The Characterisation and Regulation of T cell Immune Responses in Psoriatic Arthritis

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Abstract

Psoriatic arthritis (PsA) is a chronic inflammatory arthritis. It affects approximately 0.05% of the general population presenting as peripheral arthritis, axial disease or a combination of both. About 10% of patients with psoriasis suffer from PsA. Currently available therapies have limited efficacy with associated toxicity. PsA is therefore a common arthropathy for which novel therapies are urgently required.

Our understanding of the role of the immune system in the pathogenesis of rheumatic disease has led to the development of specific targeted therapies. The identification of tumour necrosis factor alpha (TNFα) as a key player in the inflammatory response has been a major advance in the field of rheumatology. The effects of blocking this pro inflammatory molecule have resulted in dramatic clinical effects across the spectrum of rheumatic diseases. Unfortunately however there remain a proportion of non-responders and concerns have been raised surrounding the adverse effects of these therapies (Furst, Keystone et al. 2001). It is therefore important to continue to elucidate other key players in the inflammatory cascade that can be modified to achieve clinical response.

A critical advance in understanding the immune system's role in driving inflammation has been the recognition of different functional subsets of T helper (Th) cells according to their cytokine profile. Interferon gamma (IFNγ), a potent stimulator of TNFα production, is the hallmark cytokine of the T helper type 1 (Th1) immune response whereas T helper 2 (Th2) responses are characterised by the production of anti-inflammatory cytokines such as IL-4, IL-5 and IL-10. Th1 mediated diseases such as psoriasis may benefit from therapies that direct the immune system towards a Th2 immune response (Oekenfels, Schultewolter et al.

1998). Furthermore identifying those factors that regulate Th1 immune responses may lead to specifically targeted therapies in the clinic.

Initial studies of cytokine profiles in PsA synovial fluid (SF) demonstrated an increase in Th1 cytokines (Partsch, Wagner et al. 1998) (Ritchlin, Haas-Smith et al. 1998). Subsequent data have since emerged suggesting Th1 cytokines such as IFNγ are more prominent in rheumatoid arthritis (RA) than in seronegative arthropathies including PsA (Canete, Martinez et al. 2000). The issue surrounding the predominant T cell response in PsA is controversial and clarification is required.

Techniques have evolved that enable us to achieve clarification of a true *in vivo* Th1 response by identifying cells in SF that spontaneously secrete IFNγ. In this thesis, spontaneously secreting cells were identified in SF of patients with active PsA. That such IFNγ expressing cells may have a pathogenic role in PsA is supported by the following observations:

- IFNγ is the prototype macrophage activator of TNFα that drives inflammation in psoriasis and PsA.

- IFNγ when tested as a potential treatment for psoriasis induced synovitis in a subset of patients. (O'Connell, Gerber et al. 1992)

- The injection of IFNγ into the skin induces hyperkeratosis (Fierlbeck, Rassner et al. 1990)

- Autologous Natural Killer T (NKT) cells, potent producers of IL-4 and IFNγ, are able to induce psoriasis in 'normal skin' grafted onto a SCID mouse (Nickoloff, Wrone-Smith et al. 1999).

Those factors regulating the expression of IFNγ may therefore be of considerable interest as they may offer potential targets to modify Th1 responses in PsA.

Cytokines such as interleukin 18 (IL-18) and interleukin 12 (IL-12) have been shown to regulate Th1 responses in animal models of arthritis (Leung, McInnes et al. 2000). Whether such cytokines are present and indeed induce inflammation in PsA is unknown. The first part of this thesis focuses on the characterisation of the predominant Th response in PsA and the identification of key regulatory cytokines such as IL-12 and IL-18 that may drive TNFα production and as such lead to inflammation.

The presence of other regulatory cells capable of driving the inflammatory response was explored briefly. NKT cells are a subset of T cell, which secrete IFNγ and IL-4 depending on the predominant cytokine milieu (Hafner, Falk et al. 1999). A pathogenic role for NKT cells in psoriasis has been suggested. (Bonish, Jullien et al. 2000). The ability of synovial fluid mononuclear cells (SF MC) to proliferate and secrete TNFα in response to glycolipid presented to NK T cells was investigated.

Although TNFα contributes directly to synovial inflammation, the blockade of TNFα does not protect completely against cartilage damage (Joosten, Helsen et al. 1999). IFNγ and IL-17 have opposing effects on bone resorption. IFNγ inhibits bone resorption whereas IL-17 enhances resorption through the degradation or enhancement of TNF receptor associated factor 6 (TRAF6) respectively (Chabaud, Lubberts et al. 2001). The expression of IL-17 and the functional effects of this cytokine on MMP3 expression by fibroblasts were therefore studied as a final section in this thesis.

Acknowledgements

Presentations

The work presented in this thesis has been presented in poster format at the American College of Rheumatology Scientific Conference in San Francisco November 2001, European Workshop for Rheumatology Research in Leiden February 2002 and the American College Rheumatology Scientific Conference New Orleans 2002.

Oral presentations have been presented at the Scottish Society of Experimental Medicine meeting in Glasgow June 2002 and at the Scottish Rheumatology Society meeting in Hamilton April 2002.

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Abbreviations

μg	Microgram(s)
μl	Microliter(s)
$^{\circ}C$	Degrees Centigrade
Ag	Antigen
APC	Antigen Presenting Cell
BSA	Bovine Serum Albumin
CD	cluster of differentiation
cDNA	complimentary deoxyribonucleic acid
ConA	Concavalin-A
DNA	deoxyribonucleic acid
EMSA	Electro mobility shift assay
FCS	foetal calf serum
FITC	fluorescein isothiocynate
g	Gram(s)
H&E	Haematoxylin and Eosin
H_2O_2	Hydrogen Peroxide
^{3}H-Thymidine	Tritiated thymidine
HRP	Horse Radish Peroxidase
IL-	Interleukin
IFN-γ	Interferon gamma
kg	Kilogram(s)
mAb	Monoclonal Antibody
MHC	Major Histocompatibility Complex
ml	Millilitre(s)
NK	Natural Killer Cell
NKT	Natural Killer T cell
OD	Optical Density
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
PCR	polymerase chain reaction

PsA	psoriatic arthritis
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute
RT	Room temperature
RTPCR	Reverse transcription polymerase chain reaction
SF	Synovial fluid
SM	Synovial membrane
TBE	Tris Hydrochloride, Boric Acid and EDTA
Th1	T Helper 1 subset
Th2	T Helper 2 subset
TNF–α	Tumour Necrosis factor- Alpha

Chapter 1 Introduction

1.1 Psoriatic arthritis

1.1.1 History

The earliest reports of psoriatic arthritis (PsA) come from visual, radiological and microscopy studies of skeletal remains recovered from tombs in the Byzantine Monastery in the Judean Desert. During the Byzantine period extending from 395-1453 A.D. the earlier practise of expelling those with disfiguring diseases evolved into a caring philosophy where the sick were housed and fed out of charity, often within monasteries. The presence of these skeletal remains confirms earlier suggestions that psoriasis was one of the diseases included by those in the ancient eastern Mediterranean under the term of biblical leprosy (Zias and Mitchell 1996). The earliest reports of PsA in Britain are from skeletal remains of a thirteenth century male with ankylosis of the interphalangeal joints and typical 'cup and pencil' deformity of the distal interphalangeal joints indicative of PsA (Rogers, Watt et al. 1981).

Despite observations of an association between psoriasis and arthritis in 1818 by the French physician Baron Jean Louis Alibert, it was not until 1860 that the French dermatologist Bazin coined the term PsA. Over the next 100 years many individual cases of psoriasis and arthritis were described in the literature until 1950 when PsA was accepted as a distinct clinical entity (O'Neill and Silman 1994).

1.1.2 Epidemiology and aetiology

Research into PsA is hampered by lack of a validated case definition. Epidemiological studies indicate that PsA is a unique arthropathy rather than a chance association of cutaneous and joint inflammation (Baker 1966). The prevalence of PsA in different populations varies depending on diagnostic criteria employed. Defining PsA using the American Rheumatism Association criteria for RA plus a feature typical of PsA indicates a prevalence of 0.02-0.05% in the general population (Hellgren 1970) (van Romunde, Valkenburg et al. 1984). Moll and Wright using more permissive criteria calculated a prevalence of 0.1% from a hospital study and a prevalence of psoriasis of 1.5% in the general population (Moll, Johnson et al. 1974). A population based study in Olmstead County identified 1056 patients with psoriasis of whom 6.25% had arthritis with a point prevalence of 0.1% (Shbeeb, Uramoto et al. 2000). The peak incidence of PsA occurs between the ages 20-40 years with a slight male bias except in the subset of patients with symmetrical polyarthritis where females predominate (Veale 2000).

The prevalence of psoriasis is quoted regularly to be approximately 2%. This statistic is based on rough estimates, as there have been few defined population based studies (Christophers 2001). The existence of geographical and racial differences has however been clearly defined with lower prevalence in certain ethnic groups including African, African-Americans, Latin American Indians and Orientals. Sunlight exposure could alter case acquisition or alternatively environmental factors may truly operate. The prevalence of psoriasis is similar in both men and women although women develop the disease earlier especially in the presence of a positive family history (Holgate 1975).

The precise aetiology of PsA and psoriasis remains unclear however most data suggest a combination of genetic and environmental factors.

Genetic contribution- The genetics of PsA and psoriasis are complex with polygenic, multifactorial characteristics of inheritance leading to variable penetrance, familial occurrence and age of onset. They share a common genetic background however there are few studies that have stratified psoriasis patients for the presence of PsA.

In psoriasis, familial aggregation was first noted in 1967 (Montagnani and Rossi 1969). Type 1 psoriasis, characterised by familial early age of onset, has a strong association to MHC genes whereas type 2 occurs sporadically in older individuals with no family history (Henseler 1998). Accordingly, PsA patients with type 1 psoriasis show HLA associations similar to type 1 patients without arthritis but different from those with arthritis and late onset disease (Rahman, Schentag et al. 1999).

Evidence for family and twin studies suggest that psoriasis has a strong genetic component. In a study of more than 3500 families with one, or both parents affected by psoriasis, the lifetime risk of developing psoriasis if none, one or both parents had psoriasis was calculated at 0.04, 0.28 and 0.65 respectively (Swanbeck, Incrot et al. 1997). This risk was increased if there was already an affected child in the family.

Studies on monozygotic twins have shown concordance of psoriasis of 35% to 70% compared with 12% to 20% in dyzygotic twins corresponding to a hereditability of 80% to 90% (Duffy, Spelman et al. 1993). In contrast to dizygotic twins, monozygotic twins

concordant for the disease tend to have a similar disease course, age of onset and body distribution (Brandrup, Holm et al. 1982).

There is much controversy regarding the mode of inheritance of psoriasis. Results from genome-wide linkage studies suggest that it is a multigenic disease with considerable genetic heterogeneity in different groups. In some groups, the disease appears to be caused by a dominantly acting susceptibility allele (Matthews, Fry et al. 1996), whereas in others the pattern of familial occurrence is compatible with an autosomal trait (Swanbeck, Inerot et al. 1995).

Genome wide scans using micro satellite markers have identified various chromosomal regions associated with psoriasis. HLA-B13, HLA-Cw6 and HLA-B57 were the first to be recognised in association with psoriasis. HLA-Cw6 is associated with early disease. (Bhalerao and Bowcock 1998). Subsequent family studies identified different disease associated haplotypes : Cw6-B57, Cw6-B13 and Cw8-B65 in Caucasian patients as well as Cw6-B37 in Japanese and Korean patients suggesting a common psoriasis susceptibility gene/locus close to HLA-C common to these different haplotypes (Jenisch, Westphal et al. 1999). Despite this, only 10% of HLA-Cw6 positive individuals will develop psoriasis. In non-Bantu speaking Western Africans, the incidence of psoriasis is low despite a relatively high frequency of HLA-Cw6 and HLA-B27 (Leder and Farber 1997).

Genome scans have identified a variety of susceptibility loci including chromosome 1q, 3q, 4q and 19q. Weaker associations have been identified such as 16q however this is of particular interest as markers close to chromosome16q have strong associations with ankylosing spondylitis and Crohns disease. Case controlled studies indicate the prevalence of psoriasis is increased in patients with Crohns (Lee, Bellary et al. 1990). Furthermore

patients with psoriasis and Crohns are more likely to develop spondyloarthropathy suggesting 16q may be an important susceptibility marker for PsA.

In 1973 familial aggregation was noted in PsA (Moll and Wright 1973). Data obtained in a Swedish patient cohort indicate that gene(s) located within a susceptibility region near Cw6 determine a distinct phenotype with an early age of onset (Enerback, Martinsson et al. 1997). Few specific HLA associations have been described for PsA per se however associations have been described for peripheral arthritis (B38, B39), symmetrical polyarthritis (DR4), spinal arthritis (B27) and juvenile PsA (A2) (Eastmond 1994). An association has been described between poor prognosis in PsA and HLA B39 (Gladman, Farewell et al. 1998).

The association of HLA and susceptibility to PsA may be due to NK cell interaction with their respective HLA class I ligands via killer Ig-like receptors (KIR). Independent segregation of HLA and KIR genes, along with KIR specificity for particular HLA allotypes raises the possibility that any given individual may express KIR molecules for which no ligand is present. Both activating and inhibitory KIR genes have been described. Increased susceptibility to developing PsA occurs in the presence of activating genes when HLA ligands for homologous inhibitory receptors are missing (Martin, Nelson et al. 2002). In contrast, studies of MHC and T cell receptor gene polymorphisms found no association with disease expression (Sakkas, Loqueman et al. 1990).

Functional cytokine gene polymorphisms (TNFα-302 and TNFβ+ 252) have recently been associated with age of onset of psoriasis, erosive disease and radiological progression supporting the rationale for the use of anti TNF based therapies in patients with severe disease (Balding, Kane et al. 2003).

Environmental influences on immune function are potentially wide-ranging. Infection has proved to be of major interest. Other potential influences however include stress, ultraviolet light, toxins, dietary factors, and radiation exposure. No good epidemiological studies of sufficient power have yet addressed such diversity.

Infection –Infectious environmental triggers such as β-haemolytic streptococci and HIV are closely related to PsA and psoriasis, however, a true pathogenic role for these agents is not proven (Espinoza, Berman et al. 1988) (Vasey, Deitz et al. 1982). Expression of PsA in HIV may be due to T cell dysregulation or regulatory T cell loss. Susceptibility factors for the development of psoriasis and PsA following infection have not been identified; in Sub-Saharan Africa, HIV associated PsA has no association with HLA B27 (Njobvu, McGill et al. 1998).

Trauma -The role of trauma in the aetiology of PsA has been implicated in significantly more cases compared with other forms of arthritis (Punzi, Pianon et al. 1998) (Langevitz, Buskila et al. 1990). The well-characterised Koebner phenomenon is characterised by the appearance of psoriasis following cutaneous trauma. Whether a 'deep' Koebner phenomenon could account for trauma induced PsA has not been proven. As will be discussed later this is an attractive hypothesis to explain enthesial localisation.

Neuroendocrine: Substance P (SP), a powerful vasoactive, pro-inflammatory neuropeptide, may offer an explanation for the association of trauma and arthritis or even for other neuropsychiatric effects. In one case report, a patient with long-standing hemiplegia, developed unilateral psoriatic arthritis and psoriasis on the non-hemiplegic side only

(Veale, Farrell et al. 1993). SP release requires an intact nervous system hence failure to release SP from damaged nerve endings may inhibit the inflammatory response.

In summary however it is currently likely that the best opportunity to resolve aetiology lies in fully evaluating and rationalising the pathogenesis of the psoriatic skin and synovial lesions themselves. This is a strong driver for this thesis.

1.1.3 Clinical aspects

PsA

Despite previous beliefs that PsA is a benign form of inflammatory arthritis, several data demonstrate premature mortality similar to RA (Wong, Gladman et al. 1997). Deaths are attributed to cardiovascular disease (36%), respiratory disease (21%), malignant neoplasm (17%), and to injury or poisonings (14.9%). Furthermore PsA is recognised to cause progressive functional decline associated with considerable social and financial costs (Husted, Gladman et al. 2001). Whether PsA carries the same cardiovascular risks as RA and hence shared vascular risk factors is currently unclear.

PsA develops in 5-25% of patients with psoriasis (Patel, Veale et al. 2001). In 15% of cases, the disease develops simultaneously in skin and joints. In 60% of cases the onset of psoriasis precedes arthritis and in 25% the reverse occurs (PsA sine psoriasis) (Pitzalis 1998). The clinical syndrome is therefore clearly a continuum – this in turn has major implications for pathogenic studies.

The absence of validated criteria for classification is due partly to the heterogeneous clinical features associated with the disease, and the relapsing and remitting nature of both psoriasis and arthritis. Clinical subgroups have been proposed and have proved useful in study of the disease. However, there remain inconsistencies in published data.

Moll and Wright described 5 clinical patterns of joint involvement in the late 1970s (Moll 1979). This classification has largely been abandoned as more recent studies have shown that patients can present with more than one pattern that may change over time (Khan and Gladman 2003). Alternative classifications have been suggested based on the presence of axial disease with or without peripheral joint involvement (Marsal, Armadans-Gil et al. 1999) or the presence or absence of extra osseous manifestations (Helliwell, Marchesoni et al. 1991). Clinical variants of osseous disease in PsA include onycho-pachydermo-periostitis and the SAPHO syndrome. The initial observation that osteoperiostitis of the first DIP joint of the foot was associated with nail disease and destructive joint disease of the feet led to the term psoriatic onycho-pachydermo-periostitis. This variant is characterised by painful onychopathy, soft tissue swelling and bone erosions. There have been 10 cases reported to date in France (Boisseau-Garsaud, Beylot-Barry et al. 1996). There is debate as to whether the SAPHO syndrome (synovitis, acne, pustulosis, hyperostosis and osteitis) is a true variant of PsA or an unrelated condition (Dumolard, Gaudin et al. 1999). Pustulotic arthritis more commonly involves the sternoclavicular joints with monoarticular or oligoarticular peripheral joint involvement.

Characteristic features of PsA include distal interphalangeal (DIP) involvement, enthesopathy, spondylitis and dactylitis. The presence of skin and nail involvement, the absence of rheumatoid factor (RF) and rheumatoid nodules help to differentiate it from RA. Nail lesions indicate a higher risk of psoriatic patients developing DIP involvement. (Cohen, Reda et al. 1999). Nail pitting and onycholysis are observed in 86.5% of patients affected by PsA (Lavaroni, Kokelj et al. 1994). The latter can be differentiated from a fungal infection by analysis of nail scrapings.

There are no diagnostic laboratory tests in PsA. Standard markers such as ESR and CRP are elevated in active disease in some patients only (Helliwell, Marchesoni et al. 1991). Anaemia may arise as a result of chronic disease or from secondary iron deficiency.
Both antinuclear antibodies (ANA) and RF are found in up to 10% of patients with PsA. In this latter subset, patients with symmetrical disease are difficult to differentiate from RA with co-existing psoriasis. Such antibodies also occur in patients with psoriasis uncomplicated by arthritis, and are likely to reflect immune dysregulation rather than specific autoimmunity.

The spectrum of articular presentations in PsA poses particular problems in assessing disease progress and response to drug therapies with implications for clinical trial design and for *ex vivo* pathogenic studies. Despite these drawbacks, such patterns of disease may offer clues to the pathogenesis of joint destruction. Those with severe mutilating arthritis clearly have different bone resorptive processes than those with predominantly axial disease with osteophyte formation. For example the simultaneous process of bone resorption and formation may explain the characteristic radiological 'pencil in cup'

deformities. This variation in clinical phenotype may be explained partly by variable immune pathogenic influences. The expanding field of osteo-immunology will shed light on such possibilities.

Psoriasis

Psoriasis, a chronic relapsing disorder of skin and nails, is the defining extra-articular feature of PsA. Most patients with PsA have the classic psoriasis vulgaris pattern of skin lesions, consisting of well-demarcated erythematous plaques with superficial silver scaling present in extensor aspects of the limbs and trunk. The skin lesions may be minimal or concealed especially in areas such as the scalp, ears, buttocks or umbilicus. While severe psoriasis has been associated with a higher prevalence of PsA (Stern 1985), there is no direct temporal relationship between the severity of skin and joint inflammation.

Psoriasis can be classified by lesional morphology and the presence of pustular and non-pustular forms. Acute and chronic varieties are described. As in PsA, the phenotype may change over time reflecting a spectrum of disease expression. A characteristic feature of psoriasis is the tendency to relapse. While some patients relapse within months or even weeks others may remain in remission for many years. The former tend to develop more severe disease with rapidly enlarging lesions. Disease severity is based on the extent of body involvement, degree of erythema, induration and scale. This provides a composite index of psoriasis area and severity (PASI) that is used primarily in clinical trials to assess therapeutic response.

1.1.4 Imaging in PsA

Radiographic changes in the synovial joints, tendon and ligament insertions, axial skeleton and sacroiliac joints are seen in two thirds of patients with PsA. Plain radiographs of the hands and feet yield most information with soft tissue swelling a common feature. Periarticular osteopaenia and erosions, joint space narrowing and periostitis are other characteristic features. Gross destruction of isolated joints and the occurrence of both joint lysis and ankylosis the so called 'pencil in cup' deformity, are other typical features of PsA (Gladman 1998).

In the axial skeleton, the commonest site of involvement is the sacroiliac joint. Unilateral or marked asymmetrical involvement is seen more commonly than in ankylosing spondylitis (AS) but fusion is uncommon. In the lumbar spine, osteitis and squaring are less common than in AS. In the thoracolumbar spine, paramarginal asymmetric bulky osteophytes are characteristic and may appear in the absence of sacroiliac disease.

The reported frequency of joint involvement in PsA depends on the imaging technique employed. More sensitive techniques such as magnetic resonance imaging (MRI) and ultrasound yield a higher incidence of involvement than conventional radiology (McGonagle, Conaghan et al. 1999) (Mc Gonagle, Gibbon et al. 1999) (Azouz and Duffy 1995) (Balint and Sturrock 2000). MRI is superior to conventional radiology, as it is capable of providing contrasting images of skeletal and soft tissues. The intensity of the emitted resonance signal from any given point in the patient is dependent on several factors including the hydrogen density and unique magnetic properties - T1 and T2 weighting. This property of MRI renders it capable of detecting inflammation in the adjacent bone

marrow and soft tissue around the synovial joint. The MRI appearance of enthesitis is quite distinctive characterised be marked inflammation in adjacent bone marrow and soft tissues (McGonagle, Gibbon et al. 1998). In PsA, MRI has detected enthesitis in clinically uninvolved joints suggesting that enthesitis may be the primary lesion in PsA. This is supported by the observation that enthesial inflammation may extend as far as the synovial cavity. MRI assessment of PsA hand disease has demonstrated 2 distinct patterns- synovial inflammation alone or capsular inflammation with synovitis (Jevtic, Watt et al. 1995). This suggests that PsA hand disease has either an enthesitis-related pathology or one identical to RA. Although differentiation between enthesial and synovial inflammation can be difficult in small joints due to the close proximity of the 2 lesions, fat suppressed MRI is capable of identifying bone oedema at the capsule insertion characteristic of enthesitis (Mc Gonagle, Gibbon et al. 1999). The bone enthesis interface is an area of increased vascularity rendering it susceptible to bacterial colonisation. This may offer a plausible explanation for the clinical features of enthesitis in reactive arthritis. Detailed evaluation of the enthesis from humans with seronegative arthritis may provide insights into the link between infection and arthritis and HLA responses to native antigens (McGonagle, Conaghan et al. 1999).

Ultrasound has also been usefully employed in musculoskeletal medicine since the 1970s (Cooperberg, Tsang et al. 1978). Its technology relies on echoes from pulsed sound waves to provide data for the generation of images. Real-time sonography provides a continuously updated or "live" image while the patient is being scanned. The higher the frequency of ultrasound, the sharper the resolution of the image and greater or lesser depth of tissue evaluated. Frequency is determined predominantly by the choice of the probe,

also known as the transducer. High-frequency probes (7.5 to 10 MHz) are used for scanning tissue close to the surface such as tendons and small joints. Low-frequency probes (3.5 to 5 MHz) are used to scan deep internal structures such as the hip. Ultrasound is being increasingly used as a diagnostic tool and injection aid to rheumatologists. In PsA, it has proven efficacy in the detection and treatment of small joint synovitis (Grassi, Lamanna et al. 1999) and Achilles' tendonitis (Balint and Sturrock 2000). Ultrasound also defines joint and enthesial involvement that is superior to clinical examination (Kane, Greaney et al. 1999).

Non pustular psoriasis	Pustular psoriasis
Type 1 early onset	Generalised
Type II late onset	Von Zumbusch
Guttate	Impetigo herpetiformis
Psoriatic erythroderma	Localised
Drug induced psoriasis	Palmo plantar pustular psoriasis
	Acrodermatitis continua
	Annular pustular psoriasis

Table 1.1 Clinical forms of pustular and non-pustular psoriasis.

The various forms of psoriasis described are broadly divided in to the pustular and non-pustular varieties. There is some overlap between categories and like articular disease, clinical phenotype may vary over time.

Type	Frequency (%)	Characteristics
Asymmetric oligoarticular	70	Affects MCPs, PIPs, DIPs. Sausage digits
Symmetric RA like	15	DIP involvement and negative RF help to differentiate from RA
DIP predominant	5	Nail changes almost invariable
Arthritis mutilans	5	Severely deforming
Spondylitis	5	Sacroiliitis frequently present. Symptoms may be minimal despite marked radiological features

Table 1.2 Classification of PsA (I)

MCP, metacarpophalangeal; PIP, proximal interphalangeal; DIP, distal interphalangeal.

Reproduced from Moll and Wright (1973). This classification was the earliest attempt to characterise PsA. This system has been replaced as it became evident that patterns of disease change over time.

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Peripheral arthritis	Resembling RA but commonly involving the DIP joints. Other features include dactylitis, unilateral limb oedema, enthesopathy and arthritis mutilans
Sacroiliitis and spondylitis	May be asymmetrical and spondylitic features include chunky syndesmophyte and paravertebral ossification
Extra articular manifestations	Includes SAPHO (synovitis, acne, pustulosis, hyperostosis and osteomyelitis) syndrome

Table 1.3 Classification of PsA (II)

Reproduced from Helliwell et al (1991).

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1.2   Current treatment in PsA

The use of disease modifying anti rheumatic drugs (DMARDs) for PsA has been less well studied than in other inflammatory arthritides such as RA. The problems of diagnostic criteria, different disease subsets and the marked placebo effect in this group have hindered therapeutic trials. Furthermore, outcome measures had until recently not been standardised to allow monitoring of therapeutic efficacy. To address this, the PsA response criterion (PsARC) was developed (Clegg, Reda et al. 1996). This is a composite score requiring improvement of two factors (one being joint score) and worsening of none of the following: patient and physician global assessments, tender and swollen joint scores. Prior to the PsARC, outcome measures in PsA clinical trials included a variety of patient reported (early morning stiffness, night pain and patient global assessment), clinician, laboratory (ESR and CRP) and radiographic assessments (Clegg, Reda et al. 1996).

The currently available conventional therapies for PsA include sulphasalazine, methotrexate, cyclosporin, intramuscular gold and leflunomide. These therapies often induce disappointing clinical responses and can be associated with significant toxicity. Since 1964, there have been 12 randomised-controlled trials of disease modifying agents in psoriatic arthritis involving 962 patients, 11 of which have been analysed in a recent Meta analysis (Jones, Crotty et al. 1997) (see table 1.4).

Methotrexate

Intravenous methotrexate was first used to treat PsA in a double blind study of 21 patients (Black 1964). Although the regime was effective, unacceptable toxicity precluded its use in this way. 20 years later, a trial of oral methotrexate was conducted on 37 patients

(Willkens, Williams et al. 1984). Although a significant improvement in physicians' global assessment was detected, there was no effect on swollen joint counts. This study was significantly underpowered and hence interpretation is limited. Gold and methotrexate have been compared in a retrospective analysis of 87 PsA patients (Lacaille, Stein et al. 2000). This study suggested that both agents are efficacious in PsA however methotrexate is superior to gold in terms of clinical response and the ability to reduce concomitant steroid use. This study also suggests early use of DMARDs results in better clinical response in PsA. Moreover gold is likely to be associated with higher toxicity (Hamilton, McInnes et al. 2001).

To address the efficacy and safety of methotrexate in PsA, the ARC/BSR/MRC Clinical Trials Collaberation has initiated a multicentre, placebo controlled trial of methotrexate in Psoriatic Arthritis (MIPA). This study will test the hypothesis that therapy for 6 months with methotrexate will reduce articular and cutaneous disease and improve physical function in patients with active PsA.

Sulphasalazine

Six studies have been performed to examine the efficacy of sulphasalazine in PsA. The two largest studies enrolled a total of 487 subjects (Dougados, vam der Linden et al. 1995) (Clegg, Reda et al. 1996). Those on active treatment showed only just significant improvement over the placebo group in terms of their composite indices however the placebo response was large at 44.6%. Another study revealed an improvement in pain scores but not joint count (Combe, Goupille et al. 1996).

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In a 1 year open study of 34 patients taking sulphasalazine there was an overall favourable response in 67% of patients (Farr, Kitas et al. 1988). Similarly a 6-month placebo controlled study of 39 patients suggested improvements in visual analogue score, early morning stiffness and articular index (Fraser, Hopkins et al. 1993). Another small study showed a significant improvement with sulphasalazine after only eight weeks treatment (Gupta, Grober et al. 1995).

Leflunomide

Leflunomide is a novel immunomodulatory drug that has been licensed for the treatment of active RA in several countries including the U.S.A. and Europe. The active metabolite of leflunomide, A77 1726, inhibits proliferation of activated T and B cells mainly through inhibition of protein tyrosine kinases and the enzyme dihydroorotate dehydrogenase that is involved in the de novo synthesis of pyrimidine nucleotides (Breedveld and Dayer 2000). Other in vitro activities of A77 1726 include the modulation of cytokine production in T cells and neutrophils, suppression of immunoglobulin synthesis in B cells and reduction of MC adhesion, suggesting a therapeutic potential in a broad range of inflammatory and autoimmune disorders. There has been one report of successful treatment with leflunomide in combination with prednisolone 10mg in a 56-year-old woman with a 44-year history of psoriasis vulgaris and a 15-year history of PsA (Reich, Hummel et al. 2002). The role of leflunomide in PsA is not yet clear and controlled trials are ongoing.

Penicillamine

Penicillamine has not been shown to be of benefit in PsA. However the one study performed only enrolled 11 patients therefore interpretation is limited (Price and Gibson 1986).

Gold (Auranofin)

A study of 238 patients on intramuscular gold showed a small but significant benefit in the physician's global assessment (Carette, Calin et al. 1989). A further study comparing auranofin and intramuscular gold to placebo enrolled 82 of which only 51 patients completed the study (Palit, Hill et al. 1990). Only intramuscular gold improved pain score, Ritchie articular index (RAI) and ESR.

Other agents

There is as yet no evidence for the use of azathioprine, hydroxyurea, mycophenolate mofetil or cyclosporin in the treatment of PsA.

Summary

Toxicity and sub optimal efficacy limit currently available conventional therapies in PsA. Clearly there is unmet need that requires to be addressed. Many of the conventional therapies employed thus far in PsA have poorly characterised modes of action and are used based mainly on their clinical efficacy in RA. Their less convincing efficacy in PsA suggests discrete pathogenesis requires to be understood if new therapies are to emerge for PsA. The beneficial effects emerging from anti TNF therapies in PsA and psoriasis indicate

that elucidation of the pro inflammatory pathways may yield further therapeutic targets. This provides powerful rationale for the studies contained in this thesis.

Study	Year	Drugs	Duration of disease (years)	Number of cases	Conclusion
Black	1964	MTX (iv)	2	21	Reduced SJC and ESR
Levy	1972	AZA	12	6	Intra group improvement with AZA
Wilkens	1984	MTX(oral)	3	37	Reduced PGA but o effect on SJC
Price	1986	D-Pen	4	11	No significant effect
Carnette	1989	Aur	6	238	Reduced PGA but no effect on SJC
Palit	1990	Aur vs. im gold	6	82	Pain score, RAI and ESR improved with im gold
Farr	1990	SZP	6	30	EMS, RAI and ESR improvement with SZP
Fraser	1993	SZP	6	39	Pain score and RAI improvement
Gupta	1995	SZP	2	24	EMS. PGA improvement
Dougados	1995	SZP	6	136	Reduction in pain scores
Clegg	1996	SZP	6	221	No significant difference
Combe	1996	SZP	6	117	Reduced pain scores

MTX, methotrexate; SZP, sulphasalazine; AUR, auranofin; EMS, early morning stiffness; RAI, Ritchie articular index; PGA, physician global assessment; SJC, swollen joint count.

Table 1.4 Randomised controlled trials in PsA

1.3   The role of arthroscopy in synovial biology

Insights into synovial biology have advanced rapidly since the introduction of synovial biopsy techniques. Early research using a blind needle biopsy approach provided conflicting results due to inter observer variation. For this reason the European Synovitis Study Group (ESSG) was formed at the European League Against Rheumatism (EULAR) meeting in Amsterdam in 1995. Specifically their aims were to provide guidelines for training, to develop protocols for tissue selection and preparation and to standardise methods for quantification of immunohistological and pathological features of synovial inflammation. The group is answerable to EULAR and has collaborations with North America and Australia. The synovial response to various DMARDs is emerging as a method of quantifying response to treatment and as such may become a more popular tool amongst rheumatologists in the future (Bresnihan, Tak et al. 2000).

