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CYTOKINE AND NITRIC OXIDE PRODUCTION IN INFLAMMATORY ARTHRITIS

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

To Karin

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Abstract

Rheumatoid arthritis (RA) is a chronic disease characterised by inflammatory infiltration of the synovial membrane, with concomitant destruction of adjacent cartilage and bone. Elucidation of immunoregulatory networks within the synovium offers the potential for therapeutic intervention. Two such pathways were investigated in the present study.

Interleukin-15 (IL-15) is a novel pleiotropic cytokine produced by macrophages and fibroblasts, which induces T cell migration and activation and B cell maturation and immunoglobulin production. IL-15 was identified in RA synovial fluids and synovial membrane cultures and, using immunohistochemistry, its expression was localised in the RA synovial membrane to the lining layer and T lymphocyte aggregates. Enhanced proliferation and cytokine production to IL-15 was observed in RA synovial fluid (SF) T cells in comparison to matched peripheral blood (PB) T lymphocytes, which in turn, were more sensitive to IL-15 induced proliferation than PBT cells from normal controls. Following IL-15 mediated activation, PBT cells were capable of inducing TNFα production from a macrophage cell line, from syngeneic PB monocytes, and from synovial macrophage / synoviocyte co-cultures, through a cell-contact dependent mechanism, which required no T cell cytokine synthesis. RA SFT cells exhibited similar properties, which were IL-15 dependent *in vitro*. IL-15 up-regulated CD69 expression on $CD45RO^+$ T cells and neutralisation studies determined that such CD69 expression, in combination with LFA-1 and ICAM-1, was partly responsible for cell-contact mediated macrophage activation by T cells. Finally, in a murine model, IL-15 injection induced significant local tissue T cell invasion, confirming previous *in vitro* observations of its chemotactic properties. IL-15 expression in RA synovial membrane therefore provides a mechanism whereby polyclonal T cell recruitment and activation can lead to macrophage activation and TNFα production, without T cell cytokine synthesis.

Nitric oxide (NO) is a critical immunomodulatory and microbicidal mediator in several animal models of inflammation. Production of NO in RA synovial membrane has previously not been characterised. Inducible NO synthase (iNOS) expression was localised in RA and OA synovial membrane and NO production was detected in synovial membrane cultures from both RA and OA patients, which could be up-regulated *in vitro* using bacterial superantigen. NO-donors induced TNFα production from macrophage cell lines and from synovial macrophage / synoviocyte co-cultures, indicating that NO might modify pro-inflammatory cytokine production in RA. These data provided direct evidence for NO synthesis in human synovium. However, NO mediates diverse effects *in vitro* on chondrocyte metabolism and leukocyte activation, raising doubts as to its net contribution in RA. The effect of iNOS activity were therefore investigated *in vivo* in $iNOS^{-/-}$ mice, using a model of staphylococcal infection, which mediates pathology primarily through superantigen-driven T cell activation. The incidence and severity of arthritis and septicaemia was increased in $iNOS^{-/-}$ mice compared with $iNOS^{+/-}$ controls. *Ex vivo* culture established the presence of an exaggerated Th1 cytokine response in $iNOS^{-/-}$ mice. Moreover, superantigen-induced proliferation and Th1 cytokine production was enhanced *a priori* in $iNOS^{-/-}$ animals, indicating that NO can regulate T cell cytokine production, with protective consequences *in vivo* in an arthritis model.

Neutralisation of TNFα production in RA affords significant clinical benefit. However, the mechanisms whereby TNFα production is enhanced in RA are unclear. The present study provides two novel immunoregulatory pathways which can increase TNFα production in human RA synovial membrane. However, the diverse functions of NO in host defence and immunoregulation, and consequent doubts as to its net effect in articular inflammation, may preclude therapeutic usefulness in human arthritis. In contrast, neutralisation of IL-15 offers exciting clinical potential, through inhibition of T cell activation and interruption of crucial pro-inflammatory T cell / macrophage communication within the RA synovial membrane.

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Abbreviations

APC	antigen presenting cell
bp	base pairs
cDNA	complementary deoxyribonucleic acid
CDR	complementarity-determining region
CFU	colony forming unit
CPR	cytochrome P450 reductase
CRP	C-reactive protein
DMARD	disease modifying anti-rheumatic drug
EAE	experimental allergic encephalomyelitis
EBV	Epstein Barr Virus
ELISA	enzyme linked immunosorbent assay
ENA	epithelial neutrophil activating peptide
ESR	erythrocyte sedimentation rate
FAD	flavin adenine dinucleotide
FCS	foetal calf serum
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
FMN	flavin mononucleotide
GM-CSF	granulocyte macrophage - colony stimulating factor
GROα	melanoma growth stimulating hormone
HLA	human leukocyte antigen
HVR	hyper variable region
ICAM	intercellular adhesion molecule
IDDM	insulin dependent diabetes mellitus
IFN	interferon
Ig	immunoglobulin
IL-	interleukin-
IRE-BP	iron response element binding protein
IU	international unit
kD	kilo-Dalton
LFA	lymphocyte function antigen
LPS	lipopolysaccharide
LTA	lipoteichoic acid
MCP	monocyte chemoattractant protein
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
MMP	matrix metalloproteinase

mRNA	messenger ribonucleic acid
NGS	normal goat serum
NK	natural killer
NO	nitric oxide
NOD	non obese diabetic
NOS	nitric oxide synthase
NRS	normal rabbit serum
NSAID	non steroidal anti-inflammatory drug
OA	osteoarthritis
OD	optical density
PBL	peripheral blood lymphocyte
PBMN	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PBT	peripheral blood T cell enriched
PDGF	platelet derived growth factor
PE	phycoerythrin
PFA	paraformaldehyde
PG	prostaglandin
PHA	phytohaemagglutinin
PMA	phorbol 12-myristate 13-acetate
RA	rheumatoid arthritis
RANTES	regulated upon activation normal T cell expressed and secreted
RF	rheumatoid factor
RNI	reactive nitrogen intermediates
ROI	reactive oxygen intermediates
RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
SCID	severe combined immuno-deficiency
s.d	standard deviation
s.e.m.	standard error of the mean
SE	staphylococcal enterotoxin
SF	synovial fluid
SFAC	synovial fluid adherent cells
SFMN	synovial fluid mononuclear cells
SFT	synovial fluid T cell enriched
TGF	transforming growth factor
TNF	tumour necrosis factor
TSST	toxic shock syndrome toxin
VCAM	vascular cell adhesion molecule

M	molar
mM	millimolar
mg	milligram
ml	millilitre
µg	microgram
µl	microlitre
µm	micrometre
µM	micromolar
ng	nanogram
nM	nanomolar
pg	picogram

Chapter 1

General Introduction

1.1 Rheumatoid Arthritis

1.1.1 History

The antiquity of rheumatoid arthritis (RA) is disputed in the absence of convincing records documenting its existence in ancient or mediaeval literature. Anecdotal case descriptions exist in ancient Hindu and Greek medical writings and in ancient remains in North American Indians (reviewed by Sturrock, et al, 1977; Rothschild & Woods, 1990), but frequently chronic rheumatic disease is referred to as 'gout' in mediaeval manuscripts and in references up to the 19th century. Evidence from paleopathology suggests that osteoarthritis was common in ancient and mediaeval times (Thould & Thould, 1983), but the small bones of the hands and feet are invariably missing from skeletal remains confounding a search for characteristic bony erosions of RA. The earliest clear description of RA is found in 1800 in the French literature by Landré-Beauvais who described 'goutte asthenique primitif' (reviewed by Snorrason, 1952). Subsequent reports followed before the term 'rheumatoid arthritis' was used by Garrod in 1859.

1.1.2 Epidemiology and aetiology

RA has world-wide distribution. It lacks precise clinical definition and several sets of criteria have been developed to facilitate diagnosis. Most epidemiological studies have employed the criteria defined by Ropes et al in 1958 or the most recent revision of these proposed by the American Rheumatism Association (Arnett, et al, 1988). The overall prevalence rate for RA in western Europe is about 1% with a female to male ratio of around 3:1 and peak age of onset in the fifth and sixth decades of life. Annual incidence rates vary from 30 to 300 new cases per 100,000 (Hazes & Silman, 1990; Symmons, et al, 1994). There are variations in prevalence

between ethnic groups, with low rates observed in rural African and Chinese populations.

The precise aetiology of RA remains unclear, but has been variously attributed to primary autoimmunity or environmental infectious agents, under the influence of several genetic components.

Genetic contribution RA is a polygenic disease, with overall twin concordance for community based RA of ~15% (Silman, et al, 1993). The predominant genetic influence is at the HLA-DR locus (Stastny, 1978). Around 80% of RA patients express DR4 and DR1, which share an epitope mapping to amino acids 70-74 (QKRAA) of the DRβ chain (Gregerson, et al 1987). Mutation analysis indicates that amino acid 71 confers particular susceptibility (Hammer, et al, 1995). The mechanism whereby this association influences RA severity is unclear. The QKRAA sequence at the HVR may (i) confer high affinity for an arthritogenic peptide, (ii) modify thymic education thereby prejudicing the T cell receptor repertoire, or (iii) facilitate molecular mimicry (e.g. gp110 of EBV, dnaJ or HSP73 - see below).

Microsatellite polymorphisms at cytokine loci, including TNFα, IL-1β and IL-1 receptor antagonist, are being sought in RA patients, based on observations made in other human autoimmune diseases, such as insulin dependent diabetes mellitus (IDDM; Pociot, et al, 1993). TNFα polymorphisms so far associated with RA arise from linkage with HLA-DR4 (Duff, 1994; Hajeer, et al, 1996), although a novel association has recently been identified which is independent of the HLA, which has higher frequency in patients with extra-articular disease (Field, et al, 1996). Such polymorphic variants, if confirmed, may be associated with high output of cytokine, such that specific alleles can act as 'modifier genes' on established inflammatory processes.

Infectious aetiology Many agents have been linked with RA pathogenesis, which might contribute to arthritis by persistent infection, by molecular mimicry, in which microbial proteins cross-react with host and break tolerance, or by 'immune modulation'. The latter is employed by numerous microbes to enhance survival. Thus, the generation of virus-derived 'cytokines' or their receptors, evolved for host evasion, or the generation of superantigens can directly influence immunological pathways.

Causative agents, however, have proven elusive. Raised antibody titres to *Epstein Barr virus* (EBV) have been reported in RA, which together with observed sequence homology of EBV gp110 and the HVR of HLA-DR4-(DRβ1*0401) led to suggestions of a causative link (Roudier, et al, 1989). Many RA patients, however, have no evidence of previous EBV exposure. Retroviruses offer an alternative area of some promise (Kalden & Gay, 1994). High levels of human T cell leukaemia virus-1 (HTLV-1) infection were detected in Japanese with RA, and HTLV-1 causes arthritis in mice (Iwakura, et al, 1996). However, a search for HTLV-1 conserved sequences in human RA failed to confirm an association with early RA (Di Giovine, et al, 1994). That other retroviral sequences exist undetected in the RA population remains possible. Other reports have implicated *proteus mirabilis*, parvovirus, chlamydia, mycoplasma and mycobacteria (reviewed by Griffiths, 1995). The presence of slow-growing bacterial variants of mycobacteria which evade standard detection or culture has also been postulated (McCulloch, et al, 1993). No single agent has been universally detected, although it might be that several microbial species, or their DNA or protein products, may be capable of generating related clinical syndromes, a subset of which are recognised as 'RA'.

Candidate antigens Bacteria-related products have been suggested as candidate antigens. Heat shock protein (HSP 65) is found in synovial membrane and anti-HSP 65 specific responses are detected in RA, but also at other inflammatory sites,

implying lack of specificity (De Graeff-Meeder, et al, 1990, Gaston, et al, 1990). A human hsp 70 (HSP73), similar to *E.coli* dnaK, has also recently been shown to bind motifs in the DRβ1*0401 and 1001 molecules (Auger, et al, 1996) and specific synovial T cells responses have been detected against *E.coli* dnaJ, which shares sequence homology with the shared epitope QKRAA, in early RA and juvenile RA patients (Albani, et al, 1995). Other antigens implicated are often those found within articular structures. Both T cell and antibody responses to collagen type II have been detected (Londei, et al, 1989; Ronnelid, et al, 1994), although such responses are not RA specific and may be secondary to cartilage damage. The peptide binding motif of the DR4 (DRβ1*0401) may be used to screen candidate peptides, e.g. an inflammation responsive glycoprotein (gp39) from human chondrocytes has been detected by this means which stimulates T cell responses in RA peripheral blood cells, and which, on injection into BALB/c mice, induces a relapsing, erosive arthritis (Rijnders, et al, 1996).

Other factors Several data point to a role for endocrine factors, including the female preponderance of RA and reported influence on disease activity of the oral contraceptive pill, post partum period (prolactin levels) and nulliparity. Defective hypothalamic-pituitary-adrenal responses have been detected in RA patients (Chikanza, et al, 1992). Stress, educational status and diet have also been implicated in disease severity.

1.1.3 Clinical features

RA encompasses a broad clinical spectrum and may present with systemic symptoms of malaise, low-grade fever and weight loss, associated with onset of articular disease. The latter usually fit one of three basic patterns. Most commonly, RA is onset with minimal joint involvement, which progresses slowly but inexorably over years to involve multiple joints in a severe deforming arthropathy

with associated significant functional limitation. Other patients suffer an intermittent chronic course, punctuated by acute episodes of arthritis between periods of remission. Finally, particularly in elderly patients, RA may be of explosive onset with multiple joint involvement and active synovitis, which may partially remit after around three years. Extra-articular disease complicates up to 40% of RA (reviewed by McInnes & Sturrock, 1995) and is associated with significant morbidity and mortality (Pincus & Callahan, 1990). Such disease complications should be contained in acceptable theories of aetiopathogenesis, which must explain not only intra-articular, but also extended major organ involvement in destructive pathology.

The characteristic clinical manifestations of rheumatoid disease entail the classical hallmarks of inflammation - swelling, erythema, heat and pain, presiding over progressive host tissue destruction. Radiological and gross pathological appearances demonstrate cartilage and underlying bone loss reflecting such inflammation. Magnetic resonance imaging detects erosive disease within weeks of RA onset (Heron, 1992), indicating that tissue destruction closely parallels symptom onset. The normally relatively acellular synovial membrane becomes clinically palpable ('synovitis') and often cell-rich inflammatory effusions collect within the joint space and capsule. This inflammatory symptomatology in RA provides the rationale for investigation of the processes which mediate and regulate the articular inflammatory response.

1.2 Immunopathogenesis of rheumatoid arthritis

1.2.1 Normal synovial membrane

The normal synovial membrane contains a lining layer of up to three cells thickness consisting of macrophages and fibroblasts (reviewed by Edwards, 1987).

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 (Barland, et al, 1962; Revell, et al, 1987; Revell, 1989). They exhibit secretory features and possess active golgi and represent the majority of lining layer cells (Athanasou, et al, 1988). They are approximately marked by expression of uridine diphosphoglucose dehydrogenase, VCAM-1, the β unit of prolyl hydroxylase and by synthesis of hyaluronan, and other extra cellular matrix components e.g. that identified by Mab 67 (Edwards, 1987; Lindblad & Hedfors, 1987; Stevens, et al, 1990; Edwards & Wilkinson, 1995). Type B cells form a loose boundary between lining layer and deeper highly vascular, fibrous connective tissue, which contains few cells. Those present are largely of fibroblast morphology, with some macrophages, mast cells and adipocytes present and occasional T and B lymphocytes identified, usually in perivascular cuffs (Norton & Ziff, 1966; Edwards, 1987). Although there is evidence of MHC class II expression in lining layer and some deeper macrophage-like cells (Lindblad & Hedfors, 1987) and of adhesion molecule expression (Morales-Ducret, et al, 1992), there is little to indicate the presence of ongoing inflammation in the normal synovium.

1.2.2 Synovial membrane in RA

In contrast, in RA marked synovial hyperplasia occurs. The lining layer contains increased numbers of type A macrophages as a result of increased extra-vascular migration (Edwards & Willoughby, 1982; Dreher, 1982), whereas local proliferation accounts for considerable type B fibroblast-like (synoviocyte) expansion (Revell et al, 1987; Howat, et al, 1987). Large numbers of synoviocytes, macrophages and T and B lymphocytes are found in the interstitium, as are dendritic cells, mast cells and polymorphonuclear cells, particularly neutrophils (Duke, et al, 1982; Burmester, et al, 1983; Kennedy, et al, 1988; Cush & Lipsky, 1988; Thomas, et al, 1994). The

latter are predominantly located in synovial fluid, along with T lymphocytes and macrophages. No basement membrane separates the membrane and fluid phase of synovium, indicating that eggression from the membrane is a function of adhesion molecule expression and the presence of chemotactic gradients. Profuse angiogenesis is characteristic (reviewed by Colville-Nash & Scott, 1992) and endothelial differentiation may generate 'high-endothelial venules' (Freemont, 1987; Yanni, et al, 1993; Girard & Springer, 1995), reminiscent of those found in lymph nodes. Together with the presence of lymphocytic aggregates, these histological appearances resemble an active lymphoid tissue, implying that immunological processes are important in RA pathogenesis.

The production of cytokines and their soluble receptors has been recognised to be of critical importance in RA pathogenesis (reviewed by Feldmann, et al, 1996b). Cytokines modify diverse cell functions *in vivo,* through binding to specific cell membrane receptors and considerable interest surrounds the possibility that de-regulated cytokine production can lead to autoimmune disease. In addition, high levels of adhesion marker expression are found on endothelial cells and leukocytes in RA synovial membrane and peripheral blood (Pitzalis, et al, 1988; Hale, et al, 1989; Gearing & Newman, 1993; Cronstein & Weissman, 1993; Johnson, et al, 1993; Pitzalis, et al, 1994). Together with cytokine production, these molecules comprise a complex regulatory network which determines the activation, suppression and recruitment of inflammatory cells in the RA synovial membrane.

The mechanisms whereby the above cellular infiltrate contribute to articular damage remain poorly understood. Deregulated production of matrix metalloproteinases (MMP) and tissue inhibitors of metalloproteinases (TIMPs) (Dayer, et al, 1986; Gravellese, et al, 1991; McCachren, et al, 1991; Firestein, et al, 1991), reactive oxygen intermediates (Blake, et al, 1989; Mapp, et al, 1995), and prostaglandins (Dayer, et al, 1986) by synovial membrane cells has been described. Cytokines may

also exert direct effects through modification of chondrocyte or osteoblast / osteoclast biosynthetic activity. Local production of autoantibodies by B lymphocytes leads to immune-complex formation, complement fixation, neutrophil activation and degranulation and may increase IL-1β and TNFα production (Chantry, et al, 1989; Plater-Zyberk, et al, 1992). Given such complexity, it is essential to identify the pre-eminent cell type within the synovial membrane to choose therapeutic targets with maximum potential impact. Controversy, however, surrounds the nature of the optimum target - the T cell, the macrophage (or its products) or the synoviocyte?

1.2.3 Involvement of T lymphocytes in RA

T lymphocytes lie central to the regulation of the normal immune response. The majority of synovial T cells are of the CD4 helper/inducer subset (Duke, et al, 1982; Pitzalis, et al, 1987c; Cush & Lipsky, 1988) and are found in perivascular aggregates, which occasionally also contain germinal centres (Duke et al, 1982). $CD8^+$ cells are found in the transitional zone surrounding aggregates, or scattered throughout the interstitium. Synovial T cells are predominantly $CD45RO^+$, $CD45RA^-$, CD45RBdim, $CD27^-$, indicating advanced differentiation within the memory subset (Thomas, et al, 1992; Matthews, et al, 1993; Horgan, et al, 1994). They express activation markers, including high levels of CD69 and MHC class II, particularly HLA-DR, (Pitzalis, et al, 1987a; Afeltra, et al, 1993; Fernandez-Gutierrez, et al, 1995) and several adhesion molecules, including ICAM-1 (CD54) together with the integrins, VLA-1 (CD49a/CD29), VLA-4 (CD49d/CD29) and LFA-1 (CD11a/CD18) (Pitzalis, et al 1988; Potocnik, et al, 1990; Laffon, et al, 1991; Thomas, et al, 1992; Pitzalis, et al, 1994). Moreover, CD2, CD3 and CD4 levels are suppressed. A characteristic, and as yet unexplained, feature of synovial T cells is the coincident expression of markers which normally appear in co-ordinated sequential fashion following antigen or mitogen induced activation. Thus

CD69 is coexpressed with HLA-DR, VLA-1 or VLA-4, markers which do not normally appear until after CD69 levels subside (Iannone, et al, 1994). Furthermore, the IL-2 receptor (IL-2Rα-chain - CD25) is present on only around 10% of cells (Pitzalis, et al, 1987a). Since IL-2 is the principle T cell growth factor in the normal immune response, alternative mechanisms must therefore operate to recruit and activate T cells in the synovial membrane.

Only a small number (~1%) of synovial T cells are in cell cycle (Bonvoisin, et al, 1984; Revell, et al, 1987; Qu, et al, 1994). The accumulation of synovial T cells therefore, most likely results from the preferential migration of memory T cells into the inflammatory synovium (Pitzalis, et al, 1988; Pitzalis, et al, 1991; Thomas, et al, 1992; Iannone, et al, 1994). A subpopulation of circulating $CD4^+$, $CD45RO^+$, RBdim, $CD27^-$ memory T cells possess intrinsic migratory properties allowing transmigration into endothelial-coated collagen gels (Cush, et al 1992). Such cells express CD44, CD11a, CD26 and CD49, but not CD62L (Brezinschek, et al, 1995). $CD27^-$ memory T cells are usually cutaneous lymphocyte antigen positive (CLA) and represent a tissue-homing memory T cell subset, in contrast to $CD62^+$ lymph node-seeking cells (Baars, et al, 1995). Activation is not a prerequisite for migration, and although $CD69^+$ T cells migrate more efficiently, the majority of cells with migratory potential are resting (Brezinschek, et al, 1995). Removal of the endothelium alters the migratory cell phenotype, and prior activation of the endothelial cells with TNFα induces migration of a further $CD69^+$, $CD62L^+$ population, indicating that the endothelium may regulate T cell recruitment to inflammatory sites (Brezinschek et al, 1995). Enhanced numbers of $CD45RO^+$, CD45RBdim, $CD27^-$ T cell subset were recently identified in peripheral blood of RA patients compared to controls, perhaps reflecting long-term systemic antigen stimulation (Kohem, et al, 1996).

In vivo, similar accumulation of $CD45RO^+$ T cells into PPD-induced skin blisters has been observed (Pitzalis, et al, 1991) and subsequent FACS analysis shows the early presence of VLA-1^+ and HLA-DR^+ cells which must have resulted from preferential recruitment of preactivated cells (Iannone, et al, 1994). Moreover, the interaction of T cells with endothelial cells led to up regulation of CD69 and HLA-DR expression. Thus, T cells appear to differentiate to a maturation stage at which egression from the circulation to tissue is enhanced. The mature synovial phenotype can therefore be partially explained by preferential recruitment of differentiated memory T cells, with additional activation by endothelial cell contact during extravasation.

The functional significance of cell adhesion molecule expression extends beyond cell-cell interactions. Synovial T cells express integrin receptors for extracellular matrix (ECM) components, including collagen, vitronectin and fibronectin (Rodriguez, et al, 1992; Pitzalis, et al, 1994; Kohem, et al, 1996). Membrane interactions with the ECM are capable of further activating T cells within the synovial environment (Gilat, et al, 1996). A further consequence is upregulated production of matrix-degrading enzymes by T cells, such as heparanase, which facilitate movement through inflammatory tissues and thus enhance local migration.

It has been proposed that the CD45RBdim status of synovial T cells indicates longevity within the synovial compartment, although this view has recently been challenged (Matthews, et al, 1993; Iannone, et al, 1994; Westermann & Pabst, 1996). In contrast to synovial lining layer cells, synovial T cells exhibit little evidence of apoptosis *in vivo* (Firestein, et al, 1995; Nakajima, et al, 1995). Analysis of relative levels of bcl-2, bcl-x and Bax gene expression in synovial T cells, suggests that survival may be attributable to the production of 'stromal' factors, as yet unidentified, produced by synovial fibroblasts, rather than by cytokine-mediated rescue (Akbar, et al, 1996; Salmon, M. - personal

communication). Thus, the synovial environment may represent a privileged site for memory T cell survival, contributing to the observed phenotype.

The functional capabilities of synovial T cells have been extensively investigated *in vitro*. T cell clones derived from RA synovial membrane are predominantly IFN-γ producers (Miltenberg, et al, 1992; Quayle, et al, 1993; Cohen, et al, 1995), indicating that RA may be a Th1 driven disease as has been suggested for reactive arthritis (Schlaak, et al, 1992; Simon, et al, 1994; reviewed by Sieper & Kingsley, 1996) and insulin dependent diabetes (reviewed by Liblau, et al, 1995). Many T cell clones were also capable of IL-10 production, but few produced IL-4 (Cohen, et al, 1995). However, polyclonal synovial T cell populations exhibit deficient proliferation and IFN-γ or IL-2 production in response to mitogens (Aaron, et al, 1991, Thomas, et al, 1992) and demonstrate impaired responses to recall antigens (Verwilghen, et al, 1990). Impaired helper / inducer and suppressor function *in vitro* has also been reported (Chattopadhyay, et al, 1979; McCain, et al, 1984). In contrast, the $CD45RO^+$, RBdim, $CD27^-$ subset found within synovium is capable of potent B cell help having lost the ability to suppress immunoglobulin production *in vitro* (Thomas, et al, 1992), suggesting a role in autoantibody production in RA. However, taken together with their activated phenotpye, these observations have led to the suggestion that synovial T cells are in a state of 'split anergy', (Thomas, et al, 1992; Howell, et al, 1992) or of 'frustrated activation' (Pitzalis, et al, 1987a). This might be a reflection of their maturation state, the presence of inhibitory factors in synovium, such as TGFβ, IL-10, and prostaglandins, or result from interaction with synovial antigen presenting cells on entry to the membrane with resultant anergy (Salmon & Kitas, 1989; Fava, et al, 1989; Firestein & Zvaifler, 1990; Schwarz, 1990; Katsikis, et al, 1994).

Evidence from several sources, however, strongly support a central role for T cells in RA pathogenesis (Panayi, et al, 1992; Panayi, 1993).

1. Therapy directed specifically at T cells leads to reduction in synovial inflammation. Thus, physical measures such as thoracic duct drainage, peripheral lymphocytapheresis or total lymphoid irradiation induce partial amelioration of inflammatory symptoms (Paulus, et al, 1977; Panayi & Amlot, 1982; Emery, et al, 1986; Zvaifler, 1987). Administration of monoclonal antibodies against T cell markers such as CD4 (Horneff, et al, 1991; Wendling, et al, 1991; Tak, et al, 1995), CDw52 (Campath) (Weinblatt, et al, 1995) or CD5 (Strand, et al, 1993) have yielded variable responses, with undoubted clinical improvement in some patient groups. However, patients submitted to such trials often have advanced disease, amenable only to partial remission due to extensive secondary mechanical problems, and as such perhaps underestimate the impact of T cell targeting. Furthermore, cyclosporin A treatment exhibits demonstrable benefit in early (Pasero, et al, 1996) and late RA (Harrison, 1992), indicating that T cells can play a significant role, even in established disease.

2. RA patients infected with human immunodeficiency virus (HIV) have been reported to undergo partial disease remission (Bijlsma, et al, 1988). Recently however, reports in which HIV^+ RA patients have been followed over time suggest that remission may not reflect delay in articular destruction (Müller-Ladner, et al, 1995). Moreover, several patients have now been described in whom little improvement in clinical inflammation followed HIV infection (Ornstein, et al, 1995). Since the immunological consequences of HIV extend beyond effects simply on $CD4^+$ T cells, such data require cautious interpretation.

3. Several animal models of arthritis have been shown to be T cell dependent. Collagen induced arthritis (CIA) in rodents may be induced in naive recipients by transfer of primed T cells from CIA mice, or by collagen specific T cell clones (Trentham, et al, 1978; Brahn & Trentham, 1989). Moreover, CIA is effectively prevented or ameliorated by T cell depletion if performed during immunisation (Ranges, et al, 1988; Williams & Whyte, 1996). Streptococcal cell wall arthritis, adjuvant induced arthritis and pristane induced arthritis behave similarly (reviewed by Kaklamanis, 1992; Levitt, et al, 1992; Staines & Wooley, 1994;). Thus, small numbers of specific T cells can initiate immune responses with consequent articular pathology.

4. Perhaps the strongest evidence for T cell involvement comes from the association of RA disease severity with the HLA-DR shared epitope already described (Section 1.2; Stastny, 1978). The defined function of class II molecules is to present peptide to the T cell receptor. It is therefore attractive to hypothesise that continued presentation of an arthritogenic autoantigen occurs within the synovial compartment leading to initiation and perpetuation of disease. However, it remains possible that the effect of MHC is mediated only through shaping of the T cell receptor repertoire during thymic education, or through preferential presentation of an extrinsic antigen(s) i.e. pathogen, contained on putative causative microbial agents. Nevertheless, each of these possibilities implicates the T cell at some stage in RA disease development.

5. In animal models of autoimmune disease to defined 'self' antigen, such as murine experimental encephalomyelitis (e.g. Acha-Orbea, et al, 1988) analysis of the TCR usage demonstrates restricted clonal expansion of Vβ genes, indicative of the dominant antigen-specific response. However, similar studies in RA using southern blot analysis, polymerase chain reaction or monoclonal antibody typing of T cell clones, or of IL-2 expanded synovial T cell

populations, have yielded conflicting results. Whereas some earlier studies suggested over-representation of Vβ14, indicating possible involvement of a superantigen (Paliard, et al, 1990; Howell, et al, 1991), the majority of studies, while establishing differential Vβ gene usage in synovial compared to blood T cells, have failed to provide a consistent pattern in different RA patient cohorts (Bowness & Bell, 1992; Struyk, et al, 1996). In contrast, CDR3 amino acid sequencing by DNA analysis has suggested the presence of conserved amino acid sequences within diverse Vβ repertoires (e.g. Muruyama, et al, 1993; Struyk, et al, 1994; reviewed by Struyk et al, 1996). These data indicate conserved sequences in the antigen binding domains of the T cell receptor and provide suggestive evidence for antigen-driven expansion of T cells within the synovial membrane.

Few antigen specific T cells need be present within an inflammatory lesion. In tuberculoid leprosy, fewer than 2% of T cells within the skin eruption are *M. leprae* responsive (Modlin, et al, 1988). Similarly, T cell frequency against myelin oligodendrocyte glycoprotein in blood of MS patients is only 1:7,299 and in cerebrospinal fluid, 1:450 (Sun, et al, 1991) and bacteria-specific T cells in reactive arthritis are present at a frequency of ~0.1% (Sieper, et al, 1993). The vast majority of T cells within the RA synovial membrane are therefore likely to recognise irrelevant antigens. Whether such cells contribute directly to RA pathology, or are simply functional 'bystanders', is currently unclear.

1.2.4 Fibroblasts in RA

The contribution of the synoviocyte to RA has been subject of controversy. Synovial fibroblasts produce cytokines including TGFβ, FGF, PDGF, IL-6, TNFα and IL-1β and are significant sources of MMPs and of prostaglandins (Remmers, et al, 1991; Zvaifler & Firestein, 1994; Edwards, 1995). As such they comprise an

important component of the inflammatory response in RA, but have been considered the inevitable result of chronic inflammatory infiltration. That they represent the primary lesion has also been proposed (Zvaifler & Firestein, 1994). Several animal models evolve articular erosion in the absence of T cells. *Borrelia* infection induces destructive arthritis in SCID mice (Schaible et al, 1990), and the early phase of spontaneous arthritis in MRL-MP-*lpr/lpr* mice is characterised by fibroblastic infiltration in the absence of T cells (O'Sullivan, et al, 1985). Similarly, *c-fos* transgenic mice develop antigen-induced arthritis the articular component of which proceeds independent of lymphocyte infiltration (Shiozawa, et al, 1992). Synovial fibroblasts exhibit invasive properties *in vitro* and express adhesion molecules for ECM, particularly vitronectin, which would facilitate migration into cartilage with ultimate formation of 'erosions' (Zvaifler & Firestein, 1994).

1.2.5 Macrophages in RA

Macrophages, derived from the circulating monocytes, constitute some 30-50% of the cellular pool in synovial membrane and like T cells, they exhibit features suggestive of activation. Ultrastructural examination demonstrates prominent filopodia, vacuoles, vesicles and rough endoplasmic reticulum (Athanasou, 1995) and histological studies show enhanced expression of complement receptors (C3b), Fc receptors and multiple adhesion molecules, including ICAM-1 and the β2 integrins (Theofilopoulos, et al, 1980; Burmester, et al, 1983; Athanasou & Quinn, 1991; Johnson, et al, 1993). HLA-DR up regulation has been widely reported, although the mechanism whereby this occurs is unclear in the absence of IFN-γ (Klareskog, et al, 1981; Burmester, et al, 1987; Firestein & Zvaifler, 1988). CD14 and CD68 expression have been used extensively for identification purposes. However, the former is lost during maturation in the synovial membrane, and the latter may be found in intimal synovial fibroblasts (Wilkinson, et al, 1992), raising doubts as to its specificity outwith the lining layer. Non-specific esterase therefore

probably remains the most reliable macrophage marker for histological purposes. The principle manifestation of macrophage activation however, is the presence of upregulated cytokine gene expression (Firestein, et al, 1990) and of high concentrations of macrophage-derived cytokines detectable within synovial membrane and fluid.

1.2.6 Cytokine production in RA

Bodel & Hollingsworth first described endogenous pyrogen (now 'IL-1') production by synovial cells in 1968. Subsequently, it was found that IL-1 induced leukocyte infiltration and cartilage breakdown in rabbit synovial joints (Pettipher, et al, 1986). Numerous cytokines have since been localised in the synovial membrane and some have become the focus of therapeutic intervention, as it has become established that they play a central regulatory role in articular destruction. The pattern of cytokine production, however, has led to considerable controversy as to which of the cell types present in synovial membrane is the key element in pathogenesis (Panayi, et al, 1992; Firestein & Zvaifler, 1990).

The presence of many activated synovial T cells is not reflected in high levels of T cell cytokine expression in synovial membrane (Firestein & Zvaifler, 1987; Firestein, et al, 1988; Chen, et al, 1993). Although IFN-γ, and rarely IL-2, may be detected at the mRNA level by PCR or *in situ* hybridisation (Simon, et al, 1994; Buchan, et al, 1988), immunohistochemical localisation has proven difficult, demonstrating expression in small numbers of cells only (Ulfgren, et al, 1995). Neither cytokine has been consistently detected in synovial fluid or in synovial tissue cultures by ELISA. Moreover, IL-4 has not been detected by any means. These observations have cast doubt on the role of T cells in ongoing synovitis, on the basis that T cell effects should be 'cytokine driven'. However, just as the frequency of antigen specific T cells in inflammatory sites is limited, so too is the

level of T cell cytokine expression. Thus, IFN-γ expression in leprosy skin lesions is seen in <1% of cells (Cooper, et al, 1989). Moreover, it has been recently recognised that the actual concentration of cytokine adjacent to its cell receptor *in vivo* may be markedly higher than that predicted from *in vitro* measurements (Kaplan, 1996). The level of T cell cytokines thus far detected may therefore be sufficient to sustain a small number of arthritogen-specific T cells, but is unlikely to explain the extensive T cell activation described.

In contrast, macrophage derived cytokines have been relatively easily detected. These include IL-1α, IL-1β, TNFα, IL-6, IL-8 and GM-CSF (Di Giovine, et al, 1988; Houssiau, et al, 1988; Arend & Dayer, 1990; Seitz et al, 1991; Field, et al, 1991; Chu, et al, 1991; Alvaro-Gracia, et al, 1991; Brennan, et al, 1991; Koch, et al, 1992; Deleuran, et al, 1992; Deleuran, et al, 1994) and encompass a broad range of pro-inflammatory activities. Investigations of their role in animal models and *in vitro* suggest that IL-1β and TNFα may be of prime importance (Arend & Dayer, 1995; Maini, et al, 1995b). TNFα is produced by macrophages in the lining layer and at the cartilage-pannus junction (Chu, et al, 1992). It is present in up to 50% of RA synovial fluids and is detected *in vitro* after synovial tissue culture. Its bioactivities can explain several pathological features observed in RA and include up regulation of adhesion molecule expression, activation of osteoclast-mediated resorption of bone, suppression of anabolic effects of osteoblasts and chondrocytes, promotion of angiogenesis, enhancement of MMP and prostaglandin E_2 (PGE_2) production, stimulation of fibroblast proliferation and activation of T and B lymphocytes and of endothelial cells (reviewed by Arend & Dayer, 1995; Feldmann, et al, 1996b). TNFα also upregulates other cytokine production within the synovial compartment, since the addition of neutralising anti-TNFα antibodies to synovial tissue cultures inhibits the production of IL-1β, IL-6, IL-8 and GM-CSF (Brennan, et al, 1989, Feldmann, et al, 1996b). TNFα production is enhanced in murine CIA and adjuvant arthritis, and disease development and progression are suppressed by

treatment with anti-TNFα antibodies (Williams, et al, 1992; Staines & Wooley, 1994). Moreover, a transgenic mouse which expresses the human TNFα gene, stabilised by replacement of its mRNA 3' untranslated region with that of the β-globulin gene, develops erosive polyarthritis characterised by marked synovial hyperplasia (Keffer, et al, 1991). The most persuasive evidence for a central role for TNFα, however, lies in the amelioration of clinical and laboratory parameters of disease activity in RA patients treated with 'humanised' monoclonal anti-TNFα antibodies (Elliott, et al, 1994; Rankin, et al, 1995).

The activities of TNFα overlap considerably with those of IL-1β. Thus, IL-1β upregulates MMP and PGE_2 production, enhances cartilage breakdown and upregulates osteoclast activity, promotes angiogenesis and induces fibroblast proliferation. However in comparison to TNFα, IL-1β may exert more potent effects on chondrocyte and osteoclast activity and induces higher levels of MMP production from synoviocytes (reviewed by Arend & Dayer, et al, 1995). In animal models, administration of IL-1β induces or upregulates inflammatory arthritis, and inhibition of IL-1β activity down-regulates inflammatory disease (van den Berg, et al, 1994). However, others have found IL-1 receptor antagonist (IL-1ra) less effective than anti-IL-1β antibodies, suggesting that IL-1 may be of variable importance in different models (Wooley, et al, 1993). Nevertheless, clinical trials using IL-1ra in RA have brought about clinical improvement, but remain at a preliminary stage (Campion, et al, 1996).

Addition of anti-IL-1β antibody or IL-1ra to synovial tissue cultures suppresses IL-6 and GM-CSF production, but does not influence TNFα production (Feldmann, et al, 1996b). In combination with the *in vivo* effects of TNFα neutralisation, these data indicate that TNFα lies proximal to other monokines, occupying the pivotal point in a cytokine 'cascade'. However, recent studies in streptococcal cell wall arthritis, zymosan-induced arthritis, antigen-induced arthritis and CIA have suggested that

TNFα and IL-1β may exert distinct biological effects in articular destruction. IL-1β appears more involved in cartilage degradation, whereas TNFα is implicated primarily in pro-inflammatory effects in synovium (Van de Loo, et al, 1995; Van Lent, et al, 1995; Joosten, et al, 1996). These data imply that combined neutralising therapies may be required to adequately suppress tissue damage and moreover, that functional redundancy of cytokines in the synovium is not absolute.

TGFβ, in both latent and active forms (Fava, et al, 1989; Chu, et al, 1991), and IL-10 (Katsikis, et al, 1993) have been detected in synovial membrane and are capable *in vitro* of down-regulating macrophage activation and of opposing the effects of IL-1β and TNFα. Although TGFβ may induce synovitis when injected into rodent knees (Fava, et al, 1991), its role in the majority of animal models is anti-inflammatory (Kuruvilla, et al, 1991; Brandes, et al, 1991). Increased levels of IL-1ra, and of soluble IL-1, IL-2 and TNFα receptors (p55 and p75) are also found in synovial membrane and in synovial fluid (Miossec, et al, 1990; Symons, et al, 1991; Deleuran, et al, 1992; Cope, et al, 1992; Duff, 1993; Firestein, et al, 1994). They usually inhibit the action of their respective cytokines, although in low concentration, soluble TNFαR may stabilise the bioactivity of TNFα (Aderka, et al, 1992). These molecules provide evidence of an attempt within the synovial membrane to down-regulate the inflammatory process. Their net effect in tissue will be determined by the precise local balance of cytokine and antagonist. The cytokine network in RA therefore appears to represent a balance of pro- versus anti-inflammatory (repair) processes, in which the former dominates over time.

