b) Soft-Tissue swelling (arthritis) of 3 or more joint areas observed by a physician\*
- c) Swelling (arthritis) of the proximal interphalangeal (PIP), metacarpophalangeal (MCP) or wrist joints\*
- d) Symmetric arthritis\*

- e) Subcutaneous nodules
- f) Positive test for rheumatoid factor (RF)
- g) Radiographic erosions or periarticular osteopenia in hand or wrist joints

\* present for at least 6 weeks

To be classified as having RA, a patient must meet four or more criteria. These criteria demonstrate 92% sensitivity and 89% specificity for RA when compared to control subjects with non-RA rheumatic disease (Arnett *et al* 1988).

These RA classification criteria were derived from studying a group of patients who had already been diagnosed with RA and had well-established disease. They have limited utility in routine practice and most clinicians diagnose RA without formal reference to such criteria. Many patients do not meet formal criteria particularly at onset of disease. However they are extremely useful for research purposes.

### **1.3 Aetiology**

Despite extensive research the cause of RA remains unknown. It is likely to be a multifactorial process with genetic and environmental factors playing roles. HLA-DRA4 positivity correlates strongly with RA and other susceptibility genes have recently been identified (Thomson *et al* 1993, Gregerson 1999; Mackay *et al* 2002). However, twin studies show a concordance of only 5-21% in monozygotic twins suggesting that an environmental trigger is likely (Silman *et al* 1993; Bellamy *et al* 1992).

An infectious trigger is widely postulated to lead to RA and the evidence for this includes declining incidence with successive birth cohorts and a shift towards older age at diagnosis consistent with a decline in early life infections. Clustering studies and studies of family size have displayed inconclusive results (Carty *et al* 2004).

Suggested candidates for an infectious agent include bacteria. Studies have identified fragments of bacterial DNA within the joint although no consistent relationship between organism and disease is apparent (Rook *et al* 1993; Bahr *et al* 1988).

Viruses such as Epstein Barr virus (EBV) and retroviruses have been suggested as putative causative organisms. There are high levels of EBV positivity in RA patients and evidence of EBV within synovial biopsies (Takei *et al* 1997; Takeda *et al* 2000). More recent work suggests that this may be due to abnormal T cell responses in established RA leading to persistence of infection rather than a causative role for EBV (Toussirot *et al* 2000). It is unlikely that any one infectious trigger will be identified which leads to disease in all RA patients.

#### **1.4 Pathogenesis**

The pathological processes causing RA have not been clearly elucidated although recent work has led to great advances. An uncertain pathological trigger results in dys-regulation of the immune system.

Leucocyte recruitment, predominantly that of T-lymphocytes (specifically Th1 cells) and macrophages occurs to sites of inflammation predominantly within synovial joints. Leucocyte recruitment is orchestrated by the specific pattern of chemokines produced following stimulation of cells by the up-regulation of pro-inflammatory cytokines (Tarrant *et al* 2006).

Synovial hyperplasia and inflammatory reaction of synovial tissue occurs accompanied by an inflammatory exudate within the joint cavity. Synovial fluid is produced in large quantities and is highly cellular containing a predominance of polymorphonuclear cells (PMN) with some T cells and macrophages. The normal joint lining layer consists of 1 or 2 cell layer thickness of fibroblastic-like cells without a basement membrane. Within the RA joint blood vessels multiply and populations of activated cells such as fibroblasts, T lymphocytes, plasma cells (antibody producing cells) and cells resembling macrophages are recruited. Aggregates of lymphoid tissue

resembling lymph nodes may also be found in synovial tissues (Freemont 1995; Sweeney & Firestein 2004).

Activated fibroblast and leucocytes develop into an invasive structure known as pannus which can be up to 10 cells thick. This pannus develops new blood vessels via angiogenesis allowing growth and invasion of other tissues to occur. Pannus erodes cartilage and bone directly via the secretion of mediators which breakdown cartilage and bone, e.g. Matrix Metalloproteinases (MMPs) and Aggrecanases. In addition B-lymphocytes are thought to produce pathological antibodies, e.g. rheumatoid factor. (Sweeney & Firestein 2004). Bone and cartilage damage leads to erosions which can compromise the structure and function of the joint.

#### 1.4.1 Role of Fibroblasts in Rheumatoid Arthritis Pathogenesis

Human joints can be divided up into fibrous, cartilaginous and synovial. The most common form and that which is predominantly affected by RA is the synovial joint. Examples of synovial joints include hips, knees and shoulders.

Synovial joints consist of the articular surfaces of two bones which are covered by hyaline cartilage. The joint space is enclosed by a fibrous capsule and lined by a synovial membrane which secretes synovial fluid. The synovial fluid provides lubrication to the joint and provides oxygen and nutrients to the cartilage.

The synovial membrane is composed of connective tissue which is made up of a variety of cell types. The major cell types are macrophages and modified fibroblasts (fibroblast-like synoviocytes). The synovial fibroblasts resemble fibroblasts from other organ systems but have specific ultra-structural and metabolic actions. The fibroblasts have long cytoplasmic processes which overlap and entwine and appear morphologically similar to dendritic cells. They are mesenchymal, non-vascular, non-epithelialised cells and do not express CD45 (Seki *et al* 1998; Lindhout *et al* 1999).

The specialised metabolic functions of synovial fibroblasts include synthesis of hyaluronic acid which contributes to the lubricating properties of the synovial fluid. In addition fibroblasts provide nutrients for cartilage.

The synovium has a rich blood supply and is infiltrated by a network of capillaries and venules. It is also supplied with lymphatic drainage and sympathetic nerve fibres.

In addition to their ultra structural properties fibroblasts have been shown to have high proliferative rates with loss of contact inhibition. This allows adhesion between fibroblasts and other cell types contributing to cell signalling (Kontinen 2000). Fibroblasts express cytokine mRNA and protein (Bukala *et al* 1991). Fibroblasts have been shown to release pro-angiogenic cytokines including vascular endothelial derived growth factor(VEGF) this is expressed constitutively and its excretion is up regulated in response to stimulation including hypoxia and cytokine stimulation, e.g. Interleukin 1(IL-1) (Jackson *et al* 1997). New vessel formation (angiogenesis) occurs within inflamed tissue and is a feature of the hypertrophied synovium found within RA joints.

#### 1.4.1.1 Pro-inflammatory Cytokine Production in Rheumatoid Arthritis Pathogenesis

Fibroblasts can release chemo-attractant cytokines (chemokines), e.g. macrophage inflammatory protein 1 alpha (Mip1 $\alpha$ ), monocyte chemotactic protein-1 (CCL2) in response to stimulation by pro-inflammatory cytokines which attract leucocytes into the joint. This contributes to synovial hypertrophy and continuing inflammation.

In addition the fibroblasts are capable of production of other pro-inflammatory cytokines, e.g. IL-15 which promotes T cell activation and expansion (Harada 1999), macrophage inhibitory factor (MIF) which can induce TNF- $\alpha$  production and enhance macrophage phagocytosis (Leech 1999).

#### 1.4.1.2 Matrix Degradation

Fibroblasts can produce both pro and anti- resorptive signals depending on specific conditions within the joint. IL-1 and TNF- $\alpha$  have both been shown to promote cartilage degradation via their effects on synthesis and secretion of matrix metallo-proteinases (MMPs) by fibroblasts (Migita *et al* 1996)). Fibroblasts can also produce TIMPs which are the naturally occurring inhibitors of MMPs (Wright 1991). The balance between matrix production and degradation depends upon the environment within the joint.

#### 1.4.1.3 Bone Remodelling

Erosions of bone occur as a specific feature of RA. Although fibroblasts have no direct role in bone resorption they have been shown to produce macrophage colony stimulating factor (M-CSF) and receptor activator of NF $\kappa$ B ligand (RANKL) which are necessary for the formation of osteoclasts from progenitor cells (Romas *et al* 2002; Takayanagi *et al* 2000).

#### 1.4.2 Leucocytes in Rheumatoid Arthritis

Establishment of chronic synovitis in RA is facilitated by influx of neutrophils and macrophages regulated by cell adhesion molecules. Reduction in E-selectin, ICAM-1 and VCAM-1 expression in synovial biopsies has been shown in responders to DMARD therapy (Smith *et al* 2001). Leucocyte infiltration within the synovium in particular macrophage, and lymphocytes results in a switch from acute to chronic inflammation with subsequent up-regulation of pro-inflammatory cytokine, chemokine and growth factor production. The leucocyte infiltrate is largely responsible for the degradation of the cartilage and peri-articular bone.

### 1.5 **Cytokines in Rheumatoid Arthritis**

Both TNF $\alpha$  and IL-6 are pleiotropic (act on many cell types) cytokines which exert their stimulatory effects on cells that bear appropriate cell surface receptors. Cytokines are intercellular mediators which regulate cell survival,

growth, differentiation and various effector functions. Cytokines are not stored as pre-formed molecules but are secreted by cells usually after stimulation. Each molecule is important in arthritis induction and recent observations suggest that both IL-6 and TNF $\alpha$  may be acting independently within synovial tissue. TNF $\alpha$  drives recruitment including neutrophil infiltration in the early acute phase of joint inflammation (Feldmann 1999). IL-6/sIL-6R modulates leucocyte recruitment causing a change in cells recruited from neutrophil to monocyte and lymphocyte (McLoughlin *et al* 2003; McLoughlin *et al* 2004). In addition clearance of inflammatory cells is altered by the effects of IL-6/sIL-6R signalling leading to impaired apoptosis with subsequent persistence of cellular infiltrate within the joint. This results in chronic inflammation leading to synovitis (Nowell *et al* 2003). Murine models of arthritis also provide evidence for distinct roles for TNF $\alpha$  and IL-6 in the pathogenesis and severity of arthritis (Alonzi *et al* 1998; Takagi *et al* 1998; Hata *et al* 2004).

#### 1.5.1 Role of TNF- $\alpha$ in the Pathogenesis of Rheumatoid Arthritis

TNF was initially identified in 1975 as a soluble factor that induced necrosis of tumour and was later identified as a catabolic hormone. Two forms of TNF are recognised TNF $\alpha$  and TNF $\beta$  (or lymphotoxin). TNF $\alpha$  has a half-life of 6 to 7 minutes and is produced largely by activated macrophages. Lymphotoxin is produced primarily by lymphocytes (Goeddel *et al* 1986; Beutler and Cerami 1989; Grunfeld and Palladino 1990).

Synovial fluid and serum levels of TNF $\alpha$  are increased in RA and particularly those with severe or active disease (Tetta *et al* 1990). In addition patients with high levels of TNF $\alpha$  have been shown to have higher ESR and synovial leucocyte counts (Saxne *et al* 1988); and higher DAS scores (Petrovic-Rackov 2006). Unlike TNF $\alpha$ , lymphotoxin has not been detected in synovial fluid or in serum of patients with RA (Saxne *et al* 1988). Therefore, it is likely that the TNF $\alpha$  is more important in the pathogenesis of RA than lymphotoxin.

TNF $\alpha$  is synthesised as an inactive pro-hormone and released by activated macrophages as a single unit (monomer) by the actions of TNF $\alpha$  cleavage enzyme (TACE). When activated three identical sub-units combine to form a trimer. TNF $\alpha$  signals via a cell surface receptor system (Beutler and Cerami 1989).

Two separate TNF receptors are found on the cell surface which can be divided on the basis of their molecular weight into 55 kd TNF Receptor 1 (p55 receptor) and 75 kd TNF Receptor 2 (p75 receptor). Two cell-surface receptors either p55 or p75 combine when TNF $\alpha$  binds to the cell surface. Each cell surface receptor appears to promote the same biological activities but TNF $\alpha$  binds with greater affinity to p55 receptor than p75 receptor. p55 receptor is expressed ubiquitously but p75 receptor expression appears to be confined to endothelial cells and haemopoietic cells (Kollias *et al* 1999). The 2 receptors, whilst displaying similar extra-cellular domains, signal via distinct intra-cellular pathways. p55 receptor contains a death domain not found in p75 receptor. p55 receptor signalling via MAP-kinase pathways can result in apoptosis (Bazzoni *et al* 1996). Studies from p55 receptor deficient mice and in human fibroblast cell lines demonstrate that p55 receptor is essential for formation of lymphoid tissue and defence against micro-organisms including bacteria and viruses (Wong *et al* 1992; Kollias *et al* 1999). The p75 receptor has been reported to mediate cytokine secretion together with effects on cell proliferation and cytotoxicity (Tartaglia *et al* 1993; Tartaglia *et al* 1991; Herbein *et al* 1998).

TNF $\alpha$  binding triggers a variety of biological processes which include release of other pro-inflammatory cytokines (e.g. IL-1, IL-6), trans-epithelial migration of leucocytes, activation of macrophages, T-Lymphocytes and other immune cells and release of enzymes (such as matrix metallo-proteinases and aggrecanases) which contribute to tissue breakdown (Migita *et al* 1996).

These cell surface receptors may also be shed from the cell-surface. This provides a mechanism for inhibition of TNF $\alpha$  activities as these soluble receptors (sTNF-R) competitively inhibit cell surface binding of TNF $\alpha$ .

sTNF-R levels are increased in active disease and appear to be a natural homeostatic regulator of TNF $\alpha$  activities (Aderka 1996). Murine models demonstrate that defective shedding is associated with the development of spontaneous auto-immune disease.

TNF $\alpha$  was first suggested as a prime cytokine in the pathogenesis of RA in the early 1990s following the discovery of high levels of macrophage and cytokine derived factors within the joint (Firestein *et al* 1988). Biologically active tumour necrosis factor was measured in synovial fluid of patients with several rheumatic diseases (Di Giovine *et al* 1988). Feldmann and Maini demonstrated presence of mRNA for pro-inflammatory cytokines including TNF $\alpha$  within RA synovium (Buchan *et al* 1988; Brennan *et al* 1989 a). Subsequent work by Feldmann and Maini led to the discovery that antibodies targeted against TNF $\alpha$  could also down-regulate production of other pro-inflammatory cytokines such as IL-1 and IL-6 in an RA co-culture model (Brennan *et al* 1989 b). These observations led to the use of cytokine inhibitors in animal models and latterly RA patients which have provided the majority of our insights into the function of TNF $\alpha$  in RA.

### 1.5.2 Treatment With Agents Directed Against TNF $\alpha$

Treatment with agents directed against TNF- $\alpha$  provides clinical benefit together with a reduction in tender and swollen joint counts and prevention of on-going joint destruction. Inflammatory parameters including ESR and CRP are normalised (Elliott 1993). Successful treatment is associated with a sustained reduction in levels of pro-inflammatory cytokines such as IL-1 and IL-6 (Charles *et al* 1999).

Treatment has also been demonstrated to reduce leucocyte trafficking to affected joints. This is as a result of down-regulation of adhesion molecule expression, e.g. intracellular adhesion molecule 1 (ICAM-1) and E-Selectin. This has been demonstrated by quantitative analysis of serum levels together with immuno-histochemical analysis of serial synovial biopsies (Paleolog *et al* 1996; Tak *et al* 1996).

Treatment with anti-TNF agents has also been shown to reduce levels of certain pro-inflammatory chemokines including CXCL-8 and CCL-2. Levels of vascular endothelial growth factor (VEGF) have also been shown to reduce following treatment with assumed beneficial effects in prevention of angiogenesis (Paleolog *et al* 1998).

### 1.5.3 Interleukin-6 in Rheumatoid Arthritis

Interleukin-6 is a pleiotropic cytokine which can be produced by a variety of cell types. Its molecular mass is 21-28 kDa. It is part of the family of long-chain four- $\alpha$  helical cytokines. Other members of the IL-6 family include oncostatin-M, IL-11, leukaemia inhibitory factor, cardiotrophin-12 and IL-27.

IL-6 has many varied actions and targets genes involved in differentiation, survival, apoptosis and cell proliferation. It has been demonstrated to have both pro-inflammatory and anti-inflammatory properties and is involved in the acute phase response to infection and inflammation. Studies in IL-6<sup>-/-</sup> mice suggest that IL-6 is a critical cytokine in the production of fever. IL-6 is involved in the induction of acute phase proteins such as serum amyloid A (SAA) and C reactive protein (CRP). IL-6 has also been demonstrated to play a role in auto-antibody generation. When acting as an anti-inflammatory cytokine, IL-6 provides protection against septic shock and can promote resolution of acute inflammation.

IL-6 has been postulated to be an important cytokine in RA for some time. Many investigators have demonstrated high levels of IL-6 in synovial fluid in RA and other inflammatory arthropathies (Robak *et al* 1998; Polgar *et al* 2000; Desgeorges *et al* 1997). Within the joint, many cell types including fibroblast-like synoviocytes and chondrocytes are capable of producing IL-6. Although there is some evidence of a basal level of production the majority is stimulated by other pro-inflammatory cytokines, e.g. IL-1 and TNF $\alpha$ .

IL-6 signals via a receptor complex consisting of two distinct membrane-bound glycoproteins; a cognate IL-6 receptor (80 kd) with limited cellular expression and a ubiquitously expressed gp130 signal-transducing element.

In general, resident cells within the joint lack the cognate receptor, but can be made responsive to IL-6 via a soluble IL-6 receptor (sIL-6R) (Jones 2002). This is in contrast to the actions of TNF $\alpha$  where the soluble receptor prevents TNF $\alpha$  signalling by competitive inhibition. Significantly, the sIL-6R/IL-6 complex is able to activate cells via interaction with membrane-bound gp130, allowing cells which do not express cognate IL-6 receptor to be made responsive to IL-6 (Nowell *et al* 2003). This ability of IL-6 to stimulate cells which do not express cognate receptor is known as 'trans-signalling'. gp130 activation also occurs by other IL-6 related cytokines including IL-11, oncostatin M and cardiotrophin-1 (Taga *et al* 1997). sIL-6R is found in the plasma of healthy individuals but levels are 2-3 times higher than in those with inflammatory diseases such as RA (Robak *et al* 1998; Polgar *et al* 2000; Desgeorges *et al* 1997).

Two distinct isoforms of the sIL-6R have been identified which are produced by proteolytic cleavage of the cognate receptor from the cell surface (PC-sIL-6R) or by differential mRNA splicing (DS-sIL-6R). The DS-sIL-6R isoform is found only in RA fluids and not in osteoarthritis (OA) (Nowell *et al* 2003). It appears that the release of the two different isoforms is regulated independently. Work within our group examining the biological characteristics of sIL-6R showed that a complex of IL-6 with either DS-sIL-6R or PC-sIL-6R resulted in identical pattern of chemokine induction. Both forms activate the Janus kinase-STAT pathway through gp130 and were found to be similar in their ability to regulate neutrophil infiltration (McGloughlin *et al* 2004). It is not yet known whether the two isoforms have identical actions in the pathogenesis of RA.

#### 1.5.4 IL-6 Trans-signalling in Rheumatoid Arthritis

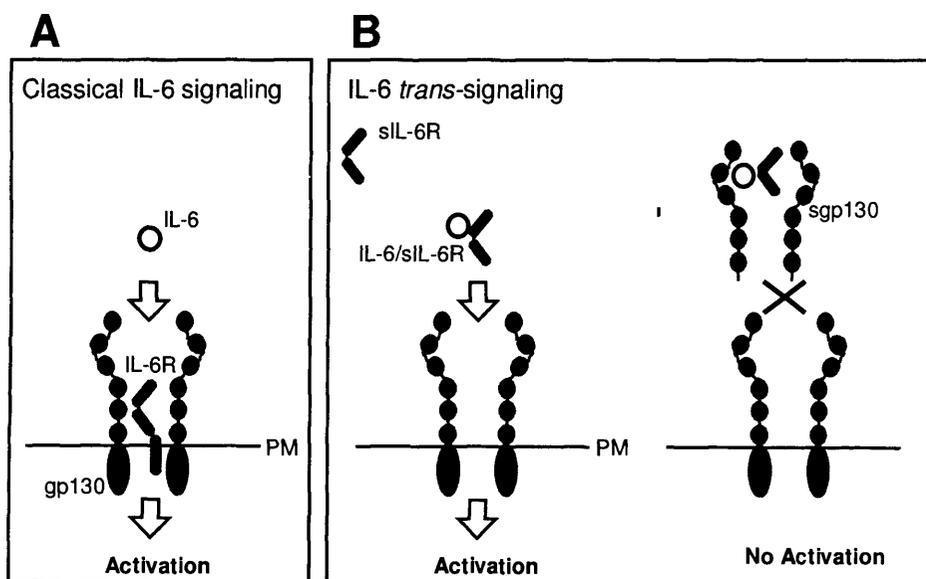
Evidence for the importance of IL-6 interactions with IL-6R in arthritis pathogenesis has come from animal models. In antigen induced arthritis (AIA), IL-6 deficient mice (IL-6<sup>-/-</sup>) have been shown to be resistant to arthritis (Ohshima *et al* 1998). Administration of HYPER-IL-6 (IL-6/sIL-6R fusion protein) restored arthritis severity to that of wild type mice (Nowell *et al*

2003). This did not occur if the mice were treated with IL-6 alone, showing that the presence of the soluble receptor is essential. Treatment of wild-type (IL-6<sup>+/+</sup>) mice with soluble gp130 (a natural antagonist to IL-6/sIL-6R complex which acts by competitive inhibition of membrane bound gp130) showed a reduction in all parameters of disease severity (Nowell *et al* 2003). This data provides indirect evidence for the role of IL-6/sIL-6R complex and gp130 in the pathogenesis of RA. Experiments using gp130 fusion proteins have been performed which prove that sgp130 exclusively inhibits IL-6 responses mediated by the soluble receptor and does not affect cognate IL-6 signalling via membrane bound receptor (Atreya *et al* 2000).

Proof of concept studies in colitis indicate that sgp130:Fc treatment reduced the severity of colitis. This provides further evidence of benefit from sgp130:Fc in other inflammatory diseases (Atreya *et al* 2000).

### Figure 1.1 - IL-6 trans-signalling and its inhibition with sgp130

(Figure reproduced and adapted with the permission of Dr S A Jones)



**A** Classical IL-6 signalling.

**B** IL-6 trans-signalling and its specific inhibition using sgp130

### 1.5.5 Treatment With Agents Directed Against IL-6 or IL-6R

Antibodies directed against IL-6 have been used in RA patients but despite initially promising results they have not reached clinical practice (Wendling *et al* 1993).

In contrast when IL-6R has been targeted results have been promising in trials in RA patients. In initial open label trials 13/15 patients obtained ACR 20 responses at 13 weeks (Nishimoto *et al* 2003). In further larger double-blind placebo-controlled trials blocking IL-6R showed significant improvements in disease activity and inflammatory markers (Choy *et al* 2002; Nishimoto *et al* 2004). The anti-IL6R antibody has been named Tocilizumab.

The results of several large phase III studies have been published. In a large, multi-centre Japanese trial patients with poorly controlled RA were treated on a 4 weekly basis with 8 mg/kg Tocilizumab infusions. At 1 year, patients in the therapy group had significantly less progression in radiographic damage than patients in the control group. Significant improvements in DAS score were also achieved (Nishimoto *et al* 2007). The results of the first and second of five large, multi-national studies have also been published recently.

The OPTION study in patients with inadequate clinical response to methotrexate found a significant improvement in ACR-20 responses at 24 weeks in patients treated with 4 or 8 mg/kg doses of Tocilizumab compared to placebo (Smolen *et al* 2008).

The TOWARD study, a multi-national double-blind trial compared Tocilizumab 8mg/kg or placebo in combination with stable DMARD therapy. At week 24, the proportion of patients achieving ACR20 was significantly greater in the tocilizumab plus DMARD group than in the control group. Secondary end points including ACR50/70 responses, DAS28, DAS28 remission responses and systemic markers including CRP and haemoglobin

showed improvement in the Tocilizumab treated patients (Genovese *et al* 2008). Tocilizumab will be available commercially in the near future.

Despite the promising results, potential side effects of blockade of cognate IL-6 signalling have become apparent. Abnormalities in liver function tests (LFT) and a rise in serum cholesterol have both been reported from the trials. 44% of patients had a rise in serum cholesterol following treatment with MRA (Tocilizumab) (Nishimoto *et al* 2004). This is likely to have detrimental implications in RA where patients already have an increased risk of cardio-vascular and cerebro-vascular disease as a result of their RA (Erb *et al* 2004; Stevens *et al* 2005). Altering the lipid profile in these patients may well further increase their cardio-vascular risks.

Malignancies were reported to have occurred in 3 patients in the treatment arm of the most recent study with none seen in the control group. No further information was given about site or likelihood of evidence that Tocilizumab was responsible (Nishimoto *et al* 2007).

As predicted, an increase in infection with IL-6R directed therapies has been reported. In the OPTION study more people receiving Tocilizumab than those receiving placebo had at least one adverse event. The most common serious adverse events were serious infections, reported by six patients in the 8 mg/kg group, three in the 4 mg/kg group, and two in the placebo group (Smolen *et al* 2008). Additionally, in the TOWARD study, grade 3 neutropenia occurred in 3.7% of patients receiving Tocilizumab and none of the patients in the control group (Genovese *et al* 2008).

Specifically targeting IL-6 trans-signalling could be considered as an alternative strategy which would spare cognate receptor signalling and may address these potential side-effects.

## **1.6 Synergistic Interactions Between TNF $\alpha$ and Other Cytokines**

Although TNF $\alpha$  and IL-6/sIL-6R have both been demonstrated to have significant importance in the pathology of RA evidence is only now beginning to emerge of their interactions.

Evidence for a synergistic effect between TNF $\alpha$  and IL-6/sIL-6R was demonstrated in a study which examined the induction of VEGF in cultured RA fibroblasts. A clear synergistic effect of the two cytokines on VEGF production was seen, which was prevented by an anti-IL-6R antibody but it could not be prevented by anti-TNF $\alpha$  alone (Nakahara *et al* 2003).

IL-1 and IL-6/sIL-6R complex also act together synergistically to up-regulate metalloproteinase production by chondrocytes (Rowan *et al* 2001; Flannery *et al* 2000).

## 1.7 Treatment of Rheumatoid Arthritis

Conventional drug therapy for RA relies on varying combinations of the following four classes of drugs:

- Non-steroidal anti-inflammatory drugs (NSAIDs)
- Analgesics
- Corticosteroids such as prednisolone and methylprednisolone
- Disease modifying anti-rheumatic drugs (DMARDs)

Current practice is that therapy with DMARDs should be started as soon as possible in order to prevent occurrence of erosions and to attempt to achieve remission (defined as 5 or more of the following criteria for 2 consecutive months: morning joint stiffness of less than 15 minutes, no fatigue, no symptoms of joint pain, no joint tenderness or pain on motion, no swelling of joints or tendon sheaths, and ESR less than 20 or 30 depending on sex) (Pinals RS *et al* 1981).

DMARDs are slow acting drugs, which provide symptomatic relief and may take several weeks or months to work. They also have the potential to induce disease remission and a potential for reducing the risk of joint damage in progressive RA.

Major aims of treatment include control of joint pain and inflammation, reduction in joint damage and disability, improvement in physical function and maintenance or improvement in quality of life (Scott *et al* 1998).

To be called a DMARD, a drug must be capable of changing the course of RA for at least 1 year as evidenced by sustained improvement in physical function, decreased synovitis and slowing or prevention of structural joint damage (Bird K 2002).

### 1.7.1 DMARD Therapy

#### 1.7.1.1 Methotrexate (dihydrofolate reductase inhibitor)

Methotrexate exerts its beneficial effects in a variety of ways. It is likely that its role as a dihydrofolate reductase inhibitor results in down-regulation of metabolically active reduced folate leading to a subsequent reduction in purine formation and prevention of cell turnover (Cutolo *et al* 2001; Smolenska *et al* 1999).

In addition it can inhibit AICAR (5-aminoimidazole-4-carboxamide riboside) transformylase which causes an increase in intracellular AICAR which stimulates the release of adenosine. Adenosine has anti-inflammatory properties and in addition is an inhibitor of neutrophil function (Cronstein *et al* 1991; Cronstein *et al* 1993).

Methotrexate is currently the standard first line therapy for RA because of its efficacy and safety profile. A meta-analysis of treatment termination has shown that continued drug use at 60 months is higher for methotrexate than other DMARDs although the median duration of methotrexate use remains only 41 months (Maetzel *et al* 2000).

#### 1.7.1.2 Sulphasalazine

Actions of sulphasalazine are less clear. Sulphasalazine is a combination of an antibiotic sulphapyridine with an anti-inflammatory linked with an azo bond. Bacterial enzymes in the large intestine digest the sulphasalazine

into its biologically active constitutive parts. Its action is largely unknown although it is known to affect gut bacteria (Neumann *et al* 1987), inflammatory cell function, cytokine and antibody production together with an increase in free radical scavenging activity (Comer 1988; Symmons *et al* 1988). Sulphasalazine concentrations within the synovial fluid have been shown to be only slightly less than that in plasma (Farr *et al* 1985).

#### 1.7.1.3 Leflunomide

Leflunomide is an inhibitor of pyrimidine synthesis and has been shown in trials to be comparable to methotrexate and sulphasalazine in reducing signs and symptoms of RA (Mladenovic *et al* 1995; Strand *et al* 1999). T and B lymphocytes have only low reserves of pyrimidine nucleotides making them very sensitive to leflunomide. Lymphocyte division is arrested at G1 phase of the cell cycle (Rückemann *et al* 1998; Cherwinski *et al* 1995). There is good clinical evidence for efficacy.

#### 1.7.1.4 Cyclophosphamide

This is an inactive pro-drug which is activated via the hepatic cytochrome p450 enzyme system to become a DNA alkylating agent. This results in cross-linking of DNA with subsequent reduction in DNA synthesis and increase in apoptosis. Its effects are most marked on rapidly dividing cells, resulting in reduction in B and T Lymphocytes. Due to its significant toxicity the use of cyclophosphamide in RA is predominately confined to those patients who develop systemic vasculitis in conjunction with their RA.

#### 1.7.1.5 Corticosteroids

Recent evidence has emerged that steroids should be considered as DMARDs because there is a significant reduction in radiological progression when given in combination with standard DMARD therapy (Kirwan and Power 2007, Goekoop-Ruiterman *et al* 2005, Verhoeven *et al* 1998).

#### 1.7.1.6 Other Traditional DMARDs

Other drugs which have been used include Azathioprine, cyclosporin, hydroxychloroquine, D-penicillamine and intra-muscular myocrisin (Gold). These medications have a variety of effects on the immune system and varying degrees of efficacy. In addition cyclosporin in particular has many side-effects including gum hypertrophy and renal impairment. Their use has been largely superseded by the more effective DMARDs listed above and biological therapies in resistant disease.

#### 1.7.2 Biologics

Biologic agents which target specific cytokines, chemokines and co-stimulatory molecules have now been developed and are rapidly becoming the gold standard for RA therapy. Agents targeting TNF $\alpha$  and Rituximab (anti CD20) which targets B cells are the only agents used in routine clinical practice but other biological therapies are in clinical trial and under development.

##### 1.7.2.1 Anti-TNF $\alpha$

Therapies directed against TNF $\alpha$  have been shown in clinical trial to be effective in both early and late stages of disease. In the UK, the British Society of Rheumatology (BSR) guidelines restrict its use. These guidelines were accepted in full by the National Institute for Clinical Excellence (NICE).

The BSR Guidelines state that anti-TNF therapies (Adalimumab, Infliximab and etanercept) may be used in patients who:

- a) Satisfy ACR (American College of Rheumatology) classification for RA;
- b) Have active disease as defined by as Disease activity Score (DAS) of 5.1 or higher. (The DAS score is a validated composite score consisting of weighted swollen and tender joint counts, ESR and patient functional visual analogue score);

- c) Have previously failed on methotrexate and one other DMARD. A minimum 6 month period on each standard therapy at clinically effective doses should be required unless withdrawal was due to toxicity;
- d) Treated patients should be entered on a central register, with drugs, dose, outcomes and toxicity reported on a quarterly basis.

Contra-indications include:

- a) Pregnancy or breastfeeding;
- b) Active infection;
- c) Patients at high risk of infection including:
  - (i) Chronic leg ulcers;
  - (ii) Previous Tuberculosis unless a full course of anti-TB therapy completed;
  - (iii) Septic arthritis within 12 months or indefinitely if a prosthetic joint remains in-situ;
  - (iv) Bronchiectasis;
  - (v) Indwelling urinary catheter;
- d) Malignancy or pre-malignancy (excluding BCC or malignancies > 10 years earlier where chance of cure is high).

#### 1.7.2.2 Infliximab

Infliximab (Schering-Plough) is a chimeric human-murine monoclonal antibody directed against TNF $\alpha$ . The TNF $\alpha$  binding region is murine and comprises 30% of the amino acid sequence of infliximab. The remainder is a human IgG1 heavy chain and kappa chain constant region.

The recommended dose of Infliximab for RA is 3 mg/kg body weight given as an intravenous infusion followed by further infusion, at the same dose, 2 and 6 weeks later. Thereafter infusions are given at 8-week intervals. Infliximab must be given with methotrexate. Infliximab binds with high affinity to cell-bound TNF $\alpha$  and soluble TNF $\alpha$  monomers and trimers and forms stable complexes. Infliximab inhibits binding of TNF $\alpha$  to TNF-R1 and TNF-R2 and it may dissociate TNF $\alpha$  already bound to TNFR. Unlike etanercept, infliximab does not bind or inhibit lymphotoxin (Jobanputra *et al* 2001).

#### 1.7.2.3 Adalimumab

Adalimumab (Abbott Laboratories) is a human-sequence antibody that binds specifically to TNF $\alpha$  and neutralizes the biological function of TNF $\alpha$  by blocking its interaction with cell-surface TNF $\alpha$  receptors. It also modulates biological responses that are induced or regulated by TNF $\alpha$ , including changes in the levels of adhesion molecules responsible for leukocyte migration. Adalimumab is licensed for the treatment of moderate to severe, active RA in adults when the response to DMARDs, including methotrexate, has been inadequate, and for the treatment of severe, active and progressive RA in adults not previously treated with methotrexate. In order to ensure maximum efficacy manufacturer's guidelines stipulate the Adalimumab should where possible be given in conjunction with methotrexate. It is generally given as a subcutaneous injection of 40 mg on alternate weeks although dosing frequency may need to be increased to once weekly particularly when given as monotherapy.

#### 1.7.2.4 Etanercept

Etanercept (Wyeth) is a combination protein consisting of the extra-cellular portion of two of the 75 kd-TNF receptors (TNF-R2) for TNF combined with a human Fc portion of human IgG1. Etanercept binds soluble TNF $\alpha$  and lymphotoxin by competing with TNF receptors (TNFR). It has a 50-fold higher affinity for TNF than monomeric TNFR, *in vitro*. It is distributed to bone, liver, spleen and kidney and probably penetrates synovial tissue. It is

administered as a twice-weekly sub-cutaneous injection of 25 mg or once-weekly 50 mg dose in RA (Personal communication from Wyeth).

#### 1.7.2.5 Efficacy of anti-TNF $\alpha$ Agents

Efficacy for all agents appears to be similar. There have now been many, high quality, randomised controlled trials examining these agents. The overall clinical response rate appears to be approximately 60%. If an adequate response is deemed to be an ACR 50 (50% reduction in number of swollen and tender joints) then approximately 20-50% of patients treated will achieve this (Maini *et al* 1999; Maini *et al* 1998; Moreland L *et al* 1997).

#### 1.7.2.6 Side-Effects of anti-TNF $\alpha$ Agents

The major side effects associated with the use of anti-TNF agents appear to be an increased risk of infection including serious infections. This has been apparent from randomised control trial data. Dixon *et al* at the BSR biologics register noted an increased risk of infection particularly serious skin and soft tissue infections with an incidence rate ratio (IRR) of 4.28. There was no significant difference in risk between therapies. Analysis of the German biologics register demonstrated a relative risk of 2.2 for all serious infections with etanercept and 2.1 with infliximab (Listing *et al* 2005). Reactivation of latent tuberculosis also continues to be a concern particularly in those treated with Infliximab.

The potential increase in tumours which was postulated has not been apparent from the BSR biologics register. However, a recent meta-analysis looking at results from long-term clinical trials found an increased risk of tumours in particular lymphomas (Relative Risk 3.3 compared to control population) (Bongartz *et al* 2006). This meta-analysis has caused controversy due, in part, to the failure of the authors to include etanercept trials and, secondly, due to the lower than expected levels of malignancy seen in the control populations in these trials (Dixon and Silman 2006).

### 1.7.3 Biologics Targeting Molecules Other Than TNF $\alpha$

#### 1.7.3.1 Anti-IL-1

Anakinra is a recombinant non-glycosylated human IL-1 receptor antagonist which inhibits the activity of IL-1. It is given by once daily subcutaneous injection in combination with methotrexate. Clinical trials show an improvement in ACR 20 response rates when compared to placebo and there is some evidence of a slowing in radiographic damage as measured by serial Sharp scores (Bresnihan *et al* 1998; Cohen *et al* 2002). However, in clinical practice results have been disappointing. NICE guidelines suggest that anakinra should not be used routinely as is likely to be significantly less effective than medications targeted against TNF $\alpha$ .

#### 1.7.3.2 Rituximab

Rituximab is a chimeric monoclonal antibody against CD20 expressed on mature B lymphocytes. The role of B Lymphocytes in RA pathogenesis is unclear but putative mechanisms include an antigen-presenting function, secretion of pro-inflammatory cytokines, production of rheumatoid factor, and co-stimulation of T cells. Clinical trials in anti-TNF non responders have demonstrated significant improvements in symptoms and ACR20 and 50 responses when compared to placebo. The dosing schedule consists of 2 IV injections on day 0 and day 15 in combination with IV Methylprednisolone and continued methotrexate (Cohen *et al* 2006). It has recently been licensed for use in RA in patients who have previously failed on anti-TNF medications. Larger clinical trials in anti-TNF naïve patients are currently underway.

#### 1.7.3.3 Anti-Cytotoxic T-lymphocyte Antigen-4 (CTLA-4)

Abatacept – a recombinant fusion protein comprising the extra cellular domain of human CTLA4 fused with a fragment of the Fc portion of human IgG1 has also been administered to patients with RA, with beneficial effects. Abatacept prevents the interactions between antigen presenting cells and T

cells at sites of inflammation in particular within the synovial joint. In phase 1 clinical trial 44% of the patients in the 2 mg/kg group and 53% in the 10 mg/kg group achieved an ACR20 response at 12 weeks (the primary outcome of the study) as compared with 32% in the placebo arm. Response was seen across all of the components in the ACR core set, with the two highest dose treatment groups for each compound exhibiting a consistently higher percentage improvement than placebo. The 10 mg/kg dose of abatacept was generally more effective than 2 mg/kg (Moreland LW 2002). In recent phase II b and III trials, abatacept produced statistically significant improvements in patients who still had active disease in spite of adequate methotrexate therapy (Schiff *et al* 2007; Kremer *et al* 2005).

#### 1.7.3.4 Anti-IL-6 Receptor Therapies

See section 1.5.5.

## 1.8 **Combination Therapies**

Combination therapy is commonly used in RA. Combined DMARDs, DMARD and biologics and combined biologics have been used.

### 1.8.1 Non-Biologics

Combination therapy with methotrexate, sulphasalazine and hydroxychloroquine in patients with long-standing RA resulted in significant improvement when compared to monotherapy (O'Dell *et al* 2002; Korpela *et al* 2004). A recent study in poor prognosis early RA in contrast failed to demonstrate any significant superiority of combination therapy over monotherapy (Proudman *et al* 2000). The sample sizes in all these cases have been small and Tugwell estimated that a sample size of 3000 would be needed in order to ensure that a study is adequately powered to detect small differences between monotherapy and combination treatments (Tugwell 1996).

Usual practice in the UK is either a step-up approach where DMARDs are added if an adequate clinical response is not achieved or alternatively switching from one DMARD to another in order to improve outcomes. There are some advocates of a step-down approach whereby a combination of DMARD with or without steroid is introduced as soon as diagnosis is made with the aim of reducing treatment later. There is little good quality evidence for this approach.

## 1.8.2 Combination Therapy With Methotrexate + Biologics

### 1.8.2.1 *Infliximab + Methotrexate*

Infliximab is always given with methotrexate in RA. This is in order to prevent the development of human anti-chimeric antibodies (HACA) which result in reduction of clinical efficacy. Maini et al in 1998 demonstrated that although initially treatment without Methotrexate resulted in improvement this effect was lost by 8 weeks. If given in combination with methotrexate efficacy continues beyond the 8 week timepoint (Maini *et al* 1998).

Results from the BEST study demonstrated that combination therapy in early arthritis with either prednisolone or Infliximab resulted in earlier functional improvement and less radiographic damage at 1 year than sequential monotherapy or step-up combination therapy (Goekoop-Ruiterman *et al* 2005).

### 1.8.2.2 *Etanercept + Methotrexate*

Weinblatt et al published results in 1999 of a randomised controlled trial comparing etanercept + methotrexate with placebo +methotrexate. ACR20 responses of 71% compared favourably with those of etanercept monotherapy where ACR20 of 59% in the 25mg dose group was achieved at 6 months (Weinblatt 1999; Moreland 1999).

Results from the TEMPO study comparing methotrexate monotherapy with etanercept monotherapy and combination of the two therapies at 2 years

demonstrated significantly lower withdrawal rates in the combination group together with significantly greater improvements in ACR20, 50 and 70 responses and a reduction in radiographic progression (van der Heijde *et al* 1992).

#### 1.8.2.3 Adalimumab + Methotrexate

The PREMIER Study (Breedveld *et al* 2006) was a 2 year multi-centre randomised control trial which demonstrated significantly better ACR responses and reduction in radiographic progression in patients treated with combination therapy when compared to adalimumab monotherapy or methotrexate monotherapy. Improved outcomes with combination therapy were also noted in a longitudinal observational study from Norway (Heiberg *et al* 2006).

#### 1.8.3 Anakinra + Anti-TNF $\alpha$

Combination therapy with etanercept and anakinra demonstrated no benefit over that of etanercept alone with no significant difference in ACR 50 response at 6 months following 6 months of standard dose therapy. There was an increase in serious infections (3.7-7.4% for combination group cf 0% in etanercept group), injection site reactions and neutropaenia (Genovese *et al* 2004). The lack of significant improvement with combination therapy is probably due to the similar modes of action of IL-1 and TNF $\alpha$ .

### 1.9 **Experimental Models of Rheumatoid Arthritis**

Animal models for RA are now well established and provide a useful strategy to test in-vitro observations prior to the use of new therapies in RA patients. Animal models are used in order to dissect further the pathophysiology of disease and to test therapeutic strategies at specific defined stages of disease.

Evaluation of disease is possible under tightly controlled circumstances which allow multiple comparisons to be made at specific time-points in

disease. Tissue sampling is easy and allows molecular, cellular and tissue variables to be studied. The use of models allows mechanisms and response to treatment to be studied without having to allow for individual variability between patients. No single model exists which can be used as a perfect model for RA. Each model has features which are similar to and different from RA. The models used most commonly are described in detail below.

#### 1.9.1 Adjuvant Arthritis

This was the first animal model for arthritis and was noted to occur in rats after the injection of spleen extracts emulsified in Freund's complete adjuvant (Stoerk *et al* 1954). This model has been described mainly in the rat but can be used in the mouse too (Knight *et al* 1992). This model was used extensively to test drugs such as aspirin, phenylbutazone and gold (Newbould 1963). The major problem with this model is the fact that disease is very short-lived. Symptoms occur around 10-12 days after disease induction but disappear as early as 20 days after induction (Billingham 1990). Secondly, drugs which display disease modifying effect in this model such as NSAIDs do not behave in this way in RA (Billingham 1990).

#### 1.9.2 Antigen Induced Arthritis

This model was first described as a model for RA following the production of synovitis in rabbits after intra-articular injection of fibrin (Dumonde and Glynn 1962). It can also be induced in mice, guinea pigs and rats. Disease in rabbits in particular appears histologically similar to human RA (Pettipher 1988). The major limitation of this model is its lack of systemic features.

#### 1.9.3 Bacterial Cell Wall Induced Arthritis

This model was first described in 1970 when chronic erosive polyarthritis was induced following intra-peritoneal injection of a suspension of cell wall extracts from group A streptococcus (Jones 1970). The arthritis is

characterized by polyarticular flares which eventually progress to joint destruction (Cromartie *et al* 1977). A mono-articular variant has also been described (Esser *et al* 1995). The major limitation of the systemic disease is the unpredictable course of disease.

#### 1.9.4 Collagen Induced Arthritis

Collagen induced arthritis was first described in 1977 and has been induced in many species including rats, mice and primates (Trentham *et al* 1977).

Murine collagen induced arthritis (mCIA) has been chosen as a model for a variety of reasons. In particular, the histological features and systemic, predictable nature of the disease which make it possible to intervene at defined time-points (Sewell and Trentham 1993). The similarities between mCIA and RA will be discussed in greater detail in table 1.1 and section 5.1.1). In mice disease is more severe and the time-course more predictable than in rats providing a better model for human disease.

**Table 1.1 – Comparison of various animal models of rheumatoid arthritis (adapted from Henderson et al)**

	<b>Adjuvant Arthritis</b>	<b>Collagen Arthritis</b>	<b>Cell wall arthritis</b>	<b>Antigen Induced Arthritis</b>	<b>Rheumatoid Arthritis</b>
<b>Species</b>	Rat	Mice/rat/monkey	Rats, mice	Rabbit mouse rat Guinea pig	Humans
<b>Genetic Linkage</b>	+	+	+	+	+
<b>Sex Predilection</b>	M/F	M/F	F	-	F
<b>Remitting/relapsing</b>	-	-	+	-	+
<b>Peripheral joints</b>	+	+	+	Local	+
<b>Axial joints</b>	+	-	-	-	+
<b>Erosions/pannus</b>	+	+	+	+	+
<b>Periosteal reaction</b>	+	-	-	-	-
<b>Antibody dependence</b>	-	+	?	+	?
<b>T-cell dependence</b>	+	+	+	+	+
<b>Antigen</b>	Mycobacterium	Type II collagen	Group A Streptococcal cell wall	Methylated Bovine serum albumin	Unknown

## 1.10 Murine Collagen Induced Arthritis

### 1.10.1 Similarities Between mCIA and Rheumatoid Arthritis

mCIA is an auto-immune polyarthritis. Immunisation with type II collagen results in a combined humoral and cellular response primarily within synovial joints. In common with RA, susceptibility is confined to specific MHC haplotypes and only certain strains of mice are affected (DBA-1 most successfully although other groups have demonstrated disease in C57BL/6 mice and other strains (Campbell 2000). Histopathologically, there is synovial proliferation with progression to pannus formation leading to marginal bone erosions and cartilage destruction (Stuart *et al* 1985). Radiographs show erosive changes. Progressive arthritis leads to joint deformity and dysfunction.

In common with RA, auto-antibodies are found (in mice to type II collagen). Levels of pro-inflammatory cytokines in particular TNF $\alpha$  are high throughout the course of arthritis (Piguet *et al* 1992; Takai *et al* 1989).

As with the clinical manifestations of RA, mice develop a poly-articular erosive disease with synovitis, periostitis, and infiltration of sub-synovial tissue with inflammatory cells pannus, exudates of inflammatory cells into the joint space, cartilage fragmentation and bony erosions (Stuart *et al* 1985).

### 1.10.2 Differences Between Murine Collagen Induced Arthritis and Rheumatoid Arthritis

In contrast to RA, disease only occurs following immunisation with type II collagen and does not occur spontaneously. No evidence of extra-articular disease, e.g. subcutaneous nodule formation and pulmonary fibrosis has been shown in mice. Unlike in RA, which is more common in females (M:F ratio 1:3), males have greater susceptibility to mCIA and testosterone appears important in its induction (Holmdahl *et al*

1986). RA distribution is different to that of mCIA where arthritis predominantly affects ankle joints and small joint of the paws. Although knee involvement has been reported the incidence appears low (Caulfield *et al* 1982).

### 1.10.3 Therapy in CIA

mCIA is well established as a model for testing therapeutics. It has been used to demonstrate efficacy of anti-TNF $\alpha$  antibodies, soluble TNF receptors, IL-1 receptor antagonists and many other therapies (Piguet *et al* 1992; Wooley *et al* 1993). This will be discussed in greater detail in chapter 5.

## 1.11 Summary

In RA it is recognised that early diagnosis and intervention improves disease management. The novel biologics such as the anti-tumour necrosis factor alpha (TNF $\alpha$ ) agents can antagonise the effects of this particular cytokine and produce significant suppression of inflammation with associated clinical improvement. Although this approach has validated specific targeting of inflammatory cytokines as a successful clinical strategy, there remains concern over the long term safety and efficacy of these agents. If one sets the response criteria as achieving an ACR50, up to 50% of patients will fail to achieve this. Agents that inhibit IL-6 or its receptor have now entered phase II clinical trials. Specifically blocking anti-IL-6 receptor antibody (Tocilizumab) has shown favourable results in clinical trials however safety data has revealed evidence of increased incidence of infection and abnormalities in lipid profile. However, it remains to be determined whether the blockade of IL-6 bioactivity offers a true advantage over anti-TNF $\alpha$  therapies.

We propose that selective blockade of IL-6 trans-signalling may facilitate resynchronisation and resolution of the inflammatory response without significant disruption of normal host defence. This project will

examine the relationship between TNF $\alpha$  and IL-6 trans-signalling in order to ascertain whether combination TNF $\alpha$  and trans-signalling blockade may offer true advantages in selected RA patient cohorts.

### **1.12 Aims of Thesis**

The ultimate aim of this thesis is to provide novel proof of concept data to support our hypothesis that combined blockade of TNF $\alpha$  and IL-6 trans-signalling may offer true advantages in selected RA patient cohorts.

The hypothesis that TNF and IL-6/sIL-6R interact to modulate leucocyte recruitment in inflammatory arthritis was addressed through the following specific aims:

Aim 1 - Establish the regulatory effects of IL-6/sIL-6R complex on TNF $\alpha$  bio-activity in-vitro.

Aim 2 - Determine the effect of combined TNF $\alpha$  and IL-6 trans-signalling blockade in experimental arthritis.

Aim 3 - Examine the effect of anti-TNF $\alpha$  treatment on IL-6 trans-signalling in RA patients.

## **2 MATERIALS AND METHODS**

### **2.1 Chemicals**

All general laboratory chemicals and reagents were obtained from Fisher scientific UK, Loughborough, LF11 5KG.

Dulbecco's modified Eagle's media (DMEM), phosphate buffered saline (PBS), L-glutamine and other reagents for tissue culture and NUNC immuno-modules were obtained from Gibco, Invitrogen, Paisley, PA4 9RF.

Foetal calf serum was obtained from Biosera, West Sussex.

Radioisotope  $^{32}\text{P}$  was obtained from Amersham Lifescience, Amersham, Buckinghamshire.

### **2.2 Antibodies and cytokines (see specific methods)**

The following antibodies were used:

MAB 227 (anti IL-6 receptor) from R and D Systems

F4/80 (rat anti mouse) from Serotec

Fox P3 (rabbit anti mouse) Santa Cruz

PE-conjugated anti-IL-6R (551850; BD Biosciences)

FITC-conjugated CXCR1 (neutrophils), CD14 (Monocytes), CD3 (T lymphocytes), CD4 (T lymphocytes) and CD19 (B lymphocytes) (BD Biosciences), FITC conjugated anti-ICAM-1(BD Pharmingen) and anti-VCAM-1. (Amersham Pharmacia Biotech)

The following cytokines were used

TNF $\alpha$ , IL-6, sIL-6R, IL-1 (R and D systems)

### 2.3 General Buffers

Distilled water for the preparation of buffers was obtained from a millipore reverse osmosis system followed by filtration through a charcoal resin and two ion exchange resin columns using a millipore Milli-Q system.

PBS used for FACS buffer was sterile and supplied diluted from Gibco, Invitrogen, Paisley. PBS used for wash buffers was supplied in tablet form by Oxoid, Basingstoke, UK. One tablet was dissolved per 100 ml of dH<sub>2</sub>O.

**FACS Buffer:** 5 g of BSA was dissolved in 500 ml PBS to make a 1% solution. 0.4 g of sodium EDTA was added and 0.01% sodium azide. The solution was stored at 4°C.

**Cell Lysis Buffer:** 2.4 g Tris and 7.56 g of ammonium chloride (NH<sub>4</sub> Cl) was dissolved in 750 ml of distilled water (dH<sub>2</sub>O). The pH of the solution was adjusted to 7.2 with HCl and then made up to 1 litre with dH<sub>2</sub>O.

**Wash Buffer:** PBS contained 0.05% or 0.1% Tween-20.

**Citrate Buffer (0.2M):** 8.4 g citric acid, was dissolved in 200 ml dH<sub>2</sub>O then pH adjusted to 3.95 by the addition of a potassium hydroxide solution.

**Tetramethylbenzadine (TMB):** 240 mg of TMB was dissolved at a concentration of (0.1M) in 5 ml of dimethyl sulphoxide (DMSO) and 5 ml of ethanol, and stored at 4°C.

**ELISA Developing Buffer:** was freshly prepared on each occasion by the addition of 10 µl Hydrogen Peroxide and 100 µl TMB to 10 ml of Citrate Buffer per 96 well plate.

**Tris Buffered Saline (X10):** was prepared by dissolving 61 g of TRis and 90 g of NaCl into 1 litre of distilled H<sub>2</sub>O. pH was adjusted to 7.6 using concentrated HCl.

**Na Citrate Buffer for antigen retrieval:** was prepared by dissolving 2.94 g of anhydrous tri-sodium citrate in 1 litre of distilled H<sub>2</sub>O. pH was adjusted to 6.0 using concentrated HCl and then 0.5 mls Tween-20 added before mixing.

## **2.4 Buffers for Western Blotting and EMSA**

**Buffer A for cytosolic protein extraction for 100mls** contained 10 mls of 100 mM HEPES pH 7.9, 10 mls of 15 mM MgCl<sub>2</sub>, 10 mls 100 mM KCl, made up to 100 mls with distilled H<sub>2</sub>O. Immediately before use protease inhibitors were added.

Dithiotrietol (DTT) to final concentration of 0.5 mM, PMSF to a final concentration of 0.5 mM, Aprotonin to a final concentration of 5 µg/ml, Pepstatin to a final concentration of 5 µg/ml, Leupeptin to a final concentration of 30 µg/ml.

**Buffer C for Nuclear Extraction** contained 20 mls 100 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) pH 7.9, 10 mls 15 mM MgCl<sub>2</sub>, 10 mls 100 mM KCl, 10 ml 2mM EDTA, 25 mls glycerol, 2.45 g NaCl, made up to 100 mls with distilled H<sub>2</sub>O. Immediately before use protease inhibitors were added Dithiotrietol (DTT) to final concentration of 0.5 mM, PMSF to a final concentration of 0.5 mM, Aprotonin to a final concentration of 5 µg/ml, Pepstatin to a final concentration of 5 µg/ml, Leupeptin to a final concentration of 30 µg/ml.

**Buffer D for Storage of Nuclear Extracts** contained 8 mM Hepes pH 7.9, 25 mM Kcl, 0.1 mM EDTA, 8% Glycerol, 0.5 mM DTT.

#### 2.4.1 Reagents for Running EMSA

**5X TBE** was prepared by dissolving 54 g Tris and 27.5 g Boric acid in 980 ml of dH<sub>2</sub>O and 20 ml of 0.5M EDTA (18.6 g in 100 ml of dH<sub>2</sub>O) was added. The solution was checked to ensure pH8.

**5X Reaction buffer stock mix** was prepared by adding 50 µl 1M HEPES, 250 µl 1M KCl, to 500 µl 100% Glycerol and 90 µl dH<sub>2</sub>O.

**5X Binding buffer** was prepared by mixing 89 µl 5X reaction buffer stock, 10 µl 10 mg/ml Acetylated BSA, 0.5 µl 1M DTT, 1.0 µl 0.1M PMSF.

#### 2.5 **Histological Buffers & Stains**

The following stock solutions were used routinely for all histological staining procedures:

**Neutral Buffered Formalin Solution (NBFS)** Tissue specimens were fixed in NBFS fixative comprised of 100 ml of 10x PBS, 100 ml of 37% w/v formaldehyde and 800 ml of dH<sub>2</sub>O. The pH was adjusted to 7.0 by the addition of a potassium hydroxide solution.

**Decalcification Buffer** Specimens were decalcified in a 10% formic acid solution, comprised of 100 ml of formic acid (Fisher, Loughborough, Leicestershire, UK), 50 ml of 37% w/v formaldehyde and 850 ml of dH<sub>2</sub>O.

**Tris Buffered Saline (TBS)** TBS was prepared by dissolving 61 g of TRIS (0.5M), 90 g of NaCl (1.5M) in 1 litre of dH<sub>2</sub>O. The pH of TBS was adjusted to pH 7.6 by the addition of HCl.

**May-Grunwald modified Wright –Giemsa stain** May-Grunwald stain was prepared by adding 25 ml May-Grunwald stain (Sigma diagnostics) to 25 ml Phosphate buffer pH 6.8 (Merck) (1/2 dilution). Giemsa was prepared by adding 45 ml Phosphate buffer to 5 ml Giemsa (Sigma diagnostics)(1/10 dilution).

**Eosin (1%)** 10 g of Eosin (Fisher, UK) dissolved in 1 litre of dH<sub>2</sub>O.

**Scott's Tap Water** 3.5g of NaHCO<sub>3</sub> and 20g of MgSO<sub>4</sub> dissolved in 1 litre of dH<sub>2</sub>O.

**Fast Green (0.02%)** 80mg of fast green (Sigma-Aldrich) dissolved in 400 ml of dH<sub>2</sub>O.

**Safranin-O (0.1%)** 400mg of safranin-O (Sigma-Aldrich) dissolved in 400 ml of dH<sub>2</sub>O.

**1% acetic acid** 4mls of glacial acetic acid (Fischer, UK) diluted in 400 ml of dH<sub>2</sub>O.

## 2.6 Cell Viability

### 2.6.1 Alamar Blue

Cell viability was assessed at the end of each fibroblast experiment using an alamar blue cell viability assay. Supernatant was removed from each well then 200 µl of alamar blue diluted 1:10 in serum free DMEM was added to each well. The samples were incubated at 37°C for 4 hours. 100µl of alamar blue was also incubated without cells.

Supernatant was transferred to a 96 well ELISA plate and read on a) 1 sec fluorescent and b) 2 wavelength settings (570 and 600 nm).

% Reduction was calculated.

$$\frac{\text{reading ...cells...treated} - \text{well...without...cells}}{\text{reading ...control...cells} - \text{well...without...cells}} \times 100$$

### 2.6.2 Trypan Blue

Cell viability for non-adherent cells was assessed using Trypan Blue exclusion. 50 µl of Trypan Blue solution was added to 50 µl cells. Total cell count and cell count of blue stained cells was performed. Non-viable cells stain blue as they are not capable of Trypan Blue

exclusion and therefore take up the blue stain. Percentage of non-viable blue cells was calculated.

## **2.7 Patient Samples**

Paired synovial fluid (SF) and serum samples were obtained from patients with RA (fulfilling the American College of Rheumatology [formerly the American Rheumatism Association] 1987 revised criteria) presenting with a joint effusion. Informed consent was obtained and ethical approval from Bro Taf and Bro Morgannwg research ethics committees was obtained (Ref numbers 02/4692, 02/4382 and 2005-035).

The samples were spun at 2000 rpm for 10 minutes at 4°C. Supernatant plasma and synovial fluid was removed and aliquots were stored at -70°C. Pellets obtained were resuspended and prepared for analysis by flow cytometry.

### **2.7.1 Collection of Samples from Patients Treated with Anti-TNF- $\alpha$ Therapies**

In order to assess whether sIL-6R levels have any bearing on response to anti-TNF $\alpha$  directed therapies, it was decided to measure sIL-6R levels in an inception cohort starting on anti-TNF $\alpha$  therapies. Baseline levels prior to commencement of therapy and week 14 samples were taken to assess whether these sIL-6R levels were altered in response to treatment. Disease activity scores (DAS) were used to assess the efficacy of treatment and to stratify patients into levels of response, i.e. good, reasonable or poor/treatment failures.

All patients starting on anti-TNF $\alpha$  therapies from May 2006 were invited to take part in this study. Any patients in agreement were consented. All patients commencing anti-TNF $\alpha$  therapies had a baseline serum sample taken prior to drug administration. Samples were spun

immediately at 2000 RPM for 10 minutes at 4°C and the serum obtained was frozen at -80°C until needed.

All patients had a DAS score at baseline which in all cases was  $\geq 6.3$  in accordance with BSR guidelines for the administration of anti-TNF $\alpha$  therapy in RA.

Further blood samples were taken at week 14 when patients attended for their initial efficacy assessment. sIL-6R ELISAS were performed as previously described.

## **2.8 Flow Cytometry**

### **2.8.1 Red Cell Lysis**

5 mls 7% dextran solution was added to 5 mls of blood mixed and left on ice for 40 mins then supernatant removed into a fresh tube. Supernatant obtained was spun at 1500 rpm for 10 minutes at 4°C. The pellet obtained was washed with cell lysis buffer (5 mls) then left on ice for 5 minutes and finally spun again. Cells were then resuspended in FACS buffer and a cell count performed.

Cells were resuspended at a concentration of  $1 \times 10^7$ /ml in a 1/10 dilution of mouse serum in FACS buffer in order to reduce non-specific staining.

### **2.8.2 Antibody Staining**

100  $\mu$ l of cells were placed in a round bottomed 96 well plate. 4  $\mu$ l of appropriate antibody was added to each well. Cells were labelled with PE-conjugated anti-IL-6R (551850; BD Biosciences). To determine the distribution of IL-6R on leucocyte sub-populations, the cells were stained with FITC-conjugated specific cell surface markers and gated according to their expression of CXCR1 (neutrophils), CD14 (monocytes), CD3 (T lymphocytes), CD4 (T lymphocytes) and CD19 (B

lymphocytes) (BDBiosciences). Appropriate isotype controls were used.

Samples were incubated for 30 minutes at 4°C in the dark. Samples were centrifuged to remove excess antibody (1300 G for 3 minutes at 4°C). Supernatant was tapped off and cells washed 3 times in FACS buffer. Cells were resuspended in 500 µl and analysed using a Becton and Dickinson Cytometer. The instrument was set to analyse appropriate cell type and compensation set using single stained wells. Data was acquired from 10,000 events and analysed using Cellquest Pro software.

## **2.9 Cytospin Preparations**

Cells were suspended at a concentration of 400 cells/mm<sup>3</sup> in isotonic saline and spun at 400 rpm for 30 minutes using a Shandon Cytospin 3. The cytopsin preparations were then allowed to dry.

### **2.9.1 Morphological Analysis of Cytospin Preparations using May-Grunwald method**

The slides were fixed in methanol for 15 minutes and then placed into May-Grunwald solution for 15 minutes, then into Giemsa solution for 15 minutes. The slides were washed in phosphate buffer for 2 minutes then air dried. The dry slides were mounted in DPX and examined under microscope.

## **2.10 Enzyme Linked Immunosorbent Assay (ELISA)**

In all cases 100 µl coating antibody solution was incubated in a 96 well plate overnight at 4°C (10 µl of capture antibody diluted in 10 mls PBS).

### **2.10.1 IL-6, CXCL8**

Plates were washed with PBS containing 0.1% Tween. 300 µl of 0.5% BSA solution was added to each well and left at room temperature for 2

hours. Following washing serial dilutions of antibody standard (sensitivity range: 1000 pg/ml-15.625 pg/ml) were added to the plate together with dilutions of samples to be tested. These were tested in duplicate or triplicate. 50 µl detection antibody diluent (4 µl of detection antibody diluted in 5.5 mls DMEM/F12) were added to each well and incubated for 2 hours at room temperature on a varishaker. Following washing 100 µl of Streptavidin HRP diluent was added to each well (1/4000 for IL6 and 1/8000 for CXCL8) and incubated for 30 minutes. After further washing 100 µl of developing buffer was added to each well. The reaction was stopped with 50 µl of 10% sulphuric acid. The plate was read in a plate reader at OD 540 nm.

#### 2.10.2 CCL5, CCL2, sIL-6R

Plates were washed with PBS containing 0.05% Tween. 300 µl of 1% BSA solution was added to each well and left at room temperature for 1 hour. Following washing serial dilutions of antibody standard (sensitivity range 2000 pg/ml - 31.5 pg/ml) were added to the plate together with dilutions of samples to be tested. These were tested in duplicate or triplicate and incubated for 2 hours at room temperature on a varishaker. The wells were then washed X3 in PBS/Tween and 100 µl of biotinylated secondary antibody added to each well at a concentration of 100 ng/ml. The plate was washed and 100µl of a 1/200 dilution of Streptavidin added to each well for 20 minutes. After further washing 100µl of developing buffer was added to each well. The reaction was stopped with 50 µl of 10% sulphuric acid. The plate was read in a plate reader at OD 540 nm.

### **2.11 Neutrophil Extraction**

In separate experiments whole blood was obtained from healthy consenting volunteers and RA patients for neutrophil extraction.

Red cells (RBC) were removed with 6% dextran on ice for 60 minutes. Leucocyte enriched plasma obtained was overlaid on Ficoll-Hypaque

(Sigma-Aldrich) and centrifuged at 400G for 30 minutes. Contaminating RBC were removed by hypotonic lysis as above.

The pellet obtained was resuspended in Hanks balanced salt solution (HBSS) and washed. Cell viability was assessed using trypan blue exclusion method. Samples which had fewer than 5% stained cells were counted as viable. Purity was confirmed using morphological analysis of cytopsin preparations.

PMN were resuspended at a concentration of  $2 \times 10^6$ /ml and stimulated with increasing concentrations of TNF $\alpha$  (0-10 ng/ml) for 30 minutes at 37°C in a humidified incubator containing 5% CO<sub>2</sub>. The cells were then separated by centrifugation. Cell surface expression of IL-6R, L-Selectin (BD Biosciences) and CXCR1 was assessed by flow cytometry using method as described above.