Synovial tissue can be obtained by 2 methods: blind needle biopsy from the suprapatellar pouch or under direct vision using a needle arthroscope. Although the latter is more expensive and time consuming, it has the advantage of providing larger samples for tissue analysis. In addition the tissue provides a better representation of the overall synovium as 5 separate biopsies are processed as a single block. It also allows complete irrigation of the joint cavity prior to steroid injection, which may result in greater efficacy (Weitoft and Uddenfeldt 2000). Finally blind needle biopsy tends to underestimate the intensity of macrophage infiltration in areas adjacent to cartilage (Youssef, Kraan et al. 1998) whereas needle arthroscopy allows direct visualisation of the cartilage pannus junction (CPJ).

1.4   Immunopathogenesis of PsA and psoriasis

1.4.1   Normal synovium

The normal SM is a discrete organ with its own circulation, lymphatic system and innervation. It contains a lining layer of up to 3 cells thickness consisting of macrophages and fibroblasts measuring 25-35 μm in depth. Superficial type A synoviocytes are CD68$^+$ and non-specific esterase (NSE) positive bone marrow derived cells of the monocyte/macrophage lineage. Type B synoviocytes are of fibroblast origin, derived from local proliferation of adjacent underlying stromal cells. Type B cells form a loose boundary between lining layer and deeper highly vascular fibrous connective tissue that contains few cells. The absence of a basement membrane allows traffic of nutrients and waste products between plasma and SF. Although there is evidence of MHC class II expression in the lining layer and deeper macrophage like cells, there is little evidence of ongoing inflammation in the normal synovium.

1.4.2   SM in PsA

In contrast, PsA synovium is characterised by a sub lining layer MC infiltration with predominantly CD4$^+$/CD45RO$^+$ and CD8$^+$/CD45RO$^+$ T cells. The lining layer is thin compared with RA but still consists of 4-6-cell depth. Increased vascularity is observed with a reduction in E-selectin expression (Veale, Yanni et al. 1993). Such differences in histopathologic features are associated with variation in cytokine expression such as IL-2

(Wong, Howell et al. 1996) (Howell, Warren et al. 1991) suggesting fundamental differences in the pathogenesis of seronegative arthropathies.

Further differences between the macroscopic vascular appearances of PsA SM compared with RA have been described. In PsA SM, blood vessels appear tortuous and bushy vessels whereas RA SM has mainly straight, branching vessels (Reece, Canete et al. 1999).

1.4.3   Cutaneous changes in psoriasis

Histologically, established psoriatic lesions demonstrate marked epidermal hyperplasia, parakeratotic scale, and microabscesses (Munro microabscesses) of neutrophils within the superficial epidermal layers (Ebling 1999). An early cellular event in the development of psoriatic lesions is the infiltration of target sites by activated memory T cells, neutrophils, macrophages and dendritic cells. This cellular infiltration in turn produces inflammatory mediators, such as IFNγ that can induce epidermal hyperplasia, and may act with keratinocytes and dermal macrophages to sustain a cycle of inflammation that finally leads to the psoriatic phenotype.

The beneficial effects of CTLA4 Ig that binds to and blocks CD28 support a probable role for co stimulation in psoriasis pathogenesis. In 43 patients who received 4 infusions of the soluble chimera protein CTLA4Ig (BMS-188667) 46% achieved a 50% or greater sustained improvement in clinical disease activity with progressively greater effects in the highest-dosing cohorts. Improvement was associated with quantitative reduction in epidermal hyperplasia, which correlated with quantitative reduction in skin-infiltrating T

cells. No markedly increased rate of intralesional T-cell apoptosis was identified, suggesting that the decreased number of lesional T cells was probably attributable to an inhibition of T-cell proliferation, T-cell recruitment, and/or apoptosis of antigen-specific T cells at extralesional sites (Abrams, Lebwohl et al. 1999) (Abrams, Kelley et al. 2000).

The temporal relationship of streptococcal infection and guttate psoriasis has led some investigators to examine the role of infection in pathogenesis (Henderson and Highet 1988). This association has generated interest in the possible role of Toll-like receptors (TLR) that identify microbial antigens as a first line of defence by the innate immune system and as such may trigger the initial event in an abnormal immune response.

1.4.4 Involvement of T lymphocytes in PsA and psoriasis

Whereas T lymphocytes lie central to the regulation of the normal immune response, dysregulation of T cell may lead to disease pathogenesis. The majority of rheumatoid synovial T cells are of $CD4^+$ helper subset found in perivascular aggregates that occasionally contain germinal centres. $CD8^+$ cells are found in the transitional zone surrounding aggregates or scattered throughout the interstitium. Synovial T cells are predominantly $CD45RO^+$, $CD45RA^-$ indicating differentiation within the memory subset. They express activation markers such as MHC class II particularly HLA-DR4 and several adhesion molecules including ICAM-1, together with integrins VLA-1, VLA-4 and LFA-1 Only a small number of synovial T cells are in cell cycle (Bonvoisin, Cordier et al. 1984). The accumulation of synovial T cells is likely to represent preferential migration of memory T cell into the inflamed synovium.

Evidence from several sources strongly indicates psoriasis and PsA are T cell mediated diseases.

- In psoriasis T cell activation and T cell receptor repertoire skewing are demonstrated in skin (Nikaein, Morris et al. 1993).

- In PsA, the SM contains large populations of activated memory T cells (CD4 and CD8 compartments). These lymphocyte populations demonstrate a predominance of CD8⁺ lymphocytes with restricted T cell Vβ gene expansions suggesting a specific response to a particular antigen (Costello, Winchester et al. 2001). (Braathen, Fyrand et al. 1979). Moreover some similarities with recognition by cutaneous T cells of the same epitope exists in studies in which T cell populations in skin and joint have been compared in the same patients (Tassiulas, Duncan et al. 1999).

- Langerhans cells isolated from psoriatic epidermis are potent APCs and weak antigen processing cells (Baadsgaard, Gupta et al. 1989). Such Langerhans cells have been shown to induce T lymphocytes to produce a range of cytokines that modulate cutaneous inflammation.

- T cell directed therapies have been shown to be of limited benefit in the treatment of psoriasis (Ortonne 1996)

- Alefacept, a fully human lymphocyte-function associated antigen 3 (LFA-3/IgG fusion protein that blocks CD2/LFA-3 interaction has beneficial effects in PsA. Treatment for 12 weeks resulted in a significant decrease in DAS associated with a reduction in synovial T cell/ macrophage infiltration in serial biopsies (Kraan, van Kuijk et al. 2002). Modified anti-CD3 therapy in PsA has reached phase II clinical trials with 75% improvement in swollen joint counts in 6 of 7 patients after 1 month of treatment (Utset, Auger et al. 2002). These data further support that T cell activation plays an

important role in pathogenesis of PsA. Benefits have also been observed in clinical trials in psoriasis (da Silva, Brickelmaier et al. 2002).

- Blockade of CTLA-4 (cytotoxic T lymphocyte-associated antigen-4 or CTLA-4), expressed on activated T cells reverses the cellular pathology of psoriatic plaques, including the activation of keratinocytes, dendritic cells, and endothelial cells (Abrams, Kelley et al. 2000).

1.4.5   Functional T cell subsets

Almost 30 years ago, functional heterogeneity in CD4$^+$ cells was recognised (Liew and Parish 1974). Subsequently Th1 and Th2 type T cells were classified based on their cytokine production (Mosmann, Cherwinski et al. 1986). Whereas Th1 cells secrete IL-1, IFNγ IL-17and TNFβ, Th2 cells secrete IL-4, IL-5, IL-10 and IL-13. Th1 responses are efficient in eliminating intracellular pathogens via macrophage activation. Th2 responses effect humoral immune responses to persistent antigen. Most immune responses involve contributions from both Th1 and Th2 cells that cross regulate each other's effector functions. Thus a balance between Th1 and Th2 cytokines can determine whether the immune response is appropriate or will result in detrimental immunopathogenesis. Overproduction of Th1 cytokines has been implicated in delayed type hypersensitivity reactions and autoimmune diseases whereas Th2 cytokines recruit eosinophils and mast cells leading to allergic and inflammatory conditions (Romagnani 1994).

The differentiation of Th1 and Th2 responses from precursor Th cells (Thp) depends on a variety of complex developmental processes. The dose of antigen, density of the antigen peptide: MHC complex and co stimulation all influence the initiation of Th differentiation. Recent data suggest vasoactive intestinal peptide may drive Th2 responses (Delgado 2003). The observation that naive antigen activated Thp could be induced to differentiate into Th1 or Th2 lineage in vitro by the addition of exogenous cytokine was an important development in understanding the regulation of Th1/Th2 responses. Cytokines (TNFα, IL-1, IL-4, IL-6, IL-12 IL-18 and IFNγ) play central roles in initiating and regulating the overall immune system. These cytokines are not only involved in regulating the innate immune system but are also critical to the development and propagation of acquired immune responses. The overlap in functions of many cytokines underscores the close relationship between innate and acquired immunity.

The ability of a Th cell to respond to both autocrine and exogenous cytokines depends on their ability to regulate expression of certain receptors e.g. IL-4 directly down regulates IL-12 receptors whereas IL-12 produced by APCs results in a positive feedback loop of IFNγ production that subsequently down regulates IL-4 receptor expression. In the absence of IL-12, IL-4 rapidly down regulates IL-12 receptors an effect amplified by IL-10. Thus Th2 cytokines serve a default pathway with Th1 responses depending on sufficient concentrations of IL-12 and IFNγ to combat IL-4 and IL10.

Transcriptional factors that regulate cytokine gene expression have also been elucidated. IL-12 via Stat-4 activation induces transcription factor T-bet whereas IL-4 via Stat-6 activation induces GATA-3. The expression of GATA-3 and T-bet during Th1 and Th2 differentiation is mutually exclusive (Rengarajan, Szabo et al. 2000). Subsequent studies

are investigating the consequences of such exclusivity since this offers an obvious therapeutic intervention for specific arms of the acquired T cell response.

The relative expression of Th1/Th2 cytokines has been employed to determine the predominant Th response in disease states. One of the difficulties in identifying a Th1 response via IFNγ detection is the low number of spontaneous IFNγ producing cells that may be directly visualised in inflammatory lesions. Consequently, functional polarity is usually inferred from relative expression of cytokine mRNA within lesions, or by quantification at the protein level of IFNγ / IL-4 / IL-5 ratios after mitogen stimulation. The ability to phenotype Th responses has been considerably advanced by the discovery of markers capable of identifying respective subsets. Th1 cells express CCR5 (Loetscher, Uguccioni et al. 1998), IL18R (Xu, Chan et al. 1998), and IL-12β2 whereas Th2 cells express predominantly CCR3, CCR8 and ST2L (Xu, Chan et al. 1998). Although expression of these markers is transient, their existence lends credence to the Th1/Th2 dichotomy in the human immune system. It is possible to critically evaluate the contribution of Th1 and Th2 cells in human disease states using these specific cells surface markers and the identification of IL-18R on RA synovial T cells indicates the utility of this approach ex vivo (Xu, Chan et al. 1998).

The foregoing is now of fundamental importance in evaluating disease state pathogenesis. The elucidation of phenotype and regulation of Th response in disease states such as psoriasis and PsA may offer therapeutic intervention. Manipulation of the Th1 response via the administration of cytokines of reciprocal activity such as IL-10 is clinically beneficial in both articular and cutaneous disease (McInnes, Illei et al. 2001). Furthermore, the

beneficial effects of fumarates in psoriasis are achieved via downregulation of IFNγ expression by circulating blood cells (Litjens, Nibbering et al. 2003).

1.4.6   Fibroblasts

The normal synovium contains mesoderm fibroblasts also known as type B lining cells. Fibroblast like synoviocytes (FLS) are prominent in the proliferating synovium during chronic synovitis. These cells are a source of proinflammatory cytokines and growth factors thus contributing to the inflammatory process within the synovium. In contrast to dermal fibroblast cultures from normal skin, synovial fibroblasts are capable of providing co stimulatory activity for activation of resting T cells (Looney, Hooper et al. 1995).

Psoriatic fibroblasts, cultured from involved skin and synovium exhibit a greater proliferative response to growth factors compared to normal fibroblast (Espinoza, Aguilar et al. 1994) and as such may be key players in the inflammatory response. Indeed RA synovial fibroblast expression of IL-18 has previously been described and such expression is up regulated by TNF-α and IL-1β (Gracie, Forsey et al. 1999). Furthermore the ability of synovial-derived fibroblasts to secret IL-6 and MMPs in response to cytokine stimulation such as IL-17 may elucidate a mechanism for cartilage damage in PsA.

1.4.7 Cytokine production in PsA and psoriasis

Large numbers of cytokines involved in regulating the acquired immune system are necessary to obtain a fine level of control to meet the immunological needs of the host to respond to a variety of external pathogens.

TNFα

The identification of TNFα as a key molecule in the proinflammatory cascade has resulted in a better understanding of how dysregulation of the immune system leads to synovial inflammation. Lesional and systemic elevated levels of TNFα have been found in psoriasis patients with and without arthritis. Furthermore, increased levels of TNFα are found in the PsA SF compared with OA but less than RA (Partsch, Steiner et al. 1997).

Clinical trials employing anti TNFα have resulted in clinical benefits in both psoriasis and PsA. Humanised anti TNFα monoclonal antibody (infliximab) was used initially in a psoriatic patient who was suffering also from an inflammatory bowel disease. A dramatic improvement in skin lesions was observed two weeks after a single infusion (Oh, Das et al. 2000). Good activity has also been reported in combination with methotrexate (Kirby, Marsland et al. 2001). Recently, the efficacy and safety of infliximab monotherapy has been demonstrated in a controlled randomised trial. 33 patients with moderate to severe plaque psoriasis received a placebo or the monoclonal antibody at two dosages intravenously at weeks zero, two and six. 82% of patients in the 5 mg /kg group and 73% in the 10 mg/kg group had at least a 75% improvement in PASI score. The mean PASI decreased from 22.1 to 3.8 (5 mg /kg) and 26.6 to 5.9 (10 mg/ kg) by week ten. The mean time to response was four weeks (Chaudhari, Romano et al. 2001).

Similar efficacy and safety data on TNFα targeted therapies are available for PsA (Mease 2002). 60 patients were assessed in a randomised double blind placebo controlled trial of etanercept. Over the 12-week study, 87% of the etanercept treated group met the response criteria (PsARC) compared with 23% in the placebo group. In addition, the severity of psoriasis as measured using the PASI, improved by 46% in the treatment group compared with 9% in the placebo group. There were approximately 30% non-responders in this group (Mease, Goffe et al. 2000).

There have been additional reports of success with anti TNFα therapy in spondyloarthropathies (Brandt, Haibel et al. 2002). An open labelled study included 21 treatment resistant patients with various subtypes of spondyloarthropathy 9 of whom had PsA. They received 3 infusions of 5mg/kg infliximab at 0, 2 and 6 weeks. Although concomitant NSAIDs and corticosteroids were allowed, other DMARDs were discontinued 4 weeks prior to the study. Rapid and significant improvements were noted throughout the treatment period and were maintained for up to 6 weeks following the last infusion (Van den Bosch, Baeten et al. 2001).

The FDA in USA has approved etanercept in PsA however UK approval is awaited. The identification of other regulatory factors in the pro inflammatory cascade may therefore offer therapeutic potential.

Interferon γ

In contrast to easily detectable macrophage derived cytokine, T cell derived cytokine has been more difficult to elucidate. Significantly elevated expression of IFNγ was demonstrated in only 2 out of 10 PsA patients (Partsch, Wagner et al. 1998). This is in contrast to other reports of cytokine production and mRNA expression in synovium in PsA compared with RA and OA (Ritchlin, Haas-Smith et al. 1998). However, the issue regarding the role of IFNγ in PsA remains controversial. One study indicated that Th1 response was of less magnitude in seronegative arthropathies than in RA (Canete, Martinez et al. 2000).

Although interferons (IFN) were initially described on the basis of their antiviral activity, these cytokines have profound effects on the immune system. IFNs can be divided into two broad categories. Numerous IFNs exist varying on the basis of their cell of origin, structure and functional effects. There are more than 30 subtypes of Type I IFNs (α and β) that virally infected cells secrete. In contrast to α and β interferons, there is only a single type of type II interferon (IFNγ) that is produced by Th1 effector cells in response to immune stimuli and by NK cells in response to IL-12. IFNγ was first identified in 1966 (Wheelock 1966). It is a polypeptide composed of 166 amino acids including a signal sequence that is cleaved to form a biologically active 143 amino acid sequence that forms a 34kD homodimer.

IFNγ interacts with a specific receptor composed of 2 chains. The α chain is a 90kD binding chain and the β chain is a 314 amino acid signalling chain. Each chain signals through Janus kinase (JAK) - α through JAK1 and β through JAK2. IFNγ initially binds to 2 α chains that dimerise resulting in transphosphorylation and reciprocal activation of the JAKs. The activated JAKs then phosphorylate the tyrosine residues on the α chains which

in turn activates signal transduction and translation proteins (STATs). STAT homodimers than translocate to the nucleus where they bind to DNA sequences encoding gamma activated sequences (GAS).

IFNγ plays an important role in T helper cell development. IL-18 (interferon-inducing factor) and IL-12 exhibit a marked synergism in IFNγ induction by T cells (Okamura, Kashiwamura et al. 1998).

IFNγ protects the host against exogenous pathogens by maximising antigen presentation via up regulation of co stimulatory molecules CD80 and CD86 on macrophages, T and B cells and dendritic cells.

T cells stimulated by IFNγ activate macrophages to produce superoxide radicals such as hydrogen peroxide and nitric oxide that have antimicrobial and anti viral properties. This results in the non-specific eradication of exogenous pathogens from the host.

As well as having a key role in regulating subsequent immune responses, IFNγ has effects on bone remodelling. Bone remodelling is a dynamic process regulated by the opposing actions of osteoclasts (bone resorption) and osteoblasts (bone formation). Osteoclastogenesis, the process of bone resorption is enhanced by the RANK/RANKL (receptor activator of NFκB) interaction. It has been demonstrated that IFNγ can inhibit osteoclastogenesis by inducing the rapid degradation of the RANK adapter protein TRAF6 (tumour necrosis factor receptor associated factor 6) (Takayanagi, Ogasawara et al. 2000). Therefore diseases in which IFNγ is over expressed may exhibit features of new bone formation.

Between 1988 and 1998, IFNγ was administered to more than 2000 patients with either RA or juvenile RA. Most were double blind placebo controlled studies. The rationale for using IFNγ as a therapy in RA was based on pre clinical trials in which IFNγ antagonised the effects of IL-1 on monocytes, chondrocytes and synovial cells including inhibiting PGE2, IL-1β and collagenase production. In addition IFNγ inhibited IL-15 induced IL-8 from human monocytes. Although 1-year data suggested an improvement in disease activity indices with reduction in corticosteroid requirements (Sprekeler, Lemmel et al. 1990), 5-year follow up data revealed that 67% of patients had withdrawn from treatment due to lack of efficacy with only 11% remaining on therapy (Lemmel, Gaus et al. 1991).

IFNγ has also been employed as a treatment for PsA (Fierlbeck and Rassner 1986). In this study, 10 of 15 patients treated with subcutaneous recombinant IFNγ had relief of joint pain within 28 days. However no further improvement was observed after the third month, and patients classified as responders in the first month showed a deterioration of the disease with continuing treatment (Fierlbeck and Rassner 1990). Furthermore, in 10 of 42 patients, psoriatic lesions developed at the injection site (Fierlbeck, Rassner et al. 1990).

Interleukin 18

Interleukin-18 (IL-18), a member of the interleukin-1 cytokine superfamily, is now recognised as an important regulator of both innate and acquired immune responses. IL-18 is expressed at sites of chronic inflammation, in autoimmune diseases, in a variety of cancers and in the context of numerous infectious diseases.

IL-18, previously known as IFN-γ Inducing Factor (IGIF), was identified as an endotoxin-induced serum factor that stimulated IFN-γ production by murine splenocytes (Nakamura,

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Okamura et al. 1989). IL-18 was cloned from a murine liver cell cDNA library generated from animals primed with heat-killed *Propionibacterium acnes* and subsequently challenged with LPS (Okamura, Tsutsi et al. 1995). Nucleotide sequencing of murine IL-18 predicted a precursor polypeptide of 192 amino acids lacking a conventional signal peptide, and a mature protein of 157 amino acids. Subsequent cloning of human IL-18 cDNA revealed 65% homology with murine IL-18 (Ushio, Namba et al. 1996) and showed that both contain an unusual leader sequence consisting of 35 amino acids at their N-terminus.

IL-18 expression has been reported in macrophages, dendritic cells (DC), Kupffer cells, keratinocytes, osteoblasts, adrenal cortex cells, intestinal epithelial cells, microglial cells and synovial fibroblasts. IL-18, like IL-1β with which it shares structural homology, is produced as a 24 kD inactive precursor lacking a signal peptide (pro-IL-18). Pro-IL-18 is cleaved after Asp35 by the endoprotease IL-1β converting enzyme (ICE, caspase-1) to generate a biologically active mature 18 kD moiety (Gu, Kuida et al. 1997) (Ghayur, Banerjee et al. 1997).

Like that of IL-1, the IL-18R complex is a heterodimer containing an α (IL-1Rrp) chain responsible for extracellular binding of IL-18 and a non-binding, signal transducing β (AcPL) chain (Torigoe, Ushio et al. 1997) (Hoshino, Tsutsui et al. 1999). Both chains are required for functional IL-18 signalling (Born, Morrison et al. 2000). IL-18R is expressed on a variety of cells including macrophages, neutrophils, NK cells, endothelial and smooth muscle cells (Hyodo, Matsui et al. 1999; Leung, Culshaw et al. 2001; Afkarian, Sedy et al. 2002). The IL-18R complex can be upregulated on naïve T cells, Th1 type cells and B cells by IL-12 (Hoshino, Tsutsui et al. 1999) (Yoshimoto, Takeda et al. 1998). IL-18Rα retention on the membrane of mature Th1 cells serves as a marker for the latter in humans

and murine systems (Xu, Chan et al. 1998). In contrast, T cell receptor (TCR) ligation in the presence of IL-4 results in downregulation of the IL-18R (Smeltz, Chen et al. 2002). Modulation of this complex is therefore likely to be functionally significant. Consistent with this, administration of anti-IL-18Rα antibody *in vivo* results in reduced LPS induced mortality associated with a subsequent shift in balance from a Th1 to a Th2 immune response (Xu, Chan et al. 1998).

Although originally identified as a factor capable of inducing IFN-γ production by murine splenocytes, the effector role of IL-18 is rapidly expanding. Consistent effects on lymphoid series, particularly Th1 lineage in combination with IL-12 have emerged (Okamura, Tsutsi et al. 1995). Thus IL-18 enhances T and NK cell maturation, cytokine production and cytotoxicity (Okamura, Tsutsi et al. 1995) (Yoshimoto, Takeda et al. 1998) (Micallef, Ohtsuki et al. 1996) (Dao, Mehal et al. 1998). IL-18 also increases FasL on NK cells and consequent Fas-FasL-mediated cytotoxicity (Dao, Ohashi et al. 1996) (Tsutsui, Nakanishi et al. 1996). IL-18 deficient mice have reduced NK cell cytolytic ability that can be restored by exogenous IL-18 (Takeda, Tsutsui et al. 1998). However, together with IL-2, IL-18 coinduces IL-13 in murine T and NK cells and in the presence of TCR activation induces T cell IL-4, IL-10, IL-13 and IFN-γ production (Hoshino, Wiltrout et al. 1999). In isolation IL-18 induces high IgE expression by B cells and in combination with IL-2, anti-CD3 and anti-CD28 markedly enhances IL-4 production by CD4^{+}T cells (Yoshimoto, Mizutani et al. 2000). When cultured alone or in combination with IL-4, IL-18 is known to induce murine T cell Th2 differentiation. This however is dependent upon genetic influences as spleen cells from BALB/c and C56BL/6 strains of mice stimulated with anti-CD3 and IL-18 exhibit enhanced Th2 and Th1 responses, respectively (Xu, Trajkovic et al.

2000). Thus IL-18 can promote Th1 or Th2 lineage maturation dependent upon underlying genetic influences and the ambient cytokine milieu.

Keratinocytes, traditionally thought to produce but not process IL-18 (Mee, Alam et al. 2000), have now been shown to secrete biologically active IL-18 when treated with dinitrochlorobenzene and pro-inflammatory mediators such as LPS (Naik, Cannon et al. 1999). In addition to keratinocytes, Langerhans cells (LC) also produce IL-18, which in turn contributes to the regulation of LC migration (Cumberbatch, Dearman et al. 2001).

IL-18 expression and effector function have now been described in inflammatory diseases across a broad range of tissues. We have focussed on recent key examples of a pro-inflammatory role for IL-18.

Data from studies in RA clearly indicate that IL-18 and its receptor system are present in inflammatory synovitis (Gracie, Forsey et al. 1999) (Yamamura, Kawashima et al. 2001) (Tanaka, Harigai et al. 2001). Its functional activities include promotion of cytokine release (particularly TNF-α, GM-CSF and IFN-γ). Marked synergy with IL-12 and IL-15 is observed in this respect. IL-18 acts not only through lymphocyte activation, but also through direct effects on macrophages. IL-18 expression is in turn upregulated in FLS by IL-1β and TNF-α, suggesting the existence of positive feedback loops linking monokine predominance in RA with innate cytokine production and Th1 cell activation in synovial immune responses. IL-18 induces NO release by RA SM *in vitro* that, since NO inhibits caspase-1 activity, provides a further potential regulatory loop. IL-18 possesses pro-degradative effects in articular cartilage. IL-18 reduces chondrocyte proliferation, upregulates iNOS, stromelysin and cycloxygenase 2 expression and increases glycosaminoglycan (GAG) release *in vitro*. Such activities may be IL-1β independent although contradictory data have also emerged (Lubberts, Joosten et al. 2001). IL-18

further promotes synovial chemokine synthesis and angiogenesis (Morel, Park et al. 2001) (Park, Morel et al. 2001). Finally, IL-18 effects are not necessarily detrimental. IL-18 inhibits osteoclast maturation through GM-CSF production by T cells, thereby retarding bone erosion (Horwood, Udagawa et al. 1998). Suppression of COX expression may also be mediated through IFN-γ production with consequent effects upon prostanoid mediated local inflammation.

The process whereby IL-18 and IL-1β are released from the cytosol are unknown. Both molecules are regulated post transcriptionally, and a particularly important aspect of regulation occurs at the level of release.

In this context, recent studies show that this release pathway can be rapidly induced by stimulation with exogenous ATP. In macrophages, exogenous ATP works via the recently cloned P2X7 receptor, a member of the P2X family of nucleotide-gated channels that are activated by extra cellular ATP.

P2X7 is a 595-amino acid polypeptide with two membrane-spanning domains and intracellular N- and C-terminal domains.

P2X7 receptor is the only pore-forming P2X family member, and its activation results in the opening of a cationic channel with increased permeability to calcium and intracellular depolarisation.

Recent studies have shown that monocytes express functional P2X7 receptors that modulate IL-1α and IL-18 processing and release in response to ATP (Mehta, Hart et al. 2001).

IL-18 has been targeted in several arthritis models *in vivo*. Upon challenge with type II collagen (CII) in CFA, IL-18-deficient mice on a DBA/1 background exhibit reduced incidence and severity of arthritis. *Ex vivo* analysis determined that both cellular and humoral responses to CII were suppressed (Wei, Leung et al. 2001) (Leung, McInnes et al. 2000). Moreover, administration of recombinant IL-18 can replace the requirement for CFA in CII induced erosive arthritis in DBA/1 mice (Leung, McInnes et al. 2000). Neutralisation of IL-18 *in vivo* using specific antibodies or IL-18BP effectively reduces developing and established rodent arthritis in both streptococcal cell wall and CIA models (Lubberts, Joosten et al. 2001) (Plater-Zyberk, Joosten et al. 2001). Such effects may operate independent of IFN-γ (Lubberts, Joosten et al. 2001). A feature of both models is suppression not only of inflammation but also of matrix destruction. These data strongly suggest that the net effect of IL-18 expression is pro-inflammatory, at least in the context of antigen-driven articular inflammation. Clinical studies to test this hypothesis in RA are awaited.

The position of IL-18 in the functional hierarchy of pro-inflammatory cytokines in chronic inflammation is not fully resolved, although there is consensus that it plays a critical early role. Nevertheless, IL-18 appears able to modulate inflammation at multiple check points, acting not only on initiation and expansion of putative auto-reactive Th1 responses but also via direct effects on multiple cellular targets, including macrophages, lymphocytes and target host tissue cells, including endothelial cells and fibroblasts. As such it may offer a therapeutic target for the treatment of inflammatory disease states.

IL-12 initially designated as natural killer cell stimulatory factor was originally identified as a product of Epstein-Barr virus transformed lymphoblastoid B cell lines that mediate several biological activities on human T and NK cells including induction of IFNγ and enhancement of cell-mediated cytotoxicity (Kobayashi, Fitz et al. 1989).

It is a 70kD disulphide-linked heterodimer consisting of 35kD (p35) and 40kD (p40) sub units. Although p35 transcripts are found in many tissue types, the p40 subunit is only found in tissues that express the whole IL-12 p70 molecule. No biologic activity is observed with either sub unit alone however the p40 subunit can be synthesised in large amounts to act as a natural inhibitor of IL-12.

IL-12 is predominantly produced by monocytes and macrophages but also by several other cell types including Langerhans cells, keratinocytes, dendritic cells and neutrophils (Trinchieri 1994).

Separate genes on separate chromosomes encode the two proteins and expression of each is independently regulated. The structure of p35 is homologous to other cytokines whereas p40 is related to members of the cytokine receptor super family.

The biologic activities of IL-12 are mediated via specific high affinity receptors that are up regulated on T and NK cells following activation. One subunit of the IL-12 receptor complex was previously cloned and when expressed in COS cells bound IL-12 with low affinity. Because of its structural homology with the β subunit of the IL-6 receptor it was termed IL12Rβ. Shortly afterwards a second subunit of the IL-12 receptor complex was cloned designated IL12Rβ2 and the previously identified subunit renamed IL12Rβ1. Co expression of both receptors in COS cells resulted in the formation of both high and low affinity binding sites (Presky, Gubler et al. 1995).

Among the biologic activities of IL-12 are its ability to promote the maturation of Th1 cells to produce IFNγ by resting and activated T, NK and NKT cells. It also enhances the lytic activity and proliferation of T cells and NK cells. Furthermore IL-12 is reported to act synergistically with IL-2 on activated B-lymphocytes to enhance immunoglobulin secretion (Jelinek and Braaten 1995).

In collagen-induced arthritis, administration of IL-12 accelerates disease onset and increases disease severity characterised by granulocyte influx increased expression of TNFα and IL1β mRNA (Joosten, Lubberts et al. 1997). In the same model, IL-12 deficient mice have less severe disease (Malfait, Butler et al. 1998).

In PsA, elevated levels of IL-12 have been found in SF and serum in association with IL-13 (Spadaro, Rinaldi et al. 2002). In psoriasis increased protein expression in association with dendritic cells, macrophages and nerve endings has been described. (Turka, Goodman et al. 1995). Enhanced IL-12 p40 mRNA signals have been detected in lesional psoriatic skin as compared with normal and non lesional psoriatic skin. Furthermore, immunoreactivity for IL-12 p70 was markedly increased in the psoriatic skin lesions and was predominantly expressed on MCs in the dermis (Yawalkar, Karlen et al. 1998).

Macrophage associated IL-12 expression has been described in RA and OA SM by immunohistochemistry (Sakkas, Johanson et al. 1998). However IL-12 p40 mRNA expression was increased in RA synovial tissues compared with OA (Morita, Yamamura et al. 1998).

The IFNγ inducing properties of IL-12 has led to its use in clinical trials in cancer and HIV to enhance tumoricidal activity of NK cells and augment the Th1 response respectively. Unfortunately toxicity was a major limiting factor requiring significant dose reduction.

In contrast anti IL-12 would seem a reasonable treatment for conditions driven by Th1 responses by limiting the production of IFNγ. Unfortunately therapies that limit IFNγ production have had little impact thus far. Interestingly there is evidence that established DMARDs (auranofin and sulphasalazine) may exert their anti inflammatory effects through inhibiting IL-12 driven Th1 mediated disease (Kim, Kang et al. 2001) (Hasko, Szabo et al. 2001) (Kang, Chung et al. 1999).

Interleukin 17

IL-17 was first identified as a rodent cDNA transcript, termed CTLA8, isolated from an activated T-cell hybridoma (Yao, Fanslow et al. 1995). It has a leader peptide and is readily secreted from T cells. As in the case of IL-10, there is a viral homologue of IL-17, which is biologically active. Viral IL-17 is part of the herpes virus-13 genome.

IL-17 is a T cell derived pro inflammatory cytokine stimulating a wide variety of cell types. From T cell clones established from RA synovium, virtually all Th0 and Th1 cells produce IL-17 whereas no Th2 clones produce IL-17 (Aarvak, Chabaud et al. 1999).