Chemokines comprise a further class of pro-inflammatory protein represented within synovium. Both C-X-C (α) chemokines, including IL-8, epithelial neutrophil activating peptide 78 (ENA 78) and melanoma growth stimulating activity (GROα), and C-C (β) chemokines, including macrophage inflammatory protein (MIP)-1α, MIP-1β, RANTES (regulated upon activation normal T cell expressed and secreted)

and monocyte chemoattractant protein (MCP)-1 have been detected in RA synovial membrane (Seitz, et al, 1991; Koch, et al, 1991; Koch, et al, 1992; Rathanaswami, et al, 1993; Koch, et al, 1994a; Koch 1994b; Deleuran, et al, 1994; Hosaka, et al, 1994). Synovial fluid therefore contains chemotactic activity which is attributable to multiple factors which, in turn, exhibit considerable redundancy (Al-Mughales, et al, 1996). Their contribution to leukocyte recruitment occurs in combination with the increased expression of adhesion molecules characteristic of the synovial environment (Johnson, et al, 1993; Cronstein & Weissman, 1993; Pitzalis, et al, 1994). Specific reorganisation of adhesion molecules, such as ICAM-3 and LFA-1, to the tips of uropods follows chemokine exposure, thereby optimising ligand interactions at the point of cell-cell contact (Angel del Pozo, et al, 1996). However, the precise role of chemokine activity in RA is unclear, since *in vitro* transepithelial migration studies of lymphocyte recruitment have demonstrated that MIP-1α and RANTES do not alter the phenotype or magnitude of peripheral blood lymphocyte invasion (Brezinschek, et al, 1995). It is therefore possible that their primary importance lies in regulating migration within the synovial membrane. In this respect, the recent demonstration that cytokine-ECM interaction can create high local concentrations of bioactive cytokine may be of relevance (Gilat, et al, 1996). An additional effect may be to potentiate neovascularisation, as demonstrated for IL-8 or ENA 78 (Koch, et al, 1991; Koch, et al, 1994b).

There is therefore little doubt that synovial macrophages occupy a central position in RA pathogenesis through cytokine secretion. Immune complexes, complement degradation products, bacterial or viral constituents and cell-cell contact have been proposed as stimulatory factors, but none is satisfactorily proven. Thus, although the effector role of TNFα in RA is established, much less is known about the events initiating and maintaining TNFα production. Nor has a satisfactory explanation been provided to link the presence and activity of the majority of T cells with macrophages in synovial membrane, beyond the putative presentation of antigen and

HLA-DR to a subset of disease-specific lymphocytes. Recently, a novel cytokine, designated IL-15, has been identified which is produced by activated macrophages and fibroblasts and exerts potent activities on T cells. This functional profile indicated that it might be highly relevant in the context of rheumatoid pathogenesis.

1.3 Interleukin 15 (IL-15)

IL-15 (synonym: 'IL-T') was simultaneously identified as a product of the simian kidney epithelial cell line, CV-1/EBNA (Grabstein, et al, 1994) and of the HTLV-1-associated HuT-102 adult T cell leukaemia cell line (Burton, et al, 1994; Bamford, et al, 1994). Relevant physical characteristics are shown in table 1.1. Although they share no primary sequence homology, IL-2 and IL-15 possess similar functional activities, as both are members of the short chain four α helix bundle cytokine family. However, whereas IL-2 synthesis is primarily restricted to activated T cells, IL-15 expression, at least at the mRNA level, has been identified in numerous normal human tissues, including skeletal muscle, placenta and kidney, and cell types, such as activated monocytes and fibroblast cell lines, but not as yet in T cells (Grabstein, et al, 1994; Tagaya, et al, 1996a). However, because IL-15 was first identified as a leukaemic T cell product, the possibility remains that T cells appropriately activated may also synthesise IL-15.

1.3.1 IL-15 receptors

The functional similarities between IL-2 and IL-15 are reflected in shared utilisation of the 70/75kD IL-2R β-chain, and the 64kD common γ-chain. Thus, the effect of IL-15 on activated T cells can be inhibited by antibodies against IL-2β or γ chains but not against IL-2Rα chain. Similar data were obtained on analysis of IL-15 binding to cells transfected with different IL-2R subunits (Giri, et al, 1994; Grabstein, et al, 1994; Bamford, et al, 1994). Subsequently, a type 1 membrane

Table 1.1 Physical characteristics of human interleukin-15

Properties	Interleukin-15
Structure	four α-helical bundle,
	two disulphide cross-links
	14-15 kD, 114 amino acids
	variable glycosylation
Primary sequence homology	97% with simian IL-15
	73% with murine IL-15
	<10% with human IL-2
Genomic structure	8 exon / 7 intron
	(variable mRNA splicing reported)
Cellular distribution of mRNA	placenta, skeletal muscle, kidney, lung,
	heart, fibroblasts, epithelial cells, monocytes
Receptor α-chain distribution	T cells, NK cells, B cells, Monocytes
T cell receptor signalling	JAK1 / JAK 3 STAT 3 / STAT 5
Mast cell receptor signalling	JAK 2 STAT 5

protein has been identified in mice which binds IL-15 with high affinity (Giri, et al, 1995). Parallel studies in humans have identified a similar receptor, designated IL-15R α-chain (Anderson, et al, 1995b; de Jong, et al 1996; Kennedy & Park, 1996). The IL-15Rα gene maps to human chromosome 10, adjacent to the IL-2Rα gene, with which it shares fragmented sequence homology. It is alternatively spliced to yield three active forms, each capable of high affinity binding to IL-15 (Anderson, et al, 1995b). Like IL-2Rα, it does not appear to transduce signals, despite the presence of a 37 amino acid intracellular domain. However, notwithstanding these similarities, the two α chains appear functionally discrete. IL-15Rα binds IL-15 with 1000-fold higher affinity than does IL-2Rα to IL-2, with no reported crossreactivity (Giri, et al, 1995). Moreover, IL-15Rα forms an unstable complex with IL-2Rβγ, in contrast to the tight IL-2Rαβγ complex which results from the stabilising effect of IL-2Rα on βγ chain binding (Minami, et al, 1993; de Jong, et al, 1996). Thus, the up regulation of IL-2Rα expression during T cell activation will effectively compete for available IL-2βγ chain, with consequent down regulation of IL-15 mediated signalling, through disaggregation of weak IL-15Rα - βγ chain interactions.

The widespread tissue distribution of IL-15Rα further distinguishes it from IL-2Rα. Whereas IL-2Rα is primarily expressed on activated T cells, IL-15Rα mRNA has been identified in numerous human tissues and cell lines, including activated T cells (Anderson, et al, 1995b). Interestingly, IFN-γ stimulated monocytes also express IL-15Rα mRNA, indicating the potential for autocrine regulation. However, since the distribution of IL-2Rβγ chain is limited, the demonstration of IL-15Rα expression alone may not be a satisfactory indicator of IL-15 responsiveness. Although IL-15 can transduce signals through βγ chain alone, it does so less efficiently than does IL-2 (Grabstein, et al, 1994; Anderson, et al, 1995b; Kumaki, et al, 1996). The functional significance of IL-15Rα expression in the absence of βγ chain is currently unknown. Possibilities include the scavenging of 'free' IL-15 at

inflammatory sites, or the presentation of IL-15 to other IL-15 receptor bearing cells, thereby increasing the efficiency of ligand-receptor interaction.

Expression of IL-2Rα and IL-15Rα appear linked. IL-15, like IL-2, upregulates the level of IL-2Rα on human B and T cells, but rapidly down-regulates the expression of IL-15Rα. Thereafter, lymphocyte responsiveness to IL-15 is reduced in comparison to IL-2 as measured by JAK3 kinase activation (Kumaki, et al, 1996). Thus, macrophage-derived IL-15 can enhance T cell activation during the early stages of an immune response, but thereafter down-regulate its own receptor, thereby enabling IL-2 to function as the principle T cell growth factor.

The observation that mast cells proliferate to IL-15 but not to IL-2, led to the discovery of a further novel receptor for IL-15, designated IL-15RX (Tagaya, et al, 1996b). This 60-65 kD protein requires neither IL-2Rβ nor γ chain for signalling and is also found on normal bone marrow mast cells. Whether a cofactor membrane protein exists, or indeed whether this receptor enjoys wider cellular distribution is not yet known. These data further demonstrate that the functional profiles of IL-2 and IL-15 are not identical.

1.3.2 Signal transduction following IL-15 receptor occupancy

Following IL-15 binding to activated T cells, functional coupling of Janus-associated kinases (JAK) 1 and JAK3 with the IL-15 receptor occurs (Johnston, et al, 1995a). JAK1 and JAK3 in turn activate and tyrosine phosphorylate insulin receptor substrate (IRS)-1 and IRS-2, with subsequent tyrosine phosphorylation and nuclear translocation of Stat 3 and Stat 5 (signal transducers and activators of transcription) (Johnston, et al, 1995b; Lin, et al, 1995). Other signalling events transduced by IL-15 include phosphorylation of p56lck and p72syk and induction of Bcl-2, with resultant protection or rescue from apoptosis (Miyazaki, et al, 1995;

Akbar, et al, 1996). The IL-15RX, in contrast, utilises a JAK2 / Stat 5 dependent pathway in mast cells which is distinct from that in activated T cells (Tagaya, et al, 1996b). Whether this pathway also exists in human T cells is unknown.

1.3.3 Biological activity of IL-15

1. *T lymphocytes* - IL-15 activates T cells as judged by several criteria. IL-15 induces proliferation of PHA activated $CD4^+$ and $CD8^+$ T cells, T cell clones and γδ T cells (Grabstein, et al, 1994), with concomitant release of soluble IL-2 receptors (sIL-2R) (Treiber-Held, et al, 1996). CD69 expression is upregulated on $CD45RO^+$, but not $CD45RA^+$, peripheral blood T cells, consistent with the distribution of IL-2Rβ chain expression on memory T cell subsets (Minami, et al, 1993; Kanegane & Tosato, 1996). IL-15 mediates cytotoxicity, through generation of both $CD8^+$ CTL and of lymphokine activated killer (LAK) cells (Grabstein, et al, 1994). IL-15 induces T cell polarization, a recognised prerequisite to activation, and migration in collagen gel or filter assays, with greater potency than either IL-8 or MIP-1α (Wilkinson & Liew, 1995). Moreover, it redistributes adhesion molecules, including ICAM-3, -1 and -2, to uropods in the presence of β-integrin ligands, such as fibronectin and VCAM-1 (Nieto, et al, 1996). Thus, IL-15 might recruit T cells and thereafter, modify homo- or heterotypic cell-cell interactions within inflammatory sites. Furthermore, IL-15 has recently been shown to rescue T cells from cytokine withdrawal-induced apoptosis, through selective induction of bcl-2 and bcl-xl (Akbar, et al, 1996), indicating that lymphocyte survival within inflammatory sites can be enhanced in the presence of IL-15.

   Given its likely early expression in immune responses, the relationship of IL-15 to T cell subset differentiation, particularly in combination with IL-12, is clearly of interest. IL-15 primes naive $CD4^+$ T cells from TCR transgenic mice for

subsequent IFN-γ, but not IL-4 production (Seder, 1996). Moreover, antigen specific responses in T cells from HIV^+ patients in the presence of high-dose IL-15 exhibit increased IFN-γ production (Seder, et al, 1995) and preliminary data indicate that IL-15 might enhance IFN-γ production if IL-12 is relatively deficient. However, IL-15 induces IL-5 production from human *dermatophagoides farinae II* specific T cell clones, implying a positive role in Th2 mediated allergic responses (Mori, et al, 1996). Whether IL-15 prejudices Th1 or Th2 responsiveness in uncommitted Th0 cells (Mossman & Coffman, 1989) therefore remains unclear. Current data indicate that its function as a T cell growth factor will likely sustain either Th1 or Th2 cells, if already committed to that differentiation pathway.

2. *Natural killer cells* - IL-15 induces NK cell activation measured either by direct cytotoxicity, antibody dependent cellular cytotoxicity (ADCC) or by production of cytokines, including IFN-γ, TNFα, GM-CSF and MIP-1α (Carson, et al 1994; Carson, et al, 1995; Bluman, et al, 1996; Warren, et al, 1996). These data imply that IL-15, rather than IL-2, may be the primary activator of NK cells during early immune responses, in combination with IL-12.

3. *B cells* - IL-15 costimulates proliferation and immunoglobulin synthesis (IgM, IgG, IgA) by human B cells activated with either CD40 ligand or immobilised anti-IgM (Armitage, et al, 1995). A role has also been proposed for IL-15 as a growth factor in B cell chronic lymphoproliferative disorders (Trentin, et al, 1996).

4. *Mast cells* - IL-15 induces mast cell proliferation and activation. The presence of the IL-15X receptor in bone marrow mast cells indicates a role in mast cell development along with IL-3 (Tagaya et al, 1996b).

5. *Thymic development* - Early reports have implicated IL-15 in T cell development. Lymphocytes from IL-2Rβ deficient mice do not proliferate to IL-15. These animals exhibit abnormal T cell development, with significantly reduced thymocyte numbers after 4 weeks (Suzuki, et al, 1995). Subsequent studies comparing the effect of IL-15 and IL-2 on thymocyte development have demonstrated that IL-15 may preferentially support differentiation of bipolar IL-2Rβ$^+$, CD4$^-$, CD8$^-$ progenitor cells towards T γδ or NK cell populations rather than to the T αβ subset (Leclercq, et al, 1996). Furthermore, IL-2 reduced thymocyte expansion *in vitro* through binding to IL-2Rα, in contrast to the proliferative effect of IL-15 through IL-15Rα expression (Willerford, et al, 1995; Leclercq, et al, 1996). Thus, IL-2 and IL-15 may exhibit differential effects in immature T / NK cell development. Recently, IL-15 has also been implicated in development of mature CD16$^+$, CD56$^+$ NK cells from CD34$^+$ haematopoietic progenitor cells (Mrozek, et al, 1996).

6. *Activity outside the immune system.* The widespread tissue distribution of mRNA for IL-15 and IL-15Rα indicates that diverse functions may exist beyond the immune system. For example, IL-15 exerts anabolic effects, but not proliferation, *in vitro* in skeletal muscle (Quinn, et al, 1995).

IL-15 thus represents a mechanism whereby local tissue cells can contribute to the early 'innate' phase of immunity, providing enhancement of NK cell and subsequently T cell responses, prior to optimal IL-2 production.

1.3.4 Regulation of IL-15 production

The factors which up regulate IL-15 synthesis are poorly understood. Using semiquantitative RT-PCR, up regulation of IL-15 mRNA levels in bone-marrow derived murine macrophages was induced by lipopolysaccharide (LPS), BCG, *M.*

tuberculosis or *T. gondii*, only after prior IFN-γ priming. Compared with mRNA for IL-12, IL-15 induction was partially resistant to down-regulation by IL-4, IL-13 and TGFβ. In contrast, IL-10 addition increased levels of IL-15 mRNA in cultures (Doherty, et al, 1996). These data were partially confirmed at the protein level using CTLL cell bioassay. Similarly, LPS / IFN-γ stimulation, or Human Herpesvirus-6 infection of peripheral blood monocytes induced IL-15 mRNA expression (Carson, et al, 1995; Bamford, et al, 1996a; Bamford, et al, 1996b; Flamand, et al, 1996). Thus, recognised macrophage activators appear to increase IL-15 expression, at least at the mRNA level.

Consistent detection of IL-15 protein synthesis, however, has not followed the widespread distribution of its mRNA. Whereas most cytokines are regulated by modification of transcription and message stabilisation (reviewed by Paul & Seder, 1994; Kishimoto, et al, 1994), significant post-transcriptional regulation has been detected for IL-15. The human IL-15 mRNA 5' UTR contains 10 AUG triplets which significantly reduce the efficiency of translation (Kozak, 1991; Bamford, et al, 1996a). Fusion of the IL-15 mRNA with an HTLV-1 R region in the HuT-102 cell line deleted the AUG rich 5'UTR sequence, leading to high levels of constitutive IL-15 secretion (Burton, et al, 1994; Bamford, et al, 1996b). Engineered removal of this sequence similarly induces increased levels of IL-15 protein expression in appropriately transfected COS cells (Bamford, et al, 1996b). In a similar system, replacement of the IL-15 signal peptide with that of IL-2, induces significantly higher levels of IL-15 production, indicating that this region too is involved in down-regulating IL-15 protein production (Tagaya, et al, 1996b). Multiple levels of regulation therefore exist to provide an available pool of mRNA, but which prevent undesirable IL-15 expression in tissues.

1.3.5 IL-15 in pathology

The corollary to this regulatory organisation is the ready resource of IL-15 mRNA in tissues in the event of infection or toxic insult. That such expression could lead to autoimmunity forms a basic tenet of this thesis. Evidence for involvement of IL-15 in human pathology, however, is sparse. A novel transcript of IL-15 has been recently identified in human small cell lung cancer cell lines, although its role in tumorigenesis is as yet unclear (Meazza, et al, 1996). IL-15 has also been identified in alveolar macrophages of sarcoid patients, from whom pulmonary $CD4^+$ T cells were found to proliferate to IL-15, implying a role in T cell alveolitis (Agostini, et al, 1996). IL-15 expression has also been detected in alveolar macrophages and epithelial cells from induced sputum samples of asthmatic patients (Leung, B.P. & McInnes I.B. - unpublished observations). Since IL-15 also induces mast cell proliferation and supports T cell clone-derived IL-5 production (Tagaya, et al, 1996b; Mori, et al, 1996), these data together indicate that IL-15 might play a role in bronchial hyperreactivity. IL-15 expression has been reported in human keratinocytes (Sorel, et al, 1996). It remains unclear whether UVB radiation upregulates IL-15 expression (Mohamadzadeh, et al, 1995), or inhibits its production, thereby contributing to UVB-mediated immunosuppression (Blauvelt, et al, 1996).

RA synovium is characterised by the presence of activated macrophages and fibroblasts, and by large numbers of activated T cells. Given its established cellular distribution and functional profile, IL-15 represents an attractive candidate cytokine for involvement in RA synovitis. Its identification in that context could provide considerable insight into the mechanisms whereby T cells are recruited and activated in RA. A second exciting possibility is that within the synovial membrane, IL-15 could direct T cell-mediated regulation of macrophage-derived cytokine production, particularly TNFα, thereby driving the pro-inflammatory response.

1.4 'Non-cytokine' mediators in RA synovitis

Non-cytokine mediated pro-inflammatory mechanisms operate in the synovial membrane. Of these, the role of free radicals in RA pathogenesis has been of particular interest. Reactive oxygen intermediates (ROI) formed through cycles of hypoxic-reperfusion can modify multiple processes within the synovial membrane by oxidation (Blake, et al, 1989). Proposed molecular targets include IgG, with consequent rheumatoid factor formation and lipids, leading to formation of lipid-radicals. Depolymerisation and fragmentation of hyaluronan after oxidation will generate fragments with immunomodulatory potential e.g. chemotactic factors. Hyporesponsiveness of synovial T cells might arise secondary to hypoxia and the formation of low molecular weight aldehydes (reviewed by Mapp, et al. 1995). Definitive confirmation of such hypotheses *in vivo* is currently lacking, although they provide stimulating alternatives to established antigen-driven mechanisms. Recently, reactive nitrogen intermediates (RNI), specifically nitric oxide ($NO^{\bullet}$), have been recognised as mediators of fundamental importance in several models of infection and inflammation (Moncada & Higgs, 1993; Nathan & Xie, 1994a). These observations have raised the possibility that similar mechanisms might operate in human inflammatory disease.

1.5 Nitric Oxide

In 1987, NO was recognised as the previously elusive endothelium-derived relaxing factor (Furchgott & Zawadski, 1980; Ignarro, et al, 1987; Palmer, et al, 1987). Subsequently, NO emerged as a key regulatory molecule in numerous and diverse physiological and patho-physiological processes.

1.5.1 NO synthase

NO is generated by isoforms of the enzyme NO synthase (NOS) which catalyses the conversion of L-arginine through hydroxy-arginine to L-citrulline. The terminal guanidino nitrogen is oxidised forming a reactive nitrogen-oxygen complex with one unpaired electron ($NO^{\bullet}$), which confers radical properties on NO (Stamler, et al, 1992). Three isoforms of NOS have been identified and their enzymology extensively studied (reviewed by Moncada & Higgs, 1993; Kiechle & Malinski, 1993; Bredt & Snyder, 1994; Nathan & Xie, 1994a; Nathan & Xie, 1994b; Marletta, 1994; Butler, et al, 1995). Endothelial NOS (eNOS) and neuronal NOS (nNOS) are constitutively expressed and are capable of rapid onset, short lived generation of low concentrations of NO (together termed cNOS). Inducible NOS (iNOS), in contrast, is present in cells only after specific up regulation. NOS isoforms share 30-40% homology with cytochrome P450 reductase (CPR), with consensus sequences for redox-active cofactors including NADPH, flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN). The homology with CPR is reflected at a functional level in the ability of NOS to generate ROI in the absence of L-arginine and presence of NADPH (Bredt & Snyder, 1994), although the biological relevance of this activity is unknown.

cNOS dimers form from a resting pool of NOS monomers, after binding of L-arginine, haem and tetrahydrobiopterin (BH_4). Calmodulin binds to this complex in the presence of elevated calcium, which results usually from agonist activity. This facilitates rapid electron transfer through FAD and FMN to haem with consequent substrate oxidation. In contrast, expression of inducible NOS (iNOS) requires novel protein synthesis and thereafter generates high concentrations of NO over prolonged periods. As with cNOS, iNOS forms dimers in the presence of BH_4, haem and L-arginine. However, calmodulin is tightly bound to a basic, hydrophobic site on

iNOS, and enzyme activity is independent of ambient calcium concentration (Cho, et al, 1992).

All three isoforms have been cloned and sequenced (Bredt, et al, 1991; Sessa, et al, 1992; Xie, et al, 1992). eNOS is 130kD and shares 58% homology with nNOS. An N-terminal myristoylation site confers the ability to bind the plasma membrane and phosphorylation or cleavage of this site renders eNOS soluble in the cytosol. The human gene is located on chromosome 7 and contains AP-1, AP-2, NF-1, acute phase reactants and shear stress regulatory consensus sites in its 5' promoter region (Marsden, et al, 1992). eNOS expression has been detected in cells outwith endothelium, including platelets, fibroblasts, vascular smooth muscle cells and polymorphonuclear cells (Nathan & Xie, 1994a). The larger human nNOS (160kD) is located on chromosome 12 (Bredt & Snyder, 1994). It is expressed in different neurone types throughout the central nervous system, in the retina and in peripheral nerve terminals, e.g. non-adrenergic, non-cholinergic signals in the gastrointestinal tract (Bult, et al, 1990). Alternatively spliced forms of murine cerebellar nNOS have been described although their functional significance is unclear.

iNOS was first cloned from murine macrophages (Xie, et al, 1992) and subsequently from human hepatocytes and chondrocytes (Geller, et al, 1993; Charles, et al, 1993), but not from human macrophages. Human iNOS shares ~50% homology with cNOS, and 80% with murine iNOS and is located on chromosome 17. Widespread tissue distribution of iNOS has been reported with expression observed in human keratinocytes, hepatocytes, osteoblasts / osteoclasts, chondrocytes, uterine smooth muscle cells, mesangial cells, dermal fibroblasts and respiratory epithelial cells (Nathan & Xie, 1994a). Expression in human tumours has also been observed, including colorectal adenocarcinoma and glioblastoma (Ellie, et al, 1996). However, considerable controversy surrounds the presence and activity of iNOS in human macrophages. Whereas some authors have demonstrated NO production

directly, or L-arginine- / NO-dependent activity (Denis, 1992; Hunt & Goldin, 1992; Reiling, et al, 1994; Pietraforte, et al, 1994; Zembala, et al, 1994; Mautino, et al, 1994; De Maria, et al, 1994; Dugas, et al, 1995; Burkrinsky, et al, 1995), others have been unable to detect any evidence of iNOS activity at all (Cameron, et al, 1990; Schneemann, et al, 1993). The required stimuli for iNOS up regulation in human macrophages appear to differ significantly from those in rodents, and where NO production has been detected, it is of an order of magnitude lower than that observed in rodent macrophages. The functional and evolutionary significance of these data are unclear.

1.5.2 Regulation of NO synthase

NO may not be stored in 'bioactive' form, thus its concentration in tissue is regulated through NOS activity. cNOS generates NO at picomolar concentrations in response to local vasoactive mediators, such as bradykinin, thrombin, histamine, acetylcholine, 5-hydroxytriptamine, to cytokines e.g. IL-1β, endothelin-3, or to physical factors, including shear stress or increased blood flow (reviewed by Lyons, 1995). Whether cNOS output may be further upregulated is unclear. Preliminary evidence for induction of 'nNOS' by IL-1β and LPS in OA chondrocytes has been reported, indicating that the delineation between low and high output NOS on the basis of calcium dependency alone may be oversimplified (Amin, et al, 1995).

The predominant source of NO in inflammatory lesions, however, is iNOS. Factors which activate iNOS *in vitro* include cytokines, microbial products, particularly LPS and superantigen toxins, picolinic acid, cAMP-elevating agents and physical factors, including UV light or trauma (reviewed by Nathan & Xie, 1994a; Nathan & Xie, 1994b). Cytokines exert the major regulatory influence *in vivo*. IFN-γ is a potent inducer of NO production by rodent macrophages and endothelial cells, in synergy with LPS (Ding, et al, 1988; Liew & Cox, 1991). IL-1β and TNFα also increase

iNOS expression in many cells, either alone or in synergistic combination (Liew, 1994). Cytokine requirements vary with the species and tissue origin of cells. Thus, rodent smooth muscle cells respond either to IL-1β alone, or in synergy with IFN-γ or TNFα, whereas human vascular smooth muscle cells require a combination of LPS, IFN-γ and TNFα for NO production. Similarly, rodent hepatocytes respond to LPS alone, whereas human hepatocytes require a combination of LPS, IFN-γ, TNFα and IL-2 (Geller, et al, 1992; Liew, 1994, Nathan & Xie, 1994b; reviewed by Lyons, 1995).

The multiplicity of activatory factors is matched by a wide range of inhibitory agents. TGFβ, IL-4, IL-8, IL-10 (indirectly through effects on TNFα production), IL-13, MIP-1α, epidermal growth factor (EGF), PDGF, and FGF all oppose iNOS activation (Liew, 1994; Nathan & Xie, 1994b; Lyons, 1995). Again species and tissue specificity appear crucial. TGFβ inhibits rodent macrophage and endothelial iNOS expression, but enhances NO production in Swiss 3T3 fibroblasts (Gilbert & Herschman, 1993). Moreover, IL-10 has been shown to increase iNOS activity in avian osteoclasts (Sunyer, et al, 1996), as has IL-4 in human macrophages (Mautino, et al, 1994). The temporal sequence of ligand binding appears important, since pre-exposure of macrophages to LPS suppresses subsequent IFN-γ induced NO production (Severn, et al, 1993). Such observations emphasise the difficulties attached to extrapolation between species and cell types. Furthermore, it has only recently been appreciated that iNOS may be 'constitutively' present in human tissues, such as lung, retina, skeletal muscle or CNS, in the absence of specific up regulatory factors (Nathan & Xie, 1994a; Park, et al, 1996). This suggests that, in addition to a postulated responsive role in inflammation, iNOS may also be involved in normal physiological regulation.

Engagement of class II MHC either by allo-specific monoclonal antibodies, or by bacterial superantigens (SEB, TSST-1), in the presence of syngeneic lymphocytes,

increases NO production by macrophages (Hauschildt, et al, 1993; Tao & Stout, 1993, Isobe & Nakashima, 1993). Similar activation of macrophage NO synthesis follows crosslinking of CD69 by antibody (De Maria, et al, 1995). Cell-cell contact between T lymphocytes of both Th1 and Th2 subsets and macrophages leads to iNOS expression mediated in part through CD40/CD40 ligand and LFA-1/ICAM-1 (Tian, et al, 1995). Thus, homo- or hetero-typic cell contact can induce NO synthesis in an inflammatory lesion. The relative contribution of such mechanisms in the context of high levels of cytokine production is currently unclear.

These diverse factors mediate regulation at multiple levels:

Transcription Two promoter / enhancer sites are found 5' to the murine iNOS gene (Xie, et al, 1993; Lowenstein, et al, 1993). Region 1 (-50 to -200 bp) contains LPS responsive elements containing AP-1, NF-IL6 and NF-κB binding sites, whereas region 2 (~1000 bp upstream) contains an interferon specific response element (reviewed by Bredt & Snyder, 1994; Nathan & Xie, 1994a, 1994b). Macrophages from IFN-γ related transcription factor-1 (IRF-1) knockout mice do not produce NO in response to IFN-γ confirming a role for IRF-1 in regulating iNOS expression, mediated partly through enhancement of LPS-dependent effects (Kamijo, et al, 1994). A hypoxia-responsive enhancer has recently been identified (-209 to -207 bp; Melillo, et al, 1996) indicating that other factors can facilitate iNOS transcription outwith cytokine-dependent elements. Transcriptional regulation of the human iNOS gene is more complex. Involvement of a NF-κB binding site (-106 to -115 bp) has been demonstrated using mutant constructs of vascular smooth muscle iNOS (Kolyada, et al, 1996) and recent studies of hepatocyte iNOS responsiveness to IL-1β, TNFα and IFN-γ revealed three cytokine responsive elements -3.8 to -16 kb upstream from the iNOS gene (De Vera, et al, 1996). This is in contrast to the shorter regulatory 5' region (~1kb) of rodent iNOS. Further characterisation of the iNOS promoter sites will elucidate inter-species differences

currently detected at the protein expression and functional level, particularly in comparison of rodent and human macrophage activation.

Post-transcription iNOS mRNA stability is increased by IFN-γ and reduced by TGFβ and IL-4 in murine macrophages. TNFα reduced the stability of eNOS mRNA in rat smooth muscle cells (Nathan & Xie, 1994a). Modification of mRNA levels by many regulatory factors is reported, but it is unclear at present whether these effects are directly mediated on mRNA stability or at earlier or later stages.

Post-translation cNOS is calcium and calmodulin dependent and may be inhibited by competitive inhibitors of calmodulin, such as trifluoperazine. iNOS requires only physiological concentrations of calcium which are present in resting cells, and as such is resistant to calmodulin antagonists (Cho, et al, 1992). NOS activity may also be modified by phosphorylation. eNOS and nNOS share consensus sequences for cAMP-dependent protein kinase phosphorylation (Bredt & Snyder, 1994). Protein kinase C, cGMP-dependent kinase, calcium / calmodulin-dependent kinase have also been implicated in cNOS phosphorylation, raising the possibility of regulatory feedback loops, whereby the reaction of NO with target enzymes may increase kinase activity, with consequent suppression of NOS activity (Bredt & Snyder 1994). However, whether significant phosphorylation occurs *in vivo* to directly modify enzymatic activity, cofactor function or iNOS compartmentalisation is unclear. Finally, haem-binding proteins are subject to down regulation by NO. Thus, direct inhibition of NOS activity by NO itself has been reported (Assreuy, et al, 1993).

Substrate availability in vitro is determined by the relative presence of L-arginine, of argininosuccinate synthetase, which converts L-citrulline to L-arginine, and of arginase in macrophages. Similarly, cofactor availability is critical. IFN-γ induces the enzyme GTP cyclohydroxylase I, which is the rate-limiting enzyme in the

biopterin synthesis pathway responsible for the generation of BH_4. Although the latter is deplete after long term culture of human endothelial cells or fibroblasts, it is unclear whether this represents *in vitro* artefact, or an *in vivo* example of coordinate function by IFN-γ.

External regulation of NOS Glucocorticoids inhibit iNOS-dependent activity. (Di Rosa, et al, 1990). The rate of iNOS transcription is reduced, and although mRNA is briefly stabilised, translation is significantly retarded and degradation of iNOS protein is enhanced (Kunz, et al, 1996). NF-κB p50 or p65 nuclear translocation is unaffected, but NF-kB and not AP-1 binding in the iNOS promoter region is prevented (Kleinert, et al, 1996). L-N^{ω}-substituted arginines also inhibit NOS activity in biological systems. L-N^{ω} methylarginine (L-NMMA) is commonly used when no isoform specificity is necessary. No isoform specific inhibitor has yet been identified, although L-N^{ω} nitroarginine (L-NNA) exhibits relative specificity for cNOS as does L-N^{ω} aminoarginine for iNOS. Aminoguanidine and N-iminoethyl-L-lysine are further 'iNOS specific' inhibitors often used in animal models (e.g. Connor, et al, 1995). However, mice in which the eNOS, nNOS and iNOS genes respectively have been specifically targeted have now been generated, allowing definitive investigation of the specific contribution of individual isoforms to be evaluated in different biological systems *in vivo* (Wei, et al, 1995; MacMicking, et al, 1995; Huang, et al, 1995; Nelson, et al, 1995).

1.5.3 Molecular targets for NO

The target interactions of NO which facilitate effector function depend largely on its redox and additive chemistry (reviewed by Stamler, 1994). NO has a half-life of 6 seconds, and can diffuse freely through membranes and within cells. NO reacts with oxygen (O_2), superoxide (O_2^-) and transition metals, to generate nitrosonium ions

(NO^+), peroxynitrite ($OONO^-$) and metal-NO adducts respectively, each capable of further reactivity with thiol groups at nucleophilic centres. NO therefore mediates effects primarily through modification of critical iron or thiol groups on protein targets. The diversity of effector function reflects the wide prevalence of such moieties in regulatory/signalling pathways, enzymes, ion channels and transcription factors (Stamler, 1994; Lyons, 1994). In conditions of oxidant stress, cell redox conditions may support the direct N-nitrosation of DNA or covalent modification of tyrosine groups. DNA may also be targeted by deamination or by induction of strand breaks (Nguyen, et al, 1992).

Reactions with metals Iron containing metals are particularly vulnerable to NO. Complex I and complex II in the mitochondrial electron transfer chain and aconitase in the tricarboxylic acid cycle, are critical targets (Stuehr & Nathan, 1989; Stamler, et al, 1992). Together with inhibition of glyceraldehyde-3-phosphate dehydrogenase, through ADP-ribosylation following activation of ADP-ribosyl transferase (Dimmeler, et al, 1992), these effects profoundly impair the capacity for energy production in the target. Aconitase serves also as an iron response element binding protein (IRE-BP) and through binding to IRE-BP, NO has been demonstrated to modify ferritin and transferrin expression and thus iron homeostasis (Weiss, et al, 1993; Drapier, et al, 1993).

The interaction of NO with haem induces functionally significant conformational changes, e.g. guanylate cyclase is activated by this means and mediates many bioactivities attributable to NO (Nathan & Xie, 1994b; Schmidt & Walter, 1994). Direct nitrosylation of the haem prosthetic group involved in catalysis is usually inhibitory. This is evident in CPR-like enzymes, including iNOS itself through attenuation of oxygenase activity, and perhaps also in ribonucleotide reductase, with subsequent modification of DNA synthesis (Stamler, 1994). In contrast, cyclooxygenase 1 and 2 are activated by this mechanism (Salvemini, et al, 1993;

Manfield, et al, 1996). Furthermore, interaction with zinc finger containing proteins has been reported, which may have functional relevance to the zinc dependent activities of matrix metalloproteinases in synovium (Murrell, et al, 1995).

Thiol group interactions Nitrosonium, peroxynitrite and metal-NO adducts are capable of S-nitrosylation with consequent modification of protein function. Target proteins undergo (i) conformational change (e.g. tissue plasminogen activator), (ii) covalent alteration by the formation of disulphide bonds (e.g. calcium-dependent potassium channels), or (iii) if cysteine residues are present in the catalytic site, may be directly inhibited by nitrosylation (e.g. neutrophil NADPH oxidase). The latter exemplifies the crucial interaction of NO and ROI production (Clancy, et al, 1992; Stamler, 1994). In circumstances where ROI are primary mediators of tissue damage, NO production may be protective by diverting oxygen species away from the formation of harmful radicals (Wink, et al, 1993; Wink, et al, 1996). In contrast, the formation of peroxynitrite has been shown in many systems to be dangerous to host tissues and in such circumstances the combination of ROI with NO generation is likely to be detrimental (e.g. Mulligan, et al, 1991). The effect of NO in a given tissue can therefore vary over time, if the presence of ROI in the tissue alters in response to other stimuli. Thus, production of NO may be initially detrimental and later protective or *vice versa.* Similarly, functional outcome of NO effects may alter as the redox environment changes over time within a cell, perhaps following activation, allowing NO to sequentially exert apparently contradictory responses on resting or activated cells. Such possibilities are of particular importance in immune responses within chronic inflammatory environments, such as those in the RA synovial membrane.

1.5.4 Functional consequences of NO production in inflammation

By virtue of its EDRF activity NO can induce vasodilatation through relaxation of vascular smooth muscle leading to erythema and increased local temperature (reviewed by Schmidt & Walter, 1994). Data from murine dextran- and carrageenan-induced models of inflammation indicate that NO also induces clinically detectable oedema formation, through alteration of endothelial permeability (Ialenti, et al, 1992; Ianaro, et al, 1994). Thus, two features of the classical inflammatory response are regulated by NO. Further roles for NO in immunoregulation, responses to infectious disease, alloreactivity and autoimmune models have been demonstrated.

(i) Immunomodulatory properties of NO

NO inhibits platelet aggregation as a function of its cardioprotective role, through production of cGMP (Schmidt & Walter, 1994). Subsequent studies of ischaemia-reperfusion in mesenteric vessels and myocardium have indicated that NO also reduces neutrophil adhesion through CD11 / CD18, ICAM-1 and P-selectin dependent pathways and by scavenging ROI, which enhance adhesion (Kubes, et al, 1993; Xin, et al, 1993). These data indicate that cellular recruitment, and in particular the crucial interaction of the leukocyte with endothelium, can be modified by NO.

NO exerts bi-phasic effects on T lymphocyte responsiveness. Initial observations described inhibition of *in vitro* antigen or mitogen driven T cell proliferation, either by NO donors or by macrophage derived NO in co-cultures (Fu & Blankenhorn, 1992; Denham & Rowland, 1992; Merryman, et al, 1993). Subsequently, it was established that low dose NO significantly enhanced peripheral blood lymphocyte activation, measured by PHA-induced proliferation, increased glucose uptake,

increased NF-κB binding activity and activation of protein tyrosine kinase $p56^{lck}$ (Lander, et al, 1993). Moreover, *in vitro* and *in vivo,* L-arginine enhances lymphocyte proliferation, and increases NK cell and lymphokine activated killer activity (Barbul, et al, 1990; Park, et al, 1991). Recent studies in mice have established that NO preferentially inhibits Th1 clonal proliferation to antigen, but has no effect on Th2 clones (Liew, et al, 1991; Taylor Robinson, et al, 1993; Taylor Robinson, et al, 1994; Wei, et al, 1995). Thus, the local concentration of NO and the developmental phenotype influence the modulatory effect of NO on T cells. Cellular immune function may be further modified by NO through induction of apoptosis (Albina, et al, 1993; Messmer, et al, 1994; Fehsel, et al, 1995).

Cytokine production is also influenced by NO. Th1 clones exhibit reduced IFN-γ production in the presence of NO, correlating with reduced proliferation, but no effect is demonstrable on Th2 cytokine generation (Taylor-Robinson, et al, 1994; Wei, et al, 1995; Huang, F.P. personal communication). Increased TNFα production from human neutrophils and from PBMN exposed to NO-donors has been detected, although the cellular origin of TNFα in the latter was not specified (Lander, et al, 1993; Dervort, et al, 1994). Production of cytokine by purified blood monocyte/ macrophages or macrophage cell lines has been variously reported to be suppressed (Fülle, et al, 1991) or enhanced (Deakin, et al, 1995) in the presence of exogenous NO. The mechanism underlying these observations is unknown, but may reflect modification of transcription factors, such as NF-κB (Huang F-P. personal communication).

(ii) Infection

Early studies detected increased nitrate generation during septicaemia (Green, et al, 1981). NO has now been implicated in the response to a large number of organisms, including intracellular bacteria, fungi, protozoa, helminths and viruses. NO

dependent innate defence has been most studied in macrophages. In most cases microbicidal activity is demonstrable *in vitro*, where it is inhibited by L^{ω}-arginine analogues, and is enhanced by addition of macrophage activating factors, such as IFN-γ or LPS. Normally *in vivo*, T cells and macrophages cooperate to regulate NO synthesis through cytokine production. Thus, host responses to *Leishmania major* are dependent on the generation of an effective Th1 response, in which IL-12 and IFN-γ production leads to NO-mediated resistance. Parasite killing activity is demonstrable in splenic macrophages *in vitro*, and treatment of infected mice with iNOS inhibitors increases lesion size and parasitic load (Liew, et al, 1990). Moreover, *L. major* infection in iNOS knockout mice is of increased severity and mortality, despite the presence of an enhanced Th1 response, indicating that NO is critical in host defence to this organism (Wei et al, 1995). However, NO-mediated parasite killing can also proceed in the absence of T cells. *Listeria monocytogenes* infected SCID mice, treated with aminoguanidine, exhibit increased mortality and enhanced recovery of viable listeria from spleens. IFN-γ production by NK cells is sufficient to confer resistance (Beckerman, et al, 1993). Thus, in rodents at least, the production of NO by activated macrophages provides a principle component of the microbicidal armature.

NO may mediate host toxicity during infection. Elevated nitrate levels are detected in animals and humans with septicaemia. NOS inhibitors can reverse the hypotension of LPS induced shock in rodents, although the effect is dose and time dependent (Kilbourn, et al, 1990), and preliminary trials in humans have indicated that some clinical features of septicaemia are NO dependent (Vallance & Moncada, 1991; Petros, et al, 1994). However, LPS-induced shock in iNOS knockout mice has yielded conflicting evidence in favour of either a protective, or a detrimental role for NO production (Wei, et al, 1995; Gross, et al, 1996), although minor differences in genetic strain between 'knockouts' may partially explain apparent discrepancies. What is clear is that NO has 'double-edged' effects (Schmidt &

Walter, 1994), and in some situations is aggressive to the host. Whether these observations can be extended to autoimmune disease in which excess NO production occurs is unclear. These data indicate that caution must be shown in the potential use of NOS inhibitors in management of autoimmune disease, in which equally complex mechanisms exist in subtle equilibrium.