## **2.12 Immuno-histochemistry on Frozen Rheumatoid Arthritis Sections**

Synovial tissue samples were taken at joint replacement and immediately frozen in preparation for histological analysis. Tissue was frozen in isopentane on dry ice and stored at -20°C. Frozen sections were then cut using a cryostat and after drying, fixed in 100% cold methanol. 2-colour immuno-fluorescence on serial sections was carried out to identify location of cell nuclei in relation to vascular markers. CD31 was used as an endothelial marker. CD90 was used as an endothelial/ fibroblast marker. DAPI was used as a nuclear stain. Using this technique it was possible to differentiate vascular structures, fibroblasts and leucocytes.

A two step process was used for antibody staining; sections were incubated with 200  $\mu$ l of a primary antibody (diluted in PBS/1% BSA with azide) at 37°C for 2 hours. Monoclonal mouse anti-CD31 and mouse anti-CD90 were used at a dilution of 1/200 (BD Biosciences) and then incubated with 200  $\mu$ l fluorescent conjugated secondary

antibody (Alexa 594 Rhodamine red fluorescent labelled antibody at a dilution of 1/1000) or 4-6-Diamidino-2-phenylindole (DAPI) blue fluorescent which was used at 1/1000 as a nuclear counter-stain for 1 hour in a humid chamber at 37°C. Slides were mounted using vectashield and checked using appropriate filters (Rhodamine used filter N2.1 and DAPI: filter A). Images were analysed using lab 309 software.

#### 2.12.1 Detection of IL-6R Expression

Serial sections were then stained for IL-6R using the same technique in order to localise the site of IL-6R expression within the joint and to identify whether IL-6R expression was confined to leucocytes. Mouse anti-IL-6R (MAB 227) (1 mg/ml) was used at dilutions from 1/10 to 1/100 using the protocol described above.

### **2.13 Synovial Fibroblast Culture**

Ethical approval was obtained from Bro-Taf Ethics Committee (Ref.02-4692). RA fibroblasts were obtained from synovial tissue of patients with RA and OA undergoing joint replacement surgery. Using a sterile scalpel and scissors the tissue was chopped into small pieces. Collagenase (750 iu/ml) (Sigma-Aldrich) was prepared and filtered through a 0.2 µm filter with a 20 ml syringe. 30 ml of Collagenase solution was added to the tissue pieces and placed in a shaking water bath at 37°C for 2 hours. After leaving to settle all liquid was pipetted into a fresh centrifuge tube and remaining tissue discarded. Cell suspension was topped up to 50 mls with Dulbecco's modified Eagle's medium (DMEM F12 (1:1)) containing 10% Fetal Calf serum (Life Technologies Invitrogen, Paisley). The cell suspension was spun at 1000 RPM for 10 minutes and cells obtained suspended in media. The cell supernatant was respun as before and further cell pellets obtained. Cells obtained were placed into T25 flasks and cultured at 37°C with 10% CO<sub>2</sub>. Cells obtained were cultured in DMEM F12 (1:1) containing 10% Fetal Calf serum, 1% L-Glutamine, 1% Insulin-transferrin-

selenium, 10 iu/ml penicillin and streptomycin. The cells were fed twice a week by replacing media with 10 ml fresh media on each occasion. Once cells reached confluence they were removed from the flask using 1/10 dilution of Trypsin-EDTA in sterile PBS. The cell suspensions were spun at 1000 RPM for 5 minutes and resuspended in culture media. Each T25 flask was placed into a T75 flask. Each T75 flask was subsequently split into 2 T75 flasks. The cells were taken to fourth passage then the fibroblasts were plated out at a standard concentration of  $5 \times 10^4$ /ml in appropriately sized plates for each specific experiment, grown to confluence then growth arrested for 48 hours in serum-free media.

## **2.14 Cytokine Production by Synovial Fibroblasts**

The cells were taken to fourth passage then the SF were plated out at  $2.5 \times 10^4$ /well in 48 well plates, grown to confluence then growth arrested for 48 hours in serum-free media.

The SF were stimulated with TNF $\alpha$  (0-1 ng/ml) and IL-6 with sIL-6R (0-200 ng/ml) (R & D Systems). Cells were stimulated by each cytokine singly and in combination. A dose response and time-course was performed for each cytokine. Viability was assessed at the end-point of each experiment using Alamar blue method. Cell supernatants were stored at -70°C until required. Cytokine production was assayed using ELISA methods detailed in section 2.10.2.

### **2.14.1 Inhibition of Cytokine Production in Rheumatoid Arthritis**

#### **Fibroblasts**

In further experiments cells were stimulated with 100 pg/ml TNF $\alpha$  and 50 ng/ml IL-6 with sIL-6R as above.

#### 2.14.1.1 TNF- $\alpha$ Inhibition

The action of TNF $\alpha$  was blocked using 0-0.1 $\mu$ g/ml etanercept (TNF receptor fusion protein) (Wyeth) in order to provide complete and incomplete blocking of TNF $\alpha$ .

#### 2.14.1.2 IL-6 Inhibition

Action of IL-6/sIL-6R complex was blocked using 50-500 ng/ml MAB227 (anti IL-6R antibody) (R&D Systems). The inhibitors were added at the same time as the stimulating cytokines.

The supernatants were removed after 20 hours and analysed using paired antibody ELISA for chemokine expression. CXCL8 (BD Pharmingen.), CCL5 and CCL2 (R&D Systems) were measured using commercially available kits as described in sections 2.10.1 and 2.10.2.

#### 2.14.2 Adhesion Molecule Expression in Synovial Fibroblasts

SF at passage four were obtained as described in section 2.13. Cells were grown to confluence in 6 well plates in order to obtain sufficient numbers for flow cytometry. Cells were stimulated with 10 pg/ml and 1 ng/ml TNF $\alpha$  and 100 ng/ml IL-6 with sIL-6R. Following removal of the supernatants cells were harvested washed and prepared for flow cytometry. Cells were resuspended at  $1 \times 10^6$ /ml. 100  $\mu$ l of cell suspension was labelled with 4  $\mu$ l FITC conjugated anti-ICAM-1 (BD Pharmingen) and 4  $\mu$ l anti-VCAM-1 (Amersham Pharmacia Biotech).

### **2.15 Electrophoretic Mobility Shift Assay (EMSA)**

EMSA techniques were used in order to identify which cell signalling cascades were stimulated by particular cytokines and the effect of blocking cytokines on cell signalling was assessed.

### 2.15.1 Nuclear Protein Extraction

Initial results obtained using a laboratory method optimised for NFκB expression produced disappointing results for STAT signalling therefore the extraction protocol was changed following discussion with colleagues.

### 2.15.2 Method 1

#### 2.15.2.1 Materials

Buffer A, C and D (see section 2.4)

Dithiotrietol (DTT) 0.5 mM, PMSF 0.5 mM, Aprotonin 5 µg/ml, Pepstatin 5 µg/ml, Leupeptin 30 µg/ml

#### 2.15.2.2 Method

Cells were stimulated for 1 hour. Stimulation was terminated by the addition of 1 ml ice cold PBS. PBS was aspirated and replaced with a further 1 ml of ice cold PBS. Cells were removed by scraping with a rubber spatula and pelleted in the micro-centrifuge (3000 rpm for 5 minutes). Pellet obtained was washed then resuspended in 200 µl buffer A with protease inhibitors added and left on ice for 5 minutes. 5 µl IPEGAL was added and mixed with a pipette. Samples were spun at 13000 RPM for 5 minutes. The supernatant obtained was the cytosolic extract which was stored at -70° C until required. The cell pellet was resuspended in 50 µl cold buffer C containing protease inhibitors and vortexed. The sample was mixed on the varishaker at 4° C for 1 hour with frequent vortexing. The sample obtained was then spun at 13000 RPM for 5 minutes and supernatant kept as nuclear extract. 150 µl buffer D containing PMSF was added to the sample for storage at -70° C.

### 2.15.3 Method 2 (Optimised for STAT)

Phosphatase inhibitors were added to Buffers A and C (2  $\mu$ l sodium orthovanadate and 10  $\mu$ l sodium fluoride were added to 1 ml of each buffer respectively).

Cytosolic extraction occurred in 20  $\mu$ l Buffer A for 10 minutes. Nuclear extraction occurred in 20  $\mu$ l buffer C for 20 minutes. The samples were stored without the addition of Buffer D. A flow diagram detailing the final method used is shown in figure 2.1.

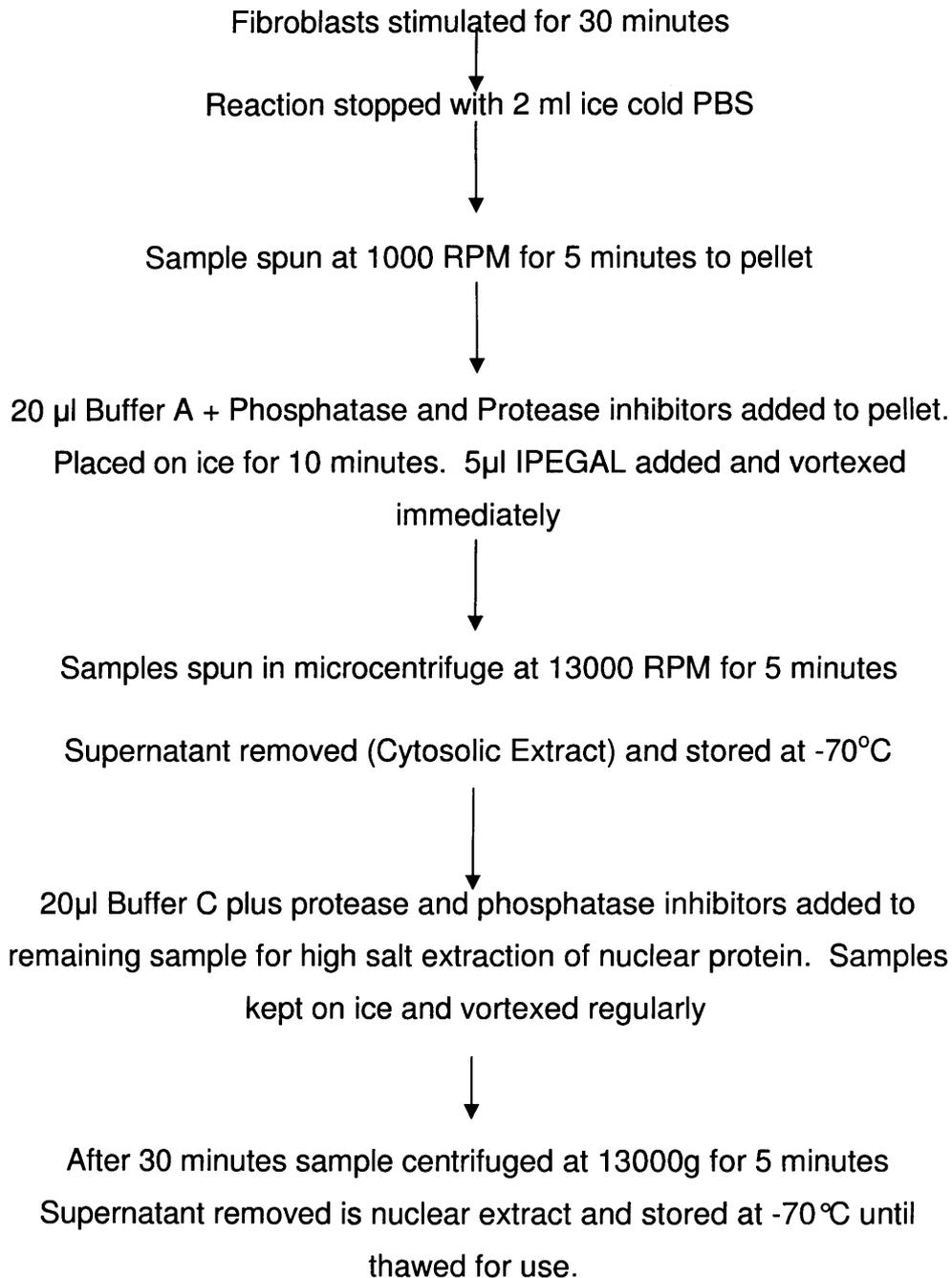
### 2.15.4 Pierce BCA Protein Assay

Protein concentrations were measured before use in order that equivalent amounts of sample could be added to a gel.

BSA standard at 2  $\mu$ g/ml was used. Doubling dilutions were performed in a 96 well plate. 10  $\mu$ l of each dilution was transferred across to the first 2 columns to act as a standard. Samples to be tested were diluted 1:2, 1:4, 1:8, 1:16, 1:32 and 1:64 in PBS.

200  $\mu$ l of BCA solution (50 parts reagent A to 1 part reagent B) was added to each standard and sample. Samples were incubated at 37°C and plate read at 30 minutes and 1 hour. High protein levels resulted in a colour change from green to purple. Samples were read in the plate reader at 540nm. A standard curve was plotted and protein concentration calculated.

**Figure 2.1 - Flow diagram showing final method for nuclear extractions**



## 2.15.5 Radio-Labeling Double-Stranded Oligonucleotide Probes for EMSA

Double-stranded oligonucleotide probes were generated by annealing complementary oligonucleotide primers with overhangs labelled with Klenow fragment of DNA polymerase. The method is described below.

### 2.15.5.1 Annealing Oligonucleotide Primers for Use as an EMSA Probe

Reverse complementary oligonucleotide primers containing the transcription factor binding site of interest or region of gene promoter to be studied were ordered containing short 5' of 3 to 4 bases overhangs containing the complement base of the label deoxynucleotide (A for  $[^{32}\text{P}]\alpha\text{-dTTP}$ ). The primers were resuspended at  $1\ \mu\text{g}/\mu\text{l}$  in double distilled water.

To anneal the primers, the following reaction mix was made in a 1.5 ml Eppendorf.

- a) Sense primer ( $1\ \mu\text{g}/\mu\text{l}$ )       $10\ \mu\text{l}$
- b) Antisense primer ( $1\ \mu\text{g}/\mu\text{l}$ )     $10\ \mu\text{l}$
- c) 1M NaCl                               $10\ \mu\text{l}$
- d)  $\text{dH}_2\text{O}$                                  $70\ \mu\text{l}$

The annealing mix was heated to  $95^\circ\text{C}$  for 10 mins in a water bath then allowed to cool to room temperature overnight. The annealed oligonucleotide probe ( $100\text{ng}/\mu\text{l}$ ) was then stored at  $-20^\circ\text{C}$ . Before use it was diluted 1:10 in  $\text{dH}_2\text{O}$  to  $10\ \text{ng}/\mu\text{l}$ .

#### 2.15.5.2 Labelling of Oligonucleotide Probe

Several hours before use the lead-lined pot containing [ $^{32}\text{P}$ ] $\alpha$ -dTTP was removed from the freezer and allowed to thaw. Labelling reaction mixture was prepared (minus radionucleotide and Klenow fragment). by adding 2.5  $\mu\text{l}$  oligonucleotide probe, 1  $\mu\text{l}$  (2.5 mM) of unlabelled mix of dNTPs (dATP, dCTP, dGTP), 5  $\mu\text{l}$  of 10x Klenow buffer, 5  $\mu\text{l}$  of 1 M NaCl to 32.5  $\mu\text{l}$  of double distilled water. Radionucleotide (3.0  $\mu\text{l}$  of  $^{32}\text{P}\alpha$ -dTTP) and 1.0  $\mu\text{l}$  Klenow fragment (2.5 U/ $\mu\text{l}$ ) were added to the labelling reaction in the hot lab workstation behind a perspex shield, using a pipette with a disposable barrel. The covered perspex box was used to hold the Eppendorf containing the labelling reaction. This made a total of 50  $\mu\text{l}$  of labelling reaction.

The mixture was then incubated at room temperature for 10-20mins. The reaction stopped by adding 2.0  $\mu\text{l}$  0.5 M EDTA (pH 8) and STE 50.0  $\mu\text{l}$ . Labelled probes were purified using probe quant micro-columns (Amersham). The end was broken off the column and the cap loosened then placed in a 1.5 ml Eppendorf without a lid and spun at 3000 rpm in a microfuge for 1 min before placing the column into a fresh 1.5 ml Eppendorf. 50  $\mu\text{l}$  labelling reaction was then added and spun at 3000 rpm for 2 mins. The column was then discarded into  $^{32}\text{P}$  waste. The labelled probe was placed into a fresh labelled Eppendorf and stored at  $-20^{\circ}\text{C}$  in a labelled yellow lead-lined container.

#### 2.15.6 Electrophoretic Mobility Shift Assay (EMSA)

Polyacrylamide gels were prepared to the following recipe, poured between an outer and inner glass plate separated by plastic spacers and left to set for approximately 15 mins. A teflon comb was inserted into the gel in order to form sample wells. Wells were washed thoroughly with 0.5X TBE buffer and filled with fresh 0.5X TBE buffer.

### 2.15.6.1 Recipe for Mini-Gel

	<u>1X</u>	<u>2X</u>
dH <sub>2</sub> O	29.5ml	44.5ml
Acrylamide stock (40%)6% or 8%	7.5ml/10ml	11.25/15ml
5x TBE	5ml	7.5ml
50% Glycerol	5ml	7.5ml
10% Ammonium Persulphate	0.5ml	0.75ml
TEMED	40μl	60μl

Radio-labelled probe was removed from lead pot behind perspex shield, placed in the covered perspex box and allowed to thaw for 30 mins.

Binding reaction was prepared by adding 10 μg of nuclear extract to a pre-mix of 5x binding buffer, 1 μl (1 mg/ml) of non-specific DNA competitor (polydIdC) and double distilled water. The resulting mixture was vortexed and incubated at room temperature for 10 mins. For supershift assays 2-10 μl antibody against proteins of interest or irrelevant control antibody were also added at this stage.

The next steps were performed behind the perspex shield with the samples in a covered perspex box. 2μl <sup>32</sup>P-labelled probe was added to samples, vortexed and incubated at room temperature for 20 mins.

Binding reaction was stopped by addition of 1/10 volume of 6X DNA loading buffer. Gels were loaded with gel loading tips then connected to the power supply and run at 180 V for 3 hrs & 30 minutes for NFκB and STAT probes. After disconnecting the power, the gel was stopped and transferred to 3M Whatman paper. The gel was covered with Saran Wrap and dried on the vacuum drier at 80°C for 2 hrs. The dried

gel was then exposed to X-ray film (overnight to 72 hrs for NFkB and STAT) and developed by autoradiography.

## **2.16 Induction of Murine Collagen Induced Arthritis**

Murine collagen induced arthritis (mCIA) was used as a model for RA in order to investigate therapeutic strategies. The model is widely recognised as the gold standard for the testing of therapies in particular biological reagents. The predictable and reproducible clinical course provides an ideal opportunity to treat animals prior to disease onset and in early or well established disease.

Male DBA/1 mice aged 7-8 weeks old were purchased from Harlan. Mice were housed in cages of up to 10 animals at Biomedical Services, Heath Park. Mice were allowed free access to food and water and kept in light/dark cycles of twelve hours.

Arthritis was induced following a published protocol with ethical approval and procedure outlined in home office licence PPL 30/1820.

### **2.16.1.1 Preparation of Freund's complete adjuvant (CFA)**

CFA was prepared by grinding 100 mg heat-killed *M. tuberculosis* (Difco laboratories Detroit) in 20 ml Incomplete Freund's Adjuvant (Sigma chemicals).

### **2.16.1.2 Preparation of Emulsion**

- a) Type II chicken collagen (MD Biosciences) at 2 mg/ml was dissolved overnight in 10 mmol glacial acetic acid at 4 °C and stirred constantly with a magnetic stirrer (8 mg collagen + 4.0 ml acetic acid).
- b) Equal volumes of dissolved chicken collagen solution and CFA were mixed in a glass syringe until a stiff emulsion was formed.

Mice were anaesthetized with isofluorane/oxygen and immunized on 2 occasions (day 0 and day 21). Mice were injected intradermally with an emulsion of type II collagen in complete Freund's adjuvant (50 µl) into 2 sites in the flank. At Day 21, animals received a second booster immunisation with type II collagen via the intradermal route (50 µl emulsion in complete Freund's adjuvant). Experiments were performed using 6 mice per condition. Clinical scores were performed daily together with paw diameter measurements using a spring-loaded micrometer. Animals were sacrificed at day 34 or earlier if severity limits were reached. Maximum severity limits allowed under terms of project licence 30/2361 were a score of 5 in any single paw or a combined score of 14 in all 4 paws.

## **2.17 Treatment of Arthritis**

### **2.17.1 Arthritis Prophylaxis Experiment**

Arthritis was initiated as above. Mice were injected with soluble-gp130:Fc fusion protein (a kind gift from Dr Stefan Rose-John, Kiel University), etanercept (10 mg/kg by I-P injection) and a control group with phosphate buffered saline (PBS) at day of first collagen injection (Day 0) and then twice-weekly until Day 21 (See section 5.2.1 for full experimental details). This experiment allowed examination of the effect of therapeutic strategies in prevention of disease onset.

Sgp130:Fc was produced by fusion of the extra-cellular portion of gp130 to the constant portion of a human IgG1 antibody protein as described by Jostock. It was demonstrated in this experiment that due to steric hinderance sgp130 had no access to membrane bound IL-6/sIL-6R and therefore specifically targeted trans-signalling. It was also shown that sgp130 provided only weak inhibition of oncostatin M and LIF responses (Jostock *et al* 2001).

The dose of etanercept was chosen as a result of a literature review. It was hoped that the dose chosen might provide incomplete resolution of

disease in order to mimic the human situation where arthritis severity may be reduced without remission occurring.

#### 2.17.2 Treatment of Established Disease with sgp130:Fc or Combination Therapy with Etanercept and sgp130:Fc

In RA, therapy would not be administered except in established disease. Therefore, in order to provide a more appropriate representation of clinical practice, therapy was not initiated until clinical signs of arthritis were present.

Arthritis was initiated as described above. When 75% of mice displayed clinical signs of arthritis (generally day 27) therapy with etanercept, sgp130:Fc fusion protein or PBS was commenced. sgp130 was added to etanercept after a few days in order to examine the effects of addition of sgp130 to therapy where TNF $\alpha$  had been partially suppressed (Experiment design is discussed in section 5.2.2).

Animals were scored then divided into 4 equal scoring groups:

- Group 1 - PBS
- Group 2 - etanercept 2.5 mg/kg/mouse on alternate days
- Group 3 - sgp130;Fc 2.5 mg/kg/mouse on alternate days
- Group 4 - Initially etanercept 2.5 mg/kg/mouse on alternate days. After 3 days sgp130:Fc (2.5 mg/kg) was added to their treatment

#### 2.17.3 Effect of High Dose sgp130:Fc Monotherapy and in Combination with Etanercept in Established mCIA

Following clinical scoring from previous experiments the protocol for therapeutic administration was altered. Animals were dosed daily and

the dose of sgp130 was increased to 5 mg/kg per mouse (experiment design discussed in section 5.2.3).

- Group 1 - PBS
- Group 2 - etanercept 2.5 mg/kg/mouse daily
- Group 3 - sgp130;Fc 5 mg/kg/mouse daily
- Group 4 - Initially etanercept 2.5 mg/kg/mouse daily. After 3 days sgp130:Fc was added to their treatment

#### 2.17.4 Early Intervention with Combination Therapy in mCIA

Having shown that combination therapy affected the clinical course of disease without necessarily affecting histological endpoints it was decided to perform an experiment in which combination therapy was tried from the outset rather than after 3 days of etanercept therapy alone. Additionally, in order to maximise the possibility that therapies could affect histological outcome, therapies were commenced in each animal when it first developed signs of arthritis (see section 5.2.4).

- Group 1 - PBS
- Group 2 - etanercept 2.5 mg/kg/mouse daily
- Group 3 - sgp130;Fc 2.5 mg/kg/mouse daily
- Group 4 – etanercept 2.5 mg/kg + sgp130:Fc 2.5 mg/kg/mouse daily

### **2.18 Assessment of Arthritis Severity**

#### 2.18.1 Clinical Gradation of Arthritis Severity.

The severity of arthritis was judged using an established in-house clinical scoring system (0-5 for any one paw) coupled with diameter measurements of paw volume using a digital micrometer.

## Paw Scores

- 0 Normal
- 1 Mild/moderate erythema and swelling
- 2 Severe erythema and swelling affecting entire paw or joint
- 3 Up to 3 joints affected by arthritis
- 4 Greater than 3 joints affected by arthritis
- 5 Deformed paw or joint with ankylosis

Severity limits were reached when a total score of 14 or 5 in any joint was reached.

### 2.18.2 Histological Assessment of Arthritis Severity

#### 2.18.2.1 Sample Preparation

At experimental end point, mice were sacrificed and the limbs prepared for histological examination.

Joints were fixed in neutral buffered saline for 1 week then decalcified with 10% formic acid in neutral buffered formaldehyde (NBFS) at 4°C for 3-4 weeks. The decalcifying solution was changed twice a week.

#### 2.18.2.2 Ammonium Oxalate Test For Decalcification

3 ml of decalcifying solution was removed from mouse joints and placed in a test tube. Fluid was neutralized by the addition of concentrated ammonia solution until just neutralised. 3 ml of saturated ammonium oxalate solution was added to the solution and left to stand for 10 minutes. Precipitate was assessed after 10 minutes. Once no precipitate was visible samples were ready for processing.

### 2.18.2.3 Shandon Tissue Processor Cycles

Murine Knee Joints: 70% alcohol (30mins), 90% alcohol (1 hour), 100% alcohol (1 hour), 100% alcohol (1 hour), 100% alcohol (1 hour), 100% Alcohol (1 hour), xylene (1 hour @ 37°C), xylene (1 hour @ 37°C), xylene (1 hour @ 45°C), wax (1 hour @ 60°C), wax (1 hour @ 60°C), wax (1 hour @ 60°C), wax (1 hour @ 60°C).

### 2.18.2.4 Tissue Embedding

Joints were removed from cassettes and embedded in paraffin wax at 60°C. Cassette tops were replaced on sample. Samples were placed on cold plate for 2 hours then stored at 4°C until sectioning.

### 2.18.2.5 Sectioning of Joints Embedded in Paraffin Wax Blocks

Sections were trimmed and 7 µm serial sections were cut using a microtome. Superfrost plus slides were used. Slides were placed in a 37°C incubator overnight then stored at room temperature until used.

## 2.18.3 Analysis of Histological Changes to the Joint

### 2.18.3.1 Haematoxylin and Eosin (H and E) Staining

H and E staining was used to assess leucocyte infiltration, synovial hyperplasia and joint and bone destruction. Slides were de-paraffinised with 3 changes of xylene (5 minutes each) then descending grades of alcohol (100% $\times$ 2, 90%, 70% for 3 minutes each) and then washed in running tap water for five minutes.

Slides were then rinsed in distilled water followed by staining in Harris's haematoxylin for 90 seconds. Slides were washed well in running water for 5 minutes then rinsed in distilled water. Slides were then blued in Scott's tap water and examined under microscope (Excess haematoxylin was removed if necessary with 1% acid alcohol).

Slides were washed well in running tap water, rinsed in distilled water then stained in eosin for 2-5 minutes, washed quickly in running tap water then dehydrated in ascending grades of alcohol (90%, 100% x3 for 2-3 minutes each). Slides were then cleared in xylene x3, 5 minutes per wash. Slides were kept in xylene until mounted in Ralmount and cover slip applied in charcoal filter extractor. Slides were then left in 45 °C oven overnight then scored.

### *2.18.3.2      Histological Scoring*

The sections were graded subjectively by 2 or 3 independent observers who were blinded to treatment allocation for each animal section. Each parameter was scored as below:

Hyperplasia (0-3)

Infiltrate (0-5)

Exudate (0-3)

Bone and Cartilage erosion (0-3)

Maximum possible score-15

The components were added to give a composite score or arthritis index (AI).

### *2.18.3.3      Safranin–O Fast Green Staining*

In order to demonstrate cartilage depletion within the joint Safranin-O fast green staining was used. Within healthy cartilage proteoglycans (PG) and glycosaminoglycans (GAGS) stain red with safranin-O. In areas of cartilage damage depletion of PG and GAGS is demonstrated by a loss of bright red staining.

Slides were de-paraffinised with 3 changes of xylene (5 minutes each) then descending grades of alcohol (100 x2, 90%, 70% for 3 minutes each) and then washed in running tap water for 5 minutes. Xylene 5

mins x3 followed by brief immersion in distilled water. Slides were then stained in haematoxylin for 2 minutes, placed in 0.05% acid alcohol for 10 seconds then washed in running water for 5 minutes. Slides were then immersed briefly in distilled water before staining in 0.02% fast green for 3 minutes, rinsed in 1% acetic acid for 10 seconds then stained in Safranin-O for 5 minutes. Slides were washed in running water for 5 minutes, immersed briefly in distilled water then dehydrated in ascending grades of alcohol (90%, 100% x3 for 2-3 minutes each). Clearing in 3 xylene washes each of 5 minutes was then carried out.

For the second alcohol run, specific Safranin-O alcohols were used in order to avoid contamination of stock alcohols. Slides were then mounted in Ralmount and left overnight at 37°C.

#### 2.18.3.4 Tartrate Resistant Acid Phosphatase (TRAP) Stain

To identify areas of osteoclast activity, staining for Tartrate resistant acid phosphatase was carried out. This is an enzyme which is produced specifically by osteoclasts within the murine joint.

Samples were deparaffinised in xylene as above then hydrated in alcohol and water.

0.2M acetate buffer (in a 50 ml coplin jar sufficient for 5 slides) was prepared by adding 0.82 g of sodium acetate and 0.58 g of L(+) tartaric acid to 50 ml of deionized water. The solution was stirred using a stir plate until dissolved, then pH was adjusted to 5 with NaOH.

Slides were placed in 0.2 M Acetate buffer at room temperature for 20 minutes. At the end of 20 minutes to the same buffer was added 0.5 mg/ml naphthol AS-MX phosphate (25 mg) and 1.1 mg/ml fast red TR salt (55 mg). Slides were incubated in this solution for 3-4 hours at 37°C until osteoclasts stained bright red. The slides were then placed in de-ionised water at 37°C for 5 minutes. Slides were counter-stained in haematoxylin for 90 seconds and then placed in dH<sub>2</sub>O for 5 minutes, followed by Scott's tap water for 30 seconds. Slides were rinsed in

running tap water then mounted with Crystalmount and a cover slip applied.

#### 2.18.4 Immuno-Histochemistry

The distribution of specific leucocyte subsets within murine joints was assessed using immuno-histochemical techniques. Macrophages were identified using an anti-F4/80 antibody, regulatory T-cells using an anti Fox-P3 antibody. In addition, an anti-CD4 antibody was tried to attempt to identify CD-4 positive T cells. All immuno-histochemical procedures were performed upon sections mounted on superfrost plus slides.

##### 2.18.4.1 F4/80 Staining

R & D Systems' rat cell and tissue staining kit was used for all steps where indicated. Sections were de-paraffinised by immersing slides in 3 changes of xylene (5 minutes), then washed in descending alcohols (100%, 100%, 90%, 70% x3 minute washes). Slides were rehydrated by washing in running tap water for 5 minutes, washed in distilled water for 5 minutes and then washed in TBS for 5 minutes. Antigen retrieval was carried out using Trypsin-EDTA 0.1% diluted 1 in 5 in TBS at 37°C for 30 minutes. Slides were washed in TBS (5 minutes x2) then 120 µl of peroxidase blocking reagent was added to the slides (5 minutes room temperature, R & D Systems). Slides were rinsed with TBS prior to gentle washing in TBS (5 minutes). Sections were incubated with 120 µl of serum blocking reagent G (15 minutes, room temperature, R & D Systems Europe). Slides were rinsed with TBS, prior to gentle washing in TBS (15 minutes). Avidin and biotin blocking steps were carried out according to the manufacturer's instructions. Following rinsing in TBS, the sections were incubated with rat anti-mouse F4/80 antibody (diluted 1/50 in TBS) or appropriate isotype control (1/400) overnight at 4°C. Sections were washed in TBS (15 minutes X3) and the excess TBS removed prior to incubating sections with biotinylated secondary antibody (rabbit anti-rat) for 1 hour at room temperature (R

& D Systems Europe). Sections were again washed in TBS (15 minutes X3) before adding 120 µl of HSS-HRP (high sensitivity streptavidin conjugated to horseradish peroxidase) for 30 minutes at room temperature. Sections were then developed using 3 drops of freshly prepared diaminobenzidine substrate (DAB) according to manufacturer's instructions and incubated for 20 minutes at room temperature. Sections were washed in distilled water, counterstained with haematoxylin and 'blued', in Scott's tap water, rinsed in dH<sub>2</sub>O then dehydrated by immersion in ascending alcohol washes (90% 1 minute, 100%, 100%, 100% 3 minutes) and xylene (2 x 5 minutes). Slides were mounted using Ralmounts.

#### *2.18.4.2 Fox-P3 Staining*

Fox-P3 was used as a regulatory T cell marker. R & D Systems' rabbit cell and tissue staining kit was used for all steps where indicated. Sections were prepared for staining as above and were incubated with rabbit anti-Fox-P3 antibody (diluted 1/200 in TBS) or appropriate isotype control (1/400) overnight at 4°C. Sections were washed in TBS (15 minutes X3) then the excess TBS removed prior to incubating sections with biotinylated secondary antibody (swine anti-rabbit) for 1 hour at room temperature (R & D Systems Europe). The streptavidin and DAB steps were then carried out as previously described before sections were counterstained, dehydrated and mounted.

#### *2.18.4.3 CD4 Staining*

CD4 staining was attempted using the previous protocol. No staining was obtained and therefore a further antigen retrieval step was attempted after the de-waxing step using sodium citrate buffer. Slides were immersed in 0.01 M citrate buffer heated to 90°C for 10 minutes, stood for 15 minutes and then allowed to cool. Unfortunately no staining was identified using this further antigen retrieval step.

## **2.19 Statistical Analysis And Presentation Of Results**

All results were expressed as the mean  $\pm$ SEM. All statistical differences determined in this study used the paired means student's t-test. p values of  $\leq 0.05$  were considered significant, with values of  $\leq 0.01$  considered highly significant.

### **3 ARE INFILTRATING LEUCOCYTES THE SOURCE OF sIL-6R IN RHEUMATOID ARTHRITIS?**

#### **3.1 Introduction**

In a normal joint synovial fluid is a hypocellular, avascular, liquid connective tissue in free connection with synovium and cartilage (Freemont and Denton 1991). In normal disease-free states fluid is present in small amounts, is viscid and contains less than 100 cells/mm<sup>3</sup>. The majority of cells in the fluid are synoviocytes or chondrocytes with low numbers of lymphocytes or macrophages seen (Freemont 1985). In diseased joints there is a large increase in the volume of synovial fluid produced and its composition and cellularity alter. During acute flares of RA, at which time the joint is most likely to be aspirated, the nucleated cell count rises to between 1500 and 50,000 cells/mm<sup>3</sup>. The majority of cells found within the joint effusion are polymorphs (55-90% of cells) together with a mixed lymphocyte population, macrophages, mast cells and synoviocytes (Davis and Freemont 1990).

Synovial leucocytes are known to be abundant during flares of RA. sIL-6R levels have been shown to correlate with leucocyte recruitment in RA. It is not clear whether infiltrating leucocytes were the source of sIL-6R in the RA joint. This chapter addresses whether leucocytes produce IL-6R on entry into the joint and whether TNF $\alpha$  may be partly responsible for this production.

It would appear that leucocytes within the synovial fluid of RA patients are different to those found at other sites of inflammation. Polymorphs containing phagocytosed immune complexes are clearly recognisable in the synovial fluid of RA patients. This specific population of polymorphonuclear cells are known as rhagocytes (Hollander *et al*

1965). Within the synovial fluid neutrophils appear resistant to normal apoptotic processes which impede their subsequent clearance from the joint. The mechanisms whereby neutrophils evade apoptosis have been investigated. Neutrophils derived directly from synovial fluid have resistance to apoptosis by neutrophils and lymphocytes even in very early disease (duration less than 3 months). The authors postulate that this is likely to be due to high levels of anti-apoptotic cytokines found within the joint in early disease (Raza *et al* 2006). Neutrophils from healthy volunteers incubated in the presence of RA synovial fluid demonstrate resistance to apoptosis which is thought to be mediated by the presence of pro-inflammatory cytokines and adenosine within the synovial fluid (Ottonello *et al* 2002).

It is still unclear whether the neutrophils within the joint are intrinsically different to circulating neutrophils and whether neutrophils obtained from healthy volunteers can be made to behave in the same way as those from RA patients. It appears that resistance to apoptosis may be partly due to the presence of synovial fluid and the unique cytokine and chemokine environment found within the inflamed joint. However, this does not exclude the possibility that neutrophils obtained from synovial fluid are structurally different to circulating cells. In addition, it is not yet clear whether this failure of normal apoptosis results in tissue damage although in support of this neutrophils can be seen to aggregate at sites of joint erosion.

Granulocyte and monocyte colony stimulating factor (GM-CSF) stimulation has been shown to rapidly increase oncostatin M production by circulating neutrophils from both RA patients and normal volunteers. However, GM-CSF could not up-regulate oncostatin-M production by SF neutrophils. The authors postulate that this is because SF neutrophils have already released and secreted oncostatin-M within the joint accounting for elevated intra-articular levels found in disease (Cross *et al* 2004).

Although the pathogenic processes underlying RA are not explained fully, the importance of leucocyte recruitment in RA pathogenesis is clear. Leucocyte recruitment within the joint is a highly regulated process controlled by chemokines (chemo-attractant cytokines) and adhesion molecule expression (Springer 1994). These processes are orchestrated by the specific cytokine environment in inflammatory disease which control the magnitude and phenotype of leucocytes recruited to the joint (Feldmann and Maini 1999; Taylor *et al* 1999; Taylor *et al* 2000; Buckley 2003a). Levels of IL-6 and soluble IL-6 receptor (sIL-6R) levels in RA have been shown to correlate with the degree of leucocyte infiltration into the joint (Desgeorges 1997; Polgar 2000). The presence of IL-6 and sIL-6R in synovial fluid would indicate the likelihood of IL-6 trans-signalling being involved in leucocyte recruitment and this would be attributable to the induction of specific chemokines (Nowell 2003).

The synovial infiltrate is predominantly composed of lymphocyte and macrophages (Sweeney and Firestein 2004). In acute synovitis with joint effusion the cell infiltrate within the effusion is mainly composed of neutrophils, with high percentage of macrophages but generally few lymphocytes (Davis *et al* 1988). Establishment of chronic synovitis in RA appears to be facilitated by influx of neutrophils, lymphocytes and macrophages together with disruption of normal apoptotic mechanisms leading to accumulation of leucocytes within the joint (Buckley 2003 b) . Leucocyte infiltration within the synovium results in chronic inflammation and causes up-regulation of cytokine, chemokine and growth factor production (Firestein *et al* 1990; Koch *et al* 1991; Kasama *et al* 2001). In addition neutrophils are partly responsible for bone and cartilage degradation within the joint by release of lysosomal enzymes and generation of oxygen free radicals (Edwards and Hallett 1997).

Nowell *et al* showed that native cells within the joint (specifically fibroblasts and chondrocytes) do not express IL-6R although these cells, particularly fibroblasts, produce the majority of IL-6 in the joint.

Therefore, the actions of IL-6 on these cells must occur via the trans-signalling mechanism (An alternative signalling mechanism which utilises sIL-6R is discussed in section 1.5.4). In order for trans-signalling to occur a source of sIL-6R is required for IL-6/sIL-6R complex generation.

To date, the source of sIL-6R in RA has not been identified conclusively. It has been shown that CRP, neutrophil activating CXC chemokines, complement components, leukotrienes (LTB<sub>4</sub>) and the lipid mediator PAF can stimulate sIL-6R production by human neutrophils through shedding of cognate IL-6R from the cell surface (Jones 1999; Jones 2001; Hurst *et al* 2001, Marin *et al* 2001, Marin *et al* 2002; McLoughlin *et al* 2004). More recently neutrophils were shown to shed IL-6R in response to apoptosis. This is less likely to be relevant within the joint where, as has been discussed already, neutrophils are relatively protected from apoptosis (Chalaris *et al* 2007). CRP levels are generally high in active RA and the level of CRP required for receptor shedding (50 µg/ml) correlates well with levels found in RA serum and synovial fluid (Rowe *et al* 1987). Although all the molecules that have been shown to cause IL-6R shedding may be implicated in RA pathogenesis; it is generally considered that TNF $\alpha$  plays a key role in leucocyte recruitment and activation in RA. The actions of TNF $\alpha$  on neutrophil IL-6R shedding have not been studied.

The specific aims of this chapter therefore were to:

1. Characterise the nature of infiltrating leucocytes within the RA joint using phenotypic markers and by morphological analysis.
2. Quantify IL-6R expression on each specific leucocyte subtype within the joint and compare this to IL-6R expression in matched whole blood samples.
3. Determine local and systemic sIL-6R levels in RA patients.
4. Assess the effects of TNF $\alpha$  on neutrophil IL-6R expression and consequent generation of sIL-6R.

## 3.2 Results

### 3.2.1 Optimisation of Protocols for Phenotypic Analysis of Leucocyte Populations

Initial experiments were undertaken to quantify IL-6R expression using circulating leucocytes obtained from healthy volunteers. Leucocyte specific phenotypic markers were used to identify leucocyte subsets. CD14 was used as a macrophage marker, CD3 and CD4 for T cell subsets, CD19 as a B cell marker and CXCR1 as a neutrophil marker. Although CXCR1 (CXCL8 receptor) is not expressed solely on neutrophils, the level of expression on neutrophils is much higher than on other leucocyte sub-groups and therefore can be used to identify this population of cells. LAP (leukocyte acid phosphatase) was also tried as a neutrophil marker but expression was low and levels were not well replicated between experiments. IL-6R expression was quantified on leucocytes using a PE conjugated antibody.

Following optimisation of single staining the distribution of IL-6R on leucocyte sub-populations in normal whole blood samples was determined. The cells were stained with FITC-conjugated specific cell surface markers and gated according to their expression of CXCR1 (neutrophils), CD14 (monocytes), CD3 (T lymphocytes), CD4 (T lymphocytes) and CD19 (B lymphocytes). Leucocyte subtypes were confirmed by morphological analysis of cytopsin preparations.

In order to assess the effects of sample storage upon expression of markers leucocytes were stained and left overnight in FACS buffer containing 10% Para formaldehyde prior to analysis by flow cytometry. When compared to fresh cells there were significant differences in cell morphology and IL-6R expression. Therefore fresh samples were used for all studies reported.

### 3.2.2 Characteristics of Rheumatoid Arthritis Patient Cohort

All patients fulfilled ACR criteria for diagnosis of rheumatoid arthritis. The mean age of the patients was 58 years and, of them, 63% were female.

For those patients whose leucocyte phenotypes had been analysed; further clinical information was obtained from medical records. Mean disease duration was approximately 8 years but 2 patients had disease duration of less than 2 years. The mean age of this patient sub-set was 59.8 years. There were equal numbers of patients with monoarticular and polyarticular flares. The mean level of CRP was 25 mg/l. The mean ESR was 44mm/hour although ESR measurements were not available for all cases. Patient medications included NSAID, oral prednisolone, sulphasalazine, methotrexate and hydroxychloroquine. Only 1 patient was treated with anti-TNF $\alpha$  therapy.

During the course of the research there was a large reduction in numbers of RA patients presenting with symptomatic joint effusions. Numbers of samples collected and stored reduced considerably during the course of the project (Table 3.1).

**Table 3.1 - Synovial fluid samples collected**

<b>YEAR</b>	<b>RA</b>	<b>OA</b>
2002	15	4
2003	27	1
2004	14	3
2005	7	
2006	1	

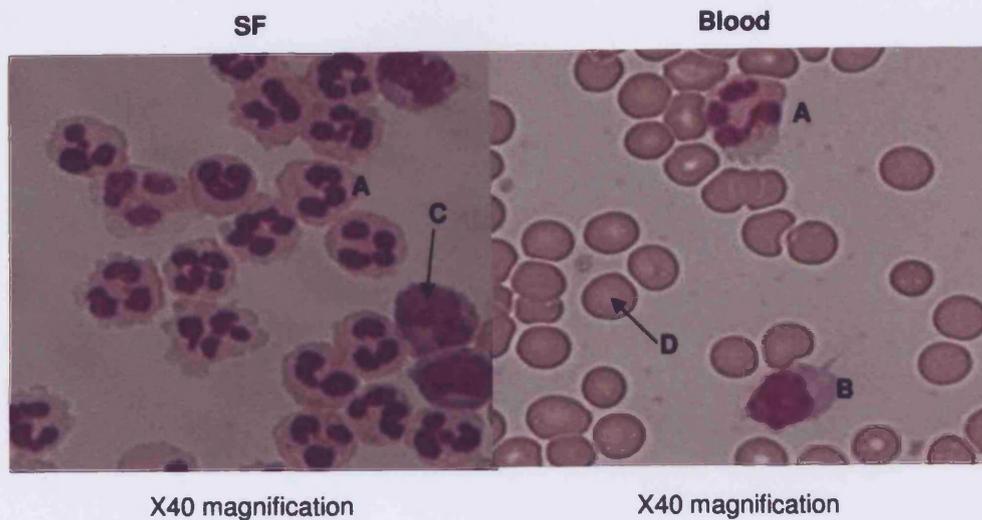
### 3.2.3 Quantification of IL-6R Expression on Local and Systemic Leucocytes Subtypes in Rheumatoid Arthritis Patients

Paired SF and blood samples were obtained from RA patients (n=12) with a symptomatic joint effusion. Samples were collected and analysed using the methods described previously in sections 2.7 and 2.8. The percentage (%) of each specific leucocyte subtype expressing IL-6R was calculated using Cell Quest Pro software. Leucocyte sub-types were confirmed by morphological analysis of cytopsin preparations (see Figure 3.1).

Neutrophils were the predominant cell type in these effusions comprising 62% of cells (range 48-75%). Macrophages accounted for 7% of cells seen (range 2-11%). CD19 and CD 4 lymphocytes were less than 3% (range 0.5-5%).

There was significant reduction in cell surface IL-6R expression (mean±SEM %) on synovial fluid CD14<sup>+</sup> macrophages and CXCR1<sup>+</sup> neutrophils (18±4% and 21±3% respectively; p≤0.05) when compared to cells obtained from peripheral blood (See Table 3.2 and Figure 3.2). There was no significant difference in IL-6R expression on CD3<sup>+</sup>, CD4<sup>+</sup> or CD19<sup>+</sup> lymphocytes between synovial fluid and blood (see Figure 3.1). Representative flow cytometry plots are shown in Figures 3.3 and 3.4.

**Figure 3.1 - Cytospins of paired blood and synovial fluid samples**



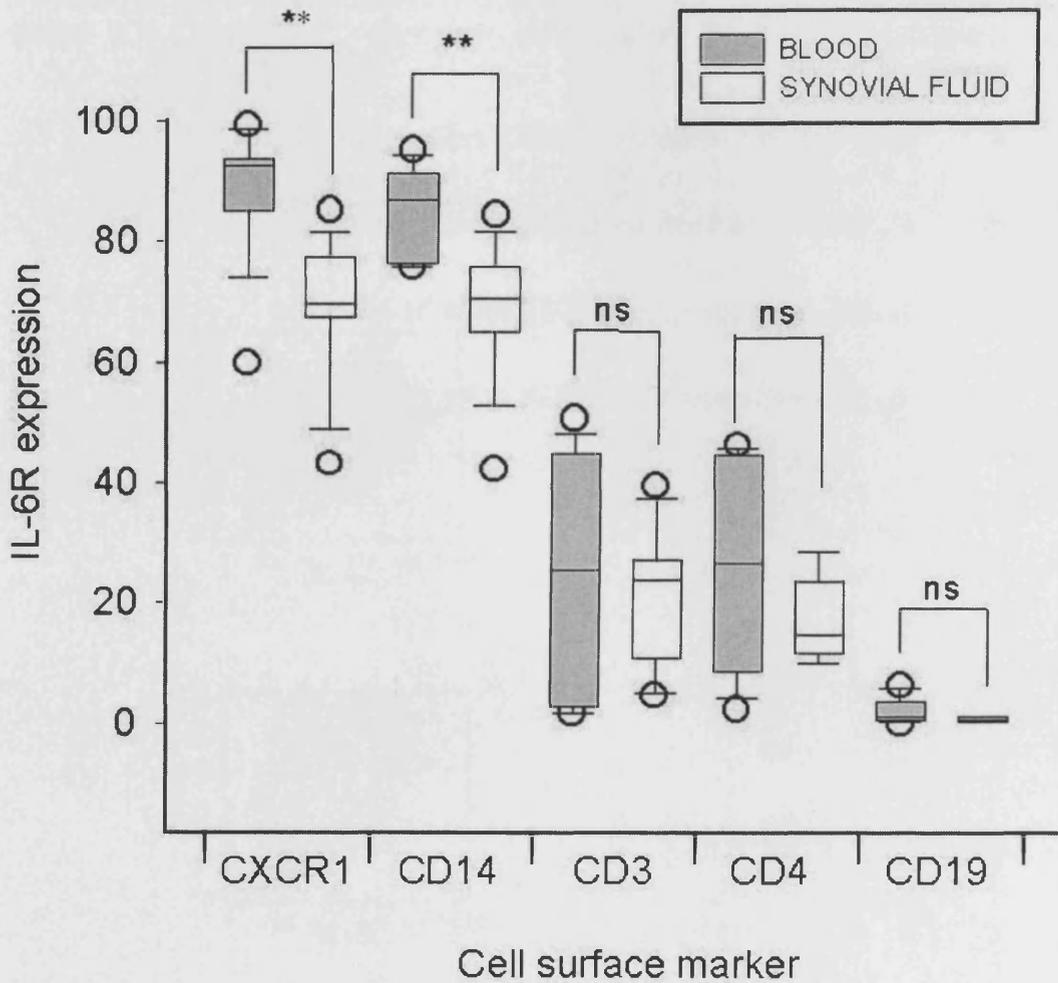
**A Neutrophil B Macrophage C Lymphocyte D Erythrocyte**

Cytospins of paired blood and synovial fluid leucocytes from an RA patient stained with a modified Wright Giemsa stain confirming cell types identified by flow cytometry

**Table 3.2 - IL-6R expression on leucocyte subsets from rheumatoid arthritis patients**

Phenotypic Marker	IL-6R Expression Blood (%)	IL-6R Expression SF (%)	<i>p</i> value
	Mean±SEM	Mean±SEM	
CXCR1	88±11	69±13	≤0.005
CD14	84±7	69±12	≤0.005
CD3	24±20	21±12	NS
CD4	26±18	17±7	NS
CD19	2±2	1±0	NS

Figure 3.2 - IL-6R expression on leucocytes obtained from paired blood and synovial fluid samples from rheumatoid arthritis patients



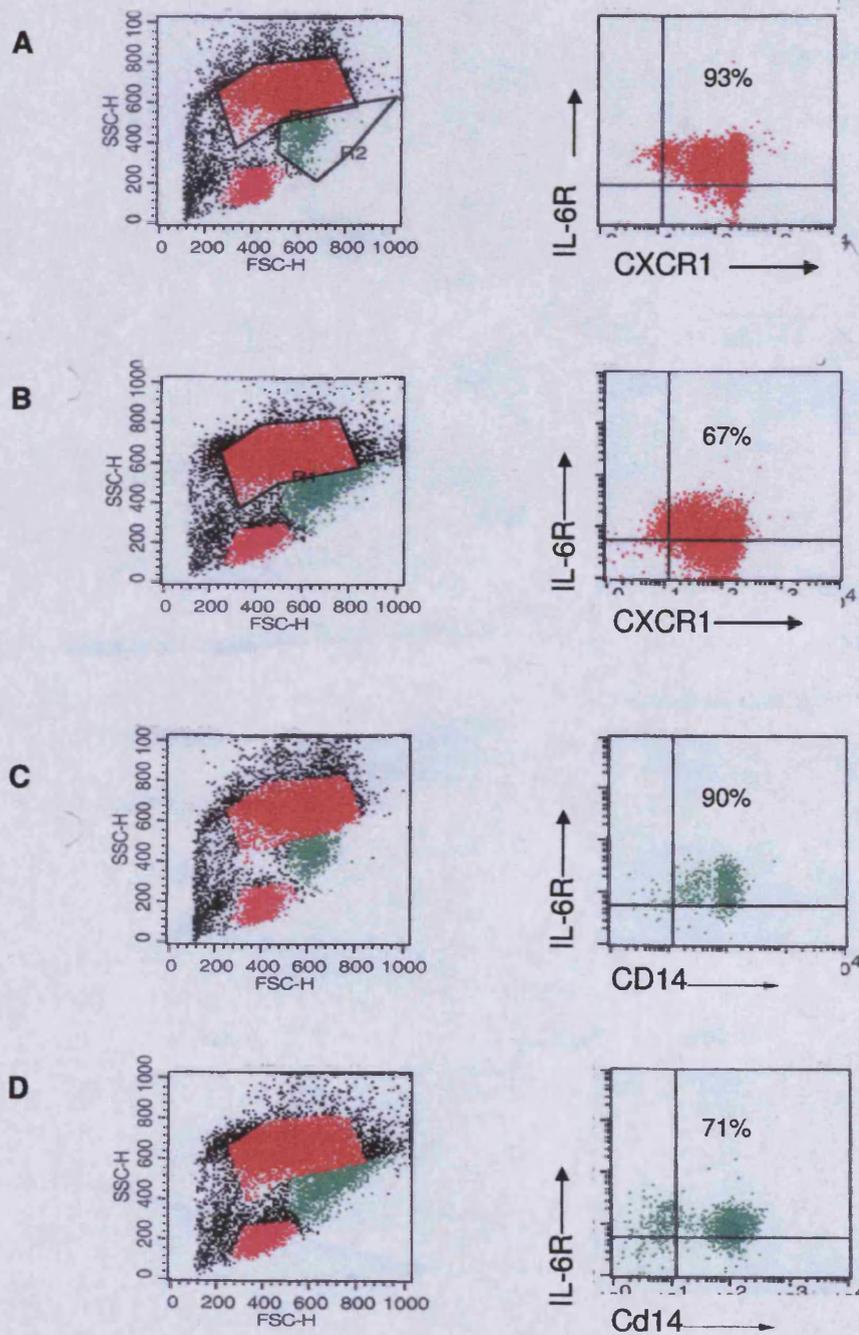
Box and whiskers plot of % IL-6R expressed on leucocyte subsets in blood and matched synovial fluid from 12 RA patients. \*\*  $p < 0.005$ , n.s. not significant). Boxes show the 25 and 75% percentile, horizontal lines within the boxes show the median and the whiskers show the 10 and 90% percentiles. Open circles indicate the upper and lower extremes.

### **Figure 3.3**

Representative flow cytometry pictures demonstrating IL-6R expression and specific phenotypic markers on leucocyte subsets in paired blood and synovial fluid samples. Total leucocyte population is gated according to cell morphology based on forward and side scatter. Gate 1(R1) (red) is neutrophil subset; Gate 2(R2)(green) is macrophage subset. PE labelling (FL-1) corresponds to IL-6R expression. FIT-C labelling (FL-2) corresponds to leucocyte specific markers within gated area. Data was acquired from 10,000 gated events.

- A IL-6R expression on CXCR1 cells in blood**
- B IL-6R expression on CXCR1 cells in SF**
- C IL-6R expression on CD14 cells in blood**
- D IL-6R expression on CD14 cells in SF**

**Figure 3.3 - Representative flow cytometry plots demonstrating IL-6R expression on neutrophils and macrophages in paired blood and synovial fluid samples**

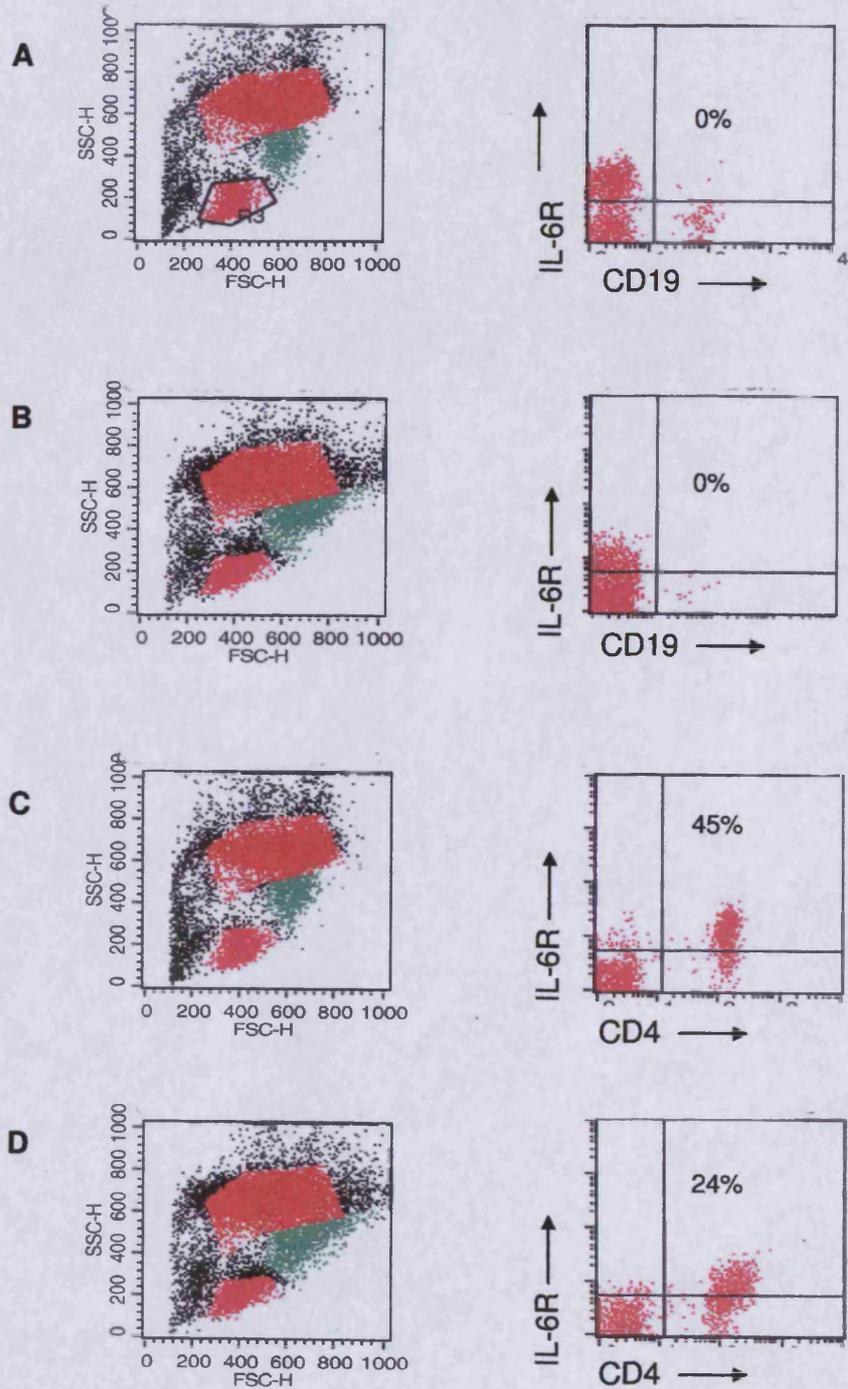


### **Figure 3.4**

Representative flow cytometry pictures demonstrating IL-6R expression and specific phenotypic markers on leucocyte subsets in paired blood and synovial fluid samples. Total leucocyte population is gated according to cell morphology based on forward and side scatter. Gate 3 (R3) (pink) is lymphocyte subset. PE labelling (FL-1) corresponds to IL-6R expression. FIT-C labelling (FL-2) corresponds to leucocyte specific markers within gated area. Data was acquired from 10,000 gated events.

- A IL-6R expression on CD19 cells in blood**
- B IL-6R expression on CD19 cells in SF**
- C IL-6R expression on CD4 cells in blood**
- D IL-6R expression on CD4 cells in SF**

**Figure 3.4 - Representative flow cytometry plots demonstrating IL-6R expression on CD19 and CD4 lymphocytes in paired rheumatoid arthritis blood and synovial fluid samples**



### 3.2.4 Measurement of IL-6R Levels in Paired Serum and Synovial Fluid Samples from Rheumatoid Arthritis Patients

After removal of leucocytes for flow cytometry, levels of sIL-6R in paired serum and synovial fluid samples (n=18) were measured by ELISA. The aim of this investigation was to determine whether the reduction in IL-6R expression on leucocytes derived from synovial fluid led to an increase in sIL-6R levels in SF and that this was attributable to shedding of receptor.

In RA patients there was no significant difference between serum and synovial fluid levels of sIL-6R (mean  $\pm$  SEM) although mean serum level was higher) ( $13.20 \pm 0.84$  ng/ml and  $11.1 \pm 0.74$  ng/ml). SF levels of sIL-6R were higher than blood levels in 3 of the 18 patients studied (see Figure 3.5A). There were no differences in mean length of effusion, number of joints affected or proportion of each leucocyte subtype noted between these patients and the RA population as a whole. However, the numbers of patients studied was small. Of note, the effusion in a single patient with highest SF sIL-6R levels had been present for 18 months. Interestingly, there was a significant correlation between sIL-6R concentrations in serum and synovial fluid using Pearson's test ( $p < 0.005$ ,  $r = 0.63561$ ) (Figure 3.5B). This shows that where serum levels are high; it is also the case for SF levels and may be a marker of disease activity or may be useful to identify those patients where trans-signalling is most important.

Having noted that mean levels of sIL-6R were unexpectedly lower in SF than in serum we speculated that sIL-6R might be binding to fibrinous components of viscous synovial fluid preventing its detection by ELISA. A review of the literature suggested that pre-treatment of SF with hyaluronidase could cause release of sIL-6R from matrix components like hyaluronic acid. Therefore, samples were assayed with and without the addition of hyaluronidase and levels of sIL-6R compared (n=4).

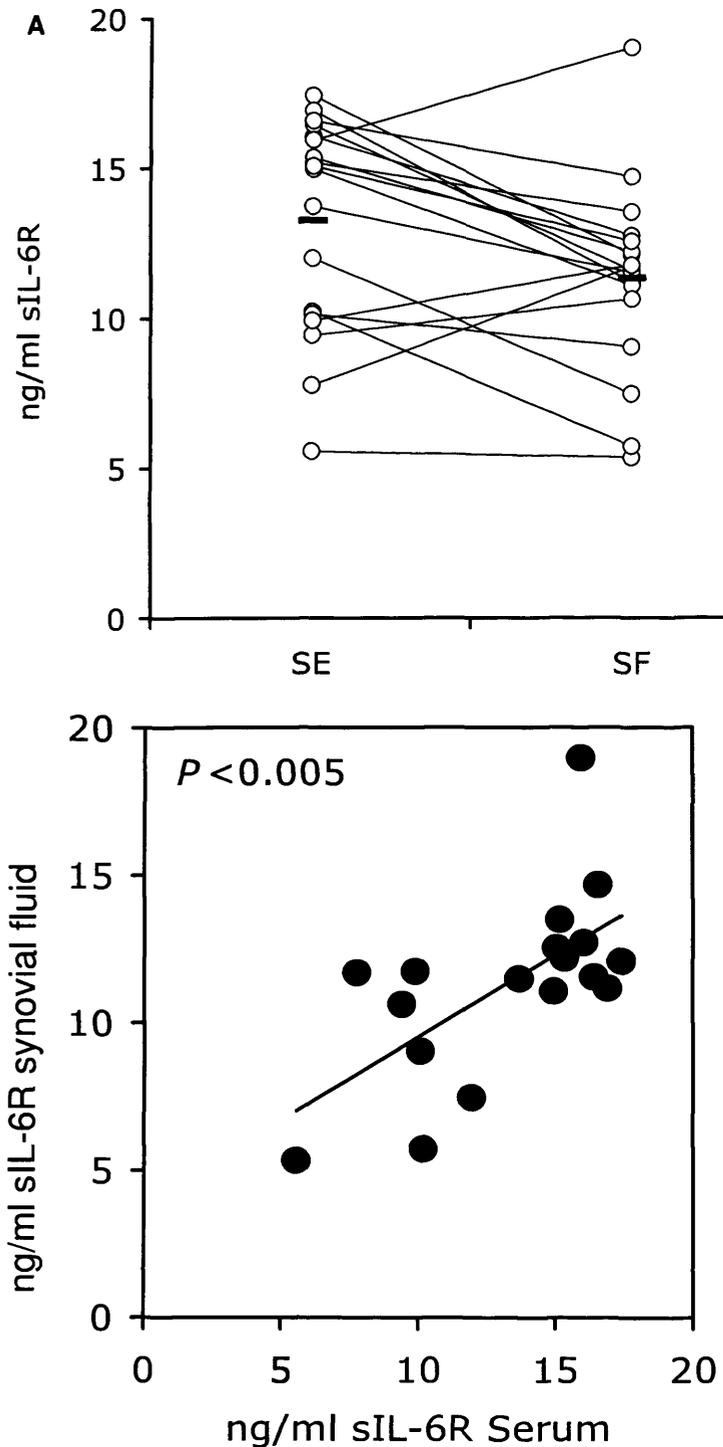
In these samples there was no significant increase in serum or SF sIL-6R levels as a result of treatment with hyaluronidase. Blood levels increased from  $532\pm 42$  pg/ml to  $594\pm 81$  pg/ml. Synovial fluid levels without hyaluronidase were  $293\pm 53$  pg/ml which increased to  $347\pm 49$  pg/ml following addition of hyaluronidase (see Figure 3.6).

#### 3.2.4.1 Detection of DS sIL-6R Isoform in Rheumatoid Arthritis Serum and Synovial Fluid

sIL-6R can be generated by 2 separate mechanisms resulting in 2 distinct isoforms. Proteolytic cleavage occurs when cognate IL-6R is cleaved from the cell surface (PC-sIL-6R). Additionally, sIL-6R may be produced by up-regulation of differential mRNA splicing (DS-sIL-6R). Previous work by our group has demonstrated that the DS-sIL-6R isoform is only found in RA fluids and not in osteoarthritis (OA). It appears that the release of the two different isoforms is independently regulated and it is not yet known whether the two isoforms have similar actions in the pathogenesis of RA. Levels of DS and total sIL-6R were therefore measured in the SF and serum of 10 RA patients.