The receptor for IL-17 is unique in that the extracellular domains are not related to any of the known cytokine receptor families. The extracellular domains contain several cysteine residues that are not usually found in cytokine receptors. The IL-17 receptor is not a member of the immunoglobulin family of receptors and is unique in possessing a long cytoplasmic domain. IL-17 receptor mRNA is present in nearly all cells.

IL-17, like other pro inflammatory cytokines, signals through NFkB and requires TRAF6. As such, embryonic fibroblasts derived from TRAF6 deficient mice fail to induce expression of IL-6 and ICAM-1 following stimulation with IL-17 despite the presence of

IL-17 receptors comparable to those on wild type control cells (Schwandner, Yamaguchi et al. 2000).

Although TNFα inhibition has led to clinical benefits in terms of inflammation, the anti erosive effects have not been as marked. IL-1 is a pivotal cytokine in cartilage destruction via the potent induction of MMPs by synovial fibroblasts. The pattern of cellular responses induced by IL-17 is similar to those of IL-1. Indeed the addition of IL-1 with IL-17 to human fibroblast cultures results in synergistic enhancement of IL-6, IL-8 and PGE2 production (Chabaud, Fossiez et al. 1998). Furthermore, IL-17 stimulates the production of IL-1β and TNFα by macrophages. In the collagen induced arthritis model, blocking IL-17 results in suppression of arthritis. In contrast over expression of IL-17 enhanced collagen arthritis. The cartilage destructive effects of IL-17 appear to function independently of IL-1β as blocking IL-1 with neutralising peptide had no effect on IL-17 induced cartilage damage (Lubberts, Joosten et al. 2001).

IL-17 exerts its osteoclastogenic effects via NFkB dependant mechanisms. Activation of NFκB requires the interaction of RANK/RANKL pathway that is dependent on the presence of TRAF6. It has been shown that whereas IL-17 induces TRAF6 expression, IFNγ inhibits TRAF6. Therefore inhibition and activation of bone resorption may be occurring in the same joint depending on the predominant cytokine milieu. This may give a plausible explanation as to some of the radiological features that are specific to PsA.

IL-17 has a variety of biological activities including induction of chemokine synthesis and stimulation of inducible nitric oxide synthase (iNOS) and prostaglandin E2 (PGE2).

It has been shown to be present in the synovium and SF of patients with RA (Chabaud, Durand et al. 1999). Furthermore, the majority of the CD4+ and CD8+ T cell clones derived from lesional psoriatic skin expressed IL-17 mRNA, suggesting that skin-

infiltrating T cells can produce this cytokine. IL-17 mRNA is detectable in biopsies from lesional psoriatic skin, but not in non lesional control biopsies suggesting that IL-17 could amplify the development of cutaneous inflammation and may support the maintenance of psoriasis through stimulation of keratinocytes to augment their secretion of proinflammatory cytokines (Teunissen, Koomen et al. 1998). The presence and role of IL-17 in PsA has not previously been studied.

IL-17 is similarly upregulated in lesional psoriatic skin, suggesting that it may play a role in the amplification and/or development of cutaneous inflammation. It may contribute to the recruitment of T cells into the epidermis by inducing expression by primary keratinocytes of the chemokine CCL20 (Homey, Dieu-Nosjean et al. 2000).

Other cytokines and chemokines - Further work has been performed comparing monocyte-derived cytokines such as IL-15 in PsA and RA SM (Danning, Illei et al. 2000). IHC demonstrated the presence of IL-15 in both the RA and PsA synovium and the presence of NFkB indicates that such cytokines are of functional significance.

The preferential expression of IL-8 and IL-8 mRNA in psoriatic synovium indicate that chemokines may regulate leucocyte traffic into the SM (Konig, Krenn et al. 1996).

Summary

The discovery of novel cytokines and associated natural inhibitory binding proteins has increased our understanding of pro inflammatory pathways and as such has given us deeper insights into inflammatory disease pathogenesis. Furthermore, the ability to inhibit such cytokines has led to the development of novel therapies in those diseases in which such cytokines have been shown to have functional significance.

1.4.8 MAP kinase signalling pathways

Inflammatory mediators activate numerous intracellular signalling cascades resulting in transcription of chemokines and pro inflammatory cytokines. These intracellular signalling cascades include the mitogen-activated protein kinase signal transduction pathways, which include the p38 mitogen-activated protein kinase (p38 MAPK) and the c-Jun N-terminal kinase. p38 MAP kinase inhibition has profound anti-inflammatory effects when administered to cells prior to stressful stimuli. Moreover, oral p38 MAP kinase inhibitors reduce TNFα production by LPS injected mice by 91% (Wadsworth, Cavender et al. 1999). In animal models of arthritis, prophylactic treatment with MAP kinase inhibitor reduced joint swelling and erosion. Furthermore, administration following onset of arthritis arrested subsequent disease progression (Jackson, Bolognese et al. 1998). Recent *in vivo* data have implicated p38 MAP kinase pathway in Borrelia burgdorferia elicited inflammation with specific inhibitors repressing spirochete production of TNFα (Anguita, Samanta et al. 1997). Although there are 4 isoforms of MAP kinase, the α isoform has been identified as a potential target for anti cytokine therapy.

p38 MAP kinase is also involved in IL-18 and IL-12 induced T cell interaction with extra cellular matrix ligands (Ariel, Novick et al. 2002).

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1.5   The role of NK and NKT cells in immune regulation

1.5.1   Natural Killer (NK)

NK cells were first described in 1976 in mice due to their tumour lysing ability leading to profound interest in their potential treatment of malignancy (Kiessling, Petranyi et al. 1976). These granular lymphocytes lack B and T cell surface markers and are characterised by an ability to secrete both Th1 and Th2 cytokines depending on their cytokine milieau. Whereas IL-12 stimulates NK cell production of IFNγ, IL-4 stimulates NK cell production of IL-5, IL-10 and IL-13. This suggests that like T cells, NK cells can differentiate into discrete functional subsets with differing effects on adaptive immunity. Because of their role in regulating the normal immune response, NK cells may also be important in auto immunity.

1.5.2   Natural Killer T (NKT) cells

A subset of T cells that express NK receptors are termed NK T cells. They were first described in 1987. NKT cells are distributed throughout the body but are found mainly in the thymus where they represent 10-20% of mature T cells, liver (30-50%) and bone marrow (20-30%). They are different from NK cells in terms of development, antigen recognition and function. These cells are characterised by the presence of a T cell receptor made up of two subunits -Vα24 and Vβ11. The cells express the TCR/CD3complex and are activated through the TCR. NKT cells recognise glycolipid antigen when presented by the APC in association with the MHC class 1 like molecule CD1d. The crystalline

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structure of CD1d differs from MHC molecule by having a large non-polar antigen-binding groove that allows it to bind the lipid moiety of certain glycolipids In addition CD1d is encoded on chromosome 1 whereas HLA class 1 is encoded on chromosome 6.

NKT cells have cell surface markers CD56 and CD161 (NKR-P1A). There is however a variety of cells within this subset resulting in functional and phenotype heterogeneity.

Initial studies performed in the mouse model, identified thymic T cells which had a TCR but were negative for CD4 and CD8 i.e. double negative (DN). The majority of these cells were found to be CD161 positive and were able to produce large amounts of IFNγ, IL-4 and TNFα. A population of CD161/CD4 positive cells with similar abilities to secrete these cytokines was also found. The development of both these subtypes was dependent on CD1d. These three populations are present in most of the tissues and are phenotypically and functionally distinct.

Biological activity

NKT cells fulfil an important immunoregulatory role in determining subsequent T cell responses. Depending upon the ambient cytokine milieau, NKT cells produce substantial amounts of IL-4 or IFNγ driving Th2 or Th1 responses respectively. The dysregulation of NKT cell function is thought to play a role in the pathogenesis of type 1 diabetes in non-obese diabetic mice (Sharif, Arreaza et al. 2001), (Hong, Wilson et al. 2001).

IL-12 and co stimulatory pathways play a critical role in modulating the activities of NKT cells (Hayakawa, Takeda et al. 2001).

In humans the DN and CD4+ subsets also exist. Both react with CD1d to produce high levels of IFNγ and IL-4 when stimulated.

The ability of the TCR of NKT cells to recognise glycolipid in association with CD1d has been used in a variety of experimental models. NKT cells can be rapidly stimulated in the murine model using a synthetic glycolipid derived from the marine sponge called alphagalactosyl ceramide (α gal/cer). Initially the production of IFNγ predominates, however, following prolonged stimulation, the profile switches to IL-4 indicating a Th2 immune response.

CD1d is present on normal keratinocytes. However its expression is up regulated in keratinocytes from psoriatic plaques (Bonish, Jullien et al. 2000). Furthermore IFNγ is able to upregulate the expression of CD1d on human keratinocytes derived from neonatal foreskins. The expression of CD1a, CD1b and CD1c but not CD1d have been described in RA synovium (Cauli, Pitzalis et al. 2000).

The ability to induce a psoriatic plaque in 'normal' skin from a psoriatic patient grafted onto the SCID mouse model by injecting the mouse with NKT cells derived from the patients' PB further supports a pathogenic role for these cells in psoriasis (Nickoloff, Bonish et al. 2000).

Reduced levels of NKT cells have been shown in the PB of patients with RA (Yanagihara, Shiozawa et al. 1999) and psoriasis (Koreck, Suranyi et al. 2002). The role of NKT cells in PsA has not been studied.

1.6   Aims of thesis

- To clarify the dominant T cell immune response in PsA

- To identify novel regulatory cytokines in different tissue compartments and assess their function in terms of regulation of Th1 responses

- Assess the function of NKT cells in PsA

- To study the expression of IL-17 in SM and fluid and its effects on MMP and IL-6 production by synovial fibroblasts

Chapter 2 Material and Methods

2.1 Patients

Glasgow Royal Infirmary Research Ethics Committee granted approval for the project in June 2000. Patients with PsA, who were greater than 18 years of age and capable of providing informed consent, were invited to take part in the project. Such patients were identified from the PsA Clinic in North Glasgow Trust, Glasgow Royal Infirmary. Demographic and disease activity data were collected and stored in a password-protected database (SPSS).

PsA was defined if the following criteria were fulfilled:

(a) Classical psoriatic skin lesion with or without nail involvement.

(b) Peripheral arthritis alone or in combination with spinal disease.

(c) Negative Rheumatoid Factor (RF) and absence of subcutaneous nodules.

2.2 Skin biopsy

Elliptical skin biopsy

Patients with active psoriatic plaques were identified. Having identified a suitable plaque avoiding the cape area, the skin was cleansed using chlorhexidine gluconate. Thereafter 1% lignocaine with 1:200000 adrenaline was injected into the subdermis. A 5mm elliptical incision was then made using a size 15 surgical scalpel blade. A skin hook and scalpel were used to lift the ellipse from the subdermis. The skin was then sutured using 2x 4.0 Ethilon sutures and a dry Mepore dressing was applied. The incision site was observed for 30 minutes for bleeding. Patients were informed to attend their practice nurse for suture removal at 7-10 days.

2.3   Synovial biopsy

2.3.1   Blind needle Biopsy

In a subset of patients this procedure was performed rather than arthroscopy due to patient preference. The patient was positioned in a semi recumbent position to relax the quadriceps group of muscles. A support was placed behind the knee for comfort. The knee was cleansed using alcohol and a drape was placed beneath the knee and over the lower leg.

5mls of 1% lignocaine with 1:200000 adrenaline were used to infiltrate the skin as previously described. SF was aspirated using a 21G green needle. The knee was then infiltrated with 20mls of 0.5% bupivicaine and rested for 10 minutes. An incision was made using a size 15-scalpel blade to the lateral aspect of the suprapatellar pouch. A needle biopsy was used to obtain at least 5 synovial biopsies providing adequate representation as per EULAR guidelines.

2.3.2   Needle arthroscopy

Preparation of patient and equipment

Patient consent was obtained prior to commencing the procedure and 20mls of blood were obtained for lymphocyte separation, DNA storage and serum. The skin over the lateral surface of the knee was shaved and Emla cream was applied to skin portals (supero lateral and infero lateral to the patella) 1 hour prior to the procedure.

The patient was positioned as for blind needle biopsy. A sterile paper drape was placed under the leg that was cleaned from thigh to ankle with Betadine solution. The leg was

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then placed in a limb bag and a sterile extremity drape placed over the lower limb. A light gauze bandage was placed around the ankle.

10-20mls of 1% Lignocaine with 1:200000 adrenaline were used to anaesthetise the portal sites and the joint capsule. Using a 20ml syringe and a 21G needle, SF was aspirated from the suprapatellar pouch. With the needle left in position, 20mls of Bupivicaine 0.5% without adrenaline were injected into the joint cavity. This was allowed to take effect over the next 10 minutes.

During this time the arthroscopy equipment was prepared. Briefly, a 1 litre bag of 0.9% Sodium Chloride was connected to an intravenous giving set for joint lavage. The arthroscope (Karl Stortz) was inserted into a sterile camera sleeve and connected to the camera and light source. Prior to arthroscopy, the light source was white balanced.

A longitudinal entry incision was made at the arthroscopy portal sites using a 15-blade scalpel. The cannula was inserted using a sharp obturator switching to a blunt obturator before advancing into the joint cavity. The blunt obturator was removed and replaced with the arthroscope. A second incision was then made at the biopsy (superior) portal and the blunt obturator was used to advance the cannula into the suprapatellar pouch. An irrigation line was connected to achieve a constant flow of normal saline through the joint cavity allowing clearer visualisation of anatomy. The joint was carefully inspected for macroscopic features of hyperaemia, vascularity, pannus and villous formation (see figure 2.1).

Using grasping forceps, synovial biopsies were taken from 6 areas – lateral, suprapatellar, medial, retropatellar, infrapatellar and the cartilage pannus junction (CPJ).

Biopsies were removed from the forceps using a sterile needle and placed on a gauze swab. The samples were then passed to the attending technician for appropriate processing. The joint cavity was inspected for any bleeding and irrigated until the saline ran clear. The portals were then closed using steri strips and a Mepore bandage. The knee was dressed with a pressure bandage and the patient was given advice regarding rest and wound care. In addition the patient was given contact numbers in the event of any problems. The patient was reviewed the following week at which time the incision sites were inspected.
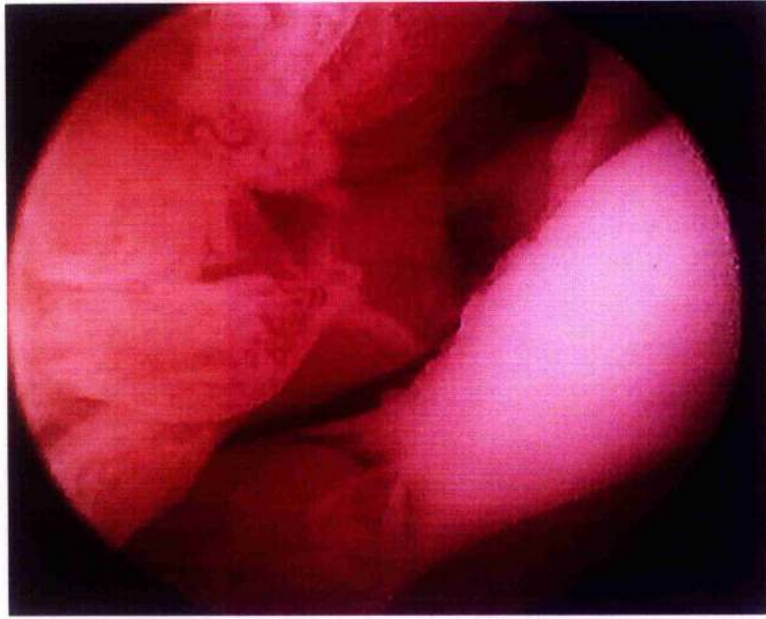
Figure 2.1 PsA SM visualised at arthroscopy.

These appearances are consistent with grade IV synovitis characterised by prominent vascularity and villous formation. Note the tortuosity of blood vessels that distinguish these appearances from RA synovitis characterised by straight branching blood vessels.

2.4.1   Tissue preparation

2.4.1   SM and skin

Tissue sections were prepared and stored for future analysis of cytokine expression by immunohistochemistry and quantitative PCR.

- To obtain frozen sections, SM and skin samples were snap frozen in liquid nitrogen after embedding in Cryo-M-Bed embedding compound (Bright Instrument Company, Cambridgeshire) on cork discs. $6\mu m$ sections were cut using a cryotome (Bright Instruments) at $-40°C$ and mounted onto silane coated slides. The sections were left at room temperature to dry for 1 hour before wrapping in foil and storing at $-70°C$.

- Paraffin embedded sections were also obtained for future IHC. Tissue was stored in formalin and transferred to the pathology department for paraffin embedding and subsequent sectioning (David Murray, chief pathology technician). Haematoxylin and eosin staining was performed on one of the serial sections to allow tissue morphology to be assessed.

- To facilitate future RNA extraction, tissue sections were homogenised using an electric homogeniser (Kinematica, Switzerland) and stored in Trizol reagent (Invitrogen, Life Technologies, Scotland) at $-70°C$ prior to processing.

- Finally, explant tissue was transferred to growth medium and cultured at $37°C$ in $5\%CO_2/95\%$ O_2 to assess tissue production of cytokine at time intervals. Fibroblasts like synoviocytes (FLS) were generated following prolonged incubation and passage of cells.

2.4.2 Cell separation from SF and peripheral blood

Peripheral blood

Wash media was prepared form RPMI containing 2 mM L-glutamine, 100 IU penicillin, and 100-μg/ml streptomycin (all obtained from Life Technologies, Paisley, U.K.). Culture media were prepared by the addition of 10% foetal calf serum (FCS) (Sigma) to wash medium. 25mls of venesected blood were transferred to 50ml heparinised container and diluted with equal volume of RPMI. Blood was carefully layered onto Lymphoprep (Nycomed, Oslo, Norway). Samples were centrifuged at 350g for 25 minutes to allow separation of the blood into its component layers. Following centrifugation the interface containing PBMCs was carefully removed using a sterile Pasteur pipette and placed in a fresh 15ml tube. This PBMC suspension was made up to 14mls with RPMI and centrifuged at 350g for 10 minutes to pellet the cells. The supernatant was discarded and the pellet resuspended and wash repeated. Cells were finally resuspended in culture media. The number of PBMCs was calculated using a haemocytometer (Weber, England). This involved making a 1 in 10 dilution of the suspension with cell counting fluid and placing 10μl on the haemocytometer under a cover slip. Using a light microscope, the number of live cells was counted over 16 squares and averaged over 4 quadrants. This gave a calculation of the number of cells times 10^4. This number was then multiplied by the dilution factor of 10 and then multiplied by the volume of the suspension to give the total number of cells. The suspension was then adjusted to 10^6/ml

SF cell isolation

SF obtained at arthroscopy or needle aspiration was transferred to heparinised universal containers. Procedure was as for PBMC isolation.

10^6 cells were placed in Trizol and stored at $-70°C$ for future RNA extraction. Subsequently, cells were used for co-culture experiments and FACS analysis to determine spontaneous IFNγ expression.

2.5   Identification of Th1 cells in PsA SF and PB

2.5.1   Flow Activated Cell Sorting (FACS)

All flow cytometric analysis was conducted using a Becton-Dickinson Fluorescence Activated Cell Scanner (FACScan). This flow cytometer measures and analyses the optical properties of single cells passing through a focused argon laser beam. Cell Quest software was used to analyse data collected.

2.5.2   Identification of IFNγ expressing cells

IFN γ secretion assay

The IFNγ secretion assay (Miltenyi, Biotec, Germany) will detect live spontaneous IFNγ producing leucocytes *ex vivo* without additional *in vitro* stimulation, thus cells are behaving as they would *in vivo* (figure 2.2)

Briefly, 10^7 cells were resuspended in 300µl RPMI and 50µl removed as negative controls. 20µl of catch reagent that binds to both CD45 antigen on the surface of cells and secreted IFNγ was added to the remainder of the cells and incubated on ice for 15minutes. 10ml of warm RPMI/FCS were added to the positive samples and incubated for 45 minutes at 37°C with continual rotation. The samples were then topped up to 14mls with cold RPMI and washed twice. 20µl of detection reagent (anti IFNγ-PE) were then added to the positive samples and 2µl to the negative controls. 8µl of CD3 FITC Pharmingen (San Diego, California) was added to all samples to allow estimation of T cell numbers and incubated on ice for 10minutes. Samples were washed with cold RPMI/FCS and resuspended in 0.5ml of RPMI for analysis on FACS.

To clarify that this assay was specific for IFNγ secreting cells, our laboratory performed control experiments. Briefly, T cells separated by a semi permeable membrane were stimulated with or without anti CD3 to induce IFNγ secretion. Although IFNγ passed easily through the membrane, no unstimulated T cells stained positive using the double antibody assay. This confirmed that the double antibody only detected IFNγ released by the CD3 stimulated T cells. Furthermore, in a second control experiment CD45 positive cells that were incapable of secreting IFNγ were mixed with IFNγ secreting CD45 positive cells. None of the non-secretors identified using a second cell surface marker stained positive with the double antibody.

Although not performed in these studies, subsequent purification of IFNγ expressing cells can be achieved by labelling with magnetic beads coated with anti PE antibody and enriched on a column placed within the magnetic field of a magnetically activated cell sorter (MACS, Miltenyi, Biotec, Germany). Thus IFNγ expressing magnetically labelled cells are retained in the column while the unlabelled cells run through.

After removal of the of the column from the magnetic field, the magnetically retained IFNγ

expressing cells can be eluted as positively selected cell fraction and can then be used for

cell culture and analysis.
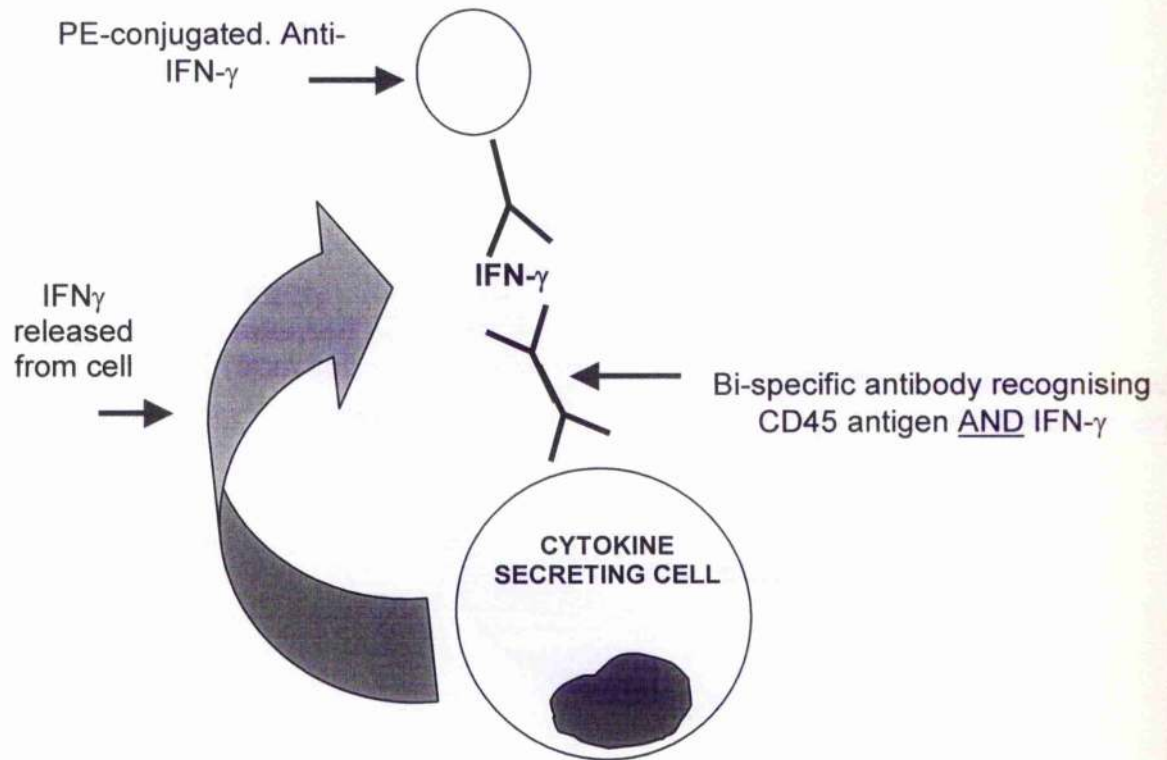
Figure 2.2 Double antibody technique for detecting IFNγ expressing cells.

2.5.3   Identification of Th1 markers CCR5 and IL18-R

The identification of stable cell surface markers such as IL-18R and CCR5 in 1998 has contributed to the identification of Th1 immune responses in disease states (Xu, Chan et al. 1998) (Loetscher, Uguccioni et al. 1998). Here we have utilised these markers in PsA PB and SF to further determine the predominant immune response.

Matched SF and PBMCs were separated as outlined in section 2.4.2. 10^5cells were transferred to appropriately labelled FACS tubes. 5µl of CCR5-FITC antibody (Pharmingen, San Diego, California) and 8µl of IL18R-PE (R&D Systems, USA) antibody were added together and alone with irrelevant PE and FITC controls. Cells were incubated on ice for 30minutes then washed with PBS and spun for 6 minutes at 350g. Cells were then resuspended in PBS and analysed by FACS.

2.6   Identification of regulatory cytokines by IHC

2.6.1   Frozen sections

Synovium obtained at needle arthroscopy and psoriatic skin tissues were snap frozen in liquid nitrogen and 6µm cryostat sections were cut onto silane coated slides using a microtome and stored at –70°C. A range of antibodies as outlined in Table 2.1 was used to label tissue using an indirect immunoperoxidase technique. Briefly, sections were rehydrated in PBS then left in methanol/ H_2O_2 for 30 minutes at room temperature. The sections were washed twice in PBS and blocked using 20% serum in PBS of the species in which the secondary antibody was raised. Sections were incubated with previously

determined optimal dilutions of monoclonal antibody overnight at 4°C. The following day the sections were washed, incubated with relevant secondary antibody for 30 minutes washed twice with PBS then incubated with substrate Vector ABC (Vector, Peterborough, UK) for 30 minutes. Sections were washed twice in PBS before developing with 0.6mg/ml 3,3'-diaminobenzidine tetrahydrochloride (Sigma) with 0.01% H_2O_2 for 5 minutes at RT until brown reaction product appeared. Sections were then washed in water and counterstained using Harris's haematoxylin (BDH Ltd. Lutterworth, Leicester, UK). Finally sections were dehydrated in ethanol, cleared in xylene then mounted in DPX mountant (both from BDH Ltd.).

2.6.2 Paraffin embedded sections

Slides were initially heated to 65°C for 35 minutes. They were then dewaxed in xylene and rehydrated through ethanol to PBS. Endogenous peroxidase activity was blocked using H_2O_2 /methanol. To expose the relevant antigen, sections were then microwaved under pressure in 1.92g of anhydrous citric acid ($C_6H_5O_7$) in 200mls of distilled water, pH 6. The sections were blocked for 1 hour at RT in 20% serum of the species in which the secondary antibody was raised. The relevant primary antibody was applied overnight at 4°C. Procedure was then followed as with frozen sections.

Antibody	Manufacturer and clone	Source	Working Concentration
CD3	Dako	mouse monoclonal	5μg/ml
CD68	Dako clone PG-M1	mouse monoclonal	3.6μg/ml
IL-18	clone E3E1, Andrew Jackson	mouse monoclonal	1μg/ml
IL-17	R&D	Goat polyclonal	5μg/ml
IL-12	clone 7.4, Trinchieri	mouse monoclonal	12.5μg/ml

Table 2.1 Antibodies for IHC

2.6.3   Quantification of Histology

Quantitative microscopy was performed using an Olympus BX-51 light microscope (Olympus Optical Co. Ltd.) with an additional 'teaching binocular eye piece that allowed two observers (Dr J.A. Gracie and the author) to quantify sections simultaneously. Synovial tissue was divided into lining layer, sublining layer and lymphoid aggregate if present. Dermal tissue was divided into dermis and epidermis. The percentage of positively stained nucleated cells were quantified in each area according to the following scoring system; 1= 1-10% of nucleated cells stained positive, 2=10-25% nucleated cells, 3=25-50% and 4=>50% positive.

2.7   Identification of cytokine mRNA

2.7.1   RNA extraction

RNA designated pipettes and workbench were initially washed with RNAase ZAP (Sigma) to avoid contamination of samples with foreign RNA. Samples stored in −70°C were thawed at room temperature (RT). 0.2mls of chloroform (Prolabo, France) were added per 1ml of Trizol reagent, shaken vigorously for 15 seconds and left at RT for 3 minutes.

The samples were centrifuged at 12000g at 4°C for 15 minutes. This resulted in the formation of an upper aqueous phase containing RNA, an interface containing proteins and a lower organic phase containing DNA. The upper aqueous phase was carefully transferred to a clean polypropylene tube and an equal volume of isopropyl alcohol (BDH Ltd. Lutterworth, Leicester, UK) added to precipitate the RNA. A further 10 minute incubation at RT was followed by centrifugation at 12000g to pellet the RNA. Following removal of the supernatant and washing in 75% ethanol (Hayman, Essex, UK), the pellet was air-dried for 5 minutes then resuspended in 22μl of sterile water and incubated at 55°C for 10 minutes.

RNA concentration was calculated by measuring the optical density (O.D) reading of a 50-fold RNA dilution at a wavelength of 260nm and 280nm using a Bio quest (Cecil Instruments, Cambridge England) spectrophotometer. This process assumes that an OD of 1 is equivalent to 40μg/ml of RNA. The purity of the sample was assessed from the ratio of the OD at 260nm to the OD at 280nm.

2.7.2 DNAse treatment of RNA

All PCR work was carried out at designated PCR bench in the laboratory using PCR designated pipettes. In certain TaqMan PCR reactions such as IL-18, genomic DNA is amplified as primers may span introns. In this case the RNA was required to be DNAse treated prior to RT PCR. This was achieved by pre incubation with RNAse free DNAse (Ambion Inc, RNA Diagnostics, Huntingdon, Cambridgeshire) for 15minutes at 37°C, 65°C for 10 minutes to inactivate the enzyme, before commencing the RT PCR reaction as above.

β Actin PCR

24μl of mastermix was made containing 2.5μl of 2mM dNTPs (Life Technologies), 2.5μl of 10xPCR reaction buffer, 1.5μl of magnesium chloride (AB Gene), 0.125μl of Taq polymerase (AB Gene), 1μl each of 5 primer and 3 primer for β actin and 15.375μl of sterile water. 1μl of cDNA was added to 24μl of mastermix. The PCR reaction was performed on a Genius PCR machine (Techne).

2.7.3 Reverse transcription of RNA

5μg of the total RNA obtained from PBMCs and SFCs was reverse transcribed to its corresponding cDNA. Initially each sample was made up to 11μl using sterile water. 1μl of random primer (Life Technologies, Invitrogen, Paisley) was added to each sample and incubated at 70°C for 10 minutes. 7μl of reaction mix containing 2 μl of PCR reaction buffer, 2 μl magnesium chloride (AB Gene House, Surrey, UK) and 2μl of 0.1M DTT

(Gibco BRL) and 1µl of 10mM dNTPs (Life Technologies) were added to each sample giving a total volume of 19µls. The samples were transferred to the Genius PCR machine (Techne Ltd.) and incubated for 5 minutes at 25°C. During a pause in the programme, 1µl of superscript (Life Technologies, Invitrogen, Paisley) was added and the programme recommenced as follows: 10 minutes at 25°C, 50 minutes at 42°C and 10 minutes at 70°C. On completion the cDNA was stored overnight at 4°C. PCR for β-actin was performed to establish successful RT PCR.

2.7.4 Agarose gel electrophoresis

A 2% agarose gel (Bioline Ltd., London) containing 0.001% ethidium bromide (Sigma) was set in a casting tray containing a 20-toothed comb. The gel once set was placed into a gel tank (Life Technologies, UK) and overlaid with 1xTBE buffer. A 1kB DNA ladder (Advanced Biotechnologies, Surrey, UK) was also loaded onto the gel to allow sizing and appropriate quantification of the products within the sample. 10µl of sample with 5µl of orange G solution containing 50% glycerol and 10% TBE were added to individual wells. The samples were then electrophoresed at 100 volts for 60 minutes to allow migration of the cDNA within the gel from cathode to anode. The gel block was then removed and visualised using camera and UVI-photo software programme.

2.7.5 TaqMan PCR

TaqMan (Applied Biosystems, California, USA) is used as a research tool for real time *in vitro* relative quantitative evaluation of gene expression. It has the advantage over standard PCR in that it identifies gene expression in small tissue samples.

1 μl of cDNA was added in triplicate to Universal PCR reaction mix (AmpliTaq Gold DNA Polymerase, AmpErase UNG, dNTPs with dUTP, passive reference and optimised buffer components) in microcentrifuge wells in a 96-well plate. The primers and probe of the gene of interest were than added to the samples. To quantify the relative expression of the gene of interest, a housekeeping gene HPRT was analysed in each sample triplicate.

The following human IL-18 primers were used: forward primer CGCCTCTATTTGAAGATATGACTGATT, probe TGACTGTAGAGATAATGC ACCCCGGACC and reverse primer CCTCTAGGCTGGCTATCCTTTATACA TACT. The PCR amplification reaction was performed (45 cycles) by ABI Prism 7700 Sequence Detection System. Products were expressed as a percentage of HPRT.