(iii) Alloreactivity

NO involvement in graft rejection is suggested by the nitrosylation of proteins during cardiac allograft rejection in rats (Lancaster, et al, 1992). iNOS expression is found at mRNA and protein levels in rejecting allografts, in both graft and host derived cells. Debate surrounds the net effect of NO production in rejection. Aminoguanidine treatment prolongs graft survival, reduces the histological grade of cellular infiltration and improves cardiac allograft contractility in rats (Worrall, et al, 1995). However, several studies have determined that iNOS inhibitors are deleterious to graft survival through removal of T cell regulatory activity attributable to NO (e.g. Langrehr, et al, 1992). Moreover, BALB/c skin allograft rejection by iNOS knockout mice is similar to that by heterozygote controls (Casey J., personal communication). The precise role of NO production in alloreactivity is therefore unclear, although the latter data imply that NO production is not obligate in graft rejection.

(iv) Autoimmune models

The evidence reviewed above clearly implicates NO as an integral component of protective host immune responses. That NO is similarly involved in deleterious autoimmune responses, either as an aggressive or protective component, has been explored in several animal models systems.

Murine disease resembling insulin dependent diabetes mellitus (IDDM) occurs following inoculation with streptozocin. NO modifies cytokine production within the pancreatic β islets and inhibition of NO production using NOS inhibitors led to delayed onset of disease, with attenuation of the pancreatic inflammatory infiltrate (Lukic, et al, 1991; Kolb & Kolb-Bachofen, 1992). Similarly, in the genetically predisposed non obese diabetic (NOD) mouse model, transfer of NOD mouse spleen cells induced diabetes in irradiated recipients. The onset of disease was significantly delayed by aminoguanidine treatment (Corbett, et al, 1993). These findings implicate NO as an aggressor in IDDM pathogenesis. However, its role in experimental allergic encephalomyelitis (EAE) is more complex. NO production is upregulated in EAE and iNOS levels have been reported to correlate with disease severity (Okuda, et al, 1995). Whereas, aminoguanidine was reported to inhibit clinical signs and progression of EAE in SJL mice and in Lewis rats (Zhao, et al, 1996), paradoxical aggravation of EAE following administration of L^{ω}-arginine analogues has also been detected (Ruuls, et al, 1996). Suppression or aggravation of EAE by NOS inhibitors may depend on the mode of disease induction, or on the choice of inhibitor in T cell-induced, or myelin basic protein-induced EAE (Zielasek, et al, 1995). Such discrepancies again emphasise the double-edged effector function of NO as an immunosuppressor, or a neurotoxin, dependent on subtle alterations of immunogen, inhibitor dosage and regimen.

NO has been implicated in immune complex mediated disease. In pulmonary alveolitis induced by intra-tracheal injection of preformed immune complexes, NOS inhibitors reduce the severity of pulmonary haemorrhage and oedema formation. Similar inhibition of dermal vasculitis is observed. Moreover, a major component of this model is dependent on intact complement function, indicating that NO synthesis may interact with complement to mediate pathology (Mulligan, et al, 1991).

Graft versus host disease (GVHD) in mice resembles the early stages of inflammatory bowel disease (IBD), or gut hypersensitivity syndromes, such as coeliac disease. (CBAxBALB/c)F_1 recipients of CBA spleen cells develop GVHD, which is significantly retarded by L-NMMA treatment, with preservation of intestinal architecture and reduced density of intra-epithelial lymphocyte infiltration (Garside, et al, 1992). However, it is unclear whether this effect operates primarily through immunoregulatory modification, or by haemodynamic effects in the mesenteric vasculature. NO production has been detected in human IBD, indicating that a role in human disease pathogenesis may exist (Middleton, et al, 1993, Broughton-Evans, et al, 1993). However, altered epithelial permeability found in IBD leads to increased exposure to bacteria and bacterial products within the lamina propria, with the potential for enhanced local NO production and consequent immunomodulation. Whether NO is ultimately protective or detrimental is therefore unclear.

1.5.5 NO in arthritis

Considerable evidence exists which indicates that NO is an important mediator in autoimmune responses. Evidence for a similar role in inflammatory articular disease has recently been derived from several animal studies.

Adjuvant arthritis in rats bears histopathological similarities to RA. iNOS is detectable in synovial membrane and elevated levels of urinary and plasma nitrite are maximal after 14 days. Continuous administration of NOS inhibitors prevents or attenuates the clinical severity of arthritis, normalises weight gain, reduces acute phase response and retards erosive articular destruction (Ialenti, et al, 1993; Stefanovic-Racic, et al, 1994a; Stichtenoth, et al, 1994; Stefanovic-Racic, et al, 1995; Connor, et al, 1995). Treatment during adjuvant priming alone is sufficient to confer subsequent reduction in disease severity (Oyanagui, 1994) and anti-

mycobacterial antigen-specific T cell responses are suppressed in treated rats. These observations indicate an immunoregulatory role for NO in this model. Similar data were obtained in streptococcal cell wall (SCW) induced arthritis in rats, in which L-NMMA inhibited the onset and progression of arthritis. Furthermore, treatment of established disease led to partial clinical amelioration (McCartney-Francis, et al, 1994). Administration of NOS inhibitors to MRL-MP-*lpr/lpr* mice suppressed the development of renal pathology and attenuated clinical and histological evidence of arthritis (Weinberg, et al, 1995; Huang, et al 1996). NO synthesis is closely linked to IL-12 production in this model, indicating that cytokine modulation by NO may complete a positive feedback loop, culminating in end-organ damage (Huang, et al, 1996). These data together implicate NO generation in articular pathology in rodents.

The role of nitric oxide production in RA, however, is less clear. Nitrite levels are elevated in synovial fluid and serum, and urinary nitrate: creatinine ratios are raised in patients with active RA, indicating that NO production might be enhanced in human articular disease (Farrell, et al, 1992, Kaur & Halliwell, 1994; Grabowski, et al, 1996; Ueki, et al, 1996). The principle source of nitric oxide production is, as yet, ill-defined. *In vitro* studies have identified iNOS expression and NO generation in rodent synoviocytes and in human and rodent chondrocytes, osteoblasts and osteoclasts (Stadler, et al, 1992; Charles, et al, 1993; Stefanovic-Racic, et al, 1994b; Rediske, et al, 1994; Ralston, et al, 1995; Murrell, et al, 1996). Given their previously recognised functional profile, neutrophils, macrophages, mast cells and endothelial cells might all contribute to NO generation within synovial membrane (Nathan & Xie, 1994a; Nathan & Xie, 1994b; Barnes & Liew, 1994; Lyons, 1995; Grabowski, et al, 1996). Moreover, the RA synovial membrane contains abundant cytokine activities, such as IL-1β or TNFα, which have been shown to up regulate iNOS *in vitro*. However, neither iNOS expression nor direct NO production within the human synovial membrane have yet been properly characterised.

1.6 Objectives

The immunopathological processes in RA resemble an ongoing immune response. Components of humoral and cell mediated arms of the immune system are represented, although their relative contributions remain controversial. Regardless of whether these processes are primary, or secondary to an unidentified insult, evidence from animal and preliminary human studies indicates that manipulation of immunoregulatory networks in synovial membrane offers exciting therapeutic potential. Two such regulatory pathways are of particular interest to the author.

(i) T lymphocytes lie central to conventional immunoregulatory paradigms. Their apparently sessile appearance in RA is therefore intriguing.

- By what means are T lymphocytes activated within RA synovial membrane in the relative absence of the recognised T cell growth factor, IL-2?
- What is the functional significance of the majority of non-arthritogen specific T lymphocytes in synovial membrane in RA?

(ii) Similarly, nitric oxide subserves multiple immunoregulatory functions in animal models, but its role in human inflammatory disease remains poorly defined.

- Where and how is NO produced by the human synovial membrane?
- What are the functional consequences of NO production within the synovial compartment?

(iii) TNFα has provided the most promising therapeutic biological target thus far in RA. Events mediating its production, however, remain obscure.

- Can either IL-15 or nitric oxide mediated activities be related to enhanced TNFα production within synovial membrane?

1.7 Aims

1. To investigate the presence and functional significance of interleukin-15 production in RA synovial membrane.

2. To provide direct evidence for nitric oxide production in RA synovial membrane, and to investigate the functional consequences of nitric oxide production in articular tissues.

Chapter 2

Materials and methods

2.1 Patients and tissue / fluid samples

Samples were obtained from patients attending the Centre for Rheumatic Diseases and University Department of Orthopaedic Surgery, Glasgow Royal Infirmary (GRI), the Rheumatology Department, Gartnavel General Hospital and the Department of Orthopaedics, Stobhill General Hospital, Glasgow. Synovial tissues were obtained at routine hip or knee arthroplasty, usually from pannus adjacent to cartilage and, in the knee, particularly around femoral condyles. Synovial biopsy material was also obtained during diagnostic arthroscopy (Dr. M. Field, Glasgow Royal Infirmary) and was used for immunohistochemical studies. Buffy coats were provided by the Blood Transfusion Service (Law Hospital, Carluke, UK) within 2 hours of donation by normal volunteer donors.

RA patients satisfied the American College of Rheumatology diagnostic criteria (Arnett, et al, 1988). The diagnosis of OA depended on characteristic clinical and radiological presentation (Altman, et al, 1986) with convincing absence of inflammatory arthritis (Erythrocyte sedimentation rate [ESR], C-reactive protein [CRP], serum urate - <460 μmol/l, no radiological erosions, rheumatoid [RF] and anti-nuclear factors [ANA] - negative). Clinical data were collected from the case record, including age, gender, disease duration, drug therapy and concurrent disease. ESR, CRP (nephelometry) and serum RF and ANA titres (ELISA) were obtained from laboratory records (Departments of Immunopathology, Haematology and Biochemistry, GRI, Glasgow, UK).

2.2 Ethical considerations

Permission from the Ethical Committee in GRI was applicable to the use of tissues from arthroscopy as part of a separate investigation (Role of gene vectors in therapeutic delivery of cytokines in inflammatory arthritis; Dr M. Field, GRI).

Synovial fluids and tissues were surplus to clinical requirements following routine therapeutic or diagnostic procedures and would otherwise have been discarded. Blood and synovial fluid samples were collected only when clinically indicated, and informed consent was obtained from patients prior to research use of samples so obtained. All animal experimentation was performed under project licences provided following UK Home Office review and were used only to address questions not amenable to *in vitro* study of human tissue. Experimentation was performed under Project Licence 60/1311, procedure 5, Project Licence 60/1475, procedure 2, and Project Licence 60/01347, procedure 6.

2.3 Reagents /buffers

The source of reagents either purchased or donated is given in the text and tables. Details of preparation of buffers and reagents, where appropriate, are contained in Appendix I.

2.4 Cell culture

All culture media and supplements were obtained from Gibco BRL, Paisley, Scotland, UK. Dulbecco's modified Eagle medium was supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 100 I.U./ml penicillin and 100 μg/ml streptomycin ('complete Dulbecco's MEM'). Supplements were stored in 5 ml aliquots (x100 final concentration) at -20°C and added to medium prior to culture. RPMI was supplemented with L-glutamine, penicillin and streptomycin as above ('complete RPMI'). Murine lymphocyte proliferation medium consisted of complete RPMI, 25 mM HEPES, 2.5 μg/ml amphoteracin B and 50 μM 2-mercaptoethanol (Sigma Chemical, Poole, UK). Iscove's modified Dulbecco's medium for whole blood culture was supplemented with penicillin, streptomycin and L-glutamine as above, and stored at -20°C in 100 ml aliquots until use. Foetal calf serum (FCS) was

mycoplasma screened by the manufacturer, heat inactivated at 56°C for 30 minutes in a circulating waterbath, then stored in 10 ml aliquots at -20°C. Culture was performed at 37°C in a humidified incubator with 5% CO_2. Cells were counted directly using a Neubauer haemocytometer (Weber Scientific International Ltd, UK) on a Nikon Labphot microscope or automatically by Coulter Z1 cell counter (Luton, UK). Viability was assessed microscopically by trypan blue exclusion (0.1% trypan blue [Sigma], 0.1% acetic acid (BDH), phosphate buffered saline[PBS]).

2.4.1 Synovial tissue culture preparation

Synovial tissue cultures were prepared as previously described (Brennan, et al, 1989). Resected tissue was harvested into cold complete Dulbecco's MEM and the synovial membrane was dissected free of fibrous connective and adipose tissue, before being cut into 2mm fragments. These were incubated in complete Dulbecco's MEM with 2.5 mg/ml collagenase (specific activity 193 u/mg, Worthington Biochemicals, NJ, USA) / 0.1 mg/ml DNAse (Boerhinger Mannheim, Germany) for 90 minutes at 37°C with gentle agitation. The digest was passed through a sterile tea strainer, washed three times and filtered through Nytex membrane (Cadisch & son, London, UK) to remove debris and cellular aggregates. Cells were washed throughout by centrifugation at 250g for 7 minutes (Mistral 3000i, MSE, UK) in 15 ml polypropylene centrifugation tubes (Costar Corporation, MA, USA). A single cell suspension was thus obtained which contained a heterogeneous synovial cell population. Cell yield was around $5x10^7$ cells / tissue, with range 10^6 to $5x10^8$ cells.

Primary cultures were established as follows. 250 µl of heterogeneous synovial cells adjusted to $2x10^6$ cells/ml in complete Dulbecco's MEM, 10% FCS, were plated into 48 well culture plates (Costar). After 1 hour, stimuli were added at different concentrations in a total volume of 250 µl, giving a final density of $1x10^6$ cells/ml in 500 µl with 5% FCS. Cultures were maintained from 24 to 120 hours. Stimuli

included staphylococcal enterotoxin B (SEB, stock 1 mg/ml, Sigma), lipopolysaccharide (LPS, stock 1 mg/ml, *Salmonella enteritidis*, Sigma), human recombinant interferon-γ (IFN-γ), human recombinant interleukin-1β (IL-1β) and human recombinant tumour necrosis factor α (TNFα). Recombinant cytokines, at various stock concentrations, were a gift from Dr G.R. Adolf, Bender Wien, Austria. Reagents were stored at -20°C as concentrated stock solutions before dilution in appropriate medium prior to culture. Nitric oxide synthase activity was inhibited in some cultures by addition of N^G-monomethyl-L-arginine hydrochloride (L-NMMA, a gift from Dr Hodson, Wellcome, Beckenham, UK). L-NMMA (mol. wt. = 225) was stored desiccated, at room temperature, and was dissolved immediately prior to use to 20 mM in appropriate medium, then filtered (Millex-GV 0.22 μm, Millipore, France). This solution was added to final culture with further 1:20 dilution to a final concentration of 1 mM *in vitro*. Culture supernatants were frozen at -70°C until assay for cytokine or nitrite production.

Cytospin preparations were made after tissue digestion to measure the proportional cellular yield. Synovial cells were suspended at $5x10^5$ cells/ml in serum free medium and spun at 500 rpm for 5 minutes onto silane coated glass slides (500 μl/slide) using a Cytospin 3 (Shandon, UK). Silane coated slides were prepared as described (section 2.6). Cytopreps were fixed in cold acetone for 15 minutes, air dried and stored at -20°C before immunohistochemical characterisation of CD3, CD19 and CD68 expression (section 2.6). Synovial cultures were typically 20-40% $CD3^+$, 30-50% $CD68^+$, <10% $CD19^+$, consistent with previously published observations (Brennan, et al, 1989).

2.4.2 Peripheral blood mononuclear cell preparation

Mononuclear cell populations were obtained by density gradient centrifugation. After collection into heparinised sterile universals (10 I.U./ml preservative free heparin, Leo

Laboratories Ltd, Bucks, UK), venous blood was diluted 1:2 in complete Dulbecco's MEM. 7 ml diluted blood was layered over 5 ml Lymphoprep (Nycomed Pharma, Oslo, Norway) in a 15ml conical tube and spun at 500g for 30 minutes at 22°C. The mononuclear fraction was collected and washed thrice in medium before adjustment to the required cell concentration. This population was designated peripheral blood mononuclear cells (PBMC). Buffy coats from the Blood Transfusion Service were diluted 1:3 in complete Dulbecco's MEM then prepared as for venous blood samples.

T lymphocyte enrichment was performed by negative selection using successive adherence steps to obtain a peripheral blood T lymphocyte fraction (PBTL). Mononuclear cell suspensions obtained above were incubated at $2x10^6$ cells/ml in 75 cm^2 tissue culture flasks (Costar) for 1 hour, then non-adherent cells were further incubated overnight in complete Dulbecco's MEM, with 10% FCS. Cells remaining non-adherent were collected and washed thrice in medium before suspension to the desired concentration. Cellular purity was assessed by FACS analysis (FACScan, Becton Dickinson) for CD3, CD14 and CD19 marker expression (section 2.5). PB monocytes were prepared by selecting adherent cells during the T cell enrichment process described above. Cells which adhered after both the 1 hour and 16 hour incubation steps were removed using a cell scraper and maintained in 10% FCS at $5x10^5$ cells/ml until required. Prior to use, they were first gently washed *in situ* to remove non-viable monocytes or residual non-adherent lymphocytes, then resuspended to the desired concentration. Cell purity was assessed by FACS analysis (section 2.5).

2.4.3 Synovial fluid mononuclear cell isolation

Synovial fluid obtained by joint aspiration from RA patients was collected with 10 I.U./ml heparin, then diluted 1:3 in complete Dulbecco's MEM. The mononuclear cell fraction was obtained using lymphoprep as before, and T cell enrichment was

performed as described above for peripheral blood. The non-adherent fraction was designated synovial fluid T lymphocytes (SFTL). The adherent fraction was retained, resuspended to $5x10^5$ cells/ml and plated in 96 well flat bottom plates (Nunclon) for cell-contact experiments (section 2.4.8). Cellular purity was assessed using cytopreps (section 2.6).

2.4.4 Proliferation and cytokine production by human cells

Proliferation assays were performed in triplicate in complete Dulbecco's MEM with 25 mM HEPES and 10% FCS. $2x10^5$ PBMC, PBTL or SFTL in 100 µl were incubated in U-bottom 96 well culture plates (Nunclon microwell, Nunc, Denmark) for 24, 48, 72 or 96 hours. Stimulatory reagents or medium alone were added in a further 100 µl at twice the desired final concentration, 30 minutes after seeding of cells to culture plates. 1 µCi of ^{3}H-thymidine (Amersham Life Science, UK) in 25 µl complete Dulbecco's MEM was added to each well during the final 6 hours of culture before harvesting onto a glass fiber filter (Packard, CT, USA) using a Micromate 196 Harvester (Packard). ^{3}H-thymidine incorporation was measured using a Matrix 96, Direct Beta Counter (Packard). Because background counts varied considerably between individuals (range from 100 cpm to 3000 cpm), proliferation was expressed as a stimulation index derived as follows:

SI = (mean counts per minute test culture) / (mean counts per minute medium alone)

Parallel triplicate cultures in identical conditions were performed in 96 well plates for analysis of cytokine production by PBMC, PBTL and SFTL following stimulation. Culture supernatants were frozen at -70°C at different time points indicated in figures and assayed for cytokine concentration by ELISA in single batches (section 2.9). Reagents included recombinant interleukin-15 (IL-15, donated by Dr D Cosman, Immunex Corporation, Seattle, USA), recombinant interleukin-2 (IL-2, R&D Systems, Oxon, UK) and phytohaemagglutinin (PHA, Murex Diagnostics Ltd, UK),

rabbit polyclonal anti-human IL-15 ('azide free', PeproTech, UK) and rabbit polyclonal anti-human IL-2 ('azide free', Genzyme Diagnostics, MA, USA).

2.4.5 Nitric oxide production by monocytes *in vitro*

Production of NO by human monocytes was investigated using adherent PBMC, prepared as above (section 2.4.2), or monocyte cell lines (U937, THP-1; European Collection of Animal Cell Cultures, Salisbury, UK). Cells were incubated in the presence or absence of stimuli in 48-well culture plates at various cell concentrations (10^6 to $4x10^6$/ml) in complete Dulbecco's MEM with 1 to 10% FCS for 24, 48, 72 or 96 hours. Supernatants were frozen at -20°C until assay for nitrite concentration using the Griess reaction (section 2.10.1). Stimuli used were SEB, LPS, IL-1β, IFN-γ and TNFα as described in section 2.4.1. In some experiments, monocytes were first matured by incubation in 10 nM phorbol myristate acetate (PMA, Sigma) for 6 - 48 hours in 25 cm^2 flasks, prior to addition of reagents.

In the absence of a recognised human monocyte / macrophage line capable of NO production, control experiments were performed using the murine macrophage cell line J774, which is known to produce nitrite after stimulation with 50 I.U./ml IFN-γ and 10 ng/ml LPS. J774 cells harvested as described (section 2.4.7) were suspended in 100 µl complete RPMI, 5% FCS, at $2x10^5$/ml in flat-bottom 96 well plates (Nunclon) and rested for 1 hour. Reagents were added in 100 µl, then cells were incubated for 24 hours before measurement of nitrite concentration in the supernatant by Griess reaction (section 2.10.1). Stimuli were SEB, LPS and murine recombinant IFN-γ (donated by Genentech Inc., CA, USA) at concentrations indicated in the text. 500 µM L-NMMA was used as an inhibitory control, to confirm the involvement of the L-arginine-dependent NOS pathway (Nathan & Xie, 1994a).

2.4.6 Murine spleen cell preparation

Murine spleens were dissected, weighed, cut into 2 mm fragments in a 10 cm petri dish (Sterilin, Middlesex, UK) and gently minced through a tea strainer, using a 10 ml syringe plunger. This cell suspension was washed three times in complete RPMI, passed through Nytex membrane to remove cellular aggregates and debris and resuspended to the desired concentration.

Proliferation assays for spleen (SC) were performed in quadruplicate in U-bottom 96 well culture plates (Nunclon) at $2x10^5$ cells/well in 100 µl proliferation RPMI with 10% FCS. Stimuli were added in 100 µl giving a final culture volume of 200 µl. 1 µCi of ^{3}H-thymidine was added during the final 6 hours of culture and plates were harvested as described in section 2.4.4. Cytokine production by SC was measured by incubating $4x10^6$ cells in 1ml of complete RPMI, 10% FCS, for various times in 48-well culture plates, in the presence or absence of stimuli. Supernatants were frozen at -70°C before cytokine assay in single batches (section 2.9). Stimuli included 0.1 to 10 µg/ml concanavalin A (Sigma) as positive control. Staphylococcal enterotoxin A (SEA), staphylococcal enterotoxin B and Toxic Shock Syndrome Toxin (TSST) -1 (all Sigma) and heat-killed *staphylococcus aureus* LS-1 (gift from Dr C. Gemmell, Dept. of Bacteriology, University of Glasgow) were used during *in vitro* studies of staphylococcal infection in iNOS deficient mice at concentrations indicated. Superantigens were resuspended to 1 mg/ml in RPMI. 10% FCS, and filter sterilised before dilution and use in culture.

2.4.7 Cell line maintenance

Human and murine monocyte (THP-1, U937 & J774) cell lines (ECACC) were maintained in complete RPMI with 10% FCS between $2x10^5$ and $5x10^5$ cells/ml with sub-culture every 2-3 days. CTLL cells (donated by Prof A. Bradley, Dept. Surgery,

University of Glasgow) were cultured in complete RPMI, 10% FCS supplemented with 10 I.U./ml murine recombinant IL-2 (Genzyme Diagnostics, MA, USA) between 10^5 and $5x10^5$ cells/ml, with sub-culture every 2-3 days. For functional assays, cells were washed twice before suspension to the desired concentration and addition of indicated stimuli. J774 cells are normally adherent and were harvested by cell scraper, then rested for at least 1 hour, after harvesting and washing, prior to addition of stimuli. Cell lines were regularly tested for *mycoplasma* (Mycoplasma PCR ELISA kit, Boerhinger Mannheim, Germany). Cells were stored in 90% FCS; 10% dimethyl sulfoxide (DMSO) in liquid nitrogen.

2.4.8 Cell contact protocols

The ability of T lymphocytes to regulate the pro-inflammatory activity of macrophages by direct cell-membrane contact may be investigated by fixing T cells after activation, but prior to co-incubation with macrophages (Vey, et al, 1992; Lacraz, et al, 1994). PBTL were cultured in 25 cm^2 flasks (Costar) for 72 hours in 5% FCS, in the presence of medium alone, rIL-15 (100 ng/ml), rIL-2 (100 ng/ml), rIL-8 (100 ng/ml; R&D Systems), rMIP-1α (100 ng/ml; donated by Dr G.J. Graham, CRC Beatson Institute, Glasgow, UK) or, as positive control, a combination of 1 μg/ml PHA, and 1 nM phorbol myristate acetate (PMA; Sigma). Cells were washed thrice then fixed for 2 hours in filter sterilised (Millex-GV, Millipore, France) 1% paraformaldehyde in phosphate buffered saline (PBS; Sigma) at 4°C with gentle agitation. SFTL were prepared in identical conditions but were cultured with or without stimulus for only 24 hours before fixation. Fixed cells were washed thrice, resuspended to $4x10^6$ cells/ml in RPMI, then immediately added in equal volume to $5x10^5$ cells/ml of either unprimed U937 cells, PB monocytes (section 2.4.2) or adherent synovial fluid derived macrophage / synoviocyte co-cultures (section 2.4.3) in 10% FCS in flat-bottomed 96 well plates (Nunclon). After a further 48 hours, supernatants were harvested and stored at -70°C prior to TNFα estimation by ELISA (section 2.9.2).

The *in vivo* capacity for cell-contact mediated macrophage activation by synovial lymphocytes was further investigated by direct fixation of freshly isolated synovial mononuclear cells (SFMC). Synovial fluid was prepared through lymphoprep as before, then SFMC were cultured for 30 mins in 10% FCS in 25 cm^2 flasks (Costar), after which the non-adherent cells were fixed in 1% PFA. $4x10^6$ fixed cells /ml were cultured with U937 cells as described above. Supernatants were collected after 48 hours for TNFα estimation.

Double chamber culture was performed in parallel experiments. PBTL were stimulated for 72 hours as described, then added without fixation at $4x10^6$ cells/ml to U937 cells at $5x10^5$ cells/ml. Identical cultures were established in which PBTL were separated from U937 cells by a culture-well insert (Falcon, Becton Dickinson) with a porous membrane, which prevented cell-contact but allowed soluble factor mediated communication. Supernatants were harvested after a further 48 hours and TNFα concentrations estimated by ELISA.

Some experiments were modified to investigate the contribution of surface markers to T cell / macrophage communication following IL-15 mediated activation of T cells (Isler, et al, 1993). Antibodies against CD69 (Becton Dickinson), LFA-1 and ICAM-1 (both donated by Dr N. Hogg, Imperial Cancer Research, London, UK) were diluted to 50 μg/ml in PBS. Fixed T cells were pelleted by centrifugation at 250g for 10 minutes, then suspended in 180 μl medium, to which was added 20 μl of neutralising antibody (final concentration 5 μg/ml), or control murine monoclonal IgG1 (DAKO, Denmark) at equivalent concentration. Cells were incubated at 4°C for 30 minutes, washed twice in cold Dulbecco's MEM and adjusted to $4x10^6$ /ml. These cells were added to monocyte preparations as before. These cultures were supplemented with 5 μg/ml human IgG (Sigma) to reduce non-specific Fc binding by cell bound antibody. In some experiments, increased concentrations of neutralising antibodies were employed up to a final concentration of 50 μg/ml.

2.4.9 Whole Blood Culture

Phenotypic maturation of peripheral blood lymphocytes to recombinant IL-15 or mitogens was investigated using FACS analysis by modification of a whole blood culture method (Bocchieri, et al, 1995). Blood was withdrawn, heparinised (10 I.U./ml) and immediately diluted 1:5 in complete Iscove's medium. After addition of rIL-15 (100 ng/ml), rIL-2 (100 ng/ml) or PHA (1μg/ml) to the diluted blood, triplicate cultures (500 μl each) in 48-well culture plates were established and incubated for 3, 6, 24, 48 and 72 hours. Unstimulated, diluted blood was withheld to serve as 'time 0' negative control and analysed within 30 minutes of venesection. FACS analysis of these cultures was performed as described below.

2.5 FACS analysis

2.5.1 Analysis of lymphocyte phenotype after whole blood culture

Double immunofluorescence staining of peripheral blood lymphocytes following stimulation with cytokines or mitogens was performed by FACS analysis. 200 μl of blood / Iscove's culture prepared and stimulated as described above were placed in a 12 x 75 mm polystyrene tube (Falcon 2052, Becton Dickinson, UK). Primary antibodies employed were as follows: CD3 (FITC and PE), CD56 (PE), CD16 (PE), CD69 (FITC and PE), CD45RA (FITC), CD45RO (PE), CD19 (FITC) (all Becton Dickinson). 10 μl of primary antibody were added to cells at 4°C for 30 minutes. Negative control primary antibodies (IgG1 FITC, IgG1 PE; DAKO) of appropriate isotype and conjugate were added to parallel tubes and were similarly processed. 2 ml FACS^R Brand Lysing Solution was added (1:10 dilution of commercial stock contained 5% diethylene glycol, 1.5% formaldehyde; Becton Dickinson), vortexed for 5 seconds, then cells were incubated for 10 minutes at room temperature in the dark to facilitate erythrocyte lysis. Leukocytes were recovered by spinning at 300g for 5

minutes, then washed with 1 ml FACSFlow (Becton Dickinson) at 200g for 5 minutes. Cells were resuspended in 200 µl FACSFlow and analysed on a FACScan (Becton Dickinson). Gates were set for lymphocytes using forward and side light scatter parameters. The percentage of FITC or PE positive cells, or of double labelled cells, was generated for lymphocytes within this region using Lysis II software (Becton Dickinson).

2.5.2 Human cell subset analysis

Cell preparations from peripheral blood, synovial fluid and tissues were analysed using FACS to determine cellular purity. Single cell suspensions were adjusted to 10^6/ml in serum free medium, then 100 µl were incubated with 5 µl of FITC or PE labelled primary antibody in separate tubes (CD3 PE, CD14 FITC, CD19 FITC; Becton Dickinson) for 15-30 minutes at 4°C in the dark. Cells were washed in 1 ml FACSFLow at 200g for 5 minutes, then analysed as described before. Both lymphocyte and monocyte regions, determined by forward and side light scatter parameters, were examined to identify cell subsets present.

2.5.3 Murine spleen cell analysis

Murine spleen cell suspensions prepared as described (2.4.6) were cultured for 24 hours with or without SEA (100 ng/ml) or TSST-1 (100 ng/ml). Cells were harvested by pipette (P1000 Gilson, UK), washed once in serum free RPMI, then 100 µl of 10^6 cells/ml were incubated with 5 µl of antibody for 20 minutes at RT in the dark. Anti-murine CD4 (PE), anti-murine CD8 (FITC) and IgG1 negative control antibodies were obtained from PharMingen (USA). After incubation, 1 ml FACS^R Brand Lysing solution was added for 10 minutes, spun at 300g for 5 minutes, than washed once in 1 ml FACSFlow at 200g for 5 minutes. Cells were analysed as before in the lymphocyte region gated by forward and side light scatter parameters.

2.6 Immunohistochemistry

Tissue from RA or OA patients undergoing arthroplasty or arthroscopy was collected in theatre and processed immediately. Approximately 5 mm fragments of synovial membrane were dissected, placed in mounting medium (Cryo-M-Bed, Bright Instrument Company Ltd, Cambs, UK) on cork disks and snap frozen in liquid nitrogen. Samples were stored at -70°C until required.

Glass microscope slides were soaked overnight in 2% Decon 90 (Decon Labs Ltd, UK), then rinsed in tap water for 3 hours before air drying. Slides were dipped in 2% silane (3-aminopropyltriethoxysilane, Sigma) in acetone for 4 minutes, rinsed in running tap water for 6 minutes, then air dried. 4-6 μm frozen sections were cut onto silane coated slides at -20°C using either a Bright 5030 Microtome, or a Leica CM1800 cryostat and immediately fixed by immersion in acetone (BDH Lab Supplies, Poole, UK) at 4°C for 15 minutes. Sections were air dried for 10 minutes and stored at -20°C in an air / moisture tight container before use.

Primary, secondary and negative control antibodies which were used, their source, species of origin, isotype and final working dilution are detailed in tables 2.1 to 2.3.

2.6.1 Alkaline phosphatase staining

Frozen sections were rehydrated in Tris buffered saline, pH 7.36 (TBS), for 10 minutes and non-specific, Fc-mediated immunoglobulin binding was blocked with 25% normal goat serum (NGS) / normal pooled human serum (PHS) (both supplied by SAPU, Scotland, UK) in TBS, for 30 minutes at room temperature. All incubation steps were performed in a humidified slide container (Shandon). For all washes, slides were placed in a slide rack in 250 ml TBS at room temperature with gentle agitation for 5 minutes. Primary antibody (Table 2.1), diluted as required in

10% NGS / PHS was then incubated in 100µl volume, overnight at 4°C. Sections were washed twice for 5 minutes before incubation with secondary biotinylated goat anti-immunoglobulin antibody of appropriate species specificity (Table 2.2), diluted in 10% NGS / PHS, for 1 hour at room temperature. After two further washes, alkaline phosphatase conjugated streptavidin, diluted 1:100 (5 µg/ml, DAKO), was added for 1 hour at room temperature. Sections were washed once and bound antibody detected by incubation under light microscopic observation for up to 10 minutes with either Fast Red solution (Vector Laboratories, UK) per the manufacturers instructions, or with fast red salts prepared as follows. Fast red salt (Sigma) was dissolved to 1 mg/ml in TBS, pH 8.2 containing 1 mg/ml naphthol-AS-MX phosphate (Sigma) and 5% N.N.-dimethyl formamide (Sigma). Levamisole (Sigma) was added to 0.001 M final concentration to inhibit endogenous tissue alkaline phosphatase activity, and the solution was filtered prior to use (Millipore). Finally, sections were dipped in Harris's haematoxylin (BDH) for 8-12 seconds to provide a nuclear counterstain and mounted using pre-warmed aqueous mounting medium (Glycergel™, DAKO).

2.6.2 Con-focal microscopy

To identify the cell subsets expressing inducible nitric oxide synthase (iNOS) within the synovial membrane, double immunofluorescence staining was performed with visualisation by con-focal microscopy. 4 µm synovial sections were cut, fixed and blocked as before. Primary rabbit anti-human iNOS antibody (NO53, Merck, Table 2.1) was incubated on sections overnight at 4°C, then with either murine monoclonal anti-CD3 or anti-CD68 antibody for 1 hour at room temperature. Sections were washed twice, incubated for 1 hour with biotinylated goat anti-rabbit immunoglobulin antibody (DAKO), washed again in TBS, and finally incubated for 1 hour with PE-conjugated streptavidin (DAKO, 5 µg/ml) in combination with FITC-conjugated Fab_2 goat anti-mouse immunoglobulin (DAKO). Sections were washed for 15 minutes in TBS and mounted in Permafluor (Immunotech SA, France), before storage at -20°C

until visualisation by con-focal microscopy. Histological fields of interest were selected by direct immunofluorescence (Nikon Optiphot-2), then con-focal images were acquired (BIORAD MRC1024, Herts, UK).

2.6.3 Non specific esterase (NSE) double staining with anti human iNOS

NSE may be visualised by incubation with α-naphthyl acetate or butyrate substrate and localises predominantly to macrophages with a characteristic cytoplasmic pattern, allowing distinction from lymphocyte expression (Meuller, et al, 1975). 4 μm sections were stained with rabbit polyclonal anti-human iNOS antiserum (Merck) as described above, but developed with fast blue (Vector, UK), without counterstain, to provide subsequent contrast with NSE staining (red-brown). Acid α-naphthyl acetate esterase stain (ANAE) was prepared as follows. Hexazotized pararosaniline was freshly prepared by mixing equal volumes of 4% sodium nitrite (BDH) with 40 mg/ml pararosaniline chloride (Sigma). The reaction mixture consisted of 10 mls 0.067 M phosphate buffer, pH 5.0 (9.85 ml 0.067 M KH_2PO_4, pH 5.0 added to 0.15 ml 0.067 M $Na_2HPO_4,2H_2O$ [appendix I]), to which was added 600 μl (6%) hexazotized pararosaniline and 100 μl (1%) 25 mg/ml α-naphthyl acetate (Sigma) dissolved in acetone (BDH) (10 mg dissolved in 400 μl). Before use this was adjusted to pH 5.8 by addition of a few drops of 2 M sodium hydroxide (BDH). Initial experiments identified the formation of non-specific, dark brown precipitates on synovial tissues. This was overcome by optimising the incubation time to 20 minutes and by centrifuging the reaction solution at 10,000g for 1 minute prior to addition to tissue. Sections were incubated with 200 μl of reaction solution for 20 minutes at 4°C in the dark, then washed extensively in running tap water before mounting in glycergel without nuclear counterstain.

2.6.4 Murine Articular and Footpad Histology

Hind limbs were removed above the knee and immediately fixed in 10% neutral buffered formalin for at least 72 hours. Paws were decalcified in 5% HNO_3 for a further 72 hours, then 6 μm sections were cut using a bone cryostat (by Mr P. Kerr, Pathology Department, University of Glasgow). Sections were stained with haematoxylin and eosin using the standard method of the Department of Pathology, University of Glasgow. Immunohistochemical characterisation of the inflammatory infiltrate following rIL-15-induced chemotaxis was performed using peroxidase detection to avoid the strong endogenous alkaline phosphatase activity observed in skin, glands, bone and cartilage in murine paw specimens. Sections prepared as above were rehydrated through xylene (BDH) and progressively dilute alcohols (100%, 90% x2, 70% x2, TBS) then treated with 0.3% hydrogen peroxide (DAKO) for 10 minutes to block endogenous peroxidase in tissues. Thereafter, tissues were stained as described in section 2.6.1, using rabbit polyclonal anti-CD3 antibody (DAKO) and horseradish peroxidase-conjugated streptavidin (DAKO), diluted 1/100, to detect bound antibody. Staining was visualised by developing for 5 minutes in 0.5 mg/ml diaminobenzidine (Sigma) containing 0.03% hydrogen peroxide, followed by haematoxylin nuclear counterstain.

2.6.5 Control Experiments

Parallel sections were stained with negative control antibodies to confirm specificity of primary antibody binding. Murine monoclonal primary antibodies were controlled with isotype matched monoclonal antibodies of irrelevant specificity (DAKO) used at equivalent concentrations (Table 2.3). Similarly, equivalent concentrations of purified rabbit immunoglobulin (DAKO) or 20% higher concentrations of non-immune rabbit serum (SAPU) served as controls for primary rabbit polyclonal antibodies and antisera respectively (Table 2.3).

Tissues were pre-blocked with normal goat serum. Goat secondary antibodies, which had been previously solid-phase absorbed against human immunoglobulins, were used to reduce background staining. The secondary antibodies utilised did not bind tissues in the absence of primary antibodies at the concentrations indicated in the above protocol. In double immunofluorescence staining for confocal studies, parallel sections were stained with several combinations of primary and secondary antibody to exclude the possibility of cross reaction. Thus, primary murine monoclonals were not detected by secondary goat anti-rabbit immunoglobulin antibodies, even in the presence of normal rabbit serum, and primary rabbit polyclonal antisera were not detected by secondary goat anti-mouse immunoglobulin antibodies. Streptavidin conjugates, either alone, or in the presence of secondary antibodies, did not bind tissues directly.

Neutralisation of primary antibody staining for IL-15 and iNOS was carried out.

(i) Human rIL-15 served as positive control for M112, which was originally raised against simian IL-15 (Grabstein, et al, 1994), and human rIL-2 (R&D Systems) was used as a likely candidate for cross-reactivity, given the similar quaternary structure and shared receptor specificity with IL-15 (reviewed by Tagaya, et al, 1996a). A dose range (0.5 - 100 μg/ml) of each cytokine was used.

(ii) Various peptides were used to test the specificity of iNOS detection. The immunising human iNOS peptide YRASLEMSAL (50 μg/ml) and iNOS peptide from a similar area of the C-terminus of rat iNOS YEEPKATRL (50 μg/ml) were gifts from Dr. R.A. Mumford, Merck Research Laboratories, USA. Recombinant human eNOS (50 μg/ml) was donated by Dr. I. Charles, Wellcome, UK. Primary antibodies were incubated with or without peptide / recombinant protein, either overnight at 4°C, or for 2 hours at 37°C, in 10% NGS / PHS in TBS, pH 7.36, before addition to sections for subsequent staining as described (section 2.6.1).