In both serum and SF samples levels of DS sIL-6R were low (mean $\pm$ SEM). In serum  $288\pm 138$  pg/ml was detected whilst in SF, the level was  $284\pm 72$  pg/ml. When hyaluronidase was added to SF samples prior to DS-sIL-6R quantification we noted a significant ( $p=0.003$ ) increase in levels to  $487\pm 70$  pg/ml (see Figure 3.7). The addition of hyaluronidase had no significant effect on mean serum levels.

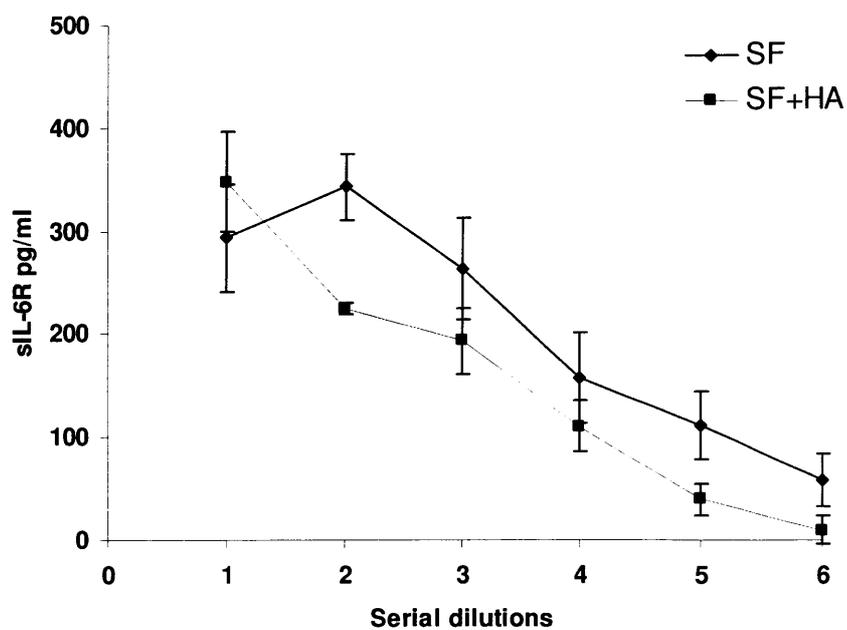
**Figure 3.5 - sIL-6R levels in paired serum and synovial fluid samples from rheumatoid arthritis patients**



**A** Matched serum and synovial fluid levels of sIL-6R from RA patients. Horizontal lines represent the mean value of 18 patients.

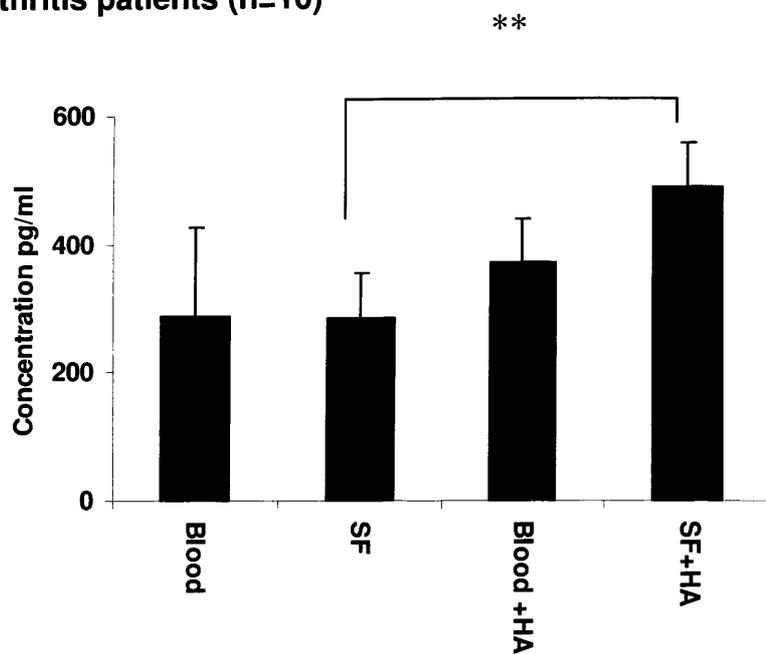
**B** Synovial fluid sIL-6R were correlated with levels of serum sIL-6R (N=18,  $r=0.63561$ ,  $t=2.604$ ,  $P<0.05$ ).

**Figure 3.6 - Detection of synovial fluid sIL-6R following addition of hyaluronidase**



Graph showing effect of addition of hyaluronidase to synovial fluid samples on detection of sIL-6R (n=4). There is no significant difference in detection of sIL-6R with the addition of hyaluronidase to samples.

**Figure 3.7 - Effect of hyaluronidase on detection of DS-sIL-6R in paired serum and synovial fluid samples from Rheumatoid Arthritis patients (n=10)**



Graph demonstrating effect of addition of Hyaluronidase to paired samples. Black bars show mean value in 10 samples. Error bars show SEM. \*\* shows significant difference between SF levels with and without hyaluronidase ( $p=0.003$ ).

### 3.2.5 Effects of TNF $\alpha$ on Leucocyte Shedding

The loss of cognate IL-6R from macrophage and neutrophil cell surface within the synovial fluid (noted in section 3.2.3) was presumed to be due to proteolytic cleavage from the cell surface. In support of this notion, the inflammatory environment within the joint contains many molecules which are known to induce proteolytic cleavage including CRP and CC chemokines. However, the effects of TNF $\alpha$  on sIL-6R generation have not been established.

We therefore assessed the effect of TNF $\alpha$  stimulation upon IL-6R shedding by neutrophils isolated from blood of healthy volunteers. In separate experiments cells were stimulated with IL-6 to test whether the effects seen with TNF $\alpha$  could be replicated with another pro-inflammatory cytokine and, specifically, whether IL-6 could induce production of its own receptor.

Neutrophils were extracted from whole blood and kept at 37°C for 30 minutes in a humidified atmosphere at constant CO<sub>2</sub> tension. Following stimulation, the cell surface expression of IL-6R was quantified. sIL-6R was analysed in the supernatant harvested at endpoint to determine whether any loss of cell surface receptor was due to shedding rather than internalisation of receptor. (Methodology was described in section 2.11).

In normal volunteers, after 30 minutes in culture, 71% of neutrophils expressed IL-6R. Following stimulation with 1 ng/ml TNF $\alpha$  there was loss of cell surface receptor and only 46% of cells expressed receptor. There was a further loss of receptor (37% expression) as stimulation dose of TNF $\alpha$  was increased to 10ng/ml (Figures 3.8 and 3.9).

In RA patients only 57% of cells expressed IL-6R after 30 minutes in culture. Stimulation with TNF $\alpha$  (1 ng/ml) resulted in further significant shedding of IL-6R: 39% of cells expressed IL-6R. There was no further

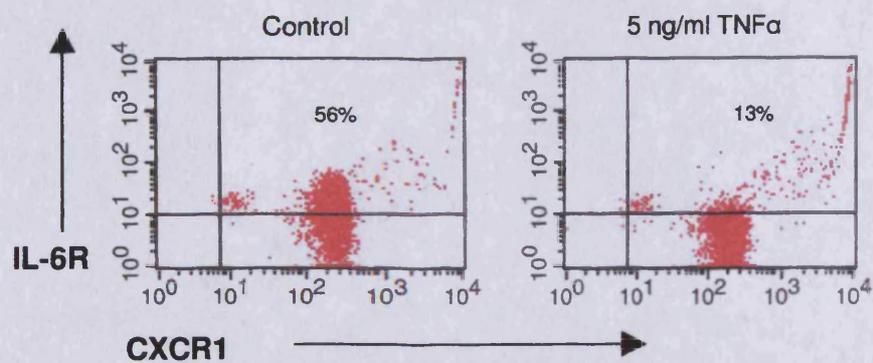
loss of cell surface receptor as a result of increase in TNF $\alpha$  stimulation dose.

IL-6R expression (mean $\pm$  SEM) on RA neutrophils and normal neutrophils was comparable at baseline (57.60 $\pm$ 19.95% and 70.86% $\pm$  12.64) (see Figures 3.8 and 3.9). Cell viability assays indicated that greater than 95% of cells were viable following stimulation.

ELISA analysis of sIL-6R levels in culture supernatants demonstrated that loss of cell surface receptor was likely to be due to shedding of cognate receptors from the cell surface as there was a dose dependent increase in supernatant levels of sIL-6R with increasing doses of TNF $\alpha$ . Neutrophils extracted from RA patients produced significantly more sIL-6R than normal neutrophils after 30 minutes in culture at 37 $^{\circ}$ C (213 $\pm$ 63 and 93 $\pm$ 27 pg/ml respectively  $p$ <0.05: figure 3.10). 10 ng/ml TNF $\alpha$  significantly increased sIL-6R generation in both RA and normal neutrophils (295 $\pm$ 63 and 198 $\pm$ 41 pg/ml respectively,  $p$ <0.05: Figure 3.10). However, sIL-6R shedding remained greater in RA samples than in normal samples.

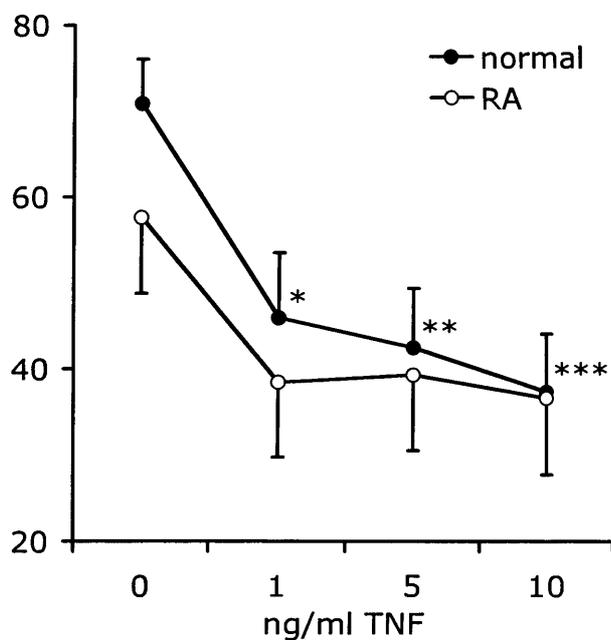
Interestingly stimulation with IL-6 did not cause any significant increase in sIL-6R generation in neutrophils from 3 normal volunteers (mean production 66.15 pg/ml). This suggests that shedding of sIL-6R is specific to TNF $\alpha$  and not the result of stimulation by any inflammatory cytokine. IL-6 is unable to cause shedding of its own receptor.

**Figure 3.8 - Effect of TNF $\alpha$  on normal neutrophil cell surface IL-6R expression**



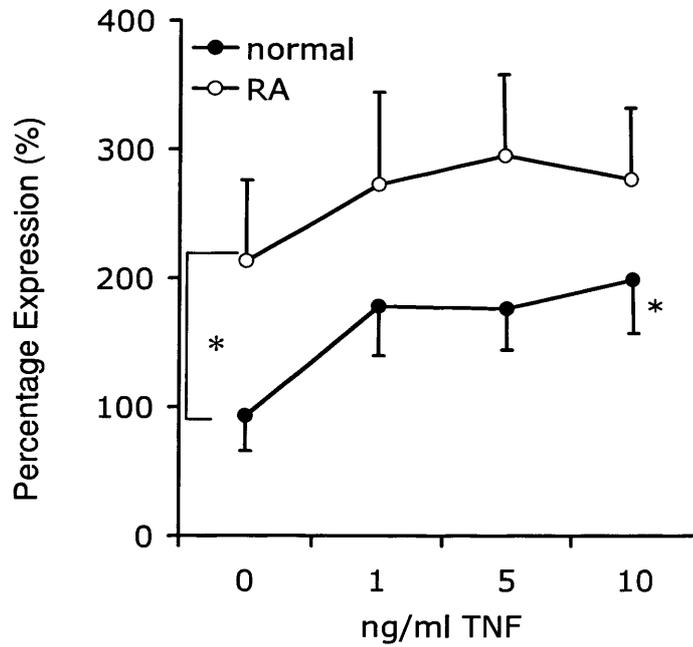
Representative flow cytometry scatter plots showing loss of surface-bound IL-6R expression on CXCR1<sup>+</sup> PMN following stimulation with 5ng/ml TNF $\alpha$ . Data was acquired from 10,000 gated events

**Figure 3.9 - Stimulation of neutrophils with TNF $\alpha$  results in loss of cell surface IL-6R expression**



Effect of TNF $\alpha$  on IL-6R expression from RA-PMN from patients with well controlled disease (N=9) and N-PMN (N=8) following 30 minutes in culture. Graph demonstrates dose-dependent loss of cell surface IL-6R from CXCR1<sup>+</sup> RA and normal PMN in response to TNF $\alpha$  quantified by flow cytometry. Data represents the mean (%)  $\pm$  s.e.m. \*p<0.05, \*\*p<0.01, \*\*\* p<0.001

**Figure 3.10 - Effect of TNF $\alpha$  on sIL-6R production by neutrophils**



Dose-dependent release of sIL-6R from CXCR1<sup>+</sup> RA (N=9) and normal PMN (N=7) in response to TNF $\alpha$  after 30 minutes. sIL-6R levels were quantified by ELISA. There is a significant difference in basal PMN-sIL-6R (0ng/ml TNF $\alpha$ ) between RA patients and normal volunteers (\*= $P$ <0.05). Data represents the mean  $\pm$  s.e.m.

### 3.3 Discussion

In this cohort of RA patients the infiltrating cells within the synovial fluid were mainly neutrophils with lesser numbers of macrophages and small numbers only of lymphocytes. This concurs with previous published work where 55-90% of cells within RA joint effusions were found to be polymorphs (Davis and Freemont 1988).

Levels of sIL-6R have been shown to be elevated in RA patients when compared to OA patients suggesting that trans-signalling may be specifically up-regulated in inflammatory arthritis (Nowell *et al* 2003). In previous work it was shown that levels of sIL-6R correlate with the severity of joint destruction (Kotake *et al* 1996). In this study loss of cognate IL-6 receptor from the cell surface of infiltrating neutrophils and macrophages within the joint was demonstrated. This is likely to be due to shedding of receptor from the cell surface. This provides a potential source of sIL-6R within the joint which would ultimately allow cells that do not express cognate IL-6 receptor to be made responsive to IL-6 via membrane-bound gp130.

Although we were unable to demonstrate a local increase in sIL-6R levels in RA synovial fluid over matched serum samples; it is likely that sIL-6R produced in this way would be used up quickly within the joint. The half-life of sIL-6R within the joint is at present uncharacterised. However, IL-6 levels in both RA and systemic JIA have been shown to be several fold higher than sIL-6R levels suggesting that the rate limiting step for trans-signalling is sIL-6R generation (Peake *et al* 2006). In JIA it has also been demonstrated that sIL-6R levels are higher systemically than within the joint (Peake *et al* 2006).

We demonstrated in PMN that cleavage of IL-6R occurs rapidly (within 30 minutes following stimulation). The majority of joint effusions aspirated in this study had been present for a week or more.

In separate work by our group looking at acute inflammation in peritoneal infection, peak levels of sIL-6R are seen at day 2 of infection with levels subsiding rapidly over the following 5 days. Soluble levels correlated with initial PMN infiltrate (Hurst *et al* 2001; Robson *et al* 2001). Therefore, it is likely that the initial PMN infiltrate providing a source of sIL-6R would have occurred some days previously.

sIL-6R is also produced by hepatocytes within the liver allowing cognate IL-6 signalling to occur. This may have implications for systemic features of disease such as CRP generation and nodule formation since RA is a systemic disease and not solely confined to the joint.

Adding hyaluronidase to SF had no significant effect on detection of sIL-6R but did increase detection of DSsIL-6R. As levels of DS were low, the small amount released by addition of hyaluronidase was significant. Other authors found no effect on Interleukin 2 detection or detection of chemotactic inhibitors (Egeland 1987; Matzner 1983). It has also been suggested that addition of hyaluronidase to SF affects mononuclear cell recovery and function (Geborek 1987). Therefore, it was felt that, on balance, addition of hyaluronidase was unnecessary.

CRP, neutrophil activating CXC chemokines, complement components and the lipid mediator PAF can stimulate sIL-6R production in human neutrophils by shedding of cognate IL-6R from the cell surface (Jones 2001 Jones 1999, McLoughlin *et al* 2004). CRP levels are generally high in active RA and the level of CRP required for receptor shedding (50 µg/ml) correlates well with levels found in RA serum and synovial fluid (Saxne *et al* 1988). It has now been demonstrated that TNF $\alpha$  can induce a similar magnitude of IL-6R shedding to CRP. Synovial fluid levels of TNF $\alpha$  are also known to be elevated in active disease (Kumon *et al* 1997). This provides an additional potential mechanism for the shedding of IL-6R seen in synovial fluid as a consequence of the inflammatory environment within the joint. TACE (ADAM 17) and probably ADAM 10 have roles in generation of IL-6R (Jones *et al*

2005). These members of the ADAM family are types of metalloproteinase which, in addition to causing shedding of IL-6R, also allow release of TNF $\alpha$  and cause shedding of other inflammatory molecules including L-selectin from cell surfaces. Levels of MMP have been shown to correlate with the severity of joint destruction in RA (Yamanaka *et al* 2000; Green *et al* 2003). However, levels of ADAM 17 have not been studied in this context.

TNF $\alpha$  is considered to be the driving cytokine in the acute inflammatory phase when neutrophil recruitment is marked. It is likely that influx of neutrophils in the presence of TNF $\alpha$  and other inflammatory mediators and cytokines leads to shedding of IL-6R from neutrophils.

IL-6 did not appear to cause an increase in sIL-6R generation in normal volunteers. This suggests that the presence of IL-6 alone is not sufficient to generate the receptor needed for trans-signalling and that other inflammatory mediators such as TNF $\alpha$  or CRP are essential for trans-signalling to occur.

In this study, RA patients who provided blood for neutrophil extraction were patients attending the methotrexate monitoring clinic and were well controlled on methotrexate monotherapy. Patients treated with anti-TNF $\alpha$  therapies were excluded. It is possible that these patients would have a different neutrophil response to TNF $\alpha$  with either up or down-regulation of shedding. These patients could be studied as a separate group in the future.

In order to compare the effects of TNF $\alpha$  on IL-6R shedding between normal volunteers and RA patients circulating blood neutrophils were used. It was shown that neutrophils within the SF have already shed IL-6R and therefore may not have responded to stimulation in the same way. It would be interesting to replicate this experiment in RA patients with neutrophils extracted from SF.

The reduction in joint effusions obtained over the course of my research has made work of this sort harder to carry out. The impact of

anti-TNF directed therapies has obviously reduced the numbers of poorly controlled patients presenting with multiple recurrent joint effusions. Additionally, the increasing use of high doses of methotrexate with rapid dose escalation has resulted in improvement in disease control. However, even newly diagnosed patients appear less likely to present with joint effusions. Possible reasons for this include earlier detection of disease with earlier referral to specialist services or alternatively a true reduction in severity of RA.

It has been suggested that the incidence of RA is falling (Silman 2002; Jacobsson 1994; Doran M 2002). This would result in fewer new referrals with RA.

Other groups suggest that RA is milder in the new millennium. This work was conducted by postal questionnaire of patients on the Oslo RA register. In its population cohort DMARD use increased from 36.5% in 1994 to 51.8% in 2004. In addition, by 2004, 11.8% of patients were on TNF-blocking agents. The authors concluded that improvement in health status was most likely due to better and more aggressive RA treatment (Uhlig T 2008). Other groups suggest that the prevalence of extra-articular manifestations of RA; which tend to be associated with more severe disease do not appear to be reducing (Turesson 2003; Turesson 2004).

In order to identify conclusively whether RA is indeed becoming a less severe disease then severity of disease over time must be assessed. The most comprehensive study of this kind looked at patients referred to the early arthritis clinic in Nijmegen. Patients presenting from 1985 - 2005 were divided into 4 sub-cohorts and severity of disease at presentation compared. In this study DAS-28 at baseline improved over time but patient perception of disease as measured by HAQ score did not. The authors concluded that RA is becoming milder over time. Unfortunately as this was a hospital-based study this may have been as a result of a change in referral patterns from the community over time with earlier referral from primary care (Welsing et al 2005).

Therefore the question of whether RA is becoming less severe remains to be answered by a long term population-based inception cohort studies.

In summary, we identified that infiltrating leucocytes within the RA joint are predominantly neutrophils with lesser numbers of macrophages and lymphocytes. Neutrophils and macrophages within the RA joint have lower levels of IL-6R expression than neutrophils and macrophages obtained from matched blood samples in the same patients. We presume that the reduction in IL-6R expression is likely to be due to shedding of IL-6R. We did not demonstrate a significant increase in SF sIL-6R as a result of this presumed shedding but did demonstrate a significant correlation between matched serum and SF sIL-6R levels. Stimulation of neutrophils with TNF $\alpha$  results in loss of cell surface IL-6R expression and was accompanied by an increase in sIL-6R levels in cell supernatants. sIL-6R production was significantly higher in neutrophils extracted from RA patients when compared to normal healthy volunteers.

# **4 EFFECT OF TNF $\alpha$ AND IL-6 TRANS-SIGNALLING UPON CHEMOKINE PRODUCTION AND ADHESION MOLECULE EXPRESSION BY RHEUMATOID ARTHRITIS SYNOVIAL FIBROBLASTS**

## **4.1 Introduction**

Having identified leucocytes as a source of sIL-6R within the RA joint and noted the effects of TNF $\alpha$  on sIL-6R production; we wished to identify whether other TNF $\alpha$  and IL-6 trans-signalling interactions occurred within the joint. Resident cells within the joint play a clear role in leucocyte recruitment. We wished to identify how interactions between TNF $\alpha$  and IL-6 trans-signalling might modulate interactions between leucocytes and resident cells, namely fibroblasts within the joint.

Evidence is accumulating that tightly controlled interactions between fibroblasts and leucocytes are responsible for the propagation of inflammation. Leucocyte recruitment is orchestrated by cytokine effects on endothelial cells leading to chemokine production, up regulation of adhesion molecule expression and alteration of leucocyte phenotype.

Leucocyte recruitment is a multi-step process which involves initial attraction under the control of cytokines followed by rolling, activation, adhesion and finally emigration from the blood vessel into the area of inflammation (the joint in RA).

The initiation of inflammation in RA occurs following an unknown insult. This results in activation of cells within the synovial lining which then

leads to up-regulation of the adaptive immune response in genetically susceptible individuals (Firestein and Zvaifler 1990). The major hallmark that distinguishes RA from acute arthritis is the transition from acute self-limiting inflammation which can be cleared by the host defences to a state of chronic inflammation with subsequent intense proliferation of the synovial lining and eventual damage to the joint.

Interactions between endothelial cells or fibroblasts with circulating leucocytes lead to further leucocyte recruitment. This process of leucocyte recruitment and subsequent synovial proliferation has been implicated in the transition to chronic inflammation. Up-regulation of interferon  $\gamma$  (IFN $\gamma$ ) production with its subsequent effects on macrophage activation is also involved in the switch from acute to chronic inflammation.

Examples of interactions that are well described include the effects of TNF $\alpha$  production by synovial macrophages. This results in fibroblast proliferation and an increase in production of other pro-inflammatory cytokines and chemokines leading to activation and recruitment of more macrophages which in turn results in further TNF $\alpha$  production. This therefore leads to an inflammatory cascade (Sweeney and Firestein 2004). High levels of TNF $\alpha$  and other pro-inflammatory cytokines cause up-regulation of IFN  $\gamma$  receptors with further increase in macrophage activation (Wijngaarden *et al* 2004).

In addition to their specialised ultra structural properties and morphology, RA synovial fibroblasts demonstrate over-expression of pro-inflammatory genes (e.g. IL-6) and matrix proteins such as metalloproteinases (e.g. MMP-3). This allows localisation of immune cells within the joint (Buckley *et al* 2003a and b). Direct cell to cell contact between fibroblasts and T lymphocytes has been shown to result in up-regulation of chemokine and adhesion molecule expression. Direct contact between the cell types appears essential for this process as separation of the cell types whilst allowing free passage

of soluble mediators prevents up-regulation occurring (Buckley 2003 a and b; Fox *et al* 1997; Bombara *et al* 1993).

Co-culture of RA fibroblasts and PMN resulted in induction of VEGF expression and proliferation of endothelial cells (Kasama *et al* 2001). This may cause neo-vascularisation of the pannus with progressive joint destruction.

Fibroblasts from RA joints, in addition to their role in cell recruitment via expression of adhesion molecules and chemokine synthesis, may also play a role in the retention of cells within the joint through effects on cell survival. T lymphocyte survival can be prolonged by co-culture with RA fibroblasts without the addition of extrinsic cytokine support. In contrast PMN survival is only marginally increased by co-culture with RA-SF. However, in the presence of pro-inflammatory cytokines (e.g. TNF $\alpha$ ), neutrophil survival is significantly enhanced. The increase in cell survival appears to be due to impaired apoptosis (Filer *et al* 2006). It appears that improvement in T lymphocyte survival may be an intrinsic property of fibroblasts. In contrast, survival of PMN is only enhanced by the presence of pro-inflammatory cytokines as may be seen during an acute flare of arthritis when neutrophil recruitment is also most marked.

TNF $\alpha$  has a well characterised role in leucocyte fibroblast interactions. It is known to cause up-regulation of chemokine production and adhesion molecule expression in addition to having effects on neutrophil survival as described above (Akahoshi *et al* 1993; Rathanaswami *et al* 1993; Taylor *et al* 2000; Filer *et al* 2006).

The role of IL-6 in this process is less clear. As stated previously, structural cells within the joint lack cognate IL-6R and therefore, all IL-6 signalling by these cells requires the presence of sIL-6R. The most likely source of sIL-6R in the RA joint appears to be shedding from infiltrating leucocytes possibly under the influence of TNF $\alpha$  as described in chapter 3.

Evidence for interplay between TNF $\alpha$  and IL-6/sIL-6R has been demonstrated recently with a clear synergistic effect of the two cytokines on VEGF production in RA fibroblasts. Using RA-SF cultured for 72 hours in the presence of IL-6/sIL-6R and TNF $\alpha$  alone and in combination it was shown that whilst IL-6/sIL-6R alone could induce production of VEGF that this production was significantly up-regulated by the addition of TNF $\alpha$ . Interestingly, TNF $\alpha$  on its own was unable to induce VEGF production (Nakahara *et al* 2003). An interaction between IL-1 and IL-6/sIL-6R has also been reported. IL-1 and IL-6/sIL-6R complex act synergistically to up-regulate proteinase production by chondrocytes (Rowan *et al* 2001; Flannery *et al* 2000). However, the effect of cytokine synergy on leucocyte- fibroblast interactions has not been assessed in these studies.

Since IL-6/sIL-6R has been shown to affect both TNF $\alpha$  and IL-1 induction of mediators we questioned whether TNF $\alpha$  and IL-6/sIL-6R could elicit additive/synergistic effect on resident cells from the joint, specifically RA synovial fibroblasts. We were particularly interested in interactions that affected chemokine and adhesion molecule expression that could lead to modulation of leucocyte trafficking within the joint.

The specific aims of this chapter were therefore:

1. To study the role of IL-6/sIL-6R and TNF $\alpha$  alone and in combination upon chemokine production by RA synovial fibroblasts (RA-SF).
2. To compare chemokine production by RA-SF with chemokine production in a non-inflammatory joint disease (i.e. Osteoarthritis) using OA-SF.
3. To quantify RA-SF adhesion molecule expression in response to cytokine stimulation with TNF $\alpha$  and IL-6/sIL-6R.
4. To assess *in vitro* whether combined inhibition of TNF $\alpha$  and IL-6 has greater efficacy for the inhibition of chemokine production and adhesion molecule expression than mono-therapy.

## 4.2 Results

### 4.2.1 Immuno-histochemistry on Frozen Rheumatoid Arthritis

#### Sections

We aimed to identify whether IL-6R could be directly visualised in frozen sections of synovium taken from RA patients in order to determine which cells within the joint expressed IL-6R.

Synovial tissue samples were taken at joint replacement and frozen immediately in preparation for histological analysis. Tissue was frozen in isopentane on dry ice and stored at -20°C. Frozen sections were then cut using a cryostat and after drying, fixed in 100% cold methanol. A 2 step process was used for antibody staining; sections were incubated with a primary antibody at 37°C for 2 hours and then incubated with fluorescent conjugated secondary antibody.

2-colour immuno-fluorescence on serial sections was carried out to identify the location of cell nuclei in relation to vascular markers. CD31 was used as an endothelial marker. CD90 was used as an endothelial/fibroblast marker. DAPI was used as a nuclear stain. Using this technique it was possible to differentiate vascular structures, fibroblasts and leucocytes (Figure 4.1 A and B).

Serial sections were then stained for IL-6R using the same technique to localise the site of IL-6R expression within the joint and to identify whether IL-6R expression was confined to leucocytes. IL-6R expression could not be visualised in frozen tissue specimens using MAB-227 (a monoclonal anti-human IL-6R antibody) using this technique. It was not clear whether the antibody was unsuitable for purpose or whether there was no IL-6R expression in frozen RA synovial tissue samples (Figure 4.1C).

In order to test whether this antibody was able to stain IL-6R; whole blood leucocytes were concentrated on to glass slides using a

cytopsin. After drying, the slides were fixed in 100% cold methanol. Sections were then incubated with MAB227 (anti-IL-6R antibody) at 37°C for 2 hours and then incubated with fluorescent conjugated secondary antibody. Whole blood leucocytes had been shown to express IL-6R by flow cytometry using this particular antibody. Primary antibody (MAB227) was added at dilutions from 1/20 to 1/200 but no positive staining was visualised. It was concluded that either the antibody available was not suitable for this technique or that leucocytes lose IL-6R cell surface expression as a result of sample preparation for this process.

#### 4.2.2 Chemokine Production by Rheumatoid Arthritis Synovial

##### Fibroblasts

RA-SF were extracted and grown to confluence as described previously (section 2.13). The cells were growth arrested for 48 hours prior to stimulation with TNF $\alpha$  (0-1000 pg/ml), IL-1 (0-1000 pg/ml) and IL-6/sIL-6R (0-200 ng/ml). IL-6 production was measured to identify whether in addition to generation of sIL-6R noted in Chapter 3 TNF $\alpha$  could also induce production of IL-6. CCL2, CXCL8 and CCL5 were measured in order to assess the effects of stimulation on macrophage, neutrophil and mixed leucocyte (including T cell) chemo-attractants.

Stimulation of RA-SF with TNF- $\alpha$  resulted in dose-dependent up-regulation of IL-6, CCL2, and CXCL8 production after 24 hours. At 24 hours, TNF $\alpha$  1000 pg/ml produced 4449.97 $\pm$ 1692 pg/ml IL-6 (Figure 4.2), 1772 $\pm$  227 pg/ml CCL2 (Figure 4.3B), 434.5 $\pm$  81pg/ml CXCL8 (Figure 4.4) and 57.91 $\pm$  11 pg/ml CCL5 (Figure 4.6A).

When cells were stimulated with IL-1 in order to compare the effects of another pro-inflammatory cytokine CCL2 production was comparable to that induced by TNF $\alpha$  (Figure 4.3B). However CXCL8 production induced by IL-1 was significantly higher than that induced by TNF $\alpha$  ( $p \leq 0.001$ ) (Figure 4.4).

Stimulation with IL-6/sIL-6R resulted in dose dependent increase in CCL2 production. IL-6/sIL-6R 100 ng/ml produced  $370 \pm 35$  pg/ml CCL2 (Figure 4.3A). Stimulation of RA-SF with IL-6/sIL-6R had no effect on CXCL8 (Figure 4.4) or CCL5 production which remained the same as in unstimulated cells.

In summary, TNF $\alpha$  stimulation resulted in induction of IL-6, CCL2, CXCL8 and CCL5 production. IL-1 stimulation resulted in up-regulation of CCL2 to levels similar to that produced by TNF $\alpha$ . IL-1 induced CXCL8 production was greater than that induced by TNF $\alpha$ . The effects of IL1 on IL-6 and CCL5 production were not assessed. IL-6/sIL-6R stimulation resulted in up-regulation of CCL2 production but had no effect on CXCL8 or CCL5 production.

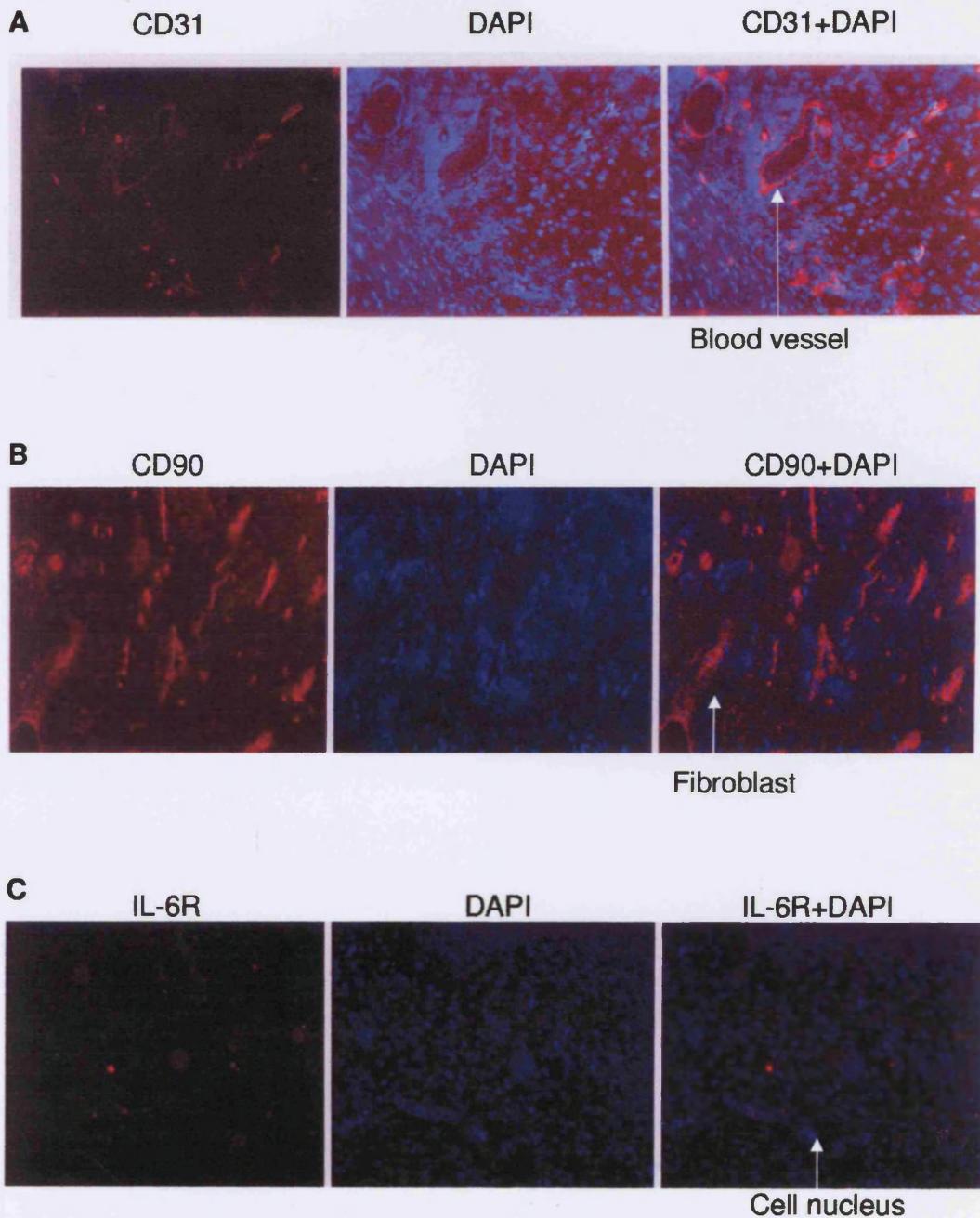
#### 4.2.3 TNF $\alpha$ and IL-6 / sIL-6R Causes Synergistic Increase in Chemokine Production by RA-SF

Combining TNF $\alpha$  (0.01-1 ng/ml) and IL-6/sIL-6R (30-200 ng/ml) resulted in significant synergistic up-regulation of CCL2, CCL5 and CXCL8 (Figures 4.5-4.7). At doses of TNF $\alpha$  1000 pg/ml + IL-6/sIL-6R 100 ng/ml, expected additive production of CCL2 was  $2013 \pm 338$  pg/ml. Actual production was  $3104 \pm 309$  pg/ml (Figures 4.5A and B).

At doses of TNF $\alpha$  1000 pg/ml + IL-6/sIL-6R 100 ng/ml, expected additive production of CCL5 was  $58 \pm 11$  pg/ml. Actual production was  $121 \pm 14$  pg/ml. Interestingly, at higher doses of IL-6/sIL-6R, the synergistic up-regulation of CCL5 by TNF $\alpha$  was lost and in fact, IL-6/sIL-6R induced a dose-dependent decrease in CCL5 production in concentrations above 50ng/ml (Figure 4.6A and B).

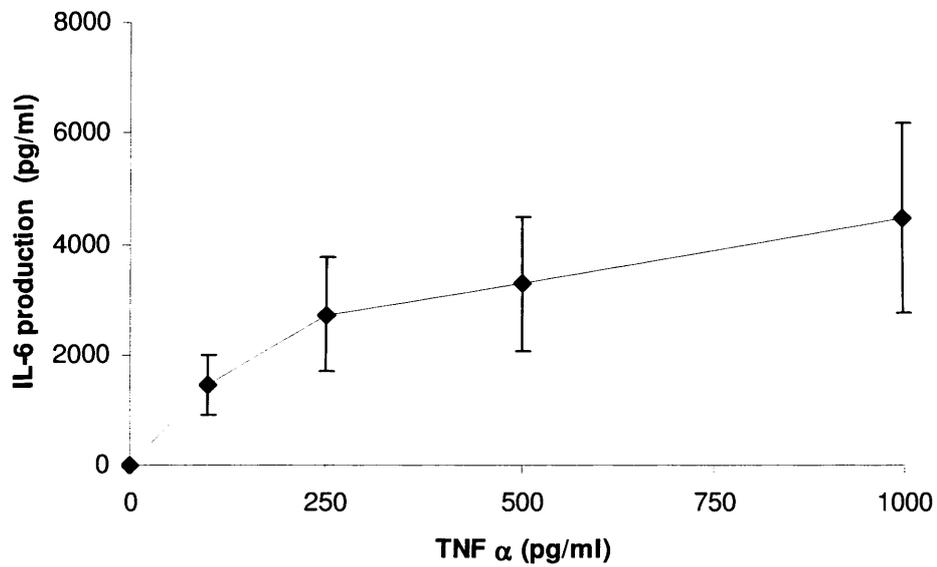
At doses of TNF $\alpha$  1000 pg/ml + IL-6/sIL-6R 100 ng/ml, expected additive production of CXCL8 was  $434 \pm 81$  vs. actual production of  $591 \pm 103$  pg/ml). However, at doses greater than 100 ng/ml, there was no further synergistic increase in CXCL8 production (Figure 4.7A and B).

**Figure 4.1 - Rheumatoid arthritis synovial tissue sections**



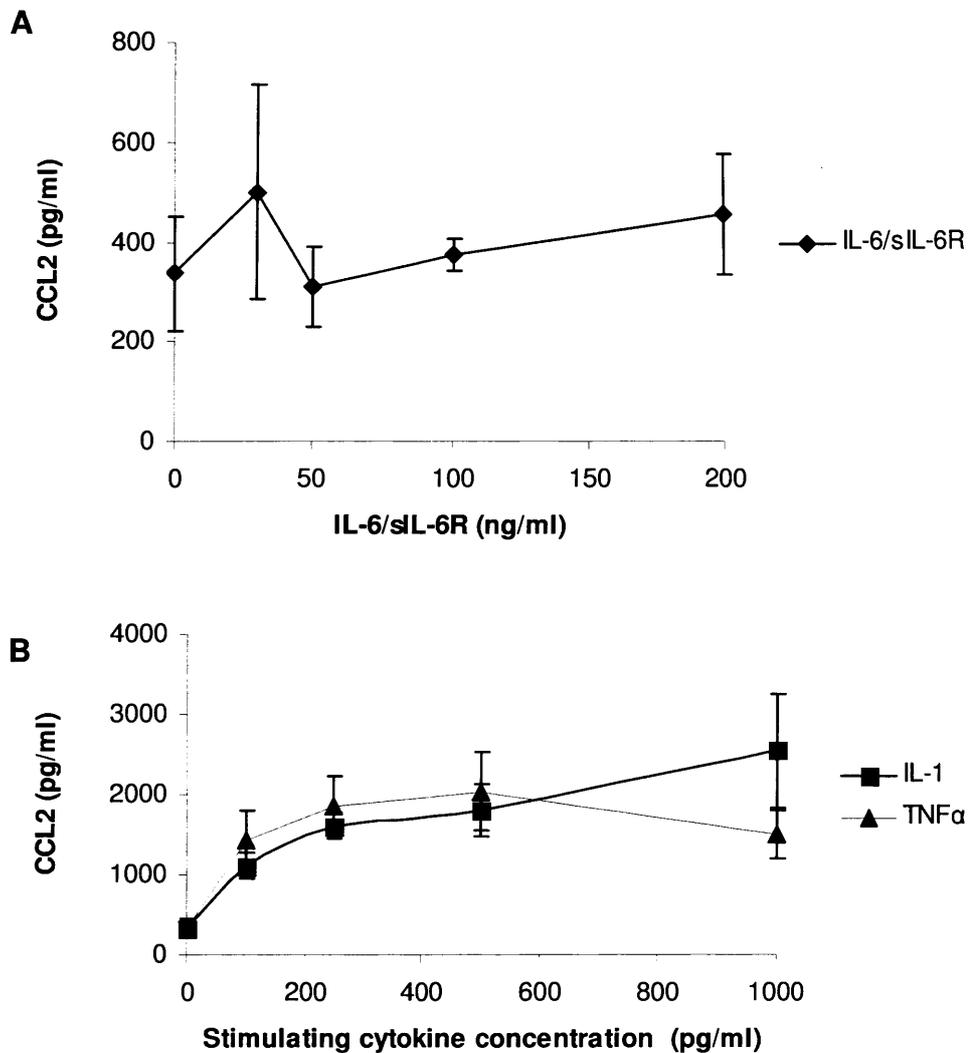
RA synovial tissue sections obtained at joint replacement. Serial sections were stained for the presence of vascular structures, fibroblasts and IL-6R. Cell nuclei were visualised using blue counter stain DAPI. CD31, CD 90 and IL-6R were counterstained with Alexa 594(Red). **A** demonstrates vascular structures within the synovium, **B** demonstrates fibroblasts within the synovium, **C** shows leucocyte infiltrate within the synovium but no staining for IL-6R is seen.

**Figure 4.2 - IL-6 production by TNF $\alpha$  stimulated fibroblasts**



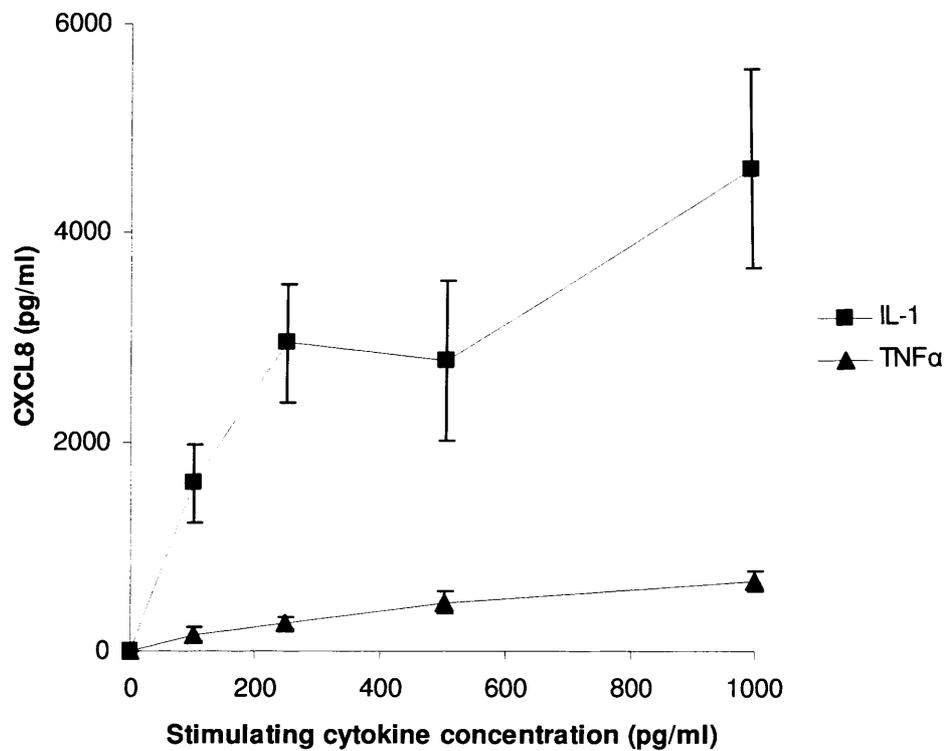
IL-6 production by RA synovial fibroblasts was quantified by ELISA. Cells were stimulated with TNF $\alpha$  (0-1000 pg/ml) in serum-free media then supernatants removed at 24 hours. Values shown are mean  $\pm$  SEM (n=4 cell lines).

**Figure 4.3 - Dose dependent up-regulation of CCL2 production following stimulation with IL-1, TNF $\alpha$  and IL-6/sIL-6R**



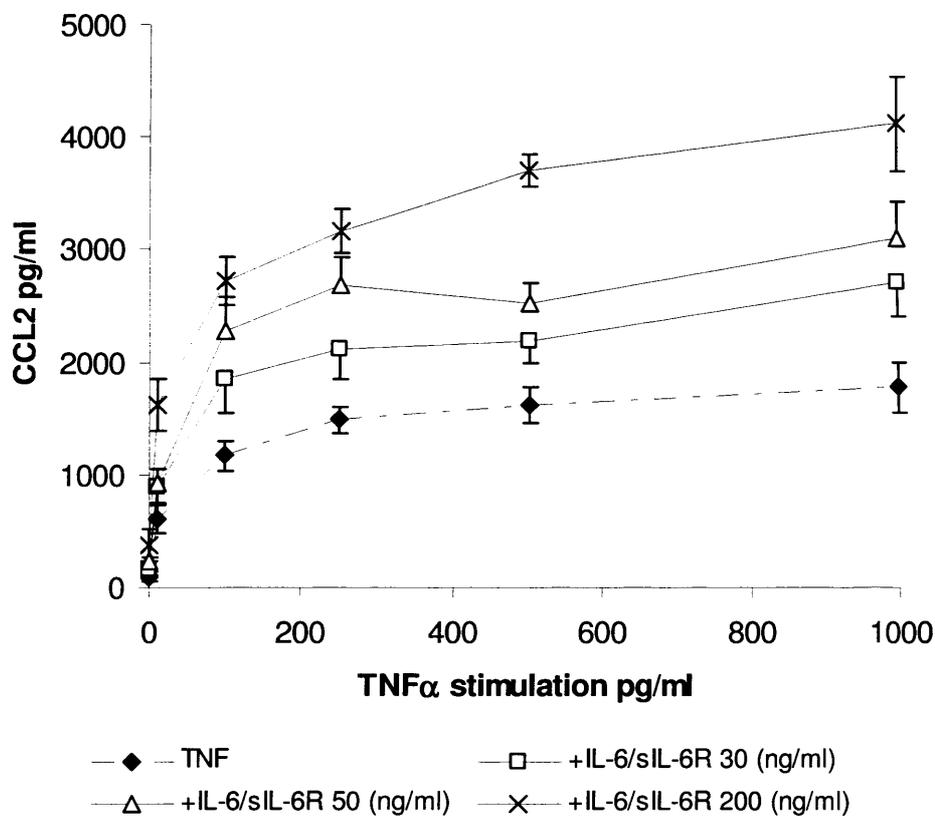
CCL2 production in RA-SF was quantified by ELISA. Cells were stimulated with A) IL-6/sIL-6R, B) IL-1 and TNF $\alpha$  respectively. Stimulations were carried out in serum free media and supernatants removed at 24 hours. Values shown are mean $\pm$ SEM (n=4 cell lines).

**Figure 4.4 - Dose dependent up-regulation of CXCL8 production following stimulation with IL-1 or TNF $\alpha$  but not IL-6/sIL-6R**



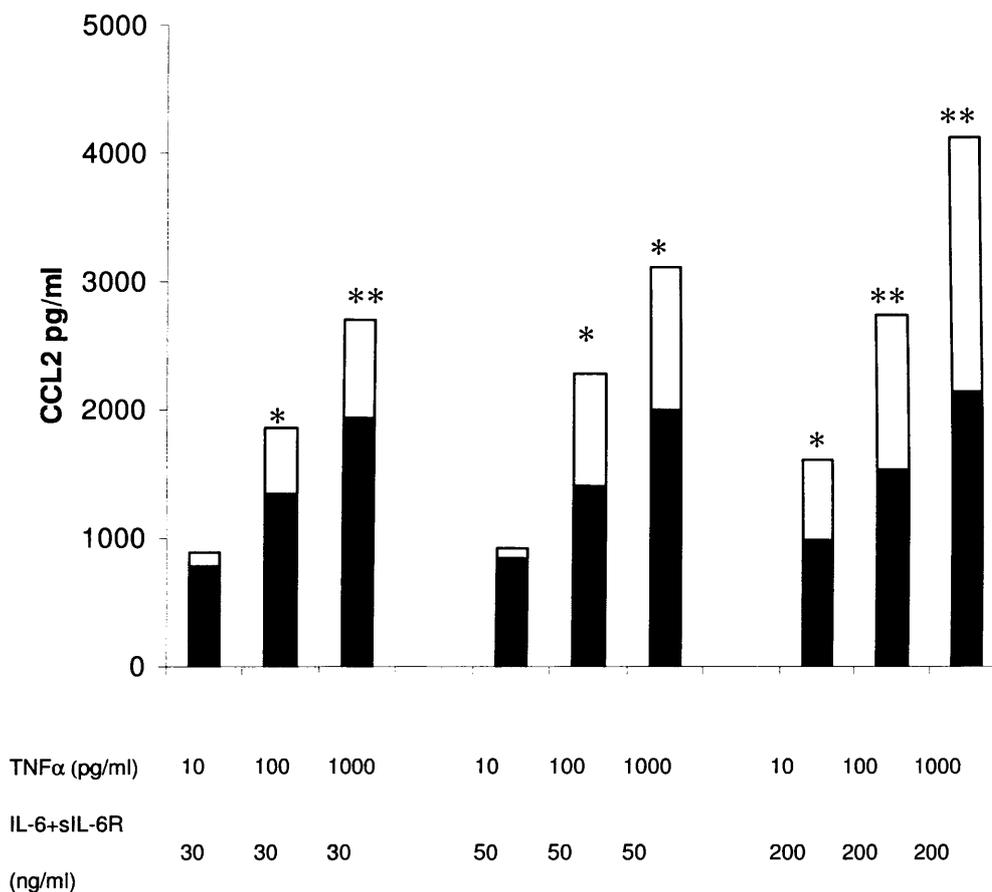
CXCL8 production in RA-SF was quantified by ELISA. Cells were stimulated with IL-6/sIL-6R, IL-1 and TNF $\alpha$  respectively. Stimulations were carried out in serum free media and supernatants removed at 24 hours. Stimulation with IL-1 or TNF $\alpha$  resulted in dose dependent up-regulation of CXCL8. Stimulation with IL-6/sIL-6R had no effect on CXCL8 production. Values shown are mean $\pm$ SEM (n=4 cell lines).

**Figure 4.5A - CCL2 production by RA-SF following stimulation with TNF $\alpha$  and IL6/sIL-6R**



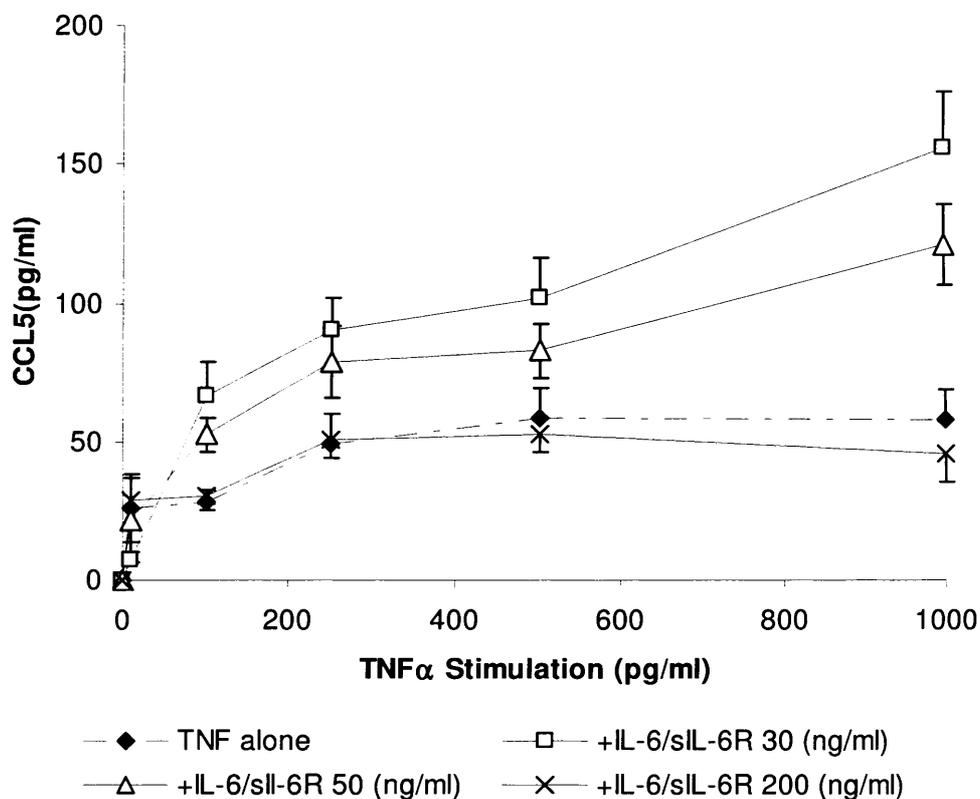
CCL2 production in RA-SF was quantified by ELISA. Cells were stimulated with IL-6/sIL-6R and TNF $\alpha$  separately and in combination. Stimulations were carried out in serum free media and supernatants removed at 24 hours. Addition of IL-6/sIL-6R to TNF $\alpha$  resulted in dose-dependent synergistic up-regulation of CCL2 production throughout the dose range. Values shown are mean $\pm$ SEM (n=5 cell lines).

**Figure 4.5B - Synergistic effect of combined cytokine stimulation on CCL2 production**



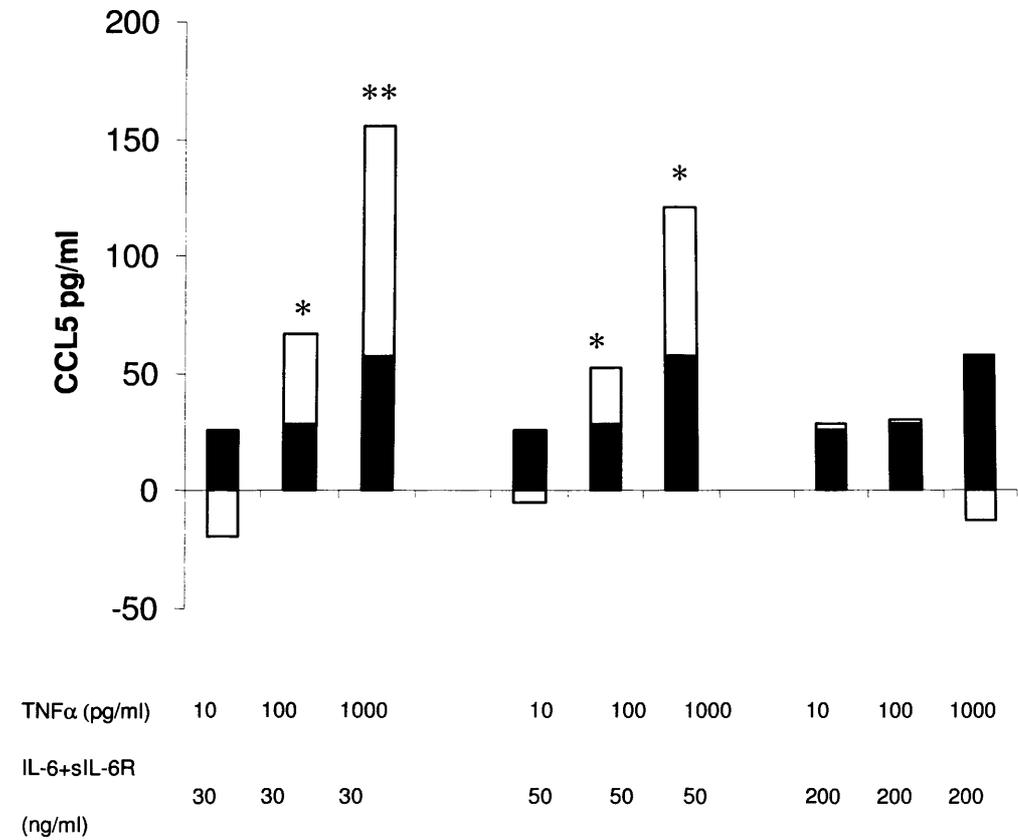
Synergistic effect of IL-6/sIL-6R on TNF $\alpha$ -induced chemokines in RA-SF. Black bars represent *expected* additive value for chemokine production, white bars show *actual* up regulation. Statistical analysis was carried between actual and expected values. (\* =  $P<0.05$ , \*\* =  $P<0.01$ )

**Figure 4.6A - Effects of combined TNF $\alpha$  and IL-6/sIL-6R stimulation on CCL5 production by RA-SF**



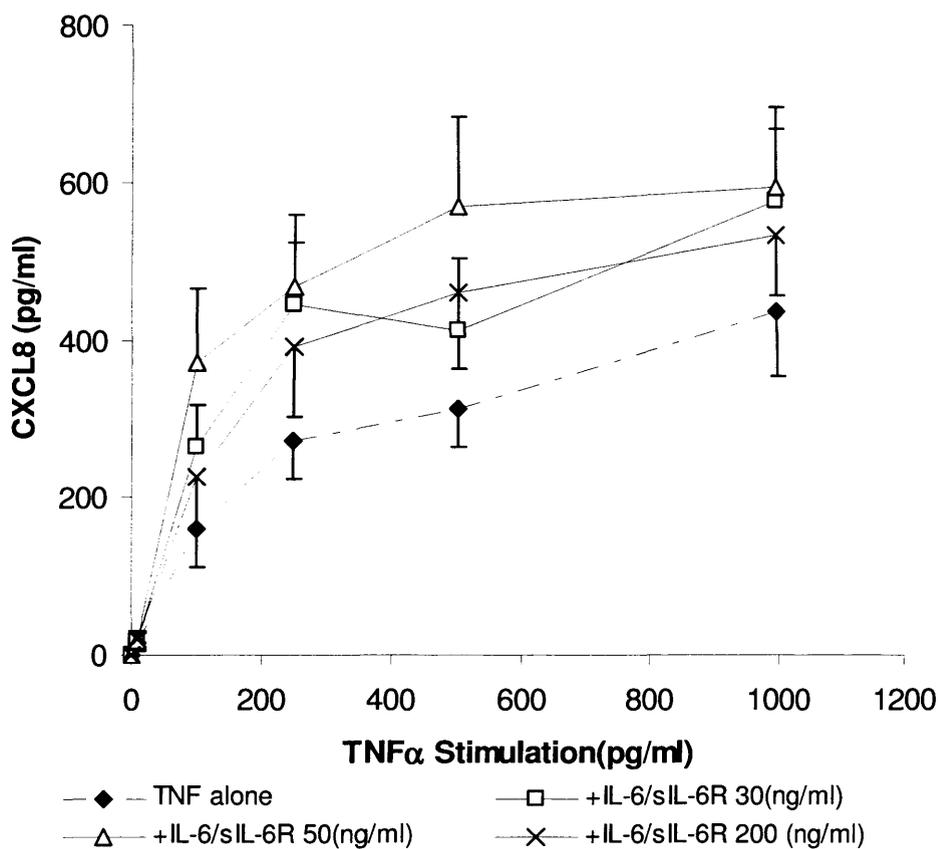
CCL5 production in RA-SF was quantified by ELISA. Cells were stimulated with IL-6/sIL-6R and TNF $\alpha$  separately and in combination. Stimulations were carried out in serum free media and supernatants removed at 24 hours. Addition of IL-6/sIL-6R to TNF $\alpha$  resulted in dose-dependent synergistic up-regulation of CCL5 production at doses of 30 and 50 ng/ml but inhibition of CCL5 production at 200 ng/ml. Values shown are mean $\pm$ SEM (n=5 cell lines).

**Figure 4.6B - Synergistic effect of combined cytokine stimulation on CCL5 production**



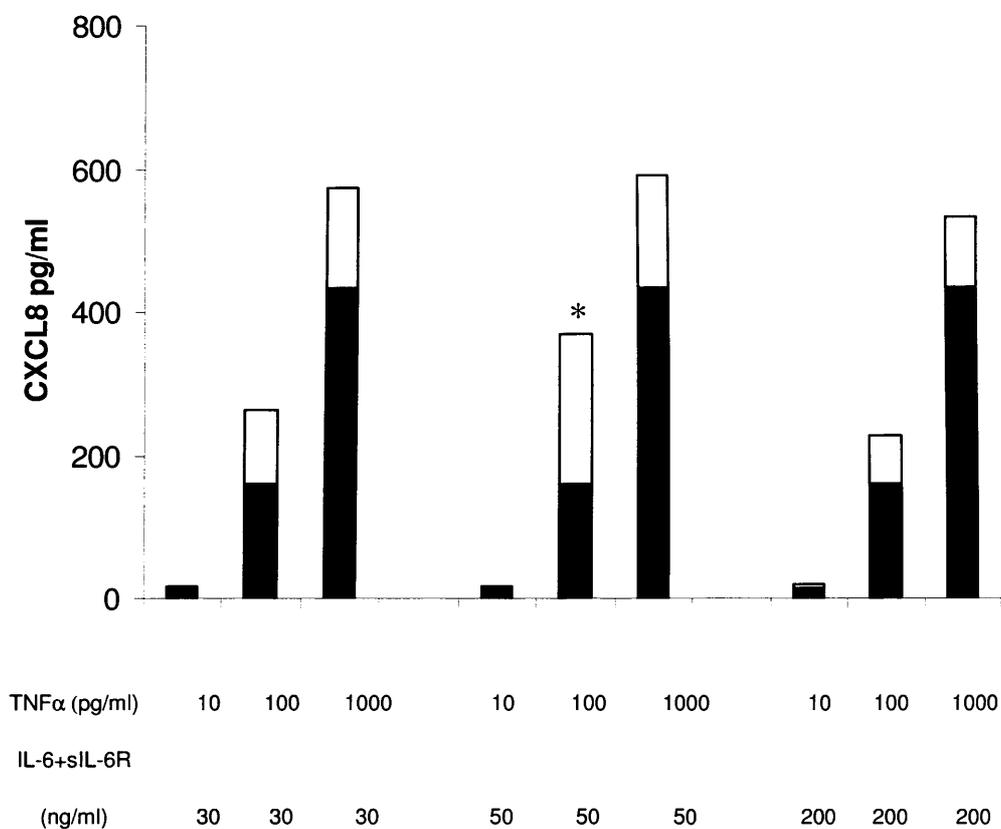
Synergistic effect of IL-6/sIL-6R on TNF $\alpha$ -induced chemokines in RA-SF. Black bars represent *expected* additive value for chemokine production, white bars show *actual* up regulation. Statistical analysis was carried between actual and expected values. (\* =  $P<0.05$ , \*\* =  $P<0.01$ )

**Figure 4.7A - Effects of combined TNF $\alpha$  and IL-6/sIL-6R stimulation on CXCL8 production by RA-SF**



CXCL8 production in RA-SF was quantified by ELISA. Cells were stimulated with IL-6/sIL-6R and TNF $\alpha$  separately and in combination. Stimulations were carried out in serum free media and supernatants removed at 24 hours. Addition of IL-6/sIL-6R to TNF $\alpha$  resulted in dose-dependent synergistic up-regulation of CXCL8 production at doses of 30 and 50 ng/ml but no further synergy increase in CXCL8 production was seen at 200 ng/ml. Values shown are mean $\pm$ SEM (n=5 cell lines).

**Figure 4.7B - Synergistic effect of combined cytokine stimulation on CXCL8 production**



Effect of IL-6/sIL-6R on TNF $\alpha$ -induced chemokines in RA-SF. Black bars represent *expected* additive value for chemokine production, white bars show *actual* up regulation. Statistical analysis was carried between actual and expected values. (\* =  $P < 0.05$ , \*\* =  $P < 0.01$ ). Although addition of IL-6/sIL-6R appears to increase CXCL8 production this is only statistically significant at a single point.