IFNγ TaqMan primers (Applied Biosystems) were provided in combination with the IFNγ probe as a pre-developed primer and probe reagent.

2.8   Analysis of cytokine expression in SF and serum

2.8.1   IL-18 ELISA

IL-18 levels were measured in SF supernatants and serum using a commercially available solid phase sandwich ELISA (Diaclone, France). Briefly, 100µl of sample were loaded into wells precoated with polyclonal specific Il-18 antibody. Following the addition of biotinylated anti IL-18 antibody, samples were incubated at RT for 3 hours. The plates were then washed thrice and 100µl of streptavidin HRP (working dilution 1:2500) were added to each well. The plates were then incubated at RT for a further 45 minutes. Following a further wash step, 100µl of tertramethyl benzidine (TMB) solution were added to each well until the reaction developed. The reaction was halted using 50µl of $0.2MH_2SO_4$. A MRX II microplate reader (Dynex) was used to determine absorbency at 450nm. A standard curve ranging from 62.5-2000pg/ml was constructed and the IL-18 content of the samples calculated. The minimum detectable dose of IL-18 by this assay is <45pg/ml.

2.8.2 IL-17 ELISA

IL-17 levels were measured in serum and SF using anti IL-17 monoclonal antibody (MAB317, R&D) as a capture antibody and biotinylated IL-17 polyclonal antibody as a detection antibody on a sandwich ELISA (BAF 317, R&D). A standard curve was constructed ranging from 31.25 to 2000pg/ml. Subsequent procedure was followed as for IL-18 ELISA.

2.8.3 IL-12p40 and IL-12p70 ELISA

IL-12p40 levels were measured in serum and SF using a commercially available ELISA (Biosource). The procedure was the same as described for TNFα ELISA (section 2.9.4).

2.9   Analysis of cytokine function

2.9.1   SFC and PBMC isolation and stimulation

SF and 30mls of matched PB were obtained at either needle arthroscopy or needle aspiration from patients with active PsA defined as the presence of detectable synovitis of the knee. SF cells (SFC) and PBMCs (PBMC) were isolated as outlined in section 2.4.2. Cell numbers and viability were determined with trypan blue exclusion. Cell numbers were adjusted to 10^6 viable cells/ml and 100μls added to each well of a 96 round bottom well plate (Cellstar). Cells were cultured in RPMI/FCS. Recombinant cytokines IL-12 (1ng/ml), IL-18 (100ng/ml), IL-15 (1ng/ml) were added singly or in combination to wells in triplicate to a final volume of 200μl/well. The working cytokine concentrations have been demonstrated to be the optimal concentration for cell stimulation in studies by this laboratory. Supernatants were harvested after 24 hours incubation at 37°C with $5\%CO_2/95\%O_2$ and assayed for TNFα and IFNγ content using specific IFNγ and TNFα ELISA (BioSource International, Hertfordshire, UK).

2.9.2   Co-culture with CD3 and CD28

The T cell receptor is expressed on T cells in association with a group of 5 invariant chains termed CD3. It is essential for TCR stabilisation and signal transduction. In cytokine co-culture experiments therefore, 96 well flat-bottomed plates were coated with anti CD3 antibody (200ng/ml) (Skybio, clone UCHT-1, UK) in PBS pH 9.6 for 24 hours at 4°C.

Wells were then washed twice with RPMI. SFC and PBMCs were adjusted to 10^6/ml in RPMI/FCS and cultured at 10^5 cells per anti CD3 immobilised wells with recombinant cytokines.

CD28, the principal receptor for B7.1 and B7.2 co stimulatory molecules expressed on APC, was added to provide additional T cell activation. Cells were cultured for 24 hours at 37°C in 5%CO_2 after which supernatants were removed and stored at -20°C for future TNFα and IFNγ expression by ELISA.

2.9.3 Proliferation after culture with recombinant cytokines

Following removal of 200μl supernatant for ELISA, 200μls of RPMI/FCS were added to the remaining cells. To assess proliferation response to recombinant cytokines, cells were pulsed with 0.5μCu ^{3}H-Thymidine (Amersham International Plc.) and incubated at 37°C in 5%CO_2/O_2 for 8 hours. Cells were then harvested onto glass fibre filter paper (Packard Instruments, Netherlands) using a cell harvester (Packard Instruments, Netherlands). ^{3}H-Thymidine uptake was determined by counting samples on a MatrixTM 96 Direct Beta counter (Packard Instruments, Netherlands).

2.9.4 Assessment of IFNγ and TNFα in culture supernatants.

TNF α ELISA

TNFα levels in culture supernatant were assessed using a commercially available solid phase human TNFα ELISA (Biosource). Briefly, the ELISA plate was coated overnight at 4°C with 100µl of anti TNFα (5µg/ml) in 0.1M NaHCO₃ pH7.4.

The following day, the plates were blocked with 2% milk in PBS for 1 hour at RT. Plates were washed thrice using PBS/0.1% Tween and standard curve for TNFα ranging 2000pg/ml to 31.5pg/ml were added to the first two columns. 100µl of sample and 50µl of biotinylated anti TNFα antibody (0.4µg/ml) were added in triplicate and incubated at room temperature for a further 2 hours. Procedure thereafter was as for IL-18 ELISA.

IFNγ ELISA

The IFNγ content of the culture supernatant was assessed using a commercially available solid phase human IFNγ ELISA (Biosource) with sensitivity of <4pg/ml. Range of detection from 15.6-1000pg/ml. Coating antibody was used at 2µg/ml. Blocking was achieved using 0.5% BSA for 2 hours at room temperature. Biotinylated secondary antibody was used at 0.4µg/ml.

Results from all ELISA experiments were transposed into graphic format using Microsoft Excel© software.

2.10   Effect of IL-17 on synovial derived fibroblasts

The ability of fibroblasts to respond to IL-17 in culture was investigated based on the observation that this Th1 derived cytokine plays an integral part in inducing MMP expression in RA (Chabaud, Garnero et al. 2000). The ability of PsA synovial derived fibroblasts to express MMP-3 and IL-6 was investigated.

2.10.1 Establishing fibroblast population

SM and SF were obtained as previously described (section 2.3). Dissected membrane and fluid were added to RPMI/FCS and incubated at 37°C in $5\%CO_2/O_2$. After 24 hours, supernatants were removed and stored for future cytokine expression by ELISA. The medium was replaced and cells cultured with regular medium changes until fibroblasts became confluent. They were washed with PBS once to remove any residual RPMI. Adherent fibroblasts were lifted into solution by adding 1x trypsin EDTA (Sigma) for 15 minutes at 37°C. The fibroblasts were then washed in RPMI by centrifugation at 350g for 5 minutes. The fibroblast pellet was resuspended in RPMI/FCS and incubated at 37°C in $5\%CO_2/O_2$. Once fibroblasts had become confluent, this process of trypsinisation was repeated until at least passage 3 at which point fibroblasts were used for co-culture experiments with recombinant cytokines.

2.10.2 Cytokine stimulation of fibroblasts

Fibroblasts at passage 3- 4 were trypsinised and washed with RPMI. Equal numbers of cells (at least 10^4) were added to 96 well flat-bottomed culture plates in triplicate. Fibroblasts were incubated at 37°C in $5\%CO_2/O_2$ for 5 days for cells to come to confluence. Recombinant IL-17 (5ng/ml) and IL-22 (300ng/ml) were then added alone or in combination to determine whether they had any anti or pro-inflammatory effects on synovial derived fibroblasts. Following 24 culture at 37°C in $5\%CO_2/O_2$, supernatants were removed and stored at $-20°C$ until measurement of MMP-3 and IL-6 by ELISA.

2.10.3 IL-6 and MMP expression by synovial fibroblasts

IL-6 ELISA

A commercially available IL-6 ELISA was used (Biosource). Protocol was identical to that of TNFα substituting for anti-IL-6 specific antibodies. Sensitivity for this assay is 2pg/ml with a range from 15-1500pg/ml.

MMP-3 ELISA

MMP-3 content of fibroblast supernatant was assessed using a commercially available ELISA kit (Amersham Pharmacia Biotec, UK), which provided plates, precoated with human anti MMP-3 antibody. Standards and samples were added to the plate and incubated for 1 hour at 4°C. After washing thrice with wash buffer provided, 100µl of peroxidase conjugate and anti MMP3 antibody were added for 2 hours at room temperature. MMP3 content of samples was determined using TMB substrate and halted using 50µl of $0.2M H_2SO_4$. A microplate reader (Dynex Technologies MRXII) was used to determine absorbency at 450nm. A standard curve was constructed starting at 120ng/ml and thereafter content of the samples calculated. The range for this assay was 3.75-120pg/ml.

2.11   IL-18 processing

2.11.1 MAP kinase inhibition

Triplicate wells of matched PBMC / SFMC (10^5 cells / well) in 10%FCS / RPMI were incubated with recombinant IL-12 (1 ng / ml) $\pm$ IL-18 (100 ng / ml) in the presence or absence of the p38 MAPK inhibitor SB203580 from Alexis (Nottingham, U.K.) or DMSO carrier control for 24 hours at 37°C. Supernatants removed after 24 hours were examined for IFN-γ and TNF-α production by ELISA. Subsequent proliferation of PBMC / SFMC was assessed at 72 hours by ^{3}H-thymidine uptake during the final 8 hours of culture.

2.11.2 ATP dependant release of IL-18 and IL-1β by SFCs

SFMC were obtained by Ficoll hypaque (Sigma) separation as previously described and suspended in RPMI/FCS. Viable cells were added to 48 well culture plate at $2x$ 10^6 cells/well and stimulated with LPS (Sigma) at 10ng/ml and 100ng/ml concentrations. After 1 or 2 hours incubation at 37°C in 5%CO_2/95%O_2 supernatants were removed and replaced with fresh medium containing 50mM Benzyl ATP (Sigma) to induce cytokine release. Following a further 20 minutes incubation, supernatants were removed and stored at -20°C for subsequent measurement of IL-1β using a commercially available ELISA (Biosource). Procedure was the same as for TNFα ELISA substituting anti TNFα antibodies with anti IL-1β antibodies. Standards were constructed ranging from 2000-37.25pg/ml with sensitivity of <10pg/ml. IL-18 levels were similarly measured in supernatants as previously described.

Chapter 3 Characterisation of PsA cohort

Although there has been considerable progress in the investigation of genetic and immunological factors in the pathogenesis of PsA, there remain major difficulties in the development of diagnostic and classification criteria. This problem has led to an international project aiming to derive definitive diagnostic criteria, that will in turn represent a major advance in future research into this complex disease. For purposes of this thesis, PsA was diagnosed in patients with an inflammatory arthritis in the presence of psoriasis. Patients with seronegative disease and definite first-degree family history of psoriasis were also included. Patients were defined as having RA with psoriasis if nodules and/or rheumatoid factor were detected.

Previous studies have indicated that community and hospital PsA cohorts differ in terms of mortality and disease severity (Wong, Gladman et al. 1997). To determine whether the hospital based PsA cohort studied in this thesis was representative of similar hospital based cohorts, demographic and disease activity data were collected from patients attending a dedicated weekly PsA clinic at Glasgow Royal Infirmary established in summer 2000. Patients attending this clinic are referred from the community by their general practitioner with a probable diagnosis of PsA or from rheumatology or dermatology hospital clinics where other consultant physicians suggested the diagnosis of PsA. Data regarding age, sex, duration of arthropathy and psoriasis, family history and smoking status were collected. DMARD use and pattern of disease was recorded. Acute phase parameters (ESR and CRP) were measured. Clinical measures of disease activity (swollen and tender joint counts) were collected over a 4-week period in January 2002. A subset of patients, prior to clinic attendance, completed a health assessment questionnaire (HAQ) (see Appendix B) to

assess disability. Originally designed for use in RA and OA, the HAQ takes approximately 5 minutes to complete and provides a score from 0 (no disability) to 3.0 (complete dependence on others). Data were stored and analysed using a password-protected database with statistical package (SPSS).

3.1   Demographic characteristics of PsA cohort

Data were collected from 180 patients over a 2-year period from August 2000 to July 2002. Approximately 25 patients were seen per week during this period by the author and Professor I.B.McInnes at Glasgow Royal Infirmary. 48% of this cohort was male and 52% female. The mean age was 44 years with a range from 17 to 75 years. Mean disease duration of PsA and psoriasis was 10 and 19 years respectively. There was a positive first-degree family history of PsA or psoriasis in 56% of cases. 70% were non-smokers, 13% ex-smokers and 17% current smokers.

ESR, CRP, swollen and tender joint counts were used to estimate disease activity. The mean ESR was 20mm/hr (range 1-74mm/hr) and the mean CRP was 17mg/l (range 6-89mg/l). 50% of patients did not exhibit an elevated acute phase response at any stage.
A cross-sectional subset of 34 patients was examined for the presence of tender and swollen joint counts over a 4-week period. The mean number of swollen joints was 6 (range 0 – 41; SD 8.6). Mean tender joint counts were 15 (range 0-64; SD 19.6). 30 patients completed a HAQ questionnaire to assess functional impact of disease. The mean for this group was 1.3 (range 0-3; SD 1.12).

	Mean	Range
Age (years)	44	17-75
PsA disease duration (years)	10	1-35
Psoriasis disease duration (years)	19	1-71
ESR (mm/hr)	20	1-74
CRP (mg/l)	17	6-89

Table 3.1 Demographics of PsA cohort.

The demographics of 180 PsA patients are shown. Mean values are shown with range of values. ESR <15mm/minute normal reference; CRP <10mg/ml normal reference.

3.2   DMARD therapy in PsA cohort

DMARD use has been characterised in other PsA cohorts and thus it was important to document DMARD use in the present study. Moreover, since these agents are themselves potentially immune modulatory and since the subject matter of the project concerned immune function it was important to establish at the outset typical DMARD use. DMARD use was captured at entry to the cohort, reflecting therefore general prescribing activities prior to the establishment of the cohort.

80% of patients attending the PsA clinic were receiving DMARD therapy. The most commonly prescribed DMARD was methotrexate (35%) followed by sulphasalazine (29%). The remainder of DMARD therapies employed were: sulphasalazine and methotrexate (2%), injectable gold (4%), hydroxychloroquine (2%). The remaining 8% were prescribed a variety of less commonly prescribed DMARDs in PsA such as penicillamine, azathioprine and cyclosporin.

3.3   Disease patterns

In the absence of validated diagnostic criteria for PsA, the concept of disease subgroups is problematic. In this study, patients were classified on the basis of peripheral joint disease alone, axial disease alone, or a combination of axial and peripheral disease. Other subgroups such as those with DIP involvement, enthesitis alone and arthritis mutilans were also recorded. The majority of patients attended with peripheral arthritis alone (52%) or in combination with axial disease (29%). The remainder of patients attended with enthesitis alone (2%), DIP involvement alone (1.3%), and arthritis mutilans (2.5%). 4 patients were diagnosed with seropositive RA and psoriasis rather than PsA. Subgroup

analysis of sex incidence in axial and peripheral disease was performed. In those with axial disease alone there was equal sex incidence. In those with a combination of peripheral and axial disease, there was a slight male predominance of 1.4:1 whereas in those with peripheral disease alone there was a female predominance of 1.3:1.
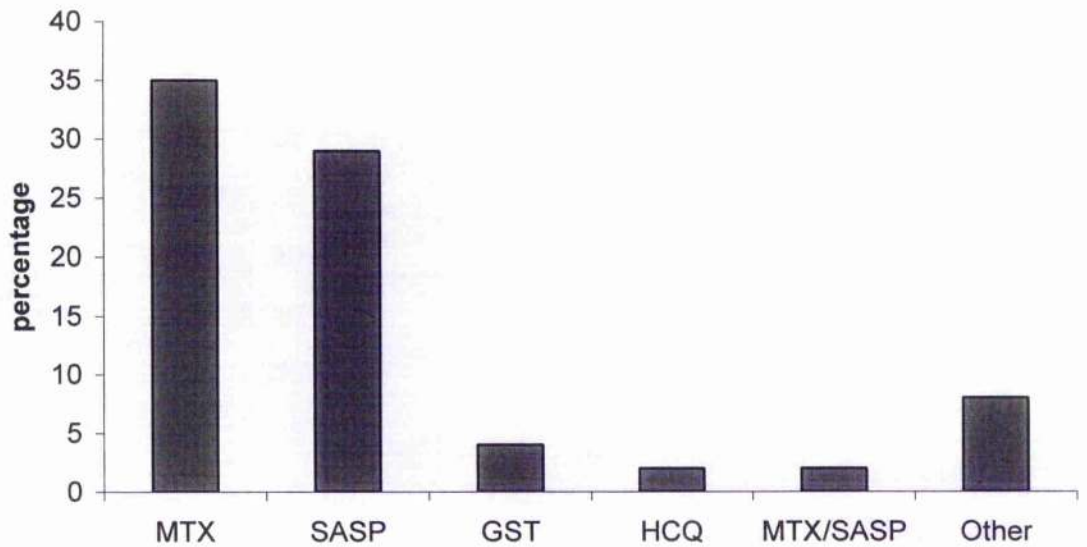
Figure 3.1 DMARD use in PsA cohort

DMARD history was obtained from 180 patients attending the PsA clinic. Methotrexate, (MTX), Sulphasalazine (SASP), Hydroxychloroquine (HCQ), gold sodium thiomalate (GST). Other therapy included penicillamine, cyclosporin or azathioprine.
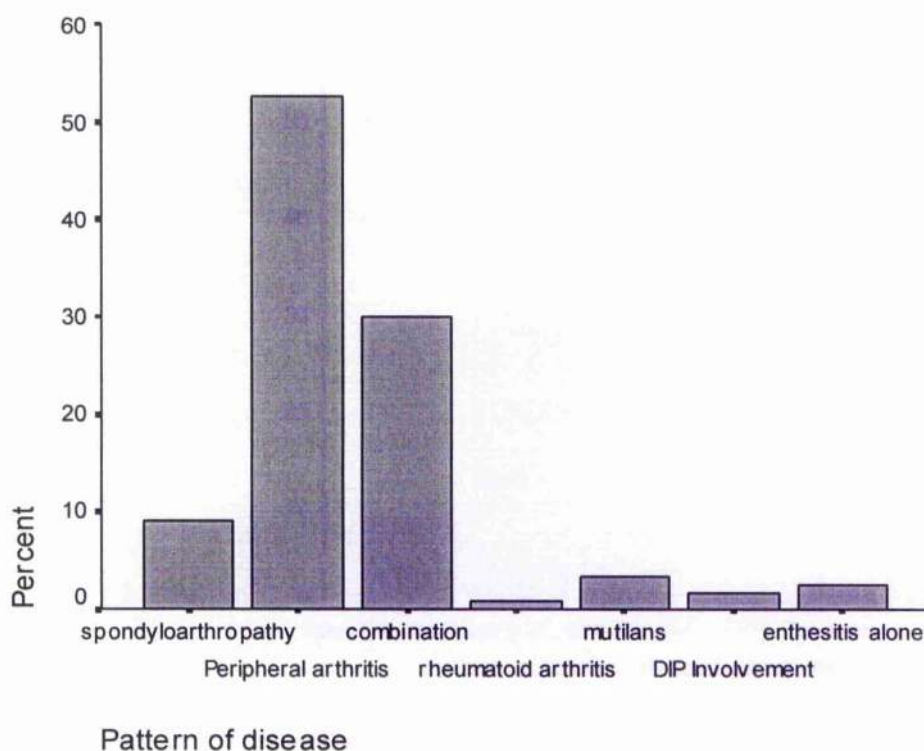
Pattern of disease

Figure 3.2 Histogram of disease patterns

Pattern of disease expression was categorised using criteria based on Helliwell. Patients

were examined for evidence of axial disease, peripheral joint involvement or a

combination of both. Patient with a positive rheumatoid factor were categorised as having

psoriasis and rheumatoid arthritis. Arthritis mutilans, distal interphalangeal involvement

(DIP) and enthesitis alone were categorised separately.

	Male	Female
Spondyloarthropathy (11)	6	5
Peripheral arthritis (63)	27	36
Combination (36)	21	15

Table 3.2 Sex incidence in disease subsets

3.4   Discussion

PsA is a heterogeneous disease characterised by joint and skin inflammation of varying severity. The heterogeneity of disease expression changes over time making this a particularly complex disease to characterise. As such, there are only two large hospital based PsA cohorts in which the clinical spectrum of disease patterns has been described.

Clinical, radiological and biochemical data from a Canadian cohort of 220 patients were studied to determine the prevalence of clinical subsets within PsA. Their results confirmed a spectrum of disease pattern and severity; polyarthritis was the most common joint pattern affecting 61% of the cohort (Gladman, Shuckett et al. 1987): 40% had a deforming, erosive arthropathy of whom 17% had five or more deformed joints; 28% exhibited an asymmetric oligoarthritis.

In a second cohort, clinical and radiological features of 100 patients with PsA were examined to determine features that may be associated with functional disability. Asymmetric oligoarthritis was the commonest presentation occurring in 43%. Symmetrical polyarthritis was documented in 33%. This latter subgroup had the highest number of erosions and deformities. Sacroiliitis was a feature in 14% with predominant spondylitis in 4%. Arthritis mutilans was rare occurring in only 2% of this population (Veale, Rogers et al. 1994).

Our data indicate that peripheral arthritis with or without axial disease accounts for the majority of cases. These findings are consistent with other hospital-based cohorts as previously described and as such are representative of the spectrum of disease patterns

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seen in hospital based PsA cohorts. Our data may not be representative of PsA as a whole as hospital based cohorts are likely to over represent the severity of either psoriasis, arthritis or both. Most patients with mild disease are often managed in the community. Patients with both psoriasis and arthritis are more likely to be referred to a secondary or tertiary centre than those with either condition alone resulting in an overestimate of the occurrence of psoriasis and arthritis. Thus extrapolation of hospital-acquired data to the general population may not be valid. In community-based cohorts, DMARD use is estimated to be approximately 1%. This is in contrast with our cohort where the DMARD use is 80% reflecting more severe disease. The spectrum of disease subtypes also differs between the hospital and community based PsA cohorts. Community studies indicate a predominance of oligoarthritis affecting 91% of patients (Shbeeb, Uramoto et al. 2000) in contrast to hospital-based cohorts where the commonest presentation is polyarthritis alone or in combination with axial disease.

Finally, increased mortality is described in hospital-based cohorts reflecting increased disease severity (Wong, Gladman et al. 1997) whereas no such increase is found in community based PsA cohorts.

The overall male to female ratio in this cohort was approximately 1:1 consistent with previous reports (Gladman 1998). The findings of a slight male predominance in the axial disease group and female predominance in the peripheral arthritis group are consistent with previous studies.

In conclusion, it would appear that our cohort is representative of other hospital based PsA cohorts and therefore is representative of the more severe end of the disease spectrum. The following studies performed on this group of patients and the conclusions drawn cannot however be extrapolated to PsA population as a whole.

Chapter 4 Characterisation of T cell immune response in PsA

There is strong evidence that T cell effector function is implicated in psoriasis and PsA. In psoriasis T cell activation, T cell receptor repertoire skewing and the beneficial clinical effects of T cell depleting therapies such as cyclosporin and PUVA (psoralen + ultraviolet A) indicate that T cells, driven by local antigen recognition, play an important role in pathogenesis (Ortonne 1996). The role of T cells in PsA is less well defined than in cutaneous disease. T cells of various phenotypic subsets are prominent in inflamed SM (Braathen, Fyrand et al. 1979) and of activated phenotype. Oligoclonality of synovial T cells further suggests that they are recognising local antigen, and that similar recognition may occur in skin and synovial compartments (Tassiulas, Duncan et al. 1999) (Borgato, Puccetti et al. 2002). Moreover, T cells detected in SF demonstrate a predominance of CD8$^+$ lymphocytes with restricted T cell Vβ gene expansion (Costello, Winchester et al. 2001). The clinical benefit of alefacept, a T cell specific agent, suggests that such T cells have a pathogenic role in PsA (Kraan, van Kuijk et al. 2002).

The effector function of T cells in psoriasis is considered to be of Th1 phenotype (Schlaak, Buslau et al. 1994). The beneficial effects of fumarates that down regulate IFNγ expression supports this hypothesis (Litjens, Nibbering et al. 2003) (Ockenfels, Schultewolter et al. 1998). I first sought to evaluate whether the same was true of the synovial T cell response in PsA.

4.1   T cell expression in PsA SM and skin

I first characterised the presence and phenotype of T cells in the SM and involved skin of patients with PsA. Parallel 6μM cryostat sections of matched, involved skin and SM obtained by skin biopsy and needle arthroscopy respectively were stained using anti-CD3 antibodies. Tonsil was used as positive control. Irrelevant primary and secondary antibodies were used to clarify specificity of staining. $CD3^+$ rich infiltrates were observed in all PsA SM (figure 4.1A) and involved skin samples examined (n=8) (Figure 4.1B). In skin, T cell expression was detected in the dermis and epidermis. In SM, there was staining detected in the sub-lining layer and in lymphocytic aggregates suggesting lymphocytic distribution in both diffuse and organising secondary lymphoid structures. No staining was detected in the SM lining layer. Positive perivascular and endothelial staining was detected likely representing lymphocytes captured during diapedesis. These data clearly demonstrate the presence of T cells within PsA SM and psoriatic skin in patterns reminiscent of those described previously, confirming the relevance of this tissue cohort for further characterisation.

4.2   Phenotype of T cell Response in PsA

I next sought to determine the functional phenotype of this T cell infiltrate. Functional heterogeneity in Th cells has been clearly established in rodents and human tissues (Mosmann, Cherwinski et al. 1986). Th1 cells produce IFNγ and IL-17, and are marked by selective expression of several membrane markers including CCR5, IL-12Rβ and IL-18Rα (Loetscher, Uguccioni et al. 1998; Xu, Chan et al. 1998). Th2 cells in contrast secrete IL-4, IL-5 and IL-13 and express CXCR3 and ST2 (Xu, Chan et al. 1998). Differential

transcription factor expression further delineates the functional subsets, such that Th1 cells express t-bet and Th2 cells GATA-3 (Rengarajan, Szabo et al. 2000)). The balance between these subsets determines disease phenotype and progression in many experimental models and potentially in clinical disease states, although definitive identification of *in vivo* activated human Th1 cells is relatively lacking in the literature, primarily due to technical constraints on analysis of human tissue derived T cell subsets. Thus elucidation of predominant Th responses and factors regulating their maturation and effector function clearly carries important implications for understanding autoimmune disease pathogenesis.

4.2.1   Expression of IFNγ mRNA in PsA SM, SF and psoriatic skin

I initially sought expression of IFNγ by SF MCs, involved skin and SM at the mRNA level. Quantitative PCR was employed (TaqMan) on cDNA derived from involved skin and SM and fluid obtained at needle arthroscopy on tissues that were immediately snap frozen. Successful reverse transcription to cDNA derived from tissue and SF cells was confirmed by RT-PCR to demonstrate the presence of β-Actin. IFNγ mRNA expression was detected in involved skin, SF MCs and SM in every sample that was examined, indicating that IFNγ expression is ubiquitous in disease derived tissues. mRNA levels of IFNγ normalised to HPRT in psoriatic skin were greatly increased compared with SM and fluid MCs (Figure 4.2).

4.2.2 Detection of IFNγ in PsA SF

IFNγ expression was next measured at the protein level in PsA SFs. In previous studies this has been used as an indicator of Th1 responses *in vivo*. Consistent detection of such protein expression has however proven elusive reflecting perhaps in part inadequate available technology. ELISA was used to detect IFNγ expression in SFs from 12 patients with active PsA undergoing needle arthroscopy or joint aspiration as clinically indicated. By this method, detectable levels of IFNγ were present above the lower limit of sensitivity (18pg/ml) in only 2 of 12 samples supernatants examined (Figure 4.3).

4.2.3 IFNγ expression in RA and PsA SF cells

Thus, although IFNγ mRNA is readily detectable, consistent protein expression has proven difficult to demonstrate. It was therefore important to identify whether IFNγ releasing cell populations were detectable in the synovial compartment. Detection of intra-cellular cytokine expression in mitogen stimulated T cells *ex vivo* in the presence of brefeldin has been widely employed. This assay however measures the mitogen responsive population rather than truly reflecting cell subsets expressing IFNγ in response to native *in vivo* stimulus. I therefore employed a novel cytokine estimation technique utilising bivalent antibody specificity / FACS analysis to identify spontaneous IFNγ producing cells *ex vivo*. The identification of such cells should provide definitive evidence that Th1 responses predominate in PsA.

SF and PBMCs from patients with active RA and PsA were labelled with a double antibody recognising CD45 antigen and IFNγ as demonstrated in Figure 2.1. The mean percentage of CD3+ T cells spontaneously expressing IFNγ in PsA was 12.56% compared with 2.5% in RA SF ($p<0.001$) (Figure 4.4). In a subset of patients CD3 negative cells, most likely NK cells, were also shown to contribute significantly to IFNγ secretion (Table 4.1). These data clarify for the first time the magnitude of the Th1 response in PsA. Importantly and unexpectedly, they clearly distinguish RA from PsA SF T cell subsets.

X20   **X40**
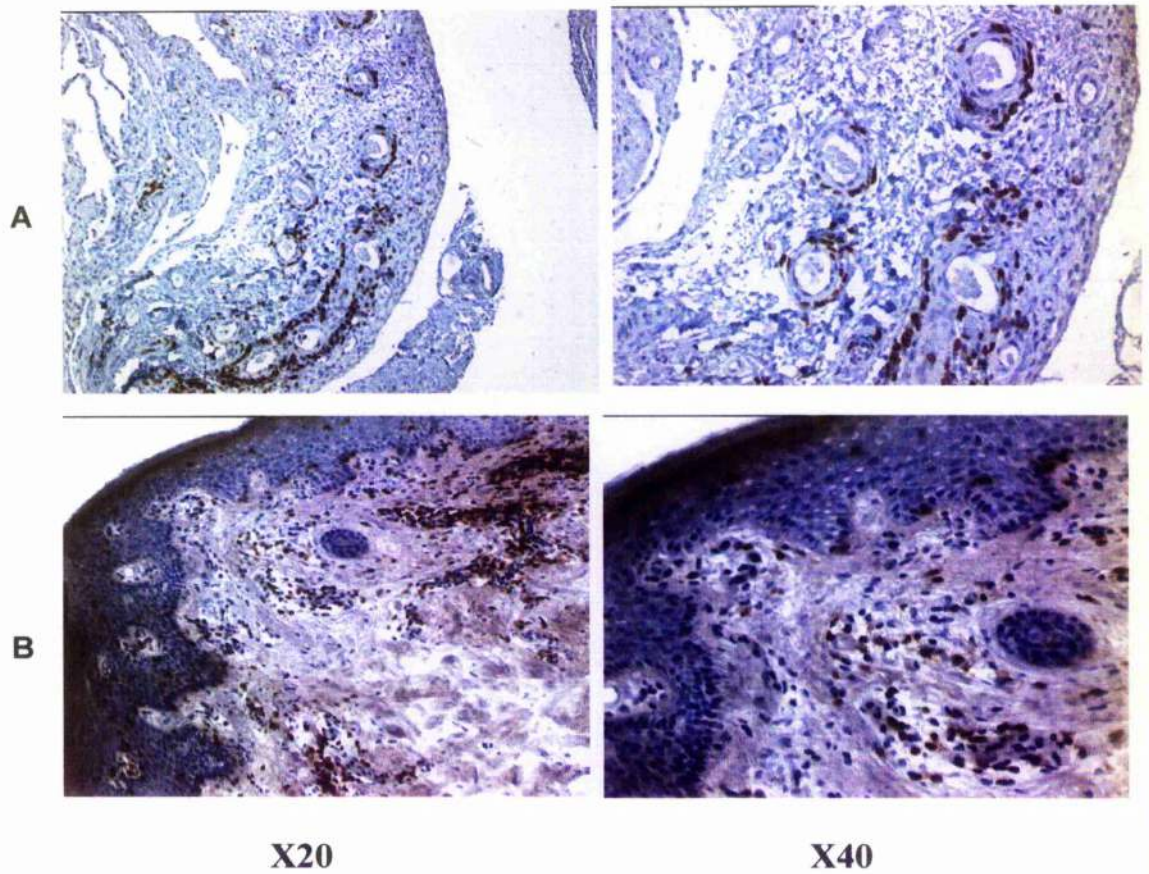
Figure 4.1 T cell infiltration in PsA SM and lesional skin

Immunohistochemical analysis of psoriatic SM (A) and involved skin (B). Cryostat sections (6µM) were acetone fixed and stained with anti-CD3 monoclonal antibody. Primary antibody was detected with biotinylated anti mouse IgG antibody and developed using the avidin/biotin complex immunoperoxidase method and diaminobenzidine for colour development (brown).
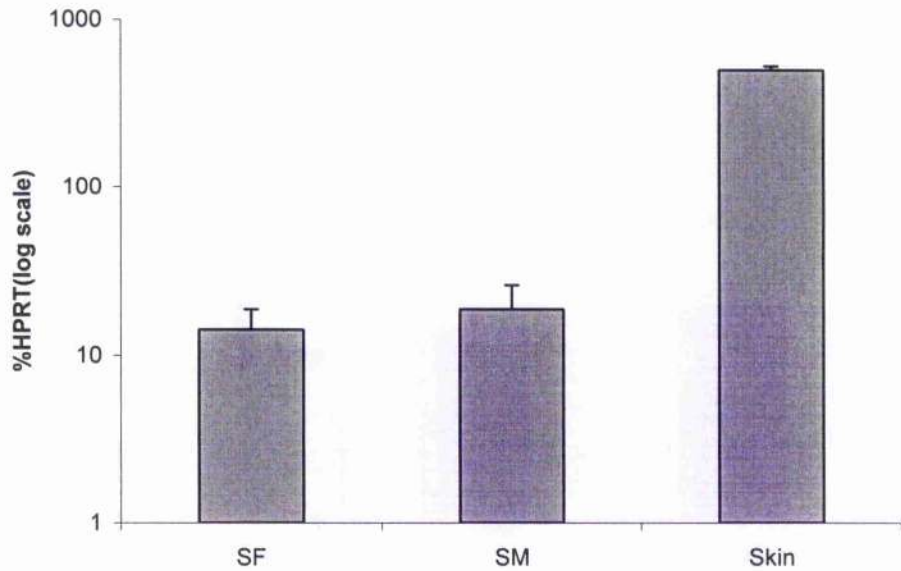
Figure 4.2 IFNγ mRNA expression in PsA and psoriasis

Real Time quantitative PCR was performed to detect human IFNγ expression in PsA SM
(n=8), skin (n=4) and SF MC lysates (n=8). Expression of IFNγ mRNA was expressed as a
percentage of HPRT. Data shown are mean ± s.e.m of triplicate assay.
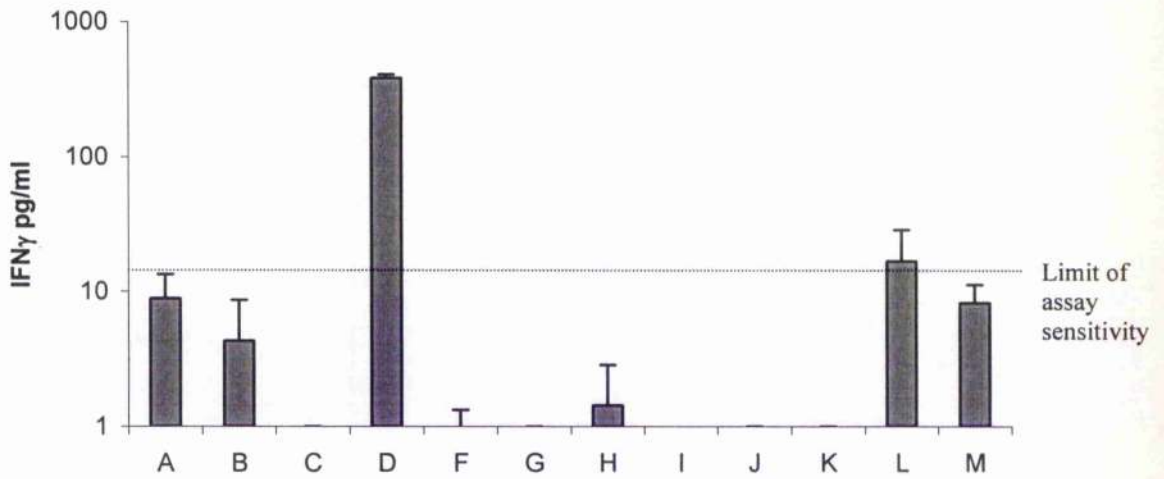
Figure 4.3 IFNγ protein levels in PsA SF

SF from PsA (n=12, patients A-M) patients were obtained at needle arthroscopy or joint aspiration. Fluids were analysed for the presence of IFNγ by ELISA. In most cases there was little or undetectable levels of IFNγ found in the SF supernatants. Data are mean ± s.e.m of triplicate assay.