2.6.6 Quantification of histology

Quantitative light microscopy was performed on a Laborlux S microscope (Leitz, Germany) with an additional 'teaching' binocular eye piece, which allowed two observers (Dr M. Field and the author) to count cells simultaneously in high power fields (x250 magnification). Synovial tissues were divided into three areas (Duke, et al, 1982; Cush & Lipsky, 1988):

- lining layer
- aggregate area
- interstitial layer

In alkaline phosphatase stained RA and OA synovial sections, representative high power fields were selected and positive cells were counted in each of the above areas as a percentage of the total number of nuclei found in that field. If possible, 200 nuclei were counted from each field and at least three fields were counted per section, with more selected in relatively acellular tissues. Observers were blind to whether positive or negative antibody was used and to the clinical diagnosis. Positive cells were those in which discrete staining could be localised to a single nucleus. Aggregate areas were not found in OA synovial tissues and direct comparison with RA in that respect was not possible. Otherwise, OA tissues were used as a disease control for RA tissues. Appropriate 'normal' synovial tissue was unfortunately unavailable and was not, therefore, included in this study. In double stained sections (NSE plus iNOS), the number of single stained cells / field was determined for each stain, then double stained cells were counted. The percentage of double stained cells for each specific cell subset was calculated as follows:

$$\frac{\text{number of double stained cells}}{\text{number of double + single stained cells for each marker}} \times 100$$

Murine footpad histology after rIL-15 injection (section 2.7.2) was quantified by determining the number of infiltrating inflammatory cells in a high power field, and

expressing this observation as a percentage of the total number of cells in the same field. CD3 positive, peroxidase stained cells in similar sections were scored as a percentage of the total number of cells in the same high power fields (x400).

2.6.7 Photo-micrography

Photo-micrographs were obtained using an Orthoplan photo-microscope (Leitz, Germany) in the Medical Illustration Department, GRI. Colour prints were made on EKTAR 25 film (Kodak, UK), and in some experiments, 35mm colour slides were made with Ektachrome 64T film (Kodak) and colour prints were subsequently developed commercially.

2.7 Animal Models of Inflammation

2.7.1 Staphylococcus aureus arthritis in mice.

MF1 Swiss mice are susceptible to induction of staphylococcal arthritis (Bremmell, et al, 1992). The iNOS gene targeted mice recently developed by Wei et al (1995) on a 129xMF1 background, allowed detailed analysis of the effect of NO deficiency in this model in the context of normal cNOS activity. Bremmell strain (LS-1) *Staphylococcus aureus* were obtained from Dr C. Gemmell (Dept. of Bacteriology, University of Glasgow) and 10^7 CFU to 10^8 CFU/mouse were injected intravenously via the tail vein in 50 µl PBS. Male and female mice aged 3-4 weeks were used (provided by Dr X.Q. Wei, Dept. of Immunology, University of Glasgow) caged in groups numbering four to six animals, fed standard chow and water *ad libitum.* Wild type 129xMF1 ($iNOS^{+/+}$) and iNOS mutant heterozygote 129xMF1 ($iNOS^{+/-}$) served as control groups for comparison with the iNOS mutant homozygote mice ($iNOS^{-/-}$). Mice were observed (blind to treatment / innoculation) for up to 14 days for development of arthritis and for clinical signs of septicaemia.

(i) Arthritis

Arthritis progression was monitored daily using the following criteria:

incidence of arthritis

weight change

number of limbs involved / mouse

footpad / inter alveolar diameter - calliper measurement (Kroeplin, Germany)

articular index - 3 points / limb, total 12 / mouse derived as follows by a treatment blinded observer:

erythema alone (1)

swelling and erythema (2)

erythema, swelling, and extension / loss of function (3)

Mice were observed for up to 14 days before sacrifice, required at that time point by Home Office guidelines.

(ii) Septicaemia

Septicaemia was assessed by the presence of the following:

stary coat (1)

hunched posture (1)

loss of spontaneous movement (1)

mucocutaneous abscess (1)

Using these signs, a summative 'septic index' (maximum 4 /mouse) was derived for each animal. This index correlated with loss of, or failure to gain, weight from base line and with mortality (Chapter 6). Further assessment by articular histology (section 2.6.4) and measurement of staphylococcal specific responses in spleen cell cultures (section 2.4.6) was performed in some experiments as described before.

Staphylococcal viability was estimated by organ culture as follows. Groups of 3 mice were sacrificed 3, 7 and 11 days after i.v. staphylococcal injection. Spleens and kidneys were removed aseptically, homogenised, then submitted to serial ten-fold

dilutions in sterile PBS. 200 µl blood was similarly diluted in PBS. Each dilution was plated out on 5% blood agar plates (Dept. of Bacteriology, GRI) and cultured overnight at 37°C. Plates in which at least 100 distinct colonies were observed were counted and the original number of CFU present in blood or tissue was derived from the dilution factor. CFU were expressed per ml of blood, or per tissue.

(iii) Staphylococcal killing assays

Staphylococcus aureus LS-1 (10^7 CFU/ml) diluted in gel-Hanks (working solution prepared as follows; 80 mls dH_2O, 10 mls 10x Hanks, 10 mls 1% gelatin [Sigma]) were opsonised by mixing with an equal volume of murine plasma prepared from $iNOS^{-/-}$ controls for 15 minutes at 37°C. Bacteria were resuspended in gel-Hanks to 10^7 CFU/ml after cenrifugation at 3000 rpm for 15 minutes. Pooled blood was obtained from 4 $iNOS^{-/-}$ mice and from 4 $iNOS^{+/-}$ controls, heparinised (10 I.U./ml), then 100 µl was added to 100 µl of opsonised bacteria. The mixture was incubated for 0, 30, 60 or 90 minutes at 37°C at which point, cells were lysed by addition of 3 mls of sterile ice cold water. The resulting solution was diluted ten-fold and plated in pairs onto 5% blood agar plates for 18 hours at 37°C. The percent killing at time *x* was calculated as follows:

$$\frac{\text{mean CFU in time } x \text{ culture}}{\text{mean CFU in time 0 culture}} \times 100$$

2.7.2 Footpad Lymphocyte Invasion Model.

The capacity of IL-15 to recruit T lymphocytes *in vivo* was investigated in a murine footpad invasion model. Male DBA/1 mice, aged 8-10 weeks, were injected intraperitoneally with 500 mg of *Corynebacterium parvum* (donated by Prof. P.C. Wilkinson, Dept. of Immunology, University of Glasgow) in 100 µl PBS and seven days later, received 500 ng rIL-15 in PBS, or PBS alone as a control, by 50 µl injection to the hind footpad. Control mice which had been given no *C.Parvum* received IL-15 or PBS injection as a further control. *C. parvum* was used because

earlier studies (Wilkinson & Liew, 1995) showed that primed T cells responded more strongly to IL-2 and IL-15 *in vitro*, in chemotactic assays. Paws were collected 16, 48 and 72 hours after injection, fixed in 10% neutral buffered formalin, decalcified and examined histologically as described in section 2.6.4. Draining lymph nodes were collected, cleaned and weighed, and then examined by light microscopy after H&E staining.

To exclude the possibility that any observed inflammatory infiltrate was due to contaminating lipopolysaccharide, rIL-15 was tested for the presence of endotoxin by limulus amebocyte lysate assay (E-toxate, Sigma, UK) as per the manufacturers instructions. 500 ng IL-15 was found to contain less than 0.0015 I.U. endotoxin in this assay.

2.8 RT-PCR for mRNA expression in synovial tissue.

Fragments of synovial tissue were snap frozen immediately after surgical excision and stored at -70°C. Synovial fluid cells were pelleted by centrifugation at 450g for 10 minutes, immediately resuspended in 400 µl RNAzol™ (Biogenesis, Bournemouth, UK) and were also snap frozen and stored at -70°C. Reverse transcription-polymerase chain reaction (RT-PCR) was performed as previously described (Kawasaki, 1990) and modified by Dr D. Xu and Mr B.P. Leung (Dept. of Immunology, University of Glasgow), whose technical assistance is gratefully acknowledged.

2.8.1 RNA extraction

Total mRNA was extracted using RNAzol™ (Biogenesis) as described (Chomczynski & Sacchi, 1987). Frozen tissue fragments, or synovial cell pellets, were disrupted in 800 µl RNAzol using a P1000 Gilson Pipette. To this was added 80 µl chloroform

(Sigma) followed by vortexing for 15 seconds and rest on ice for 5 minutes, before centrifugation at 12,000g for 15 minutes at 4°C. The aqueous phase was added to an equal volume of isopropanol (Sigma), rested at 4°C for 15 minutes, then spun at 12,000g for 20 minutes at 4°C. The pellet was resuspended in 800 µl ice-cold 75% ethanol, spun at 12,000g for 8 minutes, dried under vacuum for 10 minutes, then resuspended in 20 µl distilled water. RNA concentration was determined by its optical density at 260nm and 280nm (Sambrook, et al, 1989) per the following formula:

- $[(62 \times od_{260}) - (36 \times od_{280})] \times$ dilution of sample = "RNA" µg/ml

2.8.2 cDNA preparation

cDNA was prepared using the following mixture: 0.5 µl 40 I.U./µl RNasinR RNase inhibitor (Promega), 2 µl containing 0.5 µg random primer (Promega), 4 µl 5x RT buffer (375 mM KCl, 15 mM $MgCl_2$, 250 mM Tris-HCl, pH 8.3), 2 µl containing 0.25 µM of each dNTP (Promega), 2 µl 10 mM DTT (Promega) and 1 µl containing 200 units Moloney murine leukaemia virus reverse transcriptase (Gibco BRL). 2 µg RNA in 8.5 µl was heated to 90°C for 5 minutes, cooled on ice then added to the above mixture giving a final volume of 20 µl. After sitting at room temperature for 10 minutes, the reaction was performed at 37°C for 1 hour.

2.8.3 Polymerase Chain Reaction (PCR)

PCR was performed as follows. 2 µl of the above reverse transcription reaction mixture, or around 0.1 µg cDNA, was mixed with 10 µl 10x reaction buffer (500 mM KCl, 15 mM $MgCl_2$, 0.01% gelatine, 100 mM Tris-HCl pH 8.3), 2 µl containing 0.2-0.3 µM of each primer, 4 µl containing 40 µM of each dNTP (Promega), and was then made up to 99.5 µl with water. This mixture was heated to 95°C for 5 minutes then cooled on ice before addition of 0.5 µl Taq DNA polymerase (Promega) and was

overlaid with 50 µl mineral oil (Sigma). A Techne PHC-3 Dri-Block Cycler was programmed to give the desired cycle conditions detailed below with respective primers used. After the PCR, 20 µl of the reaction product was visualised by electrophoresis on 1% (w/v) agarose gel containing 0.5 µg/ml ethidium bromide (Sigma) with a DNA 1 kb ladder.

Oligonucleotide primers specific for human iNOS were a gift from Dr I. Charles (Wellcome, Beckenham, UK) - 5'-GCCTCGCTCTGGAAAGA-3' and 5'-TCCATGCAGCAACCTT-3' and were used to amplify a 500 bp fragment. Human chondrocyte iNOS cDNA (Dr I. Charles) and β-actin oligonucleotide primers - 5'-CCACACTGTGCCCATCTACGAGGGGT-3' and 5'-AGGGCAGTGATCTCCTTC TGCATCCT-3' (Genosys, Cambridge, UK) were used as internal controls. Reaction conditions for iNOS and β-actin PCR amplification were 95°C (35 secs), 55°C (60 secs), 72°C (120 secs) for 35 cycles. The double nucleotide sequence of the PCR product was derived by Dr X.Q. Wei (Dept. of Immunology, University of Glasgow) and found to be identical to that predicted from human chondrocyte iNOS (Charles, et al 1993; Genbank database No X73029).

2.9 Cytokine assays

2.9.1 IL-15 ELISA

IL-15 in synovial fluid and culture supernatants was detected using a sandwich enzyme linked immunosorbent assay (ELISA). Immulon 4 micro-ELISA plates (Dynatech, Virginia, USA) were coated with 50 µl monoclonal anti-IL-15 antibody (M112, 5 µg/ml) in bicarbonate coating buffer (0.1M $NaHCO_3$), pH 8.2 incubated at 4°C overnight. Between each of the following steps the plates were washed at least four times with PBS / 0.05% Tween 20. Non-specific binding was blocked with 200 µl PBS containing 1% bovine serum albumin (BSA, Sigma) and 2% heat inactivated goat serum (SAPU) for 2 hours at room temperature (blocking buffer). Subsequently, 100 µl test samples or standard recombinant human IL-15 (three-fold dilutions from 100 ng/ml to 50 pg/ml) were diluted in blocking buffer and incubated either at room temperature for 4 hours, or at 4°C overnight. Bound IL-15 was detected using 100 µl rabbit anti-human IL-15 antibody (2 µg/ml, PeproTech) for 1 hour at room temperature, followed by 100 µl alkaline phosphatase-conjugated goat anti-rabbit (1:5000, Sigma) for 1 hour, developed with p-nitrophenyl phosphate (1 mg/ml) in lM Tris, 3 mM $MgCl_2$ buffer and the optical density read at 630 nm (reference filter 405 nm) using an MR5000 ELISA reader (Dynatech, UK). The lower limit of detection of the assay was 1 ng/ml (Figure 3.1).

2.9.2 General ELISA protocol

Human TNFα and murine TNFα, IFN-γ, IL-4 and IL-6 were detected in culture supernatants or serum by ELISA, with paired capture and biotinylated detection monoclonal antibodies for each cytokine (PharMingen). Immunolon 4 micro-ELISA plates were coated overnight at 4°C with capture monoclonal antibody at 2 µg/ml in bicarbonate coating buffer. Wash steps were performed as described above. Plates

were blocked with PBS / 10% FCS (Gibco BRL) for 2 hours, incubated with test samples, or twofold standard dilutions of recombinant cytokine (10 ng/ml - 10 pg/ml) in triplicate for 2 hours, and bound cytokine was detected for 1 hour with 1 μg/ml biotinylated detection monoclonal antibody diluted in blocking buffer. Peroxidase-conjugated extravidin was added for 45 minutes (1:1000; Sigma), developed with 100 μl TMB Microwell Peroxidase Substrate (Kirkegaard & Perry Laboratories, MA, USA) and the plate was read at 630nm as above. The lower limit of detection in each assay was as follows:-

hTNFα	10 pg/ml
mTNFα	10 pg/ml
mIFN-γ	30 pg/ml
mIL-4	40 pg/ml
mIL-6	20 pg/ml

2.10 NO measurement

Nitric oxide (NO) production may be estimated by measuring the concentration of its oxidative products, nitrite or nitrate anion (Archer, 1993).

2.10.1 Griess reaction

This is a colorimetric assay for nitrite concentration (Green, et al, 1982). Reagents were prepared as follows: solution A - 0.1% α-naphthyl-amine (Sigma) in distilled water and solution B - 1% sulfanilamide (Sigma) in 5% phosphoric acid (Sigma). Both were stable stored in the dark at 4°C for 2 months. The Griess reagent was obtained by mixing equal volumes of solution A and B immediately prior to use. A sodium nitrite stock solution (10 μg/ml, Sigma) was diluted twofold to provide a standard curve (72.5 nmol/ml - 1 nmol/ml). The assay was performed in triplicate, by placing 50 μl of either test culture supernatant, or standard dilutions of nitrite in

identical medium, into a flat bottom 96 well plate, after which 50 µl Griess reagent was added to each well. The plate was incubated for 10 minutes at room temperature in the dark, and thereafter, the colorimetric reaction was measured at 570nm (reference filter at 630 nm) in an MR5000 ELISA reader (Dynatech) and the test nitrite concentration estimated from the standard curve. The lower limit of sensitivity was 2 nmol/ml.

2.10.2 Chemiluminescence assay for nitrite / nitrate

The presence of high protein concentrations in serum and synovial fluid leads to precipitation in the acid pH environment of the Griess reaction. This may interfere with colorimetric assessment, therefore, a chemiluminescent assay for nitrite was also used (Aoki, 1990; Palmer, et al, 1987). A reflux reaction was created by continuously boiling 75ml glacial acetic acid (BDH) with 25ml 6% sodium iodide (BDH) in a 250ml Pyrex reaction flask, through which was passed a low flow of nitrogen gas. 50 or 100 µl of either test sample, or standard nitrite solution prepared as above, was injected directly into the reaction flask using a Hamilton syringe (Sigma). NO_2^- in the sample is immediately reduced to $NO^\bullet$, which is carried in gaseous phase through a condenser and a cold trap, created with a glass U-tube surrounded by "dry ice" to remove acetic acid vapour, and on to a chemiluminescence NO analyser (Dasibi Environmental Corporation, Japan). NO reacts with ozone causing photoemission which may be detected and converted to a digital readout. The photomultiplier signal is proportional to the nitrite concentration in the original sample, allowing the generation of a standard curve and estimation of nitrite concentration in test samples. Sensitivity for this assay was 2 nmol/ml nitrite.

Nitrate concentration in biological fluids may be estimated by first reducing nitrate to nitrite using the enzyme nitrate reductase (Sigma). To measure 50 samples, reaction buffer was prepared as follows: 500 µl 5 mg/ml NADPH (Sigma), 500 µl 4.15 mg/ml

FAD (Sigma), 500 µl KH_2PO_4, pH 7.5, 950 µl distilled water and finally, 50 µl 34 mg/ml nitrate reductase, immediately after which 30 µl reaction buffer was added to 30 µl sample in a flat bottom 96 well plate. The reaction was incubated at 37°C for 2 hours, after which nitrite produced was measured by chemiluminescence as described above. Standard curves for sodium nitrite and sodium nitrate were included in the reductase reaction to provide a control for the efficiency of the reduction. This was calculated for the conversion of 100 µM sodium nitrate to sodium nitrite as shown below and was routinely >75%.

$$\% \text{ reduction nitrate} = (\text{ppb nitrite} / \text{ppb nitrate}) \times 100$$

2.11 Statistical Analysis

Data were collated and statistical analyses performed using Minitab software for Macintosh. Means were compared using either a two-tailed student's t-test or the Mann-Whitney test. Paired data were compared using the paired student's t-test, or using Wilcoxon's signed sum of ranks test. Tabulated data were submitted to the Chi-squared test. Significance was accepted at $p<0.05$.

Table 2.1 Primary antibodies used for immunohistochemistry

Antibody	Specificity¶	Host Species	Type	Presentation	Dilution/ concentration	Source
UCHT1	hCD3	mouse	IgG1	supernatant	1/100	DAKO
HD37	hCD19	mouse	IgG1	supernatant	1/100	DAKO
KP1	hCD68	mouse	IgG1	supernatant	1/150	DAKO
NO53	hiNOS C-term peptide	rabbit	polyclonal	neat antiserum	1/20,000	Merck Research‡
h13	hiNOS C-term peptide	rabbit	polyclonal	neat antiserum	1/10,000	Wellcome*
PA3-030	miNOS C-term peptide	rabbit	polyclonal	affinity purified	1/500 - 10,000	Cambridge Bioscience (Affiniti)
N32020	miNOS 21kD protein	mouse	IgG2a	purified ascites	1/250	Transduction Labs (Affiniti)
49M	miNOS	rabbit	polyclonal	neat antiserum	1/500 - 10,000	Wellcome*
-	hiNOS peptide	sheep	polyclonal	neat antiserum	1/200 - 10,000	Wellcome*
M112	hIL-15	mouse	IgG1	supernatant	30 µg/ml	Immunex Corporation+

¶ h - human; m - murine
‡ Donated by Dr R.A. Mumford, Merck Research Laboratories, USA.
* Donated by Dr I. Charles, Wellcome Research, Beckenham, UK.
+ Donated by Dr D. Cosman, Immunex Corporation, Seattle, WA, USA.

Table 2.2 Secondary antibodies used in immunohistochemistry

Host species	Antibody	Specificity	Conjugate	Concentration	Source
Goat	polyclonal	mouse immunoglobulins	biotin	1.4 μg/ml	DAKO
Goat	polyclonal	rabbit immunoglobulins	biotin	3 μg/ml	DAKO
Goat	Fab_2, polyclonal	mouse immunoglobulins	FITC	30 μg/ml	DAKO
Horse	polyclonal	universal (Vectastain^R)	biotin	1/20 stock	Vector

Table 2.3 Negative control antibodies for immunohistochemistry

Host species	Antibody type	Specificity	Source
Mouse	IgG1	Aspergillus niger glucose oxidase *	DAKO
Mouse	IgG2a	Aspergillus niger glucose oxidase *	DAKO
Rabbit	Serum	Non-immune	SAPU
Sheep	Serum	Non-immune	SAPU
Sheep	Immunoglobulin - affinity purified	Non-immune	Sigma

* neither constitutively nor inducibly expressed in mammalian cell systems.

Chapter 3

A role for interleukin-15 in T cell activation in rheumatoid arthritis

Introduction

IL-15 is a novel cytokine with biological functions similar to those of IL-2, but with no significant sequence homology (Grabstein, et al, 1994). It mediates its functions through the β and γ chains of the IL-2 receptor and its own unique α chain (Giri, et al, 1994; Bamford, et al, 1994; Giri, et al, 1995; Anderson, et al, 1995b). IL-15 induces T cell proliferation, enhances NK cell cytotoxicity and ADCC, up regulates production of NK cell derived cytokines, including IFN-γ, GM-CSF and TNFα (Grabstein, et al, 1994; Carson, et al, 1994), and can co-stimulate proliferation and differentiation of B cells (Armitage, et al, 1995). In addition, IL-15 stimulates locomotion and chemotaxis of normal T cells measured by an assay of locomotor shape-change (head-tail polarization), by checkerboard filter assay and by invasion into IL-15 containing collagen gels (Wilkinson & Liew, 1995). IL-15 can be detected at mRNA level in several normal human tissues including placenta, skeletal muscle and kidney. Production by epithelial and fibroblast cell lines and peripheral blood monocytes has been shown, but, unlike IL-2, IL-15 is not reported to be produced by activated T cells (Grabstein, et al, 1994). The role of IL-15 in the context of any pathological situation remains to be elucidated.

Rheumatoid arthritis (RA) is a destructive inflammatory polyarthropathy which provides an ideal opportunity to study expression of pro-inflammatory cytokines *in situ*. Chronic RA synovitis is characterised by infiltration of the normally relatively acellular synovial membrane by macrophages, T cells and plasma cells, together with the presence of activated fibroblast-like synoviocytes (Duke, et al, 1982; Burmester, et al, 1983; Cush & Lipsky, 1988). Pro-inflammatory cytokines of macrophage derivation, including IL-1, IL-6, IL-8 and TNFα, are readily detected at the protein level in RA synovial tissue, and also at the eroding cartilage/pannus junction, indicating the importance of macrophages in articular damage (reviewed by Feldmann, et al, 1996b). Nevertheless, a significant T cell infiltrate is present in RA synovial

tissue, which exhibits a memory phenotype with markers characteristic of both early and late stages of activation (Pitzalis, et al, 1987; Laffon, et al, 1991; Thomas, et al, 1992; Iannone, et al, 1994). The mechanisms governing recruitment of these T cells to the synovial membrane from the circulation remain ill-defined, but probably involve co-ordinated adhesion molecule expression and production of appropriate chemotactic factors (reviewed by Oppenheimer-Marks & Lipsky, 1995).

T cells are important in RA pathogenesis (Panayi, et al, 1992). Therapies directed at T cells, such as cyclosporin A and monoclonal antibodies against T cell surface antigens produce significant, albeit transient, clinical improvement (Horneff, et al, 1991; Wendling, et al, 1991; Pasero, et al, 1996). Several animal arthritis models can be demonstrated to be T cell-dependent (Trentham, et al, 1978; Staines & Wooley, 1994). Moreover, the association of RA with the HLA-DR shared epitope strongly implicates T cell / antigen recognition in disease aetiology (Gregerson, et al, 1987). Cytokines associated with T cell activation such as IFN-γ or IL-2 can be detected at the mRNA level (Buchan, et al, 1988; Simon, et al, 1994) and at the protein level using immunohistochemical analysis (Ulfgren, et al, 1995). However, only a relatively small number of T cells expressing these proteins can be found, and furthermore, T cell-derived cytokines are usually undetectable in synovial fluid (Firestein & Zvaifler, 1987; Firestein, et al, 1988). Whether there is sufficient IL-2 present to account for the extensive evidence of T cell activation remains uncertain and has raised controversy regarding the relative contribution of macrophages and T cells to the pathogenesis of RA (Firestein & Zvaifler, 1990; Panayi, et al, 1992). The identification of significant levels of a macrophage-derived cytokine capable of T cell chemoattraction, activation and maturation in RA synovial tissue would clearly be of considerable interest.

3.1 Detection of IL-15 in synovial fluid

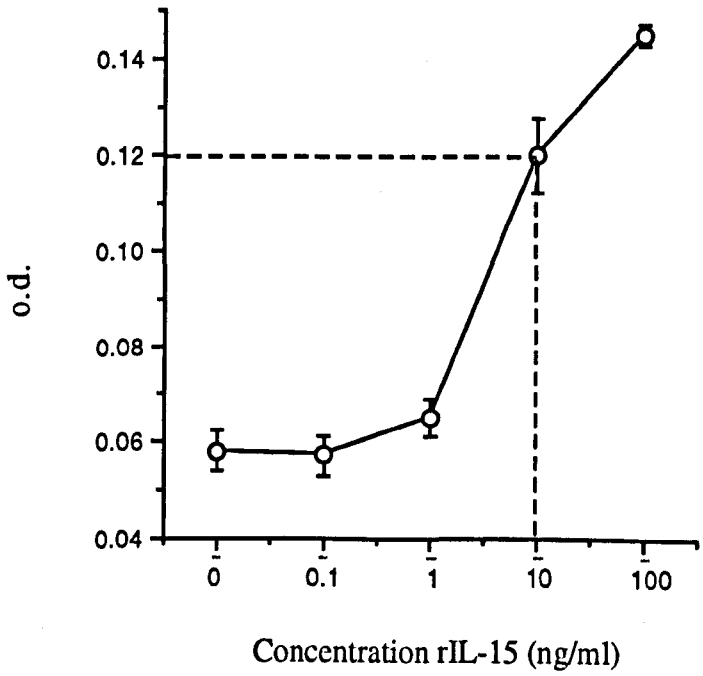
A sandwich ELISA was designed to detect IL-15 in biological solutions, using a murine monoclonal anti-simian IL-15 antibody (M112) for capture and an affinity purified rabbit polyclonal anti-human IL-15 antibody (PeproTech) for detection. Recombinant IL-2, IL-1β and TNFα were not detected in this ELISA system, nor did the level of rheumatoid factor correlate with IL-15 concentrations detected in synovial fluid. The sensitivity for recombinant human IL-15 was 1 ng/ml (Figure 3.1).

Synovial fluids were collected from 17 RA patients whose clinical and serological characteristics are shown in table 3.1 and from 6 OA patients (mean 69 years ± 11.9, range 50-79). The latter were treated with simple analgesic drugs or non-steroidal anti-inflammatory drugs. IL-15 was detected in RA synovial fluids at significantly higher levels than was observed in OA fluids ($p<0.003$, Mann-Whitney; Figure 3.2). Despite a wide variation in disease duration and acute phase response in RA patients, local synovial fluid IL-15 levels did not correlate with systemic disease activity parameters or with drug therapy (Table, 3.1; Pearson's correlation coefficient).

3.2 Synovial tissue cultures generate IL-15

To determine whether synovial membrane was the source of detectable IL-15, single cell suspensions of synovial tissue samples from 7 RA and 4 OA patients were cultured for 72 hours *in vitro*, without exogenous stimulation. IL-15 was detected in the supernatants from 4/7 RA patients, but from only 1/4 OA patients (Figure 3.3). These data indicate that rheumatoid synovial tissue is capable of generating IL-15.

Figure 3.1 Standard curve for IL-15 ELISA



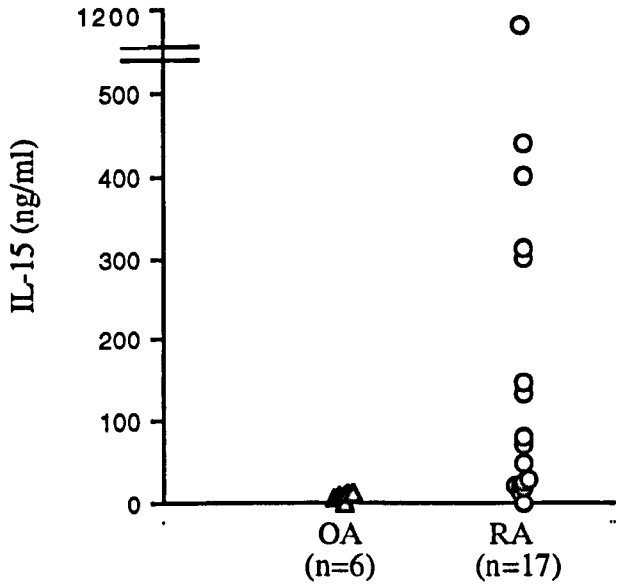
rIL-15 was diluted ten-fold in PBS / 1% BSA and submitted to assay as described in section 2.9.1. Synovial fluids were diluted in PBS then assayed as for the standard. The concentration of IL-15 in samples was calculated as shown (dotted lines). Sensitivity was typically 1 ng/ml.

Table 3.1 Demography of RA patients from whom synovial fluid samples were obtained for IL-15 analysis

	Age (years)	Disease Duration (years)	RF +/-	ESR	CRP	Drug profile
RA1	89	6	+	4	10	-
RA2	36	5	+	36	58	Gold
RA3	34	4	+	28	57	SASP
RA4	44	9	+	15	14	SASP
RA5	44	10	+	16	5	Pred, MTX
RA6	57	3	+	46	50	SASP
RA7	60	8	+	51	39	Gold
RA8	59	33	+	89	161	MTX
RA9	75	50	+	30	74	SASP
RA10	49	11	+	46	59	MTX
RA11	56	16	+	8	30	Gold
RA12	65	7	+	71	66	SASP
RA13	74	20	+	22	44	SASP
RA14	73	3	+	58	86	Gold
RA15	54	25	-	23	66	SASP
RA16	31	5	+	45	16	Gold
RA17	57	15	+	80	93	Gold

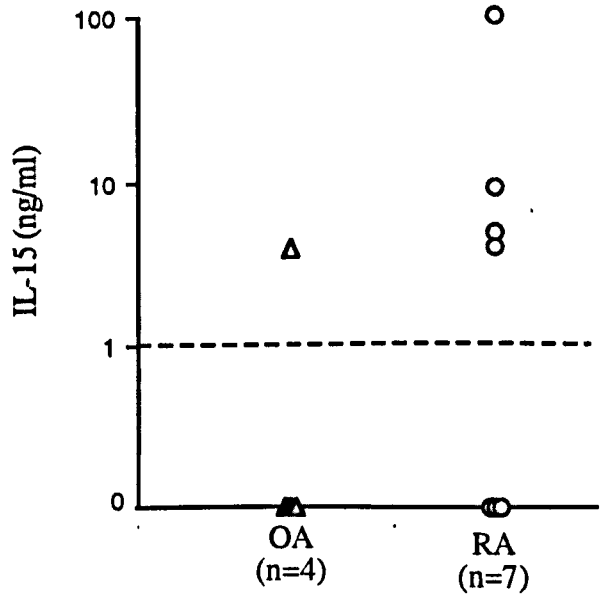
Clinical details of RA patients were collected at the time of synovial fluid aspiration. Data shown are for samples used in IL-15 ELISA. RF - rheumatoid factor, ESR - erythrocyte sedimentation rate, CRP - C-reactive protein (<10 mg/ml normal reference), SASP - sulphasalazine; Pred - prednisolone; MTX - methotrexate; Gold - intramuscular sodium aurothiomalate.

Figure 3.2 Detection of IL-15 in RA and OA synovial fluid



Synovial fluids from RA or OA patients were collected, and assayed for IL-15 presence by ELISA. Levels in RA fluids were significantly higher than in OA (p=0.0029, Mann-Whitney).

Figure 3.3 IL-15 production by synovial membrane cultures



Single cell suspensions of synovial tissue from RA and OA patients were cultured without exogenous stimulation for 72 hours. IL-15 levels in resultant supernatants were measured by ELISA (sensitivity indicated - 1 ng/ml).

3.3 Immunohistochemical localisation of IL-15 in human synovium

To further investigate the presence of IL-15 in synovial membrane, parallel cryostat sections of RA synovial tissues were stained with murine monoclonal anti-simian IL-15 (M112). Simian IL-15 shares 97% homology with human IL-15 (Grabstein, et al, 1994) and similar antibodies localised IL-15 expression in human monocytes (Carson, et al, 1995). Figure 3.4a demonstrates the tissue distribution of M112 binding in rheumatoid synovium. The pattern of cytoplasmic staining appears to represent intracellular rather than membrane-bound cytokine, implying that these cells contain, and are likely synthesising, IL-15. Staining was abolished completely by pre-incubation with recombinant human IL-15 (50 μg/ml), indicating specificity of antibody binding (Figure 3.4b). To determine which cells were expressing IL-15, parallel sections were stained with monoclonal antibodies against CD68 (tissue macrophage) and CD3 (T lymphocyte). IL-15 co-expressed with $CD68^+$ macrophages in the lining layer (Figure 3.4c), but not with adjacent $CD3^+$ T cells (Figure 3.4d). The extent of lining layer staining (mean 54 ± 13.1% of cells) indicated that type B synoviocytes may also express IL-15 (Table 3.2). IL-15 was also detected in the T cell rich aggregates. This staining may correlate with intracellular localisation in macrophages, which are found in these areas, or may reflect receptor bound IL-15. Although T cells have not thus far been shown to produce IL-15 *in vitro* (Grabstein, et al, 1994), production of IL-15 by $CD3^+$ synovial T cells cannot be excluded by these data. In keeping with the lower levels of IL-15 detected in OA synovial fluid, OA synovial sections contained fewer IL-15 expressing cells in both the lining layer and interstitial areas ($p<0.001$, Mann-Whitney; Table 3.2) suggesting that up regulation of expression of IL-15 is a feature of RA.

These data clearly demonstrate the presence of IL-15 in the RA synovial membrane. Subsequent experiments addressed the possible functional significance of this observation.

Figure 3.4 Immunohistochemical localisation of IL-15 in RA synovial membrane

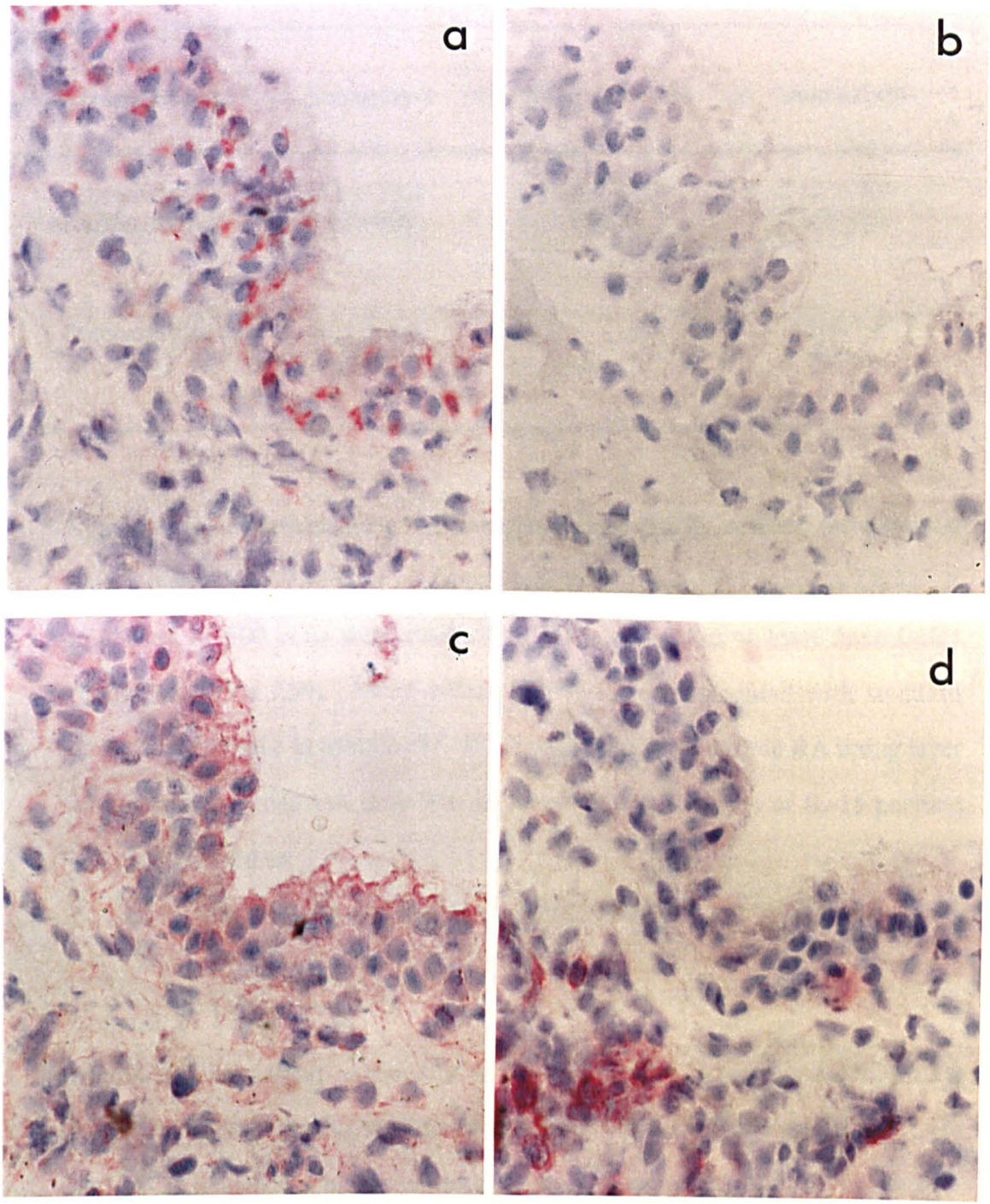


Table 3.2 Immunohistochemical localisation of IL-15 in synovial membrane

Diagnosis	% positive cells mean ± s.d. (range)		
n = number fields	Lining layer	Aggregate areas	Interstitium
Rheumatoid arthritis (n=30)	54 ± 13.1 (29-80)	24 ± 12.3 (5-55)	12 ± 7.7 (3-32)
Osteoarthritis (n=21)	6.5 ± 4.5 (1-16)	-	2 ± 1.1 (0.5-4)

Synovial tissue sections from RA (9 patients) and OA (5 patients) were stained with a monoclonal anti-IL-15 antibody (M112) as described (section 2.6.1). A minimum of 500 cells were counted in each section, in at least three fields (magnification x 250). Mean values (per field) are presented with standard deviation and range in parenthesis. IL-15 expression is greater in RA lining layer and interstitium than OA ($p<0.001$, Mann-Whitney). Percent of IL-15 positive cells is expressed as:

(positive cells /total number cells) x 100.

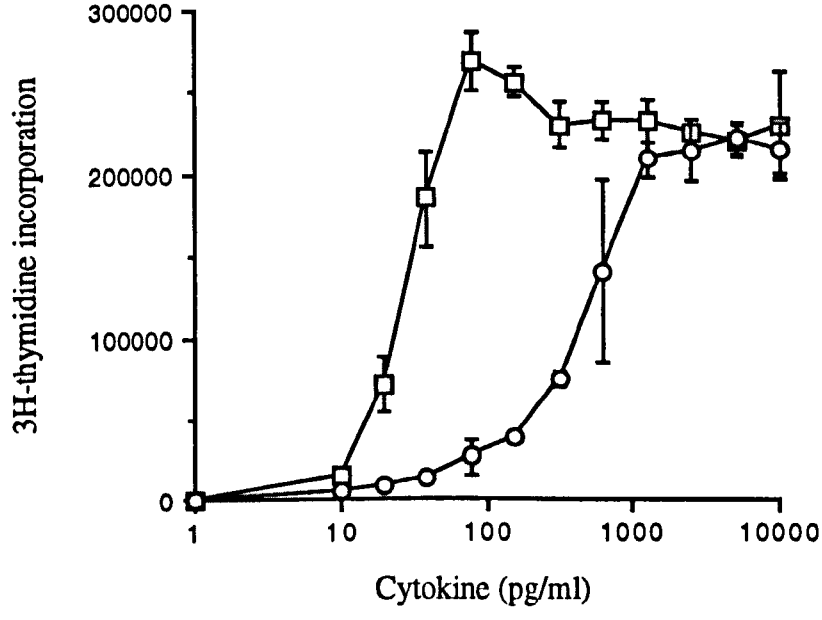
Legend to Figure 3.4

Immunohistochemical analysis of synovial tissue from a representative RA patient. Cryostat sections were fixed and stained with (a) anti-IL-15 (M112), (b) anti-IL-15 neutralised by recombinant human IL-15, (c) anti-CD68, or (d) anti-CD3. Primary antibodies were detected with biotinylated goat anti-mouse IgG, then with streptavidin-alkaline phosphatase complex and fast red salts. (Magnification x80)

3.4 IL-15 footpad injection induces inflammatory cell recruitment in DBA/1 mice.

Previous studies have shown that IL-15, like IL-2, is a potent chemoattractant for T cell polarization and migration *in vitro* (Wilkinson & Liew, 1995), raising the possibility that IL-15 may recruit T cells into local tissues during inflammatory responses. To test this hypothesis *in vivo*, male DBA/1 mice, primed 7 days previously with *C. parvum*, received either 500 ng rIL-15 (n=12), or PBS alone (n=12), subcutaneously to their hind foot pads. Human IL-15 was used because murine IL-15 was unavailable and preliminary experiments had shown that the murine T cell line, CTLL-2, proliferated to human IL-15 (Figure 3.5).