#### 4.2.4 Investigating the Role of Cytokine Synergy in Osteoarthritis

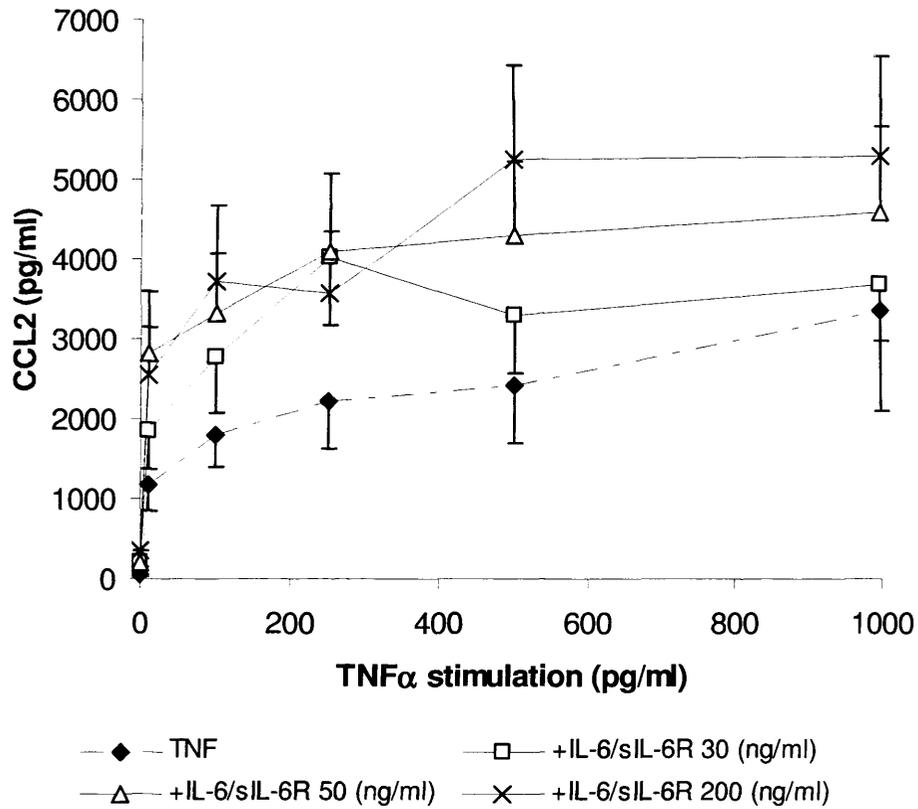
Synovial fibroblasts were cultured as described in section 2.13 from synovial tissue obtained from patients with osteoarthritis undergoing joint replacement. These cells were used as representative fibroblasts from a non-inflammatory, non-immune mediated joint disease.

At 3<sup>rd</sup> passage OA-SF were stimulated with TNF $\alpha$  over a dose range (0-1000 pg/ml) and IL-6/sIL-6R (0-200 ng/ml). After 24 hours stimulation, tissue culture supernatants were harvested and chemokine production quantified by ELISA (CCL2, CXCL8 and CCL5 were measured).

Stimulation with TNF $\alpha$  resulted in dose dependent up-regulation of CCL2, CCL5 and CXCL8 in OA fibroblasts. Maximum CCL2 production was obtained following stimulation with TNF $\alpha$  1000 pg/ml+IL-6/sIL-6R 200 ng/ml (5266 $\pm$ 1266 pg/ml compared to RA 4103 $\pm$ 414 pg/ml; Figure 4.8). Maximum CCL5 production (141 $\pm$ 48 pg/ml compared to 156 $\pm$ 20 pg/ml by RA cell lines) was obtained following stimulation with TNF $\alpha$  1000 pg/ml+IL-6/sIL-6R 30 ng/ml (Figure 4.9). Maximum CXCL8 production was stimulated by TNF $\alpha$  1000 pg/ml+IL-6/sIL-6R 50 ng/ml (782 $\pm$ 197 pg/ml compared to 591 $\pm$ 103pg/ml in RA cells; Figure 4.10). In these experiments there was no significant difference between mean amounts of chemokine produced by RA (n=5) and OA fibroblasts (n=4).

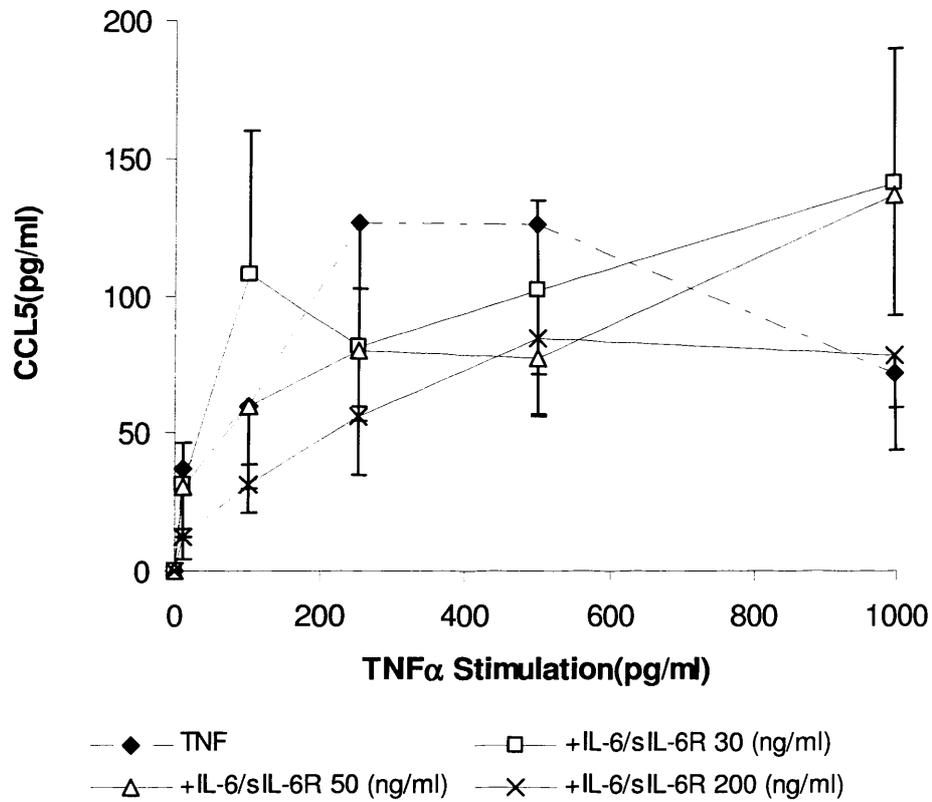
In addition there was no clear synergistic increase in chemokine production as a result of adding IL-6/sIL-6R to TNF $\alpha$ . This may be partly explained by the large variability seen in chemokine production by OA fibroblasts. In some cell lines there was minimal chemokine production as a result of cytokine stimulation. However, in other cell lines (possibly those where there was a more inflammatory component to disease) the levels of chemokine produced approached and even exceeded those produced by RA cell lines.

**Figure 4.8 - Effects of combined TNF $\alpha$  and IL-6/sIL-6R stimulation on CCL2 production by OA-SF**



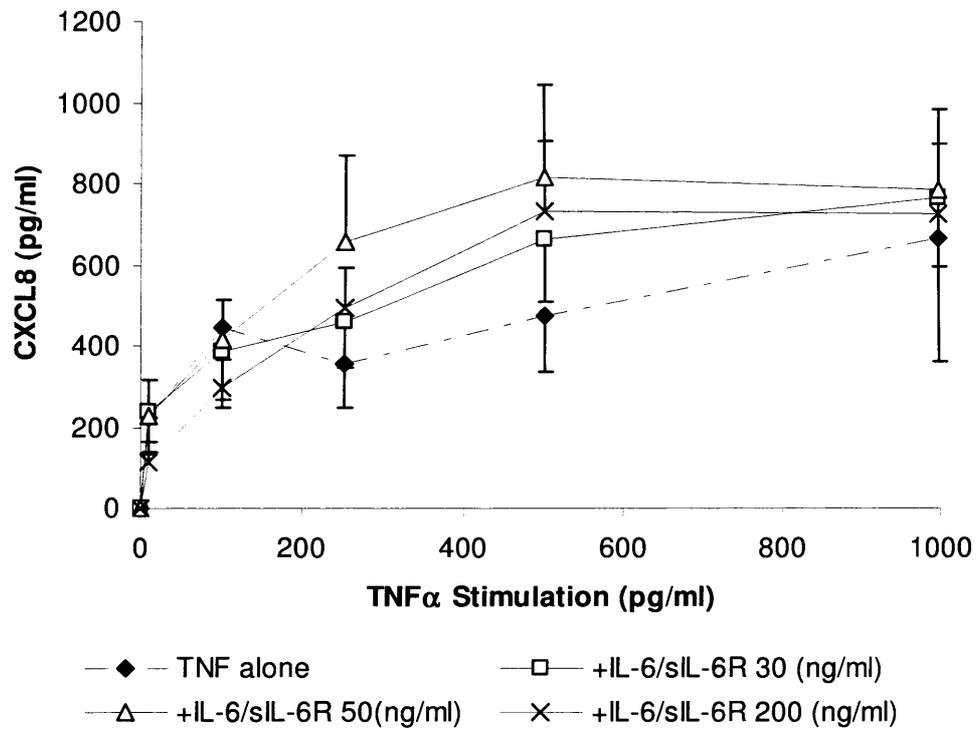
CCL2 production in OA-SF was quantified by ELISA. Cells were stimulated with IL-6/sIL-6R and TNF $\alpha$  separately and in combination. Stimulations were carried out in serum free media and supernatants removed at 24 hours. Values shown are mean $\pm$ SEM (n=4 cell lines).

**Figure 4.9 - Effects of combined TNF $\alpha$  and IL-6/sIL-6R stimulation on CCL5 production by OA-SF**



CCL5 production in OA-SF was quantified by ELISA. Cells were stimulated with IL-6/sIL-6R and TNF $\alpha$  separately and in combination. Stimulations were carried out in serum free media and supernatants removed at 24 hours. Values shown are mean $\pm$ SEM (n=4 cell lines).

**Figure 4.10 - Effects of combined TNF $\alpha$  and IL-6/sIL-6R stimulation on CXCL8 production by OA-SF**



CXCL8 production in OA-SF was quantified by ELISA. Cells were stimulated with IL-6/sIL-6R and TNF $\alpha$  separately and in combination. Stimulations were carried out in serum free media and supernatants removed at 24 hours. Values shown are mean $\pm$ SEM (n=4 cell lines).

#### 4.2.5 Inhibition of Cytokine Production in RA-SF Using Etanercept and MAB227 (anti-IL-6R antibody)

RA synovial fibroblasts were grown to confluence in 48 well plates as described in section 2.13. Cells were growth arrested for 48 hours prior to stimulation in serum free media. Initial experiments were carried out at 37°C on a varishaker in order to ensure adequate mixing of all cytokines and inhibitors. Unfortunately the lack of humidity and constant CO<sub>2</sub> led to death of cells and very low levels of chemokine production (Data not shown). Therefore, stimulation of cells was carried out in the incubator where a humidity and constant CO<sub>2</sub> controlled atmosphere was present as previously described (section 2.13). Cells were growth arrested and stimulating cytokines plus inhibitors mixed in an eppendorf for 30 minutes prior to addition of mixture to cells.

Initial dose finding experiments for anti-human IL-6R antibody (MAB227) were carried out using doses of 50 and 200 ng/ml, i.e. equal to and 4 times in excess of IL-6/sIL-6R concentration used to stimulate the cells. These doses provided dose dependent inhibition of CCL2 production and were therefore selected as appropriate for further study.

In order to provide an alternative method for neutralising IL-6 trans-signalling, sgp130 (a natural antagonist to IL-6/sIL-6R complex which acts by competitive inhibition of membrane bound gp130) was added to cells together with stimulating doses of IL-6/sIL-6R. Unfortunately no inhibition of chemokine production was seen (Data not shown). However, when sgp130 was added to fibroblasts stimulated with IL-6/sIL-6R STAT signalling was inhibited at 30 minutes but without any downstream effect on chemokine production (Figure 4.11). It is possible that despite initial reduction in STAT up-regulation that this is quickly overcome due to dynamic un-coupling of IL-6/sIL-6R and sgp130 and that over the 24 hour time-course of this experiment there

is sufficient stimulation from IL-6/sIL-6R complex to up-regulate chemokine production.

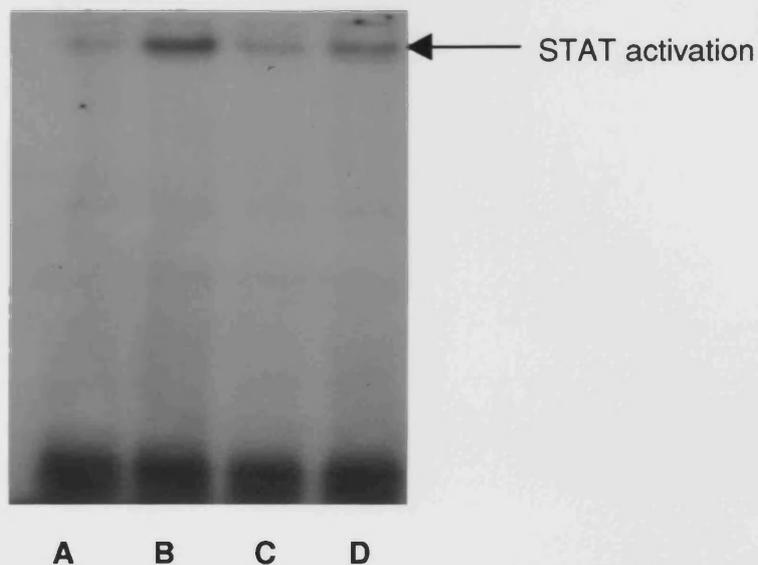
Dose finding experiments using combination etanercept and anti IL-6R were carried out. Initial doses of etanercept chosen (0.5-1 ng/ml), i.e. up to 100x in excess of stimulating TNF $\alpha$  doses resulted in almost complete inhibition of chemokine production. Therefore, further experiments were carried out using etanercept (0.001-0.01 ng/ml), i.e. up to 10x in excess of stimulating TNF $\alpha$  dose.

Once the optimum doses had been chosen, further experiments were carried out in 8 separate cell lines. Cells were grown to confluence in 48 well plates as previously described. Cells were then growth arrested for 48 hours prior to stimulation with TNF $\alpha$  (100 pg/ml), IL-6/sIL-6R (50 ng/ml)  $\pm$  etanercept (0.001 ng/ml)  $\pm$  anti IL-6R (500 ng/ml). Supernatants were removed after 20 hours and chemokine production quantified by ELISA. In these particular cell lines very little CCL5 production occurred at this time point so further analysis was not done on this chemokine.

Treatment of TNF $\alpha$  (100 pg/ml) and IL-6/sIL-6R (50 ng/ml) stimulated RA-SF with etanercept (0.001 ng/ml) resulted in significant reduction in CCL2 production (3707 $\pm$ 841pg/ml to 796 $\pm$ 146 pg/ml, Figure 4.12A  $P<0.001$ ). Treatment with anti IL-6R (500 ng/ml) also resulted in a reduction of CCL2 but was not significant (2676 $\pm$ 409 pg/ml; Figure 4.12A  $p>0.05$ ). Treatment with a combination of etanercept (0.001 ng/ml) and anti IL-6R (500 ng/ml) resulted in further additive down-regulation of combined cytokine-induced CCL2 production (666 $\pm$ 140 pg/ml; Figure 4.12A ( $p<0.001$ )).

In contrast, treatment of stimulated RA-SF with etanercept resulted in almost complete inhibition of CXCL8 production (743 $\pm$ 160 pg/ml to 14 $\pm$ 25 pg/ml  $P=0.0003$ ). Treatment with anti IL-6R had no effect on CXCL8 production (711 $\pm$ 156 pg/ml). Combination of anti IL-6R with etanercept provided no further inhibition (Figure 4.12B).

**Figure 4.11 - Anti IL-6R and sgp130 down regulate STAT activation in IL-6/sIL-6R stimulated RA-SF**



Fibroblasts were grown as previously described. At passage 3, cells were grown to confluence in flasks. Once confluent, stimulations were performed in serum free media. Cells were stimulated for 30 minutes then harvested for nuclear extraction. Protein assays were performed and 10 $\mu$ g of protein added to each well. Experiments were performed in 4 cell lines and a single representative figure is shown. Binding reactions were performed using 4 $\mu$ g of nuclear protein and  $\alpha^{32}$ -dTTP-labelled oligonucleotide containing a STAT-binding consensus sequence (SIE-m67).

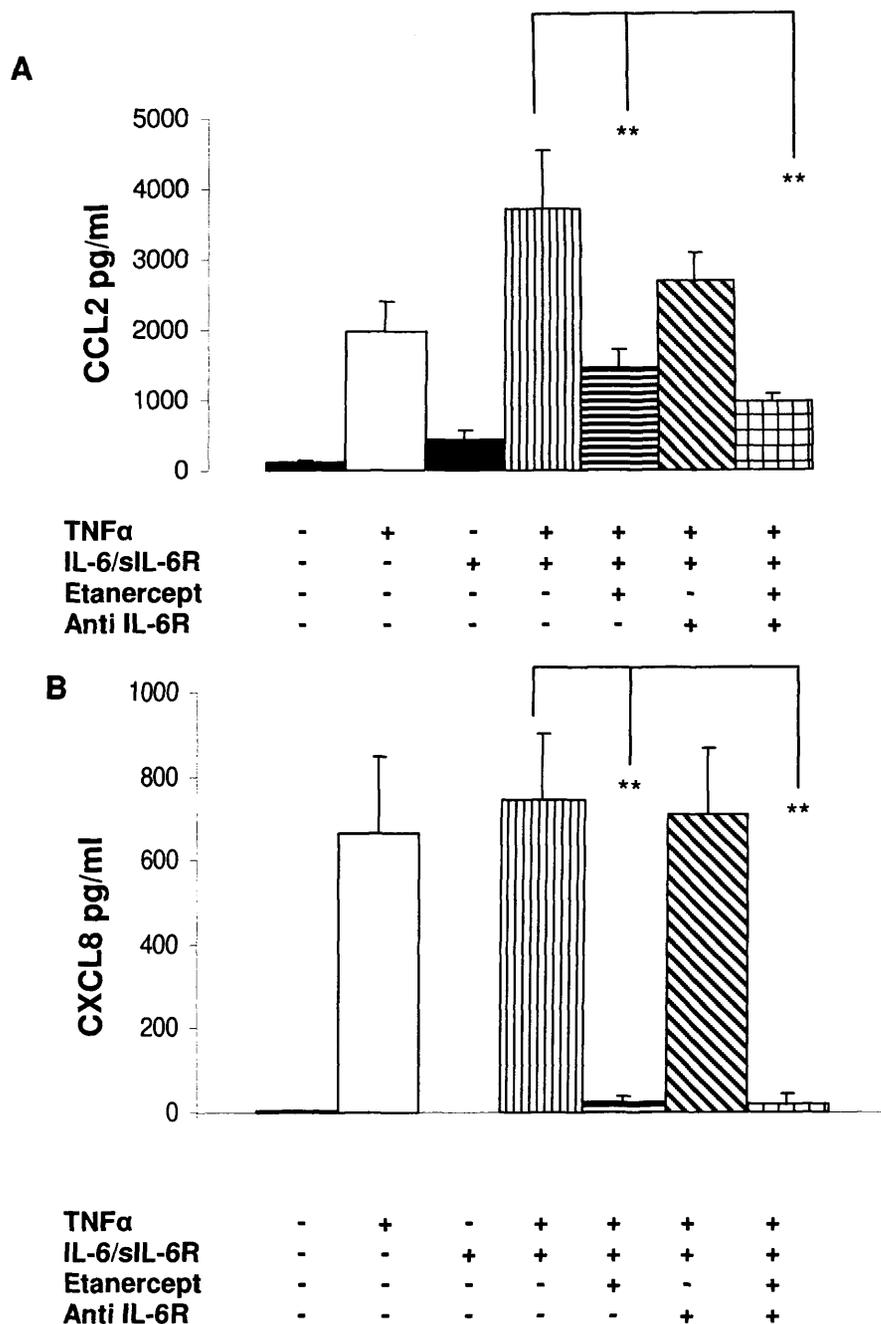
**A** = control un-stimulated cells

**B** = IL-6/sIL-6R stimulated cells

**C** = IL-6/sIL-6R+ mAb 227

**D** = IL-6/sIL-6R+sgp130

**Figure 4.12 - Effect of blockade of TNF $\alpha$  and IL-6R on chemokine production in RA-SF**



Etanercept or anti IL-6R were used respectively to block effect of TNF $\alpha$  and IL-6R on chemokine production in RA-SF (n=8). At the time of combined cytokine stimulation, growth-arrested RA-SF were co-treated with either 0.01 $\mu$ g/ml etanercept (TNF receptor fusion protein), 500 ng/ml MAB227 (anti total IL-6R antibody) or a combination of both and chemokines quantified by ELISA after 20 hours. Data represents the mean  $\pm$  s.e.m. **A** CCL2 production. **B**, CXCL8 production measured.

#### 4.2.6 Time-Course for Chemokine Production in Rheumatoid Arthritis Cell Lines

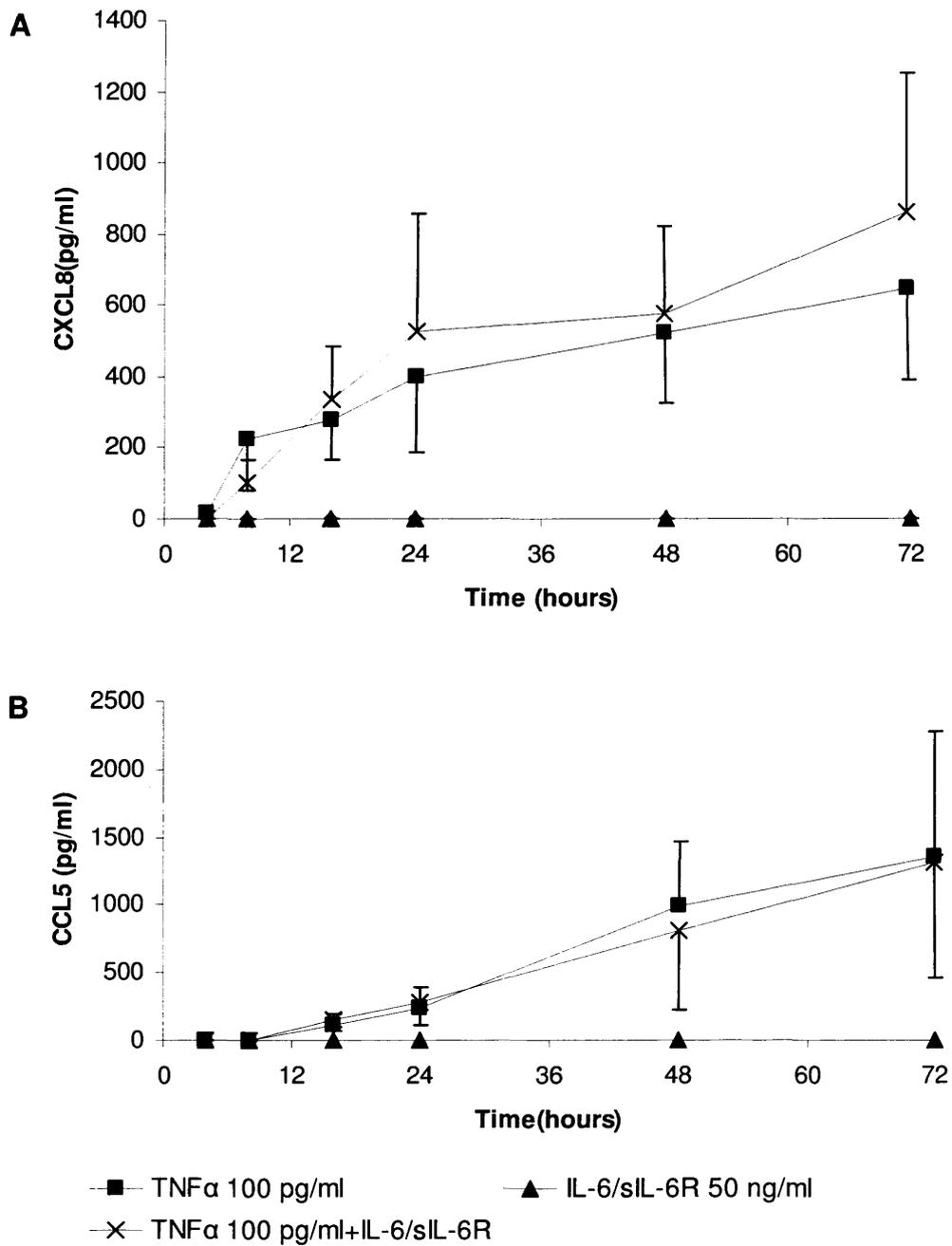
All chemokine levels were measured after 16-20 hour stimulations following on from previous work in the department which suggested that this was the optimal time point for chemokine measurement. However, at this time point CCL5 levels were very low in tissue culture supernatants making it difficult to assess the effects of cytokine inhibition on this particular chemokine. Consequently further time-course experiments were carried out using fewer doses to assess the optimal time-point for measurement of CXCL8, CCL5 and CCL2 production by growth-arrested fibroblasts.

CXCL8 was detected as early as 8 hours after stimulation with TNF $\alpha$ . At this early time point there was no synergistic increase noted by the addition of IL-6/sIL-6R to the cells. However, after 24 hours, additional CXCL8 production was noted in the wells that had been dual stimulated (Figure 4.13 A).

In contrast, CCL5 production was detected at very low levels until 48 hours after stimulation when it markedly increased. In these experiments there was no evidence of any synergy between TNF $\alpha$  and IL-6/sIL-6R in CCL5 production even at the 72 hour time-point where CCL5 production was maximal (Figure 4.13B).

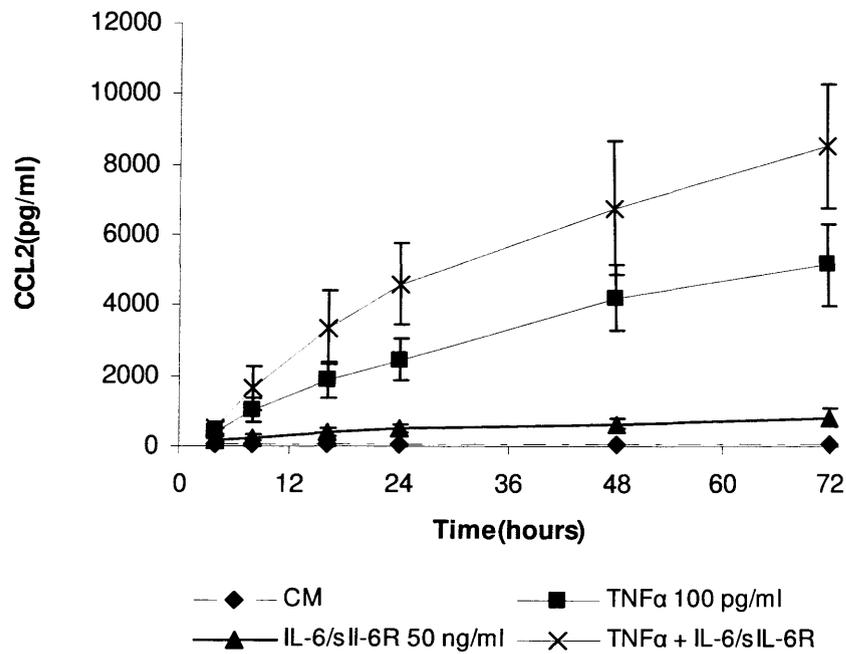
Production of CCL2 increased from 16 hours to levels above basal production. There was clear synergistic up-regulation from 24 hours and levels continued to rise up to 72 hours when the experiment was concluded (Figure 4.14).

**Figure 4.13 – Time-course of CXCL8 and CCL5 production by RA-SF**



Time-course of CXCL8 (A) and CCL5 (B) production by RA-SF quantified by ELISA. Cells were stimulated with TNFα and IL-6/sIL-6R separately and in combination. Stimulations were carried out in serum free media and supernatants removed from separate wells at 4, 8, 16, 24, 48 and 72 hours. Values shown are mean±SEM (n=5 cell lines).

**Figure 4.14 – Time-course of CCL2 production by RA-SF**



Time-course of CCL2 production by RA-SF quantified by ELISA. Cells were stimulated with TNF $\alpha$  and IL-6/sIL-6R separately and in combination. Stimulations were carried out in serum free media and supernatants removed from separate wells at 4, 8, 16, 24, 48 and 72 hours. Values shown are mean $\pm$ SEM (n=5 cell lines).

#### 4.2.7 Adhesion Molecule Expression by Rheumatoid Arthritis

##### Fibroblasts

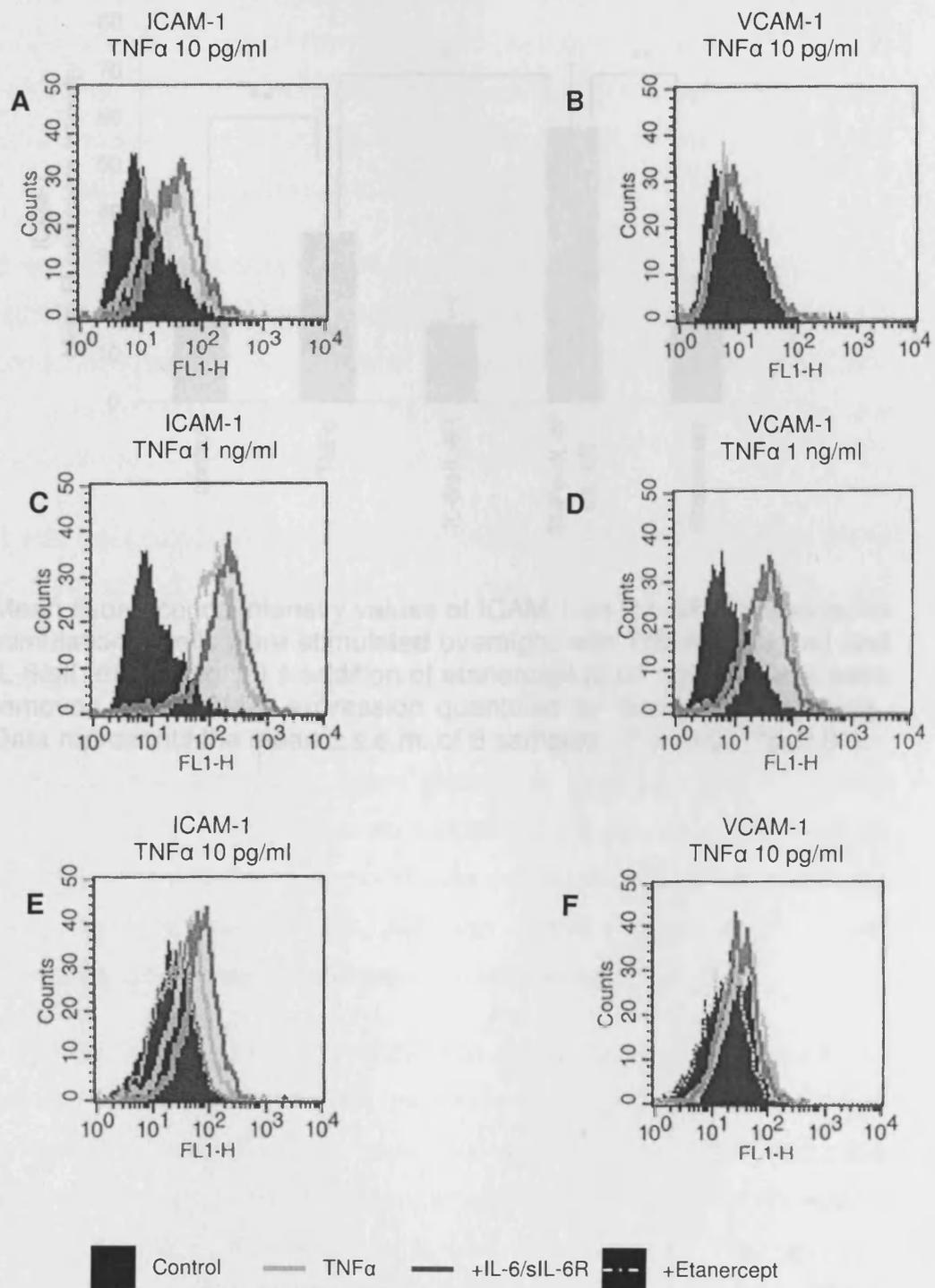
Fibroblasts were grown to confluence at passage 3 as previously described in section 2.13. Cells were then plated out into 6 well plates in order to obtain sufficient numbers for flow cytometry. Doses of TNF $\alpha$  chosen were the minimum and maximum used in chemokine stimulation experiments (10 and 1000 pg/ml). A single dose of IL-6/sIL-6R was chosen (50 ng/ml) which was in the physiological range noted for RA patients.

Stimulation of RA-SF with TNF $\alpha$  for 24 hours resulted in a dose dependent increase in ICAM-1 and VCAM-1 expression on RA-SF. Treatment with IL-6/sIL-6R (50 ng/ml) did not affect expression of either adhesion molecule. However, addition of IL-6/sIL-6R to TNF $\alpha$  resulted in a significant increase in ICAM-1 expression ( $p < 0.05$ ) (Figure 4.15 A and C) but VCAM-1 expression was unaffected (Figure 4.15 B and D). Etanercept completely inhibited the up-regulation of adhesion molecule expression induced by TNF $\alpha$  in combination with IL-6/sIL-6R (4.15 E and F and 4.16).

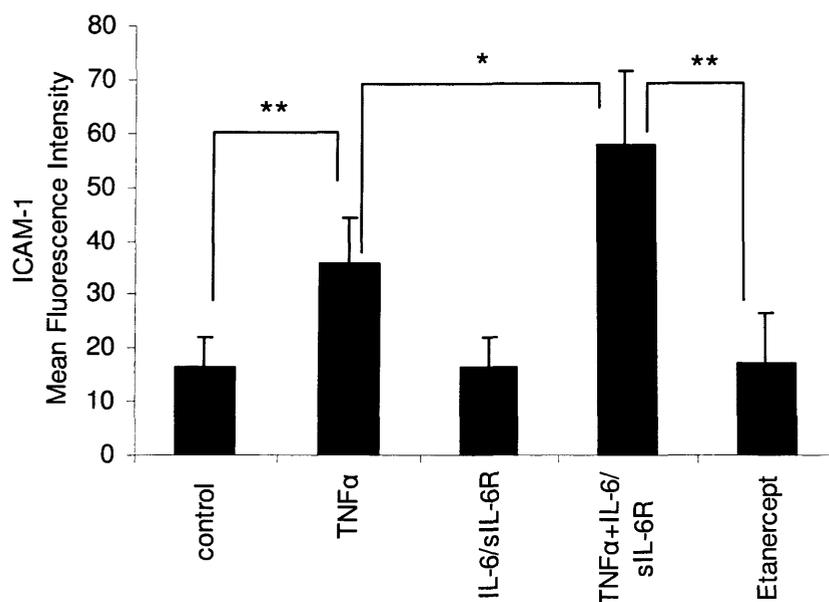
### Figure 4.15

Effect of combined cytokine stimulation and etanercept inhibition on adhesion molecule expression on RA-SF (n=8). Representative flow cytometry plots are shown. Data was acquired from 5,000 gated events. Unstimulated control RA-SF expression is represented as the filled histogram plots. **A.** ICAM-1 production following stimulation with TNF $\alpha$  10 pg/ml and IL-6/sIL-6R 50 ng/ml. **B** VCAM-1 production following stimulation with TNF $\alpha$  10 ng/ml and IL-6/sIL-6R 50 ng/ml. **C** ICAM-1 production following stimulation with TNF $\alpha$  1 ng/ml and IL-6/sIL-6R 50 ng/ml. **D** VCAM-1 production following stimulation with TNF $\alpha$  1 ng/ml and IL-6/sIL-6R 50 ng/m. **E** ICAM-1 production following stimulation with TNF $\alpha$  10 pg/ml and IL-6/sIL-6R 50 ng/ml and addition of etanercept (0.01  $\mu$ g/ml). **F** VCAM-1 production following stimulation with TNF $\alpha$  10 pg/ml and IL-6/sIL-6R 50 ng/ml and addition of etanercept (0.01  $\mu$ g/ml).

**Figure 4.15 - Effect of cytokine stimulation on adhesion molecule expression by RA-SF**



**Figure 4.16 - Mean fluorescence intensity of ICAM-1 expression on RA-SF in response to stimulation with TNF $\alpha$  and IL-6/sIL-6R**



Mean fluorescence intensity values of ICAM 1 on RA-SF in response to stimulation. Cells were stimulated overnight with TNF $\alpha$  (10 pg/ml) and IL-6/sIL-6R (50 ng/ml)  $\pm$  addition of etanercept (0.01 ng/ml). Cells were removed and ICAM-1 expression quantified by fluoroscopic analysis. Data represents the mean  $\pm$  s.e.m. of 8 samples. (\*  $p < 0.05$  \*\* $p < 0.01$ ).

### 4.3 Discussion

TNF $\alpha$  is considered to be the driving cytokine in acute inflammation when neutrophil recruitment is marked (Kumon and Loose 1997). It is likely that influx of neutrophils to the joint in the presence of TNF $\alpha$ , other inflammatory cytokines and mediators leads to shedding of IL-6R from neutrophils (as described in chapter 3).

It is probable that this sIL-6R then binds with IL-6 already within the joint leading to fibroblast activation and an increase in CCL2 and CCL5 production with a subsequent increase in macrophage and T-cell recruitment respectively. These events may contribute to the persistence of inflammation and chronic synovitis.

It was not possible to demonstrate IL-6R within the RA frozen sections or methanol fixed cytopins. This may be explained by the rapid shedding of IL-6R from leucocytes (within 30 minutes) which was demonstrated in the previous chapter. Alternatively, it may be that the antibody used was not suitable for use in immuno-histochemistry. This antibody has previously been shown to work in ELISA or flow cytometry only. There are no published reports of IL-6R receptor staining in frozen tissue sections. As no specific leucocyte stain was used it is also conceivable, although unlikely, that there were no infiltrating leucocytes within these synovial biopsies.

Although CCL2 and CXCL8 production appear to be synergistically up-regulated in a dose dependent fashion by the combination of TNF $\alpha$  and IL-6/sIL-6R, this was not the case for all chemokines. CCL5 production was inhibited when RA-SF were stimulated with TNF $\alpha$  in combination with high doses (200 ng/ml) of IL-6/sIL-6R complex. This provides further evidence for the hypothesis that TNF $\alpha$  and IL-6/sIL-6R complex have different roles in the initiation and maintenance of inflammation in RA. This observation may also be relevant to the partial response observed in RA patients following anti-TNF $\alpha$  therapy. If TNF $\alpha$  is only partially blocked, persisting low levels may interact with IL-6/sIL-6R

allowing an increase in chemokine production and further leucocyte recruitment leading to persistent synovitis.

Work published previously by our group showed down-regulation of CXCL8 (neutrophil chemo-attractant) together with synergistic up-regulation of CCL2 (macrophage stimulating chemokine) production by peritoneal mesothelial cells stimulated with combination of TNF $\alpha$  and IL-6/sIL-6R. They suggested that the influx of neutrophils facilitated formation of IL-6/sIL-6R complex leading to a switch in chemokine production from CXCL8 to CCL2 with subsequent macrophage infiltrate and resolution of acute infection (Hurst *et al* 2001).

Although we did not show inhibition of CXCL8 production the effect on CCL2 with its potential for macrophage recruitment was more marked suggesting that CCL2 was preferentially induced by the combination of TNF $\alpha$  and IL-6/sIL-6R. It is clear that chronic inflammation is present in RA. It is possible that the normal process of chemokine switching used to resolve acute inflammation is disordered in RA leading to propagation of chronic inflammation. During RA flares neutrophils and CXCL8 levels remain high within the joint suggesting that resolution of acute flare does not happen rapidly (Troughton *et al* 1996; Endo *et al* 1991). This is in contrast to CXCL8 levels in acute infection which return to normal within 2 days (Hurst *et al* 2001). In arthritis it is likely that CXCL8 serves not only as a neutrophil chemo-attractant but may have other separate roles. When CXCL8 was injected into normal rabbit knee joints rapid neutrophil recruitment occurred (by 4 hours). In addition, at 24 hours the synovium was observed to be thickened due to increase in synovial fibroblasts and macrophages (Endo *et al* 1991). It is probable that the neutrophil influx resulted in production of other cytokine and chemokines.

My time-course experiments showed that production of each chemokine is independently regulated. CXCL8 is produced early. This allows initial neutrophil infiltrate into the joint as described in other previous work (Szekanecz *et al* 1998). Production of CCL2 occurs

later after a longer more sustained exposure to TNF $\alpha$  and IL-6/sIL-6R (at approximately 24 hours in our experimental system). CCL5 production occurs even later (at 72 hours *in vitro*). Since the majority of the experiments to measure chemokines were carried out after 24 hours exposure to TNF $\alpha$  this would account for the low levels of CCL5 seen. The early production of CXCL8 and the later increase in other chemokines corresponds with the initial neutrophil influx seen in synovial fluid with the subsequent development of macrophage and lymphocyte infiltrates within pannus in the context of chronic synovitis and inflammation (Sweeney and Firestein 2004).

OA synovial fibroblasts also produced CCL2, CXCL8 and CCL5 in a dose dependent manner following stimulation with TNF $\alpha$  (Figures 4.8 - 10). There was no clear evidence of a synergistic effect on chemokine production as a result of addition of IL-6/sIL-6R, however, there is a greater variability in chemokine production by OA cell lines when compared to RA cell lines. This may mean that a true synergistic effect would not be picked up due to the large variability in chemokine production. A small synergistic effect could be missed due to the inherent variability in the system. These experiments could be repeated in further cell lines in order to clarify this point.

OA-SF have previously been shown to produce CCL2 in response to TNF $\alpha$  stimulation. There appeared to be less variability in chemokine production in this report however cells did not appear to be stimulated in serum-free media and only synovial samples deemed to be inflammatory by histological assessment were included (Fiorito *et al* 2004).

In RA cell lines treatment with etanercept resulted in significant reduction of CCL2 levels which were further reduced by the addition of anti IL-6R antibody. In contrast treatment with etanercept alone abolished all CXCL8 production again providing evidence that TNF $\alpha$  and IL-6/sIL-6R complex have different roles in the pathogenesis of RA.

Conflicting reports have occurred regarding the effect of IL-6/sIL-6R on adhesion molecule expression. Our group has shown previously that human synovial fibroblasts do not express ICAM-1 or VCAM-1 in response to stimulation with IL-6/sIL-6R complex (Nowell *et al* 2003). However, adhesion molecule expression has been reported to be IL-6/sIL-6R responsive with up-regulation of ICAM-1 in umbilical vein endothelial cells. Other reports suggest up-regulation of ICAM-1 and VCAM-1 (Modur *et al* 1997; Romano *et al* 1997). In contrast to our work these experiments were performed in serum which may have contained endogenous cytokines including TNF $\alpha$  or IL-1. It is now evident from my work that combination of IL-6/sIL-6R with TNF $\alpha$  results in greater up-regulation of ICAM-1 expression than TNF $\alpha$  alone. VCAM-1 was not further up-regulated by the addition of IL-6/sIL-6R. This represents differential regulation of these adhesion molecules.

Although VCAM-1 is up-regulated in active RA; blockade of VCAM-1 in mCIA did not result in disease improvement (Carter *et al* 2002). In contrast ICAM-1 deficient mice are resistant to mCIA. Anti ICAM-1 antibody therapy was associated with clinical improvement in phase I/II trials in RA patients suggesting that ICAM-1 may be more important in the regulation of leucocyte trafficking in RA (Bullard *et al* 1996; Kavanaugh *et al* 1996).

In summary, we demonstrated that TNF $\alpha$  and IL-6 trans-signalling interact to modulate leucocyte recruitment via effects on chemokine production most marked on CCL2 (macrophage chemo-attractant). In addition we demonstrated that TNF $\alpha$  and IL-6 trans-signalling also up-regulate ICAM-1 expression on synovial fibroblasts potentially leading to significant effects on leucocyte recruitment in the RA joint. The differing actions of these TNF $\alpha$  and IL-6/sIL-6R in leucocyte recruitment together with the evidence that combined blockade in-vitro is beneficial provides good evidence for an in-vivo study using combined cytokine blockade in an animal model of arthritis.

# 5 THERAPEUTIC EFFICACY OF COMBINED SGP130 AND ETANERCEPT THERAPY IN MURINE COLLAGEN INDUCED ARTHRITIS

## 5.1 Introduction

We have demonstrated that TNF $\alpha$  and IL-6 trans-signalling interact to modulate leucocyte recruitment via effects on chemokine production and adhesion molecule expression potentially leading to significant effects on leucocyte recruitment in the RA joint. The differing actions of these TNF $\alpha$  and IL-6/sIL-6R in leucocyte recruitment, together with the evidence that combined blockade *in-vitro* is beneficial, provides good evidence for an *in-vivo* study using combined cytokine blockade in an animal model of arthritis.

### 5.1.1 Experimental Models of Arthritis

There are a number of published experimental models of RA reported in the literature. Each model has merits and weaknesses. A brief summary of the most popular methods is provided.

Antigen induced arthritis (AIA) is induced by 2 subcutaneous injections of methylated bovine serum albumin (mBSA) in complete Freund's adjuvant 7 days apart. 14 days later arthritis is induced in a single joint by intra-articular injection of adjuvant (mBSA).. Although AIA is pathologically similar to RA, it is monoarticular and confined to the injected joint. This model is useful for assessing histological/pathological changes and can also be used for local administration of therapeutics. However, it is not a systemic disease and therefore does not provide optimal conditions for testing therapeutics which are given by parenteral means.

Pristane induced arthritis is polyarticular but primarily affects large joints, i.e. ankles and wrists. Histologically, arthritis exhibits synovitis, periostitis and erosions. However, the incidence of arthritis is low and the time course un-predictable. Even in the maximally susceptible population, only 50% of mice displayed signs of arthritis at 120 days following injection. Maximum incidence of 65% was reached at 200 days (Vigar *et al* 2000). Because the timecourse is unpredictable and the course of disease very variable, it is difficult to identify a suitable time-point for administration of therapeutics. Any experiment using pristane induced arthritis to test therapeutics would require large numbers of animals and would also take several months.

Collagen induced arthritis was felt to be the most appropriate model for this project and the reasons for choosing it are discussed below. Collagen induced arthritis (CIA) is a good model for RA. It displays the majority of features of RA including synovitis, erosions and inflammatory infiltrate (described in section 1.10). The disease is polyarticular and systemic. It can be induced in rats or mice; however the induction schedule differs between species. In rats, arthritis is generally induced by a single intra-dermal collagen injection in Freund's incomplete adjuvant. Disease is usually evident by 12 days after the intra-dermal injection but may occur at any time within a 3 week window. In mice, disease is induced by 2 intra-dermal collagen injections in Freund's complete adjuvant. The disease course is more predictable in mice and in our hands there is generally a 100% incidence by day 27. This minimises the number of animals needed in order to achieve experimental endpoints. The disease is more severe in mice than rats providing a better model for human disease. mCIA has also been well established as a model for testing therapeutics including anti-TNF $\alpha$  antibodies, soluble TNF receptors, soluble IL-15 receptor  $\alpha$ -chain therapy, soluble IL-1 receptors, IL-18 binding protein C and many other therapies (Wooley *et al* 1993 b; Ruchatz *et al* 1998; Smeets *et al* 2003).

### 5.1.2 Anti-cytokine Therapy in mCIA

mCIA has been used to demonstrate the efficacy of agents directed against TNF $\alpha$  and was essential in the development of biological therapies for RA. Williams et al in a prophylaxis experiment gave anti-TNF antibodies by intra-peritoneal (i.p.) injection once weekly for 4 weeks starting the day before the first collagen injection. This resulted in significant reduction in paw swelling and improvement in severity of joint histology. Interestingly, no effect was seen on incidence of arthritis or clinical score. Anti-TNF antibodies were then used in established disease. Animals were injected twice weekly from the first signs of clinical disease which led to a significant reduction in clinical score and improvement in histology. They did not comment on effect on leucocyte infiltrate (Williams *et al* 1992).

Piguet et al used soluble TNF receptors and anti-TNF antibodies in a prophylactic experiment to prevent development of arthritis. Their induction regime differed from ours, as collagen in CFA was given on 1 or 2 occasions 14 days apart. They described an arthritis which initially developed after 1 month and slowly increased for 2-3 months. Significant reduction in foot-pad thickness as a result of therapy was noted. No benefit was obtained from treatment in established disease (Piguet *et al* 1992).

Wooley et al used a recombinant soluble TNF receptor:Fc fusion protein by i.p injection in both preventative and therapeutic experiments. In the preventative protocol, arthritis severity and incidence were both reduced. In the therapeutic trial, the severity of arthritis was reduced. There was no histological assessment in this trial (Wooley *et al* 1993 a).

#### 5.1.2.1 Targeting IL-6 as Therapy in mCIA

IL-6 can signal in 2 separate ways. During classical IL-6 signalling, IL-6 is bound to cognate IL-6R on the cell surface (circulating leucocytes

and hepatocytes), signal transduction then occurs via gp130 signal transduction protein which is universally expressed (See section 1.5.4).

Cells which do not express the cognate IL-6R can be made responsive to IL-6 via interaction/binding to sIL-6R in a process known as trans-signalling. This occurs where IL-6 binds to sIL-6R and then forms a hetero-dimer which then signals via cell surface bound gp130.

This allows 2 separate therapeutic options:

- a) Total blockade of both classical IL-6 signalling and trans-signalling via antibodies directed against IL-6 or IL-6R; or
- b) Specifically blocking trans-signalling only using sgp130 which will bind to the IL-6/sIL-6R complex preventing activation of membrane bound gp130.

These will be discussed in detail below.

#### 5.1.2.2 Total Blockade of Both Classical IL-6 Signalling and Trans-signalling via Antibodies Directed Against IL-6 or IL-6R

Blockade of IL-6 has been tried in clinical studies with varying degrees of success. Initial murine studies using knock-out mice showed that IL-6 was essential for the development of collagen and antigen-induced arthritis (Alonzi *et al* 1998; Boe *et al* 1999). There are no published reports of therapeutic blockade of IL-6 in CIA. Antibodies directed against IL-6 have been used in RA patients but despite initially promising results they have not reached clinical practice (Wendling *et al* 1993).

Results obtained in initial animal studies using antibodies directed against IL-6R are comparable with those obtained in trials of anti-TNF therapies. In the first study using a rat anti-mouse IL-6R, antibody was administered daily for 2 weeks. Incidence of arthritis was reduced by

approximately 33% and severity of arthritis reduced. This effect was seen only when therapy was started on day 0 or 3 after collagen immunization and not in established disease. In a second experiment animals were treated with a single dose of anti mouse IL-6R antibody over a dose range (0.5 - 8mg) and at differing time points (Days 0 - 21). It was found that anti IL-6R antibody suppressed arthritis severity in a dose-dependent manner but only when given prior to the onset of clinical disease (Yoshzaki *et al* 1998; Takagi *et al* 1998; Williams *et al* 1992).

#### 5.1.2.3 Blocking Trans-signalling Specifically Using sgp130

Targeting trans-signalling would prevent IL-6 directed events within the RA joint; specifically those mediated by resident cells such as fibroblasts and chondrocytes but would allow cognate IL-6 signalling to continue unaffected. It is likely, therefore, that this will allow IL-6 mediated activities such as CRP generation and production of fever to occur as normal. Targetting trans-signalling should also prevent the occurrence of abnormalities in LFT and cholesterol seen in the trials of Tocilizumab in RA patients discussed in detail in section 1.5.5.

sgp130 is an agent that could be used to target trans-signalling specifically as this molecule will only bind to IL-6/sIL-6R which is already complexed. It cannot bind to either IL-6 or sIL-6R which is not complexed. A sgp130:Fc fusion protein has been developed in order to improve the half-life and bio-availability of sgp130 within the circulation (Atreya *et al* 2000). This sgp130:Fc was investigated as a therapeutic in Crohn's disease where IL-6 is also considered a major driving cytokine. The authors do not state what improvement in half life was obtained by adding Fc fusion protein. Local experience has shown that when producing therapeutics aimed at neutralizing complement that addition of an Fc fusion protein extends half life from 20 minutes to 33 hours (Harris *et al* 2002).

Results in an animal model of Crohn's disease showed that targeting trans-signalling by sgp130:Fc resulted in reduction in clinical features of disease (weight loss) together with an improvement in histology. There was a trend towards further improvement with a combination of antibodies directed against IL-6R and TNF $\alpha$  although this did not reach statistical significance (Atreya *et al* 2000).

Use of sgp130:Fc in arthritis has been limited to antigen induced arthritis. Nowell *et al* demonstrated a significant improvement in histological scores of disease severity in mice treated with a single dose of sgp130:Fc by i.a. injection (5 ng) at the same time as arthritis induction. Improvements were seen in synovial hypertrophy, exudate and in cellular infiltrate. A reduction in CCL2 staining was demonstrated in sections from the knees treated with sgp130:Fc compared to the arthritic knees of untreated animals (Nowell *et al* 2003).

IL-6 trans-signalling has also been targeted using recombinant protein gp130-RAPS (gp-130 of the rheumatoid arthritis antigenic peptide-bearing soluble form; a 50 kd soluble gp130 found naturally in the synovial fluid and plasma of RA patients). This peptide was initially described by Tanaka *et al* who also described presence of neutralizing antibodies to it in RA (Tanaka *et al* 2000).

Treatment of antigen induced arthritis with a single intra-articular injection of gp130-RAPS at time of arthritis induction resulted in significant improvement in histological disease severity including synovial infiltrate and cellular exudate (Richards *et al* 2006). Both these papers demonstrate the importance of trans-signalling in RA and animal models of RA.

### 5.1.3 Mechanism of Action of sgp130:Fc in Experimental Models

Nowell and Richards demonstrated a reduction in leucocyte infiltrate in antigen induced arthritis as a result of targeting IL-6 trans-signalling.

My own work has demonstrated that both CCL2 and CCL5 production are synergistically up-regulated by the combination of TNF $\alpha$  and IL-6/sIL-6R complex and that this increase is abrogated by therapy with etanercept and anti IL-6R antibody. Therefore, we surmised that the effect on leucocyte recruitment seen in AIA may be due to inhibition of chemokine production, i.e. CCL5 and CCL2 with subsequent effects on T cell and macrophage infiltration. The efficacy and mechanism of action of sgp130:Fc in mCIA are at present unknown and will be assessed in this project.

#### 5.1.4 Factors Driving Pathological Change in mCIA

##### 5.1.4.1 Macrophages in Pathogenesis of CIA

Andren et al, using an Fc $\gamma$ RIII deficient mouse which was resistant to the development of CIA, demonstrated that disease could be initiated by adoptive transfer of Fc $\gamma$ RIII positive peritoneal macrophages. It was concluded that Fc $\gamma$ RIII-expressing macrophages, producing pro-inflammatory cytokine and T helper type 1 differentiating factor, are the major effector cells in the induction of mCIA (Andren *et al* 2006).

Ogata et al, using rat collagen induced arthritis, examined the distribution of CCL2 positive cells. It was noted that MCP-1 (CCL2) peaked within the joint 2 weeks after initial collagen injection. Although MCP-1 staining was not specific for macrophages injection of a neutralizing monoclonal antibody against rat MCP-1 significantly decreased the number of exudate macrophages with no significant effect on other cell types within the joints and reduced the ankle swelling by about 30 per cent compared with controls. They suggested that MCP-1 plays a critical role in this model in the recruitment of monocytes and in the development of arthritis (Ogata *et al* 1997).

In a study investigating leucocyte recruitment in murine collagen induced arthritis it was noted that three distinct phases of leucocyte recruitment could be defined:

- 1) Early infiltration of T cells and appearance of class II expressing macrophages in the synovial lining layer;
- 2) Profound infiltration of granulocytes and oedema formation; and
- 3) Pannus formation containing activated macrophages, granulocytes, T cells and dendritic cells.

This suggested that macrophages must be important in the pathogenesis of mCIA (Holmdahl *et al* 1991).

Van Lent *et al* used clodronate to deplete macrophages from synovial lining. Using a single dose of intra-articular liposomal clodronate, a significant reduction in inflammatory cell infiltrate in the injected knees was noted. In a further experiment a reduction in chondrocyte death in clodronate injected knees was also noted in mCIA. Knee involvement is not universal in mCIA and therefore mice were also given LPS at day 28 following collagen injection. These results may not be truly representative of mCIA because of the LPS injections (van Lent *et al* 1996; van Lent *et al* 1998).

#### 5.1.4.2 Effect of Macrophage Chemokine Blockade

Other groups have shown that blockade of CCL2 (using a CCL2 antagonist at 2 mg/kg per day) in MRL-1pr mice prior to disease induction prevents arthritis but once disease is established then blockade of both CCL2 and Gro- $\alpha$  (Neutrophil chemottractant) was needed to inhibit arthritis (Gong *et al* 1997; Gong *et al* 2004). A CCR2 receptor antagonist has recently been shown to reduce macrophage infiltrate in murine-hypersensitivity when an oral CCR2 antagonist (30-100 mg/kg) was given at the same time as i.p. thioglycate. This CCR2 antagonist given orally at a dose of 100 mg/kg twice daily from day 9

after adjuvant injection; was also shown to be effective at reducing severity of rat adjuvant arthritis both clinically and histologically (Brodmerkel *et al* 2005). However, despite stating that CCR2 inhibition is effective in collagen induced arthritis; this data has never been published.

In conclusion it appears that macrophage recruitment is important in the pathogenesis of mCIA.

#### 5.1.4.3 T Cells in the Pathogenesis of mCIA

The importance of T cells in the pathogenesis of mCIA is undisputed and mCIA is generally considered to be a T cell driven disease. Using anti-CD4 antibodies, Williams *et al* demonstrated that depleting anti-CD4 antibodies were only effective in mCIA when given around the time of first collagen injection. When given prophylactically around the second collagen injection, prior to development of arthritis, therapy was ineffective (Williams RO 1996). In contrast, when anti-CD4 antibodies were given in conjunction with anti-TNF, there was a greater improvement in cellular infiltrate and histological score than when either therapy was given alone (Marinova-Mutafchieva *et al* 2000). The failure of anti-CD4 therapy may in part be due to the effect on suppressor T cells, e.g. regulatory T cells as well as effect on pathogenic T cells. Therefore, the effects of therapy on regulatory T cells should be studied.

#### 5.1.4.4 Effect of T Cell Chemokine Blockade

Blockade of CCL5 activity by a selective chemokine receptor antagonist (metRANTES) at a dose of 50 or 100 µg three times per week starting from week one post immunisation with type II collagen was shown in 1997 to reduce disease incidence and day of onset in a dose dependent manner. These animals also had significantly lower clinical scores than un-treated animals. MetRANTES was found to inhibit MIP-1α as well as RANTES (CCL5) (Plater-Zyberk *et al* 1997).

Met-RANTES has also been shown to be effective at reducing incidence and severity of rat antigen-induced arthritis when given before onset of clinical disease. Macrophage infiltrate demonstrated by anti-CD68 antibody was also significantly reduced (Shahrara *et al* 2005).

Barnes *et al*, using a RANTES specific antibody (at unspecified dose) with no chemokine cross-reactivity, demonstrated significant reduction in clinical score and histological scores, specifically leucocyte infiltration. Reduction in erosions as demonstrated by radiography was also seen (Barnes *et al* 1998).

#### 5.1.4.5 Regulatory T Cells in mCIA

Initiation of auto-immune disease requires that normal self-tolerance is lost. If reactive T cells are present in the peripheral circulation then initiation of auto-immune disease can occur. The major cell type which is usually responsible for the suppression of self-directed immune responses is known as a regulatory T cell (T-Reg). The cell surface markers of T-Regs are CD4<sup>+</sup> and CD25<sup>+</sup> and they may also be recognised by their expression of intra-cellular Fox-P3 protein (a fork head / winged-helix protein which is a transcriptional regulator crucial for murine T cell development and function).

Regulatory T cells were first discovered in animal models of auto-immune disease in 1971 when a population of T cells which suppressed other T cells were discovered. Later, Sakaguchi *et al* demonstrated that transfer of CD4<sup>+</sup> T cells which had CD25 depleted developed organ specific auto-immune disease. If CD4<sup>+</sup>/CD25<sup>+</sup> cells were co-administered then auto-immune disease did not occur. Depletion of regulatory T cells appeared to exacerbate auto-immune diseases including mCIA (Sakaguchi *et al* 1995). Absence of regulatory T cells results in faster, more aggressive development of arthritis in the K/BxN model (Nguyen *et al* 2007). Adoptive transfer of regulatory T cells has been shown to slow disease progression in

mCIA (Morgan *et al* 2005). More recently, reports have emerged that improvement in mCIA is due to induction of regulatory T cells by specific therapeutics.

### 5.1.5 Transcription Factors in mCIA

#### 5.1.5.1 NF kappa B

NFκB proteins are ubiquitously expressed transcription factors which are known to be up-regulated by pro-inflammatory signals, e.g. cytokines such as TNFα or IL-1β or alternatively by antigen receptor binding. These transcription factors are involved in the regulation of expression of numerous genes involved in the inflammatory response. Many studies have examined the contribution of the NFκB signalling pathways to the pathogenic process seen in RA including inflammation, cartilage degradation, cell proliferation, angiogenesis and pannus formation (Roman-Blas *et al* 2006; Bondeson 1999; Feldmann *et al* 2002).

NFκB activation has been shown to be critical in the initiation of mCIA. Seetharaman *et al* demonstrated that trans-genic mice expressing an NFκB inhibitor in their T cell lineage had lower incidence and less severe mCIA than their non-transgenic littermates (Seetharaman *et al* 1999).

Gerlag *et al* showed that treatment with an NFκB inhibitor (10 mg/kg per animal per day given by ip Injection from day of second intra-dermal collagen injection) resulted in significant reduction of disease severity in mCIA as measured by clinical score. Using EMSA, they demonstrated that this reduction in disease severity was associated with down-regulation of NFκB activation within murine ankle joints (Gerlag *et al* 2000).

#### 5.1.5.2 STAT Signalling

IL-6 has been demonstrated to signal via the Janus kinase and signal transducer and activator of transcription pathway (Jak-STAT). This is also the signalling target pathway of many other pro-inflammatory cytokines including Interferon  $\gamma$ ; Interleukins 12, 4, 7 and 15 together with other Interleukin -6 family members, i.e. Oncostatin-M and LIF. At present seven members of the STAT family have been identified (Walker *et al* 2006).

T cell activation in mice appears to require gp130 mediated STAT 3 activation with no evidence of involvement of STAT1 (McLoughlin *et al* 2005). Although IL-6 knock-out mice (IL-6<sup>-/-</sup>) have been shown to be resistant to the development of experimental arthritis; it is not yet clear whether this is due to impaired STAT activation (Nowell *et al* 2003).

#### 5.1.5.3 STAT Inhibition in Murine Models of Arthritis

Shouda *et al* identified that STAT3 was activated in the synovial tissue of RA patients but not those with OA. Therefore, they examined the effects of targeting STAT3 signalling in mCIA. They cloned a cytokine signal regulator SOCS3/CIS3 compound which inhibited JAK tyrosine kinase activity and had a negative regulatory role on STAT3 (CIS3). The effect of delivery of an adenovirus carrying CIS3 on mCIA was examined. mCIA was induced by immunization with bovine type II collagen in Freund's complete adjuvant on 2 occasions, 21 days apart, together with intra-peritoneal LPS at day 28. Adenovirus was administered by peri-articular injection on day 28 after first collagen injection. CIS3 injection resulted in significant reduction in clinical score and histological joint destruction - in particular, bone erosion and inflammatory cell infiltrate.

In a subsequent experiment, adenoviral gene transfer of CIS3 was administered in established disease (Day 32). CIS3 strongly blocked progression of disease as assessed by clinical score, paw thickness

and redness and was also stated to improve histology (Shouda et al 2001).

#### 5.1.6 Summary

In summary, mCIA was chosen as the most relevant model for our experiments. The efficacy of sgp130:Fc has been proven in mAIA by local injection however its efficacy in a systemic model, i.e. mCIA is not yet known. The aim of this chapter was to identify the effects of targeting trans-signalling with sgp130 by intra-peritoneal injection and to gain some insight into its mechanism of action via effects on macrophages, T cells and cell signalling pathways.

Anti-TNF therapies have been shown to be effective in mCIA. The use of anti-TNF therapies in clinical practice has, without doubt, greatly improved outcomes and quality of life for many patients with RA but there remains a cohort of patients for whom they are either inappropriate or ineffective. This has been addressed to some extent with the development of other biologics including Rituximab and Abatacept. There is a role for more biologic therapies and we hope to examine the efficacy of sgp130:Fc as a biologic for inflammatory arthritis.

Combination therapy is well recognised as a useful tool in RA management. In recent years, triple therapy with Methotrexate, Sulphasalazine and Hydroxychloroquine has been shown to be almost as effective as biological therapies in improvement of symptoms and signs of disease together with prevention of disease progression (O'Dell *et al* 2002).

Combination biological therapy has been used with agents directed against TNF $\alpha$  and IL-1. These cytokines have very similar modes of action. Unfortunately, as might be predicted from their biological actions, this resulted in an increase in side effects and specifically,

serious infections without improvement in clinical efficacy (Genovese *et al* 2004).

By modelling patients with a sub-optimal response to anti-TNF therapy the effect of addition of sgp130:Fc was studied in order to identify whether there was added benefit from combination therapy. The differing effects of IL-6 trans-signalling and TNF $\alpha$  in biological processes and disease pathology allowed us to speculate that blocking these 2 cytokine pathways could reduce disease activity and improve outcome without causing an increase in morbidity.