RA   PsA

IFN-γ

CD3
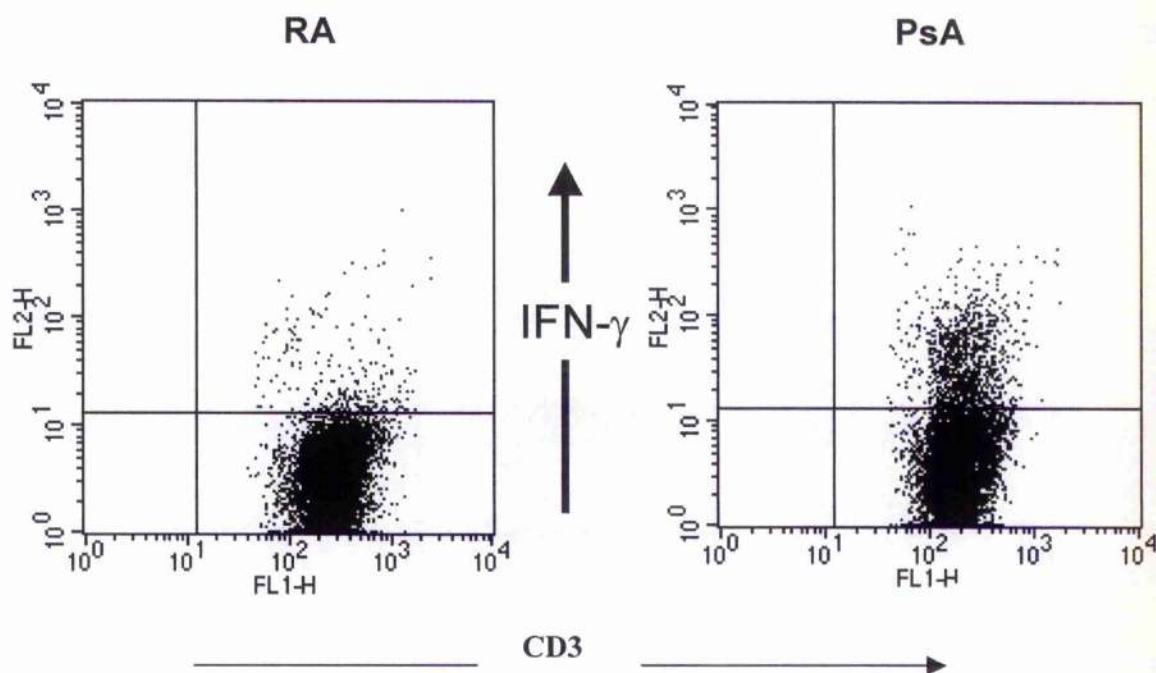
Gated Events: 10000   Gated Events: 10000

Quad	Events	% Total
UL	0	0.00
UR	284	2.84
LL	0	0.00
LR	9716	97.16

Quad	Events	% Total
UL	0	0.00
UR	1256	12.56
LL	0	0.00
LR	8744	87.44

Figure 4.4 Spontaneous IFNγ expression by SF and PBMC in RA and PsA

Double antibody FACS analysis was performed on SF cells derived from patients with active PsA and RA. Lymphocyte population was gated initially on forward and side scatter. IFNγ expression was increased in the PsA cells compared with RA cells. Data are representative of 11 experiments in the PsA group and 7 in the RA group. Data represent the percentage of gated cells with upper right being % IFNγ positive cells.

| | | Percentage IFNγ secretion | |
		CD3+ population	CD3- population
PsA 1	PBMC	7	21
	SFC	8	30
PsA 2	PBMC	2.7	1.3
	SFC	5.6	1.3
PsA 3	PBMC	8.5	14.5
	SFC	19	1.5
PsA 4	PBMC	NA	NA
	SFC	39	2.2

Table 4.1 Matched PB and SFMC in CD3+ and CD3- populations secrete IFNγ.

Matched SF and PBMCs were labelled with FITC conjugated anti CD3 antibody and PE conjugated anti IFNγ detection antibodies following incubation with IFNγ capture antibody. The percentage of CD3+ and CD3- cells that spontaneously secrete IFNγ is shown.

4.3   CCR5/IL18-R expression in PsA

IL-18Rα and CCR5 expression by PsA SF and matched PBMCs was determined to further clarify Th1 responses in PsA.

IL-18Rα is selectively and persistently expressed on Th1 cells (Xu, Chan et al. 1998) making it a useful marker for distinguishing Th1 from Th2 responses in fluid and tissue compartments in disease states. CCR5 has been detected at high levels in Th1 cells with virtual absence from Th2 cells. Immunohistochemistry demonstrates intense CCR5 expression in RA synovium T cell aggregates with relative paucity of the surface membrane Th2 marker CCR3 (Loetscher, Uguccioni et al. 1998).

SF and matched PB were obtained from patients with active PsA. MCs were obtained by density gradient centrifugation and immediately incubated with anti CCR5 and anti-IL-18R antibodies conjugated with FITC and PE respectively. Negative controls were included to allow accurate gating of positively stained cells by FACS analysis (Figure 4.5). Percentage of cells positive for CCR5 and IL-18R in PB compared with SF is demonstrated figure 4.6.
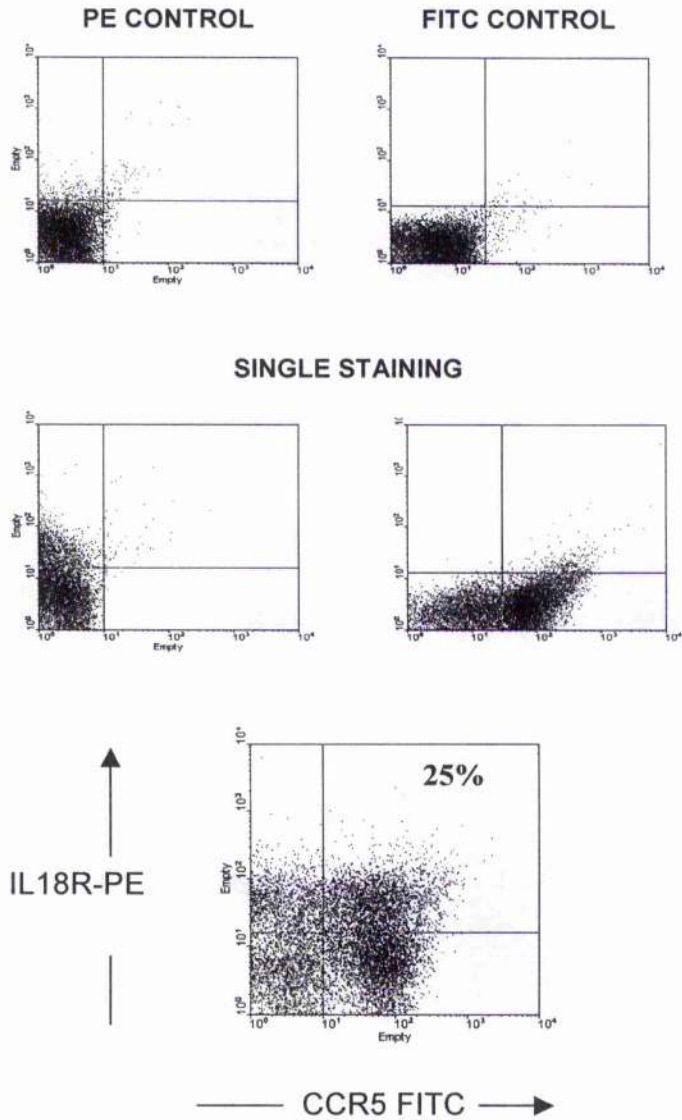
Figure 4.5 CCR5 and IL-18R expression by PsA SF MCs

FACS analysis was performed on SF cells from patients with active PsA. Data

representative of 5 experiments (presented in figure 4.6). 25% of gated cells stained with

both CCR5 and IL18-R antibodies (bottom panel).
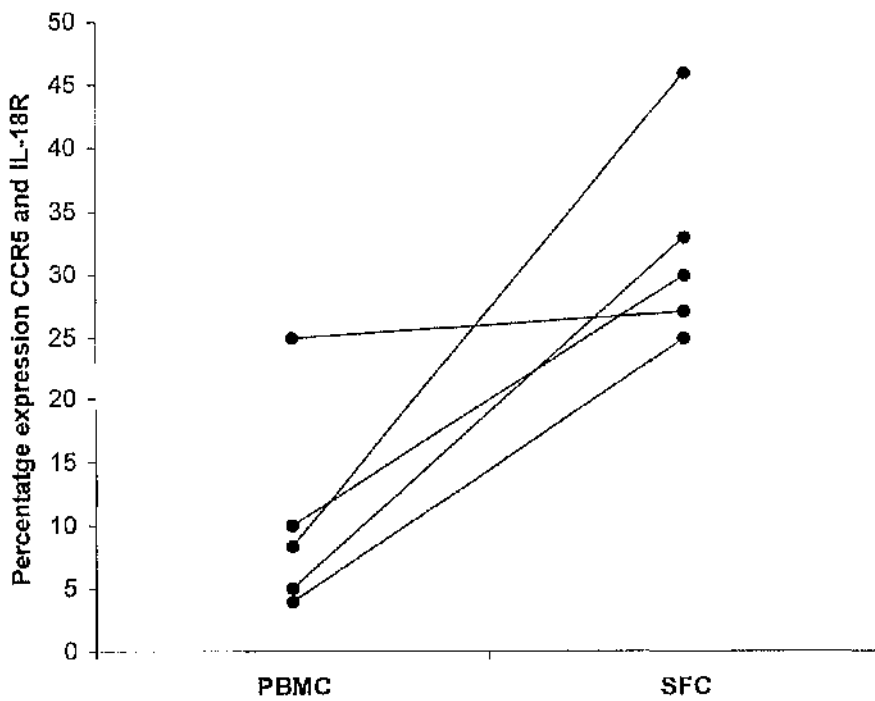
Figure 4.6 CCR5 and IL-18R expression by SF and PBMCs.

Percentage of SF and PB MCs expressing IL18R and CCR5 was calculated using PE labelled anti IL-18R and FITC labelled anti CCR5 antibodies and FACS analysis.

4.4   TNFα expression in PsA

I next sought to determine the functional significance of IFNγ expression in PsA. There are several mechanisms whereby IFNγ may induce inflammation in PsA.

- IFNγ is the prototype macrophage activator of TNFα and as such may exert its pro inflammatory effects in PsA skin and joints via induction of TNFα. The beneficial effects of TNFα blocking therapies indicate that elevated levels of TNFα in PsA are of functional significance (Mease, Goffe et al. 2000).

- Secondly IFNγ may itself be pathogenic in psoriasis and PsA. IFNγ used in clinical trials as a treatment for psoriasis resulted in the development of inflammatory arthritis that resolved with cessation of treatment. Reintroduction of IFNγ led to a relapse of symptoms (Fierlbeck and Rassner 1990). Furthermore, local psoriatic lesions occurred at the injection sites in a subset of cases (Fierlbeck, Rassner et al. 1990).

To explore whether IFNγ may induce TNFα expression in our cohort, it was important to determine whether TNFα expression was expressed in tissue and fluid compartments.

4.4.1 TNFα mRNA expression by PsA SFCs

I initially sought expression of TNFα at the message level in SF MCs. RNA was extracted from isolated SF MCs using TRIzol reagent. RT-PCR was performed to obtain cDNA. Successful RT PCR was confirmed by demonstrating the presence of β actin using standard PCR and gel electrophoresis. Samples were then assessed for the expression of TNFα mRNA.

TNFα mRNA was detected in all samples studied (Figure 4.7)

4.4.2 Spontaneous TNFα expression by PsA SFC and PBMCs

I next sought to determine spontaneous TNFα expression by PsA SF and PBMCs compared with normal PBMCs. Following cell separation, unstimulated cells were cultured in growth medium for 24 hours. TNFα levels in resultant supernatants were analysed by ELISA.

PsA SF MCs produce significantly more TNFα than matched PBMCs ($p < 0.05$). Normal PBMCs produce lower levels of TNFα than PsA PBMCs (Figure 4.8).

1 2 3 4 5 6 7 8 9

26KDa

DNA ladder
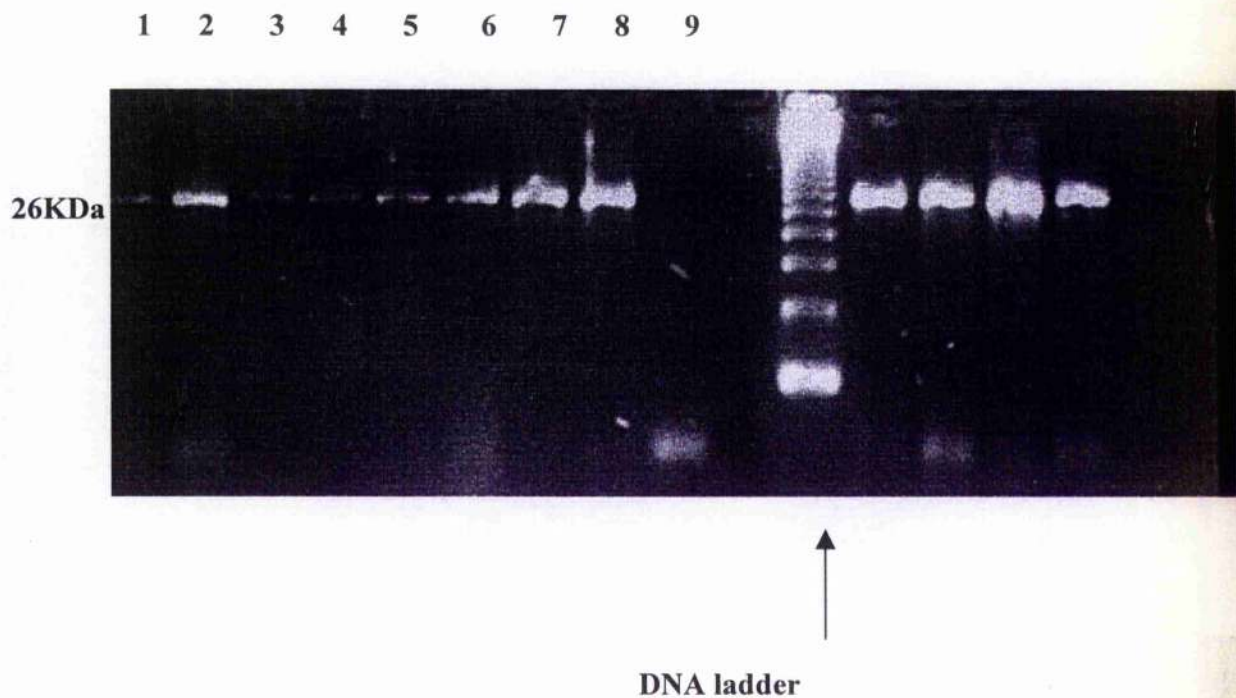
Figure 4.7 TNFα mRNA expression in PsA SF cells

RT-PCR was performed to detect TNFα mRNA in PsA SF cells. Lanes 1-7 - representative of PsA patients, lane 8-positive control, lane 9- negative control 9 (dH₂0 + reagents).   The presence of cDNA was confirmed initially by detecting β Actin in the samples.
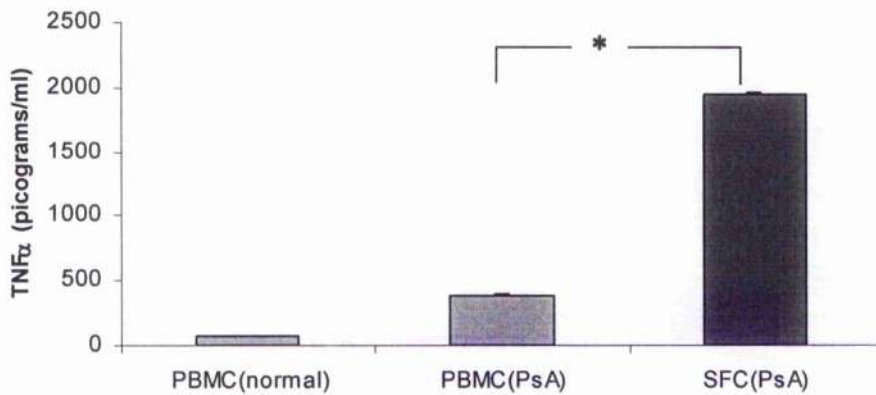
Figure 4.8 TNFα production by PB and SF MCs

Matched SF and PB were obtained from patients with active PsA. PB from a normal control was obtained for comparison. Following 24 hours incubation supernatants were measured for TNFα content by ELISA. Data are mean ± s.e.m.of triplicate assay representative of 3 separate experiments.

(∗ p <0.05, Mann-Whitney).

4.5   Discussion

The expression of CD3$^+$ cells in PsA SM and involved skin support existing data that PsA is a T cell mediated disease. The search for specific T cell clones has been elusive although similar clonal expansion of CD8+ T cells suggests that there is a response to a limited group of antigens (Costello, Winchester et al. 2001). Until such antigens are characterised, attention has focused on the dysregulation of the immune system following antigenic activation. As such, the phenotype and regulation of T cell responses following antigen stimulation may offer hope of finding novel therapeutic targets in PsA.

The characterisation of the T cell response in PsA has been inferred from the detection of elevated levels of Th1 cytokines in SF and SM compartments compared with RA and OA (Partsch, Steiner et al. 1997) (Ritchlin, Haas-Smith et al. 1998). Recent controversy however has arisen regarding the magnitude of Th1 responses in the seronegative arthropathies including PsA compared with RA (Canete, Martinez et al. 2000) hence clarification of this issue is required.

The detection of IFNγ in inflammatory lesions has previously been difficult to quantify, as levels are often low in SF possibly as a result of degradation by proteases or inadequate technology. The double antibody technique employed here to detect spontaneous IFNγ secretion is a major advance in the characterisation of Th1 responses as it permits detection of IFNγ secreting cells without prior mitogen stimulation.

IFNγ mRNA was readily identified using quantitative PCR in SF, SM and involved skin. Thereafter Th1 cells were characterised by spontaneous IFNγ secretion in SF compared with PB of greater magnitude in PsA than in RA.

These observations coupled with expression of Th1 specific cell surface markers CCR5 and IL-18R support the hypothesis that T cell responses in PsA are of Th1 phenotype.

IFNγ is known to stimulate TNFα production and is capable of inducing psoriasis and synovitis when administered subcutaneously for the treatment of cutaneous diseases. As such IFNγ can be implicated as a pro inflammatory cytokine upstream of TNFα. The use of TNFα blocking agents has already been identified as a therapeutic target in PsA and psoriasis. Determining those factors that regulate the immune response upstream of TNFα and IFNγ expression may offer novel strategies to inhibit inflammation. I therefore proceeded to explore those factors that regulate the Th1 responses in PsA.

Chapter 5 Expression of Th1 regulatory cytokines in PsA synovium

The foregoing studies confirm the presence of activated T cells in PsA SF, SM and psoriatic dermis and epidermis in our cohort. Spontaneous IFNγ secretion and CCR5/IL-18R expression clearly indicate a predominant Th1 response with the potential of driving inflammation via the induction of TNFα. Numerous mechanisms could promote and sustain Th1 responses in PsA. The type and dose of antigen, host genetic background and ambient cytokine milieu are of particular relevance. The aim of this study was to identify expression of cytokines in PsA that drive Th1 responses, namely IL-12 and IL-18.

IL-18 expression has been demonstrated by a variety of cells including macrophages, dendritic cells, keratinocytes, osteoblasts and synovial fibroblasts. Previous studies have identified the expression and functional importance of IL-18 in rheumatoid arthritis synovial tissues (Gracie, Forsey et al. 1999) and murine arthritis model systems (Wei, Leung et al. 2001). Furthermore, cutaneous expression of IL-18 by basal keratinocytes is markedly upregulated in psoriatic skin (Naik, Cannon et al. 1999). Despite these previous important observations implicating IL-18 as a relevant cytokine in inflammatory arthritis and psoriasis, the expression of IL-18 in PsA has not previously been described. IL-12 is a Th1 promoting cytokine detectable in rheumatoid and osteoarthritic SMs (Sakkas, Johanson et al. 1998). In psoriasis increased protein dermal expression in association with dendritic cells, macrophages and nerve endings has been described (Turka, Goodman et al. 1995). Enhanced IL-12 p40 mRNA has been detected in lesional psoriatic skin as compared with normal and non-lesional psoriatic skin (Yawalkar, Limat et al. 1996). Furthermore, immunoreactivity for IL-12 p70 is markedly increased in psoriatic skin lesions predominantly expressed on MCs in the dermis (Yawalkar, Karlen et al. 1998).

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The aim of this chapter was therefore to identify the expression and functional implication of IL-12 and IL-18 expression in PsA synovium.

5.1   IL-18 expression in PsA

5.1.1   Expression of IL-18 mRNA in PsA SM, skin, SF and PBMC.

I first sought evidence of IL-18 expression at the mRNA level. Quantitative TaqMan PCR was performed using RNA obtained from PBMCs, SF cells, involved psoriatic skin and inflamed SM, all obtained from PsA patients. Successful RT PCR was confirmed by elucidation of β-actin with ethidium bromide on gel electrophoresis following standard RT-PCR (Figure 5.1). There was significantly higher expression of IL-18 mRNA in PsA SF cells compared with matched PBMCs (Figure 5.2). Of interest however, although IL-18 mRNA was easily detected in the SM, this was at a lower level than that detected in dermis (Figure 5.2; p<0.05, Mann-Whitney).

5.1.2   IL-18 expression in PsA SF and serum

I next determined IL-18 protein in SF and serum from PsA patients. Sandwich ELISA was employed to detect pro and mature IL-18 in PsA SF and serum (Diaclone, France). SFs and matched serum were collected from patients with active PsA undergoing needle arthroscopy. In a subset of patients, fluid alone was obtained at needle aspirate at the time of intra-articular steroid injection as clinically indicated. Samples were spun free of cells and stored at −20°C until assay. Storage had no effect on subsequent cytokine detection (Gracie and McInnes personal communication). IL-18 was detected in all SF samples (Figure 5.3) at concentrations ranging from 18-1200pg/ml (median 172pg/ml). In contrast,

matched serum had low or undetectable levels (SF vs. serum: $p < 0.05$). These levels are comparable to those found in RA serum and SF (Gracie, Forsey et al. 1999) and strongly suggest that local production of IL-18 explains the SF levels detected.

SM   PsA skin   PsA SF
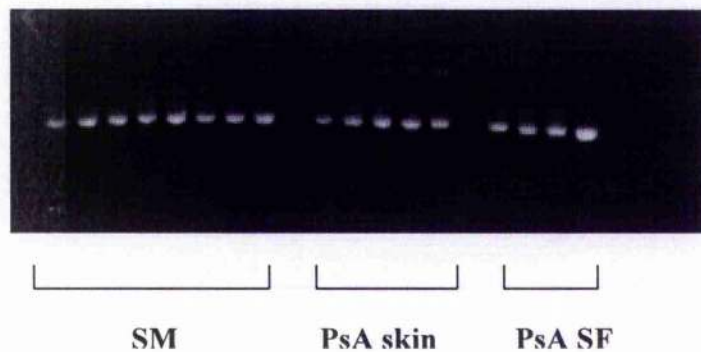
Figure 5.1 RT-PCR for β actin in PsA synovial tissue and fluids

RT-PCR was performed to detect β-actin in PsA PBMCs, SF cells, SM and involved psoriatic skin. Confirmation of the presence of β-actin was required prior to subsequent quantification of IL-18 mRNA expression by TaqMan PCR. Lanes 1-8 representative SM, lane 10-14 involved psoriatic skin, lane 16-19-SF, lane 20- negative control (dH2O + reagents).
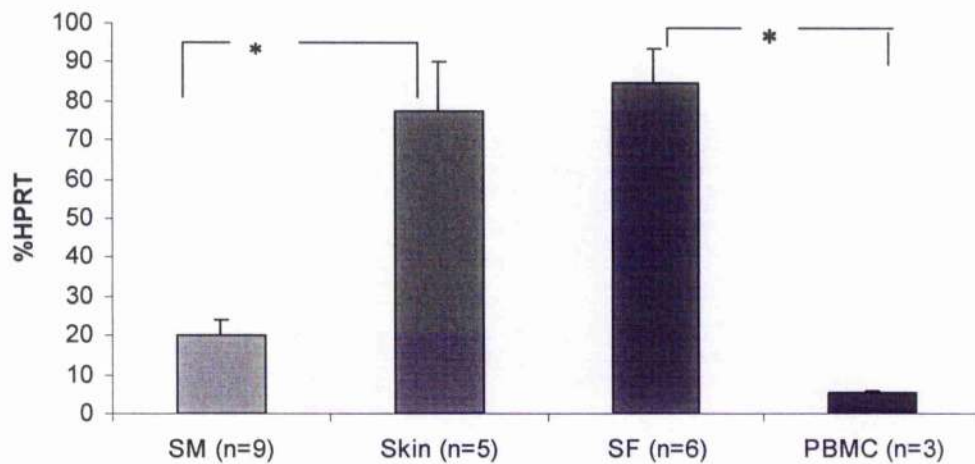
Figure 5.2 IL-18mRNA expression in PsA tissue and cell samples

Quantitative PCR (TaqMan) determined the level of human IL-18 mRNA expression in PsA SM, involved psoriatic skin, SF and PBMCs. Median levels of IL-18mRNA expressed as a percentage of HPRT are shown. n= numbers of samples analysed. Significantly higher levels of IL-18mRNA are found in SF compared with PB and in psoriatic skin compared with membrane. (* p<0.005 Mann-Whitney)
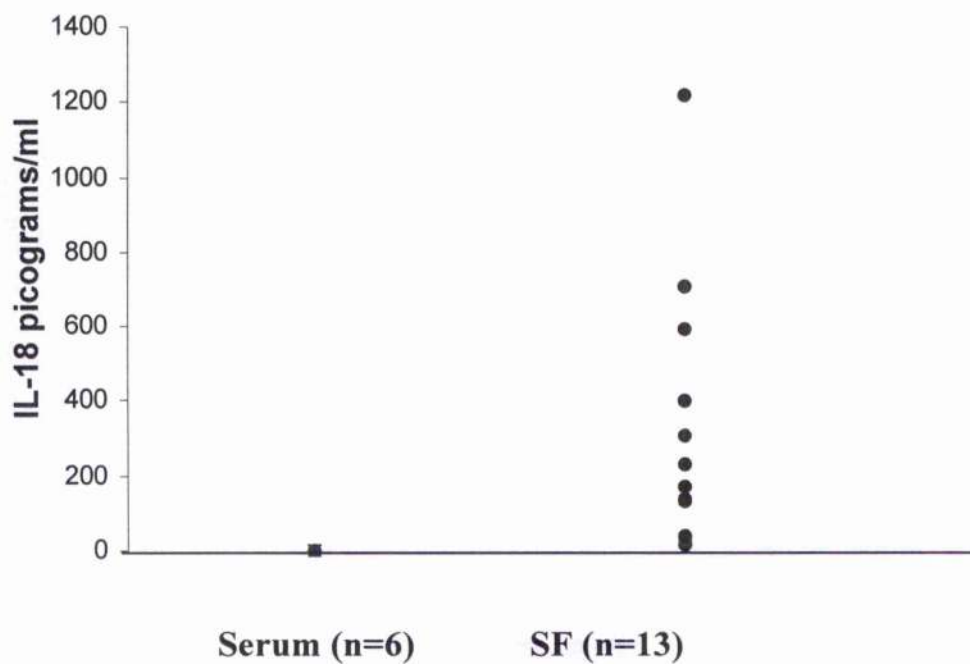
Figure 5.3 IL-18 protein expression in PsA SF and serum

Serum and SFs were collected from patients with active PsA. ELISA with sensitivity ranging of 62.5pg/ml and range up to 2000pg/ml was used to determine IL-18 levels. Levels in PsA SFs were significantly higher than in 6 versus 13 serum samples.

5.1.3 Distribution of IL-18 expression in psoriatic synovium and skin

I next sought the tissue origin and localisation of IL-18 in PsA involved skin and SM. Previous studies in this area have elucidated lining layer and interstitial expression of IL-18 by macrophages and fibroblast like synoviocytes in RA synovial tissue (Gracie, Forsey et al. 1999). It was of interest therefore to compare and contrast this expression pattern with that in PsA. To investigate the presence of IL-18 in PsA SM and skin, paraffin embedded sections of appropriate tissues were stained with murine monoclonal anti-IL-18 (E3E1). Positive staining was effectively neutralised by prior incubation of the antibody for 1 hour at 37^0C with 100 ng/ml rIL-18. Moreover in previous studies (Gracie, Forsey et al. 1999) the staining obtained with E3E1 was shown to be identical to that obtained with a broad panel of monoclonal antibodies kindly donated by Dr A Jackson (Leeds). Tonsil was used as a positive control (Figure 5.4). It is likely therefore that the staining specificity observed is properly representative of tissue IL-18 expression.

The following figures demonstrate the tissue distribution of IL-18 binding in PsA synovium (Figure 5.5) and skin (Figure 5.7). IL-18 expression was present in all tissues examined. The distribution of IL-18 was characterised by semi-quantitative measurement of expression in different tissue compartments. IL-18 was most prominent within and adjacent to lymphoid aggregates in SM, similar to the pattern of expression found in tonsil. IL-18 was also detected however in lining layer and in sublining interstitial areas, although cell numbers in these areas were lower (Table 5.1). Several cell lineages have been shown capable of IL-18 expression, including macrophages, endothelial cells, and synovial fibroblasts. Since macrophages were the most likely predominant cell subset with IL-18 expression, parallel sections were stained with monoclonal antibodies to CD68 and to IL-

18. IL-18 co-expressed with CD68$^+$ macrophages predominantly in the lining layer in SM (Figure 5.6).

For comparative purposes, IL-18 expression in psoriatic skin lesions was characterised using E3E1. Figure 5.7 demonstrates predominant staining in the epidermis, with broad expression throughout the keratinocytes in both the basal layer and in superficial areas. In the dermis, co-expression was found predominantly in CD68+ macrophages (Figure 5.6). Finally, IL-18 expression was compared in matched biopsies obtained from synovium and skin of PsA patients. Data shown in table 5.2 demonstrate the predominant expression in keratinocytes that exceeds that observed in other inflamed tissues. These data are commensurate with the higher levels of IL-18 mRNA detected in skin as compared with synovium (Figure 5.2).