Figure 3.5 CTLL cell activation by human IL-15

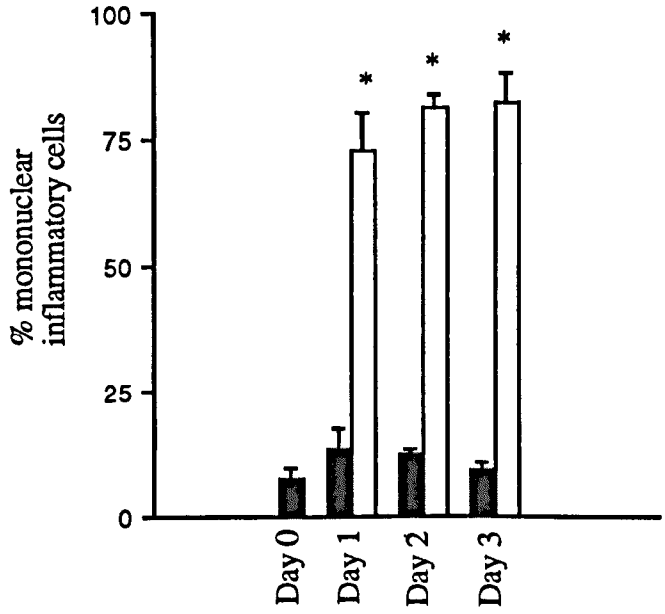


Murine T cells proliferate to human rIL-15. CTLL cells ($1x10^5$ /ml) were cultured for 24 hours with human rIL-15 (□), or human rIL-2 (O) and ^{3}H-thymidine incorporation over the last 6 hours was assessed. The response to IL-15 significantly exceeded that to IL-2 between 50 and 200 pg/ml of cytokine ($p<0.01$, Mann-Whitney).

Mice which received rIL-15 developed an inflammatory cell infiltrate in the hypodermis and muscle layers, in contrast to those which received PBS alone (Figure 3.6, $p<0.001$, Mann-Whitney; Figures 3.7a & 3.7b). Immunohistochemical staining was performed, which showed that up to 56 ± 3.6% (mean ± s.d.; n=4 tissues) of the tissue cell population after rIL-15 injection was $CD3^+$ (Figure 3.7c). Few $CD3^+$ cells were present in PBS treated limbs (<2% total cell number; n=4 tissues). Inflammatory cell recruitment was evident within 16 hours and was present up to 72 hours after cytokine injection (Figure 3.6). No change in paw thickness was detected on caliper foot-pad measurements compared with PBS injected limbs. However, the size of draining popliteal and inguinal lymph nodes was significantly increased 24 and 48 hours after rIL-15 injection (Figure 3.8). Histological examination of the lymph nodes using H&E, demonstrated marked paracortical expansion, consistent with the presence of increased numbers of T lymphocytes (Figure 3.9a & 3.9b). Thus, a single injection of rIL-15 induced a local tissue inflammatory infiltrate.

A single experiment was performed in which 10 unprimed DBA/1 mice were injected in the hind footpad with either PBS or rIL-15. No significant inflammatory infiltrate was detected in these mice (<10% inflammatory cells / high power field [HPF]), whereas *C. Parvum* primed controls developed histological appearances similar to those described above (mean 78 ± 4% inflammatory cells /HPF after 48 hours). These data suggest that an enhanced circulating pool of activated T cells was required for rIL-15-mediated recruitment in this model system.

Figure 3.6 rIL-15-induced inflammatory infiltrate in murine footpads

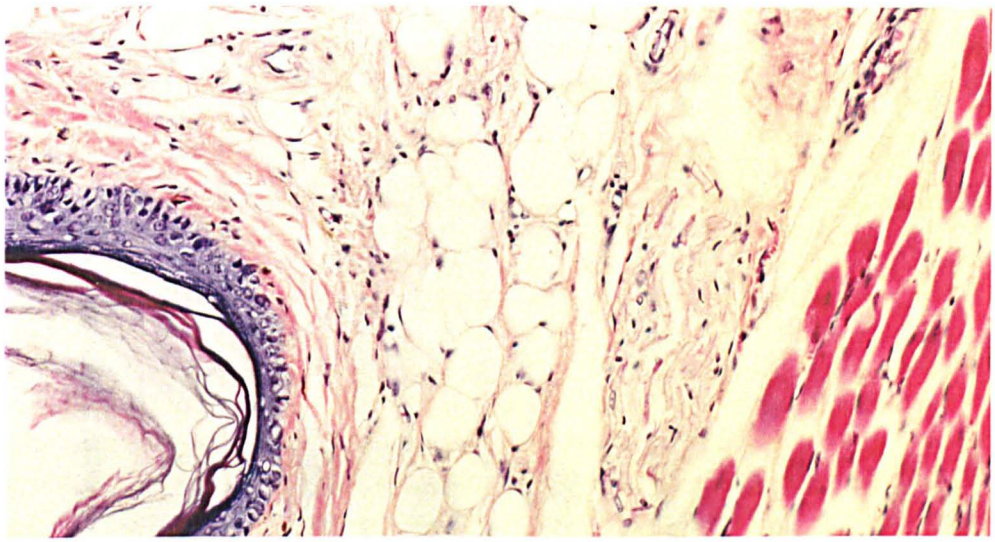


DBA/1 mice received either rIL-15 (500 ng; n=12) or PBS (n=12) by footpad injection and tissue was examined histologically daily thereafter. H&E stained sections were scored by a treatment-blinded histologist. Significantly higher numbers of infiltrating cells were detected in rIL-15 treated mice (*$p<0.002$, Mann-Whitney compared with PBS controls at same time point). Percentage infiltrating mononuclear cells was calculated for 3 high power fields (minimum 1000 cells) in each of 4 paws for each group (saline - filled bar; rIL-15 - clear bar) after 16, 48 and 72 hours. Figure shows mean value with s.e.m.

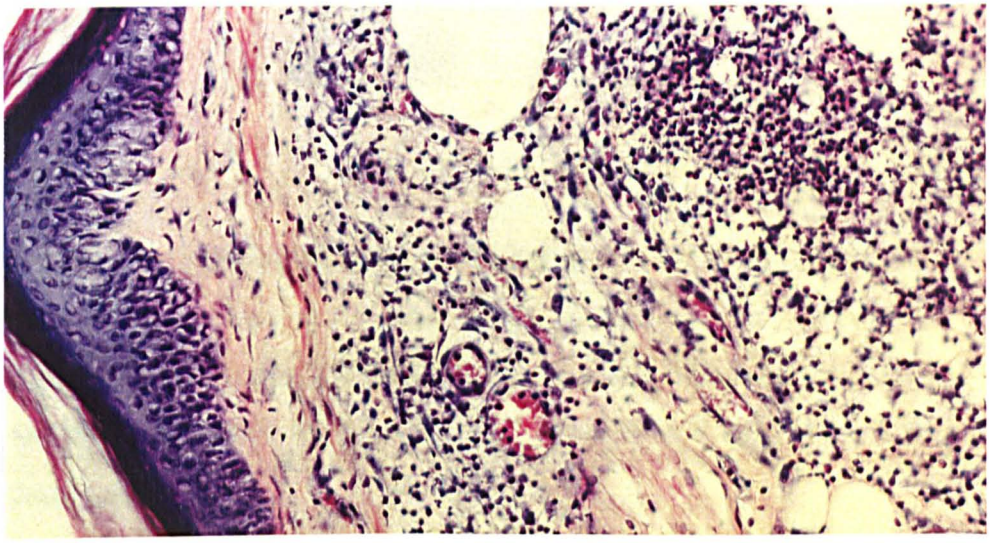
$$\frac{\text{number invading mononuclear cells}}{\text{total number cells in field}} \times 100$$

Figure 3.7 Histology of murine footpad after IL-15 injection in DBA/1 mice

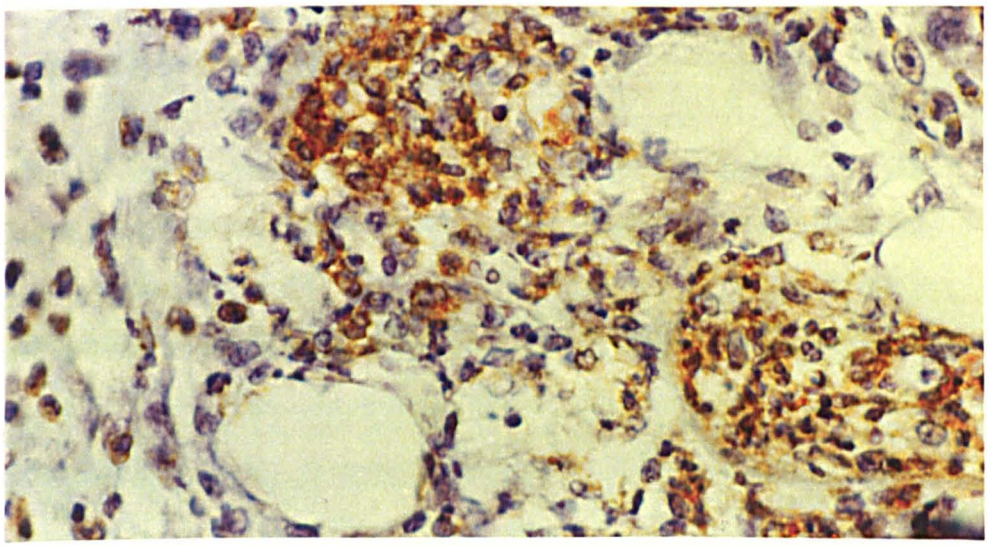
a



b

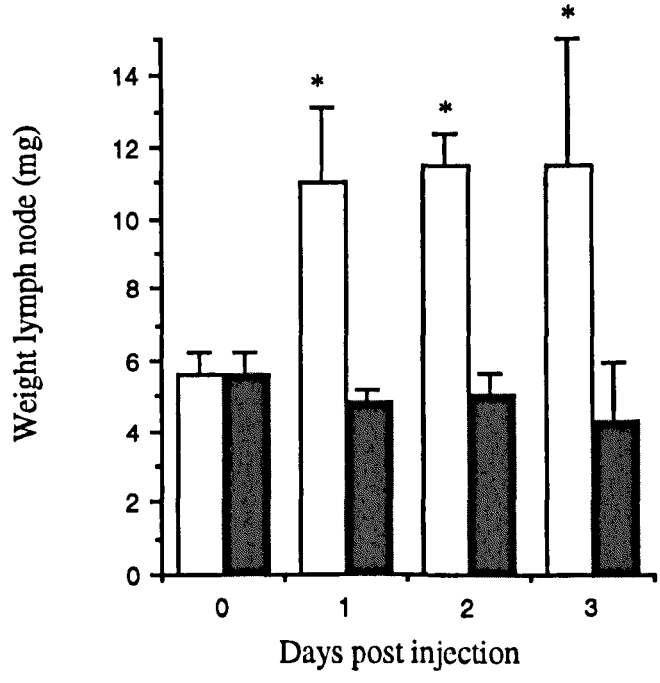


c



Legend figure 3.7 next page

Figure 3.8 Lymphadenopathy after IL-15 footpad injection in DBA/1 mice

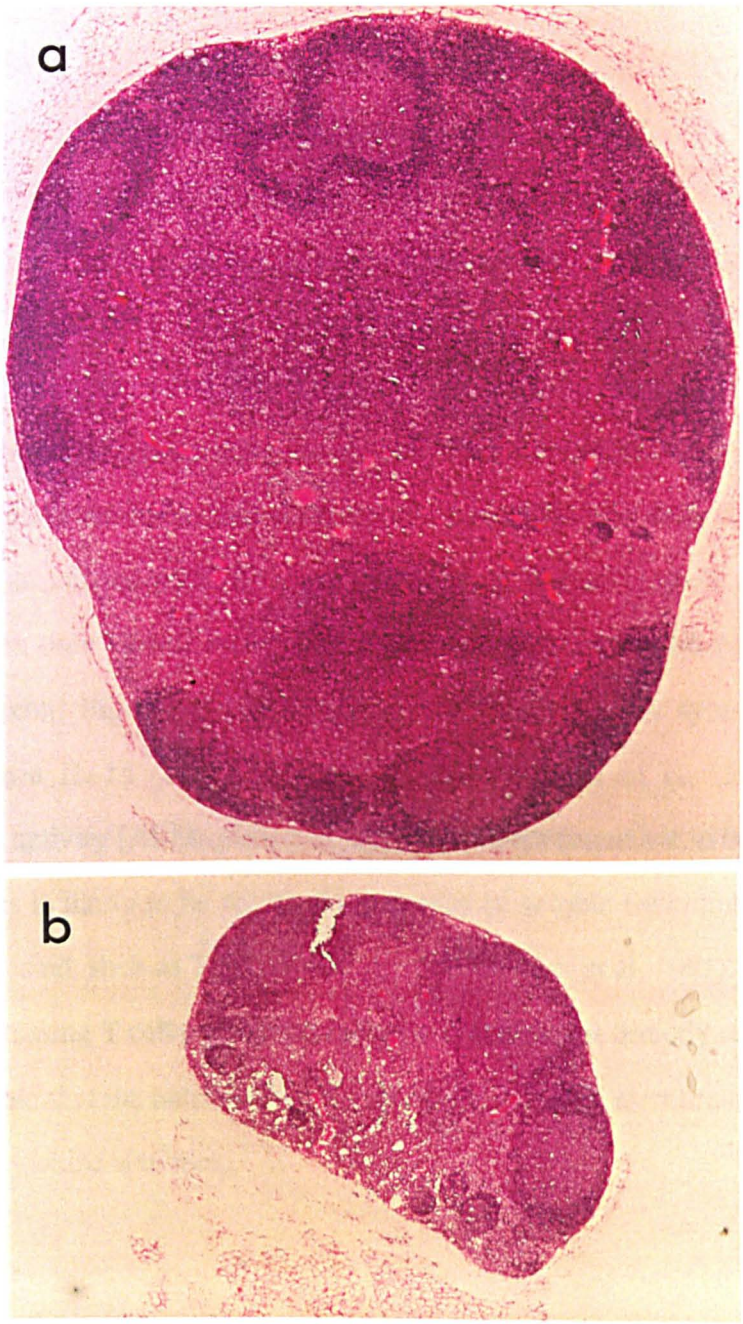


DBA/1 mice received rIL-15 (clear bar, 500 ng) or PBS (filled bar) by 50 µl footpad injection. Draining lymph nodes were removed from 4 mice in each group daily thereafter, dissected free of connective tissue and weighed. Data are mean ± s.e.m. for at least 8 lymph nodes at each time point. (*$p<0.05$, Mann-Whitney)

Legend to figure 3.7 (previous page)

Histological investigation of murine footpads was performed after injection of 500 ng IL-15 or PBS. H&E staining of formalin fixed, decalcified footpad 48 hours after (a) saline, or (b) IL-15 injection demonstrates inflammatory infiltrate in IL-15, but not in PBS recipient. (c) $CD3^+$ cells detected after 48 hours with anti-CD3 / peroxidase localisation (2.6.4). A representative field is shown from an IL-15 injected animal. Few $CD3^+$ cells (<5%) were detected in interstitial areas of saline treated controls. (Magnification a, b x50, c x250).

Figure 3.9 Histology of lymph nodes draining IL-15 injected limbs

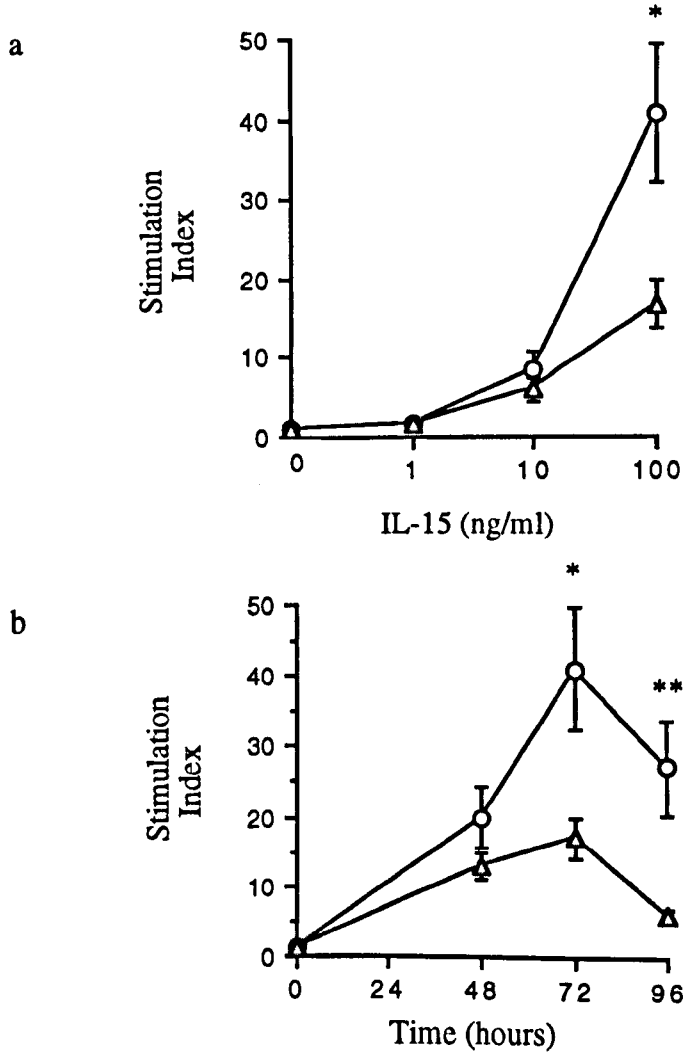


Paracortical expansion, suggestive of increased numbers of T cells, was observed in popliteal lymph nodes, draining footpads injected 48 hours previously with either (a) 500 ng IL-15, or (b) PBS. Lymph nodes were fixed in 10% neutral buffered formalin immediately after excision, and sections were stained with H&E. (Magnification x40 for each figure)

3.5 IL-15 induced peripheral blood T lymphocyte activation

T lymphocyte polarization often accompanies entry into G_1 phase of cell cycle (Wilkinson, 1986). Moreover, IL-15 induces proliferation of PHA-blasts from peripheral blood and of transformed T cell lines (Grabstein, et al, 1994). Since PB T cells polarize to rIL-15 alone (Wilkinson & Liew, 1995), it was decided to investigate whether circulating T cells were capable of cell-division in response to rIL-15. The proliferation to rIL-15 of peripheral blood mononuclear cells (PBMC) from 14 RA patients was therefore compared with 14 age and sex matched normal controls in ^{3}H-thymidine incorporation assays. RA patients were all receiving DMARD therapy, but no corticosteroids, whereas normal controls were on no regular drug therapy. RA derived PBMC, stimulated with 100 ng/ml of rIL-15, exhibited a significantly higher stimulation index than PBMC from normal controls after 72 and 96 hours culture ($p<0.05$, Wilcoxon; Figures 3.10a & 3.10b). However, 4 RA synovial fluids, known to contain IL-15 (by ELISA and by the presence of IL-15-dependent chemoattractant activity [Al-Mughales, et al, 1996]), were found not to be mitogenic for PBMC. This is likely to be due to the presence of soluble inhibitors within the synovial fluids tested, such as TGFβ (Fava, et al, 1989; Chu, et al, 1991). These data show that a circulating T cell population exists in RA which is directly responsive to IL-15, but indicate that the behaviour of T cells in the synovial membrane will likely reflect multiple cytokine activities.

Figure 3.10 Peripheral blood mononuclear cells proliferate to rIL-15



(a) Dose response of RA (O, n=14) or control (Δ, n=14) PBMC to rIL-15 after 72 hours in triplicate ^{3}H-thymidine incorporation assays. Values shown are mean ± s.e.m. Medium control ranged from 500 - 1,500 cpm. (*p<0.01, Mann-Whitney). Stimulation index calculated as:

$$\frac{\text{cpm after incubation with IL-15}}{\text{cpm medium control}}$$

(b) Time course showing stimulation index of PBMC from RA (O, n=14) or age matched controls (Δ, n=14) after addition of 100 ng/ml rIL-15 in triplicate 3H-thymidine incorporation assays. Values shown are mean ± s.e.m. (* p<0.05, ** p<0.03, Mann-Whitney) comparing RA patient with normal control group.

Discussion

This study clearly demonstrates the presence of IL-15 in the rheumatoid synovial membrane. T cell / macrophage interactions in the synovial membrane are integral to the progression of synovitis and resulting articular destruction (Feldmann, et al, 1996b). The detection of IL-15 in this context provides a mechanism for T cell recruitment and activation by a macrophage-derived cytokine in RA.

High concentrations of IL-15 were detectable in RA synovial fluid, in excess of levels previously shown to possess biological activity *in vitro* (Grabstein, et al, 1994; Burton, et al, 1994; Carson, et al, 1995). The synovial membrane was the likely source of this IL-15, since it was present in synovial cultures and could be predominantly localised immunohistochemically to the synovial lining layer. The possibility remains, however, that this represents detection and release of stored cytokine. Further experiments are required, in which the capacity for protein synthesis inhibitors to abrogate IL-15 production by synovial membrane *in vitro* is investigated. The recent development of a more sensitive ELISA system for IL-15 will allow this question to be formally addressed.

Pro-inflammatory cytokines, such as IL-1, IL-6, TNFα and IL-8, are abundant in synovial tissue and are similarly localised to the lining layer, and to cartilage / pannus junction and perivascular infiltrates, where they are recognised to be predominantly macrophage derived (reviewed by Brennan, et al, 1991; Feldmann, et al, 1996b). The high proportion of lining layer cells which expressed IL-15, and the co-staining, in parallel sections, of IL-15 with $CD68^+$ cells, made it likely that macrophages contributed significantly to IL-15 production in RA, consistent with the *in vitro* observation that IL-15 is made by cells of the monocyte series (Grabstein, et al, 1994; Carson, et al, 1995). However, IL-15 expression has been reported from the bone marrow stromal cell line, IMTLH (Grabstein, et al, 1994), therefore type B

synoviocytes, which are derived from fibroblast-like cells, may also be capable of IL-15 production. Similarly, IL-15 production by synovial T cells remains possible. IL-15 was first isolated from HuT-102 cells (Burton, et al, 1994), indicating that cells of the T lymphocytes series might also synthesise IL-15 if appropriately activated.

In common with IL-2, IL-15 induces T lymphocyte locomotion and proliferation of T lymphoblasts (Grabstein, et al, 1994; Wilkinson & Liew, 1995) raising the possibility that it is of importance in recruitment and concomitant activation of T cells at sites of chronic inflammation. The RA synovial membrane contains aggregates of polyclonal T lymphocytes which concurrently express cell surface markers normally seen sequentially through various stages of the activation pathway, such as HLA-DR, CD69 and VLA-4 (Pitzalis, et al, 1987; Burmester, et al, 1987; Laffon, et al, 1991; Iannone, et al, 1994; Fernandez-Gutierrez, et al, 1995). They also possess a mature differentiated phenotype ($CD45RO^+$, RBdim, $CD27^-$), probably reflecting preferential recruitment of recirculating memory T cells, (Pitzalis, et al, 1988; Pitzalis, et al, 1991; Thomas, et al, 1992; Matthews, et al, 1993; Kohem, et al, 1996), which may undergo further activation, initiated by endothelial interaction during extravasation, and subsequently modified by the cytokine environment within synovial tissue (Iannone, et al, 1994). Although IL-2 mRNA can be detected in RA synovial tissue (Simon, et al, 1994), and T cells containing IL-2 protein can be found immunohistochemically (Ulfgren, et al, 1995), these studies have demonstrated that most $CD3^+$ T cells in RA synovium show no evidence of IL-2 expression. Moreover, T cells derived from synovial tissues are recognised to exhibit deficient cytokine production *in vitro* to exogenous stimuli or recall antigens (Combe, et al, 1985; Verwilghen, et al, 1990; Aaron, et al, 1991; Thomas, et al, 1992) and IL-2Rα expression is limited to ~10% of cells (Pitzalis, et al, 1987). Therefore, it is unlikely that the observed T cell activation can be explained solely by the levels of IL-2 thus far detected, and other cytokine-dependent mechanisms of T cell recruitment and activation may operate in RA. IL-15, by virtue of its described functional phenotype, may fulfil such a role.

The processes whereby inflammatory cells are recruited to the synovium remain ill-defined. Synovial endothelial cells express high levels of E-selectin, ICAM-1 and VCAM-1, which can be up-regulated by IL-1β and TNFα (Abbot, et al, 1992; Morales-Ducret, et al, 1992; Wilkinson, et al, 1993). Interaction with T cell markers, such as VLA-4 and LFA-1, will thereby facilitate endothelial adhesion and transmigration (reviewed by Oppenheimer-Marks & Lipsky, 1995). These pathways operate in parallel with the activities of synovial chemokines. Al-Mughales, et al (1996) have recently demonstrated that RA synovial fluid contains chemoattractant activity for T lymphocytes which is biologically active in *in vitro* assays. Using neutralising antibodies they have identified IL-15 as an important contributory cytokine in this context (Appendix III; McInnes, et al, 1996). Recombinant IL-15, having no reported chemotactic effects on neutrophils, B cells or monocytes (Wilkinson & Liew, 1995), appears T lymphocyte specific. Its locomotor action is seen in highly purified T cells (Dixon, R. personal communication; McInnes, et al, 1996), and in $CD4^+$ and $CD8^+$ T cells subsets, both of which are represented in synovial tissue, and it is inhibited by neutralising antibody to the βγ chains of the IL-2 receptor (Wilkinson & Liew, 1995). Other macrophage-derived lymphocyte chemokines, including MIP-1α, MCP-1 and IL-8 (Koch, et al, 1991; Koch, et al, 1992; Taub, et al, 1993; Deleuran, et al, 1994; Al-Mughales, et al, 1996) have also been implicated in chemoattraction in RA, and IL-15 is therefore unlikely to operate in isolation in recruitment of lymphocytes to the synovial compartment. However, unlike other T cell chemotactic factors, IL-15 is also associated with lymphocyte proliferation, indicating a unique combination of activities which in the synovial membrane could have pathological significance by facilitating both recruitment and activation of T cells in RA.

PBMC proliferated in response to rIL-15 alone, indicating the presence of circulating primed T cells capable of cell division in response to a single signal (Janeway & Bottomley, 1994). It is of interest that this response was enhanced in RA patients.

Since increased numbers of mature $CD45RO^+$, $CD27^-$ T cells have been reported in RA peripheral blood (Kohem, et al, 1996), it is attractive to hypothesise that such cells represent an expanded memory T cell population in RA, which constitutes a pool from which the synovial membrane can recruit, partly through the activity of IL-15. Alternatively, the level of IL-15Rα expression may be increased in some PB T cells in RA patients. Currently, lack of available reagents prevents formal investigation of this latter possibility.

The chemoattractant properties of IL-15 have until now been demonstrated only *in vitro*. The murine T cell line, CTLL-2, proliferated to human IL-15 at higher levels than to murine IL-2. This unexpected finding may reflect high levels of IL-15Rα in this cell line, or perhaps represent a feature of cytotoxic $CD8^+$ T cell responses *in vitro* to IL-15. Nevertheless, this inter-species cross-reactivity permitted investigation of the role of IL-15 *in vivo* in a model of initiation of inflammation in DBA/1 mice. The demonstration of a cellular infiltrate lasting at least three days after a single subcutaneous injection of rIL-15 into murine footpads, suggests that the chemotactic activity measured *in vitro* by Al-Mughales, et al (1996) indeed has biological significance. The duration of the observed response, and presence of related lymphadenopathy, indicates that other mechanisms aside from IL-15-induced chemotaxis, are operating. The possibility that IL-15 may up-regulate adhesion molecule expression either on circulating T cells, or on endothelial cells, needs to be explored. IL-15 induces adhesion molecule redistribution on T cells (Nieto, et al, 1996), although novel expression of markers was not reported. Nevertheless, it is clear that, since up to 56% of infiltrating cells expressed CD3 three days after injection, the events initiated by IL-15 can have a prolonged action on T cell recruitment.

These data demonstrate for the first time the presence of IL-15 in a pathological setting. It was of interest to determine possible mechanisms whereby IL-15 might contribute to RA pathogenesis, beyond T cell recruitment. The relationship of IL-15 mediated T cell activation with TNFα production by macrophages in synovial membrane was therefore investigated.

Chapter 4

Interleukin-15 mediates T cell dependent regulation of tumour necrosis factor α

Introduction

IL-15 is a pleiotropic cytokine derived from several cell types, including macrophages and fibroblasts (Grabstein, et al, 1994; 1994; Tagaya, et al, 1996a), which mediates its activity through a heterotrimeric receptor consisting of a unique IL-15Rα chain with the β and γ chains of the IL-2 receptor (Giri, et al, 1995; Anderson, et al, 1995b). IL-15 can induce T cell proliferation, B cell maturation and isotype switching, NK cell cytotoxicity and cytokine generation and may protect T cells from apoptosis (Grabstein, et al, 1994; Bamford, et al, 1994; Armitage, et al, 1995; Carson, et al, 1995; Akbar, et al, 1996). IL-15 promotes inflammatory cell recruitment *in vivo* following footpad injection in mice primed with *C. Parvum* (chapter 3), and in chemotactic assays *in vitro*, induces T cell polarization and invasion into collagen gels (Wilkinson & Liew, 1995). Responding migratory T cells are mainly of the $CD45RO^+$ phenotype (Al-Mughales, et al, 1996). IL-15 can be identified immunochemically in RA synovial membrane (chapter 3) and RA synovial fluid contains potent chemotactic activity attributable, at least in part, to the presence of IL-15 (McInnes, et al, 1996; Al-Mughales, et al, 1996). Moreover, RA peripheral blood T cells exhibit enhanced proliferative responses to rIL-15. It therefore seems likely that IL-15 plays a critical role in T cell recruitment and activation in RA in the relative absence of IL-2.

The relationship of IL-15 to other cytokines in the RA synovial membrane is currently unclear. Studies in animal models and of synovial tissue *in vitro* have generated a hypothetical hierarchy of cytokine activities in RA (reviewed by Maini, et al, 1995). TNFα appears pivotal in regulating synthesis of other pro-inflammatory cytokines, particularly IL-1β, which in turn is important in enhancing chondrocyte bioactivity and the production of matrix metalloproteinases (MMP) (Dayer, et al, 1986; Arend & Dayer, 1995). Furthermore, in animal arthritis models and in RA patients, monoclonal antibodies against TNFα reduce clinical inflammation and laboratory

parameters of disease activity (Williams, et al, 1992; Elliott, et al, 1994; Rankin, et al, 1995). However, it is unclear which factors up regulate TNFα production in the synovium, particularly in the relative absence of IFNγ (Firestein & Zvaifler, 1987). Non-cytokine dependent mechanisms may therefore be important. Following mitogen stimulation *in vitro*, T lymphocytes can induce macrophage production of cytokines and matrix metalloproteinases (MMP) by cell-contact (Vey, et al, 1992; Lacraz, et al, 1994). However, it has not previously been possible to extend these observations in the context of RA, because no physiologically relevant T cell activation factor has been described prior to the identification of IL-15.

The current studies were performed to address the possibility that IL-15 might induce TNFα production in RA. They tested the hypothesis that polyclonal synovial T cells, activated by IL-15, can exert pro-inflammatory effects through a cell-contact dependent mechanism, in the absence of local antigen recognition or cytokine secretion.

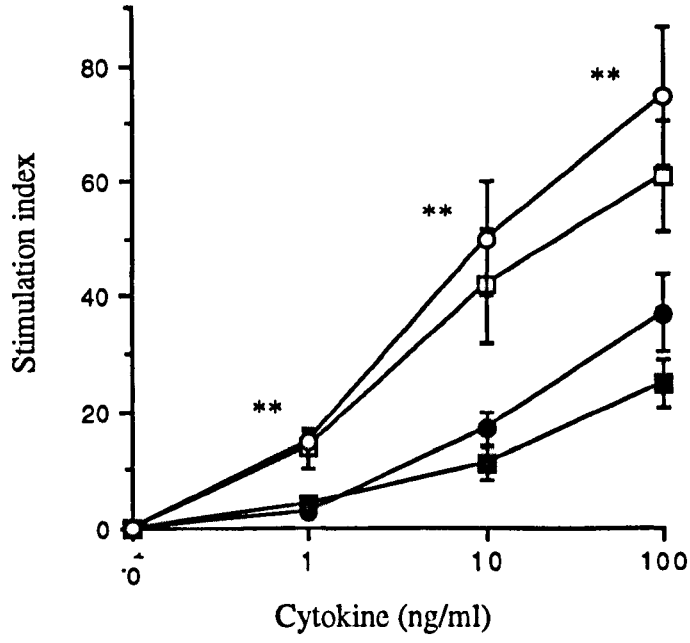
4.1 Activation of Synovial T cells by rIL-15

Previous studies have established that peripheral blood mononuclear cells from RA patients produced significantly stronger proliferative responses to IL-15 than those from normal age-matched controls (chapter 3; Figure 3.9). It was therefore of interest to compare the proliferative response to IL-15 of T cells derived from matched peripheral blood (PB) and synovial fluid (SF) samples from 15 RA patients (mean age 62.4 years [range 24 - 83]; RF^+ 90%). All patients were receiving DMARD therapy and none had received intra-articular corticosteroid within three months of sampling. Given its similar functional phenotype described thus far, IL-2 was used for control purposes. T lymphocyte enriched populations (≥90% $CD3^+$, <3% $CD14^+$ by FACS analysis of PB and ≥90% $CD3^+$, <8% $CD68^+$ cytoprep analysis of SF) from both sources proliferated vigorously to IL-15 and IL-2 in a similar dose-dependent manner (Figure 4.1a). However, the response of T cells from the SF was significantly higher than that of T cells from PB ($p<0.01$, Wilcoxon). By comparison and as previously reported (Combe, et al, 1985; Thomas, et al, 1992), the proliferative response of T cells from SF to the T cell mitogen PHA was markedly lower than that of T cells from PB (Figure 4.1b). These results therefore clearly establish that RA synovial T cells exhibit up-regulated responses to IL-15.

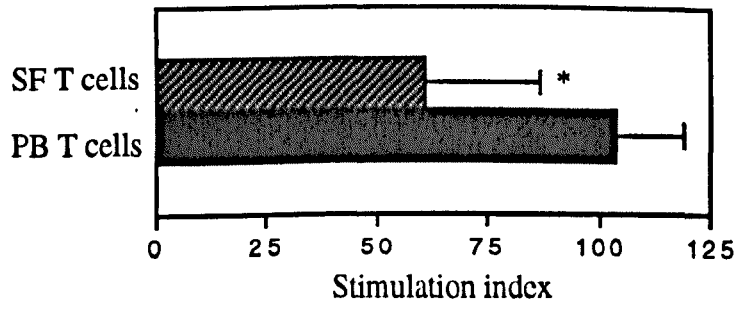
The consequence of activation of synovial T cells by IL-15 has not previously been defined. Since TNFα plays a pivotal role in the pathogenesis of RA, the possibility that IL-15 might up regulate TNFα synthesis through synovial T cells was investigated. T cells derived from PB or SF from 15 RA patients (as above) were therefore cultured with IL-15 or IL-2 and the concentrations of TNFα in culture supernatants determined by ELISA. T cell enriched cultures from SF produced significant amounts of TNFα in response to IL-15 ($p<0.005$, Wilcoxon). In contrast, IL-2 induced only low and variable levels of TNFα synthesis (Figure 4.2a). The induction of TNFα production from synovial T cells was dose-dependent (Figure

Figure 4.1 Proliferation of SF and PB lymphocytes to cytokines and mitogens

a

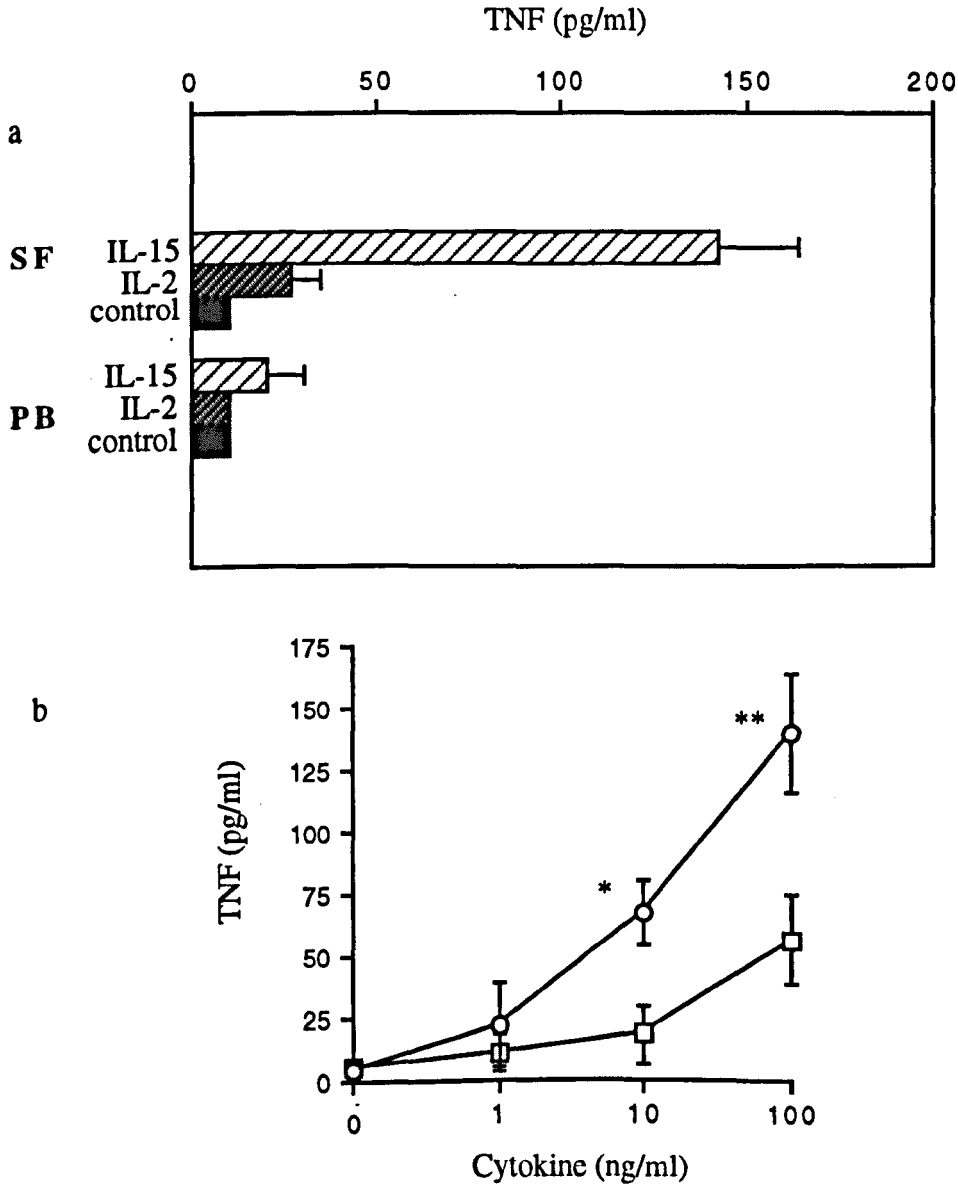


b



Synovial lymphocytes proliferate to rIL-15 (circles) and rIL-2 (squares). (a) T cells from PB (filled symbols) or SF (empty symbols) from 15 RA patients were stimulated with doses indicated of either cytokine for 72 hours. SF responses exceeded those of PB for both cytokines (**$p<0.01$). (b) In contrast, responses to PHA were diminished for SF derived, compared with PB derived, T cells (*$p<0.05$). Data are mean ± s.e.m.

Figure 4.2 Production of TNFα by PB and SF T cell-enriched cultures



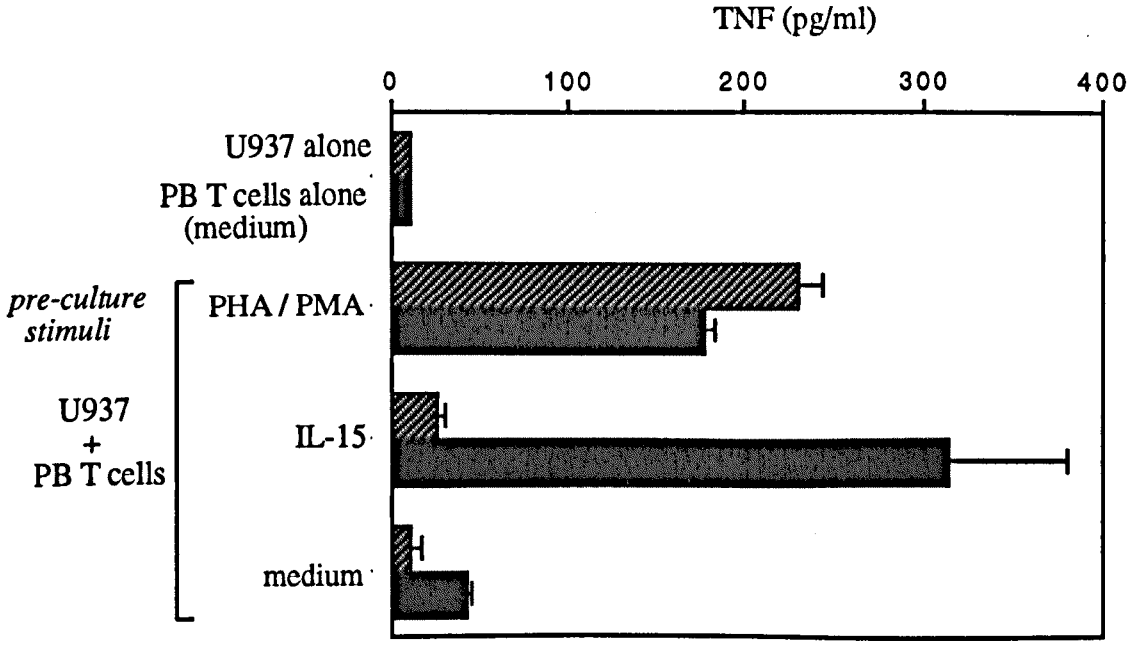
TNFα production by T cell-enriched populations from matched SF and PB. (a) 13 RA samples were stimulated with rIL-15 (100 ng/ml) or rIL-2 (100 ng/ml) for 72 hours. IL-15 induced higher levels of TNFα synthesis ($p<0.005$). (b) Dose response to either cytokine of 7 RA SF samples in which IL-2 responses were measurable. rIL-15 (circle) induced enhanced levels of TNFα synthesis from SF lymphocytes compared with rIL-2 (square) (**$p<0.003$, *$p<0.03$, Wilcoxon). Data are mean ± s.e.m.