The specific aims were:

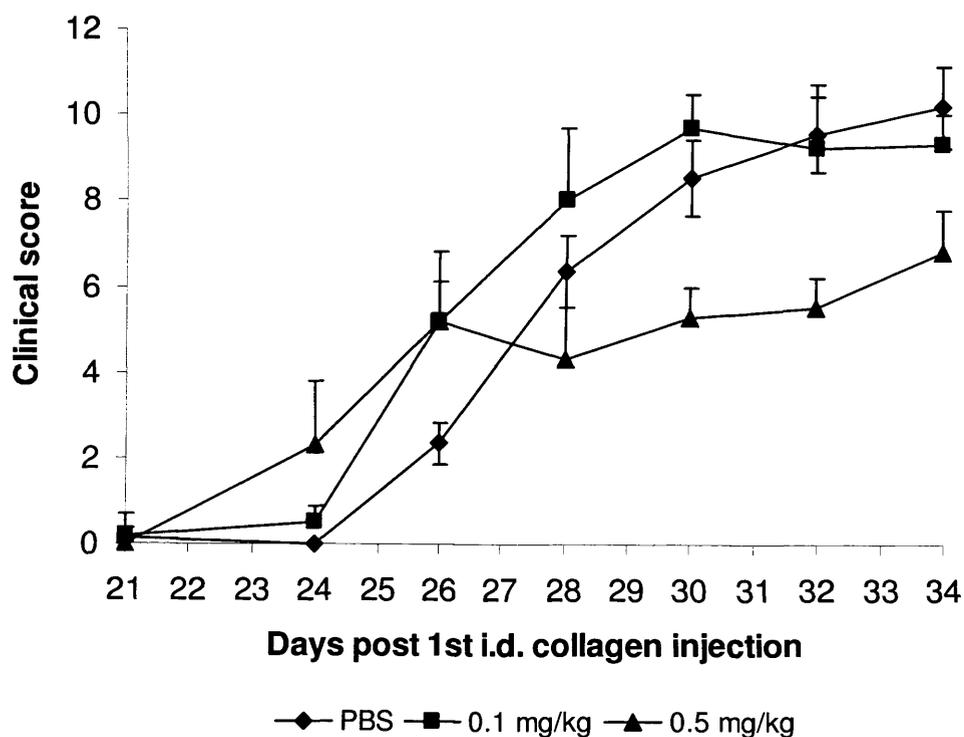
1. To establish a dose of soluble gp130:Fc that would exert significant therapeutic benefit when administered prior to disease onset in mCIA (Disease prophylaxis).
2. To identify a dose of etanercept which resulted in 50% reduction in clinical score in mCIA when compared to control animals after 7 days of therapy (to model sub-optimal response to therapy).
3. To characterise the effects of targeting trans-signalling with soluble gp130:Fc in established mCIA.
4. To assess the efficacy of combination therapy directed against TNF $\alpha$  (etanercept) and IL-6 trans-signalling (sgp130:Fc) in established mCIA.
5. To study the effects of etanercept and sgp130:FC as monotherapy and in combination upon relevant mechanisms of disease and to relate these findings to pathological outcome.

## 5.2 Results

### 5.2.1 Effect of Blockade of IL-6 Trans-signalling and Sub-optimal Dose of Etanercept on Disease Prophylaxis

Previous preliminary dose ranging experiments conducted in the laboratory demonstrated that 0.1 mg/kg sgp130:Fc had no effect upon the progression of mCIA when administered by i.p. injection on alternate days. At 0.5 mg/kg, sgp130:Fc elicited a mild but insignificant improvement in clinical score over the 34 day time course of the experiment (Figure 5.1). As a consequence of this data we speculated that increasing the dose to 2.5 mg/kg might exert significant therapeutic benefit in mCIA. Consequently, in our first experiment to determine whether sgp130:Fc could significantly improve disease outcome measures in mCIA, a dose of 2.5 mg/kg was assessed.

**Figure 5.1 - Graph showing dose response of clinical scores for mice treated with prophylactic doses of sgp130:Fc from time of arthritis induction (data courtesy of Dr P Richards)**



Arthritis (mCIA) was induced as described previously in section 2.16. Briefly, mice were injected intra-dermally with an emulsion of type II collagen in complete Freund's adjuvant (100 µl) into 2 sites in the flank. At Day 21, animals received a second booster immunisation with type II collagen via the intradermal route (100 µl emulsion in complete Freund's adjuvant). Animals received intra-peritoneal PBS (100µl) or sgp130:Fc (2.5mg/kg) in equivalent volume of vehicle on alternate days from day 21 (n=6 per treatment allocation). The disease time-course is shown in table 5.1.

Clinical scores were assessed daily together with paw diameter measurements using a spring-loaded micrometer. Animals were sacrificed at day 34 or earlier if severity limits were reached. Maximum severity limits allowed under terms of project licence 30/2361 were a score of 5 in any single paw or a combined score of 14 in all 4 paws. Typical appearance of murine paws affected by arthritis is shown in figure 5.2.

**Table 5.1 Arthritis incidence in PBS controls and sgp130:Fc therapy animals over disease time course (disease prophylaxis)**

<b>Day number</b>	<b>% Incidence- PBS</b>	<b>% Incidence- sgp130:Fc</b>
21	0	0
26	50	17
29	100	50
34	100	50

**Figure 5.2 - Animals displaying signs of arthritis**

**A**



**B**



mCIA was induced as described above. **A** depicts arthritis affecting forepaws. Left forepaw scores 3, right forepaw scores 2. **B** depicts arthritis affecting hind-paws. Left hind-paw scores 2, right hind-paw scores 3.

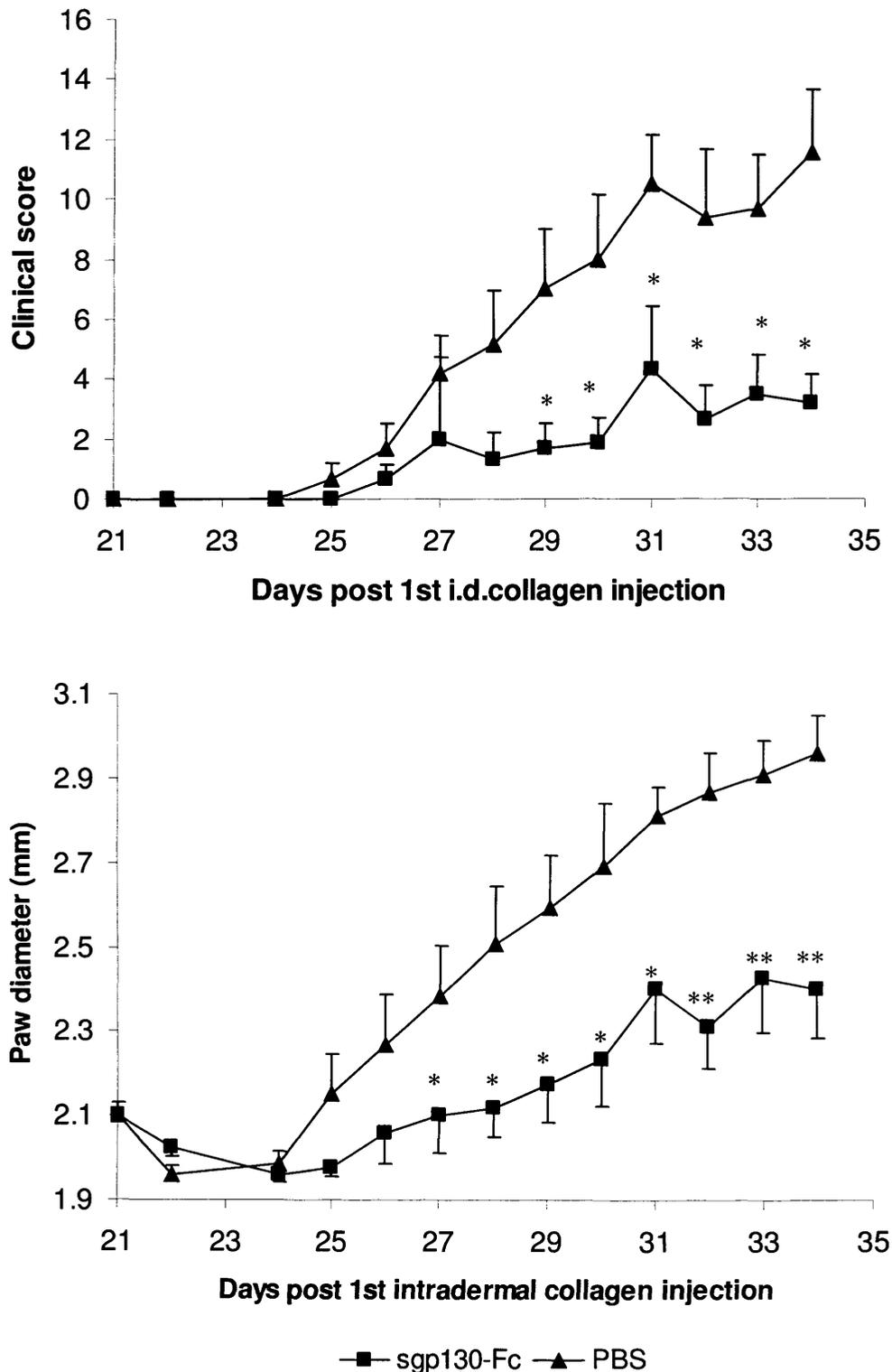
5.2.1.1 Clinical Disease Severity in sgp130:Fc Treated Animals and Control Group (Arthritis Prophylaxis Experiment)

At time of initiation of therapy on day 21, none of the animals receiving PBS or sgp130:Fc exhibited signs of arthritis and were allocated clinical score of 0. By day 26, clinical scores (mean±SEM) in PBS and sgp130:Fc treated animals had increased to  $1.67\pm 0.83$  and  $0.67\pm 0.46$ . There was no significant difference between the groups at this point. Clinical scores increased steadily in the PBS treated animals reaching  $7\pm 1.78$  on day 29. At this point clinical scores in the sgp130:Fc treated animals were significantly lower ( $1.67\pm 0.88$   $p\leq 0.05$ ). By day 34 severity limits, as defined in our PPL, had been reached which necessitated termination of the experiment. Mice receiving PBS recorded clinical scores of  $11.5\pm 2.13$  at endpoint. These were significantly higher than clinical score values in sgp130:Fc treated mice ( $3.16\pm 0.95$   $p\leq 0.05$ ).

In the PBS group, paw diameters (mean±SEM,mm) increased from  $2.1\pm 0.03$  at baseline to  $2.27\pm 0.11$  at day 26. In the gp130:Fc group, paw diameters at day 26 were  $2.06\pm 0.09$ . There was no significant difference between the groups at this point. Paw diameters in the PBS treated animals rose steadily reaching  $2.59\pm 0.15$  on day 29. At this point paw diameters in the sgp130:FC treated group were significantly lower ( $2.18\pm 0.09$   $p\leq 0.05$ ). By day 34 the severity limits defined in our PPL were reached which necessitated termination of the experiment. Mice receiving PBS recorded paw diameters of  $2.96\pm 0.09$  at endpoint. These were significantly higher than paw diameters in sgp130:Fc treated mice ( $2.4\pm 0.11$   $p\leq 0.05$ ) (Figure 5.3).

sgp130:Fc demonstrated significant efficacy in improving clinical disease severity when given prior to disease onset.

**Figure 5.3 - Assessment of arthritis by clinical score and paw swelling in animals treated with sgp130:Fc compared to control**



Graphs demonstrating clinical score and paw diameters from day 21 until experimental endpoint. Animals received intra-peritoneal PBS (100µl) or sgp130 (2.5 mg/kg) on alternate days from day of second intra-dermal injection. Graphs show mean of 6 mice per treatment allocation ± SEM. \* shows  $p \leq 0.05$ , \*\* shows  $p \leq 0.001$ .

5.2.1.2 Identification of a Dose of Etanercept which Resulted in 50% Reduction in Clinical Score

From the outset our principal aim was to determine whether sgp130:Fc therapy would provide a suitable adjunct in RA patients failing on monotherapy with a TNF $\alpha$  antagonist. We tried to model the scenario *in vivo* using mCIA. Firstly, it was necessary to determine a sub-optimal dose of a TNF $\alpha$  antagonist which would result in a 50% reduction in clinical score after 7 days of therapy. This experiment was run in parallel with the sgp130:Fc prophylaxis experiment for 2 reasons:

- a) To share control animals thereby minimising animal usage through unnecessarily high control numbers;
- b) To provide appreciation of efficacy by direct comparison with sgp130:Fc.

Etanercept was chosen because it is the most commonly used anti-TNF $\alpha$  agent. Supplies were also readily available. The doses of etanercept chosen resulted from a review of the literature of anti-TNF $\alpha$  therapy in mCIA. A variety of protocols and dosing regimens had been used so direct comparison between all trials was not possible (See table 5.2). Disease course varied between experiments as did regime for initiating disease. Doses of anti-TNF used varied from 20  $\mu$ g/ day to 1.5 mg/week. Treatment regimes included once weekly to continuous infusion via a mini-pump. The i.p. route was used in all cases (Piguet *et al* 1992, Williams *et al* 1992; Wooley *et al* 1993; Shealy *et al* 2002). The aim of these experiments had also been to abolish disease rather than to reduce disease severity. The dose of etanercept chosen of 2.5 mg/kg per mouse on alternate days (50  $\mu$ g/mouse) appeared to be an appropriate starting dose. Arthritis was initiated as described in section 2.16 and etanercept given at a dose of 2.5 mg/kg per mouse on alternate days by i.p. injection from day 21 (n=6 per treatment allocation).

**Table 5.2 - Comparison of doses of anti-TNF $\alpha$  therapies used in murine experiments**

Ref	Species	Arthritis	Drug	Prevention / Treatment	Dosing Regime	Results
Piguet 1992	DBA/1	Footpad 1/12 after collagen	Anti-TNF ab	Prevention	2 and 3 weeks after immunisation 1.5 mg i.p.	no arthritis
			TNF receptor	Prevention	Implant 15 days after immunisation i.p. minipump 20 $\mu$ g/day for 15 days	no arthritis
			Anti-TNF ab	Treatment	1.5 mg weekly from 8/52 after immunisation	No benefit
Williams et al 1992	DBA/1	Onset day 28	Anti-TNF ab	Prevention	once/week for 4/52 from day before collagen i.p. 250 $\mu$ g (10mg/kg)	6/9 arthritis Did not prevent or delay arthritis $\downarrow$ severity-joint count & swelling
				Treatment	From onset of arthritis X2/week i.p. 300 $\mu$ g/mouse 2nd expt 50, 300 or 500	$\downarrow$ clinical score & swelling, histology better
Wooley 1993	DBA/1	40% at day 45. 90% at day55	TNFR:Fc fusion protein	Prevention	50 $\mu$ g/mouse i.p. days 21 - 28	0 at day 45. 25% at day 55. Severity 50% joint count $\downarrow$
				Treatment	Disease onset 50 $\mu$ g/mouse i.p. daily for 14 days	Less severe disease, fewer paws affected. Significant at 7.5 weeks after onset
Shealy 2002	TG197	Spontaneous progressive	Anti-TNF Ab	Treatment	10 mg/kg weekly i.p. from clinical score>2	$\downarrow$ clinical score
				Prevention	10 mg/kg weekly from wk4 i.p.	$\downarrow$ clinical score

The incidence of mCIA in PBS and etanercept treated mice is shown in table 5.3.

**Table 5.3 - Incidence of arthritis in etanercept treated mice compared to PBS controls (disease prophylaxis)**

Day number	% Incidence- PBS	% Incidence- etanercept
21	0	0
26	50	17
29	100	83
34	100	83

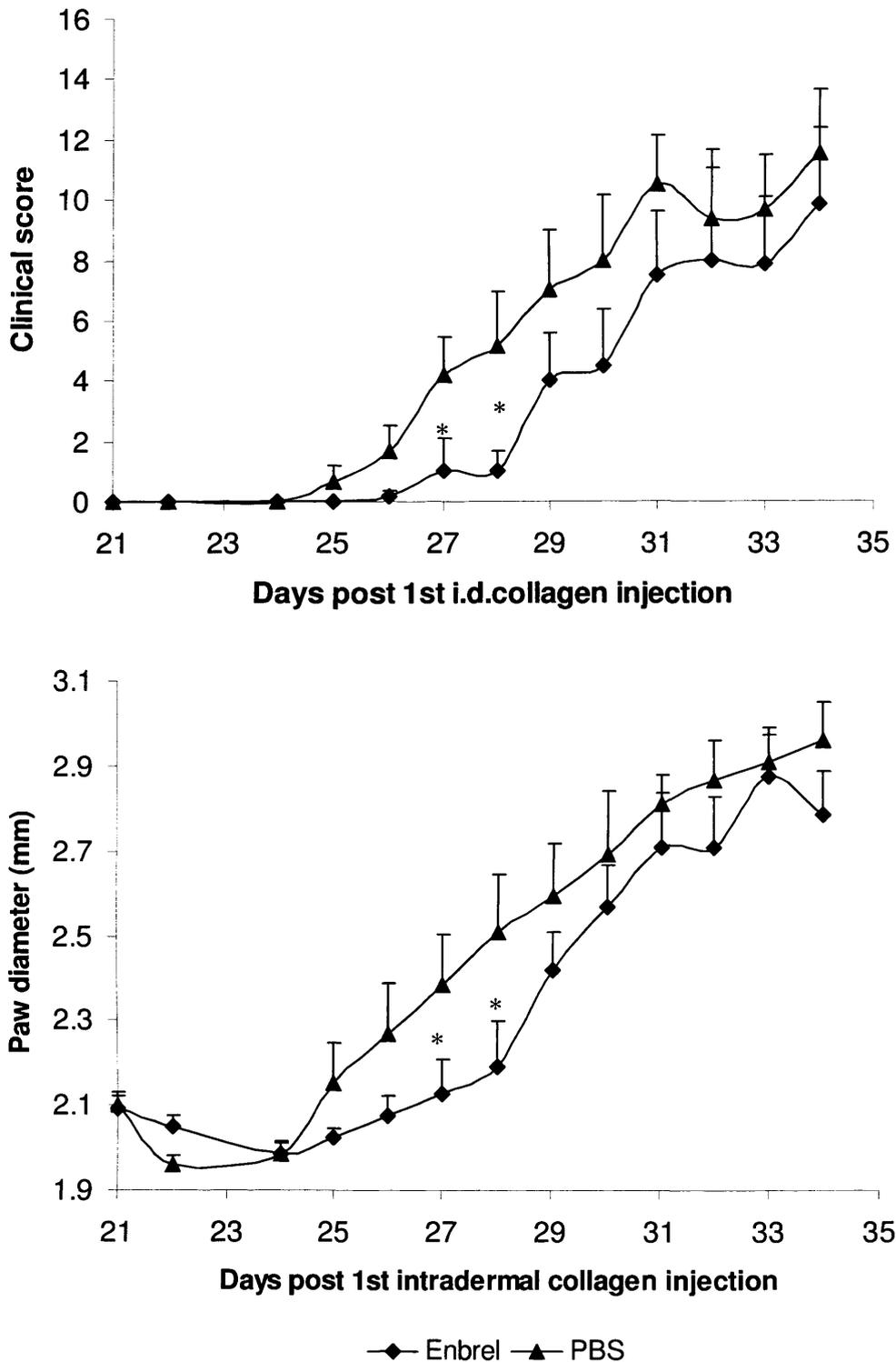
At time of initiation of therapy on day 21, none of the animals receiving etanercept exhibited signs of arthritis and were allocated clinical score of 0. By day 26, clinical scores in etanercept group had increased to  $0.17 \pm 0.18$ . At this point clinical score in the PBS treated animals were significantly higher ( $1.67 \pm 0.83$   $p \leq 0.05$ ). When the clinical scores at day 28 were compared in order to assess whether 50% inhibition in disease severity had been achieved mean score in PBS group ( $5.16 \pm 1.78$ ) remained significantly higher than in etanercept group ( $1 \pm 0.69$   $p \leq 0.05$ ). By day 34, the severity limits defined in our PPL had been reached which necessitated termination of the experiment. Mice receiving PBS recorded clinical scores of  $11.5 \pm 2.13$  at endpoint. This was not significantly higher than clinical score values in etanercept treated mice ( $9.83 \pm 2.45$  NS).

In the PBS group, paw diameters increased from  $2.1 \pm 0.03$  at baseline to  $2.27 \pm 0.11$  at day 26. In the etanercept group, paw diameters at day 26 were  $2.08 \pm 0.08$ . There was no significant difference between the groups at this point. Paw diameters in the PBS treated animals rose steadily reaching  $2.51 \pm 0.12$  on day 28. At this point paw diameters in the etanercept treated group were significantly lower ( $2.19 \pm 0.09$   $p \leq 0.05$ ). By day 34, severity limits as defined in our PPL had been reached which necessitated termination of the experiment. Mice receiving PBS recorded paw diameters of  $2.96 \pm 0.09$

at endpoint. These were not significantly higher than paw diameters in sgp130:FC treated mice ( $2.78 \pm 0.10$ ) (Figure 5.4).

The reduction in clinical score at day 28 was greater than the 50% inhibition aimed for. However when the experimental time course was assessed the dose of etanercept chosen was felt to be appropriate (Clinical score was reduced by 25-75% between days 27 and 30).

**Figure 5.4 - Graph demonstrating clinical score and paw diameters in etanercept treated mice**



Graph demonstrating paw diameters from day 21 until experimental endpoint. Animals received intra-peritoneal PBS (100µl) or etanercept (2.5 mg/kg) in equivalent volume of vehicle on alternate days from day of second intra-dermal injection. Graphs show mean of 6 mice per treatment allocation ± SEM.

5.2.1.3 Assessment of Pathological Damage in mCIA After Treatment with sgp130:Fc and Etanercept

At end-point, fore paws were frozen and hind paws prepared for histological analysis. Hind paws were fixed in NBFS for 1 week then decalcified using formic acid. Following adequate de-calcification, measured using ammonium oxalate test for decalcification (see section 2.18.2.2.), paws were processed in an automated Shandon tissue processor before being permeated with paraffin wax. Feet were then embedded in wax blocks using a Shandon histo-centre. Serial mid-sagittal (7µm) sections were cut with a microtome and placed on slides.

Haematoxylin and eosin stained sections were prepared as described in section 2.18.3.1. Sections were scored by 3 blinded observers according to an established in-house system and the mean value calculated. Each slide was scored for the following parameters: hyperplasia (0-3), infiltrate (0-5), exudate (0-3), bone and cartilage erosion (0-3). The components were added to give a composite score or arthritis index (Highest possible score - 14).

Mouse skeletal anatomy is shown in Figure 5.5A and sections from a normal mouse are shown in Figure 5.5B. In the normal joint there was no evidence of arthritis. The synovial lining was, at most, 2 cells thick with no leucocyte infiltrate. The tibio-talar joint space remained empty with no cellular exudate and there was no evidence of bone or cartilage damage. Representative sections from each treatment group are shown in Figure 5.6.

In the PBS treated group the joints showed signs of severe arthritis (Figure 5.6A). In the tibio-talar joint there was evidence of marked synovial hypertrophy with a heavy leucocyte infiltrate evidenced by dense nuclear staining. The joint space had a leucocyte rich exudate and there was marked bone and cartilage damage with loss of normal joint contours. The mean arthritis index in the PBS treated group was  $9.32 \pm 1.03$ .

In the sgp130:Fc group (Figure 5.6B), histological severity of arthritis was much reduced. There was no evidence of synovial hypertrophy; there was only mild cellular infiltrate and the joint space was free from leucocyte exudate with no bone or cartilage damage. The mean arthritis index in the sgp130:Fc fusion protein treated group was  $4.57 \pm 1.27$ .

In the etanercept treated group (Figure 5.6C), histological damage was less severe than in the PBS group. There was a degree of synovial hyperplasia with a moderate cellular infiltrate and some leucocyte exudate within the tibio-talar joint space. Cartilage damage and bony erosions were seen but without complete destruction of the joint. The mean score in the etanercept treated group was  $8.08 \pm 1.25$ .

There was a significant difference in scores between the PBS control group and the sgp130:Fc therapy group ( $p \leq 0.05$ ). There was no significant difference in arthritis index between PBS controls and etanercept treated group (Figure 5.7).

#### 5.2.1.4 Effect of Therapy with sgp130:Fc and Etanercept on Cartilage Depletion

Sections were also stained with Safranin-O/Fast Green to assess extent of cartilage damage. Proteoglycan depletion results in loss of bright red stain. Staining intensity was compared by eye between groups.

In the PBS group there was marked reduction in intensity of red staining on the articular surface as a result of almost complete proteoglycan depletion. In the sgp130:Fc therapy group there was protection from cartilage destruction assessed by intensity of safranin-O/fast green staining. In the etanercept treated animals there was reduction in intensity of staining with some evidence of proteoglycan depletion and partial protection from cartilage destruction. Representative sections are shown in Figure 5.8.

5.2.1.5 Effect of Therapy with sgp130:Fc and Etanercept on Bone Erosion

Sections were stained for tartrate resistant alkaline phosphatase (TRAP) to identify presence of osteoclasts which were used as a marker for bone erosion. Red staining intensity was compared between treatment groups. In the PBS therapy group there was evidence of osteoclast activity. In the sections taken from the sgp130:Fc treated group there was little evidence of osteoclast activation as measured by the presence of TRAP positive cells. There was some osteoclast activity in the sections from the etanercept treated group but this appeared subjectively less than in the PBS control samples. Representative sections are shown in figure 5.9.

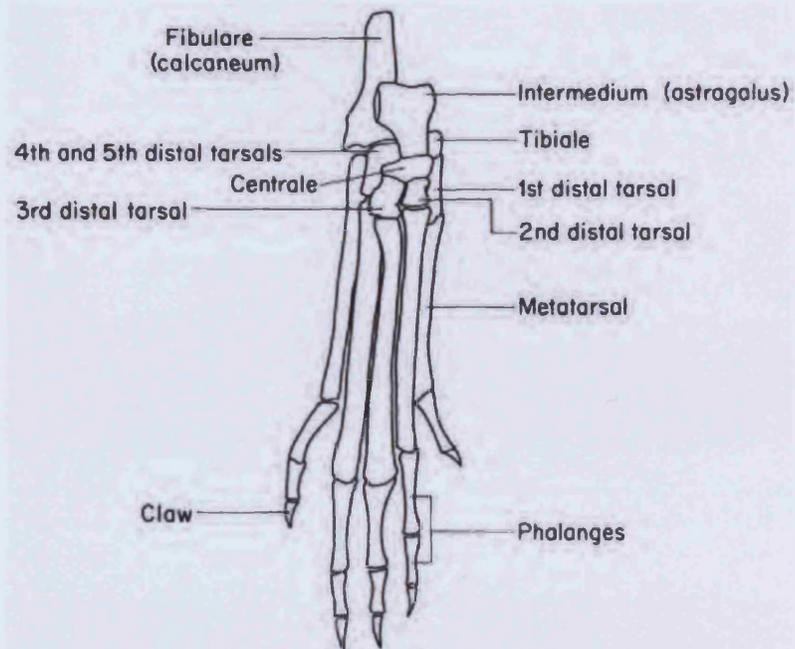
5.2.1.6 Effect of sgp130:Fc and Etanercept on NFkB and STAT Signalling Pathways in Murine Joints Affected by mCIA

EMSA was used to assess the effects of targeting IL-6 trans-signalling and TNF $\alpha$  on NFkB and STAT signalling pathways. Nuclear extracts were prepared as described in section 2.15 from frozen mouse fore paws which were taken on day 34, i.e. at experimental endpoint. Binding reactions were performed using 4  $\mu$ g of nuclear protein and  $\alpha^{32}$ -dTTP-labelled oligonucleotide containing either an NFkB or STAT-binding consensus sequence (SIE-m67).

Non-arthritic mice showed little evidence of NFkB activation in their fore paws. Endstage arthritis was associated with up-regulation of NFkB activation. This was reduced by therapy with sgp130:Fc or etanercept (see Figure 5.10A). NFkB activation correlated with clinical score, i.e. reduction in disease activity resulted in lower NFkB activation (Figure 5.11). The mean densitometry measurement was 21041 in PBS group, 11861 in sgp130:Fc and 12725 in etanercept treated group. Supershift assays showed that the P50 and to a lesser extent P65 sub-units were activated (Figure 5.10B).

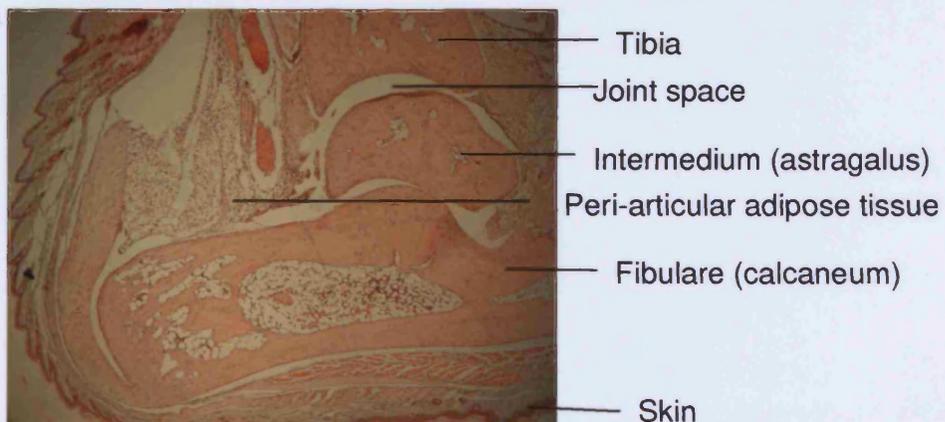
**Figure 5.5 - Anatomy and histology of mouse hind paw**

**A**



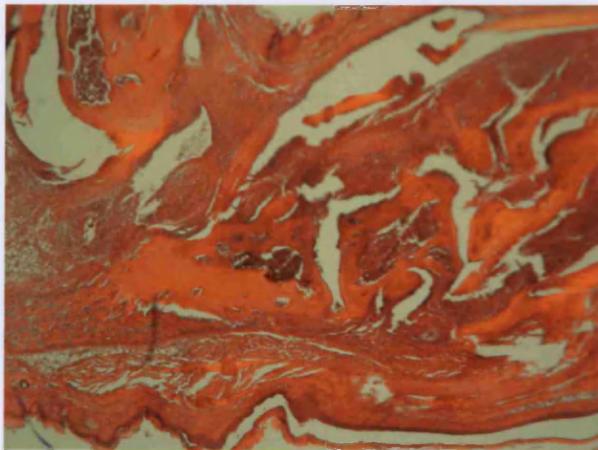
Adapted from <http://www.informatics.jax.org/cookbook/figures>

**B.-Haematoxylin and Eosin stained normal mouse hind paw with no evidence of arthritis**



**Figure 5.6 - Histological sections demonstrating key features of arthritis. Typical scoring sections for each treatment group**

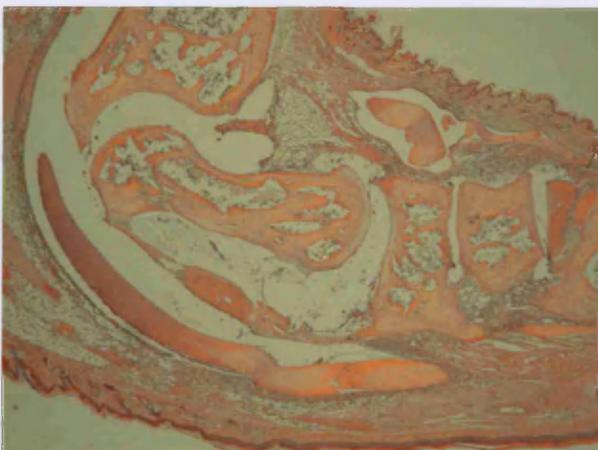
**A** PBS Control X4 magnification



Hyperplasia (3/3)  
Infiltrate (4/5)  
Exudate (2/3)  
Erosion (3/3)

Total score 12

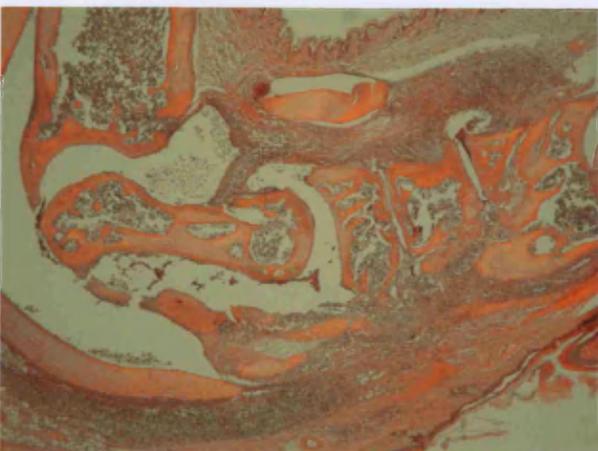
**B** sgp130:Fc treated X4 magnification



Hyperplasia (0/3)  
Infiltrate (1/5)  
Exudate (0/3)  
Erosion (0/3)

Total Score 1

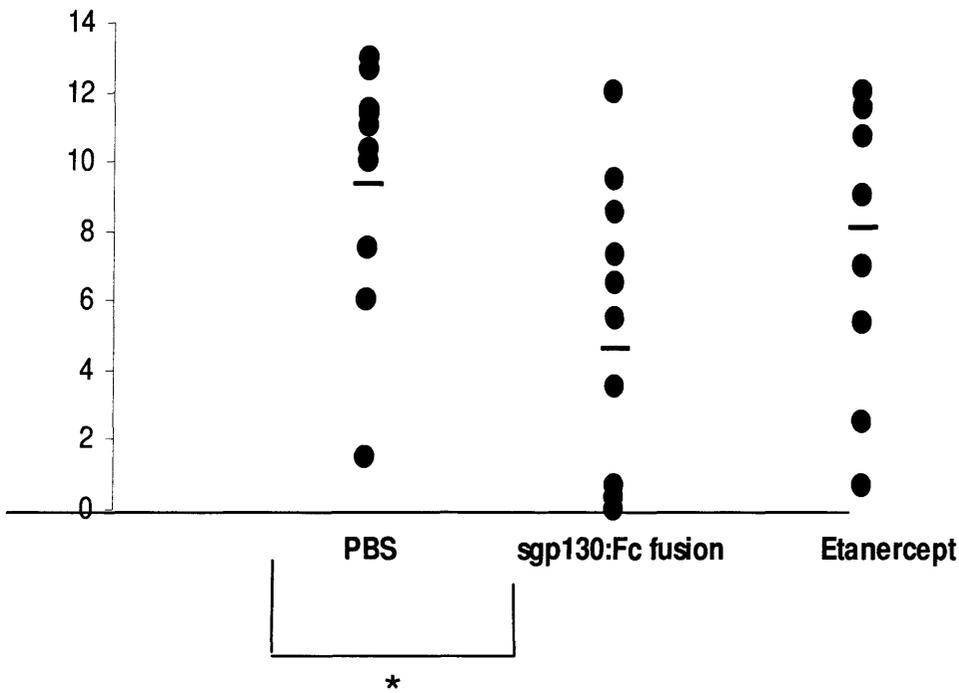
**C** etanercept treated X4 magnification



Hyperplasia (1/3)  
Infiltrate (3/5)  
Exudate (1/3)  
Erosion (1/5)

Total Score 6

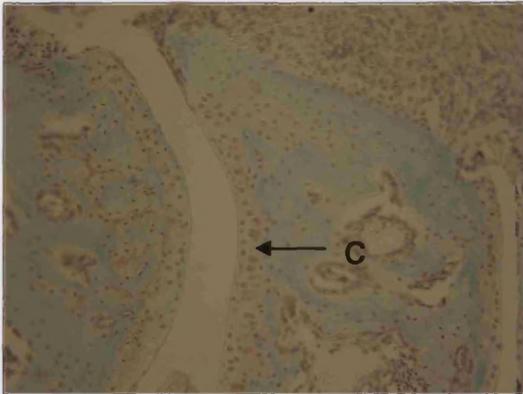
**Figure 5.7 - Mean Arthritis Index comparing PBS control group with sgp130:Fc fusion protein and etanercept treated groups.**



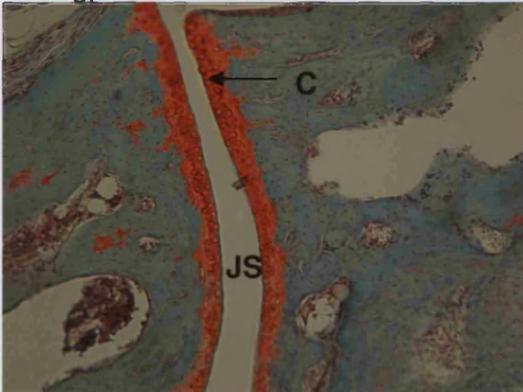
Values shown are mean of 3 independent blinded observers (n=12 sections for PBS and sgp130:Fc and n=11 for etanercept ). \* = significant difference when compared to PBS control group ( $p \leq 0.05$ ). Individual scores are shown by black circles. Mean values for group shown by horizontal line.

**Figure 5.8 - Cartilage damage shown by Safranin-O/Fast green staining**

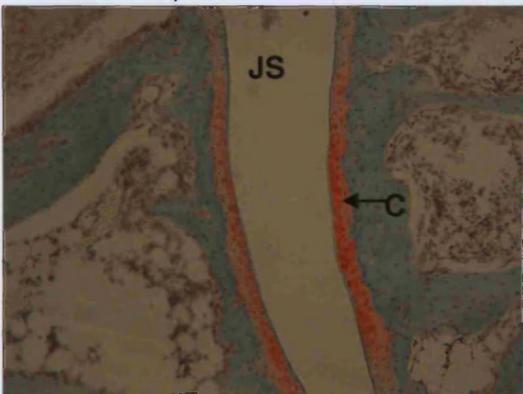
**A** PBS treated X20



**B** Sgp130:Fc treated X20



**C** Etanercept treated X20



Cartilage depletion is demonstrated by loss of Safranin-O (red stain).

**A** Strong intensity staining demonstrates little evidence of cartilage loss.

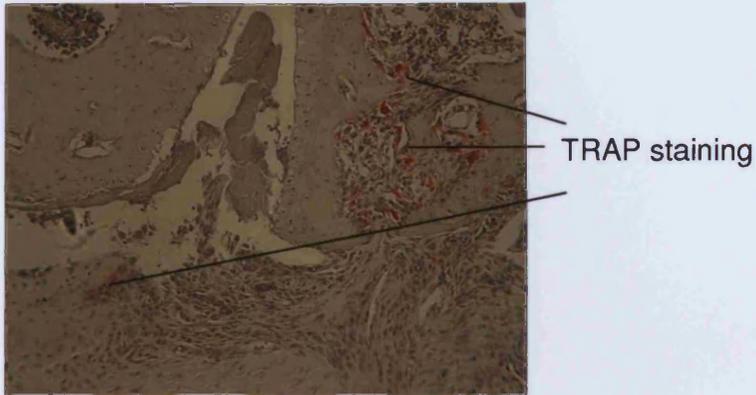
**B** Partial loss of cartilage shown by reduction in intensity of Safranin-O.

**C** Almost complete loss of cartilage.

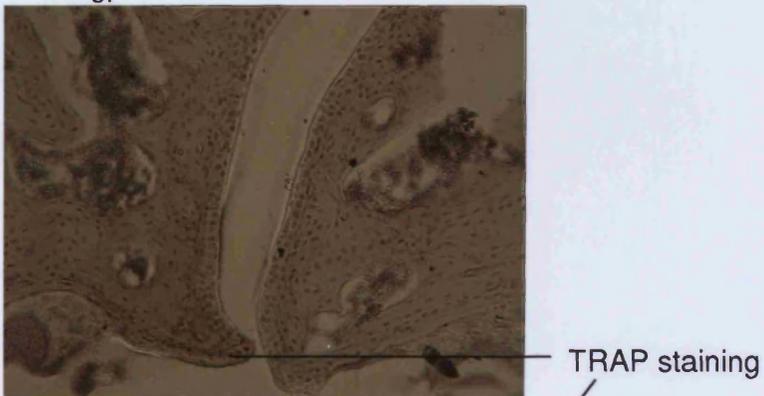
**JS** = joint space, **C** = cartilage layer.

**Figure 5.9 - Tartrate resistant alkaline phosphatase (TRAP) staining**

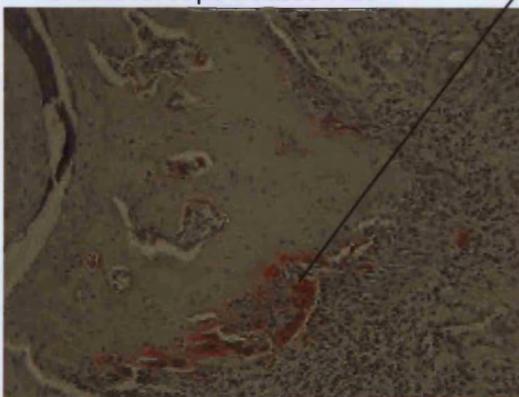
**A** PBS treated X20



**B** sgp130:Fc treated X20



**C** Etanercept treated X20



Red stain delineates areas of TRAP staining. This enzyme shows osteoclast activity and hence is a marker for bone erosion. Representative sections from each treatment group are shown.

There was no evidence of STAT activation in paws taken from control animals without arthritis. In PBS treated mice which developed mCIA there was evidence of up-regulation of STAT activation in 2 animals. Supershift showed that it was predominantly STAT3 which was activated. Although in these paw samples there was evidence of STAT activation; this was at far lower levels than seen in samples from other murine models. sgp130:Fc reduced STAT activation. It is clear that etanercept had no effect on STAT activation. There was no correlation between clinical score and level of STAT activation.

In summary in this experiment, a dose of sgp130:Fc (2.5 mg/kg) was ascertained which resulted in significant reduction in disease severity. Therapy with sgp130:Fc from time of arthritis induction resulted in improvement in clinical disease severity together with a significant reduction in histological disease severity including cartilage damage and bone erosion. Reduction in clinical disease severity was associated with a reduction in NF $\kappa$ B activity and therapy with sgp130:Fc also reduced STAT activation.

In addition, a sub-optimal dose of etanercept (2.5 mg/kg/ mouse) was chosen which resulted in reduction in disease severity when given from the time of arthritis induction. This allowed us to model the effects of addition of sgp130:Fc to animals in which arthritis had been partially treated. There was no significant reduction in arthritis index in the etanercept therapy animals although there was a reduction in cartilage depletion and bone erosion.

### **Figure 5.10**

EMSA gels demonstrating NFκB and STAT activation within murine paws affected by collagen induced arthritis. Fore paws were frozen at the termination of experiment and nuclear extracts collected as described in section 2.15. An equal amount of protein was added to each well, gels run and developed as described. Specific protein under investigation is seen as a black band at appropriate place on the gel. Molecular weight determined how far specific bands ran. Intensity of protein band translates into thicker more intense band on X-Ray. Density was assessed semi-quantitatively using Image J analysis software and correlated with paw score and treatment group.

**A** NFκB activation

**B** NFκB supershift

**C** STAT activation

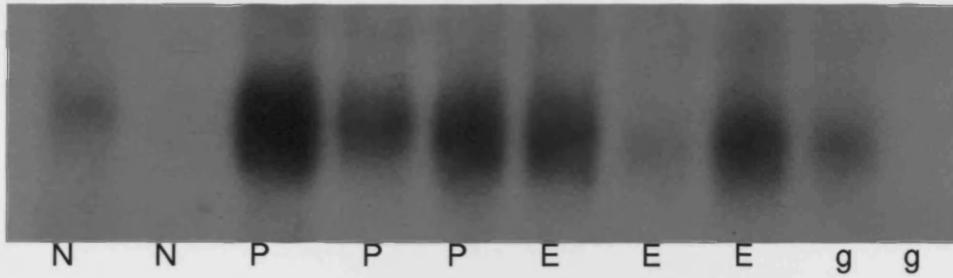
**D** STAT supershift

N - Normal control animal (no arthritis), P - PBS treated arthritis, E - etanercept treated arthritis, g - sgp130:FC treated arthritis.

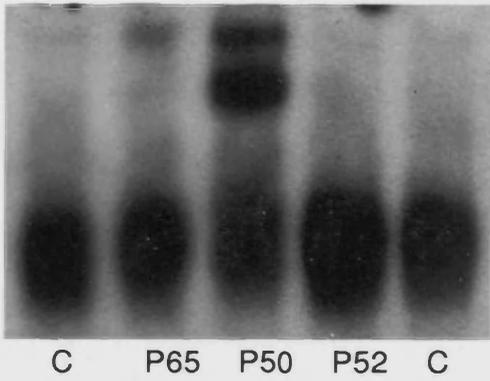
For supershifts, C = control with no antibody or irrelevant control antibody added. P50, 52 and 65 refer to NFκB sub-units, S1 - STAT1, S3 - STAT3, S5 - STAT5

**Figure 5.10 - EMSA showing NFκB and STAT activation within murine paws affected by collagen induced arthritis**

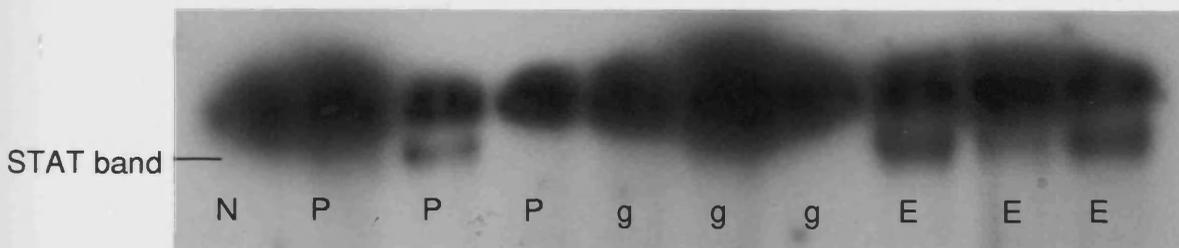
**A NFκB**



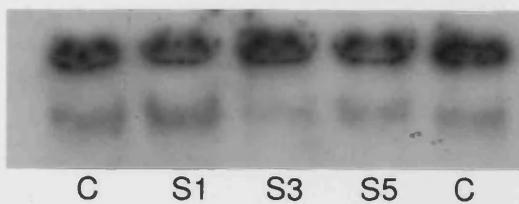
**B Supershift**



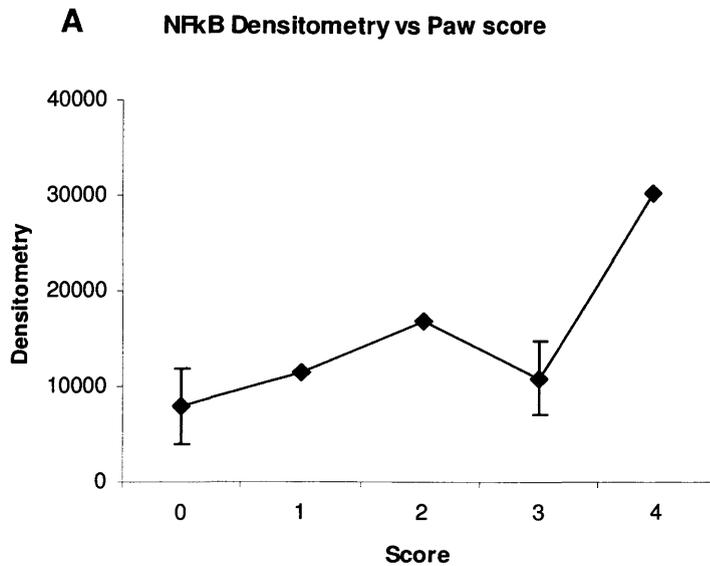
**C STAT**



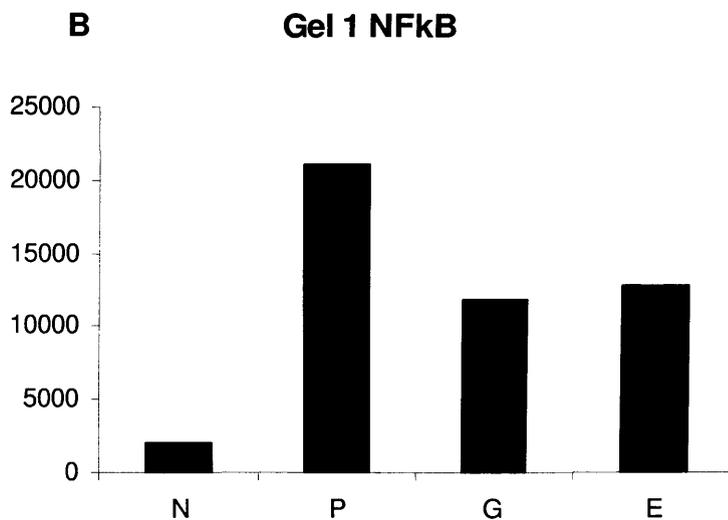
**D STAT supershift**



**Figure 5.11 - Correlation between NFkB activity and disease activity**



Graph showing densitometry of individual paws plotted against clinical paw score for each specific paw. Graph shows mean±SEM where 3 or more paws have equal clinical scores.



Bar chart showing mean densitometry plotted against treatment group for samples shown in figure 5.12A. No error bars are shown as there were less than 3 samples in some treatment groups. N = normal mice with no arthritis, P = PBS treated controls with mCIA, E = etanercept treated, G = sgp130:Fc fusion therapy.

## 5.2.2 Treatment of Established Disease with sgp130:Fc or Combination

### Therapy with Etanercept and sgp130:Fc

In the previous experiment, a dose of sgp130:Fc (2.5 mg/kg) was ascertained which resulted in significant reduction in disease severity. In addition, a sub-optimal dose of etanercept (2.5 mg/kg/ mouse) was chosen which resulted in reduction in disease severity when given from the time of arthritis induction. Intervention in established disease was proposed. The reason for using established disease was that this is more reminiscent of the clinical situation in which patients are treated once disease is apparent.

The two specific aims of this section were to identify whether sgp130:Fc had a significant therapeutic effect in established disease and to identify whether sgp130:Fc in combination with etanercept was more effective than etanercept monotherapy. Therapies were given on a daily basis by intra-peritoneal injection at a dose of 2.5 mg/kg/mouse to give the best opportunity for improving clinical score in aggressive established disease.

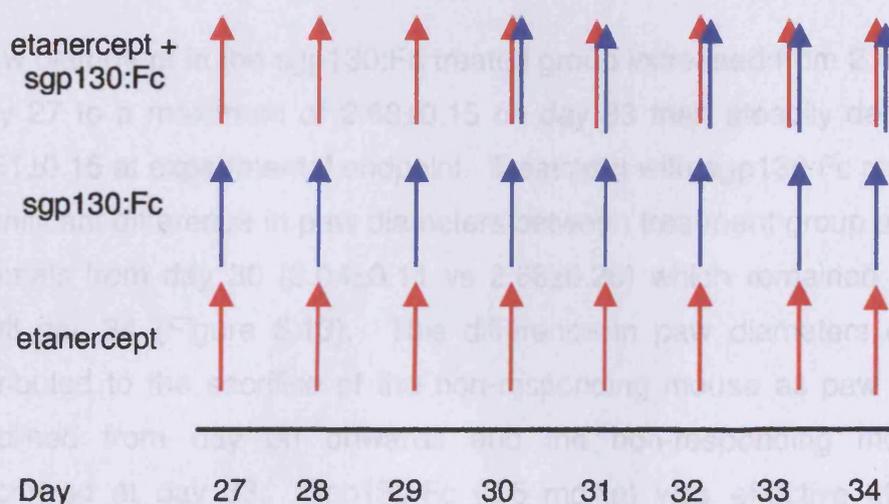
Arthritis was induced as described in section 2.16. Mice were assessed daily from day 24. At day 27 when arthritis incidence was 100% animals were divided into matched groups on the basis of clinical score. Incidence of arthritis is shown in Table 5.4

**Table 5.4 - Incidence of arthritis prior to initiation of therapy (established disease)**

<b>Day number</b>	<b>% Incidence-</b>
24	25
25	45
26	92
27	100

6 mice were assigned to PBS and sgp130:Fc treatment groups respectively. 12 mice were allocated to etanercept group, 6 to be maintained on etanercept alone and 6 had sgp130:Fc added to their treatment at day 30 which was intended to model the effect of adding sgp130:Fc in those failing on anti-TNF $\alpha$  treatment (Figure 5.12 shows pictorial representation of dosing schedule in therapy groups).

**Figure 5.12 - Pictorial representation of dosing schedule for etanercept and sgp130:Fc alone and in combination in established mCIA**



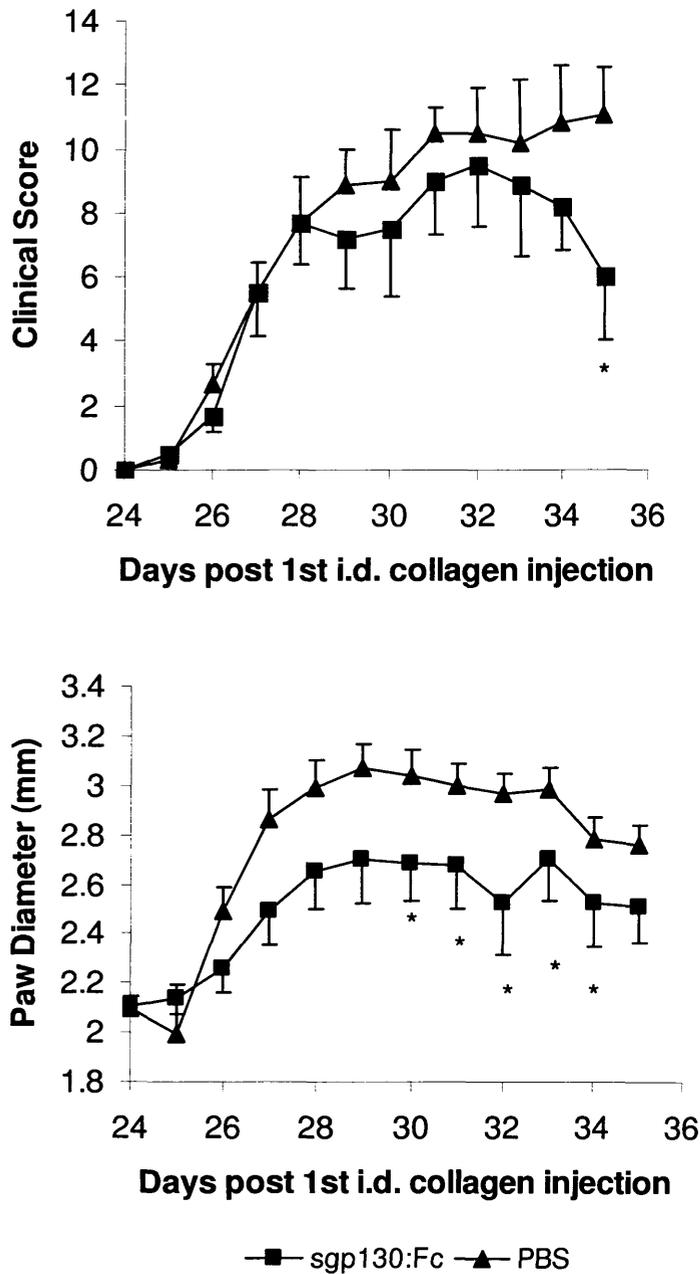
In the PBS treated controls, clinical score increased on a daily basis from  $5.5 \pm 0.96$  at day 27 on initiation of therapy to a maximum on day 35 when a score of  $11 \pm 1.54$  was recorded. On day 33 a single mouse was sacrificed because the severity limits defined in our Home Office PPL was reached. As a consequence only 5 out of original 6 controls survived to day 35. Paw diameters were also measured on a daily basis. On day 27, paw diameters of  $2.86 \pm 0.12$  were measured. Values increased steeply over the next 3 days reaching their maximum by day 29 ( $3.06 \pm 0.10$  mm). Interestingly, paw diameters then dropped slightly falling to  $2.76 \pm 0.08$  mm at endpoint.

In sgp130:Fc treated mice, clinical score on initiation of therapy was  $5.5 \pm 2.95$ ; this was not significantly different from PBS controls. Clinical score increased steadily over the next 5 days reaching a maximum of  $9.5 \pm 1.95$  on day 32. Thereafter, arthritis in the sgp130:Fc treated animals improved; indeed clinical scores decreased to  $6 \pm 1.97$  by day 35.

Unfortunately a single mouse in the sgp130:Fc therapy group did not respond to treatment reaching severity limits on day 33. Therefore, It was sacrificed at this point. It is unlikely that the significant improvement in disease activity at day 35 can be attributed entirely to the death of this animal as clinical score did decline from day 33 to day 35 after it was removed from the experiment. There was a significant reduction in clinical score at endpoint when sgp130:Fc group were compared to control animals ( $6\pm 1.97$  vs  $11\pm 1.54$   $p\leq 0.05$ ) (Figure 5.15).

Paw diameters in the sgp130:Fc treated group increased from  $2.49\pm 0.14$  on day 27 to a maximum of  $2.68\pm 0.15$  on day 33 then steadily decreased to  $2.51\pm 0.15$  at experimental endpoint. Treatment with sgp130:Fc resulted in a significant difference in paw diameters between treatment group and control animals from day 30 ( $3.04\pm 0.11$  vs  $2.68\pm 0.26$ ) which remained significant until day 34 (Figure 5.13). This difference in paw diameters cannot be attributed to the sacrifice of the non-responding mouse as paw diameters declined from day 30 onwards and the non-responding mouse was sacrificed at day 33. Sgp130:Fc (2.5 mg/kg) was effective at reducing clinical disease severity when given in established disease.

**Figure 5.13 – Time course of paw swelling in mice treated with sgp130:Fc or placebo in established disease**



Graphs demonstrating clinical scores and paw diameters in millimetres from day 24 until experimental endpoint. Graphs show mean of 6 mice  $\pm$  SEM. Therapy was initiated at day 27. Significant differences between groups are shown by \* =  $p \leq 0.05$ .

5.2.2.1 Effect of Addition of sgp130:Fc to Etanercept Monotherapy on Clinical Disease Parameters in Established mCIA

On day 27, when therapy was initiated, the clinical score (mean $\pm$ SEM) in the etanercept group was 5.42 $\pm$  1.88. There was no significant difference between groups at this point (PBS 5.5  $\pm$ 2.17, sgp130:FC 5.5 $\pm$ 2.95). Clinical score in the etanercept treated group increased slowly from day 27 reaching a score of 7.83 $\pm$ 1.87 on day 30. This was not significantly different to 9 $\pm$ 1.6 in the PBS group. Paw diameters increased from 2.65 $\pm$ 0.51 on day 27 to 2.57 $\pm$ 0.16 on day 30. This was already significantly lower than in the PBS group (3.04 $\pm$ 0.10;  $p\leq$ 0.05).

At day 30, the etanercept group was divided into those receiving etanercept alone and those to receive combination therapy. Clinical score (mean $\pm$ SEM) in combination group was 7 $\pm$  2.68 and in etanercept alone group was 7.83 $\pm$  4.17. These differences were not statistically significant. Mean paw diameter in combination group was 2.71 $\pm$ 0.36; whilst the mean diameter in etanercept alone group was 2.57  $\pm$ 0.52 (differences not significant).

The mean clinical score in the etanercept therapy group decreased from a maximum of 7.83 $\pm$ 4.17 on day 30 when groups were split to 6.17 $\pm$ 1.78 at endpoint. The addition of sgp130:Fc appeared to provide an adjunct to etanercept monotherapy as the mean clinical score in the combined therapy group decreased from 7 $\pm$ 2.68 at initiation of combined therapy to 4 $\pm$ 0.94 at experimental endpoint. A single animal was found dead on day 32. His clinical score on day 31 was 6. There was no significant difference between clinical scores at any time point. However, there were only small numbers of animals assessed with marked variability in response to therapy.

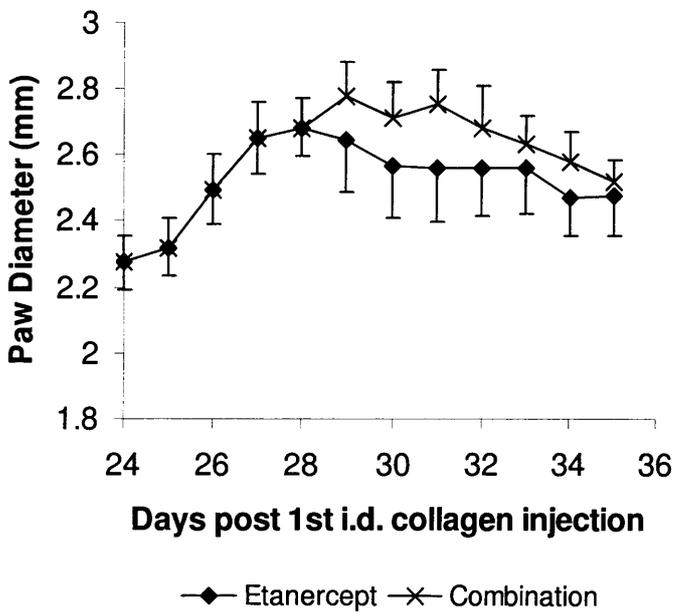
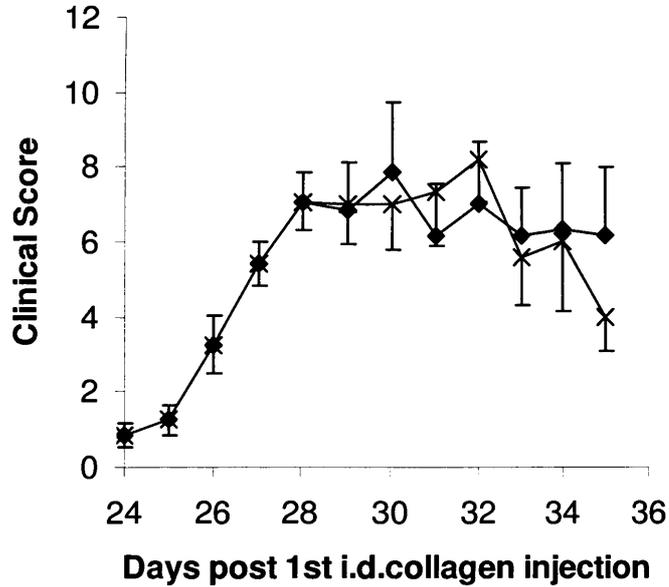
When change in clinical score from day 30 (when combination therapy was initiated) was measured; the mean fall in score in the etanercept group was -0.66 $\pm$ 0.73 vs -3.20 $\pm$ 1.19 in the combination group. There was a significant difference in change in clinical score between groups ( $p\leq$  0.05; Figure 5.15).

The mean paw diameters in the etanercept group decreased from a maximum of  $2.57\pm 0.16$ mm on day 30 to  $2.48\pm 0.12$ mm at experimental endpoint. Paw diameters in the combination therapy group increased from  $2.71\pm 0.10$ mm on day 30 to a maximum of  $2.75\pm 0.10$ mm on day 31 and then decreased to  $2.52\pm 0.06$ mm at experimental endpoint. There was no significant difference in paw diameters between the etanercept and combination therapy groups at any time point.

When sgp130:Fc monotherapy was compared to combination of etanercept and sgp130:Fc; clinical scores at endpoint were lowest in the combination therapy group ( $6\pm 1.97$  vs  $4\pm 0.94$ ). Differences did not reach significance. There was no significant difference in paw diameters between sgp130:Fc monotherapy and etanercept+sgp130:Fc groups at endpoint.

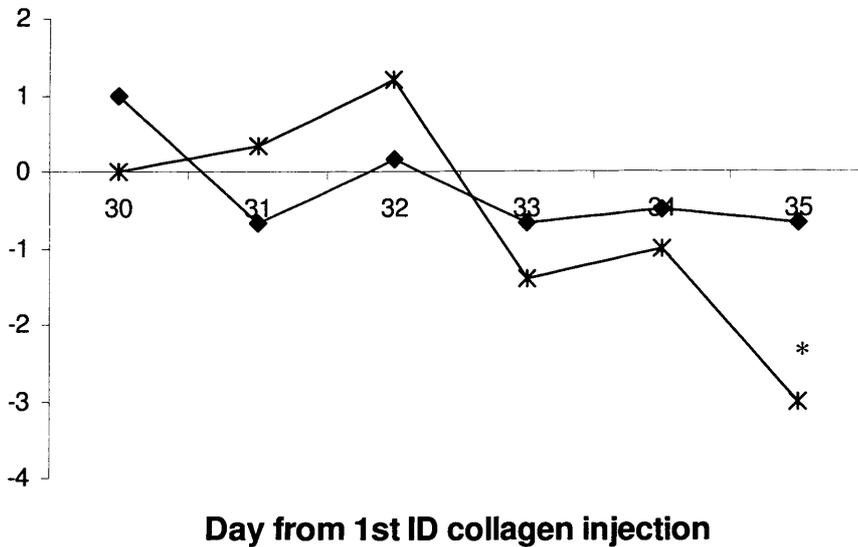
In summary, sgp130:Fc monotherapy was effective in improving clinical disease severity in mCIA. Addition of sgp130:Fc to sub-optimal etanercept therapy resulted in a significant change in clinical score when compared to continuing on etanercept monotherapy. Combination therapy did not appear to be better than sgp130:Fc. However, the timeframe for combination therapy was only 5 days and only small numbers of animals were assessed.

**Figure 5.14 – Time course of clinical score and paw swelling in mice treated with etanercept or combination of sgp130:Fc added to etanercept in established disease**



Graph demonstrating clinical score and paw diameters (mm) from day 24 until experimental endpoint. Graphs show mean of 6 mice  $\pm$  SEM. Therapy was initiated at day 27. Additional sgp130:Fc therapy was initiated at day 30 in 50% of the etanercept group. There was no significant difference in clinical score or paw swelling between etanercept and combination treated groups.

**Figure 5.15 - Graph showing daily change in mean clinical score from initiation of combined therapy**



Graph shows mean change in clinical score on a daily basis in the etanercept treated and combination therapy treated groups from day 29 when combination therapy was instituted in 50% of the etanercept treated group. ✱ shows combination therapy. ◆ shows etanercept therapy. \* shows significant change in mean score between therapy groups ( $p \leq 0.05$ ).

#### 5.2.2.2 Histological Assessment of Arthritis Severity

H and E stained sections were prepared as described in section 2.18.3.1. Sections were scored by 2 blinded observers according to an established in-house system and a mean score calculated. Each slide was scored for the following parameters: hyperplasia (0-3), infiltrate (0-5), exudate (0-3), bone and cartilage erosion (0-3). The components were added to give a composite score or arthritis index (Highest possible score - 14).

In the PBS group, the mean arthritis index from sections taken at experimental endpoint was  $9.9 \pm 1.0$ . Joints showed signs of severe arthritis. In the tibio-talar joint there was evidence of marked synovial hypertrophy with a heavy leucocyte infiltrate evidenced by dense nuclear staining. The joint space had a leucocyte-rich exudate and there was marked bone and cartilage damage with loss of normal joint contours.