Figure 5.4 IL-18 expression in human tonsil.

Paraffin embedded sections were stained with anti IL-18 antibody (E3E1). Primary antibody was detected with biotinylated goat anti mouse IgG using the avidin/biotin complex immunoperoxidase method. Colour development was achieved using diaminobenzidine. A, tonsil positive control B, tonsil negative control (Magnification x20).
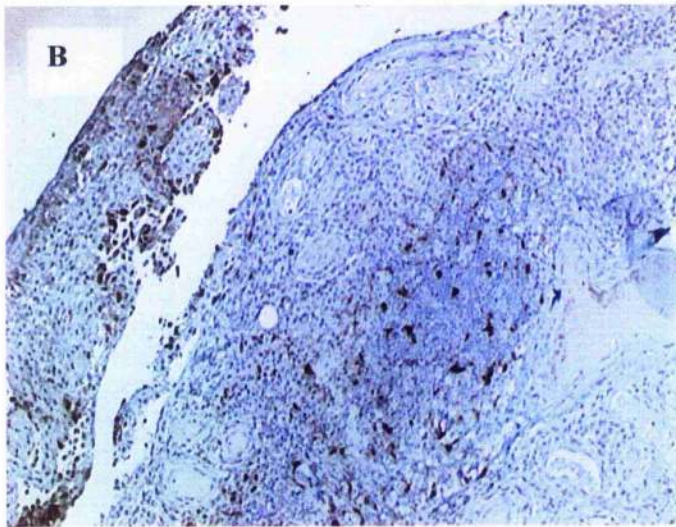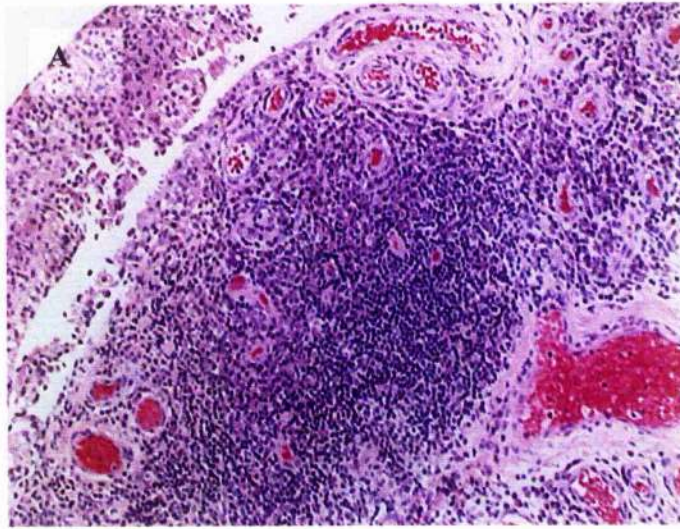
Figure 5.5 IL-18 expression in psoriatic SM.

Immunohistochemical analysis of SM from a representative PsA patient. Paraffin embedded sections were stained with H&E (A) anti IL-18 antibody (E3E1) (B) as described in legend figure 5.4. (Magnification x20).

	LL	SL	LA
PsA1	3	0	1
PsA2	3	0	1
PsA3	2	0	N/A
PsA4	0	0	2
PsA5	3	0	N/A

Table 5.1 Distribution pattern of IL-18 expression in PsA synovial tissues

Quantitative expression of IL-18 was assessed in synovial tissue lining layer (LL), sub lining layer (SL) and lymphoid aggregates if present (LA). A scoring system agreed between 2 independent observers was employed. 0 = no staining, 1= 1-10% of nucleated cells stained positive, 2=10-25% nucleated cells, 3=25-50% and 4=>50% positive. N/A, no aggregates present.
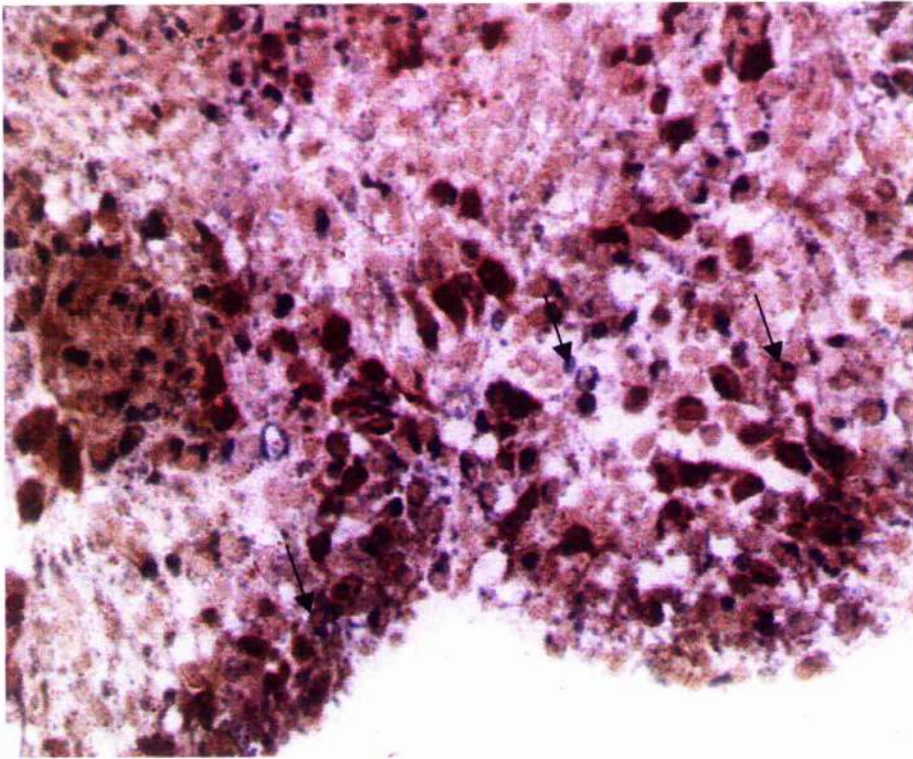
Figure 5.6 IL-18 expression in CD68$^+$ macrophages

Double staining of PsA SM is shown from a representative PsA patient. IL-18 was detected immunochemically using anti IL-18 antibody E3E1 and developed with diaminobenzidine (brown). Macrophages were then stained with anti-CD68 and developed using Vector SG (grey). Magnification x20. Double positive cells are shown by arrows.
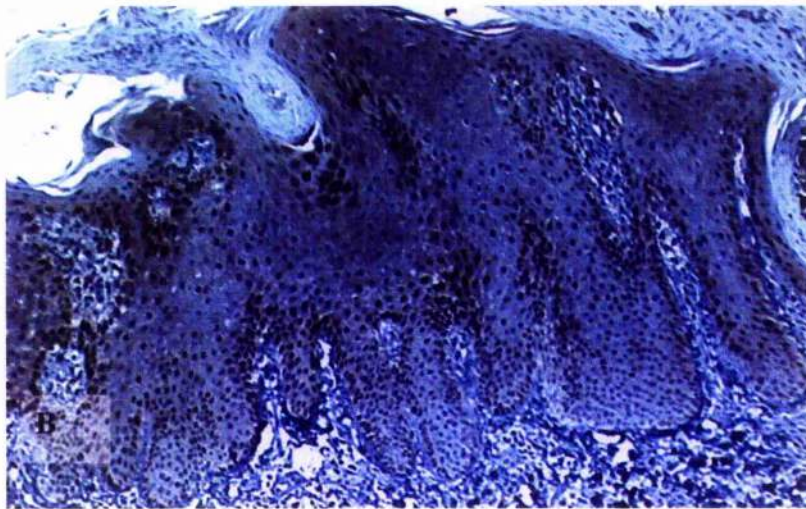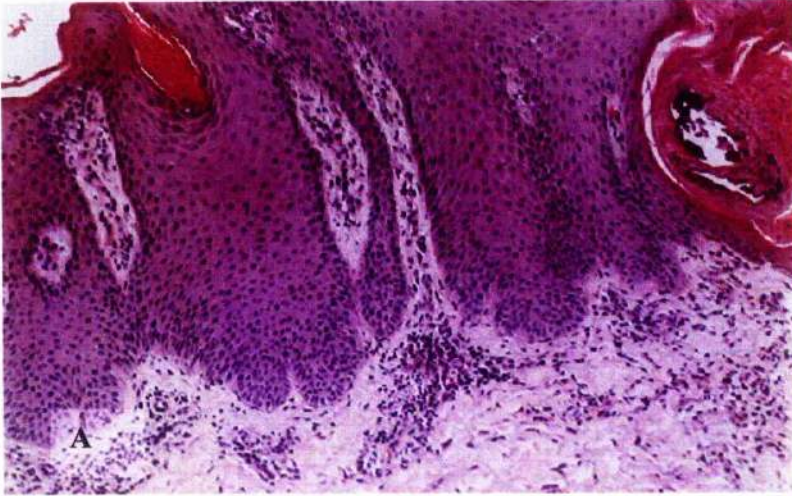
Figure 5.7 IL-18 expression in involved psoriatic skin.

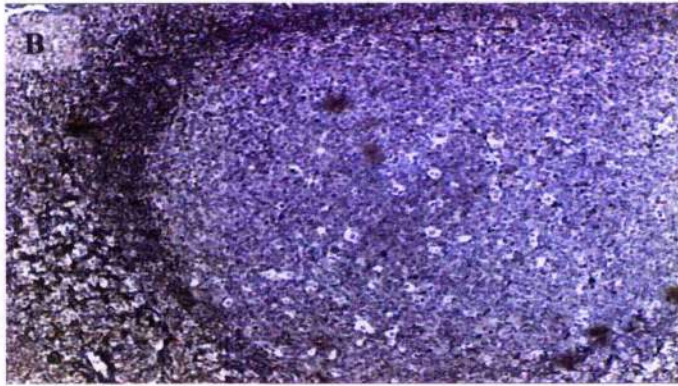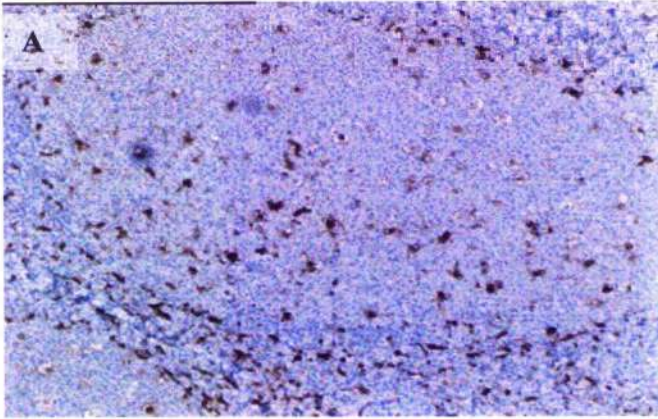Immunohistochemical analysis of involved skin from a representative PsA patient. Paraffin embedded sections were stained with anti IL-18 antibody (E3E1). Primary antibody was detected with biotinylated goat anti mouse IgG using the avidin/biotin complex immunoperoxidase method. Colour development was achieved using diaminobenzidine. A, psoriatic skin H&E; B, Psoriatic skin with anti IL-18; (Magnification x20)

	Epidermis		Subdermis		Synovial membrane	
	CD68	IL-18	CD68	IL-18	CD68	IL-18
PsA 1	0	4	2	1	2	3
PsA 2	0	4	1	1	2	3
PsA 3	0	4	3	2	3	1
PsA 4	0	4	2	1	2	2

Table 5.2 IL-18 and CD68 co-localisation in psoriatic skin and SM

Quantitative expression of IL-18 and CD68 was assessed in matched skin / synovial tissue pairs (n=4) using a scoring system agreed between 2 independent observers. 0 = no staining, 1= 1-10% of nucleated cells stained positive, 2=10-25% nucleated cells, 3=25-50% and 4=>50% positive.

5.1.4 SM and skin secrete IL-18

Finally, I investigated whether SM and skin released IL-18 in vitro. To this end I established a novel synovial biopsy explant culture system. Freshly isolated SM and involved psoriatic skin was obtained at needle arthroscopy and skin biopsy respectively. Tissue was gently disggregated with a 19G needle, then immediately transferred to culture medium and cultured for 72 hours *in vitro* without exogenous stimulation. Subsequent supernantants were assayed for the presence of IL-18 by ELISA. SM and skin explant cultures produced median 2910 pg/ml and 8799 pg/ml IL-18 respectively (figure 5.8). These data confirmed that IL-18 expression detected at mRNA level in tissue was reflected in endogenous secretory activity *ex vivo*.

5.1.5 IL-18 release via Benzyl ATP dependant pathway

Elucidation of the mechanisms whereby cytokines are released from cells may offer further potential to inhibit cytokine-mediated inflammation. It is already well established that the rapid release of IL-1β requires ATP and is a receptor-mediated event. ATP receptors such as P2X-7 are found on many cells including monocytes and macrophages. When triggered by ATP, the cell membrane becomes transiently porous with loss of membrane electrical potential. Triggering of the P2X-7 is specific for the release of mature IL-1β and release of IL-18 in LPS-stimulated whole blood cultures (Perregaux, McNiff et al. 2000). Furthermore, antagonists to P2X7 result in reduced IL-18 and IL-1β release from LPS-treated human macrophages (Ferrari, Chiozzi et al. 1997). We therefore sought to determine whether IL-18 release as observed in PsA tissue cultures is ATP dependent.

To determine optimal experimental conditions, SF MCs were stimulated with LPS at concentrations of 10μg/ml and 100μg/ml for 1 or 2 hours. Secretion of IL-1β following the addition of benzyl ATP for 20 minutes was assessed in culture supernatants by ELISA. Optimal IL-1β expression was observed following 2 hours incubation with LPS 10μg/ml (971pg/ml). Further stimulation with LPS at 100μg/ml resulted in no further IL-1β expression in the presence of Benzyl ATP (608pg/ml) (Figure 5.9).

Having identified 2 hours was optimal time for LPS stimulation; single cell adherent suspensions were cultured at $2x10^6$/ml in the presence of 2 different concentrations of LPS (10μg/ml of 100μg/ml). Fresh culture medium was added with or without benzyl ATP and resultant supernatants were assayed for the presence of IL-18 by ELISA.

IL-18 levels in SF cell supernatants were generally low despite stimulation with LPS 10μg/ml and 100μg/ml. The addition of Benzyl ATP to SF cells stimulated with LPS at both concentrations for 2 hours resulted in an increase of IL-18 production (Figure 5.10).
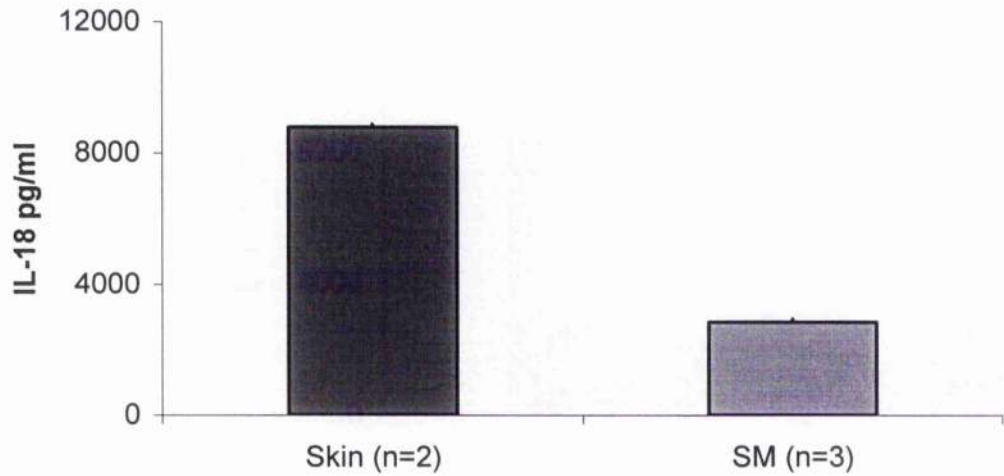
Figure 5.8 IL-18 production by psoriatic skin and SM *ex vivo*

Lesional psoriatic skin and SM were obtained from patients by elliptical skin biopsy and needle arthroscopy respectively. Tissue was disaggregated with a 19G needle and subsequently cultured without exogenous stimulation for 72 hours. IL-18 levels in resultant supernatants were measured by ELISA.

A

B
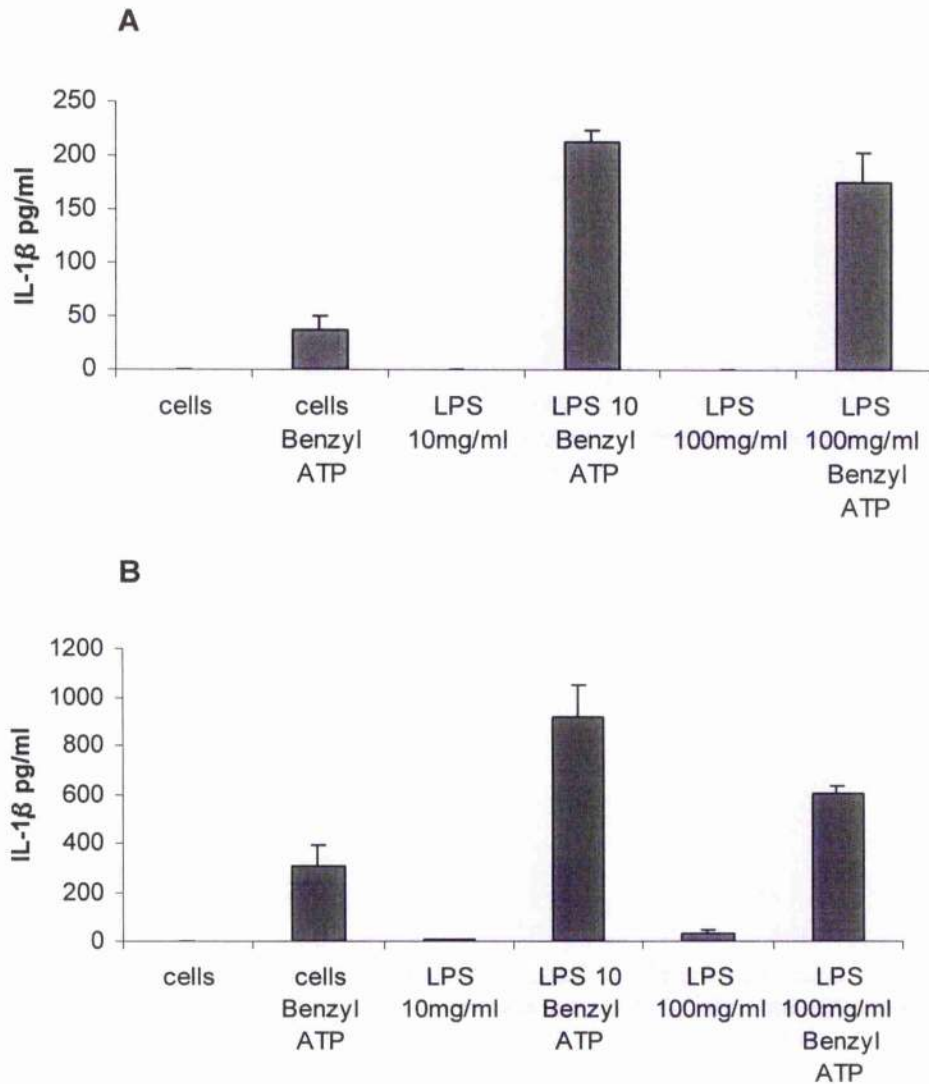
Figure 5.9 Benzyl ATP release of IL-1β from LPS stimulated PsA SF MCs

PsA SF cells were cultured in the presence of LPS at concentration of 10µg/ml and 100µg/ml for 1 hour (A) or 2 hours (B). Supernatants were removed after 1 and 2 hours then replaced with fresh culture medium plus or minus the addition of Benzyl ATP. Supernatants were then harvested after 20minutes and assessed for IL-1β secretion by ELISA.
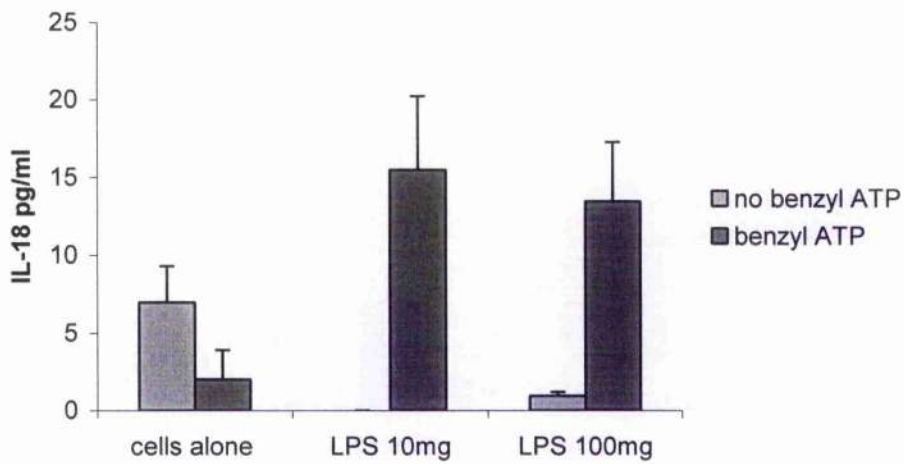
Figure 5.10 IL-18 release from LPS activated PsA SF MCs with Benzyl ATP

Benzyl ATP was added to cultures containing SF MCs stimulated with LPS at 10mg/ml

and 100mg/ml for 2 hours. Supernatants were harvested after 20 minutes for detection of

IL-18 by ELISA.

5.2   IL-12 expression in PsA

We have previously shown that IL-18 can drive Th1 and Th2 responses depending on host genetic background and ambient cytokine milieu (Xu, Chan et al. 1998). Therefore the defining cytokine functional phenotype may depend not primarily upon IL-18 expression but upon the context of such expression. IL-12 is critical to this process since IL-12 in synergy with IL-18 drives Th1 responses *in vivo* (Okamura, Kashiwamura et al. 1998). Increased levels of IL-12p70 have been described in PsA SFs (Spadaro, Rinaldi et al. 2002). I therefore defined IL-12 expression in PsA tissues, which had not previously been described. Parallel studies performed by our collaborators (Dr David Kane and colleagues, Dublin) have identified the presence of IL-12 mRNA in PsA SM samples.

5.2.1 IL-12 p70 and IL-12 p40 expression in PsA SF and serum

To determine SF and serum levels of total IL-12 p40 and of IL-12 p70 heterodimers, sandwich ELISA was performed. Serum and SFs were obtained from patients with active PsA disease at the time of needle arthroscopy or joint injection as clinically indicated. ELISA detected elevated levels of IL-12 p40 up to 334pg/ml in SFs in 11 patients with active PsA. Matched serum was available in 5 patients that was found to contain comparable levels ranging from 25pg/ml to 94pg/ml (SF vs. serum: p=0.3). The median concentration of IL-12 p40 in SF was 45pg/ml and 40pg/ml in serum (Figure 5.11).

IL-12 p70 levels were also detected in SF from PsA patients (Figure 5.12), whereas lower levels were detected in SF from RA patients with active disease prior to commencement of DMARD administration (gift of Dr D Porter, TICORA study sera). Surprisingly however IL-12p70 expression was detected in serum from normal, healthy volunteers at higher levels. This experiment was repeated with identical results to confirm the levels detected. The significance of this is not clear but could reflect consumption of IL-12p70 in actively inflamed patients leading to spuriously low levels detectable as free cytokine as compared to 'non-inflamed' healthy volunteers.

5.2.2 IL-12 expression in PsA SM

I next determined the pattern of tissue expression of IL-12 in PsA SM and skin. Paraffin embedded tissue sections were stained with murine monoclonal anti IL-12 p35 (kindly gift of Dr S Porcelli, Wistar Institiute, Philidelphia). Figure 5.13 demonstrates tissue expression of IL-12p35 in PsA SM and skin. IL-12 p35 subunit was detected in all SM and skin sections examined (n=6). In SM, staining was found predominantly in the sub-lining layer, with few lining layer cells found to contain IL-12. In skin sections, IL-12 p35 was expressed by keratinocytes in the epidermis. There was additional expression in the dermis but to a lesser degree. Serial sections were subsequently stained for the expression of IL-12p70 using IL-12p70 monoclonal antibody (R&D clone 24945.11). No positive staining was detected using this antibody in SM or skin sections. Thus, the epitope on the IL-12 p40/p35 heterodimer that is expressed and amenable to antibody binding in tissue, may be differentially detected by discrete monoclonal antibody clones. It is also possible that p35 could be present as part of a novel cytokine complex with other homologues of the IL-12 family e.g. IL-27, EBI3, thereby yielding signal without concomitant IL-12p70 detection (Pflanz, Timans et al. 2002; Brombacher, Kastelein et al. 2003).
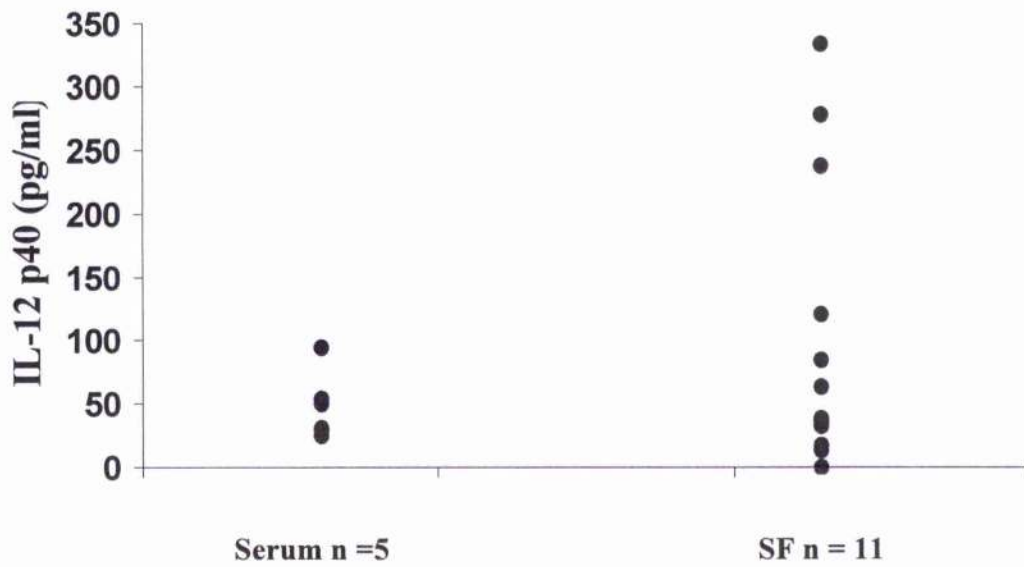
Figure 5.11 IL-12 p40 expression in PsA serum and SF

Serum and SFs from patients with active PsA were obtained and assayed for the presence of IL-12p40 by ELISA. Levels in SFs were higher than serum however this did not reach statistical significance (p=0.3, Mann Whitney)
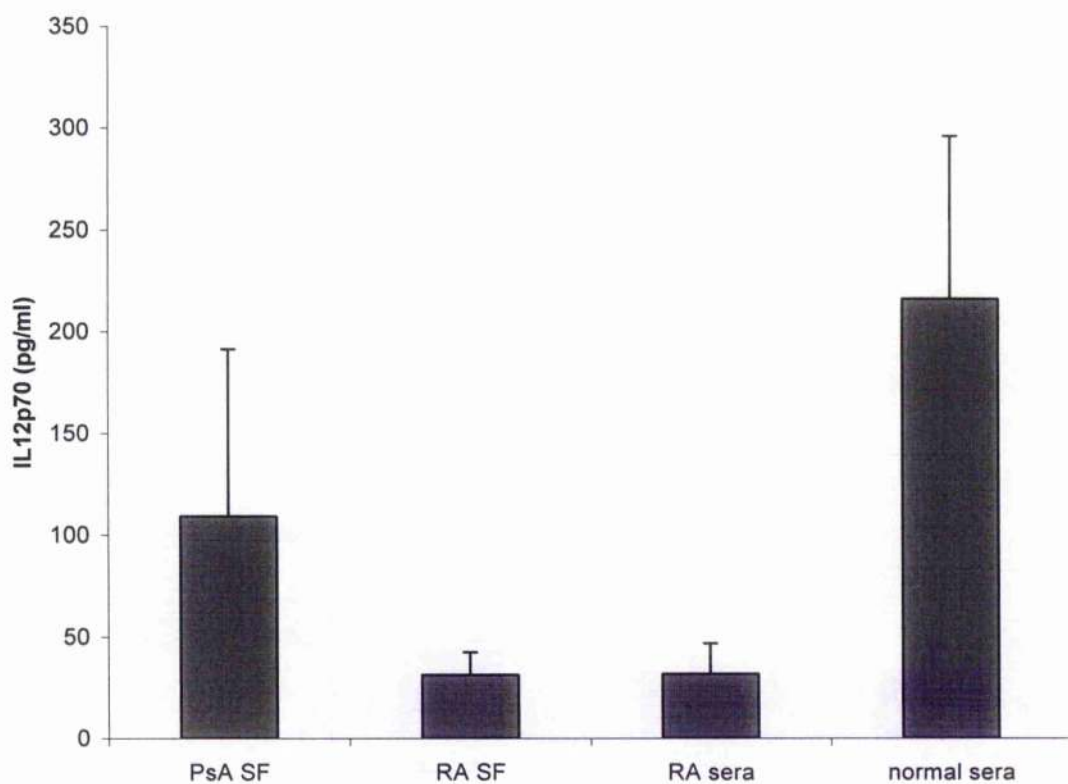
Figure 5.12 IL-12 p70 expression in PsA serum and SF

SF was obtained from patients with active PsA (n=19) and RA (n=20). Sera were similarly

obtained from RA (n=20) and normal individuals (n=19) for comparison. IL-12p70 levels

were analysed by ELISA.
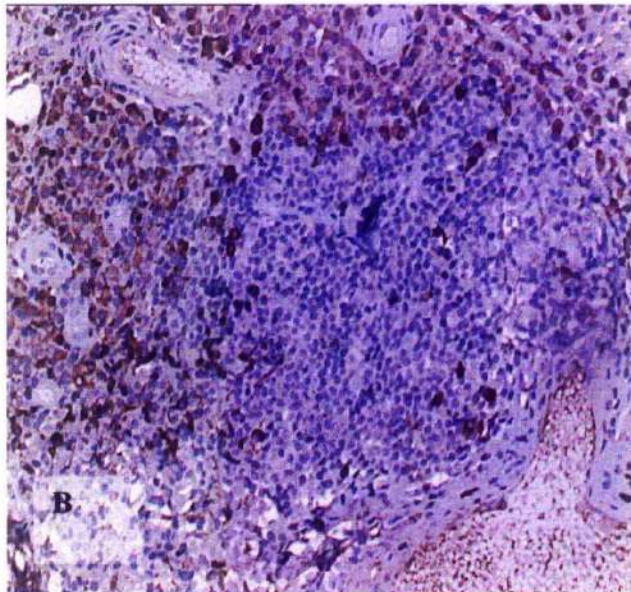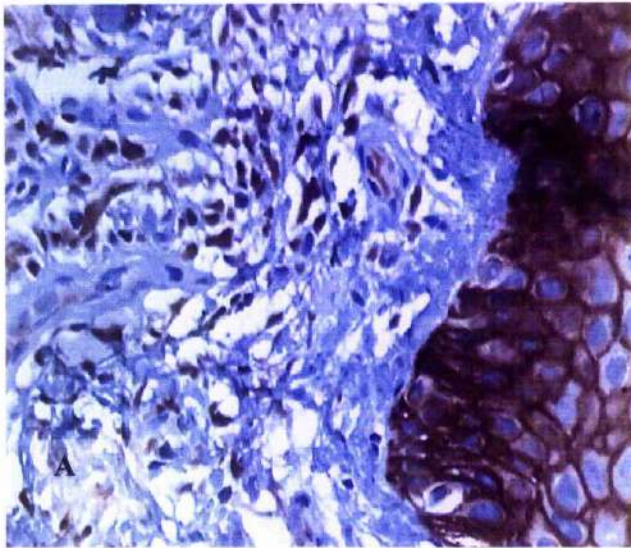
Figure 5.13 IL-12 p35 expression in PsA SM and skin

Immunohistochemical analysis of skin (A) and synovial tissue (B) from a representative PsA patient. Paraffin embedded sections were fixed in acetone and stained with anti IL-12p35 (clone C7.4). Primary antibodies were detected using biotinylated goat anti mouse IgG then with streptavidin–alkaline phophatase complex and diaminobenzidine. A, PsA skin and B, PsA SM. (Magnification x 20)

5.3 Discussion

This study clearly demonstrates the presence of regulatory cytokines IL-18 and IL-12 in psoriatic SM, skin and SFs. IL-18 and IL-12 are critical regulators of Th1 responses. The detection of IL-12 and IL-18 in this context provides a possible mechanism for the predominant Th1 responses indicated in the previous studies.