4.2b), thus the reduced response to IL-2 was unlikely to be due to an altered dose-response. Neither cytokine, however, induced significant TNFα synthesis by T cells derived from PB (Figure 4.2a). In comparison, PHA induced TNFα synthesis by lymphocytes from both SF and PB lymphocytes, but that from PB exceeded that from SF (386 ± 110 pg/ml v 184 ± 39 pg/ml respectively [mean ± s.e.m], $p<0.03$, Wilcoxon). These results therefore demonstrate that synovial T cells can contribute directly to TNFα production in the RA synovial membrane in response to IL-15. Moreover, they show that the functional effects of IL-15 and IL-2 on this pathological T cell population may be different.

4.2 IL-15-dependent upregulation of TNFα production from macrophages

As they are the major source of TNFα in RA synovitis (Chu, et al, 1991), the effect of IL-15 on TNFα production by macrophages was investigated. Addition of IL-15 (1 - 100 ng/ml) to cultures of unprimed macrophage cell lines (U937 and THP-1) or blood-derived monocytes (n=3) from normal donors failed to induce TNFα production. These data implied that in RA, IL-15 might mediate its effects on macrophages primarily via T cells. Therefore, the possibility that IL-15-activated T cells could induce TNFα synthesis by macrophages / monocytes was addressed. Optimal PB proliferative responses to IL-15 having been obtained after 72 hours culture (Figure 3.10), PB T cells from normal donors were stimulated with 100 ng/ml IL-15 or mitogen for 72 hours, then washed thoroughly before co-culture in double-chamber wells, either in contact with, or separated by a semi-permeable membrane from U937 cells. TNFα production was observed only in cultures in which IL-15-stimulated T cells and macrophages were in direct contact (Figure 4.3). As expected, PHA / PMA-stimulated T cells induced TNFα production in the presence or absence of cell contact (Figure 4.3; section 4.1). These data indicated that a cell-contact event

Figure 4.3 Double chamber culture of PB T and U937 cells



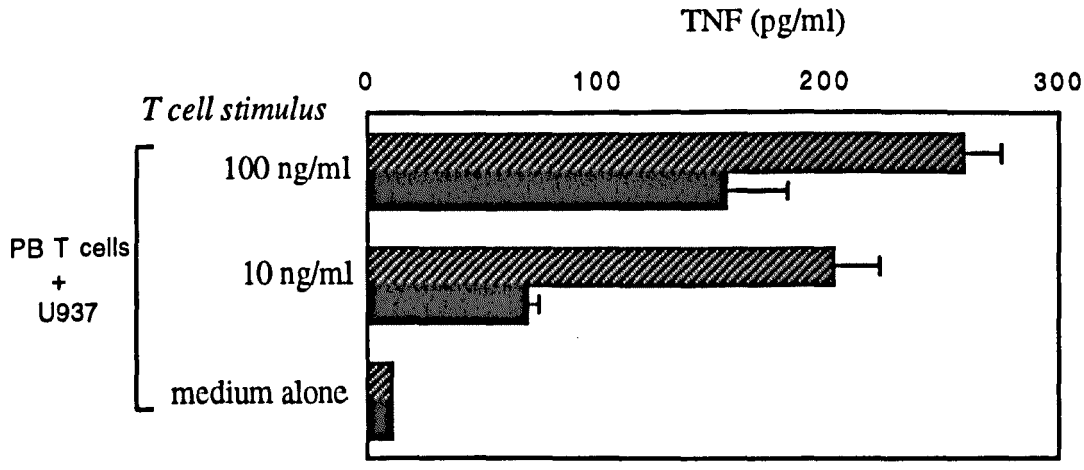
T cell / macrophage cell-contact is required for IL-15-induced TNFα production. PHA (1 μg/ml)/ PMA (1 nM) or IL-15 (100 ng/ml) activated PB T cells were cultured in contact with (solid bars), or separated by semi-permeable membrane from (hatched bars), unprimed U937 cells. IL-15-activated T cells required cell-contact to induce TNFα production by U937 cells. Data are mean ± s.e.m. of triplicate cultures, representative of 3 similar experiments.

between IL-15-activated T cells and macrophages was capable of inducing TNFα production.

To clarify whether T cell cytokine production was required in addition to cell-contact, and to confirm that macrophages were the primary source of TNFα in these mixed cultures, T cells were fixed in 1% paraformaldehyde (PFA) before co-culture with macrophages. PFA fixation is known to prevent T cell cytokine production but preserves cell-membrane integrity providing a simple experimental system for investigation of cell-contact mediated effects (Vey, et al, 1992; Lacraz, et al, 1994). T cells from PB of normal individuals (n=10) were therefore cultured for 72 hours with medium alone, cytokines or, as positive control PHA / PMA, then fixed in 1% PFA. They were thoroughly washed, then co-cultured with U937 cells for a further 48 hours (Lacraz, et al, 1994). Preliminary experiments established that pre-stimulation with 100 ng/ml IL-15 was optimal for induction of T cell-mediated macrophage activation, at a ratio of 8:1 T cells:U937 (Figure 4.4). PFA fixed-T cells, previously stimulated with IL-15, consistently induced production of significant concentrations of TNFα by U937 cells (Figure 4.5a). In contrast, similar prior activation of T cells with IL-2 was effective in inducing TNFα production only in some donors, and did so at lower levels (Figure 4.5a, $p<0.003$ compared with IL-15-activated PB T cells, Wilcoxon). This is unlikely to be due to a dose-response effect, since the concentration of IL-2 used (100 ng/ml) was supra-optimal, and because IL-2 and IL-15 had identical induction profiles for T cell proliferation (Figure 4.1a) and polarization (Wilkinson & Liew, 1995).

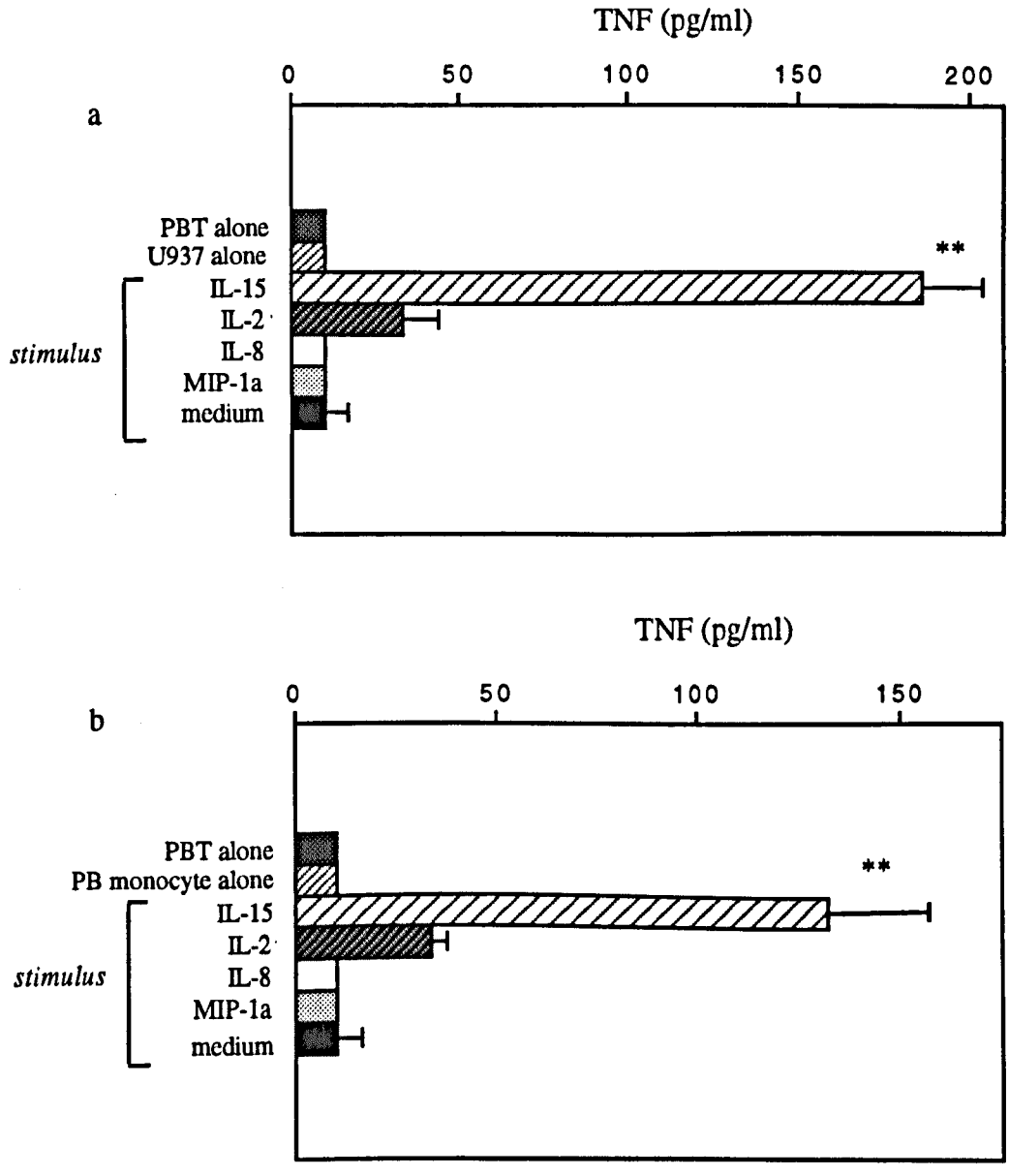
To investigate whether other T cell chemotactic factors might possess similar activity to IL-15, PB T cells were stimulated with IL-8 or MIP-1α at doses known to induce polarization and migration (Wilkinson & Liew, 1995), but no TNFα production was detected in the above culture system (Figure 4.5a). IL-15-activated, PFA-fixed T cells alone were unable to produce TNFα, even after addition of further PHA

Figure 4.4 Dose response of IL-15 mediated U937 cell activation by peripheral blood T cells



PBT cells were stimulated with increasing concentrations of IL-15, or medium alone, after which they were PFA fixed, then co-cultured with unprimed U937 cells for 48 hours at a ratio of either 4:1 (solid bar) or 8:1 (hatched bar). TNFα production was highest with 100 ng/ml IL-15 pre-stimulation, and a final T cell:U937 ratio of 8:1. Data are mean ± s.e.m. for one normal donor, representative of four similar experiments.

Figure 4.5 Activated PB T cells induce TNFα production by U937 and monocytes



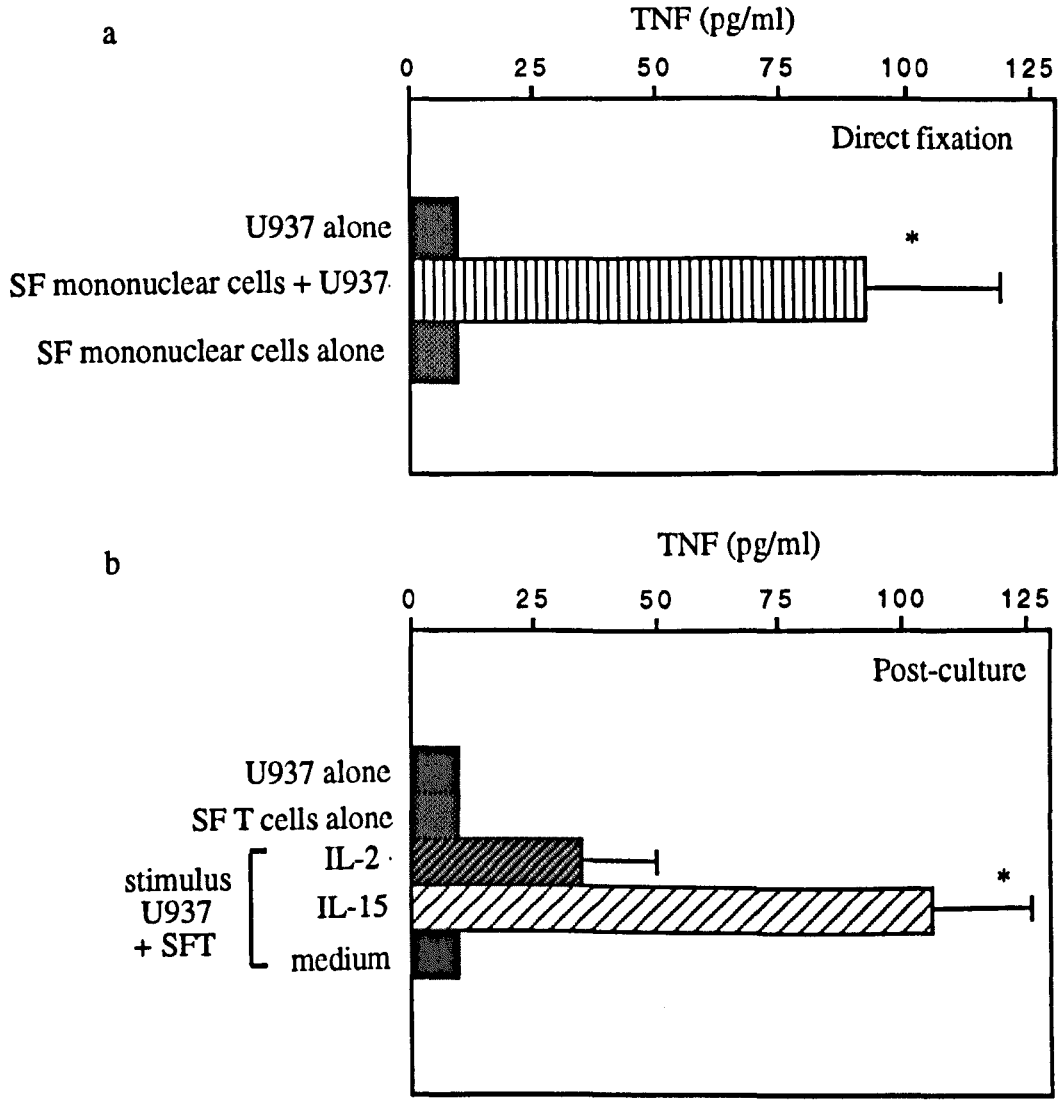
IL-15-activated T cells induce TNFα synthesis by macrophages. PFA-fixed IL-15 (100 ng/ml)-activated PB T cells from normal donors induced TNFα production by (a) U937 cells (n=10), or (b) syngeneic PB monocytes (n=3), at significantly higher levels than did IL-2 (100 ng/ml)-activated T cells (**$p<0.003$). IL-8 (100 ng/ml) or MIP-1α (100 ng/ml) activated T cells (n=2) were ineffective. Sensitivity of TNFα ELISA was 10 pg/ml. Data are mean ± s.e.m.

(1 μg/ml), indicating that macrophages were the source of TNFα in the co-cultures. Identical results were obtained when highly purified syngeneic peripheral blood-derived monocytes (>92% $CD14^+$, <2% $CD3^+$ by FACS analysis) were used instead of U937 cells (Figure 4.5b), demonstrating that these results are unlikely to be due to an allogeneic effect between T cells and macrophages, nor could it be a unique feature of an immortalised macrophage cell line. Finally, separation of IL-15-activated, PFA-fixed T cells from U937 cells in double chamber wells prevented induction of TNFα production confirming that a soluble factor was not involved after fixation.

4.3 Induction of TNFα production by cells of synovial origin.

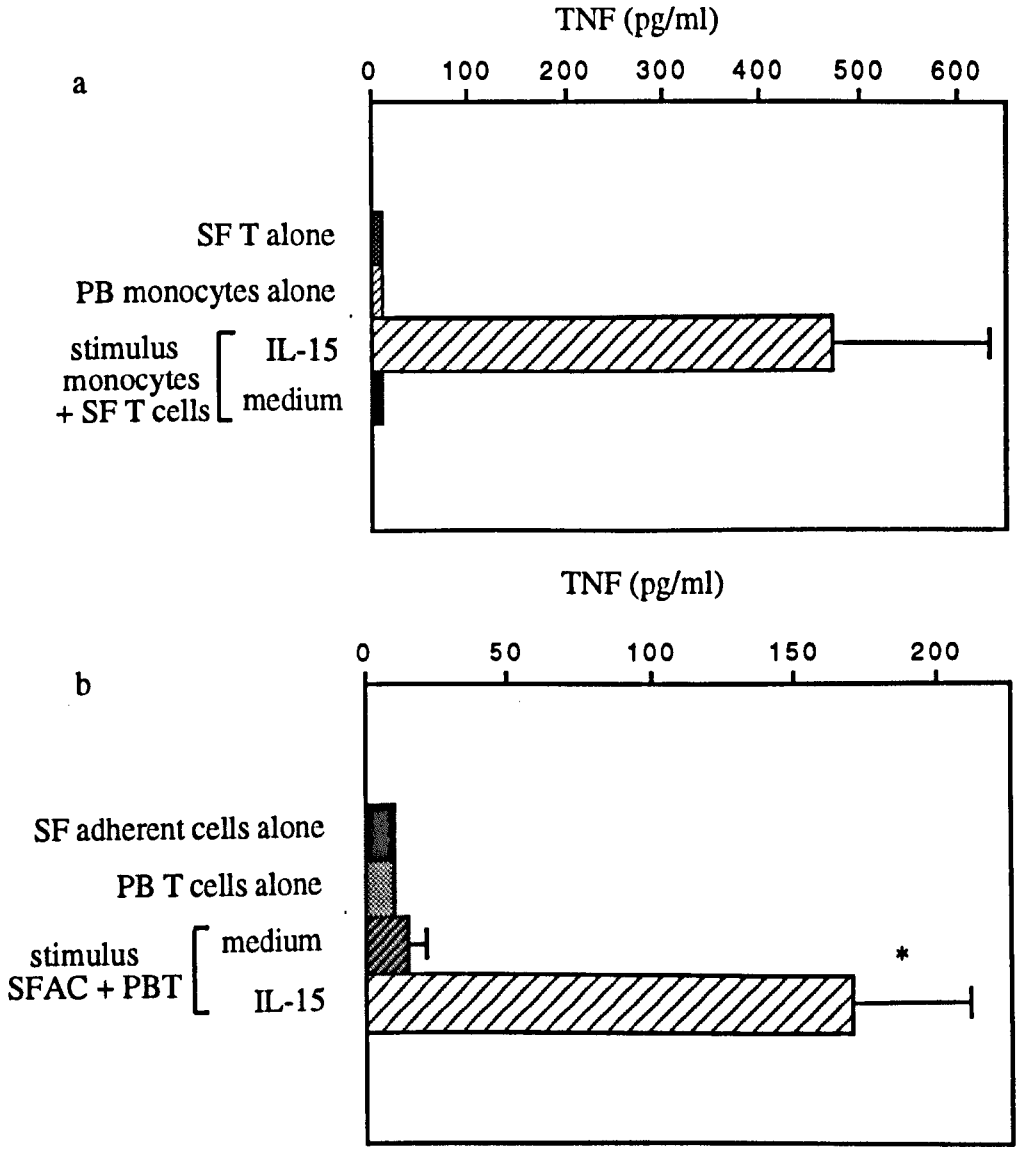
To determine whether a similar mechanism might operate *in vivo* in RA, freshly isolated synovial fluid mononuclear cells from RA patients (n=8) were fixed with 1% PFA without prior stimulation and then added directly to U937 cells. Significant TNFα production was observed after 48 hours in all culture supernatants (Figure 4.6a), demonstrating that synovial T cells may have been sufficiently activated *in vivo* to induce TNFα production by macrophages. It was of interest to determine whether IL-15 was required to maintain this ability of synovial T cells to induce macrophage TNFα synthesis. Synovial T cells were cultured for 24 hours with medium alone, IL-15 or IL-2, then fixed with PFA, before co-culture with U937 cells. TNFα was produced only when synovial T cells were maintained in IL-15 or, to a significantly lesser extent, in IL-2 ($p<0.01$, Figure 4.6b). Similar results were obtained using syngeneic peripheral blood monocytes (instead of U937 cells) which were obtained from each RA patient at the time of joint aspiration (Figure 4.7a). To confirm that synovial macrophage TNFα synthesis could be enhanced by this pathway, PB T cells from 4 RA patients were added to syngeneic synovial macrophage / synoviocyte co-cultures (>75% $CD68^+$, <3% $CD3^+$ by cytoprep analysis). TNFα synthesis was observed only with prior activation of PB T cells by IL-15 (Figure 4.7b). Together, these data demonstrate that synovial T cell-mediated upregulation of TNFα production

Figure 4.6 Synovial T cells induce TNFα production via cell contact



IL-15 is required to sustain synovial T cell mediated cell-contact induction of TNFα synthesis. PFA fixed RA SF T cells induced TNFα synthesis by U937 cells, whether (a) fixed immediately (n=8), or (b) after culture for 24 hours (n=10) in the presence of IL-15 (100 ng/ml). Culture with IL-2 (100 ng/ml) induced significantly less TNFα synthesis (*$p<0.01$ compared with IL-15). Data are mean ± s.e.m.

Figure 4.7 Cell-contact regulates TNFα production by blood monocytes and synovial macrophages



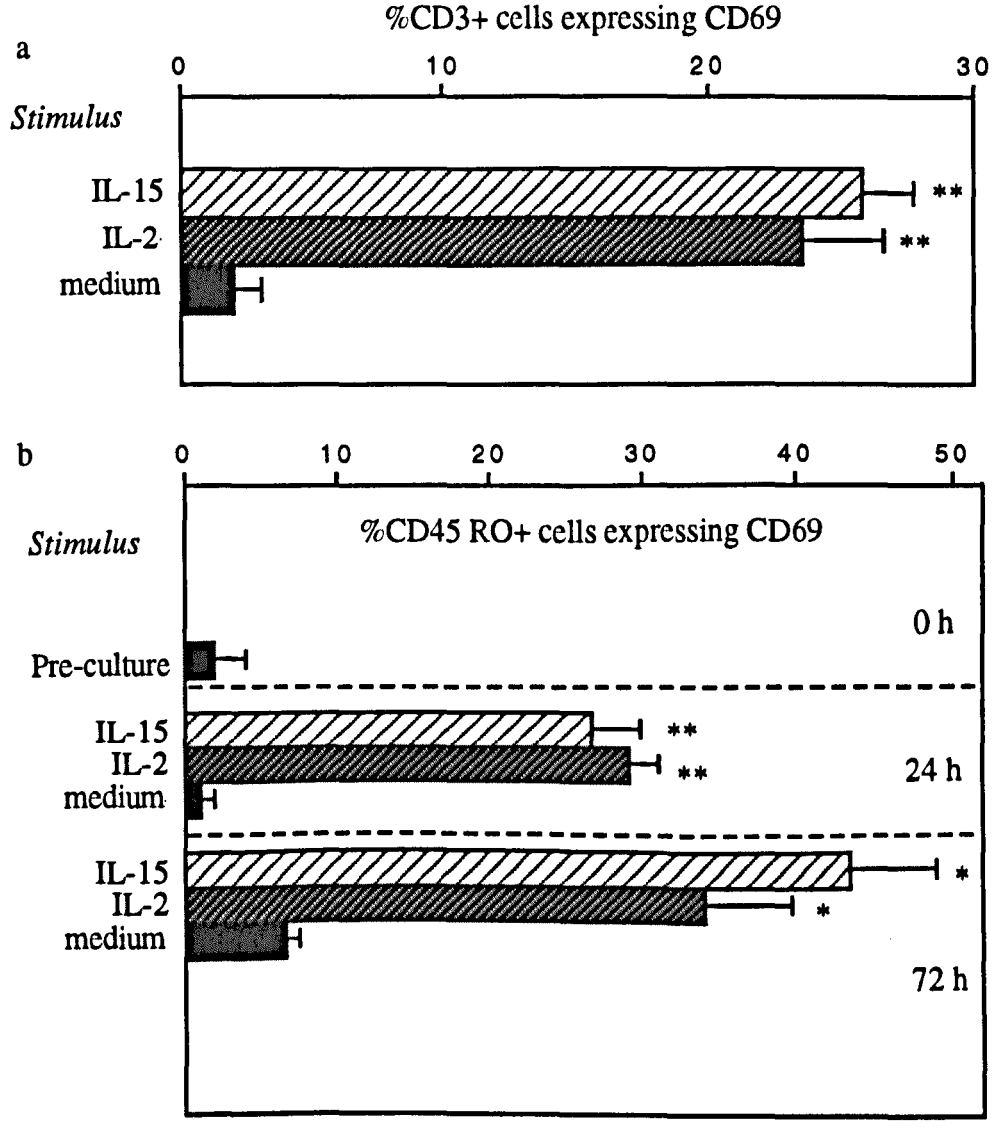
Blood-derived and synovial monocyte / macrophages produce TNFα after cell-contact activation. (a) PFA-fixed RA SF T cells activated by IL-15 (100 ng/ml) induced TNFα synthesis by RA blood monocytes from the same donor patients (n=3). (b) IL-15-activated T cells induced TNFα synthesis by synovial macrophage / synoviocyte co-cultures (*$p<0.01$). PB T cells from RA patients (n=4) were stimulated with medium alone or IL-15 (100 ng/ml) for 72 hours, then PFA fixed prior to addition to synovial macrophage / synoviocyte co-cultures. Data are mean ± s.e.m.

by macrophages through cell-contact can occur in RA, and indicate that IL-15 within the synovial membrane enhances this activity.

4.4 Effect of IL-15 on PB lymphocyte phenotype

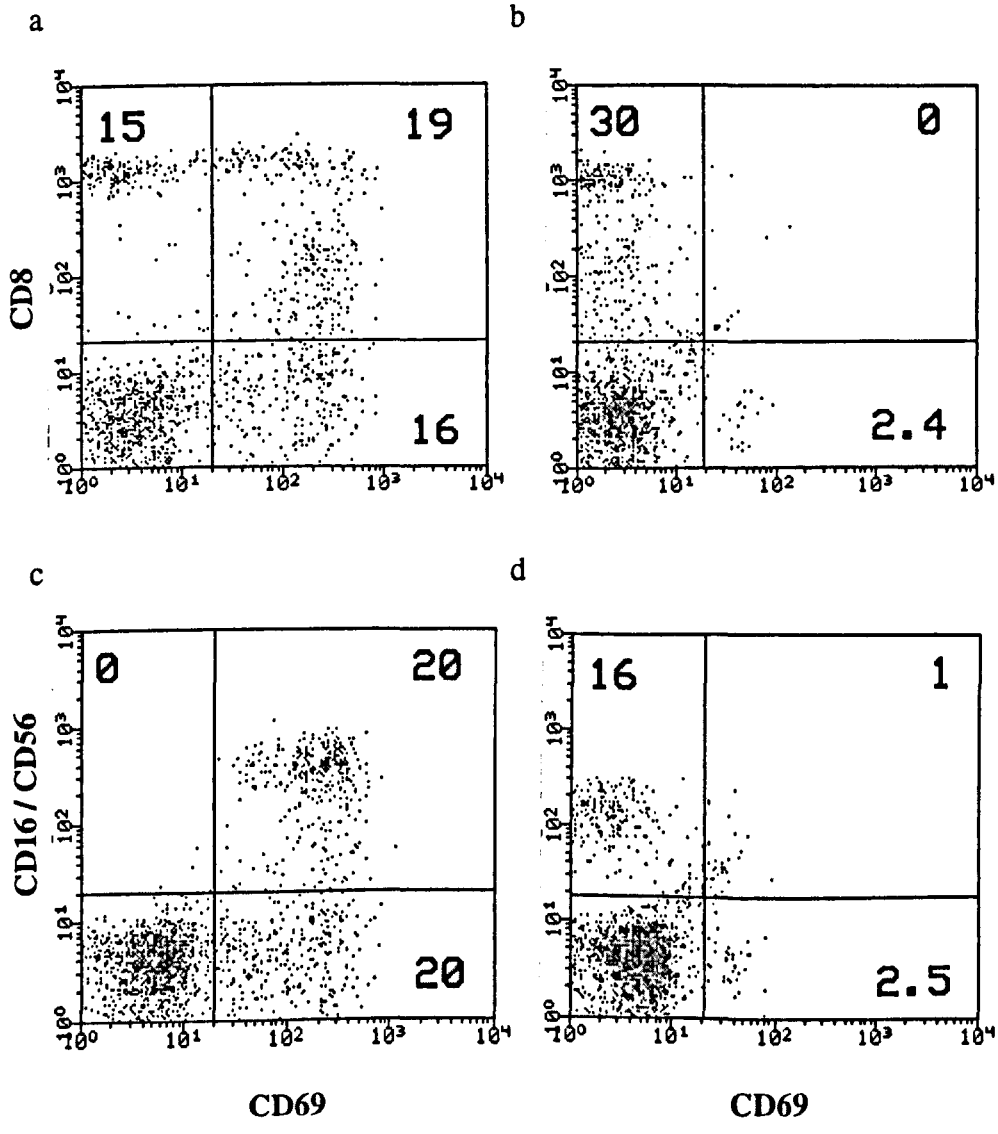
The ability of single cytokines to induce cell division and to sustain cell-contact mediated macrophage activation suggested that the responding cells were of a primed, differentiated T cell subset. Thus, experiments were performed to characterise alterations in T cell-surface phenotype following activation with IL-15, compared with IL-2, on different peripheral blood lymphocyte populations. Whole blood cultures were employed to avoid T cell activation during purification. Both IL-15 and IL-2 up-regulated CD69 expression on $CD3^+$ lymphocytes, which was detectable in whole blood cultures up to 72 hours ($p<0.005$, Figure 4.8a). Since circulating T cells recruited to synovial membrane are predominantly of 'memory' phenotype, the effect of IL-15 on CD69 expression in the $CD45RO^+$ subset was next examined. CD69 expression was elevated within 24 hours, and enhanced seven-fold in $CD45RO^+$ PB T cells by 72 hours after addition of IL-15 *in vitro* ($p<0.02$, Figures 4.8b). In contrast, $CD45RA^+$ cells demonstrated only two-fold enhancement of CD69 levels (Table 4.1), which was probably accounted for by elevated CD69 expression on CD8dim, $CD16^+$, $CD56^+$ NK cells (Figure 4.9), which are recognised to reside in the CD45RA population (Prince, et al, 1992). Modest elevation of CD69 on a small subset of $CD19^+$ B cells was observed (Table 4.1).

Figure 4.8 FACS analysis of PB lymphocytes following cytokine induced activation



IL-15 upregulates expression of CD69 on PB T cells. (a) CD69 expression on $CD3^+$ PB T cells stimulated with IL-15 (100 ng/ml), or IL-2 (100 ng/ml) *in vitro* for 72 hours (n=6, $^{**}p<0.005$ compared with medium alone). (b) CD69 expression was enhanced in $CD45RO^+$ T cells up to 72 hours ($^{*}p<0.02$, $^{**}p<0.002$ compared to medium alone) by addition of IL-15 (100 ng/ml) or IL-2 (100 ng/ml) *in vitro*. No significant difference between IL-15 and IL-2 was detected. Data are mean ± s.e.m.

Figure 4.9 IL-15 upregulates CD69 expression on NK cells



Double label FACS analysis was performed after 24 hours whole blood culture from normal volunteers. CD69 (FITC) expression was increased in $CD8^{dim}$ cells (PE) after culture with (a) rIL-15 (100 ng/ml), but not with (b) medium alone. In the same cultures, $CD16^{+}$, $CD56^{+}$ NK cells (PE) were entirely $CD69^{+}$ after addition of (c) IL-15 (100 ng/ml), but not (d) medium alone. Data are representative of four similar experiments. Figures represent the percentage of gated cells.

Table 4.1 CD69 upregulation by IL-15 and IL-2 *in vitro*

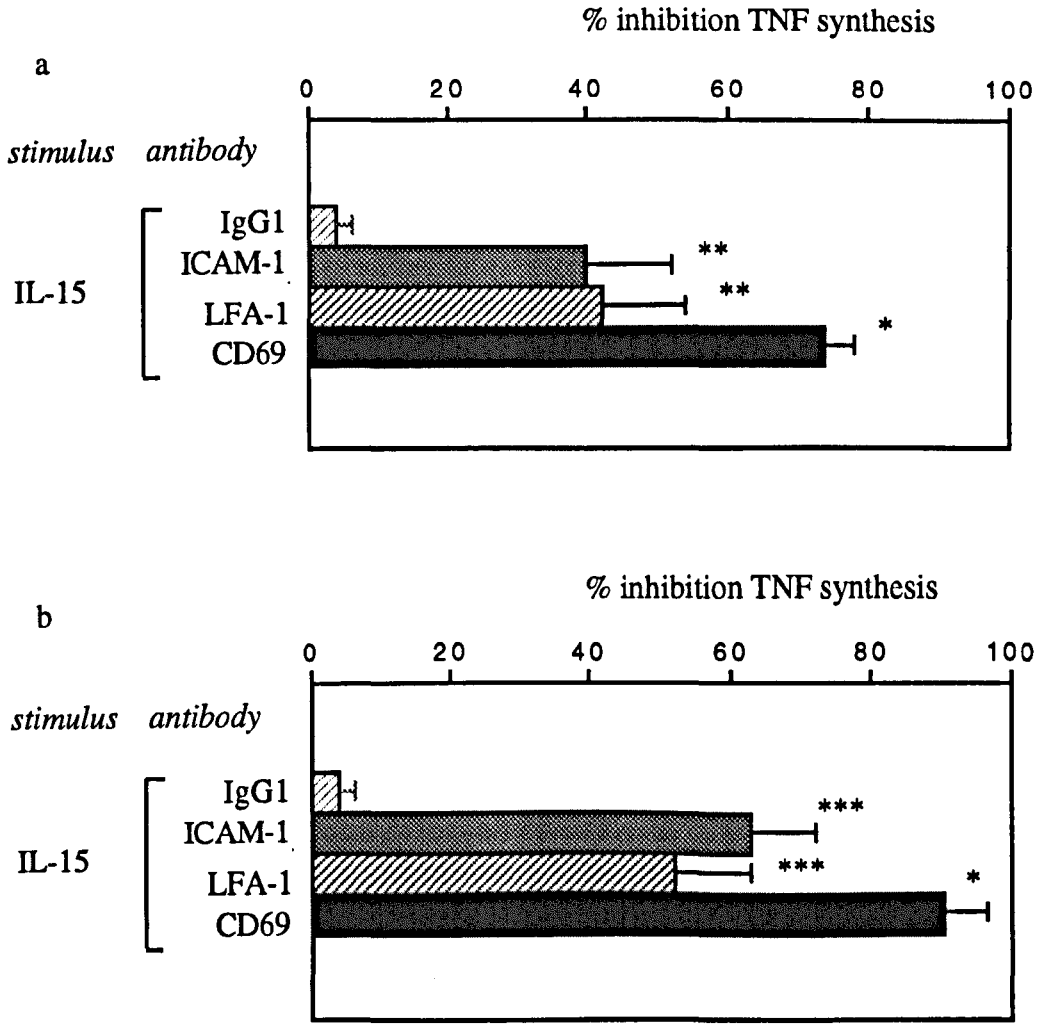
	medium	IL-2	IL-15
n=5		% $CD45RA^+/69^+$	
0 h	<2	<2	<2
24 h	4.8 (0.7)	25 (4)*	25 (4.5)*
72 h	10.4 (4)	25 (4.9)**	24 (4.8)**
n=6		% $CD19^+/69^+$	
0 h	<2	<2	<2
24 h	<2	<2	<2
72 h	<2	5.1 (2.3)	5.1 (2.3)

Whole blood cultures from normal volunteers were maintained up to 72 hours in the presence of medium alone, IL-2 (100 ng/ml) or IL-15 (100 ng/ml). At time points indicated, FACS analysis was performed to determine the percentage of either $CD45RA^+$ cells, or of $CD19^+$ B lymphocytes, expressing CD69. n = number of donor samples cultured *$p<0.05$ comparing IL-15 or IL-2 stimulated cultures vs medium alone, ** not significant, Wilcoxon. Data are mean ± s.d. in parenthesis.

4.5 Molecules involved in T lymphocyte / macrophage cell-contact

Many surface receptors have been implicated in T cell / macrophage membrane contact, including the LFA-1 and CD69 (Vey, et al, 1992; Isler, et al, 1993). The contribution of these markers to the production of TNFα by monocytes after contact with IL-15-activated T cells was therefore investigated. TNFα production by PB T cell / U937 co-cultures (n=4) was significantly reduced by neutralisation of LFA-1 ($p<0.02$) or ICAM-1 ($p<0.03$) and almost completely abrogated by addition of anti-CD69 antibody ($p<0.001$, Figure 4.10a). Increasing the concentration of neutralising antibody (from 5 μg/ml up to 50 μg/ml) did not further inhibit TNFα induction. Parallel experiments (n=4) demonstrated similar involvement of CD69 ($p<0.001$), LFA-1 and ICAM-1 (both $p<0.01$) on the production of TNFα by blood-derived monocytes induced by IL-15-activated T cells (Figure 4.10b). Addition of human IgG (5 μg/ml) to minimise Fc receptor binding on U937 cells and blood monocytes by T cell-surface bound antibody did not reverse the observed inhibition of TNFα production by macrophages. Thus, at least CD69 and the LFA-1 / ICAM-1 pathway were implicated in IL-15-activated T cell-mediated macrophages activation.

Figure 4.10 Inhibition of T cell / macrophage activation by neutralising antibodies



Inhibition of cell-contact induced TNFα synthesis by antibodies to cell surface markers. PFA-fixed IL-15-activated PB T cells were cultured with (a) U937 cells, or (b) PB monocytes. Pre-incubation of the fixed T cells with neutralising antibodies to CD69, LFA-1 or ICAM-1 significantly inhibited TNFα synthesis compared to IgG1 control antibody treated cells (*$p<0.001$, **$p<0.03$, ***$p<0.01$). Human IgG (5 μg/ml) was present in all cultures. Data are mean ± s.e.m. from four separate experiments.

% inhibition was calculated as follows:

$$100 - \left(\left[\frac{\text{TNF}\alpha \text{ synthesis with neutralising antibody present}}{\text{TNF}\alpha \text{ synthesis without neutralising antibody}}\right] \times 100\right)$$

Discussion

Many pro-inflammatory cytokines have been detected in synovial membrane including TNFα, IL-1β, IL-6, IL-8 and GM-CSF (Feldmann, et al, 1996b). Intervention using monoclonal anti-TNFα therapy in murine collagen-induced arthritis (Williams, et al, 1992) and in clinical trials in RA (Elliott, et al, 1994; Rankin, et al, 1995) leads to significant amelioration of joint inflammation. These observations therefore confirm the clinical relevance of such cytokine production within the synovial membrane and, in combination with previous *in vitro* reports, establish the central importance of TNFα in the cytokine cascade which regulates synovitis (Maini, et al, 1995). At present, however, the factors which up regulate TNFα production in RA remain unclear. Data presented here demonstrate that IL-15 is critical in this process.

IL-15-stimulated proliferation and direct TNFα production in T cells derived from synovial fluid was enhanced compared to those from blood, thereby establishing that T cell responsiveness to IL-15 was upregulated *in vivo*. However, T cells represent only a minor source of TNFα compared with macrophages in RA synovium (Chu, et al, 1991). IL-15-activated PB T cells induced significant TNFα production from either unprimed U937 cells, syngeneic blood-derived monocytes, or RA synovial macrophage / synoviocyte cultures, by a cell-contact dependent mechanism. Thus, freshly recruited IL-15-activated T cells may contribute to TNFα synthesis by activating macrophages within the synovial membrane. Furthermore, freshly isolated SF T cells behaved like IL-15 activated PB T cells, indicating that this pathway could operate *in vivo* in RA. IL-15 was required to maintain this activity *in vitro*, because synovial T cells cultured in the absence of IL-15 for 24 hours lost this ability. This is unlikely to be simply the result of apoptosis due to the absence of IL-15, because IL-2, which can rescue T cells from apoptosis *in vitro* through IL-2Rγ chain binding (Akbar, et al, 1996), was incapable of maintaining synovial T cell-contact activity. Thus, synovial T cells under the control of IL-15 are clearly able to activate RA blood

monocytes to produce TNFα. Since recently recruited CD14$^+$ monocyte / macrophages constitute a major source of TNFα in the synovial membrane (Chu, et al, 1991), these data together provide compelling evidence for T cell-contact mediated upregulation of TNFα synthesis by macrophages, driven by IL-15 produced in the synovial membrane.