The mean arthritis index in the sgp130:Fc fusion protein treated group was  $8.4 \pm 1.73$ . Similar histological features of arthritis were seen with large numbers of infiltrating cells, synovial membrane hypertrophy together with a leucocyte rich exudate within the joint space. Bone erosions were seen. There was no significant difference in scores between PBS controls and sgp130:Fc therapy.

The mean score in the etanercept treated group was  $7.08 \pm 1.33$ . Histological damage was less severe than in the PBS group. There was a degree of synovial hyperplasia with a moderate cellular infiltrate and some leucocyte exudate within the tibio-talar joint space. Some cartilage damage and bony erosions were seen but without complete destruction of the joint. Etanercept therapy was significantly better than PBS at preventing joint destruction ( $p \leq 0.05$ ).

In contrast, the mean score in the etanercept +sgp130:Fc group was  $10.6 \pm 1.33$ . The histological changes seen were similar to the PBS group with large numbers of infiltrating cells shown by synovial membrane hypertrophy and a dense cellular infiltrate together with a leucocyte-rich

exudate within the joint space. Etanercept therapy alone was superior to combination therapy ( $p \leq 0.05$ ).

There was no difference seen between PBS and sgp130:Fc treated or combination groups (Figure 5.17). Representative pictures for each experimental group are shown (Figure 5.16).

When arthritis index was divided into respective components and mean score for inflammatory infiltrate (total leucocyte infiltrate) compared between groups, there was no significant difference in leucocyte infiltrate. My *in vitro* work in chapters 3 and 4 suggested that TNF $\alpha$  and IL-6/IL-6R together led to synergistic up-regulation of specific macrophage and T cell chemo-attractants, i.e. CCL2 and CCL5. This led us to surmise that although total leucocyte numbers did not appear to be affected by therapy; there may have been an alteration in leucocyte phenotype. Therefore, specific leucocyte markers were analysed by immuno-histochemistry to determine whether therapy had an effect on leucocyte sub-sets.

### 5.2.2.3 Immuno-histochemical Analysis of Leucocyte Infiltrate

Antigen retrieval was carried out using Trypsin-EDTA retrieval. Peroxidase and biotin were blocked (method is described in full in section 2.18.4). Sections were stained with anti-F4/80 (1/50) as a macrophage marker or anti-Fox-P3 (1/200) as a regulatory T cell marker overnight at 4°C and antibody binding detected using biotin-conjugated rabbit anti-rat and swine anti-rabbit IgG followed by StrepABcomplex. Sections were then developed using diaminobenzidine substrate, counterstained with haematoxylin.

Numbers of F4/80 positive cells (macrophages) per high power field were counted for 3 animals per therapy group. In the PBS group the mean number ( $\pm$ SEM) of F4/80 positive cells was  $10 \pm 6.16$ . In the sgp130:Fc treated group there were a complete absence of F4/80 positive cells. In the etanercept therapy group there were a mean number of  $10.67 \pm 1.63$  F4/80 cells per field. In the combination group there were  $0.67 \pm 0.82$  F4/80 positive cells per high power field. There was a significant difference in

number of F4/80 positive cells in the sections taken from animals treated with sgp130:Fc either alone or in combination with etanercept ( $p \leq 0.05$ ). Etanercept monotherapy had no effect on macrophage infiltrate (Figure 5.18).

Numbers of Fox-P3 positive cells (Regulatory T cells) per high power field were counted for 3 animals per therapy group. In the PBS group, the mean number ( $\pm$ SEM) of Fox-P3 positive cells was  $2.5 \pm 1.2$ . In the sgp130:Fc treated group there were  $4 \pm 2.5$  cells. Sections from etanercept therapy animals had  $2 \pm 1.22$  cells per field and in the combination group there were  $3 \pm 0$  positive staining cells per high power field. Although Fox-P3 positive cells could be identified, the numbers of cells present were low. In this small sample there was no significant difference seen in Fox-P3 expression between treatment groups although there appeared to be a trend towards an increase in the sgp130:Fc therapy groups (see Figure 5.19).

In summary, we had identified a dose of sgp130:Fc which significantly improved clinical outcome in established disease. This improvement in clinical outcome was associated with a significant reduction in macrophage infiltrate but this did not result in an overall improvement in disease histology. It was clear that because of the aggressive nature of arthritis that sgp130:Fc therapy was less effective in established disease than when given prior to the onset of clinical disease. Therefore, further experiments were designed to assess whether a higher dose of sgp130:FC or therapy given earlier in disease might be even more effective.

**Figure 5.16 - Representative histological sections for each therapy group (treatment of established disease)**

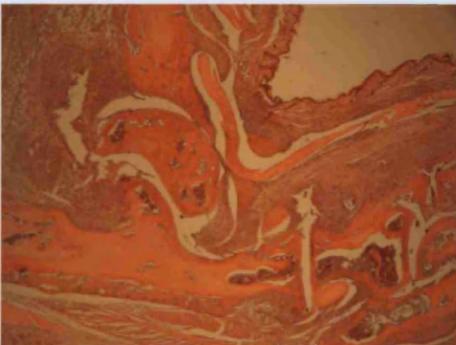
**A PBS x4 magnification**



Hyperplasia (2/3)  
Infiltrate (4/5)  
Exudate (3/3)  
Erosion (3/3)

Total score 12

**B sgp130:Fc X4 magnification**



Hyperplasia (3/3)  
Infiltrate (5/5)  
Exudate (3/3)  
Erosion (3/3)

Total score 14

**C etanercept X4 magnification**



Hyperplasia (1/3)  
Infiltrate (2/5)  
Exudate (1/3)  
Erosion (0/3)

Total score 4

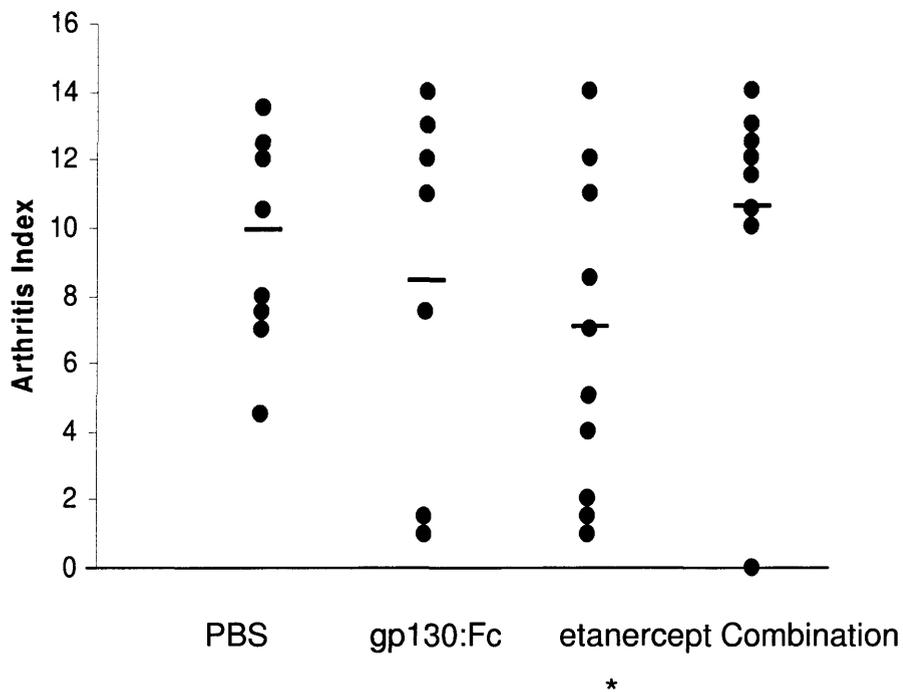
**D etanercept + sgp130:Fc X4 magnification**



Hyperplasia (1/3)  
Infiltrate (2/5)  
Exudate (2/3)  
Erosion (2/3)

Total score 7

**Figure 5.17 - Mean arthritis index comparing PBS control group with sgp130:Fc and etanercept monotherapy to combination therapy**



Histology scores show mean of 2 blinded observers for each treatment group (n=10 for PBS and sgp130:Fc, n=11 for combination group and n=12 for etanercept group). Individual paw scores are shown by black circles and mean of each treatment condition by a horizontal line. \* shows significant difference in mean when compared to PBS control group.

### **Figure 5.18**

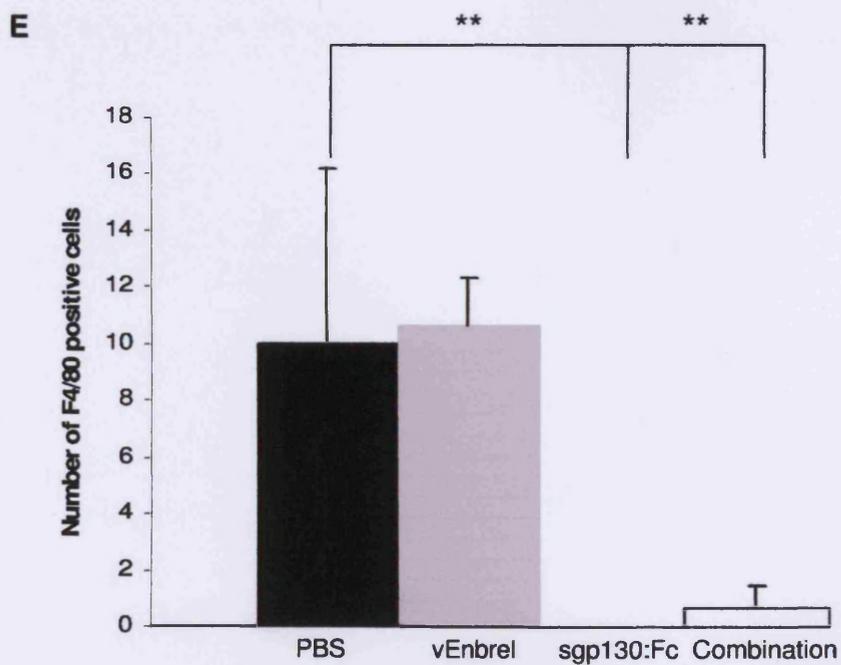
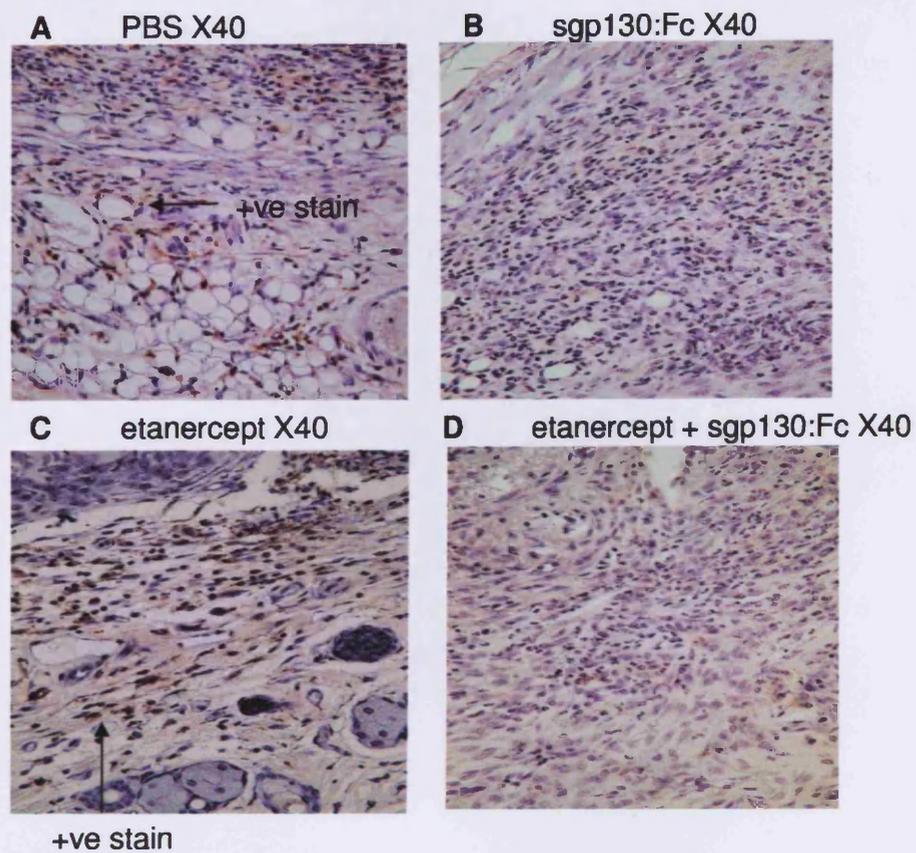
Representative sections from each therapy group demonstrating F4/80 expression within synovial infiltrate. Sections were stained with F4/80 rat anti-mouse antibody following antigen retrieval then antibody detected using biotin-conjugated rabbit-anti rat antibody. Sections were developed using diaminobenzidine substrate counterstained with haematoxylin. F4/80 positive cells are stained brown. Sections shown are original magnification X 40

- A)** PBS control
- B)** sgp130:Fc therapy
- C)** etanercept therapy
- D)** etanercept + sgp130:Fc
- E)** Numbers of F4/80 positive cells per 20 infiltrating leucocytes.

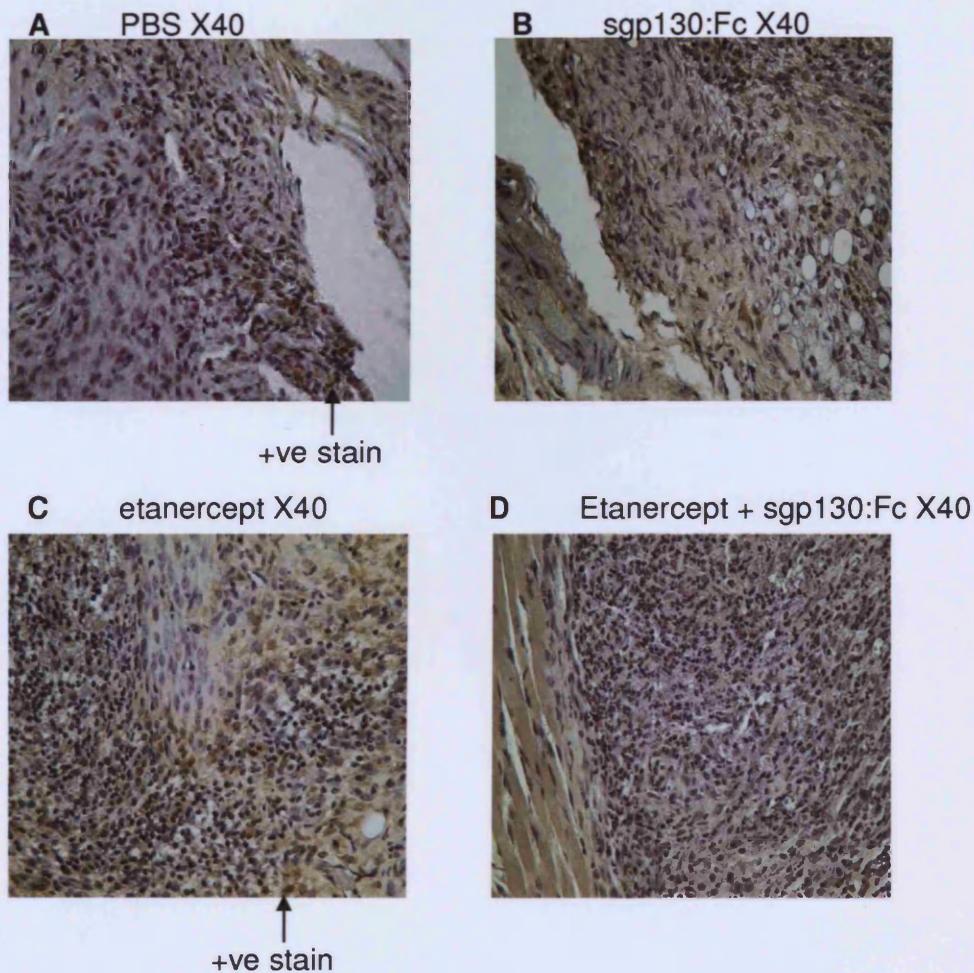
Graphs show mean of 3 sections  $\pm$  SEM for each treatment condition.

\*\*demonstrates significant difference in number of F4/80 cells when compared to PBS control group ( $P \leq 0.001$ ).

**Figure 5.18 – Immuno-histochemical staining showing F4/80 expression within the joints of mice affected by mCIA.**



**Figure 5.19 – Immuno-histochemical staining showing regulatory T cell distribution demonstrated by the presence of Fox-P3 positive cells.**



Representative sections from each therapy group demonstrating FOX-P3 expression within synovial infiltrate. Sections were stained with FOX-P3 rabbit anti-mouse antibody following antigen retrieval then antibody detected using biotin-conjugated swine-anti rabbit. Sections were developed using diaminobenzidine substrate counterstained with haematoxylin. Fox-P3 positive cells are shown by nuclear brown staining.

**A)** PBS control      **B)** sgp130:Fc therapy      **C)** etanercept therapy  
**D)** etanercept + sgp130:FC

When analysis of numbers of FOX-P3 positive cells was carried out there was no significant difference in numbers of Regulatory T cells between treatment groups.

### 5.2.3 Effect of High Dose sgp130:Fc Monotherapy and in Combination with Etanercept in Established mCIA

A further experiment was carried out in established mCIA using sgp130:Fc at 5 mg/kg per mouse in order to determine whether a higher treatment dose improved clinical and histological scores of arthritis. Arthritis was initiated as described previously. Mice were assessed daily from day 21. Incidence of arthritis prior to initiation of therapy is shown in Table 5.5.

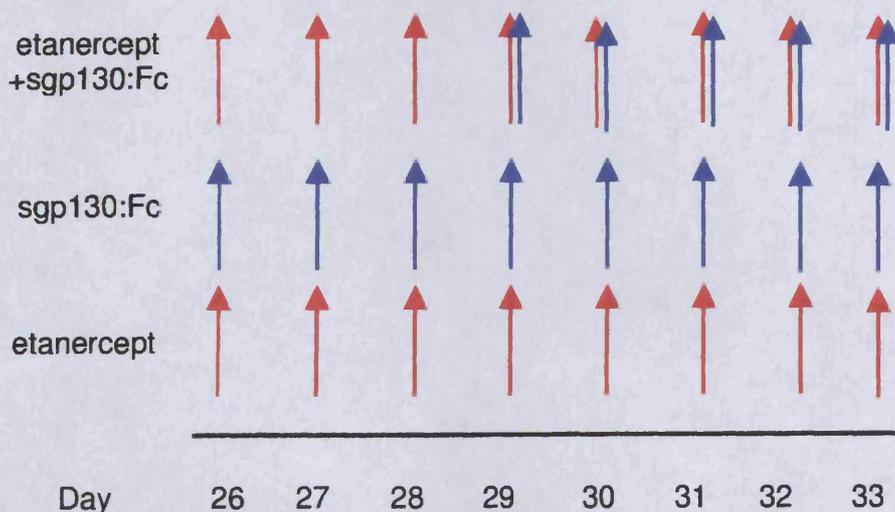
**Table 5.5 Incidence of arthritis in high dosesgp130:Fc experiment**

Day number	% Incidence-
21	21
23	21
25	71
26	75

In this experiment, therapy was started from day 26 as the arthritis appeared more severe and incidence was higher at this point than in the previous experiment. Animals were divided into matched groups on the basis of clinical score. 6 mice were assigned to PBS and sgp130:Fc treatment groups respectively. 12 mice were allocated to etanercept group, 6 to be maintained on etanercept alone and 6 had sgp130:Fc added to their treatment at day 29 which was intended to model the effect of adding sgp130:Fc in those failing on anti-TNF $\alpha$  treatment (Figure 5.20).

Etanercept and sgp130:Fc were administered daily by intra-peritoneal injection at a dose of 5 mg/kg/mouse (sgp130:Fc) and 2.5 mg/kg/mouse (etanercept). Clinical scores and assessments were carried out daily. The experiment was terminated at day 34 in accordance with project licence terms. Hind paws were fixed for histological assessment. Fore paws were frozen in liquid nitrogen for future protein extraction.

**Figure 5.20 - Pictorial representation of dosing schedule for etanercept (2.5 mg/kg) and sgp130:Fc (5 mg/kg) alone and in combination in established mCIA**



There was no significant difference between treatment groups in clinical scores at start of treatment. Clinical score (mean $\pm$ SEM) in PBS group was 2.33 $\pm$ 1.22; 3 $\pm$ 1.57 in sgp130:Fc group; 4.16 $\pm$ 1.88 in etanercept group and 4.16 $\pm$ 1.63 in combined therapy group.

At day 29 when sgp130:Fc was added to etanercept in the combination therapy group mean paw diameter in combination group was 2.68 $\pm$ 1.3 whilst the mean diameter in etanercept alone group was 2.86 $\pm$ 0.08. Mean clinical score in combination group was 7.5 $\pm$ 2.25 and the mean score in etanercept alone group was 6 $\pm$ 0.69. These differences were not statistically significant.

#### 5.2.3.1 Effect of sgp130:Fc on Clinical Parameters of Disease Severity in Established mCIA

In the PBS treated controls, clinical score increased on a daily basis from 2.8 $\pm$ 1.38 at day 26 on initiation of therapy to a maximum of 9 $\pm$ 1.41 on day 31 then decreased to 7.6 $\pm$ 1.4 at experimental endpoint. Paw diameters were also measured on a daily basis. Diameters increased from 2.31 $\pm$ 0.18

on day 27 to a maximum mean paw diameter of  $2.93 \pm 0.07$  mm at day 32 and then decreased to  $2.76 \pm 0.05$  mm at endpoint.

In the sgp130:Fc group, mean clinical score on initiation of therapy was  $4.5 \pm 1.67$ . This was not significantly different from PBS controls. Clinical score increased steadily over the next 5 days to a maximum of  $7.5 \pm 1.91$  on day 31. Thereafter, arthritis in the sgp130:Fc treated animals improved and clinical score decreased to  $6.33 \pm 1.78$  at experimental endpoint. Paw diameters increased from  $2.43 \pm 0.17$  on day 26 to a maximum of  $2.79 \pm 0.13$  on day 31 and then decreased to  $2.64 \pm 0.13$  at experimental endpoint. There was no significant difference between PBS and sgp130:Fc treated animals in measurements of clinical score or paw diameter at any time point (Figure 5.21).

#### 5.2.3.2 Effect of Addition of High Dose sgp130:Fc to Etanercept Monotherapy on Clinical Disease Parameters in Established mCIA

On day 27 when therapy was initiated the mean clinical score in the etanercept group was  $4.16 \pm 1.13$ . There was no significant difference between groups at this point (PBS  $2.8 \pm 1.39$ , sgp130:Fc  $3 \pm 1.57$ ). Clinical score in the etanercept treated group increased slowly from day 27 reaching a score of  $6 \pm 1.59$  on day 29 which was not significantly different to the score of  $7.2 \pm 1.35$  obtained in the PBS group. Paw diameters increased from  $2.65 \pm 0.08$  on day 26 to  $2.87 \pm 0.11$  on day 29. This was already significantly lower than in the PBS group ( $2.75 \pm 0.10$ ;  $p \leq 0.05$ ).

At day 29 when etanercept group were divided into those receiving etanercept alone and those to receive combination therapy, clinical score (mean  $\pm$  SEM) in combination group was  $7.5 \pm 2.25$  and in etanercept alone group was  $6 \pm 0.69$ . These differences were not statistically significant. The mean clinical score in the etanercept therapy group increased from a score of  $6 \pm 1$  on day 29 when groups were split to a maximum score of  $6.33 \pm 0.69$  on day 30. Mean score then decreased to  $4.5 \pm 0.62$  at endpoint. The

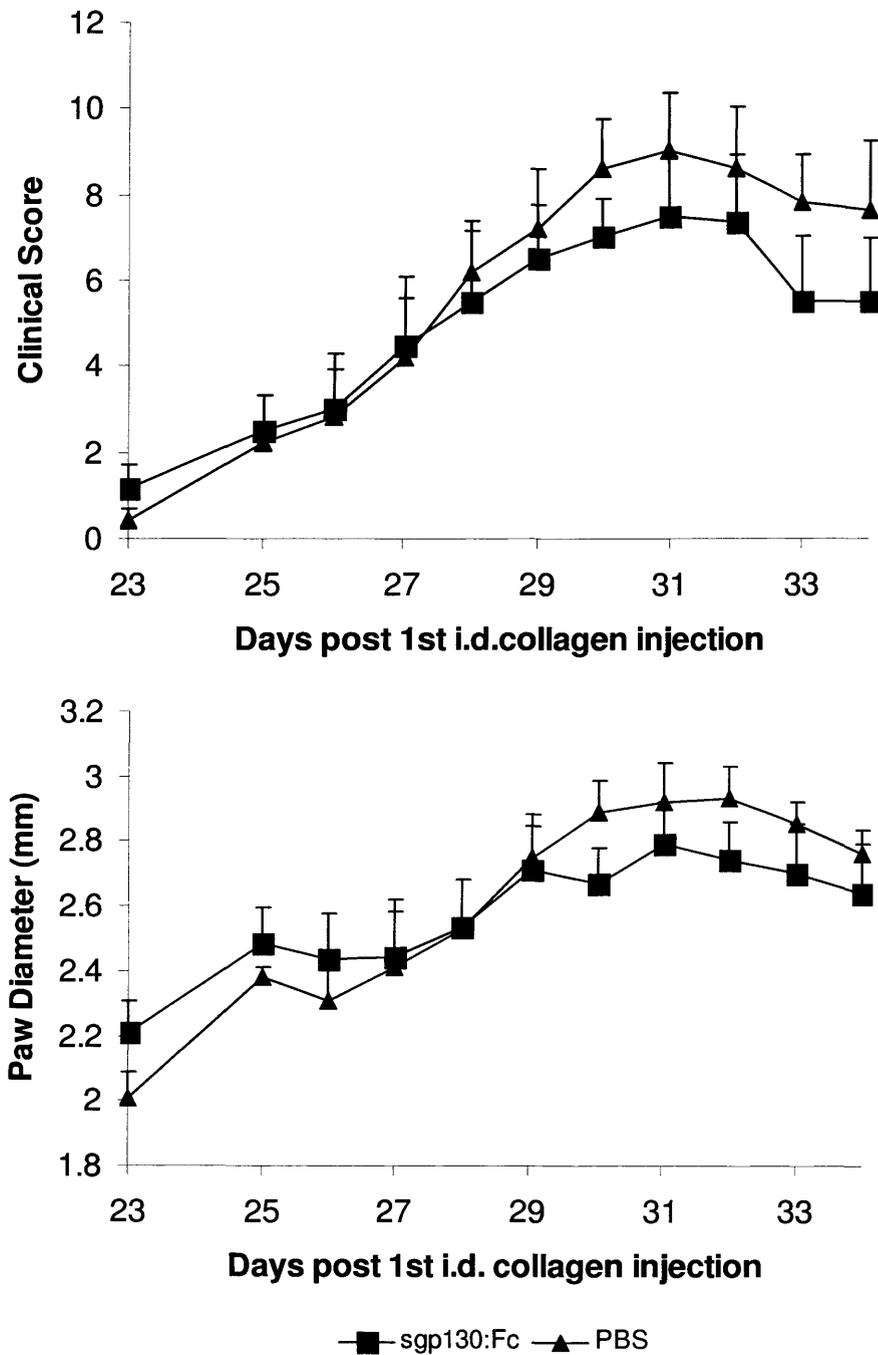
addition of sgp130:Fc appeared to provide an adjunct to etanercept monotherapy as the mean clinical score in the combined therapy group decreased from  $7.5 \pm 2.25$  at initiation of combined therapy to  $5.33 \pm 1.71$  at experimental endpoint. There was no significant difference in clinical score between groups at any time point.

The mean paw diameters in the etanercept group increased from  $2.87 \pm 0.11$  mm at initiation of combined therapy to a maximum of  $2.89 \pm 0.08$  mm on day 30 and then decreased to  $2.77 \pm 0.08$  mm at experimental endpoint. Paw diameters in the combination therapy group increased from  $2.68 \pm 0.13$  mm to a maximum of  $2.72 \pm 0.13$  mm on day 30 and then decreased to a minimum of  $2.46 \pm 0.10$  mm at experimental endpoint. There was a significant difference between paw diameters ( $2.76 \pm 0.08$  mm in etanercept group vs  $2.45 \pm 0.1$  mm in combination group) at end point. However, the mean paw diameter in the etanercept group was also lower in the combination therapy group at the time sgp130:Fc was initiated. This difference was not significant ( $2.87 \pm 0.11$  mm vs  $2.68 \pm 0.13$  mm NS). The groups were matched on the basis of clinical score but could not be completely matched for paw diameters (Figure 5.22).

When sgp130:Fc monotherapy was compared to combination of etanercept and sgp130:Fc there was no significant difference in clinical scores at endpoint ( $5.5 \pm 1.28$  mm vs  $5.34 \pm 1.71$  mm). There was no significant difference in paw diameters between sgp130:Fc monotherapy and etanercept + sgp130:Fc groups at endpoint ( $2.63 \pm 0.13$  mm vs  $2.45 \pm 0.11$  mm NS).

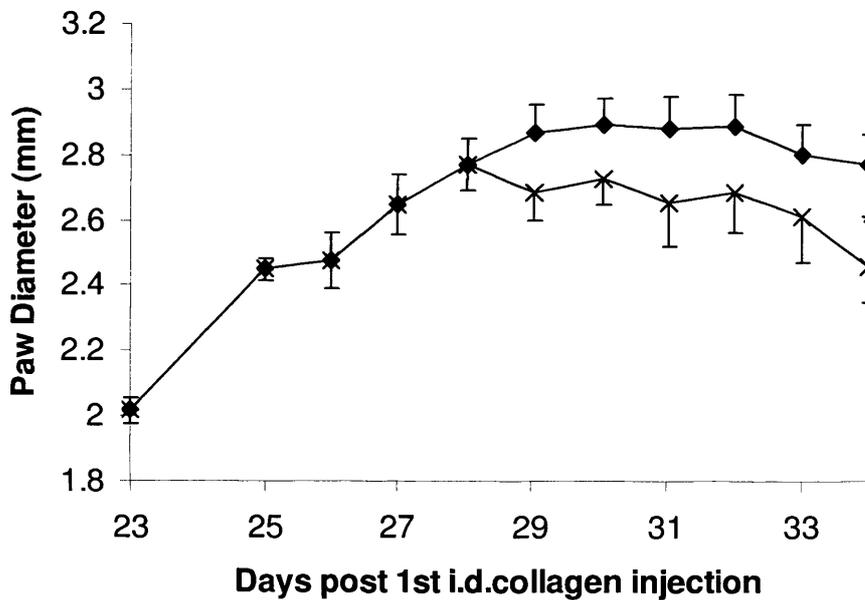
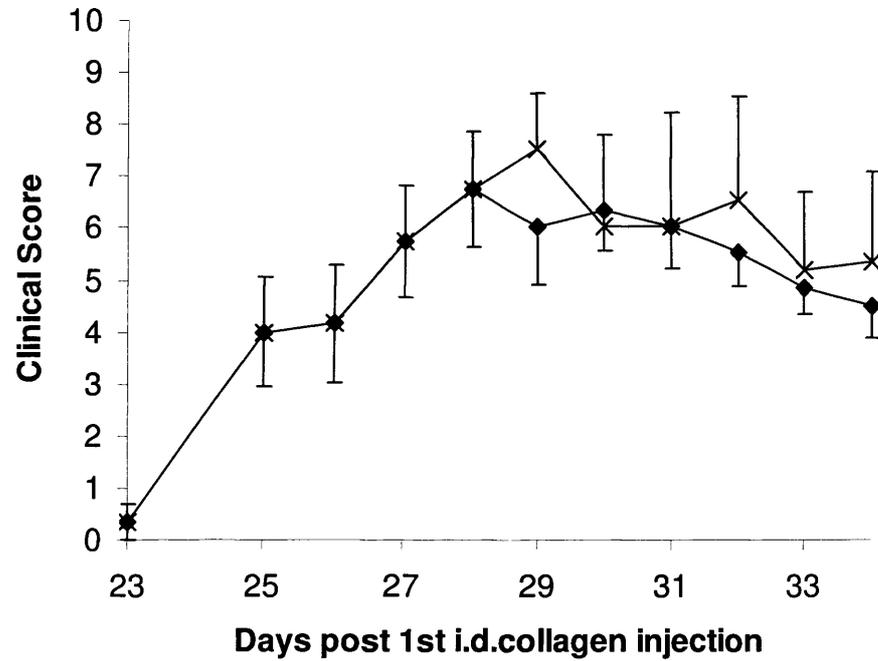
In summary, this experiment showed sgp130:Fc monotherapy (5 mg/kg) was not effective in improving clinical disease severity in mCIA. Addition of sgp130:Fc to sub-optimal etanercept therapy did not result in a significant change in clinical score when compared to continuing on etanercept monotherapy. Combination therapy did not appear to be better than sgp130:Fc.

**Figure 5.21 – Time course of clinical score and paw swelling in mice treated with high dose sgp130:Fc or placebo in established disease**



Graph demonstrating clinical score and paw diameters in millimetres from day 23 until experimental endpoint. Graphs show mean of 6 mice  $\pm$  SEM. Therapy was initiated at day 26. There was no significant difference in clinical score or paw swelling at any time point in the therapy group when compared to PBS controls.

**Figure 5.22 – Time course of paw swelling in mice treated with etanercept or high dose sgp130:Fc added to etanercept in established disease**



◆ E ✕ E+G

Graphs demonstrating paw diameters from day 23 until experimental endpoint. Graphs show mean of 6 mice  $\pm$  SEM. Therapy was initiated on day 26. Additional sgp130:Fc therapy was initiated at day 29 in 50% of the etanercept group. Paw swelling at endpoint was significantly lower in combination therapy treatment groups than in etanercept alone therapy group. Shown on graph by \* ( $p \leq 0.05$ ).

### 5.2.3.3 Histological Analysis of Disease Severity

Sections were cut and stained as described previously. Sections were scored by a single blinded observer using in-house scoring system as described above. In the PBS group, the mean arthritis index from sections taken at experimental endpoint was  $9.2 \pm 3.6$  (values shown are mean  $\pm$  SD). Joints showed signs of severe arthritis. In the tibio-talar joint there was evidence of marked synovial hypertrophy with a heavy leucocyte infiltrate evidenced by dense nuclear staining. The joint space had a leucocyte rich exudate and there was marked bone and cartilage damage with loss of normal joint contours.

The mean arthritis index in the sgp130:Fc fusion protein treated group was  $9 \pm 4.6$ . Histological features of severe arthritis were seen with synovial hypertrophy, leucocyte infiltration and exudate within the joint space together with bone and cartilage erosion. There was no significant difference in scores between PBS controls and sgp130:Fc therapy.

The mean score in the etanercept treated group was  $8.5 \pm 2.5$ . Similar histological features of arthritis were seen with large numbers of infiltrating cells shown by synovial membrane hypertrophy and a dense cellular infiltrate together with a leucocyte rich exudate within the joint space. Cartilage and bone erosions were seen.

The mean score in the combination therapy group was  $6.5 \pm 3.6$ . Combination therapy was significantly better than PBS control in preventing histological damage. Although arthritis was still present, histological changes were less marked with a reduction in the degree of synovial hyperplasia together with a reduction in leucocyte infiltrate and bone and cartilage damage.

Etanercept and sgp130:Fc monotherapy did not prove significantly better than control therapy. There was no significant difference between etanercept monotherapy and combination therapy in preventing histological damage (representative sections shown below in Figure 5.23).

**Figure 5.23 - Representative sections for each treatment group (high dose sgp130:Fc experiment)**

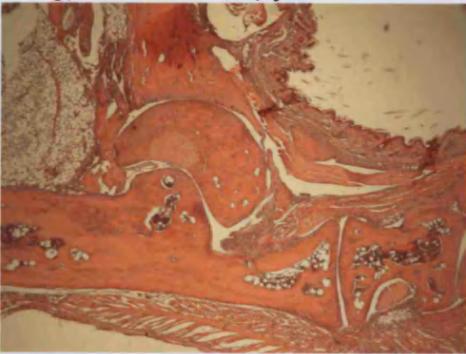
**A PBS control**



Hyperplasia (3/3)  
Infiltrate (4/5)  
Exudate (3/3)  
Erosion (3/3)

Total score 13

**B gp130:Fc therapy**



Hyperplasia (2/3)  
Infiltrate (3/5)  
Exudate (3/3)  
Erosion (2/3)

Total score 10

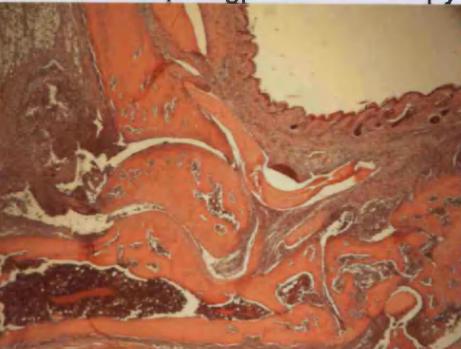
**C etanercept therapy**



Hyperplasia (1/3)  
Infiltrate (2/5)  
Exudate (2/3)  
Erosion (1/3)

Total score 6

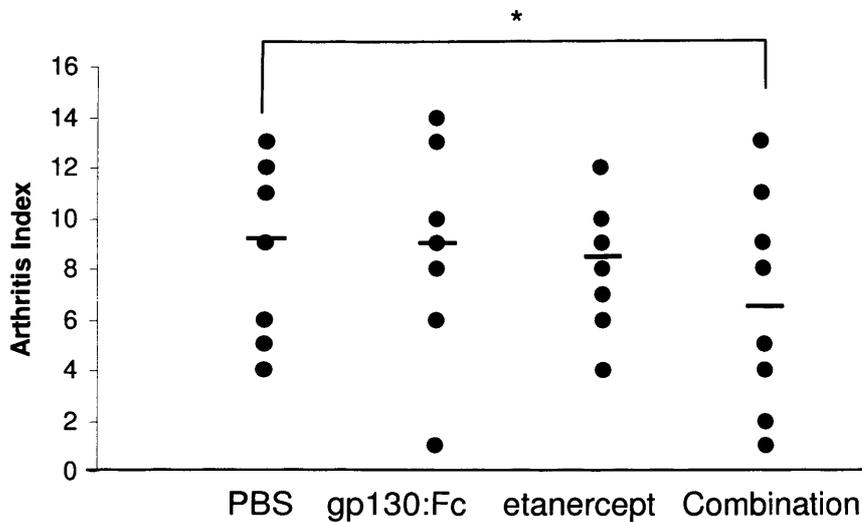
**D etanercept+sgp130:Fc therapy**



Hyperplasia (0/3)  
Infiltrate (2/5)  
Exudate (2/3)  
Erosion (1/3)

Total score 5

**Figure 5.24 - Mean Arthritis Index comparing PBS control group with sgp130:Fc fusion protein (high dose) and etanercept monotherapy to combination therapy.**



Histology scores show results of 1 blinded observer for each treatment group. (n= 10 for PBS and n=12 for all other treatment groups). Individual paw scores are shown by black circles and mean of each treatment condition by a horizontal line. \*  $p \leq 0.05$  shows significant difference in mean when compared to PBS control group.

5.2.3.4 Effect of sgp130:Fc and Etanercept Therapy in Established Disease on NFκB and STAT Signalling Pathways in Murine Joints Affected by mCIA

EMSA was used to assess the effects of targeting IL-6 trans-signalling and TNFα on NFκB and STAT signalling pathways. Nuclear extracts were prepared as described from frozen mouse fore-paws. Paws were taken from mice at experimental end-point. Binding reactions were performed using 4 μg of nuclear protein and α<sup>32</sup>-dTTP-labelled oligonucleotide containing either an NFκB or STAT-binding consensus sequence (SIE-m67) (Method described in section 2.15.6).

Non-arthritic mice showed little evidence of NFκB activation in their fore-paws. Endstage arthritis was associated with up-regulation of NFκB activation. Up-regulation in PBS controls and sgp130:Fc treated animals was seen (sample EMSAs shown in Figures 5.25A and B). Therapy with etanercept abolished NFκB activation. Interestingly, addition of sgp130:Fc appeared to negate the effect of etanercept and combination therapy animals had high levels of NFκB activation (sample EMSA shown in Figure 5.25A).

In contrast to the disease prophylaxis experiment; in this experiment there was no correlation between clinical score and NFκB activation. There was no correlation between treatment group and activation. However, control mice with no arthritis had low levels of activation as did animals treated with etanercept alone (Figure 5.27A). Supershift assays showed that the P50 and P65 sub-units were activated (Figure 5.25C)

Positive control samples taken from mice with SES induced peritonitis had high levels of STAT activation (Figure 5.26A). In mice which developed mCIA there was evidence of up-regulation of STAT activation but this was not as prominent as in peritonitis. Supershift showed that STAT activation was predominantly STAT1 and STAT3 in this particular experiment (Figure 5.26C). Animals treated with combination of etanercept and sgp130:Fc

therapy showed activation of STAT. Etanercept therapy alone did not affect STAT activation. There was a reduction in STAT activation in some mice treated with sgp130:Fc monotherapy (Figure 5.26B). There was no correlation between clinical score or treatment group and STAT activation when intensity of STAT band was compared by densitometry.

In summary, this experiment showed sgp130:Fc monotherapy (5 mg/kg) was not effective in improving clinical disease severity in mCIA. There was no improvement in disease histology as a result of sgp130:Fc therapy.

Addition of sgp130:Fc to sub-optimal etanercept therapy did not result in a significant change in clinical score when compared to continuing on etanercept monotherapy. Combination therapy did not appear to be better than sgp130:Fc alone. Combination therapy was significantly better than PBS control in preventing histological damage.

Assessment of cell signalling pathways showed that endstage arthritis was associated with up-regulation of NF $\kappa$ B activation. Therapy with etanercept abolished NF $\kappa$ B activation. Interestingly, addition of sgp130:Fc appeared to negate the effect of etanercept and combination therapy animals had high levels of NF $\kappa$ B activation. There was no correlation between clinical score or treatment group and STAT activation when intensity of STAT band was compared by densitometry.

### **Figure 5.25**

EMSA gels demonstrating NF $\kappa$ B activation within murine paws affected by collagen induced arthritis. Forepaws were frozen at the termination of experiment and nuclear extracts collected as described in section 2.15. An equal amount of protein was added to each well, gels run and developed as described. Specific protein under investigation is seen as a black band at appropriate place on the gel. Molecular weight determines how far specific bands run. Intensity of protein band translates into thicker more intense band on X-Ray.

**A** NF $\kappa$ B activation

**B** NF $\kappa$ B activation

**C** NF $\kappa$ B supershift

P - PBS treated arthritis, E - etanercept treated arthritis, g - sgp130:Fc treated arthritis. C - control mouse with no arthritis.

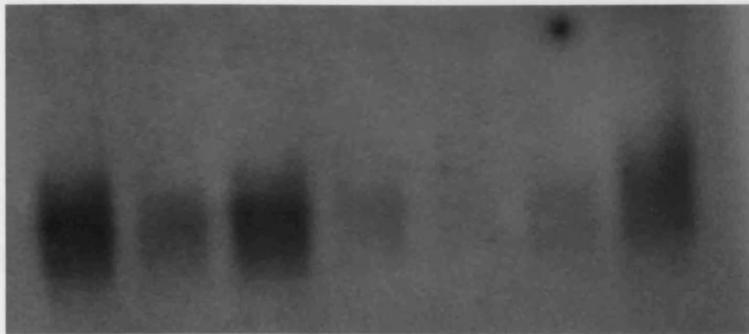
In this experiment there was no correlation between clinical score and NF $\kappa$ B activation. There was no correlation between treatment group and activation. However, control mice with no arthritis had low levels of activation as did animals treated with etanercept alone.

For supershifts, C = control with no antibody or irrelevant control antibody added. P50, 52 and 65 refer to NF $\kappa$ B subunits.

**C** supershift showing that NF $\kappa$ B activation activation is predominantly P50 and P65 subunits.

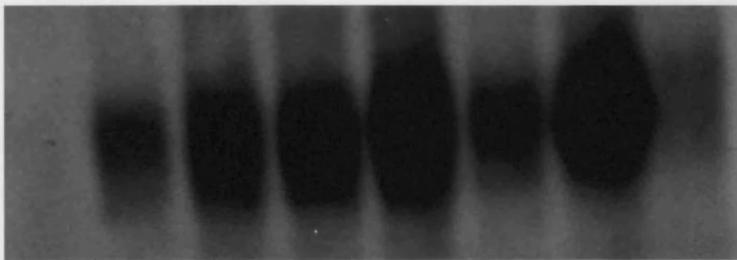
**Figure 5.25 - NFκB activation from forepaws of mice affected with murine collagen induced arthritis**

**A NFκB**



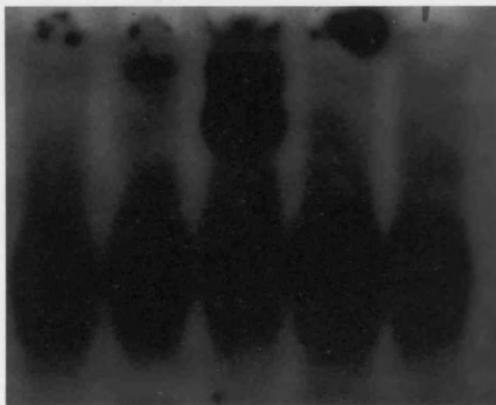
E+g E+g E+g E E E P

**B NFκB**



g g g P P P C

**C NFκB supershift**



C P65 P50 P52 C

## **Figure 5.26**

**A and B** STAT activation in fore paws from mice with and without arthritis.

P = PBS treated, E = etanercept treated, g = sgp130:Fc treated, C = control mouse with no arthritis, +ve = control sample from SES peritonitis where STAT is known to be up-regulated.

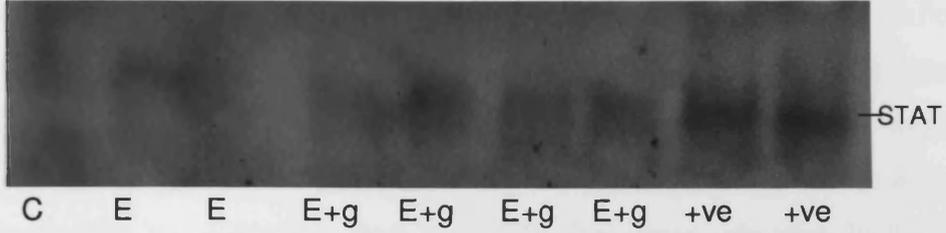
In this experiment there was no correlation between clinical score and STAT activation. There was no correlation between treatment group and activation. However, control mice with no arthritis had low levels of activation. SES peritonitis mice had higher levels of activation.

**C** Supershift showing that STAT activation is predominantly STAT1 and STAT3 in this particular experiment.

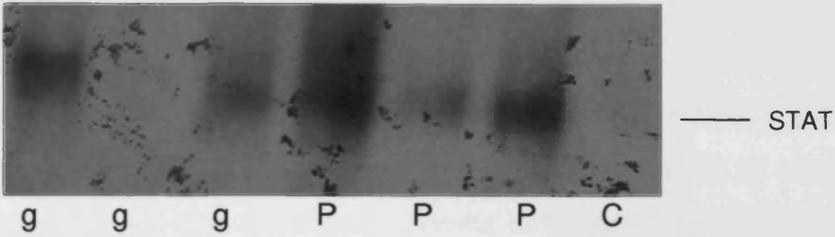
**S1-STAT1, S3-STAT3, S5-STAT5**

**Figure 5.26 - STAT activation in forepaws of mice affected by murine collagen induced arthritis**

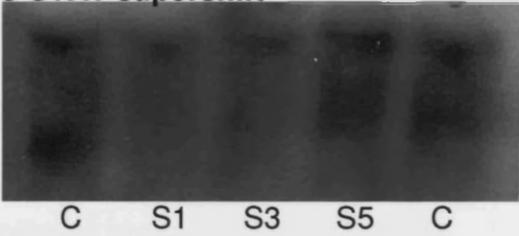
**A STAT**



**B STAT**



**C STAT supershift**



#### 5.2.4 Early Intervention with Combination Therapy in mCIA

Despite initially promising results with sgp130:Fc in disease prophylaxis experiments; using sgp130:Fc in established disease did not prove as beneficial as we had predicted. Increasing the dose of sgp130:Fc did not improve clinical or histological outcomes and it was decided to intervene in early disease to maximise the effect of therapy in mCIA which is an aggressive disease. The original dose of 2.5 mg/kg sgp130:Fc was used as there was no evidence that 5 mg/kg dose was effective in the previous experiment.

In this experiment combination therapy was given from the initiation of therapy to assess the effect of combination therapy given from disease onset. This is in contrast to modelling the effects of adding sgp130:Fc to animals with sub-optimal response to etanercept described in the previous experiments.

We wondered whether severe damage had already occurred to the joints by day 27. This might provide an explanation for the failure of therapy initiated at day 27 to make an impact on disease histology. The first 6 mice that developed arthritis were sacrificed at this time-point in order to compare histological damage comparison at the time-point in which therapy had been initiated in previous experiments.

Therapy was initiated in each mouse at the first sign of clinical arthritis and continued for 7 days in total. It was not possible to match animals for clinical score as some animals developed arthritis in more than one joint when arthritis first occurred. Animals were assigned randomly to each treatment group in turn: sgp130:FC (2.5 mg/kg); etanercept (2.5 mg/kg); and combination therapy (sgp130:FC + etanercept each at 2.5 mg/kg and PBS control. A single animal did not develop arthritis and therefore there were only 5 animals in the PBS control group.

**Table 5.6 Range of clinical scores at initiation of therapy (early intervention)**

Day number	Number of mice developing arthritis	Range of clinical scores
24	1	2
25	2	1
26	12	1-8
27	8	1-10
28	3	2-3
29	2	2
30	2	1

At baseline there was no significant difference between groups in paw diameter or clinical score.

*5.2.4.1 The Effect of Early Intervention with sgp130:Fc Compared to PBS Control*

At initiation of therapy in the PBS treated controls, clinical score was  $3.8 \pm 1.29$  which increased on a daily basis to a maximum of  $11.8 \pm 2.25$  prior to dose 7 and remained at  $11.8 \pm 2.48$  at experimental endpoint. In the gp130:Fc group, mean clinical score increased on a daily basis from  $2.33 \pm 0.83$  at initiation of therapy to a maximum of  $10.67 \pm 1.85$  at experimental endpoint. There was no significant difference between PBS and sgp130:Fc treated animals in measurements of clinical score at any time point (Figure 5.27).

In the PBS group, paw diameter increased from  $2.18 \pm 0.14$ mm at initiation of therapy to a maximum mean paw diameter of  $2.75 \pm 0.18$ mm prior to dose 6 and decreased to  $2.66 \pm 0.13$ mm at endpoint. In the sgp130:FC treated animals paw diameters increased from  $2.08 \pm 0.08$ mm at initiation of therapy to a maximum of  $2.65 \pm 0.16$ mm prior to dose 6 and decreased to

2.61±0.10mm at endpoint. There was no significant difference in paw diameters between control and sgp130:Fc treated animals at any time point. There was, therefore, no significant improvement in disease severity as a result of sgp130:Fc therapy given in early arthritis.

#### 5.2.4.2 Etanercept Monotherapy Compared to Combination Therapy in Early Disease

In the etanercept therapy group, clinical score increased on a daily basis from 3.33±1.0 on initiation of therapy to a maximum of 6.83±1.11 prior to dose 6 then decreased to 5.67±0.92 at endpoint.

In the combined therapy group, mean clinical score on initiation of therapy was 1.83±0.34. This was not significantly different from etanercept monotherapy group. Clinical score increased to 4.5±1.32 prior to dose 3 then remained steady until dose 6 when a maximum score of 4.5±1.67 was reached. Clinical score then reduced to 3.83±1.83 at experimental endpoint.

Paw diameters were also measured on a daily basis. The mean paw diameters in the etanercept group increased from 2.01±0.10mm at initiation of combined therapy to a maximum of 2.28±0.13mm at experimental endpoint. Paw diameters in the combination therapy group increased from 1.98±0.09mm to a maximum of 2.24±0.14mm prior to dose 6 and then decreased to 2.17±0.14mm at experimental endpoint (Figure 5.28).

Although there was a trend towards improvement in all parameters of clinical disease in the combination group compared to etanercept alone, this did not reach statistical significance at any time point. However, when individual paw scores were compared there was a significant improvement in paw score when compared to etanercept alone (1.13±0.25 vs 1.71±0.24 prior to dose 6 and 0.96±0.25 vs 1.54±0.23 prior to dose 7;  $p \leq 0.05$ ; data not shown).

When combination therapy was compared to sgp130:Fc monotherapy, there was a significant difference in clinical scores from prior to dose 4 (4.16±1.25

vs  $9.33 \pm 0.92$ ;  $p \leq 0.05$ ) which persisted until experimental endpoint when scores were  $3.84 \pm 1.43$  in the combination therapy group compared to  $10.67 \pm 1.85$  in sgp130:Fc therapy group ( $p \leq 0.05$ ).

When sgp130:Fc monotherapy was compared to combination of etanercept and sgp130:Fc, there was significant difference in paw diameters from prior to dose 4 ( $2.06 \pm 0.12$ mm vs  $2.55 \pm 0.14$ mm;  $p \leq 0.05$ ) which persisted until experimental endpoint when paw diameters were  $2.16 \pm 0.14$ mm and  $2.61 \pm 0.10$ mm respectively ( $p \leq 0.05$ ).

In conclusion, there was a trend towards clinical improvement in all parameters in the combination group compared to etanercept alone. Although this was not statistically significant, it is likely that if greater animal numbers were used there would be a significant difference. Etanercept and sgp130:Fc in combination were significantly better than sgp130:Fc monotherapy.

#### 5.2.5 Histological Assessment of Effect of Intervention in Early Arthritis

Sections were cut and stained as previously described. Sections were scored by a blinded observer using an in-house scoring system as previously described (section 2.18).

In the samples from PBS control mice sacrificed at day 27 the mean arthritis index was  $8.25 \pm 1.61$  (Values shown are mean  $\pm$  SD). By day 27 after initiation of disease there was already evidence of cellular infiltrate with leucocyte infiltrate and exudate into the joint space. There was significantly less synovial hypertrophy when compared to PBS treated animals sacrificed at experimental endpoint ( $1.67 \pm 0.37$  vs  $2.8 \pm 0.21$   $p \leq 0.05$ ). In addition there appeared to be a reduction in bone and cartilage erosion ( $1.75 \pm 0.39$  compared to  $2.3 \pm 0.32$ ) although this did not reach statistical significance. There was no significant difference in arthritis index between animals sacrificed at day 27 and animals sacrificed at endpoint.

In the samples from animals sacrificed at experimental endpoint mean arthritis index for PBS controls was  $11.2 \pm 1.18$ . There was severe arthritis in all joints with evidence of marked synovial hypertrophy with a heavy leucocyte infiltrate evidenced by dense nuclear staining. The joint space had a leucocyte rich exudate and there was marked bone and cartilage damage with loss of normal joint contours.

Changes seen in the sgp130:Fc therapy group were similar with marked synovial hypertrophy and a heavy leucocyte infiltrate evidenced by dense nuclear staining. The joint space had a leucocyte rich exudate and there was marked bone and cartilage damage with loss of normal joint contours. The mean arthritis index was  $11 \pm 0.71$  which was not statistically different from arthritis index in the PBS group.

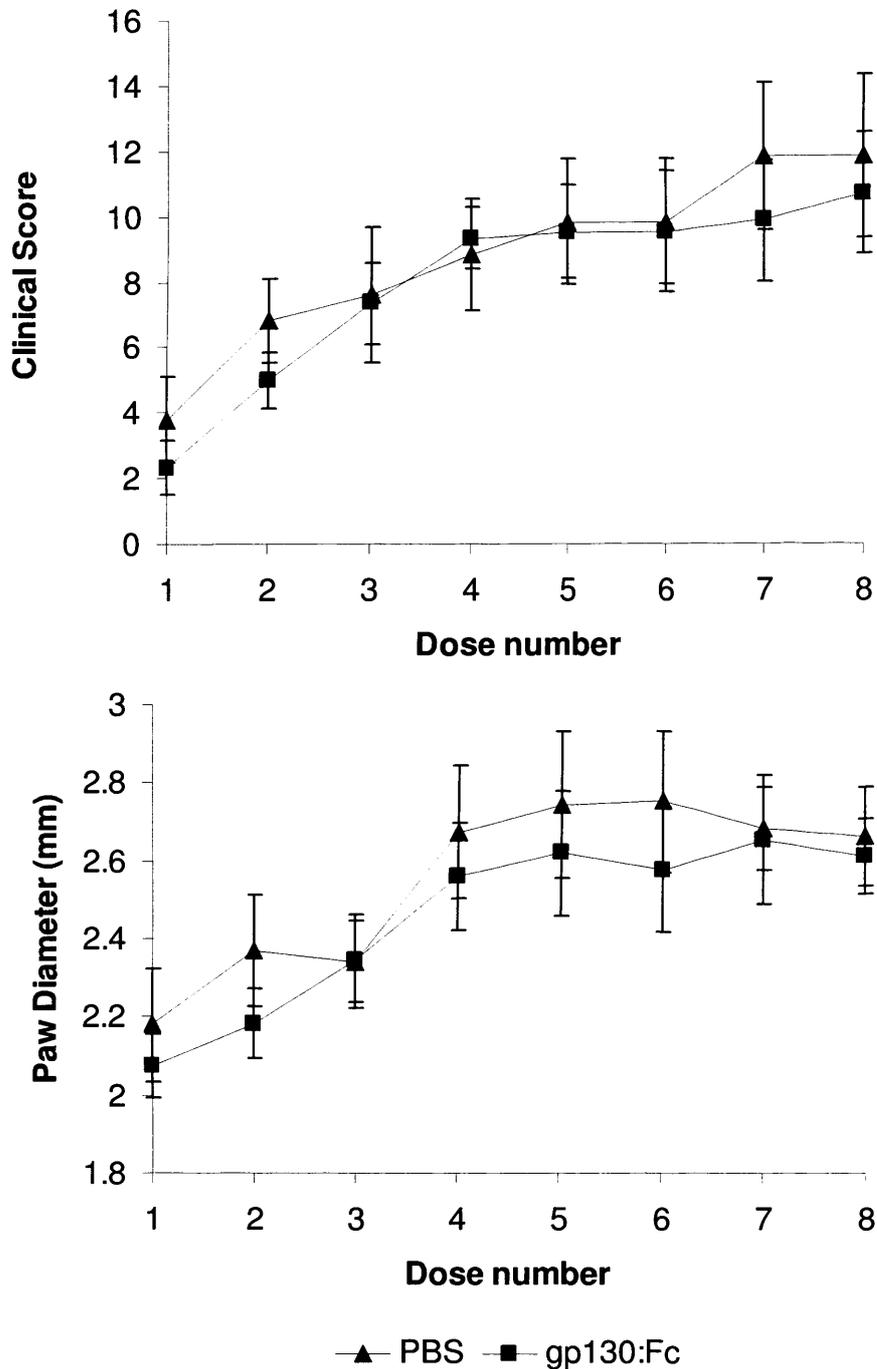
In contrast, in the etanercept group, arthritis was milder with an arthritis index of  $6.75 \pm 1.41$ . Joints showed a reduction in all parameters of histological damage with only mild synovial hypertrophy, less leucocyte infiltrate and a reduction in bone and cartilage erosion. The arthritis index was significantly lower than in the PBS controls ( $p \leq 0.05$ ).

In the combination therapy group, the mean arthritis index was also significantly lower than in the PBS controls ( $6.55 \pm 1.72$ ;  $p \leq 0.05$ ). Arthritis appeared milder with less synovial hypertrophy, less leucocyte infiltrate and a reduction in bone and cartilage erosion.

Both etanercept monotherapy and combination therapy were significantly better than PBS control in preventing histological damage. There was no significant difference between etanercept monotherapy and combination therapy in preventing histological damage (Figure 5.30). Representative sections are shown below in Figure 5.29.

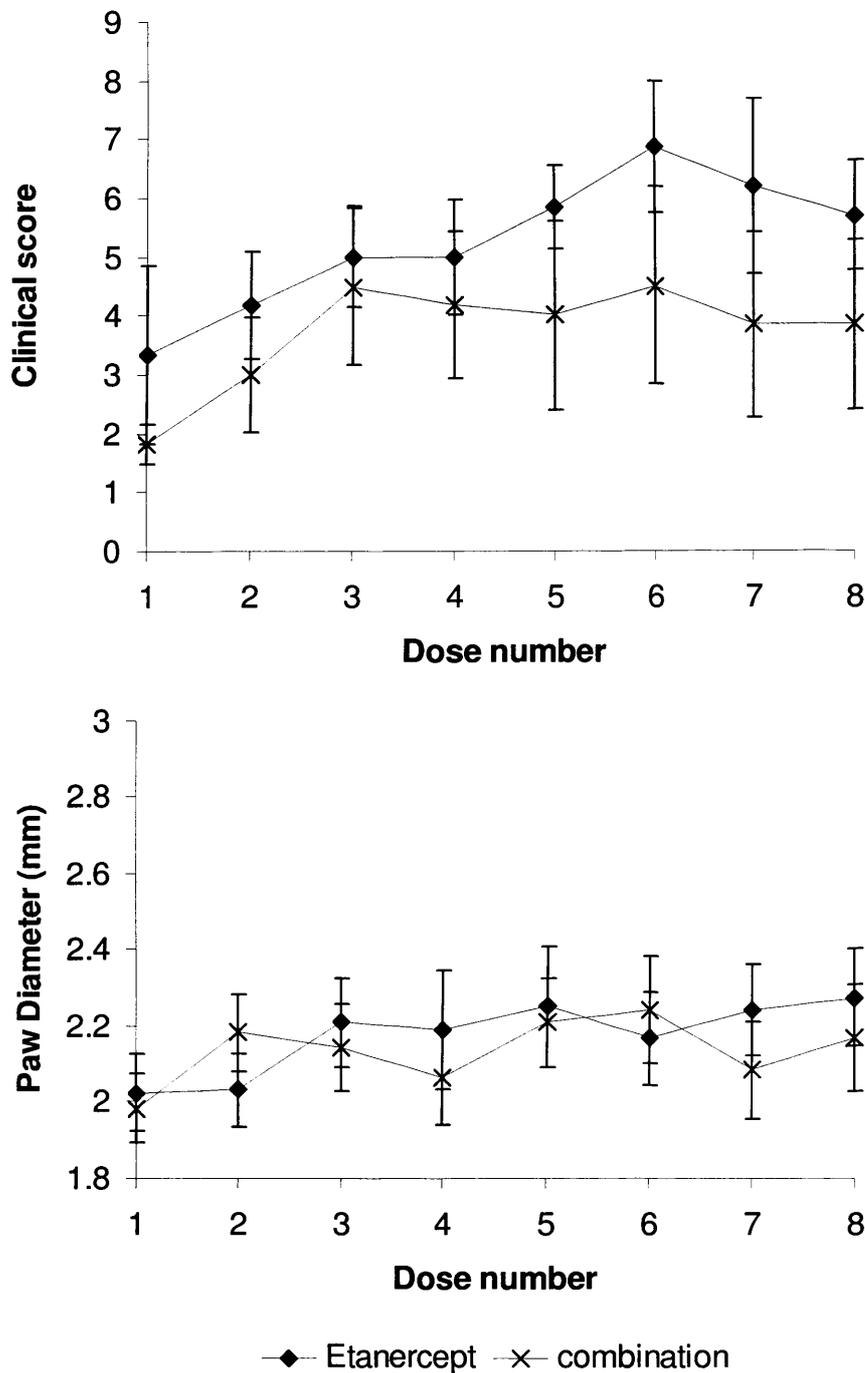
In conclusion, combination therapy with etanercept and sgp130:Fc shows a trend towards improving clinical score when compared to etanercept monotherapy and significantly improves disease pathology, as shown by improvement in all parameters of histological assessment and arthritis index when compared to PBS controls.

**Figure 5.27 - Effect of sgp130:Fc on clinical score and paw swelling in early arthritis**



Graph demonstrating clinical score and paw swelling in millimetres from day of first clinical signs of arthritis until experimental endpoint when mice had received 7 doses of therapy or severity limits reached. Mice were treated with 2.5 mg/kg sgp130:Fc on a daily basis or PBS. Graphs show mean of 6 mice  $\pm$  SEM in treatment group and mean of 5 mice  $\pm$  SEM in PBS control group. There was no significant difference seen between groups.

**Figure 5.28 - Effect of low dose etanercept alone and in combination with sgp130:Fc on clinical score and paw swelling in early arthritis**



Graph demonstrating clinical score and paw swelling in millimetres from day of first clinical signs of arthritis until experimental endpoint when mice had received 7 doses of therapy or severity limits reached. Mice were treated with 50 µg of etanercept alone or in combination with 2.5mg/kg sgp130:Fc on a daily basis. Graphs show mean of 6 mice±SEM per treatment condition. There was no significant difference seen between groups.