The expression of IL-18 mRNA in PsA SF and membrane is a novel observation. This study revealed markedly up regulated cutaneous IL-18 mRNA expression in involved skin commensurate with previous studies indicating that human keratinocytes constitutively express IL-18 and secrete IL-18 after treatment with pro-inflammatory mediators (Naik, Cannon et al. 1999).

Variable levels of IL-18 were detected in PsA SF compared with absence of cytokine in matched sera. This finding contrasts with those in RA where levels of IL-18 were detected in one third of serum samples (Gracie, Forsey et al. 1999). SM was the likely source of IL-18 as it was present in synovial cultures and could be predominantly localised immunohistochemically to the synovial lining layer. The release of IL-18 appeared to be benzyl ATP dependant as LPS stimulation in the presence of benzyl ATP resulted in a significant increase in cytokine detection. This observation confirms previous studies that indicate release of both IL-1β and IL-18 are ATP dependant. Such observations have clinical implications as IL-1β and IL-18 release can be completely blocked using a P2X7 receptor antagonist. There is clearly the possibility that IL-18 levels detected in synovial culture may represent release of stored cytokine and further experiments are required to determine whether IL-18 inhibitors can reduce synthesis *in vitro*.

IL-18 expression in SM and skin was localised to the lining layer and keratinocytes respectively. Other pro inflammatory cytokines such as IL-1, IL-6 TNFα that are abundant in synovial tissue are similarly localised to the synovial lining layer where they are predominantly macrophage derived. The intensity of lining layer cells that expressed IL-18 and the double staining with CD68+ cells make it likely that macrophages contribute to the synovial production of IL-18 in PsA. However IL-18 is expressed by a wide variety of cells and further localisation of cellular expression using dual staining is required.

The biological profile of IL-18 in RA and animal models implicates it as a key pro inflammatory cytokine in other inflammatory autoimmune diseases such as psoriasis and PsA. Localised expression of IL-18 by SM in this study may have implications for mode of delivery of IL-18 targeted therapies.

Although IL-18 is capable of promoting synovial inflammation independently of IL-12, co administration of IL-12 and IL-18 promote more severe disease in animal models of arthritis (Leung, McInnes et al. 2000). Transient gene transfer for IL-12 in animal models accelerated disease progression and increased severity of arthritis (Parks, Strieter et al. 1998). Neutralisation of IL-12 resulted in delayed disease onset and attenuation of disease. Furthermore, IL-12, independent of IFNγ, is able to induce pathogenic, inflammatory T cells that are able to induce psoriasis in mice (Hong, Chu et al. 1999). The expression of IL-12 was therefore a logical progression to place IL-18 expression in context. IL-12 induces IFNγ via up regulation of IL-18R. Studies in chapter 4 demonstrated IL-18R expression on PsA SF cells suggesting that this mechanism may be occurring within the synovial compartment.

IL-12 p35 staining was detected in all membrane and skin samples analysed. Higher levels of IL-12 p40 subunit protein were detected in the SF compared with matched serum suggesting local production of cytokine. The high levels of IL-12p70 in normal serum compared with SF and serum of patients with inflammatory arthritis is unexpected. The emerging biology of IL-12 may explain some of the findings in this study. On activation, heterodimeric IL-12 is found in small amounts, whereas free p40 is produced in excess. Besides IL-12, other p40-dependent molecules exist that orchestrate Th1 responses. Homodimeric p40 can act as an IL-12 antagonist by competing for its receptor. Recent data also reveal potential immunostimulatory functions of p40. In addition, p40 can be covalently linked to a p35-related protein p19. This heterodimer is known as IL-23, a novel cytokine, that has activities on memory T cells. Finally, IL-27 is a heterodimer composed of the p40-related protein EBI3 (Epstein-Barr virus-induced gene 3) and the p35-related protein p28. IL-27 is involved in early Th1 initiation (Brombacher, Kastelein et al. 2003).

Having previously defined that PsA is a Th1 mediated disease and that regulatory cytokines such as IL-12 and IL-18 are expressed in SM and fluid, it was therefore crucial to determine the functional significance of such cytokine expression.

Chapter 6 Regulation of Th1 responses in PsA

A critical aspect of host immune defence against infection is mediated via the effector function of Th cells. Following antigen presentation, Th cells differentiate into functionally distinct subsets characterised on the basis of their effector cytokine release (chapter 1). Most immune responses involve contributions from both Th1 and Th2 cells that cross regulate effector functions - a balance between Th1 and Th2 cytokine could therefore determine whether responses are appropriate or detrimental (immunopathogenesis).

The differentiation of Th1 and Th2 responses from precursor Th cells (Thp) depends on a variety of complex developmental processes including dose of antigen, density of the antigen peptide: MHC complex, co stimulation, genetic background and critically the ambient cytokine milieu. In chapter 4 I demonstrated some evidence for the presence of Th1 cells that were activated and differentiated *in vivo* in PsA. Thereafter in chapter 5 I have demonstrated the presence of cytokines IL-12 and IL-18 that are known to promote the development of Th1 cells. However to confirm that these phenomena are functionally linked it was important to test the activities *ex vivo* of IL-12 and IL-18 in PsA derived cell lineages. To this end, I have utilised PsA PB and SF derived cell cultures to measure the effector activity of IL-12 and IL-18 in the context of plausible Th1 mediated function. The aim of this chapter was therefore to determine the functional effects of regulatory cytokines *in vitro* thereby placing IL-12, IL-18 and IFNγ expression in context. Preliminary data exploring intracellular signalling and cytokine release mechanisms are described.

6.1 Effects of CD3, CD28 and cytokines on PsA SF MC proliferation

Optimal T cell activation requires the presence of a co-stimulatory signal that may be generated via the interaction between CD80/CD86 on the APC and CD28 expressed on the T cell. To confirm that SF MCs were activated by this mechanism, proliferation in response to anti-CD3 (plastic adherent) /anti-CD28 (soluble) stimulation in the presence of the innate cytokines IL-12 and IL-18 was determined using ^{3}H-thymidine incorporation assays. IL-15 was added in addition as a further positive control (McInnes, al-Mughales et al. 1996). Cytokine concentrations were derived from previously published experiments in the laboratory that showed synergistic effects on RA derived synovial cultures (Gracie, Forsey et al. 1999). Proliferation was significantly increased by the addition of anti-CD3 / anti-CD28 in comparison to resting cultures (p <0.05, Mann Whitney). However, the addition of recombinant cytokine IL-12, IL-15 or IL-18 had no additional effect on proliferation (Figure 6.1). Since IL-15 has previously been shown to induce high levels of RA and PsA SF lymphocyte proliferation in isolation, this suggested that the mitogen antibody cocktail employed had induced maximal proliferative responses that were unlikely to be further enhanced by cytokine addition.

6.2 Synergistic effect of IL-12 and IL-18 in IFNγ production by PsA SF cells

Although no additional response was measured in terms of proliferation, it remained possible that alterations in cytokine output could occur. Anti-CD3 / anti-CD28 stimulated SF cells were therefore cultured with IL-12 and IL-18 alone or in combination for 24 hours as before and cytokine release was measured by ELISA. Addition of IL-18 alone induced

minimal IFNγ release. In contrast, and commensurate with previous observations in human T cell clones (Ahn, Maruo et al. 1997), IL-12 was able to induce IFNγ release. Addition of IL-18 together with IL-12 led to further enhancement of IFNγ release suggesting a cooperative / synergistic role for IL-18 in Th1 effector function (Figure 6.2).

6.3   Effect of IL-12 and IL-18 on TNFα production

The observation that more T cells in PsA than in RA synovium were IFNγ positive (chapter 4) suggested that IFNγ could play a distinct role in TNFα regulation in PsA. TNFα release in the above culture system was therefore examined to determine whether IL-12 and IL-18 provoked similar TNFα secretion patterns. Anti-CD3 / anti-CD28 induced basal TNFα expression that was higher in SF MCs than in PB MCs (252pg/ml vs. 46pg/ml; Figure 6.3). Unlike IL-12, IL-18 could promote TNFα release in PB cultures. (IFNγ release is not easily detected in primary PB cultures and was not performed in these experiments (Gracie, Forsey et al. 1999). Both IL-12 and IL-18 induced significant increases in TNFα production by SF MCs (p<0.05, Mann Whitney). However IL-18 induced significantly higher levels of TNFα release than IL-12. There was no synergistic induction of TNFα in PBMCs with IL-12 and IL-18 combined. Since these cultures did not contain measurable IFNγ (Figure 6.2) it is probable that IL-18 was operating via an alternative pathway. To test this formally a neutralising anti-IFNγ antibody would be required – unfortunately this was not available at the time of performing these experiments. As a further control, no significant increase in TNFα production was detected by SF MCs

cultured with IL-15 alone (Figure 6.3) suggesting that the effects noted above might be specific to given innate cytokine subsets.
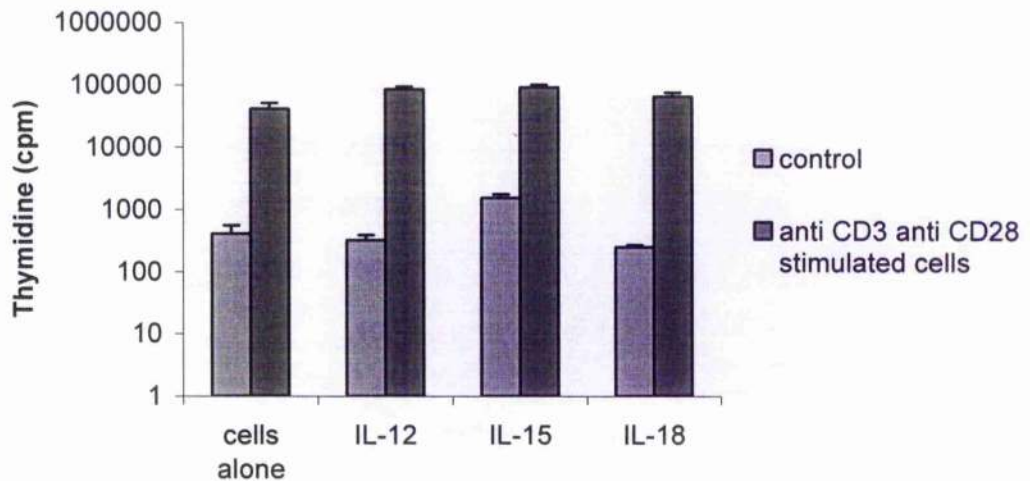
Figure 6.1 SF cell proliferation with anti-CD3 and anti-CD28

PsA SF MCs were cultured for 24 hours with recombinant cytokines IL-12 (1ng/ml), IL-18 (100ng/ml), IL-15 (1ng/ml) and ^{3}H-thymidine incorporation was measured over the last 6 hours of culture. Data shown are mean ± s.e.m of triplicate culture. (*P<0.05, Mann Whitney). Data shown are representative of 4 similar experiments.
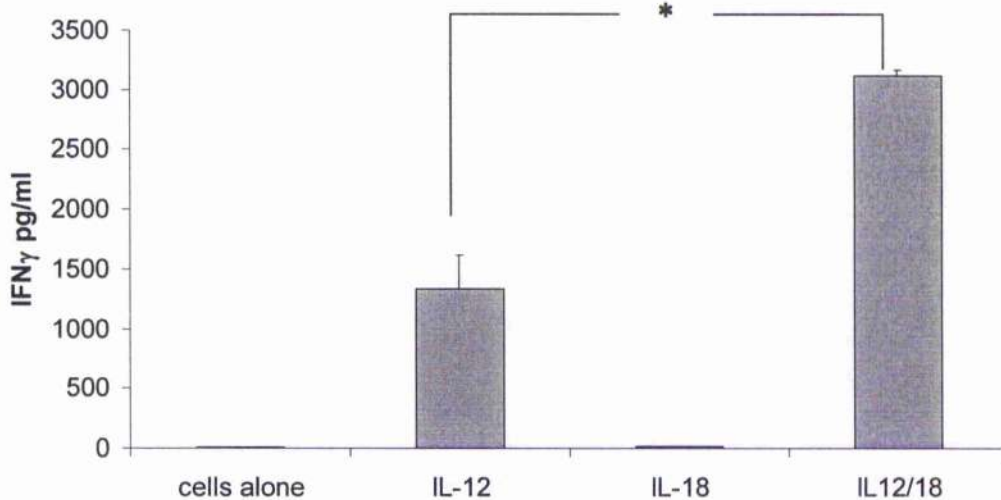
Figure 6.2 IL-12 and IL-18 induction of IFNγ by PsA SF cells

PsA derived SF cells activated with anti-CD3 and anti-CD28 antibody were cultured in the presence of IL-12 and IL-18 alone or in combination for 24hours. Concentration of IFNγ was measured by ELISA. There was significant increase in IFNγ secretion in cells stimulated with IL-12 alone that was further increased by addition of IL-18 (*P<0.05, Mann Whitney). Data are representative of 5 patients in which similar results were obtained. Data expressed as mean ± s.e.m of triplicate cultures.
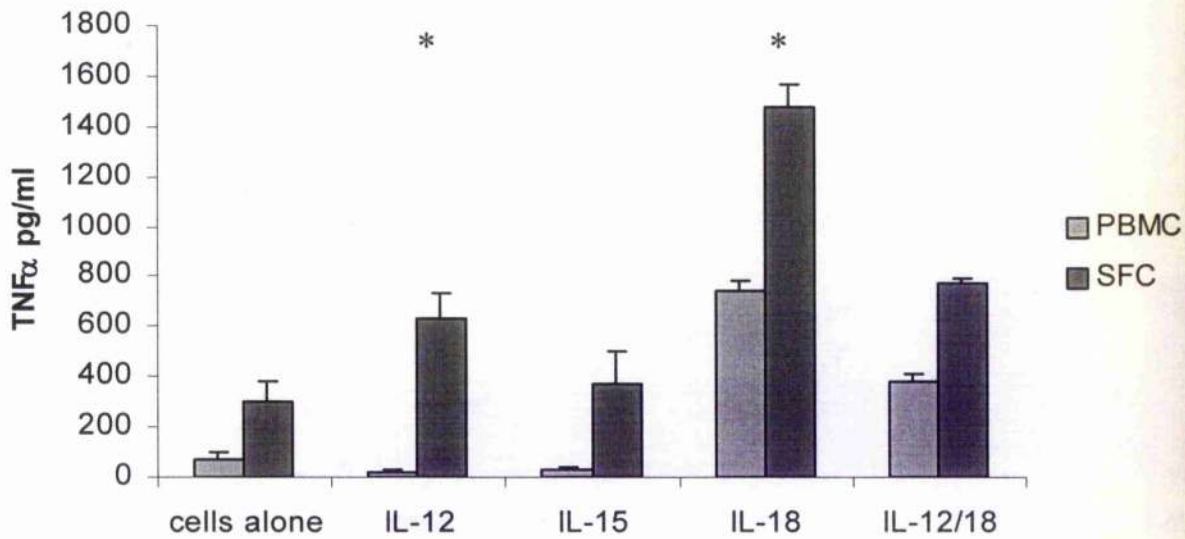
Figure 6.3 Effect of innate cytokines on production of TNFα

Matched PB and SF MCs were obtained from patients with active PsA. Cells activated with anti-CD3 and anti-CD28 antibody were cultured in the presence of recombinant cytokine for 24 hours. Levels of TNFα were measured in supernatants by ELISA. (*P<0.05, Mann Whitney SF alone vs. SF with IL-12 or IL-18). Data shown mean ± s.e.m. of triplicate cultures and are representative of 5 similar experiments obtained from individual patients.

6.4 Presence and Functional role of Natural Killer T cells in PsA

The primary objective in my studies was to investigate effector Th1 cell responses in PsA. The stimulatory responses demonstrated thus far were dependent on the presence of anti-CD3 suggesting that T cells may be the source of resultant IFNγ or TNFα production. However, a further possibility was that CD3$^+$ NKT cells might in some way contribute to the cytokine release detected in the above assay system. Activated NK T cells can produce variable amounts of IL-4 and IFNγ depending on the ambient cytokine milieu making them directly relevant in determining Th1/Th2 responses in human disease states (Hafner, Falk et al. 1999). Moreover, a pathogenic role for NK T cells has been implicated in animal studies of psoriasis (Nickoloff, Bonish et al. 2000).

6.4.1   Presence of NKT cells in PsA synovium

I thereafter sought NK T cells in PsA SF compared with matched peripheral blood. PB and SF MCs were obtained from patients with active PsA. NK T cells were identified using anti-Vβ11 and anti-CD3 by FACS analysis. CD3/TCR Vβ11 double positive cells were identified in both synovial and PB compartments (Figure. 6.4). Of interest there was no difference in the relative expression in each compartment, in contrast to previous studies in RA (Yanagihara, Shiozawa et al. 1999). This suggested that there was no particular selection or accumulation of NK T cells in the synovium, or less likely that recruited NK T cells altered their characteristic phenotype upon tissue entry.

6.4.2 Effect of α galactosyl ceramide on PsA SF and PB MC proliferation

Since these data suggested that NKT cells are indeed present in synovium, I investigated their functional significance. Glycosylceramides, particularly αgalcer, presented by CD1d molecules on APC are native NKT ligands that promote subsequent effector function. I sought to determine whether PB or SF NK T cells proliferated to αgalcer. Basal proliferation by SF cells was greater than in PB cells. The addition of α galcer, either alone or in the presence of IL-15 (a critical NK cell activating factor used as control) or IL-18 did not significantly increase proliferation of either PB or SF MCs (Figure 6.5)

6.4.3 IFNγ and TNFα production by PsA SFC in response to α galcer and recombinant cytokines

I next measured expression of TNFα and IFNγ following addition of αgalcer to SF MCs in the presence of IL-12, IL-15 and IL-18. DMSO was added as a diluent control to a similar final concentration. In no cultures was I able to measure any IFNγ. Although SF MCs produced variable amounts of TNFα in response to individual cytokines, no significant increase of TNFα was observed with the addition of αgalcer. These observations strongly suggested that NKT cells when stimulated with their native TCR ligand were not substantial contributors to the IFNγ and TNFα expression reported above. Future studies will be required to determine the wider expression profile of NKT cells following stimulation since this would likely include Th2 cytokines such as IL-4. Since the latter is

not detected in PsA synovium there was no obvious rationale for pursuing such experiments in the context of my studies.
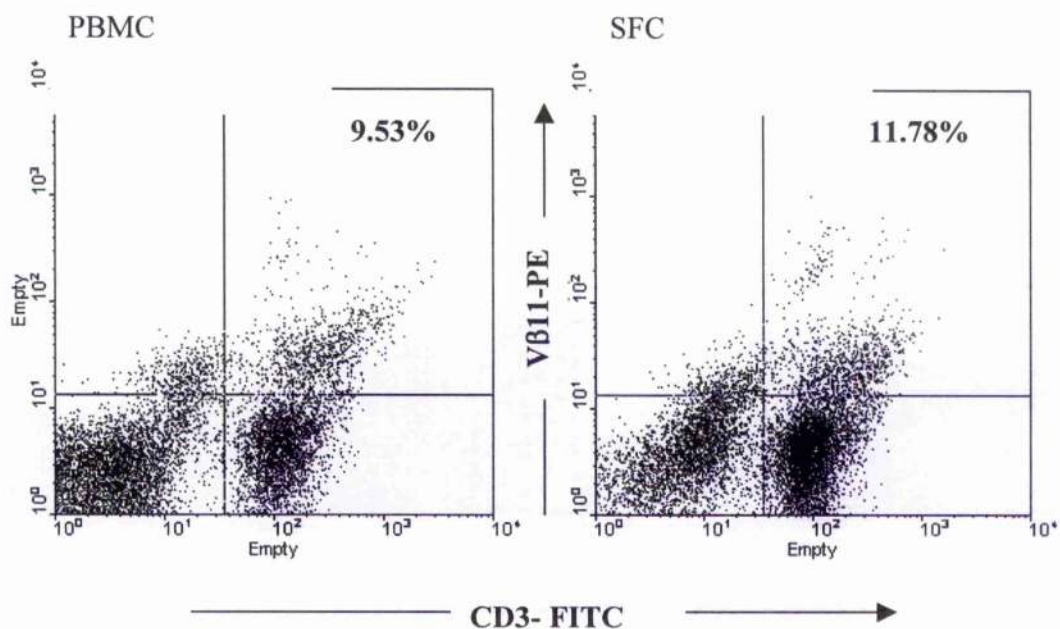
Figure 6.4 NKT cell expression in PsA PBMC and SFC

Matched PBMC and SF MCs were obtained by Ficol-hypaque separation Cells were stained with FITC conjugated anti-CD3 antibody and PE conjugated TCR Vβ11 antibody. Gating on forward and side scatter identified lymphocyte population. Percentage of cells with positive staining for CD3 and Vβ11 are shown above. There was no significant increase in double positive cells in SF compared with PB. Data are representative of 3 different patients.
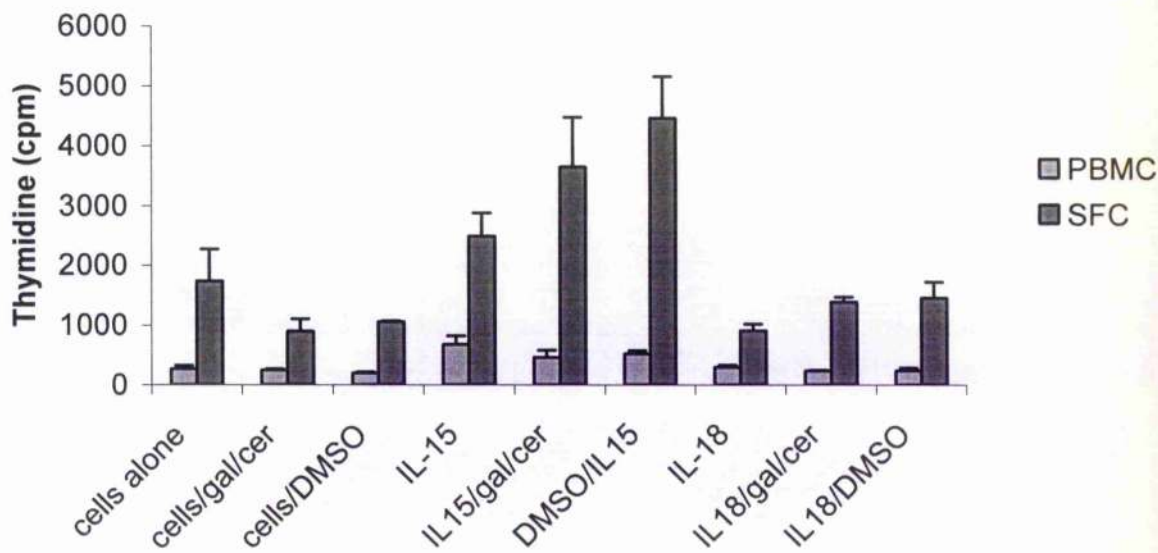
Figure 6.5 SF and PB MC proliferation in response to α galcer

Matched SF and PB MCs were cultured for 24 hours with recombinant cytokines and ^{3}H-thymidine incorporation over the last 6 hours. Although an increase was detected in the IL-15 stimulated cells in both compartments, there was no additional increase in proliferation in the presence of αgalcer. Values shown are mean ± s.e.m. of triplicate cultures. Data are representative of 5 individual experiments.
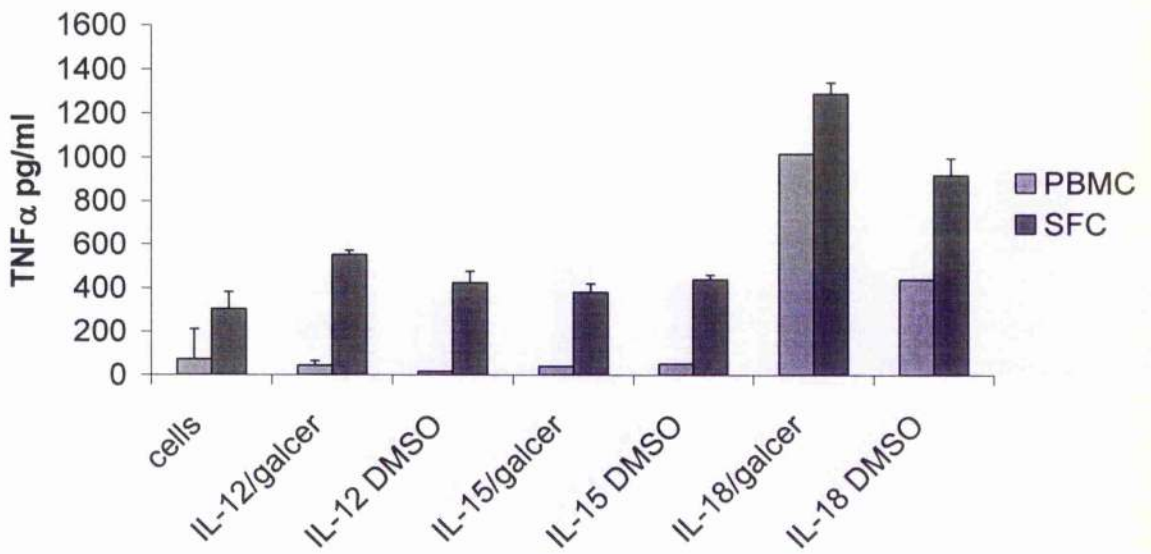
Figure 6.6 TNFα production by PsA synovial MCs in response to αgalcer

SF MCs were cultured with recombinant cytokines with or without αgalcer for 24 hours. Resultant supernatants were assessed for TNFα by ELISA. No increase in TNFα production was detected with addition of αgalcer. Data are representative of three patients and are mean s.e.m. of triplicate cultures.

6.5   Functional role of p38 mitogen activated protein kinase in PsA

The forgoing experiments clearly indicate that IL-12 and IL-18 were capable of promoting IFNγ release from T cell subsets in PsA synovium. I next performed preliminary studies to explore possible signal pathways involved in such expression. These were intended to pump prime future detailed investigations. Previous studies indicate that IFNγ expression by activated Th1 cells is MAP kinase dependant (Rincon, Enslen et al. 1998). Moreover IL-12 was shown to activate p38 MAPK activity via pathways independent of stat-4 phosphorylation (Zhang and Kaplan 2000). p38 MAPK may be an important in vivo therapeutic target. These published data suggest that p38 represents a logical point for initial studies and therefore I utilised SB 203580, a selective inhibitor for p38 MAP kinase in PsA PB and SF MC cultures.

PB and SF MCs were cultured with anti-CD3 / anti-CD28, together with recombinant IL-12 / IL-18 in combination with or without SB203580 for 24 hours. DMSO was used as a diluent control for SB203580. There was little production of IFNγ by SF MCs in the absence of cytokine stimulation consistent with previous observations. SF MCs produced IFNγ to IL-12 / IL-18 that was inhibited after the addition of SB203580 (SB203580 vs. DMSO control; p<0.05) (Figure 6.7). Although clearly of a preliminary nature these data provide an early indication that p38 dependent pathways may offer an opportunity to suppress Th1 cell activation in PsA synovium.
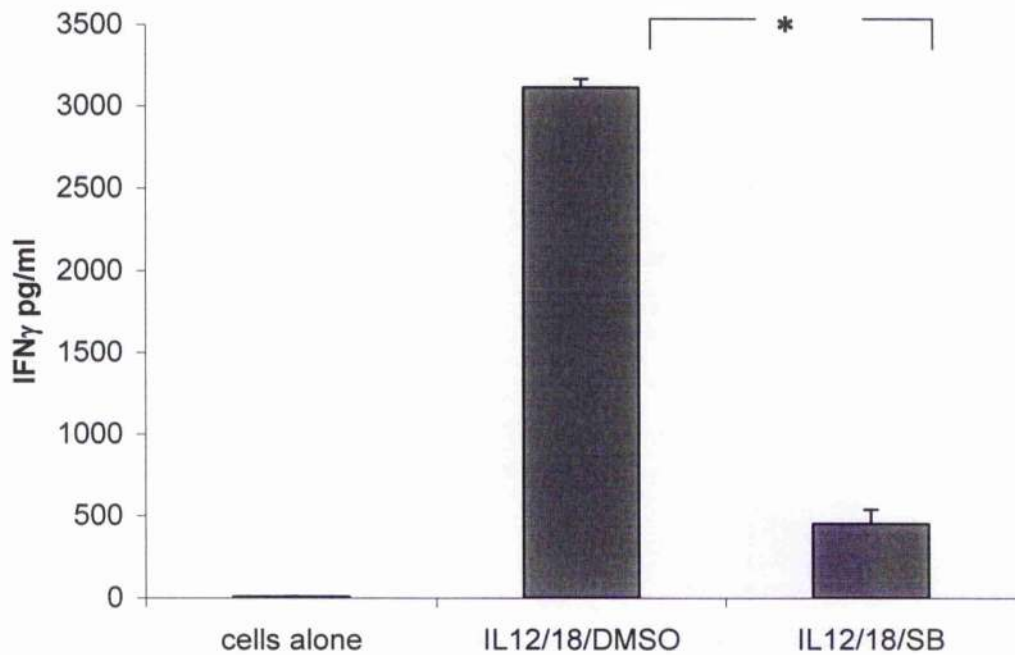
Figure 6.7 Effect of MAP kinase inhibitor on cytokine induced IFNγ

SF MCs were obtained from patients with active PsA. Cells were cultured with anti-CD3 and anti-CD28, together with recombinant cytokines IL-12 and IL-18 in the presence of the specific p38MAP kinase inhibitor, SB 203580 or DMSO alone (as carrier diluent control). IFNγ release into supernatants was assayed by ELISA. (* P <0.05, Mann Whitney). Data are representative of three similar experiments from independent patients.

6.6   Discussion

Having identified the presence of IL-12 and IL-18 in PsA SF and membrane, the functional significance of these cytokines alone or in combination is demonstrated in these studies.

Proliferation of PsA synovial derived MCs was increased following anti-CD3 and anti-CD28 stimulation. No additional proliferation was observed by the addition of recombinant cytokines. Subsequent co culture experiments with IL-12 and IL-18 induced regulation of individual cell expression rather than simply an increase in cell numbers In subsequent experiments therefore, matched SF and PBMCs were stimulated with anti-CD3 and anti-CD28 in the presence of cytokine to determine whether cytokines induced TNFα and IFNγ expression.

SF MCs spontaneously produce TNFα compared with PB commensurate with previous studies. The addition of IL-18 alone to SF MCs induced significant TNFα production. There was no significant increase in SF MCs stimulated with recombinant IL-15. SF MCs produce significantly elevated levels of IFNγ after stimulation with IL-12 and IL-18 in combination compared with no cytokine stimulation or with IL-12 alone. SF cells alone even in the presence of anti-CD3 and anti-CD28 produced no detectable IFNγ suggesting that cytokine stimulation is necessary for IFNγ secretion. Elucidation of possible mechanisms whereby cytokines induce TNFα and IFNγ was a logical progression from this work

The subsequent experiments in this chapter suggest that IL-18/IL-12 induces IFNγ expression via at least MAP kinase dependant pathways.

Although there is considerable evidence implicating NK T cells in the pathogenesis of psoriasis, the evidence for their role in PsA has not been studied. Given that PsA and psoriasis form a clinical spectrum of one disease, it was logical to assume that NK T cells may have a pathogenic role in driving inflammation in PsA particulary as we observed a population of NK T cells in the SF of patients with active disease.

NK T cells are a potential source of IFNγ or IL-4 depending on the ambient cytokine milieu. I did not observe an increase in proliferation, TNFα or IFNγ production following stimulation with the synthetic glycolipid αgalcer in the presence of cytokines IL-12 or IL-18. These limited data do not support a functional role for NK T cells in PsA.

Chapter 7 Expression and function of IL-17 in PsA

Previous studies focused largely on identifying those factors that may perpetuate synovial and cutaneous inflammation via T cell activation in PsA. Resultant joint damage in PsA is characterised by specific radiological changes that include both bone formation and resorption. This spectrum of radiological patterns is as yet unexplained. Recent studies have indicated that IL-17, a Th1 cell derived pro inflammatory cytokine, and IFNγ may have opposing effects on osteoclastogenesis (Takayanagi, Ogasawara et al. 2000). Since I have initially demonstrated IFNγ expression, I next sought to characterise IL-17 expression and functional activity.

Most data derived from T cell clones suggest that IL-17 is Th1 predominant (Aarvak, Chabaud et al. 1999). IL-17 mediates effects in both leukocyte and stromal cell lineages. IL-17 can induce IL-6, IL-8, TNFα and IL-1β release by leukocytes. Other varied effects include stimulation of PGE$_2$, inducible NOS and chemokine synthesis by leukocytes and fibroblasts. Direct and indirect effects on chondrocytes have been reported including up regulation of matrix metalloproteinase expression (Cai, Yin et al. 2001). Finally, when incubated with osteoclast progenitor cells and osteoblasts, IL-17 induces osteoclastogenesis. (Kotake, Udagawa et al. 1999). IL-17 may therefore play an important upstream role in T cell mediated inflammation by stimulation of stromal cells to promote local tissue remodelling and damage. IL-17 blockade results in suppression of collagen-induced arthritis whereas over expression enhances disease. These deleterious effects on cartilage may function independently of IL-1β as blocking IL-1 with neutralising peptide

had no effect on IL-17 induced cartilage damage (Lubberts, Joosten et al. 2001) in vivo and in vitro.