Pro-inflammatory cytokine and metalloproteinase production following cell contact-mediated activation of macrophages and fibroblasts by T cells and T cell clones stimulated with non-physiological mitogens (PHA, PMA, OKT3) has been reported (Vey, et al, 1992; Lacraz, et al, 1994; Li, et al, 1995; Miltenburg, et al, 1995). Here a cytokine has been employed which is widely distributed in the RA synovial membrane. The synovial T cell population is predominantly CD45RO$^+$, RBdim, CD27$^-$, implying advanced differentiation (Thomas, et al, 1992; Kohem, et al, 1996). They often simultaneously express early (CD69), mid (HLA-DR) and late (VLA1) markers of activation, perhaps initiated by interactions with endothelium during extravasation of mature memory T cells (Burmester, et al, 1987; Pitzalis, et al, 1987; Pitzalis, et al, 1988; Potocnik, et al, 1990; Laffon, et al, 1991; Iannone, et al, 1994; Fernandez-Gutierrez, et al, 1995), and subsequently modified by the cytokine environment within synovial tissue, particularly IL-15. The finding that IL-15-induced CD69 expression was primarily restricted to CD45RO$^+$ T cells is compatible with these observations, and with a recent report comparing the effect of IL-15 on naive and memory T cell CD69 expression (Kanegane, et al, 1996). Despite extensive efforts to determine the synovial T cell repertoire, no consensus has emerged for oligoclonal T cell receptor Vβ gene expression between RA patient cohorts, although some amino-acid conservation of CDR3 regions has been reported, raising the possibility that some synovial T cells are antigen driven (reviewed by Struyk, et al, 1996). The majority of synovial T cells, however, are polyclonal. T cell contact-mediated macrophage activation, driven by IL-15, is therefore consistent with the recognised phenotype and functional profile of synovial T cells. It provides

a pathological role, but implies no local antigen recognition and requires no cytokine secretion, consistent with the polyclonality of synovial T lymphocytes, and their relative absence of cytokine expression.

The cell-contact pathway for macrophage activation by T cells in synovium is likely to utilise multiple cell surface molecules. As expected, the LFA-1 / ICAM-1 pathway was involved. Following chemokine-induced polarization, T lymphoblasts redistribute adhesion molecules, such as ICAM-1 or ICAM-3, to the tips of uropods and IL-15 has recently been shown to exhibit similar properties (Nieto, et al, 1996; Angel del Pozo, et al, 1996). However, since it was not possible to induce macrophage activation by T cells maintained with the T cell chemotactic factors, IL-8 or MIP-1α, it seems probable that IL-15 must exert effects beyond ligand redistribution on the T cell surface. The data also implicated CD69 in cell-contact. Increased CD69 expression occurs *in vivo* on RA synovial T cells (Laffon, et al, 1991; Fernandez-Gutierrez, et al, 1995) and has been reported in other autoimmune diseases, such as multiple sclerosis and chronic active hepatitis at sites of inflammation (Garcia-Monzon, et al, 1990; Perrella, et al, 1993). CD69 appears following TCR / antigen interaction, although IL-2 alone may up regulate expression on NK cells and $CD45RO^+$ lymphocytes (Testi, et al, 1994). Data presented here show that IL-15 shares this activity with IL-2 and is therefore likely to account for the continued expression of CD69 characteristic of synovial T cells, in the relative absence of IL-2 in synovial tissue. Although CD69 has previously been shown to mediate mitogen-induced T cell / macrophage contact (Isler, et al, 1993), the current observations demonstrate that it might subserve this function in RA. Both IL-2 and IL-15 upregulated CD69 expression equally in PB lymphocytes, but IL-2 was significantly less efficient than IL-15 at inducing T cell-dependent macrophage activation, either in PB cells, or in synovial cells. This implies that other receptors which are involved in cell-contact are preferentially upregulated by IL-15, compared to IL-2, and also indicates that the precise combination of surface markers present is

likely to be of critical importance. The widespread expression of CD69 on other circulating cells, such as platelets (Testi, et al, 1990), requires that such additional levels of regulation be present.

The bioactivities described thus far for IL-15 have been broadly similar to those of IL-2 (Grabstein, et al, 1994; Burton, et al, 1994; Tagaya, et al, 1996a). The differential distribution of IL-2Rα and IL-15Rα, however, implies that this may not always be the case (Anderson, et al, 1995b), although functional evidence to support this proposal is currently sparse. Whereas SF T cells proliferated equally to IL-15 and IL-2, differential direct TNFα production was observed. In combination with the divergent effects of IL-15 and IL-2 in generating PB and SF T cell-induced macrophage activation, these findings show, in an immunopathological setting, that IL-15 can exhibit effects distinct from IL-2 on the same target cell population. This has implications for IL-15 receptor expression within the synovial membrane. It is possible that IL-15Rα-chain binding modifies the signal generated by IL-2Rβ or common γ chain occupancy, although the α-chain has no apparent direct role in signal transduction (Giri, et al, 1994; Giri, et al, 1995: Anderson, et al, 1995b). The distribution of IL-15R α-chain compared to IL-2R α-chain in the synovium however, is currently unknown and discordant expression in different subsets may partially explain these data. Recently, however, a novel receptor for IL-15 has been described on mast cells (Tagaya, et al, 1996b), and it remains possible that expression of this or related, as yet undescribed, receptors may explain the divergent functional profile for IL-15 and IL-2 in the synovial T cell population.

In the absence of a clearly identified antigen, non-antigen driven processes within the RA synovial membrane offer the best targets for therapeutic intervention (Miossec, et al, 1996). It is attractive to hypothesise that macrophage or fibroblast derived IL-15 recruits and further activates circulating memory T cells in the synovial membrane. Under the continuing influence of IL-15, newly arrived T cells within the synovial

membrane can up regulate TNFα production by macrophages, predominantly through cell membrane contact. This may generate a positive feedback loop, whereby IL-15 produced by activated macrophages maintains T cell-induced synthesis of TNFα, which can further activate macrophages to produce IL-15. This proposal predicts that anti-T cell therapies which not only inhibit T cell activation, but also deplete T cells from the synovial compartment will be most successful. Anti-T cell monoclonal antibody therapies in RA have been less successful than direct anti-cytokine approaches, perhaps reflecting inadequate T cell depletion within the synovial compartment. It is of interest that RA clinical improvement following anti-CD4 therapy correlates with synovial T cell coating with anti-CD4 (Tak, et al, 1995; Choy, et al, 1996). Anti-IL-15 antibody or anti-IL-15Rα antagonists therefore represent potential approaches to attenuating RA and perhaps other inflammatory diseases. Further efforts are now required to characterise other cell surface molecules involved in T cell / macrophage contact and to establish which factors up regulate IL-15 production, thereby generating further rational targets for novel therapy.

Chapter 5

Nitric oxide production within human synovial membrane

Introduction

Critical immuno-regulatory and effector activities have been attributed to nitric oxide (NO) in several models of infection and inflammation (Nathan & Xie, 1994a; Lyons, 1995). NO is produced constitutively in small amounts by endothelial NO synthase (eNOS), or by neuronal NO synthase (nNOS) and in higher concentrations by inducible NO synthase (iNOS) following stimulation by bacterial products, including LPS, or by cytokines, including IFN-γ, TNFα and IL-1β (Bredt & Snyder, 1994; Nathan & Xie 1994b). All NOS isoforms may generate NO in inflammatory lesions, although in the chronic phase, iNOS is likely to provide the principal contribution.

Considerable circumstantial evidence indicates that NO production is important in autoimmune pathology. Inducible NOS expression and increased NO generation have been implicated in murine streptozocin-induced diabetes (Lukic, et al, 1991; Kolb & Kolb-Bachofen 1992) and experimental allergic encephalomyelitis (Zhao, et al, 1996). Similarly, articular pathology in MRL-*lpr/lpr* mice (Weinberg, et al, 1995; Huang, et al, 1996) and streptococcal cell wall and adjuvant arthritis models in the rat are dependent on NO production. iNOS activity can be detected within the synovial membrane by northern blot analysis and NO is produced by synovial explant culture (McCartney-Francis, et al, 1993; Ialenti, et al, 1993; Stefanovic-Racic, et al, 1994a).

Their observed phenotype and cytokine production profile indicates that activated synovial macrophages are of central importance in RA pathogenesis (Feldmann, et al, 1996a). Whereas, in animal models, NO synthesis occurs predominantly in macrophages, the capacity for NO synthesis by human macrophages is currently controversial. This has raised doubts as to the origin of NO in human diseases in which macrophage activation is a central feature, such as RA. NO generation within inflammatory foci may differ between rodents and humans, and data from animal models therefore require cautious interpretation. However, several other cell types

normally found in RA synovium are capable of inducible NO production. Rodent synoviocytes (Stefanovic-Racic et al 1994b) and rodent and human neutrophils, chondrocytes, osteoblasts and mast cells (Stadler, et al, 1991; Charles, et al, 1993; Stefanovic-Racic, et al, 1993; Barnes & Liew, 1994; Ralston, et al, 1995) have been clearly shown to generate significant levels of NO *in vitro*.

Evidence for NO production within human synovium has, until now, been indirect. Preliminary studies indicate that nitrite concentrations are higher in synovial fluid than in serum in RA patients (Farrell, et al, 1992). 3-nitrotyrosine is detectable in synovial fluid and serum from RA, but not from OA patients or normal donors (Kaur & Halliwell, 1994) and elevated urinary nitrate:creatinine ratios are found in active RA patients (Grabowski, et al, 1996). These data, however, do not address either the precise tissue or cellular location of NO synthesis. In particular, the relative contribution of cartilage and synovial membrane remains unclear since direct NO production *in vitro* has not been reliably demonstrated from human synovial tissue.

In this context, it was clearly important to establish whether human synovial membrane was indeed capable of significant NO production and thereafter, to determine the cellular location of such synthesis, if present.

5.1 Assay for nitrite in biological fluids

NO production was estimated by the concentration of its oxidative products nitrite and nitrate ions in culture supernatants, serum and synovial fluid. Two methods were employed.

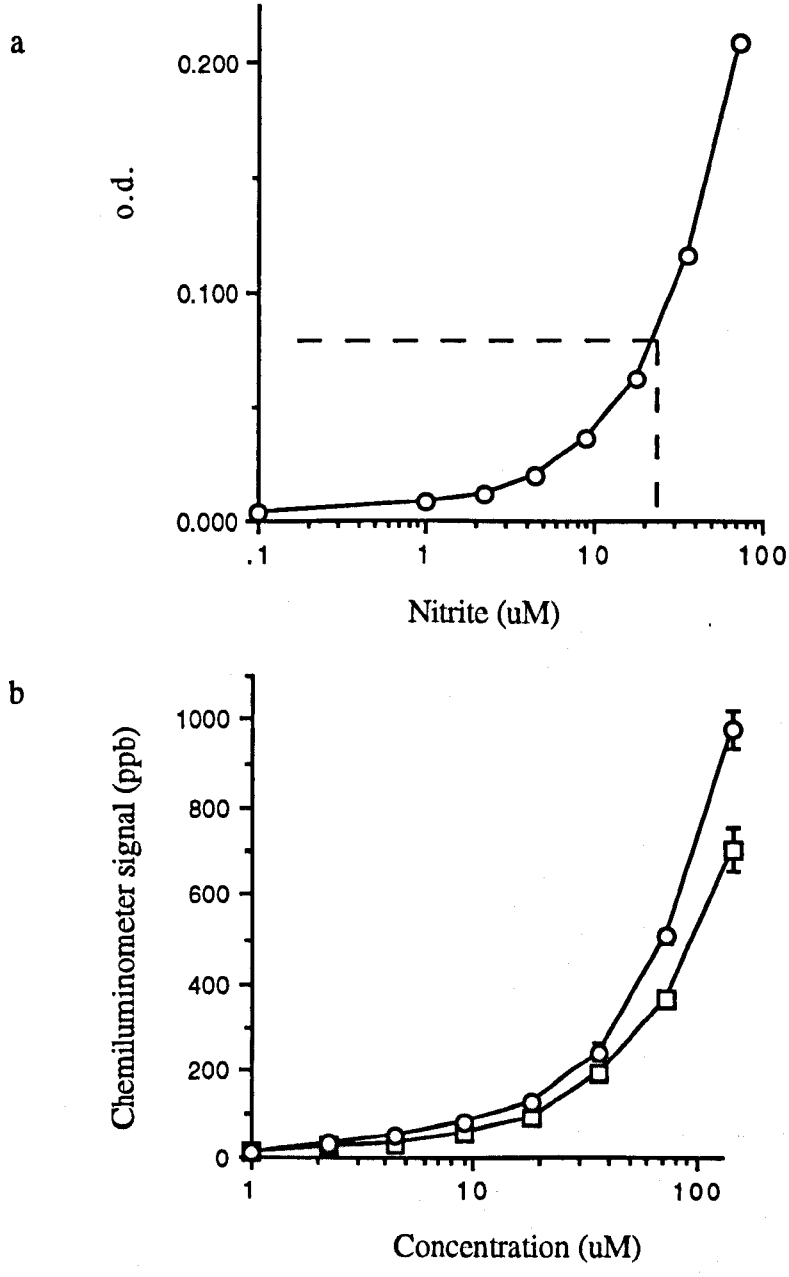
(a) The Griess reaction was performed to detect nitrite in either fresh, or previously frozen (-20°C) culture supernatants, with typical sensitivity of 2 μM (Figure 5.1a).

(b) The Griess reaction develops at acid pH raising the possibility of protein precipitation in test samples. Serum or synovial fluid nitrite levels were therefore measured by chemiluminescence, after reduction of nitrate to nitrite using nitrate reductase. A typical standard curve is shown in Figure 5.1b, demonstrating sensitivity of 2 μM.

5.2 Nitrate / nitrite in rheumatoid synovial fluid and serum

Matched cell-free synovial fluid and serum samples were collected from 17 patients with RA and stored at -70°C prior to assay for nitrite / nitrate concentration using chemiluminescence. Figure 5.2a shows that the concentration of nitrite, representing total nitrite / nitrate concentration after enzymatic nitrate reduction, was not significantly different in paired RA serum or synovial fluid (Wilcoxon). The mean concentration of either was not different from total nitrite / nitrate levels measured in serum from 17 age and sex matched normal controls (Figure 5.2b; Mann-Whitney). Neither the Griess reaction, nor the chemiluminescence assay was sufficiently sensitive to detect nitrite concentration alone in serum or synovial fluid samples, without prior conversion of nitrate, and it was not therefore possible to confirm or refute the data of Farrell et al (1992). Moreover, patient samples were usually collected on routine outpatient visits, with no prior dietary modification, and it is therefore likely that the effect of dietary nitrate rendered the detection of measurable or significant differences improbable (Green, et al, 1982; Knight, et al, 1987). The

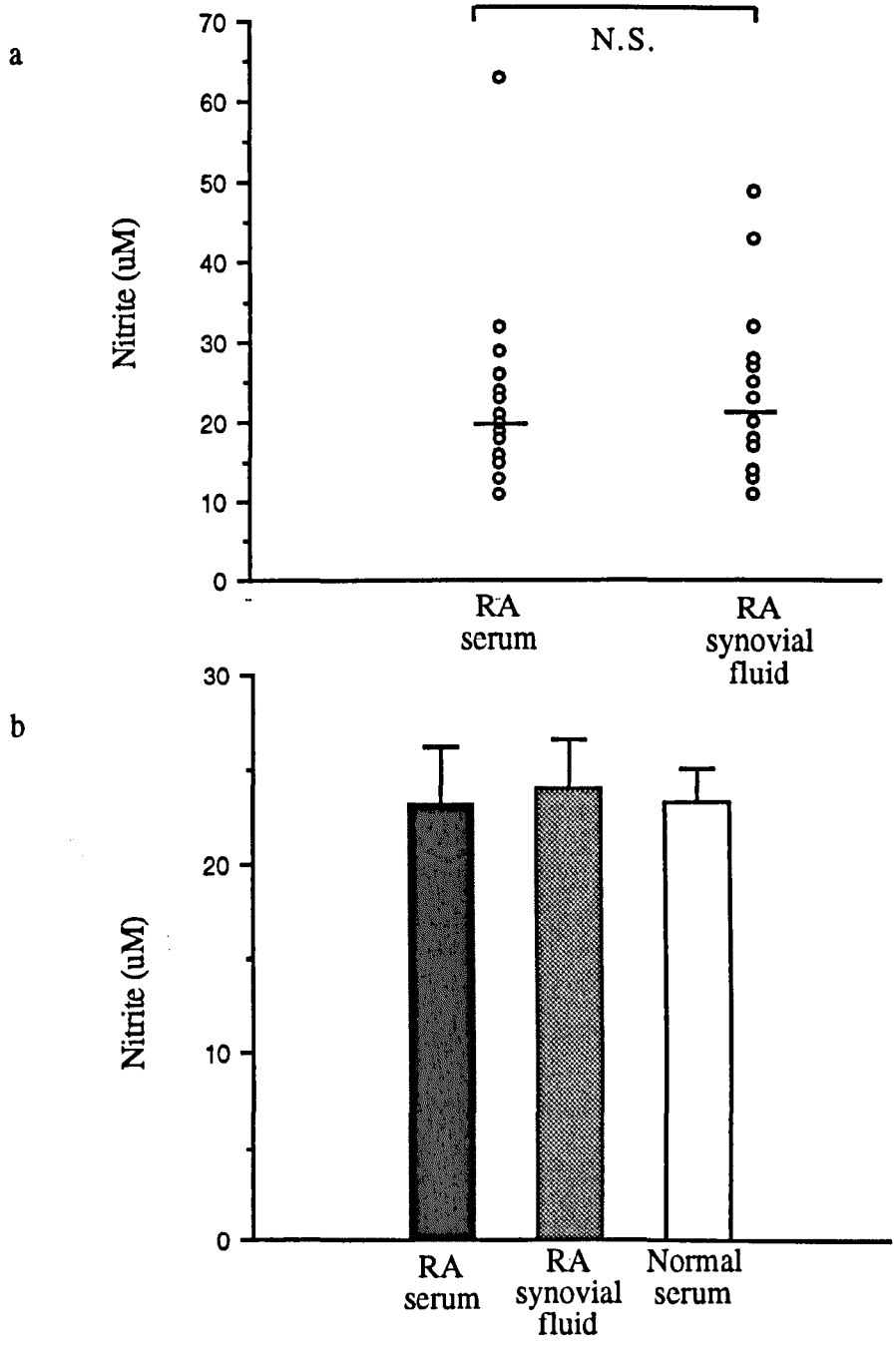
Figure 5.1 Standard curves for nitrite / nitrate estimation



(a) Standard curve derived from triplicate sodium nitrite dilutions in Dulbecco's MEM estimated by the Griess reaction as described in section 2.10.1. Calculation of sample nitrite concentration was as shown (dotted lines).

(b) Standard curves derived from dilutions of sodium nitrite (O) and sodium nitrate (□) in Dulbecco's MEM estimated by chemiluminescence as described in section 2.10.2.

Figure 5.2 Nitrate concentration in serum and synovial fluid in RA



Total nitrate / nitrite levels were assayed using chemiluminescence (section 2.10.2).

(a) Paired synovial fluids and serums from 17 RA patients were compared for nitrate / nitrite levels. No significant difference between serum and synovial samples was observed (paired t-test).

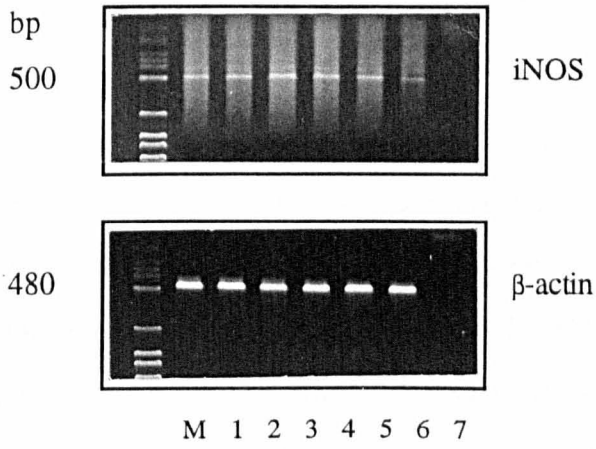
(b) Nitrate / nitrite levels were compared in serum from RA and age / sex matched controls (n=17). No difference between the group means was observed (Mann-Whitney). RA synovial fluid group mean is shown for comparison.

required low nitrate diet was investigated, but proved unpalatable, and was not pursued for ethical reasons. Moreover, Grabowski, et al (1996) subsequently developed a urinary nitrate:creatinine ratio for clinical follow-up, which clearly indicated that nitrate turnover was elevated in active RA, which obviated further investigation.

5.3 Expression of iNOS mRNA in RA synovial membrane

Inducible NOS expression in human synovium was determined first at the mRNA level. RT-PCR was performed using primers specific for human chondrocyte iNOS (Charles, et al, 1993). Preliminary experiments using snap-frozen leukocyte pellets from freshly isolated synovial fluid from 5 RA patients contained no iNOS mRNA, although satisfactory positive control amplification was observed from a plasmid containing human iNOS cDNA. RT-PCR was therefore performed, using the same primers, on 4 RA synovial membrane samples frozen immediately after surgical excision (Figure 5.3). In all 4 tissues, PCR generated the expected 500 bp product, consistent with that predicted from human chondrocyte iNOS. This product was sequenced by Dr X.Q. Wei (Dept. of Immunology, University of Glasgow) and found to be identical to human iNOS (Charles et al 1993), thereby confirming the specificity of the reaction. For control, β-actin was amplified, as was cDNA prepared from OA synovial tissue. The latter also contained iNOS mRNA (Figure 5.3). Thus, iNOS expression was detectable at the mRNA level in both RA and OA synovial tissue.

Figure 5.3 RT-PCR for iNOS in human synovial membrane



RT-PCR was performed to detect human iNOS mRNA expression in freshly isolated RA and OA synovial tissue samples. Lanes 1-4 - representative RA patients, lane 5 - representative OA patient, lane 6 - positive control (human iNOS cDNA), lane 7 - negative control (dH_2O + reagents). Subsequent sequencing showed that the PCR product was identical to human iNOS. β-actin mRNA was amplified for control.

5.3 Nitric oxide production in RA synovial membrane

The principal objective of this study was to obtain direct evidence for NO production by human synovial tissue. Primary heterogeneous cultures were therefore established from samples obtained at primary hip or knee arthroplasty. Synovial membranes were collected from 13 patients with RA (mean age 62 years [range 28-83], mean disease duration 14.7 years [range 5-22], 78% rheumatoid factor positivity) and single cell suspensions were cultured at $2x10^6$/ml before nitrite measurement using the Griess reaction. Spontaneous nitrite generation was measured in 9 of 13 RA culture supernatants after 72 hours (Table 5.1). The level of nitrite production varied considerably (mean ± s.e.m. 9.6 ± 3.6 μM [range 3.2 - 51]) presumably reflecting clinical and cellular heterogeneity. No obvious association with specific DMARD therapy, nor correlation with age, disease duration or serum acute phase response (ESR, CRP) was observed (Pearson's correlation coefficient). To confirm that bacterial contamination of the collagenase preparation was not responsible for 'spontaneous' NOS expression, $4x10^6$ /ml J774 cells were incubated with 5 mg/ml collagenase for 2 hours at 37°C, then cultured for a further 24 hours. Although J774 cells are capable of high output of NO, no nitrite production was detected under these conditions. These data demonstrate that NO production is a feature of RA synovial tissue and indicate that iNOS activity has been initiated *in vivo*.

5.4 Induction of NO production *in vitro* in RA synovial membrane

In contrast to the situation in rodents, the precise cytokine requirements for activation of iNOS in human pro-inflammatory cells remain unclear. The superantigen, staphylococcal enterotoxin B (SEB), induces T lymphocyte proliferation and cytokine secretion through interaction with the T cell receptor and can also up regulate mononuclear cell cytokine expression following MHC class II binding (Marrack & Kappler, 1990; Trede, et al, 1993). Moreover, SEB stimulates NO production by

Table 5.1 Production of nitric oxide by primary human synovial cultures

	Disease Features		Nitric oxide production			
Patients	Duration (yrs)	DMARD	Unstimulated		SEB†	
			nitrite (μM)			
RA1	14	H	9.3	(3.0)	33	(7)*
RA2	20	M	13	(1.7)	34	(0.5)
RA3	5	M+H+P	51	(2.7)	131	(6)
RA4	13	G	<2		6	(2.2)
RA5	9	M	10.5	(1.3)	16.2	(0.5)
RA6	19	A+P	<2		11.7	(3.2)*
RA7	22	S+P	10.1	(0.3)	32	(5.9)
RA8	20	-	<2		<2	
RA9	15	D	10.4	(2)	8.8	(1.0)*
RA10	20	-	5.2	(1.2)	6.7	(2.8)*
RA11	10	-	3.2	(0.5)	44	(8)
RA12	10	M	4.3	(0.5)	41	(3.9)
RA13	15	-	<2		19	(2.5)

Production of nitrite (μM /10^6 cells) by RA synovial cells *in vitro*. Values are mean of triplicate nitrite assay of each of triplicate cultures (s.d.) after 72 hours. SEB stimulated RA synovial tissues produce enhanced nitrite levels ($p<0.01$, Wilcoxon).

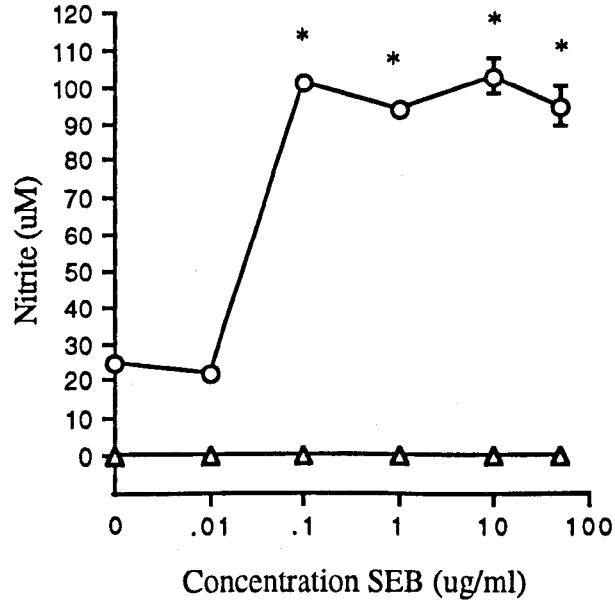
†Data shown are for optimal concentration of SEB (range 1-10 μg /ml) for each patient. * 1 μg/ml LPS present in culture.

Disease modifying anti-rheumatic drugs (DMARD): - G - IM sodium aurothiomalate; S - sulphasalazine; M - methotrexate; H - hydroxychloroquine; D - penicillamine; A - azathioprine; P - prednisolone.

murine macrophages in the presence of T lymphocytes (Isobe & Nakashima, 1992). It was first necessary to clarify whether this effect was through direct iNOS activation, or through T cell activation and subsequent IFN-γ production. Preliminary experiments were performed using J774 cells which showed that SEB addition alone did not induce NO production (Figure 5.4). However, SEB augmented the production of nitrite by IFN-γ primed J774 cells in the absence of LPS (Figure 5.4). This finding established that SEB can act directly on appropriately primed target cells to induce NOS activity, presumably through MHC class II binding. Since several synovial cell species express high levels of MHC class II (Burmester, et al, 1987; Pitzalis, et al, 1987), the effect of SEB on NO production by RA primary synovial cultures was therefore investigated.

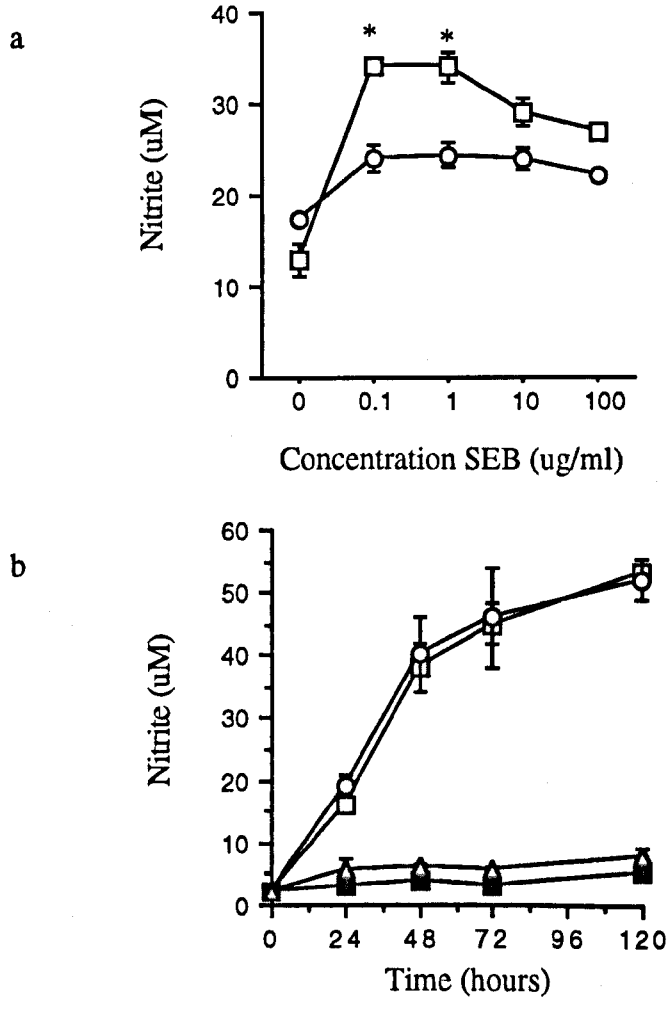
SEB induced dose dependent production of nitrite in RA synovial cultures (Figure 5.5a) which increased up to 72 hours, but not thereafter (Figure 5.5b). All patient samples were therefore compared for peak nitrite production at 1 or 10 μg/ml SEB after 72 hours. The addition of SEB induced novel or significantly increased nitrite production in 12 of 13 RA tissues examined ($p<0.01$, Wilcoxon, Table 5.1). LPS (1 μg/ml) alone was less effective than SEB in inducing nitrite production (Figure 5.5a, Table 5.2), indeed, addition of LPS (0.1-10 μg/ml) inhibited SEB-mediated enhancement in 7 of 7 RA patients studied (Figure 5.5b, Table 5.2; $p<0.02$). This may reflect the synthesis of inhibitory cytokines, such as IL-10 or TGFβ. Production of nitrite *in vitro* was completely inhibited by addition of 1 mM L-NMMA. L-NMMA did not significantly reduce synovial cell viability by trypan blue exclusion - >80% at 72 hours in presence or absence of 1mM L-NMMA. These observations demonstrate that enhancement of basal NO production through an L-arginine dependent pathway is possible within the inflamed human synovial membrane in RA patients.

Figure 5.4 SEB induces NO production by IFN-γ stimulated J774 cells



The production of NO by J774 cells in response to SEB was investigated either alone (Δ), or in combination with 40 I.U./ml IFN-γ (O). Triplicate culture supernatants were frozen after 24 hours and nitrite measured by Griess reaction. Representative of three similar experiments. Data are mean ± s.e.m. *$p<0.005$ comparing SEB + IFN-γ with IFN-γ alone.

Figure 5.5 Production of NO by RA synovial membrane cultures



(a) Dose response for SEB-induced nitrite production, with or without LPS, by heterogeneous synovial cells from representative patient RA12 (**$p<0.005$ compared with basal levels). LPS addition reduced SEB-induced NO production (*$p<0.05$ compared with SEB alone). □ SEB alone; ○ SEB + LPS 1 μg/ml

(b) Time course of nitrite production by synovial tissue from RA11. Parallel cultures were established and harvested at the time points indicated, and stored prior to simultaneous nitrite assay. Production with SEB was significantly greater than basal levels ($p<0.001$, Mann-Whitney) at all time points from 24 hours. Data are mean ± s.e.m., representing 4 similar experiments.

□ SEB 1μg/ml, ○ SEB 1μg/ml + LPS 1μg/ml,

■ SEB 1μg/ml + L-NMMA 1mM, △ LPS 1μg/ml

Table 5.2 Effect of LPS on RA synovial membrane NO production

Patient	Unstimulated	LPS¶	SEB†	SEB + LPS*
		nitrite (μM)		
RA2	13 (1.7)	17 (2.3)	34 (0.5)	25 (1.4)
RA3	51 (2.7)	87 (2)	131 (6)	111 (3.5)
RA4	<2	3 (0.7)	6 (2.2)	3.1 (0.8)
RA5	10.5 (1.3)	8.9 (1.8)	16.2 (0.5)	14.6 (1)
RA7	10.1 (0.3)	21 (6)	32 (5.9)	28 (2.5)
RA11	3.2 (0.5)	6 (0.4)	46 (8)	41 (3.7)
RA12	4.3 (0.5)	8 (1.4)	41 (3.9)	34 (2.9)

Production of NO (μM /10^6 cells) by RA synovial cells *in vitro*. Values are mean of triplicate nitrite assay of each of triplicate cultures (s.d.) after 72 hours. ¶LPS (1 μg/ml) alone induced significantly less nitrite production than SEB alone ($p<0.04$, Wilcoxon). *LPS also significantly inhibited SEB enhanced nitrite levels ($p<0.02$, Wilcoxon). †Data shown are for optimal concentration of SEB (range 1-10 μg /ml) for each patient.

5.5 Cross-reacting antibodies against iNOS in synovial membrane

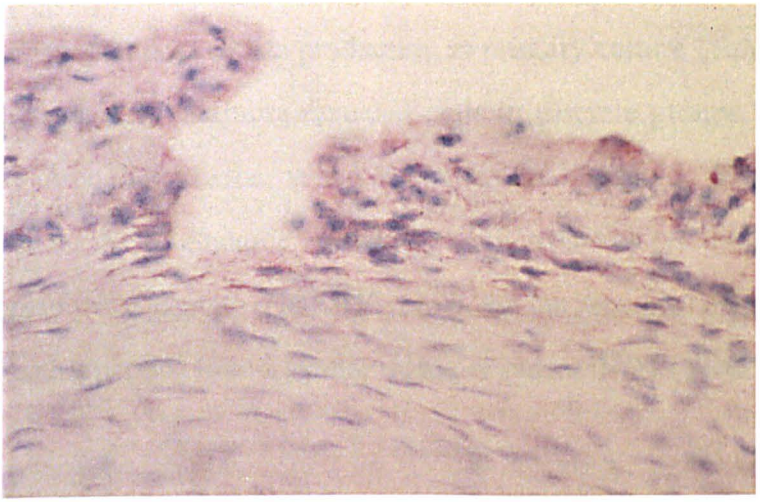
Initial efforts to localise iNOS in human synovial tissues employed cross-reacting antibodies developed against rodent iNOS, raising the possibility of target non-specificity or of cross-reactivity with other NOS isoforms, or with P450 reductase enzymes (Bredt & Snyder, 1994). The antibodies used and ICC conditions are detailed in table 2.1. Both monoclonal and polyclonal anti-murine iNOS antibodies exhibited extensive synovial lining layer and blood vessel staining (Figure 5.6a). Non-specific nuclear cross-reactivity was observed with the rabbit polyclonal anti-murine iNOS from Affiniti (Figure 5.6b). Similarly, rabbit (h13) and sheep anti-human iNOS products (both Wellcome) exhibited widespread lining layer staining. However, since none of the staining patterns observed was neutralised by prior incubation with 50 μg/ml human recombinant iNOS (riNOS) protein, and because no consistent staining pattern emerged, considerable doubt remained as to the specificity of the available reagents.

5.6 Tissue localisation of iNOS in RA synovial membrane

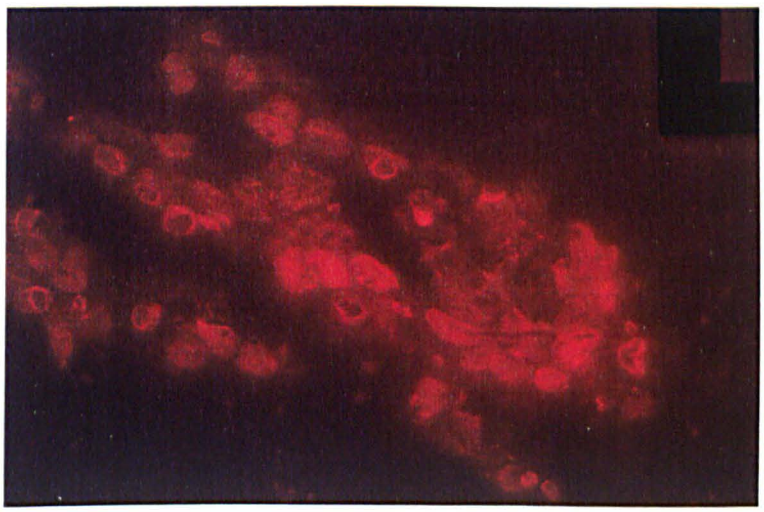
To resolve such concern, a rabbit antiserum raised specifically against the C-terminal heptamer peptide (YRASLEMSAL) of human iNOS was obtained (NO53; Nicholson, et al, 1996). This sequence is not present on either eNOS or cNOS, nor on rodent iNOS. This antiserum detected human iNOS in ELISA at a dilution of 1:400,000 (personal communication Dr. H.R. Williams, Merck Research Laboratories, NJ, USA) and recognised neither human eNOS nor the C-terminal of rodent iNOS by ELISA. Preliminary antibody concentration-range studies were performed which established positive staining of human RA synovial tissue down to 1:40,000 serum dilution, although 1:20,000 dilution gave optimal tissue localisation and was used subsequently.

Figure 5.6 Anti-rodent iNOS antibodies bind non-specifically in human RA synovial membrane

a



b



RA synovial sections were stained with (a) polyclonal anti-murine iNOS (Wellcome) and localised with fast red. Extensive lining layer staining was detected which could not be neutralised by prior incubation with human iNOS (50 μg/ml). (b) Nuclear cross-reactivity was evident following staining with anti-murine iNOS (Affiniti) localised with lissamine rhodamine.

(Magnification x 250).

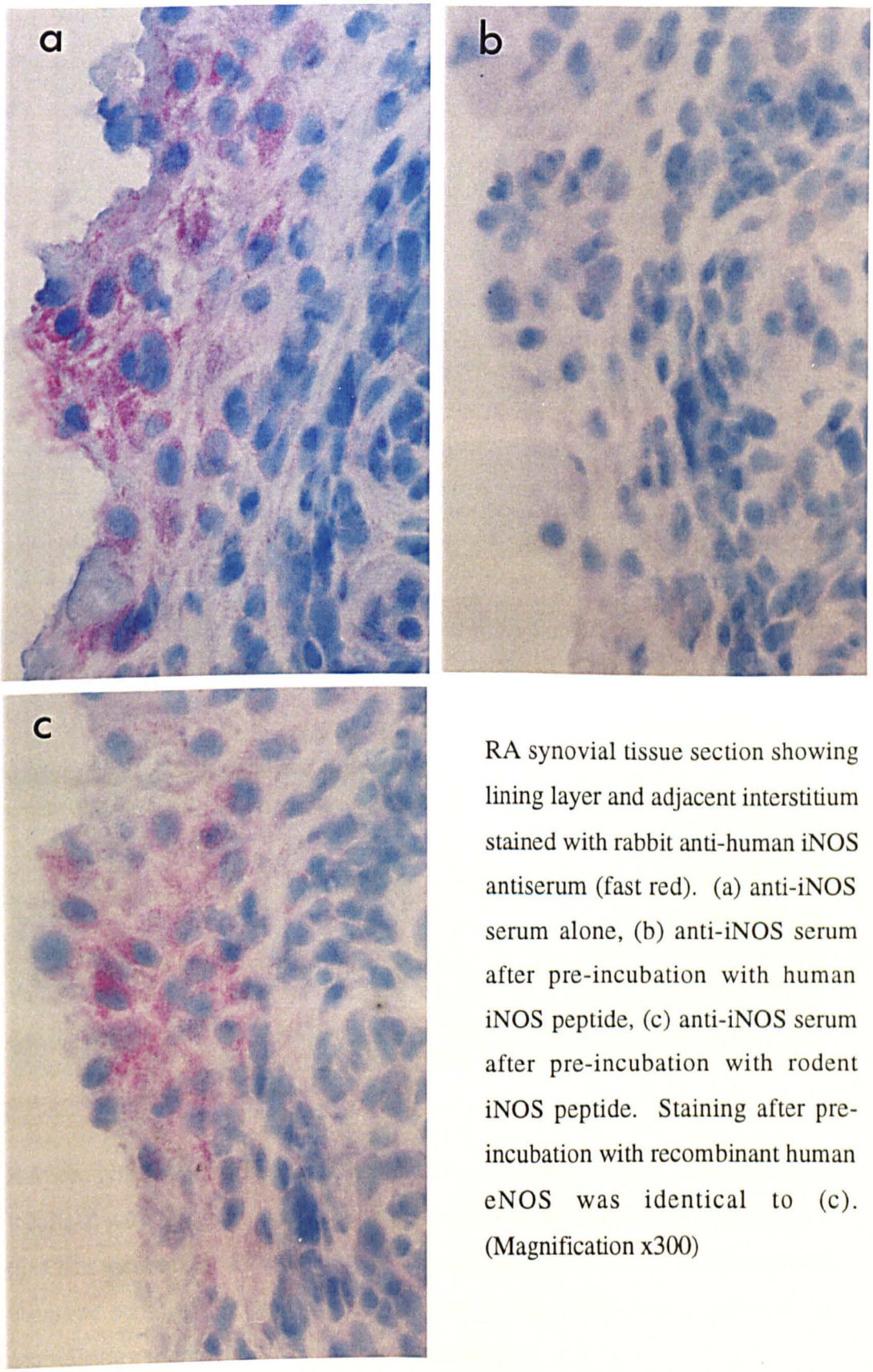
Light microscopy with this antibody demonstrated intra-cytoplasmic staining in cells both within, and deep to, the synovial lining layer (Figure 5.7a). The lining layer contained significantly greater numbers of $iNOS^+$ cells than interstitial or lymphocytic aggregate areas (Table 5.3, $p<0.01$, Mann Whitney). The frequency of positive cells for each area varied considerably between different patients, as predicted by the heterogeneity of spontaneous nitrite production in primary culture (Table 5.1), and within individual tissues. Staining revealed cells in discrete groups, rather than spread diffusely through the tissue, as had been a feature of staining with antibodies raised against rodent iNOS. Staining for iNOS was abolished by prior addition of the immunising human iNOS peptide (NO54; Figure 5.7b), but remained unchanged after pre-incubation with a peptide from a similar region of rodent iNOS (YEEPKATRL)

Table 5.3 Histological distribution of iNOS in RA synovial membrane.