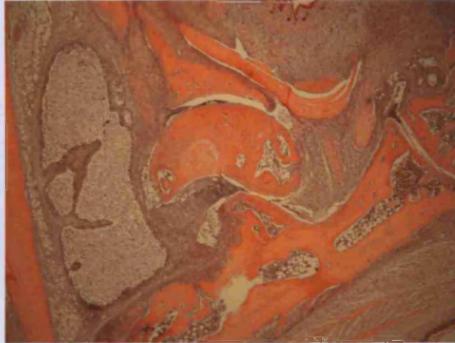
**Figure 5.29 - Representative histological sections from each therapy group following intervention in early arthritis.**

**A PBS day 27**



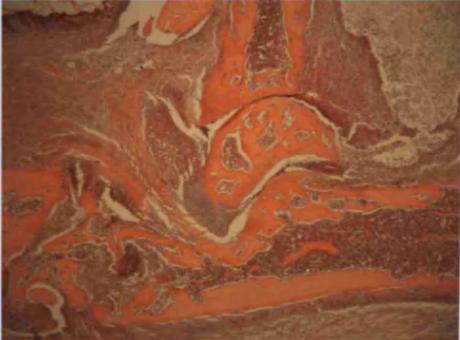
**Arthritis index- 6**

**B PBS endpoint**



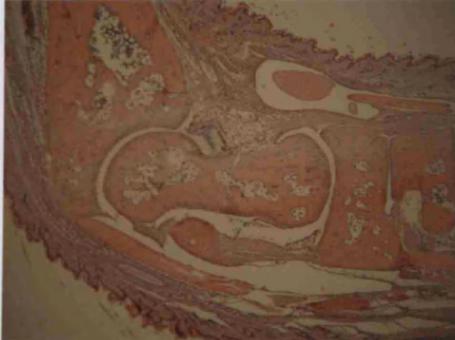
**Arthritis index- 11**

**C sgp130:Fc endpoint**



**Arthritis index- 14**

**D etanercept endpoint**



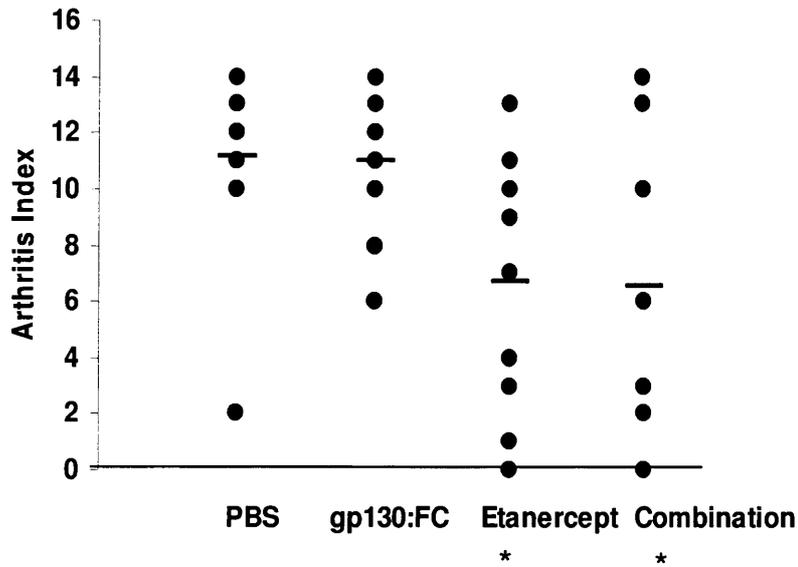
**Arthritis index- 4**

**E gp130:Fc+etanercept endpoint**



**Arthritis index- 2**

**Figure 5.30 - Effect of early intervention with sgp130:Fc and etanercept singly and in combination on histological parameters of disease**



Histology scores show results for each treatment group scored by a blinded observer. Individual paw scores are shown by black circles and mean of each treatment condition by a horizontal line. \* shows significant difference in mean ( $p \leq 0.05$ ) when compared to PBS control group. There was no significant difference between etanercept and combination therapy groups.

### 5.3 Discussion

sgp130:Fc therapy given from time of arthritis induction led to a 50% reduction in incidence of mCIA. This compares favourably with results obtained in anti-TNF $\alpha$  therapeutic trials where 66% of animals developed disease (Williams *et al* 1992). The results are similar to those described by Wooley *et al* where incidence of arthritis in anti-TNF treated animals was 55% by day 55. In their control group there was 40% incidence at day 45 and 90% incidence at day 55 (Wooley *et al* 1993). The time course of mCIA described by Wooley is very different to our local experience where 100% incidence is generally seen by day 28 and severity limits are reached with termination of experiment normally between day 35 and 40.

In this prophylaxis experiment, disease severity in those animals which did develop arthritis was significantly reduced. Treatment with sgp130:Fc resulted in a significant improvement in all parameters of joint degradation when assessed histologically, i.e. Infiltration, exudate, hyperplasia and bone and cartilage erosion. Therefore, Aim One (which was to establish a therapeutic dose of soluble gp130:Fc for disease prophylaxis) was achieved.

The second aim of this chapter was to identify a dose of etanercept which resulted in 50% inhibition in disease activity in mCIA. The dose of etanercept chosen (2.5 mg/kg) reduced incidence of arthritis to 83%. Clinical scores were reduced by 25-75% between days 27 and 30. Therefore, Aim Two was achieved with the selection of an appropriate dose deemed to be suitable for further combination therapy experiments.

The mechanism of therapeutic efficacy was assessed using histological staining. Tartrate resistant acid phosphatase staining (TRAP) was demonstrated at joint margins in sites of bony erosions but also within the bone marrow. This suggests that osteoclasts can be formed both locally at erosion site and also within neighbouring bone marrow. TRAP activity was also demonstrated within the bone marrow by other groups in murine

inflammatory arthritis (Cawston *et al* 2005). Erosions may occur both from the joint surface inwards and also from the bone marrow outwards.

Both NFκB and STAT signalling pathways are up-regulated in the presence of arthritis as measured by EMSA in nuclear extracts obtained from mouse fore paws. NFκB activation appeared to be predominantly that of P50 and to lesser extent P65 sub-units. NFκB activation correlated fairly well with clinical score and was also reduced by etanercept treatment even in paws where clinical score remained high. Reduction in disease severity has been shown previously to be associated with reduction in NFκB activation within murine ankle joints but specific sub-units were not assessed (Gerlag DM *et al* 2000).

STAT activation appeared to be predominately STAT 3. Levels of activation were low even in markedly arthritic paws (scoring 4). Therefore, it appears likely that, in end-stage disease, STAT activation is less important than NFκB activation in mCIA. It would be useful to assess signalling at other earlier time-points. It is known that IL-6 levels in mCIA peak at day of first collagen injection, i.e. prior to onset of clinical disease. This is likely to be the time at which STAT activation is also maximal (Takagi *et al* 1998).

The effect of therapy on STAT activation was less clear cut. STAT activity was not up-regulated in all arthritic paws. However, sgp130:Fc appeared to be more effective at reducing STAT activation than etanercept therapy.

Richards *et al* showed reduction in STAT-3 staining by immunohistochemistry in paraffin wax sections from mice with AIA treated with gp130-RAPS (an alternative method for blocking IL-6 trans-signalling) (Richards *et al* 2006). It is likely therefore that therapeutic effects of etanercept occur via inhibition of NFκB activity, whilst blockade of IL-6 trans-signalling is likely to occur via its effects on STAT 3 activity.

Atreya *et al* demonstrated a reduction in STAT 3 activation *in-vitro* in T cells from patients with Crohn's disease cultured with anti IL-6R antibody. They also demonstrated a reduction in STAT3 activity in spleen cells obtained from colitic T cell reconstituted severe combined immunodeficiency (SCID)

mice following treatment with anti IL-6R. It is not clear from their paper at what stage in disease STAT 3 was measured (Atreya *et al* 2000). This would suggest that blockade of IL-6 directly also results in down-regulation of STAT 3 signalling and that this is not a pathway activated solely by trans-signalling mechanism.

In RA, STAT 1, 3 and 4 have been shown to be up-regulated in the synovium. STAT 3 has been shown to promote survival of RA synovial fibroblasts (Krause 2002). Treatment of RA synovial fibroblasts with IL-6/sIL-6R also results in significant up-regulation of STAT 1 and STAT 3 when measured by EMSA (Deon *et al* 2001). Therefore, a therapy which down-regulates STAT 3 may well be effective in RA.

Following the successful use of sgp130:Fc in prophylaxis of arthritis; the effect of sgp130:Fc in established disease was then assessed. Therapy was initiated at day 27 when arthritis incidence was 100%. Mice were treated with either PBS (control), sgp130:Fc or etanercept. At day 30, 50% of the etanercept group had sgp130:Fc added to their therapy in order to model the addition of a second therapy in those who may be achieving sub-optimal benefit from anti-TNF $\alpha$  therapy.

sgp130:Fc therapy resulted in significant improvement in clinical disease parameters. The improvement was comparable to that achieved in trials of anti-TNF directed therapies in established disease. Wooley *et al* reported a reduction of approximately 30% in clinical score in established disease with soluble TNF receptor (Wooley *et al* 1993).

When combination therapy was compared to etanercept monotherapy, there was a trend towards improvement in clinical scores at endpoint. When change in arthritis index was compared from time of initiation of combination therapy, the combination therapy group had significantly less disease progression. Unfortunately, the improvement in clinical scores did not result in improvement in gross histology. Only etanercept treated animals had significantly lower histology scores than the PBS control group.

Reduction in macrophage numbers and staining intensity were seen in sgp130:Fc and combination therapy treatment groups when compared to PBS controls as assessed by immuno-histochemistry. Numbers of macrophages were decreased significantly in sgp130:Fc treated animals when compared to control or etanercept treated groups even when total leucocyte numbers did not appear to be affected. This correlates with my data shown in chapter 3 where anti-IL6R therapy does down regulate production of CCL2 (macrophage chemo-attractant). Interestingly, from my *in-vitro* studies, etanercept also appeared to down-regulate CCL2 but this has not translated into a reduction in macrophage infiltrate in mCIA.

The effects on regulatory T cell numbers were less clear cut. There appeared to be an increase in regulatory T cell numbers in sgp130:Fc treated animals although this was not significant. There did not appear to be an increase in regulatory T cell numbers in etanercept monotherapy or combined therapy groups. It was not possible to stain for any other T cell markers so it is still unclear whether the possible change in T reg numbers was due to an alteration in total T cell numbers or due to specific induction of T regs by sgp130:Fc treatment. As functional activity was not assessed, it was not possible to determine whether the increase in T-Reg numbers led to improvements in functional activity, i.e. suppression of cytokine production.

Work from Doganci et al looking at the effect of IL-6R blockade on regulatory T cell function in asthma showed that regulatory T cell numbers could be locally up-regulated using inhaled antibodies against IL-6R but again found no difference in T-Reg numbers after administering sgp130:Fc systemically. Therefore, sgp130:Fc may not exert its beneficial action via effects on regulatory T cell numbers (Doganci A *et al* 2005). In recent work Dominitzki et al identified that addition of hyper IL-6 to stimulated T cells obtained from murine splenocytes prevents induction of Fox-P3. However, they did not look at the capacity of sgp130:Fc to block this IL-6 trans-signalling capacity (Dominitzki et al 2007). Further work is therefore needed

to assess the ability of sgp130:Fc to affect regulatory T cell numbers and function in mCIA.

The function of regulatory T cells in RA has also been studied. Conflicting reports exist regarding the proportion of T Regs in the peripheral circulation in RA compared to normal. This appears to depend on whether all CD25+ cells were analysed or whether only CD25 bright cells were included (Van Amelsfort 2004; Ehrenstein *et al* 2004). There does appear to be a significant correlation between peripheral T Reg numbers, ESR and CRP (Van Amelsfort 2004). T Reg numbers have been shown to be increased in synovial fluid when compared to peripheral blood (Cao *et al* 2003).

Ehrenstein *et al* suggested that although T Reg numbers in RA are increased; paradoxically their function is compromised and they are unable to suppress production of pro-inflammatory cytokines. They demonstrated that, following anti-TNF $\alpha$  treatment, the capacity of cells to inhibit cytokine function was restored. Treatment resulted in a significant rise in peripheral blood levels of T regs (Ehrenstein *et al* 2004). However, this increase in regulatory T cell numbers was not noted in other studies (Dombrecht *et al* 2006; Vigna-Perez *et al* 2005). More recently, Nadkarni *et al* showed that whilst Infliximab therapy induced a new population of T regulatory cells lacking CD-62 ligand that natural CD62L<sup>+</sup> cells remained deficient (Nadkarni *et al* 2007).

Recent work has shown a possible role for IL-6/sIL-6R in the function of regulatory T cells. Using an animal model of asthma, Doganci *et al* demonstrated induction of regulatory T cells following treatment with anti-IL-6R antibody (Doganci *et al* 2005). Using an in-vitro assay of regulatory T cell function, addition of hyper-IL-6 (soluble IL-6 receptor/IL-6 fusion protein) resulted in marked reduction of suppressor capacity of regulatory T cells (Oberg *et al* 2006).

Therefore, whilst T Regs appear to display a suppressive role in the development of murine models of arthritis; their role in RA appears more complex. The presence of large numbers of cells in the synovium in the

absence of a clear suppressor role may be due to overwhelming of this host mechanism by large numbers of reactive T cells or alternatively to specific conditions within the joint in human disease which prevent the action of these T Regs. Further work is obviously needed to determine the role of regulatory T cells in RA and the effect of therapy on their function.

Whilst sgp130:Fc was effective in established disease, the aggressive nature of the condition meant that the dose of 2.5 mg/kg, which was very effective when given for disease prophylaxis, was less effective in established disease. A further experiment was therefore planned using 5 mg/kg sgp130:Fc to assess whether a higher dose might be more clinically effective. Unfortunately, sgp130:Fc was no more effective than PBS control in this experiment. The course of disease seen in this experiment was different to that seen normally. Arthritis was earlier in onset, i.e. 5/24 mice had signs of arthritis on day 21. The incidence was 71% at day 25 and 2 mice had a score of 12 at this point. It was necessary to dispatch 2 mice before the end of the experiment due to severity limits being reached which affected the final analysis. After initial severe disease onset, there was some improvement in the control group disease severity before the end of the experiment meaning that it was harder to pick out improvements due to therapy.

Interestingly, when anti-IL6R antibodies were used in established disease in mCIA, it was also found that there was only therapeutic benefit when therapy was administered shortly after collagen immunisation rather than in established disease (Yoshizaki et al 1998). Work from this group showed that IL-6 levels peaked at day one following first collagen injection and had a second smaller peak at day 28. This may explain why IL-6 targeted therapy is more effective at disease initiation as this would coincide with timing of IL-6 peaks (Takagi et al 1998). It has also been shown previously that IL-6 is essential for the development of collagen-induced arthritis (Alonzi et al 1998) and, therefore, if IL-6 is sufficiently suppressed at time of arthritis induction then mCIA will not develop.

There was also no improvement in paw scores or clinical scores when etanercept and sgp130:Fc were given in combination compared to etanercept monotherapy. In contrast, there was an improvement in histological joint damage in the combination therapy group when compared to PBS which did not occur in either monotherapy group. When individual components of arthritis index were assessed, it appeared that this improvement was mainly due to a reduction in cartilage and bone erosion and exudate. Similar levels of hypertrophy and infiltrate were seen in all therapy groups.

Direct comparisons with histological outcomes in other studies are difficult as different scoring systems are used in each experiment. Williams *et al* showed reduction in severe histological damage characterised by synovitis, extensive erosions and disruption of joint architecture but did not separate into specific components (Williams *et al* 1992). Other groups did not look at histology (Piguet *et al* 1992; Wooley *et al* 1993).

mCIA resulted in up-regulation of NF $\kappa$ B primarily P65 sub-unit with lesser P52 involvement. Etanercept monotherapy abolished this up-regulation. Combination therapy and sgp130:Fc did not prevent up-regulation. The failure of sgp130:Fc to suppress disease and the loss of etanercept effect on NF $\kappa$ B may be due to inefficacy of sgp130:Fc. Alternatively, it is possible that this batch of sgp130:Fc may have been contaminated with a substance, e.g. LPS which worsened arthritis and resulted in NF $\kappa$ B activation.

In this experiment STAT 1 and 3 both appeared to be up-regulated in active arthritis. This was less than the level of activation which occurs in SES induced peritoneal inflammation. Activation appeared lower in all therapy groups than in PBS controls. STAT activation appeared higher in the combination therapy group than in animals treated with etanercept or sgp130:Fc monotherapy. As discussed earlier in the chapter, day 35 is probably not the best time point to assess STAT activity in mCIA.

Interactions between IL-1 or TNF $\alpha$  and IL-6/sIL-6R resulting in down-regulation of STAT activation have been described (Deon D *et al* 2001).

This provides an explanation for low levels of STAT activation seen at disease end-point. TNF $\alpha$  is assumed to be up-regulated in end-stage disease (Wooley *et al* 1993). This may explain why high STAT levels were seen in the combination therapy group where TNF $\alpha$  activity was reduced therefore removing inhibitory effect of TNF $\alpha$  on STAT activity.

In a final experiment designed to maximise the effects of therapy, treatment was started at the first sign of clinical disease in order to model intervention in early RA as opposed to end-stage disease when it was likely that much joint damage had already occurred. Both therapies were given simultaneously to assess the effects of combination therapy rather than modelling inadequate response to TNF $\alpha$  monotherapy.

sgp130:Fc did not improve clinical disease severity. Both etanercept and combination therapy were significantly better than PBS at improving clinical markers of disease severity.

Combination therapy was superior to monotherapy at all time points in reducing paw swelling, paw scores and arthritis index but this reached significance at doses 6 and 7 only. It is likely that if greater numbers of animals had been treated that statistical significance would have been reached at endpoint.

Histological analysis again showed significant improvement in all parameters when etanercept and combination were compared to PBS controls. There was no significant difference between etanercept monotherapy and combination with sgp130:Fc. It is possible that if greater numbers of animals had been treated that statistical significance would have been reached.

In summary, although sgp130:Fc did not appear to be effective when given in established disease; the results were not dissimilar to the effects of anti IL-6R in mCIA which did not have any benefit when given later than 3 days after disease initiation (Takagi *et al* 1998). Although this was the case, anti IL-6R therapies have been shown to be effective in established disease in humans (Nishimoto *et al* 2004; Choy *et al* 2002). It is likely that IL-6 levels

are more dynamic in RA than in mCIA. Indeed both serum and synovial fluid IL-6 levels have been shown to undergo diurnal variation. Levels in serum and SF appear to be independently regulated (Perry *et al* 2006). IL-6 levels are high in active disease, even in long-standing arthritis (Charles *et al* 1999). There is, therefore, a reasonable expectation that targeting IL-6 trans-signalling in established RA may well be beneficial.

The efficacy of combination therapy and its mechanisms of action were studied in these experiments. It is likely that sgp130:Fc exerts some of its effect by modulating leucocyte recruitment, specifically macrophage recruitment. There may be an additional effect on T cell function which could not be fully assessed in this experimental model. Although it appears that NFκB and STAT activation are affected in this model, further experiments at different time points would be necessary to fully elucidate the effects of therapy on these signalling pathways.

Combination therapy for mCIA appeared to be tolerated by mice and resulted in reduction in clinical disease severity. Its effects may be mediated by modulating macrophage recruitment preventing chronic inflammatory changes. It is likely that therapy would need to be started early in disease to prevent histological damage. It appears that there is now proof of concept for the theory that combination therapy with etanercept and sgp130:Fc may be effective in selected patients with rheumatoid arthritis.

## **6 IMPLICATIONS FOR IL-6 SIGNALLING IN RESPONSE TO ANTI-TNFA THERAPY**

### **6.1 Introduction**

In previous chapters I demonstrated that the functions of IL-6/sIL-6R and TNF $\alpha$  are closely linked in the pathogenesis of RA. TNF $\alpha$ , in levels similar to that found in the RA joint, elicited shedding of IL-6 from the surface of infiltrating leucocytes which would allow trans-signalling to occur within the joint. TNF $\alpha$  and IL-6 trans-signalling activities then cause up-regulation of chemokine and adhesion molecule expression by RA fibroblasts with further effects on leucocyte recruitment and propagation of inflammation. Having demonstrated that functions of TNF $\alpha$  and IL-6 trans-signalling within the joint are closely linked; we speculated that response to therapy with TNF $\alpha$  antagonists may also be affected by IL-6 trans-signalling in RA.

The majority of patients commenced on anti-TNF $\alpha$  therapies have an excellent response to treatment. However, there are a significant minority who fail to respond adequately or, who after initially promising response, have disease relapse. Studies have been performed to assess whether it may be possible to identify those patients who are less likely to have a good clinical response.

This would be useful both to prevent wastage of expensive drugs in a situation where they are unlikely to succeed and also to choose the most appropriate therapy which is likely to help an individual patient.

Proteomics, with its focus on comparing individual gene and protein expression patterns with clinical features, may eventually be of use in predicting which therapies are most likely to be effective in each individual patient. However, at present, research in this area is not far enough advanced to utilise this technology.

In RA, clinical response can be defined in several ways. For the purposes of BSR/NICE recommendations, a fall in DAS score of 1.2 or to below 3.2 is needed in order to stay on therapy.

ACR response criteria have been used in the majority of clinical trials. Most commonly, ACR 20 (20% improvement from baseline) as the primary endpoint but ACR 50 and 70 have also been assessed.

When considering anti-TNF $\alpha$  treatment, the following factors which might be considered to predict clinical response have been investigated;

a) Clinical predictors

Work from the BSR registry looking retrospectively at clinical data and outcomes in 2879 patients concluded that a higher baseline HAQ score correlated with a lower response rate; whilst a better response was associated with the current use of NSAIDs and the use of methotrexate (MTX). There was a lower response rate among current smokers. Age, disease duration, rheumatoid factor and the previous number of disease-modifying anti-rheumatic drugs (DMARDs) did not predict response to anti-TNF therapy. Females were less likely to achieve remission. They suggested that the inability of other baseline disease characteristics to predict the outcome suggests that other factors, including potential genetic differences in drug metabolism, may be influencing the response to anti-TNF-alpha therapies (Hyrich *et al* 2006).

b) Genetic predictors

Many other studies have focussed on the role of TNF $\alpha$  gene polymorphisms in predicting response to anti-TNF $\alpha$  therapies. There were no consistent findings between these studies which used a variety of therapies and a variety of methods for assessing outcome. Seitz *et al* found that those patients with a G/G phenotype were more likely to have a good response to therapy irrespective of the underlying disease (Seitz *et al* 2007). An earlier study from 2003 also concluded that patients with a TNF $\alpha$  -308G/G

genotype were better infliximab responders than are patients with A/A or A/G genotypes (Mugnier *et al* 2003).

In contrast, another study from 2004 compared patients with G/G and A/G phenotypes. Both groups showed a significant improvement with treatment in all variables studied (Cuchacovic *et al* 2004).

Finally, a meta-analysis incorporating six studies showed a significant association between the TNF $\alpha$  promoter -308 A/G polymorphism and responsiveness to anti-TNF therapy, suggesting that the individuals with RA who carry the A allele have a poorer response to anti-TNF therapy than those with the G allele (Lee *et al* 2006).

Polymorphisms associated with other cytokine genes were assessed in a single study. 123 patients with active RA were commenced on etanercept. Genotyping was carried out for TNF (-308 TNFA), interleukin 10(IL-10), transforming growth factor Beta 1(TGF $\beta$ 1), and IL-1 receptor antagonist (IL-1RA). Response was defined by ACR20 or DAS 28 response criteria. 20% of patients did not achieve response. None of the recorded alleles was associated with responsiveness to treatment. However, a certain combination of alleles (-308 TNF1/TNF1 and -1087 G/G) was associated with good responsiveness to etanercept ( $p < 0.05$ ). In addition, a combination of alleles influencing interleukin 1 receptor antagonist (IL1Ra) and TGF $\beta$ 1 production (A2 allele for IL1RN and rare C allele in codon 25 of TGF $\beta$ 1 gene) was associated with non-responsiveness (Padyukov *et al* 2003).

#### c) Laboratory measurements predicting response

A Scandinavian group studied 35 RA patients initiating treatment with Infliximab. They defined response to treatment as an improvement of at least 20% in ACR 20 at 2 weeks, i.e. after a single infusion of Infliximab. They noted significantly higher numbers of CCR3 positive CD8 cells and higher numbers of CCR5 positive CD4 cells at baseline in non-responders.

Responders had increased numbers of CD3 cells following treatment. This increase was not seen in non-responders. No other difference between responders and non-responders was seen (Nissinen *et al* 2004).

In 2006, a cohort was described in which anti-CCP antibodies were measured prior to treatment with Infliximab. Anti-CCP and IL-6 levels decreased in those patients who responded to treatment but initial anti-CCP levels did not correlate with likelihood of response to treatment (Braun-Moscovi *et al* 2006). These findings were disputed by a second study assessing response to Adalimumab where there did not appear to be any relationship. The positive finding from this study was that patients with low serum cartilage oligomeric protein (COMP) levels (<10 U/l) at baseline showed a significantly higher ACR70 response (>50%,  $p < 0.02$ ) within 3 months, and also at 6 months, than patients with higher COMP values (ACR70 < 20%) (Morozzi G *et al* 2007).

It therefore appears that at present the best predictor of response to therapy is being a non-smoker with ability to tolerate methotrexate. Whilst there is some evidence for genetic polymorphisms predicting response to therapy, other bio-markers would be useful.

IL-6 appears important in RA pathogenesis and it has been shown that IL-6 falls rapidly following treatment with Infliximab (Charles *et al* 1999). However, in initial clinical trials of patients receiving low dose Infliximab therapy (1 mg/kg) there was a subsequent increase in IL-6 levels over the following 28 days (Charles *et al* 1999).

There is no published data on the effect of anti-TNF directed therapies on IL-6 levels at week 14. Since this is the point at which decisions are taken regarding whether therapy should be continued or not it would be interesting to assess IL-6 levels at this point. The effect of anti-TNF directed therapies on sIL-6R levels has not been assessed.

We identified previously that stimulation of neutrophils with TNF $\alpha$  results in loss of cell surface IL-6R expression and was accompanied by an increase in sIL-6R levels in cell supernatants. TNF $\alpha$  and IL-6 trans-signalling interact

to modulate leucocyte recruitment via effects on chemokine production most marked on CCL2 (macrophage chemo-attractant). Additionally, we demonstrated that TNF $\alpha$  and IL-6 trans-signalling also up-regulate ICAM-1 expression on synovial fibroblasts; potentially leading to significant effects on leucocyte recruitment in the RA joint. We also identified that combination therapy with sgp130:Fc and Etanercept results in improvement of clinical and histological features of mCIA. This led us to surmise that TNF $\alpha$  and IL-6 trans-signalling are closely linked in the pathogenesis of RA and that measurement of IL-6/sIL-6R levels may be useful in predicting response to anti-TNF $\alpha$  therapies.

The hypothesis that IL-6/sIL-6R may be useful markers to predict response to anti-TNF $\alpha$  therapies was examined through the following specific aims:

1. To study an inception cohort of patients commencing anti-TNF $\alpha$  therapy;
2. To measure serum IL-6 and sIL-6R levels pre and post anti-TNF $\alpha$  therapy;
3. To identify whether there was any correlation between response to anti-TNF $\alpha$  therapy and IL-6/sIL-6R levels.

## 6.2 Results

All patients commencing anti-TNF $\alpha$  therapy from July 2006 at UHW were invited to take part in this study to identify whether there is any correlation between IL-6/sIL-6R levels and response to anti-TNF $\alpha$  directed therapies. Any patient starting a second biologic drug, or who had a diagnosis other than RA was excluded. Samples were collected at baseline and at 14 week assessment. DAS scores were calculated prior to treatment on 2 separate occasions 1 month apart and again at 14 weeks in accordance with BSR guidelines. Previous DMARD therapy was noted together with age and sex. Therapy was chosen as a result of consultant and patient preference. The gender, therapy and age of the 9 patients utilised in this small pilot study are tabulated below (Table 6.1).

Sample collection is ongoing but results of first 9 patients were assessed to identify whether there is any correlation between IL-6/sIL-6R levels and response to anti-TNF $\alpha$  directed therapies.

**Table 6.1 - Patient characteristics of anti-TNF $\alpha$  inception cohort**

ID	1	2	3	4	5	6	7	8	9
<b>Sex</b>	M	M	F	F	F	M	M	F	F
<b>Therapy</b>	Infliximab	Etanercept	Etanercept	Etanercept	Humira	Humira	Infliximab	Humira	Etanercept
<b>Age</b>	33	75	55	55	44	44	62	57	45

### 6.2.1 DAS Scores

The mean pre-treatment DAS score was  $6.7 \pm 0.7$ . All patients had a mean pre-treatment DAS score of greater than 5.1, in accordance with BSR guidelines for prescription of anti-TNF $\alpha$  therapy. Mean DAS score at week 14 assessment was  $4.65 \pm 0.90$ . BSR guidelines define response to therapy

as a DAS score of  $\leq 3.2$  or a fall in DAS of greater than 1.2. This occurred for all patients except patient 6 who had a reduction of 0.19 from mean pre-treatment DAS. Patient 7 had a fall in DAS of 1.13 from mean although a fall of 1.53 from final pre-treatment DAS. Mean fall in DAS score for the entire patient population was  $2.05 \pm 0.92$  (Figure 6.1).

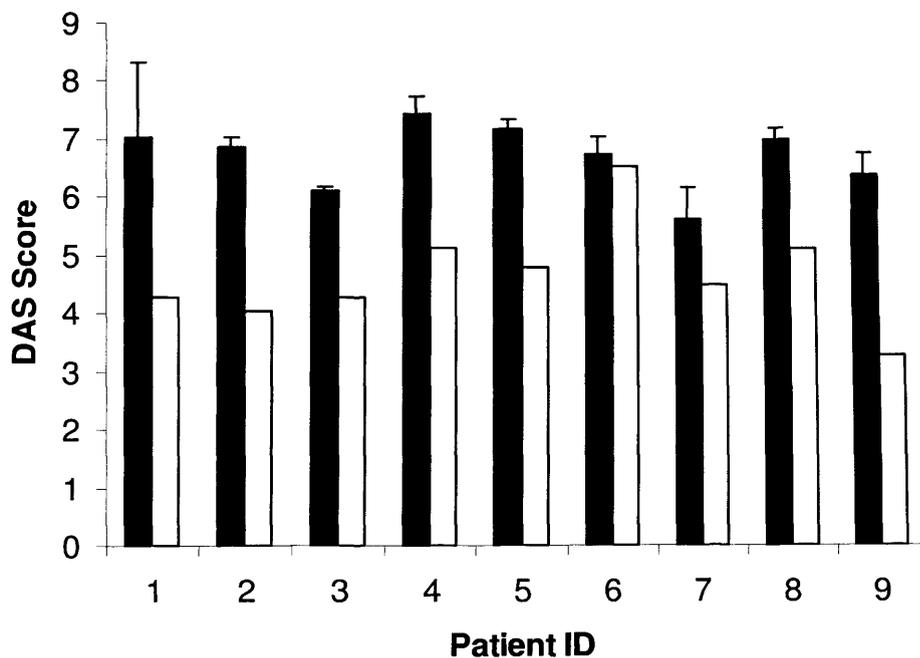
### 6.2.2 Serum IL-6 Levels

In this patient population the mean pre-treatment IL-6 level was  $569 \pm 330$  pg/ml with week 14 levels rising to  $747 \pm 483$  pg/ml. IL-6 levels were detectable in serum of all but one of the patients pre-treatment. Following anti-TNF $\alpha$  treatment, IL-6 was not detectable in sera of three patients. Levels of IL-6 fell in five patients; remained undetectable in one patient and rose in three. This did not correlate with response to therapy. However, the patient with highest initial level of IL-6 (2828 pg/ml) also had the poorest response to therapy and the largest rise in levels following therapy (to 4030 pg/ml). IL-6 levels are shown in Figure 6.2A.

### 6.2.3 Serum sIL-6R Levels

sIL-6R was detectable in the sera of all the patients pre and post treatment. Mean pre-treatment sIL-6R level was  $48 \pm 6.67$  ng/ml with post treatment levels of  $51 \pm 3.38$  ng/ml. Levels following anti-TNF therapy rose in five patients and fell in four patients. There were too few patients to identify whether sIL-6R levels were associated with response to therapy, however levels did appear lower in non-responders (Figure 6.2B).

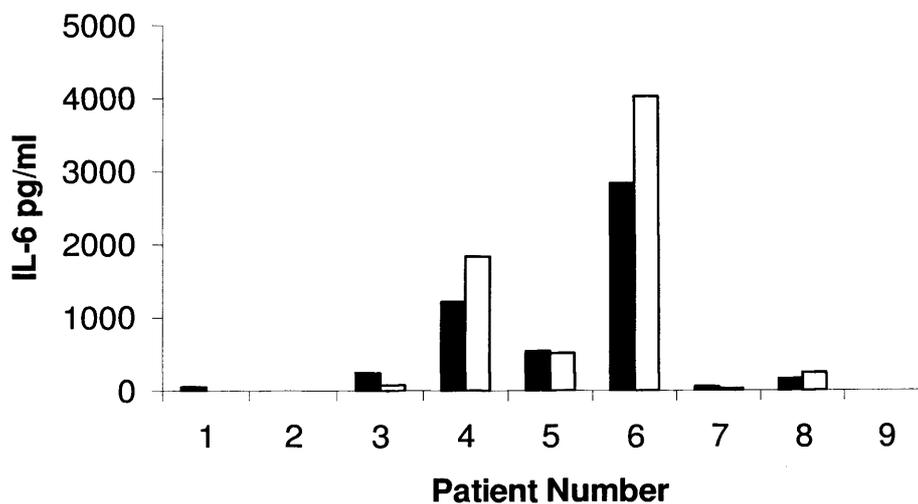
**Figure 6.1 - DAS scores pre and post anti-TNF $\alpha$  therapy**



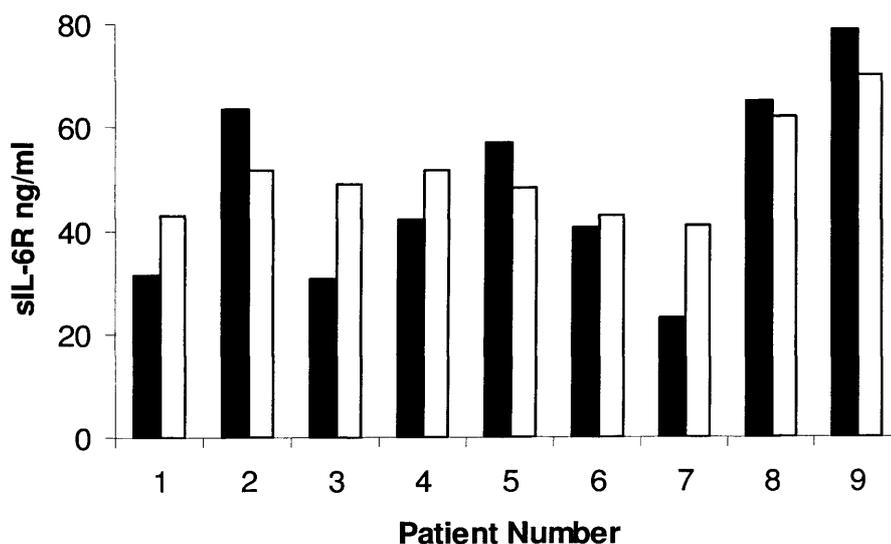
Graph showing mean pre-treatment DAS score  $\pm$  SD and week 14 DAS score. Black bars show pre-treatment score and white bar week 14 score. Patient 6 did not have a fall in DAS greater than or equal to 1.2 (fall from mean DAS = 0.19). Patient 7 had a fall in DAS of 1.13 from mean although a fall of 1.53 from final pre-treatment DAS. Mean fall in DAS score for patient population was  $2.05 \pm 0.92$ .

**Figure 6.2 - IL-6 and sIL-6R levels pre and post anti-TNF $\alpha$  therapy**

**A**



**B**



Graphs showing pre and post treatment serum IL-6 and sIL-6R levels. Black bars show pre-treatment levels and white bar week 14 levels. Mean pre-treatment IL-6 level was  $569 \pm 330$  pg/ml with post treatment levels of  $747 \pm 483$  pg/ml. Mean pre-treatment sIL-6R level was  $48 \pm 6.67$  ng/ml with post treatment levels of  $51 \pm 3.38$  ng/ml.

#### 6.2.4 Correlation Between Response to Therapy and Serum Levels of IL-6 and sIL-6R

Response to therapy was calculated according to NICE/BSR guidelines, i.e. a fall in DAS score of 1.2 or to below 3.2. Patients 6 and 7 were classified as non-responders according to the BSR guidelines. There was no clear pattern between response to therapy and IL-6 or sIL-6R levels pre-therapy. However, the numbers of patients, in particular non-responders, were low (Table 6.2 shows levels in responders compared to non-responders). There was no correlation between type of anti-TNF therapy used and effect on IL-6 or sIL-6R levels although greater numbers of patient samples would need to be examined.

**Table 6.2 - Correlation between response to therapy and serum levels of IL-6 and sIL-6R**

	<b>Responders</b>	<b>Non responders</b>
<b>Pre-treatment sIL-6R (ng)</b>	52.72±18.34	31.82±12.35
<b>Post-treatment sIL-6R</b>	53.69±9.64 NS	41.86±1.24 NS
<b>Pre-treatment IL-6 (pg)</b>	320±438	1440±1963
<b>Post-treatment IL-6</b>	382±670 NS	2025±2835 NS

### 6.3 Discussion

Levels of IL-6 and their variability measured in our patient population were similar to those noted in previous published work. Robak et al noted mean IL-6 levels of  $52.7 \pm 53.2$  pg/ml in their patient population (Robak *et al* 1998). Desgeorges et al noted levels of IL-6 of  $82.7 \pm 71$  pg/ml in their patient population (DesGeorges *et al* 1997). Although the mean level in our study was 569 pg/ml, there was high disease activity in all patients as evidenced by their need for anti-TNF $\alpha$  therapy. The median pre-treatment value in our series was 168 pg/ml and post-treatment 80 pg/ml. The work of Feldmann et al suggests that patients entering trials of Infliximab had elevated levels of IL-6 in the region of 100 pg/ml (Feldmann *et al* 1998).

Trials of anti-TNF therapies suggest a rapid normalisation of IL-6 levels in most patients by day one which appears to last up to 28 days. However, levels past this point have not been reported and only the change in median values were quoted (Charles *et al* 1999). Although there was a drop in IL-6 levels in some patients in our series, this was by no means universal. However, there was a change in median IL-6 values at week 14.

Levels of sIL-6R seen in our patient series also correlate well with those in the published literature: DesGeorges - approx 25 ng/ml; Robak et al -  $49.7 \pm 14.5$  ng/ml (DesGeorges *et al* 1997; Robak *et al* 1998). Mean pre-treatment values in our cohort were  $48 \pm 6.67$  ng/ml with post treatment levels of  $51 \pm 3.38$  ng/ml.

In practice, there is great variability in IL-6 and sIL-6R levels. Perry et al demonstrated circadian variation in serum and SF levels of IL-6 in patients with RA with an overnight variability of approximately 2 fold in serum values (Perry *et al* 2006; Perry *et al* 2008). IL-6 levels, and more recently sIL-6R and sgp130 levels, have also been shown to increase with exercise (Gray *et al* 2008). Sleep strongly enhances sIL-6R levels with an increase of up to 70% with peak levels shortly after morning wakening (Dimitrov *et al* 2006). Therefore, in order to accurately compare IL-6 and sIL-6R levels over time and between patients further information would be useful regarding exercise

and sleep patterns. If possible, samples should be taken at the same time of day for all patients.

Although in this patient population it has not been possible to identify IL-6 or trans-signalling factors which may be used to identify response to anti-TNF $\alpha$  directed therapies; it would be useful to continue this study in a larger patient cohort to identify whether any true differences do exist. In addition, the failure of patients with high IL-6 levels to reduce these with therapy suggests that there may well be a role for IL-6 directed therapies singly or in combination in selected patient cohorts.

## 7 GENERAL DISCUSSION

Rheumatoid arthritis (RA) is a chronic inflammatory disorder characterised by persistent, symmetrical inflammation of synovial tissue. Much is known about the processes which lead to the recruitment of cells into synovium, the maintenance of chronic inflammation and the destruction of bone and cartilage. Chemokines, cytokines and proteolytic enzymes have been identified although their precise contribution to RA pathogenesis is not fully known.

Recent advances in the therapeutic management of rheumatoid arthritis have identified that selective targeting of inflammatory cytokines represents a valid approach to treatment of rheumatoid arthritis. Although blockade of the inflammatory response at its inception by anti-TNF $\alpha$  agents has shown considerable clinical promise, this approach is not without its drawbacks. Consequently, identification of novel therapeutic strategies is essential. In recent clinical trials, favourable results have been found with modalities that block interleukin 6 (IL-6) signalling. However, it is unclear whether blockade of IL-6 bioactivity offers a true advantage over anti-TNF $\alpha$  therapies, and raises the possibility of combination therapies for selected patient cohorts. To provide insight into the validity of this approach we investigated the hypothesis that TNF $\alpha$  and IL-6/sIL-6R interact to modulate leucocyte recruitment in inflammatory arthritis. This hypothesis was addressed in order to provide proof of concept for the possibility that combination TNF $\alpha$  and IL-6 blockade may offer true advantages in selected RA patient cohorts.

In the studies presented in chapter 3, neutrophils were identified as the predominant cell type in RA joint effusions comprising 62% of cells. Macrophages were the next most prevalent cell type and small numbers of lymphocytes were also seen. There was significant reduction in cell surface IL-6R expression on synovial fluid macrophages and neutrophils when compared to cells obtained from peripheral blood. In RA patients there was no significant difference between serum and synovial fluid levels of sIL-6R although mean serum level was higher. Stimulation of neutrophils, obtained

from peripheral blood, with TNF $\alpha$ , resulted in loss of cell surface IL-6R expression and was accompanied by an increase in sIL-6R levels in cell supernatants. sIL-6R production was significantly higher in neutrophils extracted from RA patients when compared to normal healthy volunteers. In summary, the experiments detailed in chapter 3 demonstrate that infiltrating leucocytes within the RA joint are the most likely source of sIL-6R needed for trans-signalling within the joint and that the high levels of TNF $\alpha$  found during RA flares are likely to contribute to the production of sIL-6R.

In chapter 4 we sought to address aim 1 which was to establish the regulatory effects of IL-6/sIL-6R complex on TNF $\alpha$  bio-activity in-vitro. Using RA synovial fibroblasts, experiments were carried out to assess the effects of TNF $\alpha$  and IL-6/sIL-6R on resident cells from the joint and to assess whether these interactions lead to modulation of leucocyte trafficking within the joint.

We demonstrated that TNF $\alpha$  stimulation of RA fibroblasts led to up-regulation of CCL2, CCL5 and CXCL8. IL-6/sIL-6R led to up-regulation of CCL2 production. Interactions between TNF $\alpha$  and IL-6/sIL-6R modulated chemokine production with a synergistic increase in CCL2 production most markedly and, to a lesser extent, CXCL8 production. Effects on CCL5 production were more complex with synergistic up-regulation at low levels of IL-6/sIL-6R and down-regulation of CCL5 production at higher doses.

We also conducted parallel experiments using OA fibroblasts. In these experiments there was no significant difference between mean amounts of chemokine produced by RA and OA fibroblasts. In OA, there was no clear synergistic increase in chemokine production as a result of adding IL-6/sIL-6R to TNF $\alpha$ . This may be partly explained by the large variability seen in chemokine production by OA fibroblasts. In some cell lines there was minimal chemokine production as a result of cytokine stimulation. However, in other cell lines, possibly those where there was a more inflammatory component to disease, the levels of chemokine produced approached and even exceeded those produced by RA cell lines.

Adhesion molecule expression in RA fibroblasts was also studied. Combination of IL-6/sIL-6R with TNF $\alpha$  in RA fibroblasts resulted in greater up-regulation of ICAM-1 expression than TNF $\alpha$  alone. VCAM-1 was not further up-regulated by the addition of IL-6/sIL-6R. This indicated differential regulation of these adhesion molecules in RA synovial fibroblasts by TNF $\alpha$  and IL-6 trans-signalling.

Having identified up-regulation of chemokine and adhesion molecule expression in RA synovial fibroblasts by TNF $\alpha$  and IL-6 trans-signalling, the effect of inhibiting TNF $\alpha$  and IL-6/sIL-6R was then assessed. Treatment of TNF $\alpha$  and IL-6/sIL-6R stimulated RA fibroblasts with etanercept resulted in significant reduction in CCL2 production. Treatment with anti-IL-6R also resulted in a reduction of CCL2 but was not significant. Treatment with a combination of etanercept and anti IL-6R resulted in further additive down-regulation of combined cytokine-induced CCL2 production. In contrast, treatment of stimulated RA-SF with etanercept resulted in almost complete inhibition of CXCL8 production. Treatment with anti IL-6R had no effect on CXCL8 production. Combination of anti IL-6R with etanercept provided no further inhibition. Adhesion molecule up-regulation was completely abolished by etanercept monotherapy.

In order to provide an alternative method for neutralising IL-6 trans-signalling sgp130 was added to cells together with stimulating doses of IL-6/sIL-6R. Unfortunately, no inhibition of chemokine production was seen. However, when sgp130 was added to fibroblasts stimulated with IL-6/sIL-6R STAT signalling was inhibited at 30 minutes but without any downstream effect on chemokine production. It is possible that despite initial reduction in STAT up-regulation, this is quickly overcome due to dynamic un-coupling of IL-6/sIL-6R and sgp130. Over the time-course of the experiments there was sufficient stimulation from IL-6/sIL-6R complex to up-regulate chemokine production.

Aim 1 of the thesis was achieved by the work described in chapters 3 and 4. We identified a source of sIL-6R receptor within the joint and identified that

TNF $\alpha$  and IL-6/sIL-6R had the potential to interact to modulate leucocyte recruitment in RA via effects on chemokine and cell adhesion molecules.

We then tested whether these observations could be translated *in vivo*. Aim 2 of the thesis (to determine the effect of combined TNF $\alpha$  and IL-6 trans-signalling blockade in experimental arthritis) was investigated using an experimental model of RA (murine collagen induced arthritis).

mCIA was used to assess *in vivo* the effects of targeting trans-signalling with sgp130:Fc and to gain some insight into its mechanism of action via effects on macrophages, T cells and cell signalling pathways. By modelling patients with a sub-optimal response to anti-TNF therapy, the effect of addition of sgp130:Fc was studied to identify whether there was added benefit from combination therapy.

A dose of sgp130:Fc (2.5 mg/kg/mouse/day) was identified that exerted significant therapeutic benefit when administered prior to disease onset in mCIA. Following a review of relevant literature, a dose of etanercept was chosen to model sub-optimal response to therapy. This dose, although resulting in greater than 50% reduction in clinical score in mCIA when compared to control animals after 7 days of therapy, was felt to be a suitable dose for further experiments. Using EMSA to assess the effect of therapy on cell signalling pathways demonstrated that NF $\kappa$ B was up-regulated in active disease and was down-regulated by etanercept therapy. NF $\kappa$ B up-regulation was also less in those animals treated with sgp130:Fc who had low clinical scores. This suggests that a reduction in disease activity is the best predictor of NF $\kappa$ B activity. In contrast, STAT activation was less marked in active disease than might have been expected. Further review of the literature suggested that STAT activation is most marked in the 10 days after disease onset and that therefore disease endpoint (day 35) is probably not a good time point at which to examine STAT activity (Shouda *et al* 2001; Sharara *et al* 2003).

When trans-signalling was targeted with sgp130:Fc in established mCIA; sgp130:Fc monotherapy was effective in improving clinical disease severity

in mCIA. There was no improvement in overall disease histology. There was a significant difference in macrophage infiltration in joints of animals treated with sgp130:Fc. The effect of therapy on regulatory T cells was assessed by staining for Fox-P3. Although Fox-P3 positive cells could be identified, the numbers of cells present were low. In this small sample there was no significant difference seen in Fox-P3 expression between treatment groups although there appeared to be a trend towards an increase in the sgp130:Fc therapy groups.

Addition of sgp130:Fc to sub-optimal etanercept therapy resulted in a significant change in clinical score when compared to continuing on etanercept monotherapy. Combination therapy did not appear to be better than sgp130:Fc. However, the timeframe for combination therapy was only 5 days and only small numbers of animals were assessed. There was no improvement in disease histology as a result of any therapy other than etanercept monotherapy. There was a significant difference in macrophage infiltration in joints of animals treated with sgp130:Fc in combination with etanercept.

A further experiment was carried out with an increased dose of sgp130:Fc which we anticipated would further improve disease outcome. Unfortunately, when this higher dose (5 mg/kg) was used, sgp130:Fc monotherapy (5 mg/kg) was not effective in improving clinical disease severity in mCIA. Addition of sgp130:Fc to sub-optimal etanercept therapy did not result in a significant change in clinical score when compared to continuing on etanercept monotherapy. When histological parameters were compared, targeting trans-signalling had no effect on pathology. Combination therapy did reduce histological damage although arthritis was still present. There was a reduction in the degree of synovial hyperplasia together with a reduction in leucocyte infiltrate and bone and cartilage damage.

A final experiment was carried out to mimic intervention in early arthritis which we predicted would provide the best chance of preventing pathological damage. Targeting trans-signalling alone had no effect on

clinical or histological disease severity. There was a trend towards clinical improvement as a result of combination therapy with sgp130:Fc and etanercept compared to etanercept alone. Although this was not statistically significant, it is likely that if greater animal numbers were used there would be a significant difference. Etanercept and sgp130:Fc in combination were significantly better than sgp130:Fc monotherapy. There was also significant improvement in disease pathology, as shown by improvement in all parameters of histological assessment, when combination therapy was compared to PBS control. Thus, aim 2 of the thesis was achieved.

Having demonstrated that functions of TNF $\alpha$  and IL-6 trans-signalling within the joint are closely linked, we speculated that response to therapy with TNF $\alpha$  antagonists may also be affected by IL6 trans-signalling in RA. This led to the development of aim 3 of the thesis, which was to examine the effect of anti-TNF $\alpha$  treatment on IL-6 trans-signalling in RA patients.

In chapter 6, experiments were described in which all patients commencing anti-TNF $\alpha$  therapy from July 2006 at UHW were invited to take part. Blood samples were collected at baseline and at 14 week assessment. DAS scores were calculated prior to treatment on 2 separate occasions 1 month apart and again at 14 weeks in accordance with BSR guidelines.

A pilot study assessing small numbers of patients found that IL-6 was detectable in the serum of all the patients bar one prior to treatment with anti-TNF $\alpha$ . Following anti-TNF $\alpha$  treatment, IL-6 was not detectable in the sera of three patients. Levels of IL-6 fell in five patients; remained undetectable in one patient and rose in three. This did not correlate with response to therapy. However, the patient with highest initial level of IL-6 (2828 pg/ml) also had the poorest response to therapy and the largest rise in levels following therapy (to 4030 pg/ml). sIL-6R was detectable in the sera of all the patients pre and post treatment. Levels following anti-TNF $\alpha$  therapy rose in five patients and fell in four patients. There were too few patients to identify whether sIL-6R levels were associated with response to therapy. However, levels did appear lower in non-responders.

In practice, there is great variability in human IL-6 and sIL-6R levels. Perry *et al* demonstrated circadian variation in serum and SF levels of IL-6 in patients with RA with an overnight variability of approximately two fold in serum values (Perry *et al* 2006; Perry *et al* 2008). IL-6 levels and more recently sIL-6R and sgp130 levels have also been shown to increase with exercise (Gray *et al* 2008). Sleep strongly enhances sIL-6R levels with an increase of up to 70% with peak levels shortly after morning waking (Dimitrov *et al* 2006). Therefore, in order to accurately compare IL-6 and sIL-6R levels over time and between patients further information would be useful regarding exercise and sleep patterns. If possible, samples should be taken at the same time of day for all patients. Future work could be done using questionnaires providing more detailed information from patients including information on sleep patterns, exercise and other therapies taken for conditions other than RA.

The work described in chapter 6 to address aim 3 of the thesis showed that further work is needed to identify whether IL-6 trans-signalling is implicated in the response to anti-TNF $\alpha$  therapies.

In conclusion, achievement of aims 1, 2 and 3 of this thesis provides proof of concept that combination TNF $\alpha$  and IL-6 blockade may offer true advantages in selected RA patient cohorts.

## 8 FUTURE DIRECTIONS FOR RESEARCH

Other resident cells within the joint are important in RA pathogenesis apart from fibroblasts and leucocytes. It would be interesting to assess the effect of combined TNF $\alpha$  and trans-signalling on other resident cells within the joint specifically chondrocytes. IL-1 and IL-6/sIL-6R complex interact to up-regulate metalloproteinase production by chondrocytes but the effect on production of other mediators responsible for bone and cartilage pathology is as yet unstudied (Rowan *et al* 2001; Flannery *et al* 2000).

In order to identify fully the mechanism by which sgp130:Fc exerts its actions, cell signalling at earlier time points in disease, i.e. disease induction and very early arthritis must be studied. Assessment of the effect of therapy on regulatory T cell function would also be very useful.

Recent reports have identified interactions between IL-6 trans-signalling and TGF $\beta$  in the induction of regulatory T cells (Dominitzki *et al* 2007). It would be interesting to identify whether further interactions between TGF $\beta$  and IL-6 trans-signalling may occur. This would have relevance for diseases in which TGF $\beta$  appears prominent in pathogenesis, i.e. scleroderma where IL-6 levels have also been shown to be elevated (Scala *et al* 2004). sIL-6R and gp130 have also been shown to be elevated in those patients with limited cutaneous scleroderma (Nagaoka *et al* 2000).

Despite the promising results obtained with combination therapy, our results were not statistically significant. It would be useful to repeat the early intervention study with an increased number of animals to determine whether combination therapy offers a significant advantage over etanercept monotherapy. In addition, I would like to repeat the experiments with a higher (therapeutic) dose of etanercept to identify if there is advantage in combination therapy with what is assumed to be a full therapeutic dose of etanercept. Obviously, the eventual aim of this work would be to test sgp130:Fc as a therapeutic in RA. More work would be needed in terms of studies of toxicity, increased animal numbers and testing in healthy

volunteers before this could take place. However, this thesis provides proof of concept that combination TNF $\alpha$  and IL-6 blockade may offer true advantages in selected RA patient cohorts.

## 9 REFERENCES

Aderka D. The potential biological and clinical significance of the soluble tumor necrosis factor receptors. *Cytokine Growth Factor Rev.* 1996 Oct;7(3):231-40. Review.

Akahoshi T, Wada C, Endo H, Hirota K, Hosaka S, Takagishi K, Kondo H, Kashiwazaki S, Matsushima K. Expression of monocyte chemotactic and activating factor in rheumatoid arthritis. Regulation of its production in synovial cells by interleukin-1 and tumor necrosis factor. *Arthritis Rheum.* 1993 Jun;36(6):762-71.

Alonzi T, Fattori E, Lazzaro D, Costa P, Probert L, Kollias G, De Benedetti F, Poli V, Ciliberto G. Interleukin 6 is required for the development of collagen-induced arthritis. *J Exp Med.* 1998 Feb 16;187(4):461-8.

Alonzi T, Fattori E, Cappelletti M, Ciliberto G, Poli V. Impaired Stat3 activation following localized inflammatory stimulus in IL-6-deficient mice. *Cytokine.* 1998 Jan;10(1):13-8.

Andrén M, Xiang Z, Nilsson G, Kleinau S. FcγRIII-expressing macrophages are essential for development of collagen-induced arthritis. *Scand J Immunol.* 2006 Apr;63(4):282-9.

Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, Healey LA, Kaplan SR, Liang MH, Luthra HS, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum.* 1988 Mar;31(3):315-24.

Atreya R, Mudter J, Finotto S, Müllberg J, Jostock T, Wirtz S, Schütz M, Bartsch B, Holtmann M, Becker C, Strand D, Czaja J, Schlaak JF, Lehr HA, Autschbach F, Schürmann G, Nishimoto N, Yoshizaki K, Ito H, Kishimoto T, Galle PR, Rose-John S, Neurath MF. Blockade of interleukin 6 trans signaling suppresses T-cell resistance against apoptosis in chronic intestinal inflammation: evidence in crohn disease and experimental colitis in vivo. *Nat Med.* 2000 May;6(5):583-8.

Bahr GM, Rook GA, al-Saffar M, Van Embden J, Stanford JL, Behbehani K. Antibody levels to mycobacteria in relation to HLA type: evidence for non-HLA-linked high levels of antibody to the 65 kD heat shock protein of *M. bovis* in rheumatoid arthritis. *Clin Exp Immunol.* 1988 Nov;74(2):211-5.

Barnes DA, Tse J, Kaufhold M, Owen M, Hesselgesser J, Strieter R, Horuk R, Perez HD. Polyclonal antibody directed against human RANTES ameliorates disease in the Lewis rat adjuvant-induced arthritis model. *J Clin. Invest.* 1998 Jun;101(12):1910-1919.

Bazzoni F, Beutler B. The tumor necrosis factor ligand and receptor families. *N Engl J Med.* 1996 Jun 27;334(26):1717-25.

Bellamy N, Duffy D, Martin N, Mathews J. Rheumatoid arthritis in twins: a study of aetiopathogenesis based on the Australian Twin Registry. *Ann Rheum Dis.* 1992 May;51(5):588-93.

Beutler B and Cerami A. The biology of cachectin/TNF- $\alpha$  primary mediator of the host response. *Annu Rev Immunol.* 1989;7:625-55.

Beutler B, Cerami A. The history, properties, and biological effects of cachectin. *Biochemistry.* 1988 Oct 4;27(20):7575-82.

Billingham MEJ 1995 Adjuvant arthritis the first model -in (Henderson B, Edwards JCW, Pettipher ER) Mechanisms and models in Rheumatoid Arthritis. Academic press, Harcourt Brace & company,389-411.

Billingham ME, Carney S, Butler R, Colston MJ. A mycobacterial 65-kD heat shock protein induces antigen-specific suppression of adjuvant arthritis, but is not itself arthritogenic. *J Exp Med.* 1990 Jan 1;171(1):339-44.

Bird K, O'Dell J 2002. Systemic anti-rheumatic drugs- in (West S) Rheumatology secrets. Hanley and Belfus, 579-587.

Boe A, Baiocchi M, Carbonatto M, Papoian R, Serlupi-Crescenzi O. Interleukin 6 knock-out mice are resistant to antigen-induced experimental arthritis. *Cytokine* 1999;11:1057-64.

Bombara MP, Webb DL, Conrad P, Marlor CW, Sarr T, Ranges GE, Aune TM, Greve JM, Blue ML. Cell contact between T cells and synovial fibroblasts causes induction of adhesion molecules and cytokines. *J Leukoc Biol.* 1993 Nov;54(5):399-406.

Bondeson J, Foxwell B, Brennan F, Feldmann M. Defining therapeutic targets by using adenovirus: blocking NF-kappaB inhibits both inflammatory and destructive mechanisms in rheumatoid synovium but spares anti-inflammatory mediators. *Proc Natl Acad Sci U S A.* 1999 May 11;96(10):5668-73.

Bongartz T, Sutton AJ, Sweeting MJ, Buchan I, Matteson EL, Montori V. Anti-TNF antibody therapy in rheumatoid arthritis and the risk of serious infections and malignancies: systematic review and meta-analysis of rare harmful effects in randomized controlled trials. *JAMA.* 2006 May 17;295(19):2275-85. Review.

Braun-Moscovici Y, Markovits D, Zinder O, Schapira D, Rozin A, Ehrenburg M, Dain L, Hoffer E, Nahir AM, Balbir-Gurman A. Anti-cyclic citrullinated protein antibodies as a predictor of response to anti-tumor necrosis factor-alpha therapy in patients with rheumatoid arthritis. *J Rheumatol.* 2006 Mar;33(3):497-500.

Breedveld FC, Weisman MH, Kavanaugh AF, Cohen SB, Pavelka K, van Vollenhoven R, Sharp J, Perez JL, Spencer-Green GT. The PREMIER study: A multicenter, randomized, double-blind clinical trial of combination therapy with adalimumab plus methotrexate versus methotrexate alone or adalimumab alone in patients with early, aggressive rheumatoid arthritis who had not had previous methotrexate treatment. *Arthritis Rheum.* 2006 Jan;54(1):26-37.

Brennan FM, Chantry D, Jackson AM, Maini RN, Feldmann M. Cytokine production in culture by cells isolated from the synovial membrane. *J Autoimmun.* 1989 Jun;2 Suppl:177-86 a)

Brennan FM, Chantry D, Jackson A, Maini R, Feldmann M. Inhibitory effect of TNF alpha antibodies on synovial cell interleukin-1 production in rheumatoid arthritis. *Lancet*. 1989 Jul 29;2(8657):244-7. b)

Bresnihan B, Alvaro-Gracia JM, Cobby M, Doherty M, Domljan Z, Emery P, Nuki G, Pavelka K, Rau R, Rozman B, Watt I, Williams B, Aitchison R, McCabe D, Musikic P. Treatment of rheumatoid arthritis with recombinant human interleukin-1 receptor antagonist. *Arthritis Rheum*. 1998 Dec;41(12):2196-204.

Brodmerkel CM, Huber R, Covington M, Diamond S, Hall L, Collins R, Leffet L, Gallagher K, Feldman P, Collier P, Stow M, Gu X, Baribaud F, Shin N, Thomas B, Burn T, Hollis G, Yeleswaram S, Solomon K, Friedman S, Wang A, Xue CB, Newton RC, Scherle P, Vaddi K. Discovery and pharmacological characterization of a novel rodent-active CCR2 antagonist, INCB3344. *J Immunol*. 2005 Oct 15;175(8):5370-8.

Brownlie RJ, Myers LK, Wooley PH, Corrigan VM, Bodman-Smith MD, Panayi GS, Thompson SJ. Treatment of murine collagen-induced arthritis by the stress protein BiP via interleukin-4-producing regulatory T cells: a novel function for an ancient protein. *Arthritis Rheum*. 2006 Mar;54(3):854-63.

Buchan G, Barrett K, Turner M, Chantry D, Maini RN, Feldmann M. Interleukin-1 and tumour necrosis factor mRNA expression in rheumatoid arthritis: prolonged production of IL-1 alpha. *Clin Exp Immunol*. 1988 Sep;73(3):449-55.

Buckley CD 2003 Michael Mason prize essay 2003. Why do leucocytes accumulate within chronically inflamed joints? *Rheumatology (Oxford)*. 2003 Dec;42(12):1433-44. a)

Buckley CD. Why does chronic inflammatory joint disease persist? *Clin Med*. 2003 Jul-Aug;3(4):361-6. b)

Bucala R, Ritchlin C, Winchester R, Cerami A. Constitutive production of inflammatory and mitogenic cytokines by rheumatoid synovial fibroblasts. *J Exp Med*. 1991 Mar 1;173(3):569-74.

Bullard DC, Hurley LA, Lorenzo I, Sly LM, Beaudet AL, Staite ND. Reduced susceptibility to collagen-induced arthritis in mice deficient in intercellular adhesion molecule-1. *J Immunol*. 1996 Oct 1;157(7):3153-8.

Campbell IK, Hamilton JA, Wicks IP. Collagen-induced arthritis in C57BL/6 (H-2b) mice: new insights into an important disease model of rheumatoid arthritis. *Eur J Immunol*. 2000 Jun;30(6):1568-75.

Cao D, Malmström V, Baecher-Allan C, Hafler D, Klareskog L, Trollmo C. Isolation and functional characterization of regulatory CD25<sup>bright</sup>CD4<sup>+</sup> T cells from the target organ of patients with rheumatoid arthritis. *Eur J Immunol*. 2003 Jan;33(1):215-23

Carter RA, Campbell IK, O'Donnel KL, Wicks IP. Vascular cell adhesion molecule-1 (VCAM-1) blockade in collagen-induced arthritis reduces joint involvement and alters B cell trafficking. *Clin Exp Immunol*. 2002 Apr;128(1):44-51

Carty SM, Snowden N, Silman AJ. Should infection still be considered as the most likely triggering factor for rheumatoid arthritis? *J Rheumatol*. 2003 Mar;30(3):425-9

Caulfield JP, Hein A, Dynesius-Trentham R, Trentham DE. Morphologic demonstration of two stages in the development of type II collagen-induced arthritis. *Lab Invest*. 1982 Mar;46(3):321-43

Chalaris A, Rabe B, Paliga K, Lange H, Laskay T, Fielding CA, Jones SA, Rose-John S, Scheller J. Apoptosis is a natural stimulus of IL6R shedding and contributes to the proinflammatory trans-signalling function of neutrophils. *Blood*. 2007 September;110(6):1747-1755

Charles P, Elliott MJ, Davis D, Potter A, Kalden JR, Antoni C, Breedveld FC, Smolen JS, Eberl G, deWoody K, Feldmann M, Maini RN. Regulation of cytokines, cytokine inhibitors, and acute-phase proteins following anti-TNF-alpha therapy in rheumatoid arthritis. *J Immunol.* 1999 Aug 1;163(3):1521-8.

Cherwinski HM, Cohn RG, Cheung P, Webster DJ, Xu YZ, Caulfield JP, Young JM, Nakano G, Ransom JT. The immunosuppressant leflunomide inhibits lymphocyte proliferation by inhibiting pyrimidine biosynthesis. *J Pharmacol Exp Ther.* 1995 Nov;275(2):1043-9

Choy EH, Isenberg DA, Garrood T, Farrow S, Ioannou Y, Bird H, Cheung N, Williams B, Hazleman B, Price R, Yoshizaki K, Nishimoto N, Kishimoto T, Panayi GS. Therapeutic benefit of blocking interleukin-6 activity with an anti-interleukin-6 receptor monoclonal antibody in rheumatoid arthritis: a randomized, double-blind, placebo-controlled, dose-escalation trial. *Arthritis Rheum.* 2002 Dec;46(12):3143-50.

Cohen S, Hurd E, Cush J, Schiff M, Weinblatt ME, Moreland LW, Kremer J, Bear MB, Rich WJ, McCabe D. Treatment of rheumatoid arthritis with anakinra, a recombinant human interleukin-1 receptor antagonist, in combination with methotrexate: results of a twenty-four-week, multicenter, randomized, double-blind, placebo-controlled trial. *Arthritis Rheum.* 2002 Mar;46(3):614-24.

Cohen SB, Emery P, Greenwald MW, Dougados M, Furie RA, Genovese MC, Keystone EC, Loveless JE, Burmester GR, Cravets MW, Hessey EW, Shaw T, Totoritis MC; REFLEX Trial Group. Rituximab for rheumatoid arthritis refractory to anti-tumor necrosis factor therapy: Results of a multicenter, randomized, double-blind, placebo-controlled, phase III trial evaluating primary efficacy and safety at twenty-four weeks. *Arthritis Rheum.* 2006 Sep;54(9):2793-806.