IL-17 has been studied in a variety of human models of inflammation. IL-17 is detected in RA SFs and membrane (Chabaud, Durand et al. 1999). In psoriasis, the majority of CD4+ and CD8+ T cell clones derived from lesional psoriatic skin express IL-17 mRNA compared with none in non lesional skin suggesting that IL-17 may be involved in the development of cutaneous inflammation. IL-17 can directly promote keratinocyte secretion of pro inflammatory cytokines (Teunissen, Koomen et al. 1998). Thus far, the presence and role of IL-17 in PsA has not been studied.

7.1   Expression of IL-17 in PsA SF and serum

I initially sought to determine IL-17 levels in serum and SFs of patients with active PsA. SF and blood was collected at needle arthroscopy or joint aspiration as clinically indicated. Samples were spun and stored at $-20^{\circ}C$ until assay. IL-17 in serum and SFs was measured by ELISA. Elevated levels of IL-17 were detected in all SF samples across a broad range from 4pg/ml to 5175pg/ml. Matched serum in 13 patients contained no detectable levels of IL-17 (SF vs. serum: $p<0.05$). The median concentration of IL-17 in SF was 46pg/ml (Figure 7.1).

7.2   Immunohistochemical detection of IL-17 in PsA SM

Previous studies have indicated that IL-17 is expressed by RA synovium and is produced spontaneously in RA synovial cultures (Chabaud, Lubberts et al. 2001). I used immunohistochemistry to characterise expression of IL-17 in PsA SM tissue obtained at needle arthroscopy and fixed in formalin prior to paraffin embedding. Paraffin embedded tonsil was used as control tissue (figure 7.2). Biotinylated polyclonal goat anti-human IL-17 antibody was used for IL-17 detection and amplified using ABC system. IL-17 staining was detected in 6 of 10 SM samples (Figure 7.3). Positive staining was observed predominantly in the sublining layer. Quantitation of positive staining cells was performed on the 6 positive sections (Table 7.1).
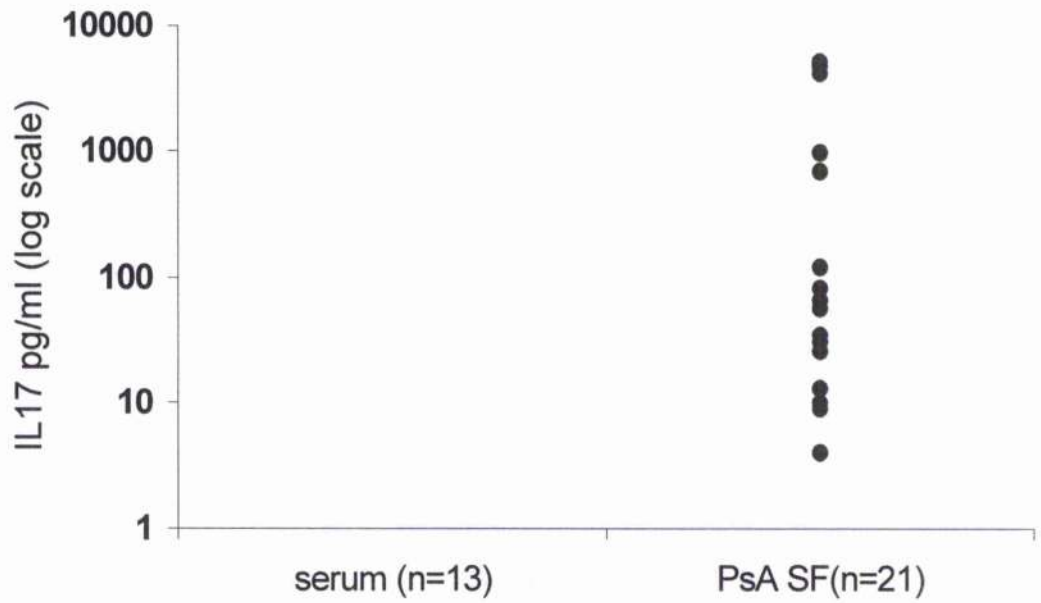
Figure 7.1 IL-17 levels are raised in PsA SF

Serum and SFs were obtained from patients with active PsA and assayed for the presence of IL-17 by ELISA. Levels in SFs were significantly higher than serum. IL-17 values are expressed on a log scale.
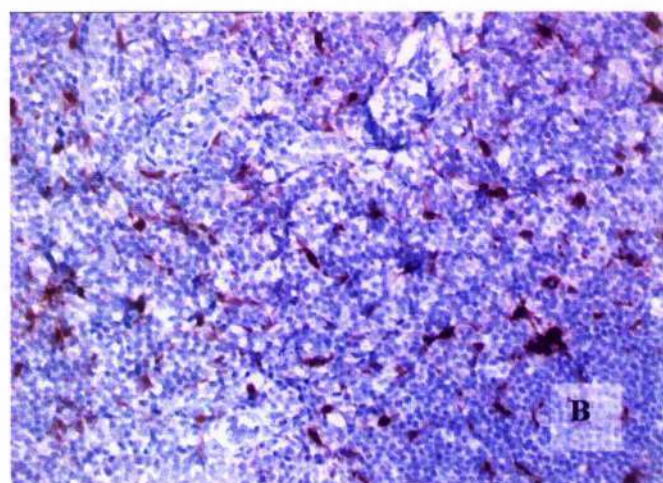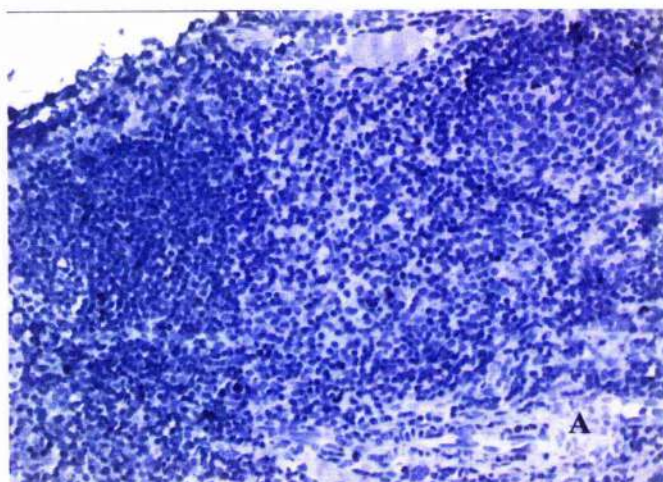
Figure 7.2 Immunohistochemical analysis of IL-17 in tonsil

Paraffin embedded sections were fixed and stained with biotinylated anti IL-17 antibody or irrelevant primary as control (goat IgG). Primary antibody was detected using avidin/biotin complex immunoperoxidase method and diaminobenzidine for colour development.

A, Tonsil negative control (magnification x40) B, Tonsil positive control (magnification x40).
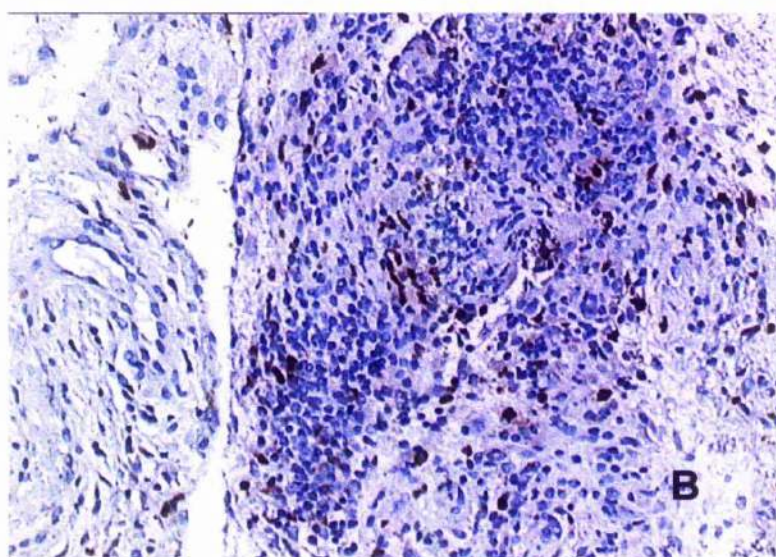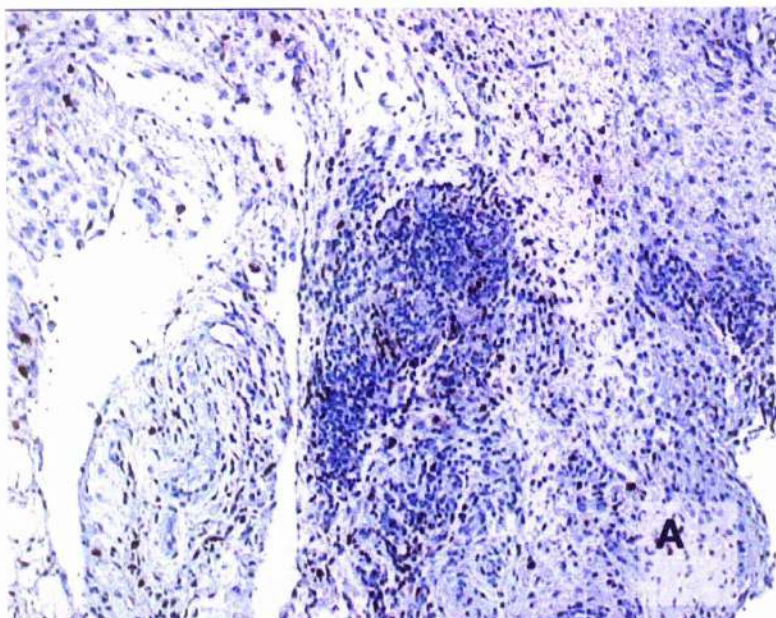
Figure 7.3 IL-17 expressions in psoriatic SM

Immunohistochemical analysis of synovial tissue from a representative PsA patient. Paraffin embedded sections were fixed and stained with biotinylated anti IL-17. Primary antibody was detected using avidin/biotin complex immunoperoxidase method and diaminobenzidine for colour development.

A, PsA SM (magnification x20) B, PsA SM (magnification x40).

	LL	SL	LA
PsA 1	0	1	na
PsA 2	1	2	2
PsA 3	0	1	na
PsA 4	0	2	1
PsA 5	0	2	1
PsA 6	0	1	1

Table 7.1 Quantification of synovial expression of IL-17

Quantitative expression of IL-17 was assessed in tissue samples using a scoring system

agreed between 2 independent observers. 0 = no staining, 1= 1-10% of nucleated cells

stained positive, 2=10-25% nucleated cells, 3=25-50% and 4=>50% positive.

7.3. Functional effects of IL-17 in PsA

Based on the hypothesis that IL-17 might form a link from Th1 cell to tissue destruction via fibroblast activation, I next determined the functional role of IL-17 in PsA. To this end, I performed a series of experiments with synovium-derived fibroblast like synoviocytes (FLS) examining their potential to synthesise cytokines and MMPs following stimulation with IL-17.

7.3.1 IL-17 induced IL-6 production by synovial fibroblasts

I initially sought to determine whether IL-17 was capable of activating synovial FLS cytokine release *in vitro*. FLS were grown as described in chapter 2 from SM obtained at needle arthroscopy. FLS were used from passage 3 at which point $CD3^+$ and $CD14^+$ cells were both <1% by FACS analysis. Single cell suspensions of fibroblasts from the third passage were then cultured in the presence of recombinant human IL-17 or medium alone. FLS alone produced a median 1094pg/ml of IL-6 over 24h. IL-6 production was significantly increased by the addition of 5ng/µl IL-17 (FLS alone vs. IL-17; $p<0.05$, Mann Whitney) (Figure 7.4). There has been considerable recent interest in novel IL-10-like cytokines that may be T cell derived and that may modulate downstream effector responses from stromal cells. IL-22 represents one such novel cytokine that is Th1 cell derived and that is capable of activating STAT3 phosphorylation in RA and PsA FLS (Dr A. Crilly unpublished observations). I therefore added IL-22 together with, or in the absence of IL-17 to evaluate potential synergy or cytokine interactions. However, over three repetitions,

there was no demonstrable effect of IL-22 either upon spontaneous IL-6 release or on IL-17 induced IL-6 release (Figure 7.4).

7.3.2   MMP3 in PsA – regulation by IL-17

MMP3 is a critical regulator of extracellular matrix integrity that is upregulated in a number of inflammatory arthropathies. I initially confirmed the baseline expression of MMP3 in the PsA SFs using ELISA. In 6 PsA SF supernatants MMP3 was easily detected with median concentration 1079ng/ml (769ng/ml to 1333ng/ml) (Figure 7.5). To further investigate this relationship *in vitro*, PsA SM derived FLS were cultured in the presence or absence of rIL-17 and MMP3 release was measured after 24h. Median baseline production of MMP-3 by PsA FLS was 1.7ng/ml. The addition of 5ng/μl IL-17 to FLS cultures resulted in an increase of MMP-3 production to 22ng/ml. This increase in MMP-3 production by exogenous IL-17 was inhibited by 50% in FLS when cultured in the presence its neutralising anti IL-17 antibody ($p < 0.05$) (Figure 7.6).
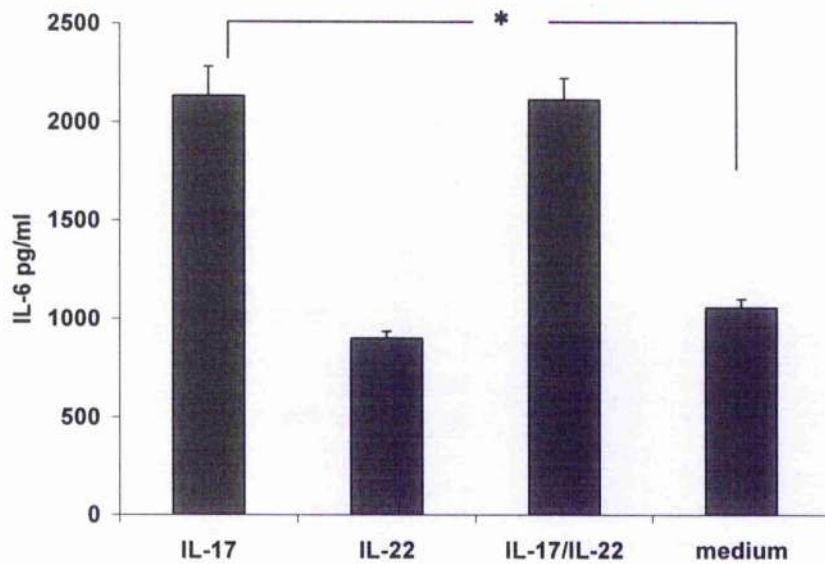
Figure 7.4 IL-6 production by fibroblasts stimulated with IL-17 and IL-22

SM derived fibroblasts were grown in 10%FCS and RPMI. Passage 3 fibroblasts were then cultured in the presence of recombinant human IL-17 and IL-22 singly or in combination. Medium alone was used to control for spontaneous IL-6 production by fibroblasts. (* $p<0.05$, Mann Whitney) Representative of 5 experiments.
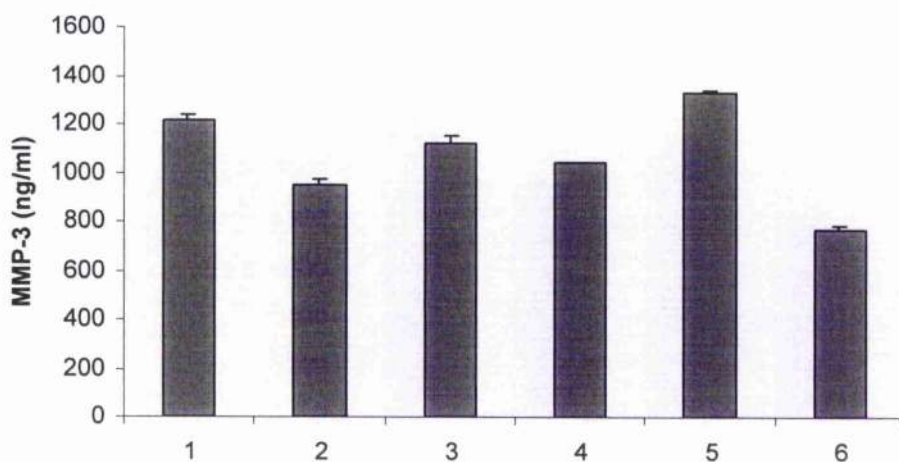
Figure 7.5 PsA SF supernatant MMP-3 levels

MMP-3 content was assessed in SFs from 6 patients with active PsA by ELISA. SF was spun free of cells and stored at -20°C until assay. In all 6 patients there was substantial levels of MMP3 detected.
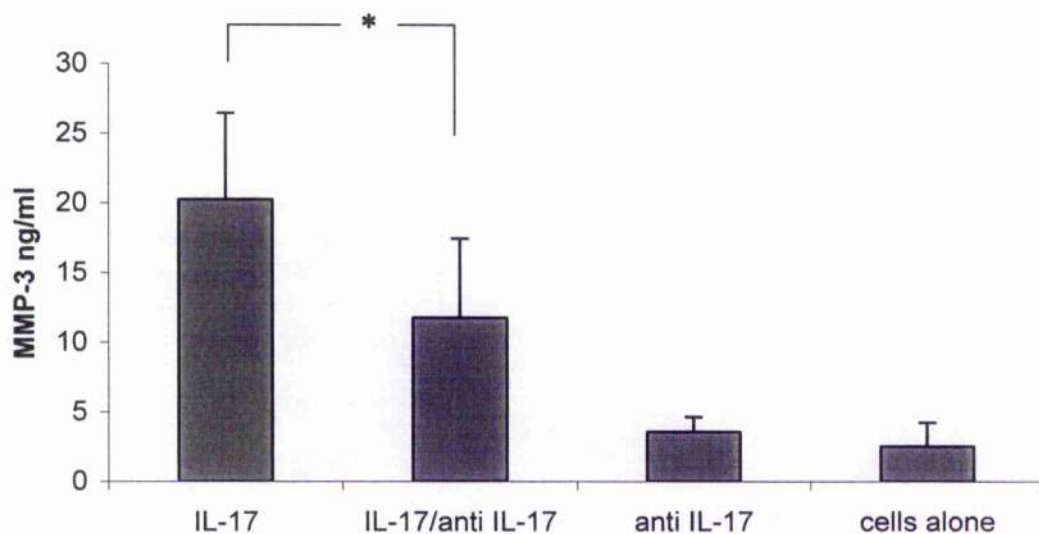
Figure 7.6 Effect of IL-17 on MMP3 production by PsA FLS

FLS were cultured in the presence of IL-17 with or without prior neutralision. Supernatants were harvested after 24 hours and MMP-3 levels were detected using standard sandwich ELISA. (* $p < 0.05$, Mann Whitney) representative of 5 experiments.

7.4 Discussion

The detection of IL-17 protein expression in PsA SM and fluid is a novel finding. PsA is a unique arthropathy that has features of both bone formation and bone destruction occurring in the same joint simultaneously. The identification of IL-17 is an important observation as IL-17 has opposing effects to IFNγ on osteoclastogenesis. IL-17 is associated with increased expression of TRAF-6 that is required for RANK/ RANKL interactions that ultimately lead to osteoclastogenesis. The reverse occurs in the presence of IFNγ which causes rapid degradation of TRAF6 leading to overall increased bone formation. It is possible that the pattern of joint involvement seen in different patients with PsA is a reflection of the predominant cytokine in the environment. Correlation of radiological appearances with relative synovial expression of IFNγ and IL-17 would have been of value in confirming this hypothesis but was unfortunately beyond the scope of this project.

The regulation of connective tissue degradation in the presence of synovial inflammation is largely due to elevated levels of MMPs secreted by synoviocytes, chondrocytes and inflammatory cells. Previous studies have already shown that serum level of MMP1, MMP3 and MMP9 are increased in patients with RA, particularly with follicular synovitis, compared with osteoarthritis (Klimiuk, Sierakowski et al. 2002). Furthermore, it has recently been reported that psoriatic skin and synovium express MMP-9 and that this expression is greater than in RA synovium (Hitchon, Danning et al. 2002) indicative that MMPs are important regulators in synovial and cutaneous inflammation in PsA. However the direct contribution of T cells to joint destruction is a matter of debate. The definition of IL-17 as a T cell derived pro inflammatory cytokine sharing properties with IL-1 has led to

speculation that such IL-17 may also contribute to joint destruction. Studies of RA synovium have demonstrated that IL-17 is capable of inducing MMP-1 release by synovial fibroblasts indicating a potential mechanism of joint damage. As destruction remains the major therapeutic target in inflammatory synovitis, the observations that IL-17 may induce synovial derived MMP-3 expression may offer an attractive therapeutic target. Although the preliminary data suggest that neutralisation of IL-17 resulted in a reduction of MMP-3 release by synovial derived FLS, further clarification would be required using escalating doses of neutralising peptide.

Chapter 8 Discussion

PsA is a chronic inflammatory arthropathy of unknown aetiology. The combination of deformity and disability pose unique difficulties for those affected. Disease progression with time and an increased mortality rate is now recognised. Despite this there is significant unmet clinical need manifest by paucity of understanding of disease pathogenesis, lack of safe and effective therapies and lack of rationally designed therapies. PsA is easily diagnosed on the basis of inflammatory arthropathy in the presence of co-existing skin inflammation and hence should be easier to define and study compared with other inflammatory arthropathies. It is therefore rather surprising that it is less well studied in terms of pathogenesis and therapeutic interventions than RA.

Recent studies using biologic agents such as etanercept, infliximab and alefacept appear promising. Interestingly however, etanercept and infliximab, were used to treat psoriasis and PsA before TNFα was identified in involved tissues. Their beneficial effects do however indicate that the synovial immune response offers an attractive therapeutic target (Mease, Goffe et al. 2000). Consequently, current unmet need may be resolved by developing a better understanding of immune events in the joint and beyond. It is well established that synovial and cutaneous inflammation in PsA results from an intense infiltrate of activated T cells (Ortonne 1996) (Braathen, Fyrand et al. 1979). Unfortunately however, T cells modulating therapies such as cyclosporin have failed to produce significant clinical benefit particularly in PsA. Furthermore, long-term use is hindered by nephrotoxicity in both psoriasis and PsA (Olivieri, Salvarani et al. 1997).

The true phenotype of T cell response in PsA remains controversial. Although the majority of evidence points towards a Th1 driven disease, conflicting data regarding the magnitude

of such Th1 responses has hindered further developments in this area. I set out to initially clarify the phenotype of Th responses in PsA to clarify this issue. Such studies combined with the identification of regulatory factors for T cell activation were performed to contribute to the understanding of disease immunopathogenesis and subsequent generation of rationally designed therapies.

Previous to this work, analyses of SF in seronegative arthropathies has revealed relative paucity of IFNγ, the hallmark of Th1 response, compared with RA (Canete, Martinez et al. 2000). Although IFNγ was readily detectable at mRNA level, protein detection by ELISA in SFs was seldom present (Ritchlin, Haas-Smith et al. 1998). To address this discrepancy in expression, I employed a double antibody technique to detect spontaneously secreting IFNγ cells in the SF and PB of patients with PsA and RA by FACS analysis. This is the first time that cells actively secreting IFNγ cells have been identified in a human disease state. The presence of such a population of IFNγ secreting cells without prior mitogen simulation reflects a true Th1 response *in vivo*. This response was of greater magnitude in PsA than in RA reflecting differences in terms of clinical expression and pathogenesis. The presence of Th1 response in PsA was further explored by analysing the presence of other Th1 cell specific surface markers CCR5 and IL-18R (Loetscher, Uguccioni et al. 1998; Xu, Chan et al. 1998). Such dual expression by SF MCs compared with PB further support the predominance of Th1 response in PsA. Clearly one criticism of these studies would be that although IFNγ secreting cells were identified, are they truly pathogenic? The ability to study these cells is possible using magnetic bead labelling prior to FACS analysis. This was a technique that I failed to perform successfully during the time period of this study but would have contributed significantly to the proposed hypothesis. By isolating the IFNγ

secreting cells one could phenotype with up to 5 separate cell surface markers using spectrophotometry. This would confirm whether the IFNγ secreting cells co- expressed CCR5 and IL-18R. The technique of spectrophotometry in my host laboratory was in early stages of development at the time of these studies. Furthermore, by isolating such cells one could proceed to study their functional response to cytokine stimulation in terms of TNFα secretion. The results of these studies would clarify or refute that these cells are truly pathogenic in PsA.

The regulation of Th1 responses provides a further means of regulating the inflammatory immune response. I next therefore sought to identify the presence of key Th1 regulatory cytokines IL-12 and IL-18 in the synovial and skin compartments in patients with active PsA. Several methods of targeting regulatory cytokines have been described particularly in the case of IL-18 as it serves as a fine tuner of the Th1 response. Soluble receptors, IL-18 binding protein and IL-18 neutralising antibody are potential agents in blocking IL-18 induced effector responses. The recently designed 'cytokine traps' can potently block cytokines *in vitro* and *in vivo*. Traps consist of fusion between the Fc region of IgG and the extracellular domains of two distinct cytokine receptor components involved in binding. They therefore represent a substantial advance in creating novel therapeutic candidates for cytokine-driven diseases (Economides, Carpenter et al. 2003).

IL-18 was easily detected at the protein and message level in synovial compartment and matched skin from patients with active PsA. Positive double staining using CD68 indicated that IL-18 was generated in resident macrophages. Both membrane and skin explant cultures were capable of producing significant levels of IL-18. These tissue culture experiments repeated in the presence of P2X7 inhibitors would be of interest as IL-18

release was shown to be ATP dependant in SF cell culture. One inhibitor (AR-C134170AA) resulted in no inhibition of IL-18 release detected when added to SF MC cultures. As IL-18 fine tunes Th1 responses and induces TNFα release alone, it is a more attractive target than IL-12 that is a critical regulator of effector Th1 responses. Finally, IL-18 in synergy with IL-12 induced IFNγ production that was inhibited in the presence of p38 MAP kinase inhibitor suggesting that oral MAP kinase inhibitors may be of potential benefit in PsA.

Having identified IL-18 expression in PsA SM and SFs, my host department has now commenced clinical trials of anti IL-18 therapy in *vivo*.

Having confirmed that Th1 responses were likely to be predominant in PsA, I sought further clarification by determining the expression of IL-17, a novel Th1 cytokine. IL-17 protein was readily detected in PsA SM and fluids of patients with active disease. The presence of mRNA will be explored in future studies however these data are not available at the time of writing. The co expression of IL-17 and IFNγ may have implications for some of the unique radiological features seen in PsA. IL-17 mediates osteoclastogenesis via activation of RANK/RANKL via TRAF6 whereas IFNγ causes rapid degradation of TRAF6 thereby inhibiting bone resorption (Takayanagi, Ogasawara et al. 2000). Our preliminary studies indicate that cartilage degradation may occur via IL-17 induced MMP-3 production by fibroblasts as described in other rheumatic disease (Ribbens, Andre et al. 2000). Significant baseline levels of MMP-3 were detected in all PsA SF samples tested. IL-17 was capable of inducing MMP3 and IL-6 release by synovial derived fibroblasts that was reversed using anti IL-17 neutralising antibody.

To clarify whether relative expression of IL-17 and IFNγ could affect disease phenotype, I would have liked to correlate radiological features with levels of IFNγ and IL-17 in a subset of patients over time. Those with arthritis mutilans would be a particularly interesting group as one would expect high levels of IL-17 in the presence of relatively low levels of IFNγ. To extend this theory, the differential frequency and phenotype of Th1 cells observed in PsA compared with RA may explain the observed differences in disease expression such as the typical 'pencil in cup' deformity that is classical feature of PsA.

Contrary to the literature in psoriasis implicating a role for NKT cells in disease pathogenesis, I did not detect an increase in this population within the SF of PsA patients compared with peripheral blood. There are several markers of NKT cell that can be employed to detect expression in tissue and fluids. I measured TCR Vβ11, however time constraints prevented the exploration of other NKT cell specific markers such as TCR Vα24 or CD161. The inability of αgalcer a synthetic glycolipid recognised by NKT cells, to stimulate proliferation, TNFα of IFNγ production by PsA SF cells further supported that NKT cells do not seem to play a major role in PsA pathogenesis.

PsA and psoriasis are diseases that affect a significant proportion of our population however those factors triggering onset and perpetuation of skin and joint inflammation have yet to be defined. Clearly the most critical discovery would be the identification of the triggering antigen. This may be achievable in the future however until such times, it is essential that we acquire a better understanding of disease pathogenesis to provide a rational basis for the development of future treatments. Although the data presented in this thesis are suggestive of an association of Th1 responses with psoriasis and PsA, the

numbers of tissue samples studied were small. The clinical phenotype of the cohort, disease duration and disease modifying therapy may alter the results obtained. Clearly more work is required in this field to correlate the preliminary findngs with a more defined cohort.

The work outlined in this thesis has contributed to the understanding of this disease and as such will hopefully lead to the development of novel therapies in the future.

Appendix A

1. Phosphate buffered saline (PBS, x10 stock)

    80g NaCl

    2g KCL

    11.6g NaH_2PO_4

    2g KH_2PO_4

    Adjust to pH7.4

    Autoclave and store at room temperature

2. PBS/Tween

    0.5ml Tween

    1000ml PBSx1

3. Coating buffer (ELISA)

    0.1M $NaHCO_3$, pH 8.2

4. TBS

    900ml 0.9% NaCl

    100ml 50mM Tris-HCl, pH 3.6

    50mM Tris-HCl

    Add conc HCl to 25ml 0.2M tris-HCl to pH 7.36

    Make up to 100ml with dH_2O

Appendix B

HEALTH ASSESSMENT QUESTIONNAIRE

Name _____

Today's date [ ][ ] / [ ][ ] / [2][0][0][3]

MONTH   DAY   YEAR

Please tell us how your arthritis affects your ability to carry out your daily activities.

Please place an "X" in the box ☒ which best describes your usual abilities OVER THE PAST WEEK:

DRESSING & GROOMING	WITHOUT ANY DIFFICULTY	WITH SOME DIFFICULTY	WITH MUCH DIFFICULTY	UNABLE TO DO
Are you able to:				
Dress yourself, including shoelaces and buttons?	☐	☐	☐	☐
Shampoo your hair?	☐	☐	☐	☐

ARISING				
Are you able to:				
Stand up from a straight chair?	☐	☐	☐	☐
Get in and out of bed?	☐	☐	☐	☐

EATING				
Are you able to:				
Cut your meat?	☐	☐	☐	☐
Lift a full cup or glass to your mouth?	☐	☐	☐	☐
Open a new milk carton?	☐	☐	☐	☐

WALKING				
Are you able to:				
Walk outdoors on flat ground?	☐	☐	☐	☐
Climb up five steps?	☐	☐	☐	☐

Please check any AIDS OR DEVICES that you usually use for any of the above activities:

☐ Devices Used for Dressing   ☐ Built up or special utensils   ☐ Crutches
    (button hook, zipper pull, etc.)   ☐ Cane   ☐ Wheelchair
☐ Special or built up chair   ☐ Walker

Please check any categories for which you usually need HELP FROM ANOTHER PERSON:

☐ Dressing and Grooming   ☐ Arising   ☐ Eating   ☐ Walking

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Please place an "X" in the box ☒ which best describes your usual abilities OVER THE PAST WEEK:

HYGIENE	WITHOUT ANY DIFFICULTY	WITH SOME DIFFICULTY	WITH MUCH DIFFICULTY	UNABLE TO DO
Are you able to:				
Wash and dry your body?	☐	☐	☐	☐
Take a tub bath?	☐	☐	☐	☐
Get on and off the toilet?	☐	☐	☐	☐

REACH	WITHOUT ANY DIFFICULTY	WITH SOME DIFFICULTY	WITH MUCH DIFFICULTY	UNABLE TO DO
Are you able to:				
Reach and get down a 5 pound object (such as a bag of sugar) from above your head?	☐	☐	☐	☐
Bend down to pick up clothing from the floor?	☐	☐	☐	☐

GRIP	WITHOUT ANY DIFFICULTY	WITH SOME DIFFICULTY	WITH MUCH DIFFICULTY	UNABLE TO DO
Are you able to:				
Open car doors?	☐	☐	☐	☐
Open previously opened jars?	☐	☐	☐	☐
Turn faucets on and off?	☐	☐	☐	☐

ACTIVITIES	WITHOUT ANY DIFFICULTY	WITH SOME DIFFICULTY	WITH MUCH DIFFICULTY	UNABLE TO DO
Are you able to:				
Run errands and shop?	☐	☐	☐	☐
Get in and out of a car?	☐	☐	☐	☐
Do chores such as vacuuming or yard work?	☐	☐	☐	☐

Please check any AIDS OR DEVICES that you usually use for any of these activities:

☐ Raised toilet seat   ☐ Bathtub bar   ☐ Long-handled appliances for reach

☐ Bathtub seat   ☐ Long-handled appliances in bathroom   ☐ Jar opener (for jars previously opened)

Please check any categories for which you usually need HELP FROM ANOTHER PERSON:

☐ Hygiene   ☐ Reach   ☐ Gripping and opening things   ☐ Errands and chores

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How much pain have you had because of your arthritis IN THE PAST WEEK:

PLACE A SINGLE <u>VERTICAL MARK</u> THROUGH THE LINE TO INDICATE THE SEVERITY OF THE PAIN

NO
PAIN

SEVERE
PAIN

100

0669014400

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