Comer SS, Jasin HE. In vitro immunomodulatory effects of sulfasalazine and its metabolites. *J Rheumatol.* 1988 Apr;15(4):580-6.

Cromartie WJ, Craddock JG, Schwab JH, Anderle SK, Yang CH. Arthritis in rats after systemic injection of streptococcal cells or cell walls. *J Exp Med.* 1977 Dec 1;146(6):1585-602.

Cronstein BN, Eberle MA, Gruber HE, Levin RI. Methotrexate inhibits neutrophil function by stimulating adenosine release from connective tissue cells. *Proc Natl Acad Sci U S A.* 1991 Mar 15;88(6):2441-5.

Cronstein BN, Naime D, Ostad E. The antiinflammatory mechanism of methotrexate. Increased adenosine release at inflamed sites diminishes leukocyte accumulation in an in vivo model of inflammation. *J Clin Invest.* 1993 Dec;92(6):2675-82.

Cross A, Edwards SW, Bucknall RC, Moots RJ. Secretion of oncostatin M by neutrophils in rheumatoid arthritis. *Arthritis Rheum.* 2004 May;50(5):1430-6.

Cuchacovich M, Ferreira L, Aliste M, Soto L, Cuenca J, Cruzat A, Gatica H, Schiattino I, Pérez C, Aguirre A, Salazar-Onfray F, Aguilón JC. Tumour necrosis factor-alpha (TNF-alpha) levels and influence of -308 TNF-alpha promoter polymorphism on the responsiveness to infliximab in patients with rheumatoid arthritis. *Scand J Rheumatol.* 2004;33(4):228-32.

Cutolo M, Sulli A, Pizzorni C, Seriola B, Straub RH. Anti-inflammatory mechanisms of methotrexate in rheumatoid arthritis. *Ann Rheum Dis.* 2001 Aug;60(8):729-35.

Davis MJ, Denton J, Freemont AJ, Holt PJ. Comparison of serial synovial fluid cytology in rheumatoid arthritis: delineation of subgroups with prognostic implications. *Ann Rheum Dis.* 1988 Jul;47(7):559-62.

Deon D, Ahmed S, Tai K, Scaletta N, Herrero C, Lee IH, Krause A, Ivashkiv LB. Cross-talk between IL-1 and IL-6 signaling pathways in rheumatoid arthritis synovial fibroblasts. *J Immunol.* 2001 Nov 1;167(9):5395-403.

Dequeker J. Arthritis in Flemish paintings (1400-1700). *Br Med J.* 1977 May 7;1(6070):1203-5.

Desgeorges A, Gabay C, Silacci P, Novick D, Roux-Lombard P, Grau G, Dayer JM, Vischer T, Guerne PA. Concentrations and origins of soluble interleukin 6 receptor-alpha in serum and synovial fluid. *J Rheumatol*. 1997 Aug;24(8):1510-6.

Di Giovine FS, Nuki G, Duff GW. Tumour necrosis factor in synovial exudates. *Ann Rheum Dis*. 1988 Sep;47(9):768-72.

Dimitrov S, Lange T, Benedict C, Nowell MA, Jones SA, Scheller J, Rose-John S, Born J. Sleep enhances IL-6 trans-signaling in humans. *FASEB J*. 2006 Oct;20(12):2174-6.

Dixon WG, Watson K, Lunt M, Hyrich KL, Silman AJ, Symmons DP; British Society for Rheumatology Biologics Register. Rates of serious infection, including site-specific and bacterial intracellular infection, in rheumatoid arthritis patients receiving anti-tumor necrosis factor therapy: results from the British Society for Rheumatology Biologics Register. *Arthritis Rheum*. 2006 Aug;54(8):2368-76.

Doganci A, Eigenbrod T, Krug N, De Sanctis GT, Hausding M, Erpenbeck VJ, Haddad el-B, Lehr HA, Schmitt E, Bopp T, Kallen KJ, Herz U, Schmitt S, Luft C, Hecht O, Hohlfeld JM, Ito H, Nishimoto N, Yoshizaki K, Kishimoto T, Rose-John S, Renz H, Neurath MF, Galle PR, Finotto S. The IL-6R alpha chain controls lung CD4+CD25+ Treg development and function during allergic airway inflammation in vivo. *J Clin Invest*. 2005 Feb;115(2):313-25.

Dombrecht EJ, Aerts NE, Schuerwegh AJ, Hagendorens MM, Ebo DG, Van Offel JF, Bridts CH, Stevens WJ, De Clerck LS. Influence of anti-tumor necrosis factor therapy (Adalimumab) on regulatory T cells and dendritic cells in rheumatoid arthritis. *Clin Exp Rheumatol*. 2006 Jan-Feb;24(1):31-7.

Dominitzki S, Fantini MC, Neufert C, Nikolaev A, Galle PR, Scheller J, Monteleone G, Rose-John S, Neurath MF, Becker C. Cutting edge: trans-signaling via the soluble IL-6R abrogates the induction of FoxP3 in naive CD4+CD25 T cells. *J Immunol*. 2007 Aug 15;179(4):2041-5.

Doran MF, Pond GR, Crowson CS, O'Fallon WM, Gabriel SE. Trends in incidence and mortality in rheumatoid arthritis in Rochester, Minnesota, over a forty-year period. *Arthritis Rheum.* 2002 Mar;46(3):625-31.

Dumonde DC and Glynn LE. The production of arthritis in rabbits by an immunological reaction to fibrin. *Br J Exp Pathol.* 1962 Aug;43:373-83.

Edwards SW and Hallett MB. Seeing the wood for the trees: the forgotten role of neutrophils in rheumatoid arthritis. *Immunol Today.* 1997 Jul;18(7):320-4.

Egeland T, Lund H. Immunoregulatory lymphokines in rheumatoid joints. I. Search for interleukin 2 in synovial fluid. *Scand J Immunol.* 1987 Jan;25(1):101-6.

Ehrenstein MR, Evans JG, Singh A, Moore S, Warnes G, Isenberg DA, Mauri C. Compromised function of regulatory T cells in rheumatoid arthritis and reversal by anti-TNFalpha therapy. *J Exp Med.* 2004 Aug 2;200(3):277-85.

Elliott MJ, Maini RN, Feldmann M, Long-Fox A, Charles P, Katsikis P, Brennan FM, Walker J, Bijl H, Ghayeb J. Treatment of rheumatoid arthritis with chimeric monoclonal antibodies to tumor necrosis factor alpha. *Arthritis Rheum.* 1993 Dec;36(12):1681-90

Endo H, Akahoshi T, Takagishi K, Kashiwazaki S, Matsushima K. Elevation of interleukin-8 (IL-8) levels in joint fluids of patients with rheumatoid arthritis and the induction by IL-8 of leukocyte infiltration and synovitis in rabbit joints. *Lymphokine Cytokine Res.* 1991 Aug;10(4):245-52.

Erb N, Pace AV, Douglas KM, Banks MJ, Kitis GD. Risk assessment for coronary heart disease in rheumatoid arthritis and osteoarthritis. *Scand J Rheumatol.* 2004;33(5):293-9.

Esser RE, Hildebrand AR, Angelo RA, Watts LM, Murphey MD, Baugh LE. Measurement of radiographic changes in adjuvant-induced arthritis in rats by quantitative image analysis. *Arthritis Rheum.* 1995 Jan;38(1):129-38.

Farr M, Brodrick A, Bacon PA. Plasma and synovial fluid concentrations of sulphasalazine and two of its metabolites in rheumatoid arthritis. *Rheumatol Int.* 1985;5(6):247-51.

Feldmann M, Andreakos E, Smith C, Bondeson J, Yoshimura S, Kiriakidis S, Monaco C, Gasparini C, Sacre S, Lundberg A, Paleolog E, Horwood NJ, Brennan FM, Foxwell BM. Is NF-kappaB a useful therapeutic target in rheumatoid arthritis? *Ann Rheum Dis.* 2002 Nov;61 Suppl 2:ii13-8. Review.

Feldmann M, Maini RN, Bondeson J, Taylor P, Foxwell BM, Brennan FM. Cytokine blockade in rheumatoid arthritis. *Adv Exp Med Biol.* 2001;490:119-27. Review.

Feldmann M, Maini RN. The role of cytokines in the pathogenesis of rheumatoid arthritis. *Rheumatology (Oxford).* 1999 Nov;38 Suppl 2:3-7. Review.

Feldmann M, Charles P, Taylor P, Maini RN. Biological insights from clinical trials with anti-TNF therapy. *Springer Semin Immunopathol.* 1998;20(1-2):211-28.

Fex E, Larsson BM, Nived K, Eberhardt K. Effect of rheumatoid arthritis on work status and social and leisure time activities in patients followed 8 years from onset. *J Rheumatol.* 1998 Jan;25(1):44-50.

Filer A, Parsonage G, Smith E, Osborne C, Thomas AM, Curnow SJ, Rainger GE, Raza K, Nash GB, Lord J, Salmon M, Buckley CD. Differential survival of leukocyte subsets mediated by synovial, bone marrow, and skin fibroblasts: site-specific versus activation-dependent survival of T cells and neutrophils. *Arthritis Rheum.* 2006 Jul;54(7):2096-108.

Fiorito S, Magrini L, Adrey J, Mailhé D, Brouty-Boyé D. Inflammatory status and cartilage regenerative potential of synovial fibroblasts from patients with osteoarthritis and chondropathy. *Rheumatology (Oxford).* 2005 Feb;44(2):164-71.

Firestein GS, Zvaifler NJ. How important are T cells in chronic rheumatoid synovitis? *Arthritis Rheum.* 1990 Jun;33(6):768-73. Review.

Firestein GS, Xu WD, Townsend K, Broide D, Alvaro-Gracia J, Glasebrook A, Zvaifler NJ. Cytokines in chronic inflammatory arthritis. I. Failure to detect T cell lymphokines (interleukin 2 and interleukin 3) and presence of macrophage colony-stimulating factor (CSF-1) and a novel mast cell growth factor in rheumatoid synovitis. *J Exp Med.* 1988 Nov 1;168(5):1573-86.

Flannery CR, Little CB, Hughes CE, Curtis CL, Caterson B, Jones SA. IL-6 and its soluble receptor augment aggrecanase-mediated proteoglycan catabolism in articular cartilage. *Matrix Biol.* 2000 Nov;19(6):549-53.

Fox DA. The role of T cells in the immunopathogenesis of rheumatoid arthritis: new perspectives. *Arthritis Rheum.* 1997 Apr;40(4):598-609. Review.

Freemont AJ, Denton J. Disease distribution of synovial fluid mast cells and cytophagocytic mononuclear cells in inflammatory arthritis. *Ann Rheum Dis.* 1985 May;44(5):312-5.

Freemont AJ, Denton J, Chuck A, Holt PJ, Davies M. Diagnostic value of synovial fluid microscopy: a reassessment and rationalisation. *Ann Rheum Dis.* 1991 Feb;50(2):101-7.

Freemont AJ 1995. Histopathology of the rheumatoid joint-in (Henderson B, Edwards JCW, Pettipher ER) Mechanisms and models in Rheumatoid Arthritis. Academic press, Harcourt Brace & company, 83-115.

Garrod AB. The great practical importance of separating rheumatoid arthritis from gout. *Lancet.* 1892;2:1033-1037.

Geborek P, Truedsson L, Sandberg M, Wollheim FA. Hyaluronidase treatment of synovial fluid affects the recovery of mononuclear cells and their in vitro immunoglobulin synthesis. *Clin Exp Rheumatol.* 1987 Jul-Sep;5(3):255-7.

Genovese MC, Cohen S, Moreland L, Lium D, Robbins S, Newmark R, Bekker P. Combination therapy with etanercept and anakinra in the treatment of patients with rheumatoid arthritis who have been treated unsuccessfully with methotrexate. *Arthritis Rheum.* 2004 May;50(5):1412-9.

Genovese MC, McKay JD, Nasonov EL, Mysler EF, da Silva NA, Alecock E, Woodworth T, Gomez-Reino JJ. Interleukin-6 receptor inhibition with tocilizumab reduces disease activity in rheumatoid arthritis with inadequate response to disease-modifying antirheumatic drugs: the tocilizumab in combination with traditional disease-modifying antirheumatic drug therapy study. *Arthritis Rheum.* 2008 Oct;58(10):2968-80.

Gerlag DM, Ransone L, Tak PP, Han Z, Palanki M, Barbosa MS, Boyle D, Manning AM, Firestein GS. The effect of a T cell-specific NF-kappa B inhibitor on in vitro cytokine production and collagen-induced arthritis. *J Immunol.* 2000 Aug 1;165(3):1652-8.

Goeddel DV, Aggarwal BB, Gray PW, Leung DW, Nedwin GE, Palladino MA, Patton JS, Pennica D, Shepard HM, Sugarman BJ. Tumor necrosis factors: gene structure and biological activities. *Cold Spring Harb Symp Quant Biol.* 1986;51 Pt 1:597-609.

Goekoop-Ruiterman YP, de Vries-Bouwstra JK, Allaart CF, van Zeben D, Kerstens PJ, Hazes JM, Zwinderman AH, Ronday HK, Han KH, Westedt ML, Gerards AH, van Groenendael JH, Lems WF, van Krugten MV, Breedveld FC, Dijkmans BA. Clinical and radiographic outcomes of four different treatment strategies in patients with early rheumatoid arthritis (the BeSt study): a randomized, controlled trial. *Arthritis Rheum.* 2005 Nov;52(11):3381-90

Gong JH, Ratkay LG, Waterfield JD, Clark-Lewis I. An antagonist of monocyte chemoattractant protein 1 (MCP-1) inhibits arthritis in the MRL-lpr mouse model. *J Exp Med.* 1997 Jul 7;186(1):131-7.

Gong JH, Yan R, Waterfield JD, Clark-Lewis I. Post-onset inhibition of murine arthritis using combined chemokine antagonist therapy. *Rheumatology (Oxford)*. 2004 Jan;43(1):39-42.

Gonzalez-Rey E, Chorny A, Varela N, O'Valle F, Delgado M. Therapeutic effect of urocortin on collagen-induced arthritis by down-regulation of inflammatory and Th1 responses and induction of regulatory T cells. *Arthritis Rheum*. 2007 Feb;56(2):531-43.

Gonzalez-Rey E, Chorny A, O'Valle F, Delgado M. Adrenomedullin protects from experimental arthritis by down-regulating inflammation and Th1 response and inducing regulatory T cells. *Am J Pathol*. 2007 Jan;170(1):263-71

Gray SR, Robinson M, Nimmo MA. Response of plasma IL-6 and its soluble receptors during submaximal exercise to fatigue in sedentary middle-aged men. *Cell Stress Chaperones*. 2008 Mar 5; [Epub ahead of print]

Green MJ, Gough AK, Devlin J, Smith J, Astin P, Taylor D, Emery P. Serum MMP-3 and MMP-1 and progression of joint damage in early rheumatoid arthritis. *Rheumatology (Oxford)*. 2003 Jan;42(1):83-8.

Gregersen PK. Genetics of rheumatoid arthritis: confronting complexity. *Arthritis Res*. 1999;1(1):37-44.

Grunfeld C, Palladino MA Jr. Tumor necrosis factor: immunologic, antitumor, metabolic, and cardiovascular activities. *Adv Intern Med*. 1990;35:45-71.

Harada S, Yamamura M, Okamoto H, Morita Y, Kawashima M, Aita T, Makino H. Production of interleukin-7 and interleukin-15 by fibroblast-like synoviocytes from patients with rheumatoid arthritis. *Arthritis Rheum*. 1999 Jul;42(7):1508-16.

Harris CL, Williams AS, Linton SM, Morgan BP. Coupling complement regulators to immunoglobulin domains generates effective anti-complement reagents with extended half-life in vivo. *Clin Exp Immunol.* 2002 Aug;129(2):198-207.

Hata H, Sakaguchi N, Yoshitomi H, Iwakura Y, Sekikawa K, Azuma Y, Kanai C, Moriizumi E, Nomura T, Nakamura T, Sakaguchi S. Distinct contribution of IL-6, TNF-alpha, IL-1, and IL-10 to T cell-mediated spontaneous autoimmune arthritis in mice. *J Clin Invest.* 2004 Aug;114(4):582-8.

Heiberg MS, Rødevand E, Mikkelsen K, Kaufmann C, Didriksen A, Mowinckel P, Kvien TK. Adalimumab and methotrexate is more effective than adalimumab alone in patients with established rheumatoid arthritis: results from a 6-month longitudinal, observational, multicentre study. *Ann Rheum Dis.* 2006 Oct;65(10):1379-83.

Herbein G, Mahlknecht U, Batliwalla F, Gregersen P, Pappas T, Butler J, O'Brien WA, Verdin E. Apoptosis of CD8+ T cells is mediated by macrophages through interaction of HIV gp120 with chemokine receptor CXCR4. *Nature.* 1998 Sep 10;395(6698):189-94.

Hückel M, Schurigt U, Wagner AH, Stöckigt R, Petrow PK, Thoss K, Gajda M, Henzgen S, Hecker M, Bräuer R. Attenuation of murine antigen-induced arthritis by treatment with a decoy oligodeoxynucleotide inhibiting signal transducer and activator of transcription-1 (STAT-1). *Arthritis Res Ther.* 2006;8(1):R17.

Hollander JL, McCarty DJ Jr, Rawson AJ. The "R.A. cell", "ragocyte", or "inclusion body cell". *Bull Rheum Dis.* 1965 Sep;16(1):382-3.

Holmdahl R, Jansson L, Larsson E, Rubin K, Klareskog L. Homologous type II collagen induces chronic and progressive arthritis in mice. *Arthritis Rheum.* 1986 Jan;29(1):106-13.

Holmdahl R, Tarkowski A, Jonsson R. Involvement of macrophages and dendritic cells in synovial inflammation of collagen induced arthritis in DBA/1 mice and spontaneous arthritis in MRL/lpr mice. *Autoimmunity*. 1991;8(4):271-80.

Hui W, Cawston TE, Richards CD, Rowan AD. A model of inflammatory arthritis highlights a role for oncostatin M in pro-inflammatory cytokine-induced bone destruction via RANK/RANKL. *Arthritis Res Ther*. 2005;7(1):R57-64.

Hurst SM, Wilkinson TS, McLoughlin RM, Jones S, Horiuchi S, Yamamoto N, Rose-John S, Fuller GM, Topley N, Jones SA. IL-6 and its soluble receptor orchestrate a temporal switch in the pattern of leukocyte recruitment seen during acute inflammation. *Immunity*. 2001 Jun;14(6):705-14.

Hyrich KL, Watson KD, Silman AJ, Symmons DP; British Society for Rheumatology Biologics Register. Predictors of response to anti-TNF-alpha therapy among patients with rheumatoid arthritis: results from the British Society for Rheumatology Biologics Register. *Rheumatology (Oxford)*. 2006 Dec;45(12):1558-65.

Jackson JR, Minton JA, Ho ML, Wei N, Winkler JD. Expression of vascular endothelial growth factor in synovial fibroblasts is induced by hypoxia and interleukin 1beta. *J Rheumatol*. 1997 Jul;24(7):1253-9.

Jacobsson LT, Hanson RL, Knowler WC, Pillemer S, Pettitt DJ, McCance DR, Bennett PH. Decreasing incidence and prevalence of rheumatoid arthritis in Pima Indians over a twenty-five-year period. *Arthritis Rheum*. 1994 Aug;37(8):1158-65.

Jacoby RK, Jayson MI, Cosh JA. Onset, early stages, and prognosis of rheumatoid arthritis: a clinical study of 100 patients with 11-year follow-up. *Br Med J*. 1973 Apr 14;2(5858):96-100.

Jobanputra P, Barton P, Bryan S, Fry-Smith A, Burls A. The clinical effectiveness and cost-effectiveness of new drug treatments for rheumatoid arthritis: etanercept and Infliximab. Assessment for NICE September 2001.

Jones JM, Schwab JH. Immune response to cell wall and tissue antigens in rabbits injured with streptococcal cell wall fragments. *Int Arch Allergy Appl Immunol.* 1970;39(4):445-8.

Jones SA, Novick D, Horiuchi S, Yamamoto N, Szalai AJ, Fuller GM. C-reactive protein: a physiological activator of interleukin 6 receptor shedding. *J Exp Med.* 1999 Feb 1;189(3):599-604.

Jones SA, Horiuchi S, Topley N, Yamamoto N, Fuller GM. The soluble interleukin 6 receptor: mechanisms of production and implications in disease. *FASEB J.* 2001 Jan;15(1):43-58. Review.

Jones SA, Rose-John S. The role of soluble receptors in cytokine biology: the agonistic properties of the sIL-6R/IL-6 complex. *Biochim Biophys Acta.* 2002 Nov 11;1592(3):251-63. Review.

Jones SA, Richards PJ, Scheller J, Rose-John S. IL-6 transsignaling: the in vivo consequences. *J Interferon Cytokine Res.* 2005 May;25(5):241-53.

Jonuleit H, Schmitt E, Stassen M, Tuettenberg A, Knop J, Enk AH. Identification and functional characterization of human CD4(+)CD25(+) T cells with regulatory properties isolated from peripheral blood. *J Exp Med.* 2001 Jun 4;193(11):1285-94.

Jostock T, Müllberg J, Ozbek S, Atreya R, Blinn G, Voltz N, Fischer M, Neurath MF, Rose-John S. Soluble gp130 is the natural inhibitor of soluble interleukin-6 receptor transsignaling responses. *Eur J Biochem.* 2001 Jan;268(1):160-7

Kasama T, Shiozawa F, Kobayashi K, Yajima N, Hanyuda M, Takeuchi HT, Mori Y, Negishi M, Ide H, Adachi M. Vascular endothelial growth factor expression by activated synovial leukocytes in rheumatoid arthritis: critical involvement of the interaction with synovial fibroblasts. *Arthritis Rheum.* 2001 Nov;44(11):2512-24.

Kavanaugh AF, Davis LS, Jain RI, Nichols LA, Norris SH, Lipsky PE. A phase I/II open label study of the safety and efficacy of an anti-ICAM-1 (intercellular adhesion molecule-1; CD54) monoclonal antibody in early rheumatoid arthritis. *J Rheumatol.* 1996 Aug;23(8):1338-44.

Kirwan J, Power L. Glucocorticoids: action and new therapeutic insights in rheumatoid arthritis. *Curr Opin Rheumatol.* 2007 May;19(3):233-7.

Koch AE, Kunkel SL, Burrows JC, Evanoff HL, Haines GK, Pope RM, Strieter RM. Synovial tissue macrophage as a source of the chemotactic cytokine IL-8. *J Immunol.* 1991 Oct 1;147(7):2187-95.

Kollias G, Douni E, Kassiotis G, Kontoyiannis D. On the role of tumor necrosis factor and receptors in models of multiorgan failure, rheumatoid arthritis, multiple sclerosis and inflammatory bowel disease. *Immunol Rev.* 1999 Jun;169:175-94. Review.

Konttinen YT, Li TF, Hukkanen M, Ma J, Xu JW, Virtanen I. Fibroblast biology. Signals targeting the synovial fibroblast in arthritis. *Arthritis Res.* 2000;2(5):348-55. Review.

Korpela M, Laasonen L, Hannonen P, Kautiainen H, Leirisalo-Repo M, Hakala M, Paimela L, Blåfield H, Puolakka K, Möttönen T; FIN-RACo Trial Group. Retardation of joint damage in patients with early rheumatoid arthritis by initial aggressive treatment with disease-modifying antirheumatic drugs: five-year experience from the FIN-RACo study. *Arthritis Rheum.* 2004 Jul;50(7):2072-81.

Kotake S, Sato K, Kim KJ, Takahashi N, Udagawa N, Nakamura I, Yamaguchi A, Kishimoto T, Suda T, Kashiwazaki S. Interleukin-6 and soluble interleukin-6 receptors in the synovial fluids from rheumatoid arthritis patients are responsible for osteoclast-like cell formation. *J Bone Miner Res.* 1996 Jan;11(1):88-95.

Krause A, Scaletta N, Ji JD, Ivashkiv LB. Rheumatoid arthritis synoviocyte survival is dependent on Stat3. *J Immunol.* 2002 Dec 1;169(11):6610-6.

Kremer JM, Dougados M, Emery P, Durez P, Sibilia J, Shergy W, Steinfeld S, Tindall E, Becker JC, Li T, Nuamah IF, Aranda R, Moreland LW. Treatment of rheumatoid arthritis with the selective costimulation modulator abatacept: twelve-month results of a phase iib, double-blind, randomized, placebo-controlled trial. *Arthritis Rheum.* 2005 Aug;52(8):2263-71.

Kumon Y, Loose LD, Birbara CA, Sipe JD. Rheumatoid arthritis exhibits reduced acute phase and enhanced constitutive serum amyloid A protein in synovial fluid relative to serum. A comparison with C-reactive protein. *J Rheumatol.* 1997 Jan;24(1):14-9.

Lee YH, Rho YH, Choi SJ, Ji JD, Song GG. Association of TNF-alpha -308 G/A polymorphism with responsiveness to TNF-alpha-blockers in rheumatoid arthritis: a meta-analysis. *Rheumatol Int.* 2006 Dec;27(2):157-61.

Leech M, Metz C, Hall P, Hutchinson P, Gianis K, Smith M, Weedon H, Holdsworth SR, Bucala R, Morand EF. Macrophage migration inhibitory factor in rheumatoid arthritis: evidence of proinflammatory function and regulation by glucocorticoids. *Arthritis Rheum.* 1999 Aug;42(8):1601-8.

Leipe J, Skapenko A, Lipsky PE, Schulze-Koops H. Regulatory T cells in rheumatoid arthritis. *Arthritis Res Ther.* 2005;7(3):93-99.

Lindhout E, van Eijk M, van Pel M, Lindeman J, Dinant HJ, de Groot C. Fibroblast-like synoviocytes from rheumatoid arthritis patients have intrinsic properties of follicular dendritic cells. *J Immunol.* 1999 May 15;162(10):5949-56.

Listing J, Strangfeld A, Kary S, Rau R, von Hinueber U, Stoyanova-Scholz M, Gromnica-Ihle E, Antoni C, Herzer P, Kekow J, Schneider M, Zink A. Infections in patients with rheumatoid arthritis treated with biologic agents. *Arthritis Rheum.* 2005 Nov;52(11):3403-12.

MacKay K, Eyre S, Myerscough A, Milicic A, Barton A, Laval S, Barrett J, Lee D, White S, John S, Brown MA, Bell J, Silman A, Ollier W, Wordsworth P, Worthington J. Whole-genome linkage analysis of rheumatoid arthritis susceptibility loci in 252 affected sibling pairs in the United Kingdom. *Arthritis Rheum.* 2002 Mar;46(3):632-9.

Maetzel A, Wong A, Strand V, Tugwell P, Wells G, Bombardier C. Meta-analysis of treatment termination rates among rheumatoid arthritis patients receiving disease-modifying anti-rheumatic drugs. *Rheumatology (Oxford).* 2000 Sep;39(9):975-81.

Maini RN, Breedveld FC, Kalden JR, Smolen JS, Davis D, Macfarlane JD, Antoni C, Leeb B, Elliott MJ, Woody JN, Schaible TF, Feldmann M. Therapeutic efficacy of multiple intravenous infusions of anti-tumor necrosis factor alpha monoclonal antibody combined with low-dose weekly methotrexate in rheumatoid arthritis. *Arthritis Rheum.* 1998 Sep;41(9):1552-63.

Maini R, St Clair EW, Breedveld F, Furst D, Kalden J, Weisman M, Smolen J, Emery P, Harriman G, Feldmann M, Lipsky P. Infliximab (chimeric anti-tumour necrosis factor alpha monoclonal antibody) versus placebo in rheumatoid arthritis patients receiving concomitant methotrexate: a randomised phase III trial. ATTRACT Study Group. *Lancet.* 1999 Dec 4;354(9194):1932-9.

Marin V, Montero-Julian FA, Grès S, Boulay V, Bongrand P, Farnarier C, Kaplanski G. The IL-6-soluble IL-6Ralpha autocrine loop of endothelial activation as an intermediate between acute and chronic inflammation: an experimental model involving thrombin. *J Immunol.* 2001 Sep 15;167(6):3435-42.

Marin V, Montero-Julian F, Grès S, Bongrand P, Farnarier C, Kaplanski G. Chemotactic agents induce IL-6Ralpha shedding from polymorphonuclear cells: involvement of a metalloproteinase of the TNF-alpha-converting enzyme (TACE) type. *Eur J Immunol.* 2002 Oct;32(10):2965-70.

Marinova-Mutafchieva L, Williams RO, Mauri C, Mason LJ, Walmsley MJ, Taylor PC, Feldmann M, Maini RN. A comparative study into the mechanisms of action of anti-tumor necrosis factor alpha, anti-CD4, and combined anti-tumor necrosis factor alpha/anti-CD4 treatment in early collagen-induced arthritis. *Arthritis Rheum.* 2000 Mar;43(3):638-44.

Matzner Y, Partridge RE, Babior BM. A chemotactic inhibitor in synovial fluid. *Immunology.* 1983 May;49(1):131-8.

McLoughlin RM, Witowski J, Robson RL, Wilkinson TS, Hurst SM, Williams AS, Williams JD, Rose-John S, Jones SA, Topley N. Interplay between IFN-gamma and IL-6 signaling governs neutrophil trafficking and apoptosis during acute inflammation. *J Clin Invest.* 2003 Aug;112(4):598-607.

McLoughlin RM, Hurst SM, Nowell MA, Harris DA, Horiuchi S, Morgan LW, Wilkinson TS, Yamamoto N, Topley N, Jones SA. Differential regulation of neutrophil-activating chemokines by IL-6 and its soluble receptor isoforms. *J Immunol.* 2004 May 1;172(9):5676-83.

McLoughlin RM, Jenkins BJ, Grail D, Williams AS, Fielding CA, Parker CR, Ernst M, Topley N, Jones SA. IL-6 trans-signaling via STAT3 directs T cell infiltration in acute inflammation. *Proc Natl Acad Sci U S A.* 2005 Jul 5;102(27):9589-94.

Migita K, Eguchi K, Kawabe Y, Ichinose Y, Tsukada T, Aoyagi T, Nakamura H, Nagataki S. TNF-alpha-mediated expression of membrane-type matrix metalloproteinase in rheumatoid synovial fibroblasts. *Immunology*. 1996 Dec;89(4):553-7.

Mladenovic V, Domljan Z, Rozman B, Jajic I, Mihajlovic D, Dordevic J, Popovic M, Dimitrijevic M, Zivkovic M, Campion G. Safety and effectiveness of leflunomide in the treatment of patients with active rheumatoid arthritis. Results of a randomized, placebo-controlled, phase II study. *Arthritis Rheum*. 1995 Nov;38(11):1595-603.

Modur V, Li Y, Zimmerman GA, Prescott SM, McIntyre TM. Retrograde inflammatory signaling from neutrophils to endothelial cells by soluble interleukin-6 receptor alpha. *J Clin Invest*. 1997 Dec 1;100(11):2752-6.

Moreland LW, Schiff MH, Baumgartner SW, Tindall EA, Fleischmann RM, Bulpitt KJ, Weaver AL, Keystone EC, Furst DE, Mease PJ, Ruderman EM, Horwitz DA, Arkfeld DG, Garrison L, Burge DJ, Blosch CM, Lange ML, McDonnell ND, Weinblatt ME. Etanercept therapy in rheumatoid arthritis. A randomized, controlled trial. *Ann Intern Med*. 1999 Mar 16;130(6):478-86.

Moreland LW, Baumgartner SW, Schiff MH, Tindall EA, Fleischmann RM, Weaver AL, Ettlinger RE, Cohen S, Koopman WJ, Mohler K, Widmer MB, Blosch CM. Treatment of rheumatoid arthritis with a recombinant human tumor necrosis factor receptor (p75)-Fc fusion protein. *N Engl J Med*. 1997 Jul 17;337(3):141-7.

Moreland LW, Alten R, Van den Bosch F, Appelboom T, Leon M, Emery P, Cohen S, Luggen M, Shergy W, Nuamah I, Becker JC. Costimulatory blockade in patients with rheumatoid arthritis: a pilot, dose-finding, double-blind, placebo-controlled clinical trial evaluating CTLA-4Ig and LEA29Y eighty-five days after the first infusion. *Arthritis Rheum*. 2002 Jun;46(6):1470-9.

Morgan ME, Flierman R, van Duivenvoorde LM, Witteveen HJ, van Ewijk W, van Laar JM, de Vries RR, Toes RE. Effective treatment of collagen-induced arthritis by adoptive transfer of CD25+ regulatory T cells. *Arthritis Rheum.* 2005 Jul;52(7):2212-21.

Morozzi G, Fabbroni M, Bellisai F, Cucini S, Simpatico A, Galeazzi M. Low serum level of COMP, a cartilage turnover marker, predicts rapid and high ACR70 response to adalimumab therapy in rheumatoid arthritis. *Clin Rheumatol.* 2007 Aug;26(8):1335-8.

Mugnier B, Balandraud N, Darque A, Roudier C, Roudier J, Revirion D. Polymorphism at position -308 of the tumor necrosis factor alpha gene influences outcome of infliximab therapy in rheumatoid arthritis. *Arthritis Rheum.* 2003 Jul;48(7):1849-52.

Nadkarni S, Mauri C, Ehrenstein MR. Anti-TNF-alpha therapy induces a distinct regulatory T cell population in patients with rheumatoid arthritis via TGF-beta. *J Exp Med.* 2007 Jan 22;204(1):33-9.

Nagaoka T, Sato S, Hasegawa M, Ihn H, Takehara K. Serum levels of soluble interleukin 6 receptor and soluble gp130 are elevated in patients with localized scleroderma. *J Rheumatol.* 2000 Aug;27(8):1917-21

Nakahara H, Song J, Sugimoto M, Hagihara K, Kishimoto T, Yoshizaki K, Nishimoto N. Anti-interleukin-6 receptor antibody therapy reduces vascular endothelial growth factor production in rheumatoid arthritis. *Arthritis Rheum.* 2003 Jun;48(6):1521-9.

Narazaki M, Yasukawa K, Saito T, Ohsugi Y, Fukui H, Koishihara Y, Yancopoulos GD, Taga T, Kishimoto T. Soluble forms of the interleukin-6 signal-transducing receptor component gp130 in human serum possessing a potential to inhibit signals through membrane-anchored gp130. *Blood.* 1993 Aug 15;82(4):1120-6.

Neumann VC, Shinebaum R, Cooke EM, Wright V. Effects of sulphasalazine on faecal flora in patients with rheumatoid arthritis: a comparison with penicillamine. *Br J Rheumatol*. 1987 Oct;26(5):334-7

Newbould BB. Chemotherapy of arthritis induced in rats by mycobacterial adjuvant. *Br J Pharmacol Chemother*. 1963 Aug;21:127-136.

Nguyen LT, Jacobs J, Mathis D, Benoist C. Where FoxP3-dependent regulatory T cells impinge on the development of inflammatory arthritis. *Arthritis Rheum*. 2007 Feb;56(2):509-20.

Nishimoto N, Yoshizaki K, Maeda K, Kuritani T, Deguchi H, Sato B, Imai N, Suemura M, Kakehi T, Takagi N, Kishimoto T. Toxicity, pharmacokinetics, and dose-finding study of repetitive treatment with the humanized anti-interleukin 6 receptor antibody MRA in rheumatoid arthritis. Phase I/II clinical study. *J Rheumatol*. 2003 Jul;30(7):1426-35.

Nishimoto N, Yoshizaki K, Miyasaka N, Yamamoto K, Kawai S, Takeuchi T, Hashimoto J, Azuma J, Kishimoto T. Treatment of rheumatoid arthritis with humanized anti-interleukin-6 receptor antibody: a multicenter, double-blind, placebo-controlled trial. *Arthritis Rheum*. 2004 Jun;50(6):1761-9.

Nishimoto N, Hashimoto J, Miyasaka N, Yamamoto K, Kawai S, Takeuchi T, Murata N, van der Heijde D, Kishimoto T. Study of active controlled monotherapy used for rheumatoid arthritis, an IL-6 inhibitor (SAMURAI): evidence of clinical and radiographic benefit from an x ray reader-blinded randomised controlled trial of tocilizumab. *Ann Rheum Dis*. 2007 Sep;66(9):1162-7.

Nissinen R, Leirisalo-Repo M, Peltomaa R, Palosuo T, Vaarala O. Cytokine and chemokine receptor profile of peripheral blood mononuclear cells during treatment with infliximab in patients with active rheumatoid arthritis. *Ann Rheum Dis*. 2004 Jun;63(6):681-7.

Nowell MA, Richards PJ, Horiuchi S, Yamamoto N, Rose-John S, Topley N, Williams AS, Jones SA. Soluble IL-6 receptor governs IL-6 activity in experimental arthritis: blockade of arthritis severity by soluble glycoprotein 130. *J Immunol.* 2003 Sep 15;171(6):3202-9.

O'Dell JR, Leff R, Paulsen G, Haire C, Mallek J, Eckhoff PJ, Fernandez A, Blakely K, Wees S, Stoner J, Hadley S, Felt J, Palmer W, Waytz P, Churchill M, Klassen L, Moore G. Treatment of rheumatoid arthritis with methotrexate and hydroxychloroquine, methotrexate and sulfasalazine, or a combination of the three medications: results of a two-year, randomized, double-blind, placebo-controlled trial. *Arthritis Rheum.* 2002 May;46(5):1164-70.

Oberg HH, Wesch D, Lenke J, Kabelitz D. An optimized method for the functional analysis of human regulatory T cells. *Scand J Immunol.* 2006 Sep;64(3):353-60.

Ogata H, Takeya M, Yoshimura T, Takagi K, Takahashi K. The role of monocyte chemoattractant protein-1 (MCP-1) in the pathogenesis of collagen-induced arthritis in rats. *J Pathol.* 1997 May;182(1):106-14.

Ohshima S, Saeki Y, Mima T, Sasai M, Nishioka K, Nomura S, Kopf M, Katada Y, Tanaka T, Suemura M, Kishimoto T. Interleukin 6 plays a key role in the development of antigen-induced arthritis. *Proc Natl Acad Sci U S A.* 1998 Jul 7;95(14):8222-6.

Otonello L, Cutolo M, Frumento G, Arduino N, Bertolotto M, Mancini M, Sottofattori E, Dallegri F. Synovial fluid from patients with rheumatoid arthritis inhibits neutrophil apoptosis: role of adenosine and proinflammatory cytokines. *Rheumatology (Oxford).* 2002 Nov;41(11):1249-60.

Padyukov L, Lampa J, Heimbürger M, Ernestam S, Cederholm T, Lundkvist I, Andersson P, Hermansson Y, Harju A, Klareskog L, Bratt J. Genetic markers for the efficacy of tumour necrosis factor blocking therapy in rheumatoid arthritis. *Ann Rheum Dis.* 2003 Jun;62(6):526-9.

Paleolog EM, Hunt M, Elliott MJ, Feldmann M, Maini RN, Woody JN. Deactivation of vascular endothelium by monoclonal anti-tumor necrosis factor alpha antibody in rheumatoid arthritis. *Arthritis Rheum.* 1996 Jul;39(7):1082-91.

Paleolog EM, Young S, Stark AC, McCloskey RV, Feldmann M, Maini RN. Modulation of angiogenic vascular endothelial growth factor by tumor necrosis factor alpha and interleukin-1 in rheumatoid arthritis. *Arthritis Rheum.* 1998 Jul;41(7):1258-65.

Peake NJ, Khawaja K, Myers A, Nowell MA, Jones SA, Rowan AD, Cawston TE, Foster HE. Interleukin-6 signalling in juvenile idiopathic arthritis is limited by proteolytically cleaved soluble interleukin-6 receptor. *Rheumatology (Oxford).* 2006 Dec;45(12):1485-9.

Perry MG, Richards L, Harbuz MS, Jessop DS, Kirwan JR. Sequential synovial fluid sampling suggests plasma and synovial fluid IL-6 vary independently in rheumatoid arthritis. *Rheumatology (Oxford).* 2006 Feb;45(2):229-30.

Perry MG, Jessop DS, Hunt LP, Kirwan JR. Overnight variations in cortisol, interleukin-6, tumor necrosis factor {alpha} and other cytokines in people with rheumatoid arthritis. *Ann Rheum Dis.* 2008 Mar 28; [Epub ahead of print].

Petrovic-Rackov L, Pejnovic N. Clinical significance of IL-18, IL-15, IL-12 and TNF-alpha measurement in rheumatoid arthritis. *Clin Rheumatol.* 2006 Jul;25(4):448-52.

Pettipher ER, Henderson B. The relationship between cell-mediated immunity and cartilage degradation in antigen-induced arthritis in the rabbit. *Br J Exp Pathol.* 1988 Feb;69(1):113-22.

Piguet PF, Grau GE, Vesin C, Loetscher H, Gentz R, Lesslauer W. Evolution of collagen arthritis in mice is arrested by treatment with anti-tumour necrosis factor (TNF) antibody or a recombinant soluble TNF receptor. *Immunology*. 1992 Dec;77(4):510-4.

Pinals RS, Masi AT, Larsen RA. Preliminary criteria for clinical remission in rheumatoid arthritis. *Arthritis Rheum*. 1981 Oct;24(10):1308-15.

Plater-Zyberk C, Hoogewerf AJ, Proudfoot AEI, Power AC, Wells TNC. Effect of a CC chemokine receptor antagonist on collagen induced arthritis in DBA/1 mice. *Immunology letters*. 1997;57:117-120.

Polgár A, Brózik M, Tóth S, Holub M, Hegyi K, Kádár A, Hodinka L, Falus A. Soluble interleukin-6 receptor in plasma and in lymphocyte culture supernatants of healthy individuals and patients with systemic lupus erythematosus and rheumatoid arthritis. *Med Sci Monit*. 2000 Jan-Feb;6(1):13-8.

Proudman SM, Conaghan PG, Richardson C, Griffiths B, Green MJ, McGonagle D, Wakefield RJ, Reece RJ, Miles S, Adebajo A, Gough A, Helliwell P, Martin M, Huston G, Pease C, Veale DJ, Isaacs J, van der Heijde DM, Emery P. Treatment of poor-prognosis early rheumatoid arthritis. A randomized study of treatment with methotrexate, cyclosporin A, and intraarticular corticosteroids compared with sulfasalazine alone. *Arthritis Rheum*. 2000 Aug;43(8):1809-19.

Rathanaswami P, Hachicha M, Wong WL, Schall TJ, McColl SR. Synergistic effect of interleukin-1 beta and tumor necrosis factor alpha on interleukin-8 gene expression in synovial fibroblasts. Evidence that interleukin-8 is the major neutrophil-activating chemokine released in response to monokine activation. *Arthritis Rheum*. 1993 Sep;36(9):1295-304

Raza K, Scheel-Toellner D, Lee CY, Pilling D, Curnow SJ, Falciani F, Trevino V, Kumar K, Assi LK, Lord JM, Gordon C, Buckley CD, Salmon M. Synovial fluid leukocyte apoptosis is inhibited in patients with very early rheumatoid arthritis. *Arthritis Res Ther.* 2006;8(4):R120.

Richards PJ, Nowell MA, Horiuchi S, McLoughlin RM, Fielding CA, Grau S, Yamamoto N, Ehrmann M, Rose-John S, Williams AS, Topley N, Jones SA. Functional characterization of a soluble gp130 isoform and its therapeutic capacity in an experimental model of inflammatory arthritis. *Arthritis Rheum.* 2006 May;54(5):1662-72.

Robak T, Gladalska A, Stepień H, Robak E. Serum levels of interleukin-6 type cytokines and soluble interleukin-6 receptor in patients with rheumatoid arthritis. *Mediators Inflamm.* 1998;7(5):347-53.

Robson RL, McLoughlin RM, Witowski J, Loetscher P, Wilkinson TS, Jones SA, Topley N. Differential regulation of chemokine production in human peritoneal mesothelial cells: IFN-gamma controls neutrophil migration across the mesothelium in vitro and in vivo. *J Immunol.* 2001 Jul 15;167(2):1028-38.

Roman-Blas JA, Jimenez SA. NF-kappaB as a potential therapeutic target in osteoarthritis and rheumatoid arthritis. *Osteoarthritis Cartilage* 2006 Sep;14(9):839-48.

Romano M, Sironi M, Toniatti C, Polentarutti N, Fruscella P, Ghezzi P, Faggioni R, Luini W, van Hinsbergh V, Sozzani S, Bussolino F, Poli V, Ciliberto G, Mantovani A. Role of IL-6 and its soluble receptor in induction of chemokines and leukocyte recruitment. *Immunity.* 1997 Mar;6(3):315-25.

Romas E, Gillespie MT, Martin TJ. Involvement of receptor activator of NFkappaB ligand and tumor necrosis factor-alpha in bone destruction in rheumatoid arthritis. *Bone.* 2002 Feb;30(2):340-6. Review.

Rook GA, Lydyard PM, Stanford JL. A reappraisal of the evidence that rheumatoid arthritis and several other idiopathic diseases are slow bacterial infections. *Ann Rheum Dis.* 1993 Mar;52 Suppl 1:S30-8.

Rothschild BM, Woods RJ. Symmetrical erosive disease in Archaic Indians: the origin of rheumatoid arthritis in the New World? *Semin Arthritis Rheum.* 1990 Apr;19(5):278-84.

Rowan AD, Koshy PJ, Shingleton WD, Degnan BA, Heath JK, Vernallis AB, Spaul JR, Life PF, Hudson K, Cawston TE. Synergistic effects of glycoprotein 130 binding cytokines in combination with interleukin-1 on cartilage collagen breakdown. *Arthritis Rheum.* 2001 Jul;44(7):1620-32.

Rowe IF, Sheldon J, Riches PG, Keat AC. Comparative studies of serum and synovial fluid C reactive protein concentrations. *Ann Rheum Dis.* 1987 Oct;46(10):721-6.

Ruchatz H, Leung BP, Wei XQ, McInnes IB, Liew FY. Soluble IL-15 receptor alpha-chain administration prevents murine collagen-induced arthritis: a role for IL-15 in development of antigen-induced immunopathology. *J Immunol.* 1998 Jun 1;160(11):5654-60.

Rückemann K, Fairbanks LD, Carrey EA, Hawrylowicz CM, Richards DF, Kirschbaum B, Simmonds HA. Leflunomide inhibits pyrimidine de novo synthesis in mitogen-stimulated T-lymphocytes from healthy humans. *J Biol Chem.* 1998 Aug 21;273(34):21682-91.

Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol.* 1995 Aug 1;155(3):1151-64.

Saxne T, Palladino MA Jr, Heinegård D, Talal N, Wollheim FA. Detection of tumor necrosis factor alpha but not tumor necrosis factor beta in rheumatoid arthritis synovial fluid and serum. *Arthritis Rheum.* 1988 Aug;31(8):1041-5.

Scala E, Pallotta S, Frezzolini A, Abeni D, Barbieri C, Sampogna F, De Pità O, Puddu P, Paganelli R, Russo G. Cytokine and chemokine levels in systemic sclerosis: relationship with cutaneous and internal organ involvement. *Clin Exp Immunol*. 2004 Dec;138(3):540-6.

Scallon BJ, Trinh H, Nedelman M, Brennan FM, Feldmann M, Ghrayeb J. Functional comparisons of different tumour necrosis factor receptor/IgG fusion proteins. *Cytokine*. 1995 Nov;7(8):759-70.

Schiff M, Keiserman M, Coddling C, Songcharoen S, Berman A, Nayiager S, Saldate C, Li T, Aranda R, Becker JC, Lin C, Cornet PL, Dougados M. Efficacy and safety of abatacept or infliximab versus placebo in ATTEST: a phase III, multicenter, randomized, double-blind, placebo-controlled study in patients with rheumatoid arthritis and an inadequate response to methotrexate. *Ann Rheum Dis*. 2007 Nov 29 [Epub ahead of print].

Scott DL, Shipley M, Dawson A, Edwards S, Symmons DP, Woolf AD. The clinical management of rheumatoid arthritis and osteoarthritis: strategies for improving clinical effectiveness. *Br J Rheumatol*. 1998 May;37(5):546-54.

Scott DL, Steer S. The course of established rheumatoid arthritis. *Best Pract Res Clin Rheumatol*. 2007 Oct;21(5):943-67.

Seetharaman R, Mora AL, Nabozny G, Boothby M, Chen J. Essential role of T cell NF-kappa B activation in collagen-induced arthritis. *J Immunol*. 1999 Aug 1;163(3):1577-83.

Seitz M, Wirthmüller U, Möller B, Villiger PM. The -308 tumour necrosis factor-alpha gene polymorphism predicts therapeutic response to TNFalpha-blockers in rheumatoid arthritis and spondyloarthritis patients. *Rheumatology (Oxford)*. 2007 Jan;46(1):93-6.

Seki T, Selby J, Häupl T, Winchester R. Use of differential subtraction method to identify genes that characterize the phenotype of cultured rheumatoid arthritis synoviocytes. *Arthritis Rheum*. 1998 Aug;41(8):1356-64.

Sewell KL, Trentham DE. Pathogenesis of rheumatoid arthritis. *Lancet*. 1993 Jan 30;341(8840):283-6.

Sharara S, Amin MA, Woods JM, Haines GK, Koch AE. Chemokine receptor expression and in vivo signalling pathways in the joints of rats with adjuvant-induced arthritis. *Arthritis Rheum* 2003 December;48(12):3568-3583.

Shahrara S, Proudfoot AEI, Woods JM, Ruth JH, Asif Amin M, Park CC, Haas CS, Pope RM, Haines GK, Zha YY, Koch AE. Amelioration of rat adjuvant-induced arthritis by Met-RANTES. *Arthritis Rheum* 2005 June;52(6):1907-1919.

Shealy DJ, Wooley PH, Emmell E, Volk A, Rosenberg A, Treacy G, Wagner CL, Mayton L, Griswold DE, Song XY. Anti-TNF-alpha antibody allows healing of joint damage in polyarthritic transgenic mice. *Arthritis Res*. 2002;4(5):R7.

Shouda T, Yoshida T, Hanada T, Wakioka T, Oishi M, Miyoshi K, Komiya S, Kosai K, Hanakawa Y, Hashimoto K, Nagata K, Yoshimura A. Induction of the cytokine signal regulator SOCS3/CIS3 as a therapeutic strategy for treating inflammatory arthritis. *J Clin Invest*. 2001 Dec;108(12):1781-8.

Silman AJ. Reproductive events and the risk of development of rheumatoid arthritis. *Scand J Rheumatol Suppl*. 1998;107:113-5.

Silman AJ, MacGregor AJ, Thomson W, Holligan S, Carthy D, Farhan A, Ollier WE. Twin concordance rates for rheumatoid arthritis: results from a nationwide study. *Br J Rheumatol*. 1993 Oct;32(10):903-7.

Silman AJ. The changing face of rheumatoid arthritis: why the decline in incidence? *Arthritis Rheum*. 2002 Mar;46(3):579-81.

Smeets RL, van de Loo FA, Arntz OJ, Bennink MB, Joosten LA, van den Berg WB. Adenoviral delivery of IL-18 binding protein C ameliorates collagen-induced arthritis in mice. *Gene Ther*. 2003 Jun;10(12):1004-11.

Smith MD, Slavotinek J, Au V, Weedon H, Parker A, Coleman M, Roberts-Thomson PJ, Ahern MJ. Successful treatment of rheumatoid arthritis is associated with a reduction in synovial membrane cytokines and cell adhesion molecule expression. *Rheumatology (Oxford)*. 2001 Sep;40(9):965-77.

Smolen JS, Beaulieu A, Rubbert-Roth A, Ramos-Remus C, Rovensky J, Alecock E, Woodworth T, Alten R; OPTION Investigators. Effect of interleukin-6 receptor inhibition with tocilizumab in patients with rheumatoid arthritis (OPTION study): a double-blind, placebo-controlled, randomised trial. *Lancet*. 2008 Mar 22;371(9617):987-97

Smoleńska Z, Kaznowska Z, Zarówny D, Simmonds HA, Smoleński RT. Effect of methotrexate on blood purine and pyrimidine levels in patients with rheumatoid arthritis. *Rheumatology (Oxford)*. 1999 Oct;38(10):997-1002.

Springer TA. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell*. 1994 Jan 28;76(2):301-14. Review.

Steinbrocker O, Traeger CH, Batterman RC. Therapeutic criteria in Rheumatoid arthritis. *JAMA* 1949;June 25:140(8)659-662.

Stevens RJ, Douglas KM, Saratzis AN, Kitas GD. Inflammation and atherosclerosis in rheumatoid arthritis. *Expert Rev Mol Med*. 2005 May 6;7(7):1-24.

Stoerk HD, Bielinski TC, Budzilovich T. Chronic polyarthritis in rats injected with spleen adjuvants. *Am J Pathol* 1954;30:616-630.

Strand V, Tugwell P, Bombardier C, Maetzel A, Crawford B, Dorrier C, Thompson A, Wells G. Function and health-related quality of life: results from a randomized controlled trial of leflunomide versus methotrexate or placebo in patients with active rheumatoid arthritis. Leflunomide Rheumatoid Arthritis Investigators Group. *Arthritis Rheum*. 1999 Sep;42(9):1870-8.

Stuart JM, Townes AS, Kang AH. Type II collagen-induced arthritis. *Ann N Y Acad Sci.* 1985;460:355-362.

Sweeney SE, Firestein GS. Rheumatoid arthritis: regulation of synovial inflammation. *Int J Biochem Cell Biol.* 2004 Mar;36(3):372-8.

Symmons DP, Salmon M, Farr M, Bacon PA. Sulfasalazine treatment and lymphocyte function in patients with rheumatoid arthritis. *J Rheumatol.* 1988 Apr;15(4):575-9.

Szekanecz Z, Strieter RM, Kunkel SL, Koch AE. Chemokines in rheumatoid arthritis. *Springer Semin Immunopathol.* 1998;20(1-2):115-32.

Taga T, Kishimoto T. gp130 and the interleukin-6 family of cytokines. *Annu Rev Immunol.* 1997;15:797-819.

Tak PP, Taylor PC, Breedveld FC, Smeets TJ, Daha MR, Kluin PM, Meinders AE, Maini RN. Decrease in cellularity and expression of adhesion molecules by anti-tumor necrosis factor alpha monoclonal antibody treatment in patients with rheumatoid arthritis. *Arthritis Rheum.* 1996 Jul;39(7):1077-81.

Takagi N, Mihara M, Moriya Y, Nishimoto N, Yoshizaki K, Kishimoto T, Takeda Y, Ohsugi Y. Blockage of interleukin-6 receptor ameliorates joint disease in murine collagen-induced arthritis. *Arthritis Rheum.* 1998 Dec;41(12):2117-21.

Takai Y, Seki N, Senoh H, Yokota T, Lee F, Hamaoka T, Fujiwara H. Enhanced production of interleukin-6 in mice with type II collagen-induced arthritis. *Arthritis Rheum.* 1989 May;32(5):594-600.

Takayanagi H, Iizuka H, Juji T, Nakagawa T, Yamamoto A, Miyazaki T, Koshihara Y, Oda H, Nakamura K, Tanaka S. Involvement of receptor activator of nuclear factor kappa B ligand/osteoclast differentiation factor in osteoclastogenesis from synoviocytes in rheumatoid arthritis. *Arthritis Rheum.* 2000 Feb;43(2):259-69.

Takeda T, Mizugaki Y, Matsubara L, Imai S, Koike T, Takada K. Lytic Epstein-Barr virus infection in the synovial tissue of patients with rheumatoid arthritis. *Arthritis Rheum.* 2000 Jun;43(6):1218-25.

Takei M, Mitamura K, Fujiwara S, Horie T, Ryu J, Osaka S, Yoshino S, Sawada S. Detection of Epstein-Barr virus-encoded small RNA 1 and latent membrane protein 1 in synovial lining cells from rheumatoid arthritis patients. *Int Immunol.* 1997 May;9(5):739-43.

Tanaka M, Kishimura M, Ozaki S, Osakada F, Hashimoto H, Okubo M, Murakami M, Nakao K. Cloning of novel soluble gp130 and detection of its neutralizing autoantibodies in rheumatoid arthritis. *J Clin Invest.* 2000 Jul;106(1):137-44.

Tarrant TK, Patel DD. Chemokines and leukocyte trafficking in rheumatoid arthritis. *Pathophysiology.* 2006 Feb 21;13(1):1-14.

Tartaglia LA, Weber RF, Figari IS, Reynolds C, Palladino MA Jr, Goeddel DV. The two different receptors for tumor necrosis factor mediate distinct cellular responses. *Proc Natl Acad Sci U S A.* 1991 Oct 15;88(20):9292-6.

Tartaglia LA, Rothe M, Hu YF, Goeddel DV. Tumor necrosis factor's cytotoxic activity is signaled by the p55 TNF receptor. *Cell.* 1993 Apr 23;73(2):213-6.

Taylor PC, Peters AM, Glass DM, Maini RN. Effects of treatment of rheumatoid arthritis patients with an antibody against tumour necrosis factor alpha on reticuloendothelial and intrapulmonary granulocyte traffic. *Clin Sci (Lond).* 1999 Jul;97(1):85-9.

Taylor PC, Peters AM, Paleolog E, Chapman PT, Elliott MJ, McCloskey R, Feldmann M, Maini RM. Reduction of chemokine levels and leucocyte traffic to joints by tumor necrosis factor  $\alpha$  blockade in patients with rheumatoid arthritis. *Arthritis Rheum.* 2000 Jan;43(1):38-47.

Tetta C, Camussi G, Modena V, Di Vittorio C, Baglioni C. Tumour necrosis factor in serum and synovial fluid of patients with active and severe rheumatoid arthritis. *Ann Rheum Dis.* 1990 Sep;49(9):665-7.

Toussirot E, Despaux J, Wendling D. Decreased T cell precursor frequencies to Epstein-Barr virus glycoprotein Gp110 in peripheral blood correlate with disease activity and severity in patients with rheumatoid arthritis. *Ann Rheum Dis.* 2000 Jul;59(7):533-8.

Trentham DE, Townes AS, Kang AH. Autoimmunity to type II collagen an experimental model of arthritis. *J Exp Med.* 1977 Sep 1;146(3):857-68.

Trentham DE and Dynesius-Trentham R 1995. Collagen-induced arthritis-in (Henderson B, Edwards JCW, Pettipher ER) Mechanisms and models in Rheumatoid Arthritis. Academic press, Harcourt Brace & company, 447-457.

Troughton PR, Platt R, Bird H, el-Manzalawi E, Bassiouni M, Wright V. Synovial fluid interleukin-8 and neutrophil function in rheumatoid arthritis and seronegative polyarthritis. *Br J Rheumatol.* 1996 Dec;35(12):1244-51.

Tugwell P. Combination therapy in rheumatoid arthritis: meta analysis. *J Rheumatol Suppl.* 1996 Mar;44:43-6.

Turesson C, O'Fallon WM, Crowson CS, Gabriel SE, Matteson EL. Extra-articular disease manifestations in rheumatoid arthritis: incidence trends and risk factors over 46 years. *Ann Rheum Dis.* 2003 Aug;62(8):722-7.

Turesson C, McClelland RL, Christianson TJ, Matteson EL. No decrease over time in the incidence of vasculitis or other extraarticular manifestations in rheumatoid arthritis: results from a community-based study. *Arthritis Rheum.* 2004 Nov;50(11):3729-31.

Uhlig T, Heiberg T, Mowinckel P, Kvien TK. Rheumatoid arthritis is milder in the new millennium: Health status in RA patients 1994-2004. *Ann Rheum Dis.* 2008 Jan 24 [Epub ahead of print].

van Amelsfort JM, Jacobs KM, Bijlsma JW, Lafeber FP, Taams LS CD4(+)CD25(+) regulatory T cells in rheumatoid arthritis: differences in the presence, phenotype, and function between peripheral blood and synovial fluid. *Arthritis Rheum.* 2004 Sep;50(9):2775-85.

van der Heijde DM, van Riel PL, van Leeuwen MA, van 't Hof MA, van Rijswijk MH, van de Putte LB. Prognostic factors for radiographic damage and physical disability in early rheumatoid arthritis. A prospective follow-up study of 147 patients. *Br J Rheumatol.* 1992 Aug;31(8):519-25.

van Lent PL, Holthuysen AE, van den Bersselaar LA, van Rooijen N, Joosten LA, van de Loo FA, van de Putte LB, van den Berg WB. Phagocytic lining cells determine local expression of inflammation in type II collagen-induced arthritis. *Arthritis Rheum.* 1996 Sep;39(9):1545-55.

van Lent PL, Holthuysen AE, Van Rooijen N, Van De Putte LB, Van Den Berg WB. Local removal of phagocytic synovial lining cells by clodronate-liposomes decreases cartilage destruction during collagen type II arthritis. *Ann Rheum Dis.* 1998 Jul;57(7):408-13.

Verhoeven AC, Bibo JC, Boers M, Engel GL, van der Linden S. Cost-effectiveness and cost-utility of combination therapy in early rheumatoid arthritis: randomized comparison of combined step-down prednisolone, methotrexate and sulphasalazine with sulphasalazine alone. COBRA Trial Group. *Combinatietherapie Bij Reumatoïde Artritis.* *Br J Rheumatol.* 1998 Oct;37(10):1102-9.

Vigar ND, Cabrera WH, Araujo LM, Ribeiro OG, Ogata TR, Siqueira M, Ibañez OM, De Franco M. Pristane-induced arthritis in mice selected for maximal or minimal acute inflammatory reaction. *Eur J Immunol.* 2000 Feb;30(2):431-7.

Vigna-Pérez M, Abud-Mendoza C, Portillo-Salazar H, Alvarado-Sánchez B, Cuevas-Orta E, Moreno-Valdés R, Baranda L, Paredes-Saharopulos O, González-Amaro R. Immune effects of therapy with Adalimumab in patients with rheumatoid arthritis. *Clin Exp Immunol.* 2005 Aug;141(2):372-80.

Walker JG, Ahern MJ, Coleman M, Weedon H, Papangelis V, Beroukas D, Roberts-Thomson PJ, Smith MD. Changes in synovial tissue Jak-STAT expression in rheumatoid arthritis in response to successful DMARD treatment. *Ann Rheum Dis.* 2006 Dec;65(12):1558-64.

Weinblatt ME, Kremer JM, Bankhurst AD, Bulpitt KJ, Fleischmann RM, Fox RI, Jackson CG, Lange M, Burge DJ. A trial of etanercept, a recombinant tumor necrosis factor receptor:Fc fusion protein, in patients with rheumatoid arthritis receiving methotrexate. *N Engl J Med.* 1999 Jan 28;340(4):253-9.

Welsing PM, Fransen J, van Riel PL. Is the disease course of rheumatoid arthritis becoming milder? Time trends since 1985 in an inception cohort of early rheumatoid arthritis. *Arthritis Rheum.* 2005 Sep;52(9):2616-24.

Wendling D, Racadot E, Wijdenes J. Treatment of severe rheumatoid arthritis by anti-interleukin 6 monoclonal antibody. *J Rheumatol.* 1993 Feb;20(2):259-62.

Wijngaarden S, van de Winkel JG, Jacobs KM, Bijlsma JW, Lafeber FP, van Roon JA. A shift in the balance of inhibitory and activating Fcγ receptors on monocytes toward the inhibitory Fcγ receptor IIb is associated with prevention of monocyte activation in rheumatoid arthritis. *Arthritis Rheum.* 2004 Dec;50(12):3878-87.

Wildin RS, Freitas A. IPEX and FOXP3: clinical and research perspectives. *J Autoimmun.* 2005;25 Suppl:56-62.

Williams RO, Feldmann M, Maini RN. Anti-tumor necrosis factor ameliorates joint disease in murine collagen-induced arthritis. *Proc Natl Acad Sci U S A.* 1992 Oct 15;89(20):9784-8.

Williams RO, Whyte A. Anti-CD4 monoclonal antibodies suppress murine collagen-induced arthritis only at the time of primary immunisation. *Cell Immunol.* 1996 Jun 15;170(2):291-5.

Wong GH, Tartaglia LA, Lee MS, Goeddel DV. Antiviral activity of tumor necrosis factor is signaled through the 55-kDa type I TNF receptor [corrected]. *J Immunol.* 1992 Nov 15;149(10):3350-3.

Wooley PH, Dutcher J, Widmer MB, Gillis S. Influence of a recombinant human soluble tumor necrosis factor receptor FC fusion protein on type II collagen-induced arthritis in mice. *J Immunol.* 1993 Dec 1;151(11):6602-7. a).

Wooley PH, Whalen JD, Chapman DL, Berger AE, Richard KA, Aspar DG, Staite ND. The effect of an interleukin-1 receptor antagonist protein on type II collagen-induced arthritis and antigen-induced arthritis in mice. *Arthritis Rheum.* 1993 Sep;36(9):1305-14. b).

Wright JK, Clark IM, Cawston TE, Hazleman BL. The secretion of the tissue inhibitor of metalloproteinases (TIMP) by human synovial fibroblasts is modulated by all-trans-retinoic acid. *Biochim Biophys Acta.* 1991 Dec 3;1133(1):25-30.

Yagi H, Nomura T, Nakamura K, Yamazaki S, Kitawaki T, Hori S, Maeda M, Onodera M, Uchiyama T, Fujii S, Sakaguchi S. Crucial role of FOXP3 in the development and function of human CD25+CD4+ regulatory T cells. *Int Immunol.* 2004 Nov;16(11):1643-56.

Yamanaka H, Matsuda Y, Tanaka M, Sendo W, Nakajima H, Taniguchi A, Kamatani N. Serum matrix metalloproteinase 3 as a predictor of the degree of joint destruction during the six months after measurement, in patients with early rheumatoid arthritis. *Arthritis Rheum.* 2000 Apr;43(4):852-8.

Yoshizaki K, Nishimoto N, Mihara M, Kishimoto T. Therapy of rheumatoid arthritis by blocking IL-6 signal transduction with a humanized anti-IL-6 receptor antibody. *Springer Semin Immunopathol.* 1998;20(1-2):247-59.