	% cells iNOS positive		
n=number fields	Lining layer n=36	Interstitium n=24	Aggregate n=31
mean ± s.d. (range)	19 ± 13.1 * (2-56)	5 ± 5.6 (0-20)	7 ± 7.8 (0-37)

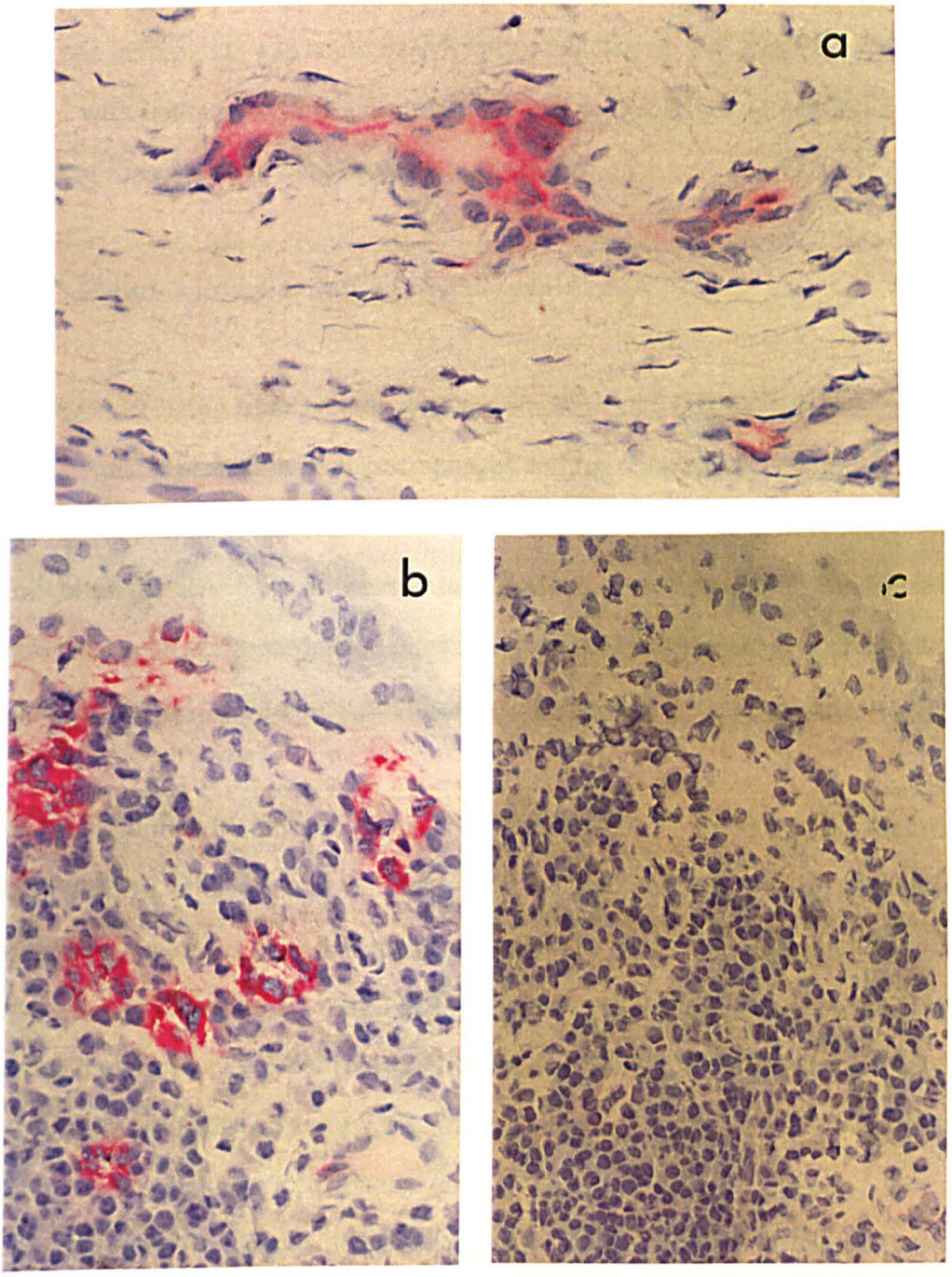
Synovial sections from 10 RA patients stained with anti-human iNOS antiserum were assessed by two histologists counting at least 500 cells in at least 3 high power fields per section area (magnification x400). Data are mean ± s.d. (range) for total number of fields counted. iNOS expression is greater in lining layer compared to either aggregate or interstitial areas (* $p<0.001$). Percent of iNOS positive cells is expressed as - [(positive cells)/(total number cells in field)] x 100

Figure 5.7 Immunohistochemical localisation of iNOS in human RA synovial membrane



RA synovial tissue section showing lining layer and adjacent interstitium stained with rabbit anti-human iNOS antiserum (fast red). (a) anti-iNOS serum alone, (b) anti-iNOS serum after pre-incubation with human iNOS peptide, (c) anti-iNOS serum after pre-incubation with rodent iNOS peptide. Staining after pre-incubation with recombinant human eNOS was identical to (c). (Magnification x300)

Figure 5.8 iNOS expression in endothelial cells in RA synovial membrane



RA synovial membrane was stained with anti-human iNOS antiserum and localised with fast red. (a & b) Endothelial cells were localised by morphology. (c) Prior incubation of section b with recombinant human iNOS neutralised the observed staining. In contrast recombinant human eNOS was ineffective in neutralising experiments (appearances similar to [b])

or recombinant human eNOS (Figure 5.7c), demonstrating that the pattern of staining was specific for human iNOS. iNOS positive smooth muscle and endothelial cells were also identified around blood vessels in 8 of 10 RA tissues examined (Figure 5.8a). As before, this was neutralised by prior incubation with human iNOS peptide, but not with human eNOS (Figure 5.8b) indicating that iNOS upregulation is also a feature of activated synovial endothelial cells in RA.

5.7 Cellular localisation of iNOS expression

The degree of lining layer staining with anti-human iNOS antibody implied that iNOS was potentially present in both macrophages and fibroblast-like synoviocytes. It was therefore important to establish which of these was the major source of inducible NO production in RA. To address this question, double staining for iNOS was performed with the macrophage markers, non-specific esterase (NSE) and murine monoclonal anti-CD68 by light microscopy and confocal microscopy respectively.

Double staining with NSE and anti-iNOS (Figure 5.9) revealed that the majority of iNOS positive cells either in the lining layer, or the interstitium, were NSE negative (mean ± s.e.m, 89 ± 5%) and therefore, unlikely to be macrophages (Table 5.4). Only 5% of synovial cells were NSE^+ / $iNOS^+$. These data were confirmed using confocal microscopy. Figure 5.10a demonstrates that most iNOS positive cells were not double-labelled with anti-CD68, although co-staining of anti-iNOS and CD68 in lining layer cells was occasionally observed (Figure 5.10b), indicating that some macrophages were capable of iNOS expression. Together these data showed that the majority of NSE^+ and $CD68^+$ cells in the lining layer were iNOS negative, indicating that most macrophages were not directly involved in NO production (Figure 5.10a, Table 5.4). *In vitro*, T cells do not normally synthesise NO and consistent with this, synovial T cells detected with murine anti-CD3 were all $iNOS^-$ (Figure 5.10c).

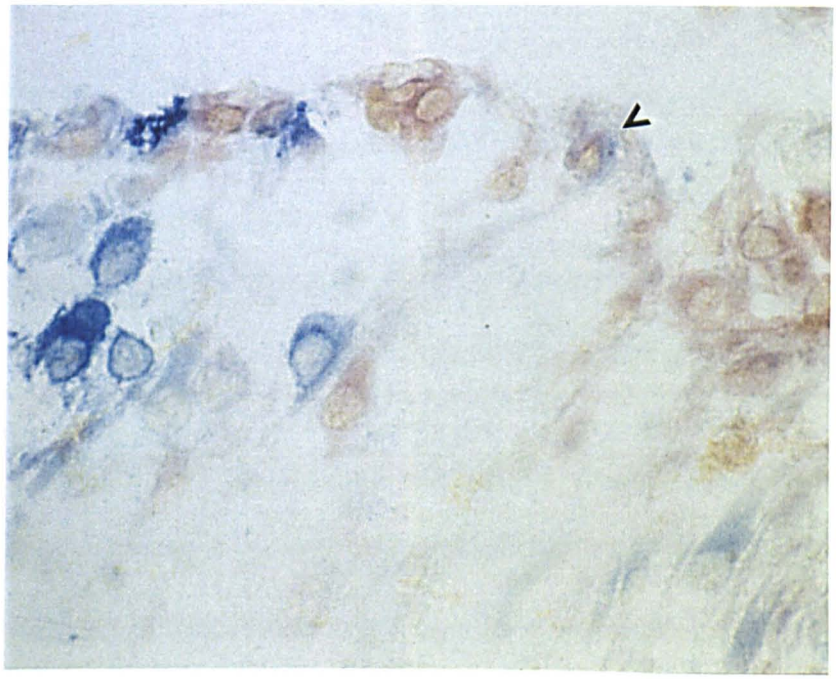
Because murine anti-CD3 monoclonal shares isotype and species origin with anti-CD68 antibody, this finding serves as an additional specificity control for the positive double staining observed with anti-iNOS and anti-CD68.

Table 5.4 Co-localisation of iNOS with NSE in RA synovial membrane.

	% positive cells		
phenotype	NSE^+ / $iNOS^-$	NSE^- / $iNOS^+$	NSE^+ / $iNOS^+$
mean ± s.d (range)	49 ± 24 (0-80)	45 ± 23 (16-93)	5 ± 3.1 (0-12.5)

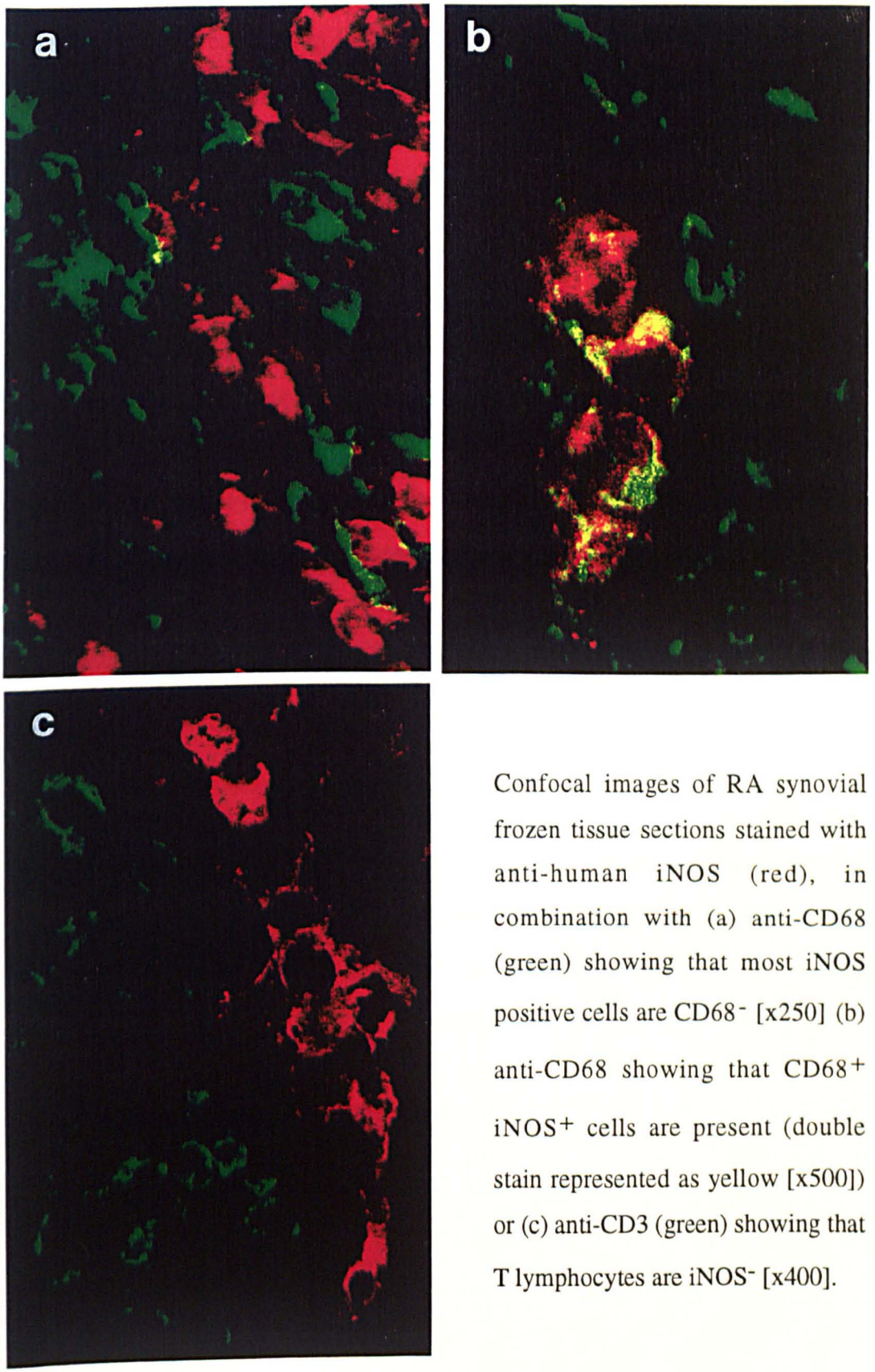
Sections from 4 RA patients were double stained with anti-human iNOS antiserum and non-specific esterase (NSE). At least 250 lining layer and adjacent interstitial area cells positive for NSE and / or iNOS were counted per section and the number of NSE^+, $iNOS^+$, or double positive cells were expressed as a % of the total number of cells counted.

Figure 5.9 Co-localisation of non-specific esterase and iNOS in RA synovial membrane



RA synovial sections were stained with anti-human iNOS antiserum, localised with fast blue, then with NSE (brown). No nuclear counterstain was used. The majority of $iNOS^+$ cells were NSE^-, although occasional double labelled cells were observed (arrow). (Magnification x200)

Figure 5.10 Con-focal microscopic localisation of iNOS in RA synovial membrane



Confocal images of RA synovial frozen tissue sections stained with anti-human iNOS (red), in combination with (a) anti-CD68 (green) showing that most iNOS positive cells are $CD68^-$ [x250] (b) anti-CD68 showing that $CD68^+$ $iNOS^+$ cells are present (double stain represented as yellow [x500]) or (c) anti-CD3 (green) showing that T lymphocytes are $iNOS^-$ [x400].

5.8 NO production in OA synovial tissue

In order to test whether NO synthesis was a specific property of inflammatory arthritides, NO production in OA synovial tissues was investigated. RT-PCR had earlier demonstrated the presence of iNOS mRNA in an OA synovial membrane indicating that increased NO production might be a feature of OA (Figure 5.3). Single cell suspensions from 7 OA synovial membranes were therefore cultured for 72 hours and nitrite generation measured. Spontaneous nitrite production was measured in 5 of 7 tissues examined, with mean concentration produced of 19 ± 9 μM (Table 5.5 & Figure 5.11). As with RA synovial membrane, addition of SEB (1-10 μg/ml) lead to novel or significantly increased levels of nitrite production in 7 of 7 patients *in vitro* (Table 5.6, $p<0.05$, Wilcoxon). No significant difference in either spontaneous or induced NO production was observed between the group means of RA and OA patient samples. The remarkable level of NO generated in the absence of exogenous stimuli in patient sample OA8 provoked clinical reassessment from which emerged a history of congenital hip disease, with a possible seronegative inflammatory arthritis. These data were therefore excluded from statistical analysis.

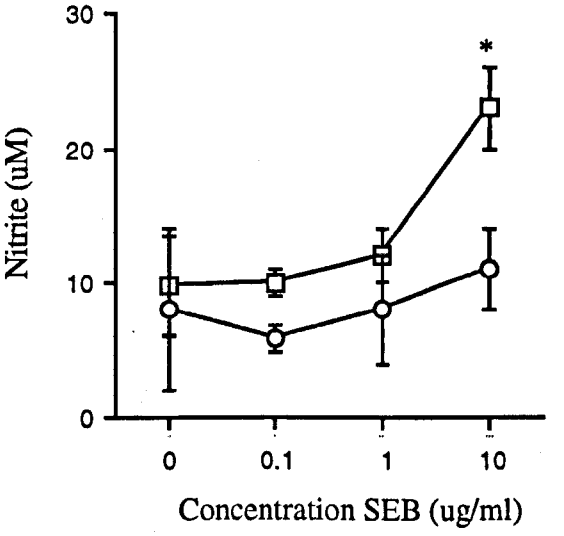
The distribution of iNOS in OA synovial membrane was investigated by immunohistochemistry, using rabbit anti-human iNOS antiserum (NO53) as described. iNOS expression was detected mostly in lining layer, with occasional interstitial cell staining (Table 5.6). Taken together, these data provide direct evidence that iNOS expression and NO production is a feature of OA synovial membrane.

Table 5.5 Production of nitric oxide by primary human synovial cultures

Disease Features		Nitric oxide production			
Patients	**Duration (yr)**	**Unstimulated**		**SEB†**	
		nitrite (μM)			
OA1	10	15	(0.5)	32	(3.6)
OA2	7	<2	-	3.3	(1.0)*
OA3	5	<2	-	3.2	(1.0)*
OA4	10	22	(2.5)	57	(8)
OA5	5	10	(3.3)	24	(3)
OA6	7	6	(2)	19	(4)
OA7	8	76	(9)	112	(8)
OA8	3	128	(26)	98	(8)

Production of NO (μM /10^6 cells) by OA synovial cells *in vitro*. Values are mean of triplicate nitrite assay of each of triplicate cultures (s.d.) at 72 hours. SEB stimulated OA synovial tissues produce enhanced nitrite levels ($p<0.02$, Wilcoxon). †Data shown are for optimal concentration of SEB (range 1-10 μg /ml) for each patient. * 1 μg/ml LPS present in culture. Patients were all receiving non-steroidal anti-inflammatory drugs.

Figure 5.11 Production of NO by OA synovial membrane culture



Dose response for SEB-induced nitrite production, with or without LPS, by heterogeneous synovial cells from a representative patient, OA06. NO production with SEB was significantly greater than basal levels, or with added LPS (*$p<0.001$, Mann-Whitney). Data are mean ± s.e.m., representing 3 similar experiments.

□ SEB, O LPS (1 μg/ml)

Table 5.6 Immunohistochemical distribution of iNOS in OA synovial membrane

	% cells iNOS positive	
n=number fields	Lining layer n=28	Interstitium n=25
mean ± s.d. (range)	11 ± 13 (0 - 53)	3 ± 3.5 (0 - 15)

Synovial sections from 10 OA patients stained with anti-human iNOS antiserum were assessed by two histologists counting at least 500 cells in at least 2 high power fields per section area (magnification x400). Data are mean ± s.d. (range) for total number of fields counted. iNOS expression is greater in lining layer compared to interstitial areas (*$p<0.006$, Mann-Whitney). Percent of iNOS positive cells is expressed as - [(positive cells)/(total number cells in field)] x 100

5.9 Do similar culture conditions induce human monocytes to produce NO?

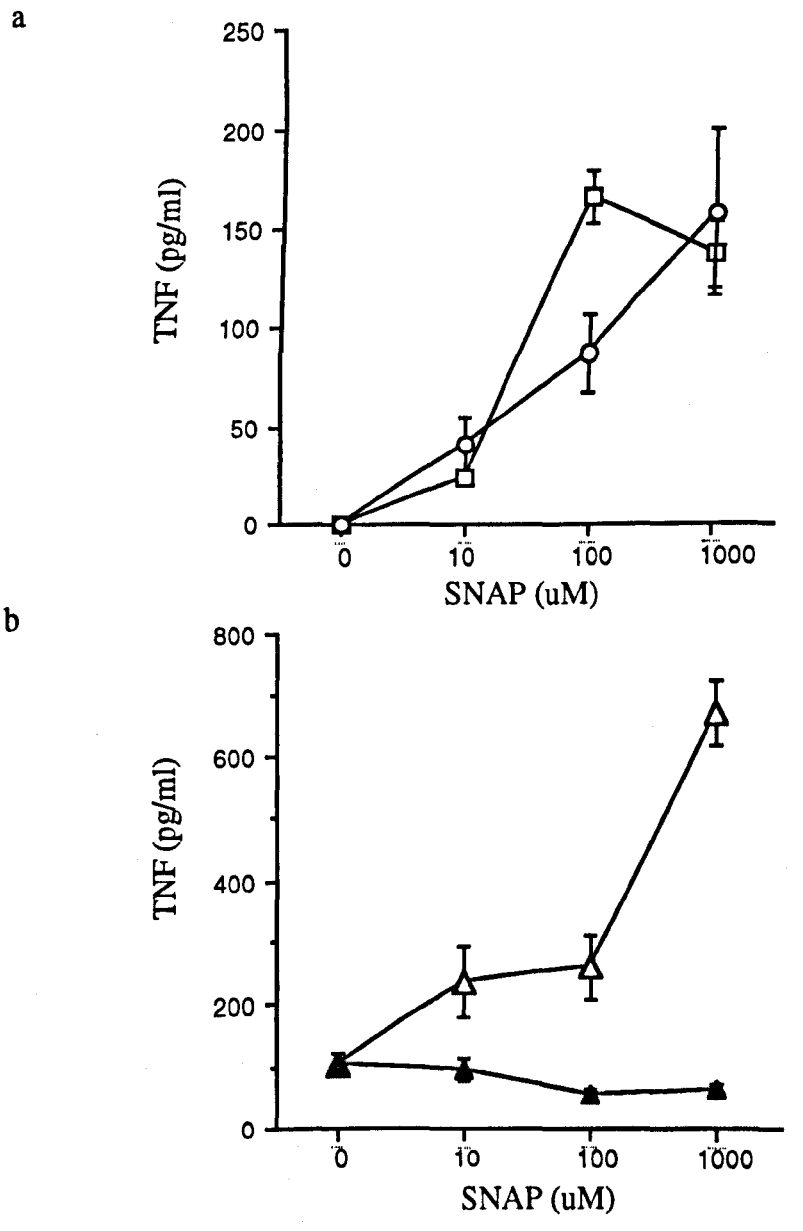
The ability of human macrophages to produce NO is currently controversial (Schneemann, et al, 1993; Dugas, et al, 1995). Light and con-focal microscopic data presented here indicate that human macrophages activated in a chronic inflammatory environment can indeed express iNOS, although in the context of RA, they may be of secondary importance in quantitative terms. Nevertheless, the enhancement of NO production by SEB in both human synovial cultures and J774 cells raised the possibility that this agent might up regulate iNOS expression in human monocytes. THP-1 cells, U937 cells, or unfractionated PBMC and purified blood monocytes (>90% $CD14^+$, <3% $CD3^+$, <3% $CD19^+$ by FACS analysis) from 4 normal donors and 4 RA patients were cultured between 10^6 / ml and $4x10^6$ / ml for 1, 3, 5 and 7 days with various doses and combinations of SEB (1 - 200 μg/ml) and LPS (0.01 - 1 μg/ml) with or without prior maturation for 24 hours with 10 nM PMA. In some experiments, reagents were added to culture wells 24 hours after cell seeding to wells and in others, the concentration of FCS was reduced to 0.1%. No combination of reagents or *in vitro* conditions was capable of consistent NO production measured by the Griess reaction (sensitivity of 2 μM).

5.10 NO dependent regulation of TNFα production.

Since TNFα production constitutes an important pro-inflammatory pathway in RA pathogenesis, it was of interest to determine whether NO might regulate its production in RA synovium. Synovial tissues or fluids were collected and adherent single cell suspensions prepared (>75% $CD68^+$, <5% $CD3^+$, <2% $CD19^+$, by cytoprep analysis). These were cultured at $2 x10^6$ /ml in the presence of graded concentrations of the NO donor, S-nitroso-N-acetylpenicillamine (SNAP), and the levels of TNFα in the culture supernatant determined 48 hours later by ELISA. Cells cultured with

medium alone produced low concentrations of TNFα. This was markedly enhanced by the presence of SNAP in a dose-dependent manner (Figure 5.12a). In parallel experiments, SNAP also increased the production of TNFα by the macrophage cell line U937 which had been primed by prior culture in 1 nM PMA for 24 hours (Figure 5.12b). The analogue N-acetylpenicillamine (NAP) which does not release NO, induced no TNFα synthesis. Neither SNAP, nor NAP, contained detectable LPS, using the Limulus amoebocyte assay (<0.0015 I.U. /mM stock solution).

Figure 5.12 Nitric oxide enhances TNFα synthesis *in vitro*



TNFα production (mean ± s.e.m.) to SNAP by (a) adherent cells from RA synovial fluid (SFAC) (O, n=4) or RA synovial tissue (□, n=2) or (b) PMA matured U937 cells (Δ SNAP, ▲ NAP; representative of 3 similar experiments). NAP did not induce TNFα production from SFAC (n=3). 2 mM SNAP contained <0.0015 I.U. LPS by limulus amebocyte lysis assay. Data are mean ± s.e.m.

Discussion

These observations provide direct evidence for NO production by human synovial membrane. In contrast to reports in animal experimental systems, in which cartilage was the predominant source of NO (Murrell, et al, 1996), synovial tissue appears capable of significant NO generation in human inflammatory arthritis. Evidence from *in vivo* model systems suggests that such NO synthesis is likely to have pathological significance.

The current data constitute a detailed *ex vivo* study of the cellular origin of NO production in RA synovial membrane. As such they significantly extend previous reports which provided largely indirect evidence for NO production in RA. Synovial fluid nitrite levels are reported to exceed those in serum in RA (Farrell, et al, 1992; Ueki, et al, 1996). This observation was not confirmed in the present study because the sensitivity of the chemiluminescence method for nitrite estimation employed here was inadequate to detect isolated elevation of nitrite in the presence of normal daily nitrate intake (Knight, et al, 1987). Moreover, Renoux, et al, (1995) recently detected higher levels of nitrite in OA synovial fluid than in RA. Dietary influences and possible variations in sample acquisition or methodology for nitrite estimation therefore cast doubt on the validity of nitrite as an index of NO production in RA. However, the fasting urinary nitrate:creatinine ratio, which avoids dietary effects, is increased in active RA (Grabowski, et al, 1996) and elevation of urinary nitrite excretion is reduced by prednisolone therapy (Stichtenoth, et al, 1995). 3-nitrotyrosine levels detected by electron-spin trapping are also elevated in RA synovial fluid (Kaur & Halliwell, 1994). These data indicate that articular tissues are a likely source of NO. The present study showed clearly, that the synovial membrane itself can contribute significantly to NO production during inflammatory arthritis, in combination with cartilage and bone derived cells.

Several recent reports have addressed the articular source of NO production. Whereas bovine or human chondrocyte cultures produced high levels of NO induced by cytokines, including IL-1β and TNFα, parallel cultures of synovial fibroblasts made only minimal levels of NO (Rediske, et al, 1994; Murrell, et al, 1996). In contrast, comparing NO production by human chondrocytes, osteoblasts and synovial fibroblasts, Ralston et al (1996) confirmed chondrocyte production of NO, but showed *in vitro* and using RT-PCR, that NO was produced by passaged synovial fibroblasts, after stimulation with IL-1β, TNFα and IFN-γ. However, unstimulated synovial fibroblasts and synovial fluid-derived leucocytes were iNOS$^-$ by RT-PCR. Nitrite production by human RA synovial explant cultures and preliminary evidence for enhanced NO production by IL-1β, TNFα and LPS has also been reported which correlated with levels of CD14 expression (Sakurai et al 1995). However, synovial fibroblasts in the latter study did not synthesise NO even after activation by cytokine combinations. Data presented here clearly establish that primary heterogeneous RA synovial cultures spontaneously generate NO. That such production was induced by bacterial product contamination during the enzymatic tissue digestion process seems unlikely, since iNOS expression was detectable by RT-PCR and immunohistochemistry in freshly isolated tissue. Moreover, J774 cells were resistant to NOS induction by a similar incubation protocol. These data indicate that NO production is upregulated *in vivo*, and as such constitutes a component of the inflammatory response in RA synovial membrane.

Immunohistochemical localisation of iNOS indicated that synovial fibroblasts constitute the predominant source of NO *in vivo*, with lesser contributions from macrophages and endothelial cells. The anti-iNOS antibody employed here also detects iNOS in alveolar macrophages in pulmonary tuberculosis patients (Nicholson et al 1996) and in asthmatic alveolar macrophages (Leung, B.P. & McInnes, I.B. unpublished observations). Neutralisation studies confirmed that it did not cross-react with either rodent iNOS or human eNOS. The iNOS staining pattern observed

contrasts with that reported using antibody raised against murine iNOS, which suggested more extensive iNOS expression, found mainly in $CD14^+$ synovial macrophages (Sakurai, et al, 1995). This discrepancy may relate to the use of anti-rodent iNOS antibodies with attendant problems of cross-reactivity or non-specificity clearly documented in the current study.

There is currently considerable controversy as to the capacity of human macrophages to produce NO (e.g. Denis, 1991; Schneemann, et al, 1993; Zembala, et al, 1994; Dugas, et al, 1995). The *in vitro* culture conditions optimal for the induction of iNOS in murine macrophages are apparently distinct from those required for the induction of iNOS in human macrophages. While macrophage cell lines (J774 or RAW) or thioglycollate stimulated peritoneal macrophages are used in most rodent systems, peripheral blood mononuclear cells are usually employed in human monocyte /macrophage experiments perhaps representing a different maturation stage. However, even in reports where the production of NO by human monocyte /macrophages was detectable (Zembala, et al, 1994; Dugas, et al, 1995), the levels are an order of magnitude lower than those produced by murine macrophages. This implies that cellular delegation of specific functions may differ between species in inflammatory lesions. Results from the present study show clearly that, although in the minority, some synovial macrophages from RA patients can indeed express high levels of iNOS. The latter observation was based not only on the presence of $CD68^+$ / $iNOS^+$ cells, since CD68 expression in synoviocytes in the interstitium has been reported (Wilkinson, et al, 1992), but also on the identification of NSE^+ / $iNOS^+$ cells on light microscopy. The reason why only a limited number of macrophages is activated to produce NO in RA is at present unclear.

There is, however, little doubt that RA synovial macrophages are activated, as demonstrated by the production of pro-inflammatory cytokines, such as IL-1, TNFα, IL-6 (Feldmann, et al, 1996b). Evidence from clinical intervention studies using

monoclonal anti-TNFα therapy have suggested that such cytokine generation is critical in ongoing synovitis (Elliott, et al, 1994; Rankin, et al, 1995). The factors responsible for TNFα upregulation remain unclear. Data reported here show clearly that, in contrast to their central position in cytokine production, synovial macrophages may express iNOS, but are not likely to be the principle producers of NO. In the context of inflammatory arthritis, this property appears to lie within the fibroblast compartment. Therefore, a reciprocal role for NO may exist whereby NO produced by synovial fibroblasts enhances pro-inflammatory cytokine production by macrophages, which in turn may up regulate iNOS expression, thereby generating a positive feedback loop. Certainly, TNFα is a recognised inducer of iNOS expression in animal systems (Nathan & Xie, 1994b; Liew, 1994). Further experiments examining the effect of NOS inhibitors on spontaneous cytokine production *in vitro* by synovial membrane cultures will help to address this possibility.

NO is likely to influence other immunoregulatory pathways in RA synovitis. The levels of NO spontaneously generated in synovial cultures are sufficient to inhibit T cell proliferation (Fu & Blankenhorn, 1992; Merryman, et al, 1993) and may therefore contribute to the 'frustrated activation' phenotype characteristic of synovial T lymphocytes (Pitzalis, et al, 1987). LPS-stimulation-dependent generation of angiogenic activity by human macrophages requires an L-arginine-dependent NO-synthase effector mechanism that may be independent of the generation of TNFα and IL-8 (Leibovich, et al, 1994), indicating that NO may contribute to neovascularisation in RA synovial membrane. Moreover, NO can up regulate production of metalloproteinases (Murrell, et al, 1995) and of prostaglandins (Manfield, et al, 1996), and has been implicated in IL-1β-mediated inhibition of proteoglycan biosynthesis (Hauselmann, et al, 1994; Jarvinen, et al, 1995; Fukuda, et al, 1995), implying a pro-inflammatory effect. However, an acute chondroprotective role has recently been proposed for endogenous NO in bovine cartilage (Stefanovic-Racic, et al, 1996). Thus, in contrast to the situation in rodent arthritis models, in which NO is

usually detrimental (Ialenti, et al, 1993; McCartney-Francis, et al, 1994), the net effect of NO generation in the human situation remains unclear.

It was not clear whether SEB-induced NO production was mediated through direct effects on MHC class II^+ synoviocytes / macrophages, or rather through 'surrogate' cytokine production by T lymphocytes activated by Vβ region / TCR binding (Marrack & Kappler, 1990). The induction of NOS activity by SEB in IFN-γ primed J774 cells indicated that a direct effect on macrophages was possible in the presence of adequate co-stimuli. The presence of the latter would, in any case, reflect concomitant T cell activation *in vivo* and in the context of heterogeneous RA synovial cultures, a combination of mechanisms seems likely. Further investigation of highly purified subsets of synovial cells will be required to address this question. Nevertheless, the ability of SEB to induce NO production in OA tissues, in which fewer T lymphocytes were present, perhaps indicates that a direct effect does exist. The addition of LPS alone induced low levels only of NO synthesis, and addition with SEB did not further increase SEB-induced NO production, but rather led to inhibition. This may reflect the generation of inhibitory cytokines, such as IL-10, from $CD14^+$ synovial macrophages. However, SEB, with or without LPS, was unable to induce NO production by peripheral blood monocytes, even after prior maturation with IFN-γ or PMA, or the presence of SEB-responsive PB lymphocytes. These data indicate that the complex signals priming macrophages within an inflammatory site are not as easily mimicked in the human, as in the rodent situation.

The apparently contradictory evidence which exists regarding NO production by synovial fibroblasts can be resolved by the current study. Previous discrepancies may reflect various stages of fibroblast differentiation *in vitro*, cell passage used, or the culture conditions to confluence employed by different groups (Rediske, et al, 1994; Ralston, et al, 1996; Murrell, et al, 1996). By providing immunohistochemical evidence for fibroblast iNOS expression, the difficulties attached to interpretation of

in vitro data can be avoided. Thus, NO synthesis can be added to the list of synoviocyte bioactivities which includes, MMP production, cytokine generation and PG synthesis. This provides further evidence indicating a crucial role for fibroblasts in RA pathogenesis (reviewed by Zvaifler & Firestein, 1995).

It is of interest also that both RA and OA patient synovia were capable of NO generation. OA synovial tissues contain areas of inflammatory infiltrate, with macrophages present, particularly in the lining layer, and low levels of monokines detectable, including IL-1β and TNFα (Gordon, et al, 1984; Brennan, et al, 1989; Brennan, et al, 1991). High levels of NO production have been detected in OA chondrocytes (Amin, et al, 1995) and indeed, higher levels of nitrite have been reported in OA than in RA synovial fluid (Renoux, et al, 1995). The clinical OA syndrome, however, is not considered to be of primarily inflammatory origin. NO production is therefore unlikely to be a property of the inflammatory arthritides alone, but may reflect a non-specific response to injury or inflammation with potential protective or pathological consequences. In particular, given its extensive microbicidal activities, the role of NO in synovial defence against infection merits consideration. This may have important therapeutic consequences when specific iNOS inhibitors come to clinical trial.

In summary, these results demonstrate directly the production, and further induction, of NO by synovial cultures from RA and OA tissue and provide evidence of iNOS expression in synovial inflammatory cells. These *in vitro* and immunohistochemical data significantly extend the previous reports of NO production in synovial membrane, by clarifying its cellular origin and confirming that the production of NO may be inducible *in vitro*.

Chapter 6

Staphylococcus aureus arthritis in mice lacking inducible nitric oxide synthase.

Introduction

High concentrations of NO are generated by inducible NOS (iNOS) regulated by pro-inflammatory cytokines, particularly TNFα, IL-1β and IFN-γ and by bacterial components, including lipopolysaccharide (LPS) and staphylococcal enterotoxins (Isobe & Nakashima, 1992; Bredt & Snyder, 1994; Nathan & Xie, 1994a). iNOS activity constitutes the predominant source of NO in inflammatory lesions. Several observations implicate NO in articular inflammation. NOS inhibitors retard or abrogate streptococcal cell wall and adjuvant-induced arthritis in rats indicating that, in rodents at least, NO synthesis is required for autoimmune joint destruction (Ialenti, et al, 1993; MacCartney-Francis, et al, 1994; Stefanovic-Racic, et al, 1994). Elevated synovial fluid nitrite levels and urinary nitrite excretion provide indirect evidence for NO synthesis in humans in RA (Farrell, et al, 1992; Grabowski, et al, 1996). Furthermore, data presented in chapter 5 clearly demonstrate NO production by fibroblasts and macrophages in the synovial membrane of RA patients (McInnes, et al, 1996b), which can be further enhanced *in vitro* by addition of staphylococcal enterotoxin B (SEB).

The net effect *in vivo* of NO synthesis in human synovial membrane, however, is subject to considerable controversy. NO induces matrix metalloproteinase production, cyclooxygenase activation and can enhance synovial macrophage derived TNFα synthesis (Murrell, et al, 1995; Manfield, et al, 1996). It also promotes macrophage driven neovascularisation and has been implicated in IL-1β mediated inhibition of proteoglycan biosynthesis and thus, cartilage degradation (Leibovich, et al, 1994; Hauselmann, et al, 1994; Jarvinen, et al, 1995; Fukuda, et al, 1995). In contrast, an acute chondro-protective role for NO has recently been proposed (Stefanovic-Racic, et al, 1996) and immunomodulatory effects on T cell activation may partially account for the 'frustrated activation' exhibited by T cells in RA inflammatory synovium (Pitzalis, et al, 1987). Thus, in inflammatory arthritis, NO

may exhibit bi-potential properties. However, high levels of NO production can also be demonstrated in joints of patients with OA (chapter 5; Amin, et al, 1995; Renoux, et al, 1995). NO synthesis in the synovial membrane most likely represents a non-specific response to diverse challenges. In evolutionary terms, the principle role for NO in immunity appears to be microbicidal, with immunomodulatory effects designed to assist in regulation of anti-microbial cellular and cytokine responses. This raises the possibility that NO production may be important in synovial defence against infection. This is of particular importance if iNOS inhibitors are to provide effective novel anti-inflammatory therapies.

Septic arthritis commonly results from gram positive bacterial infection, particularly by *Staphylococcus aureus* (Rosenthal, et al, 1980; Goldenberg & Reed, 1985; Goldenberg, 1989; Ostensson & Geborek, 1991). Whereas NO has been clearly shown to mediate cytotoxicity against intracellular pathogens, including protozoa, viruses and fungi (Nathan & Xie, 1994b), its role in killing extra cellular bacterial is poorly defined. Reactive nitrogen intermediates (RNI) have been implicated in staphylococcal killing after neutrophil phagocytosis (Malawista, et al, 1992). In a cell free system, NO exerted delayed bactericidal activity, but in contrast to the action of reactive oxygen intermediates (ROI), exerted no immediate killing effect (Kaplan, et al, 1996).

Septic arthritis can be induced by intravenous inoculation of mice with exo-toxin producing *Staphylococcus aureus* (Bremmell, et al, 1991; Bremmell, et al, 1992). The resulting articular lesion exhibits synovial hyperplasia, containing neutrophils, macrophages and a prominent T lymphocyte infiltrate, with associated erosion of underlying cartilage and bone (Bremmell, et al, 1992; Abdelnour, et al, 1994a). T cell activation by bacterial superantigens leads to high levels of cytokine production, particularly IFN-γ and TNFβ (Abdelnour, et al, 1994b). The course of staphylococcal infection in IFN-γ receptor knockout and T cell receptor transgenic

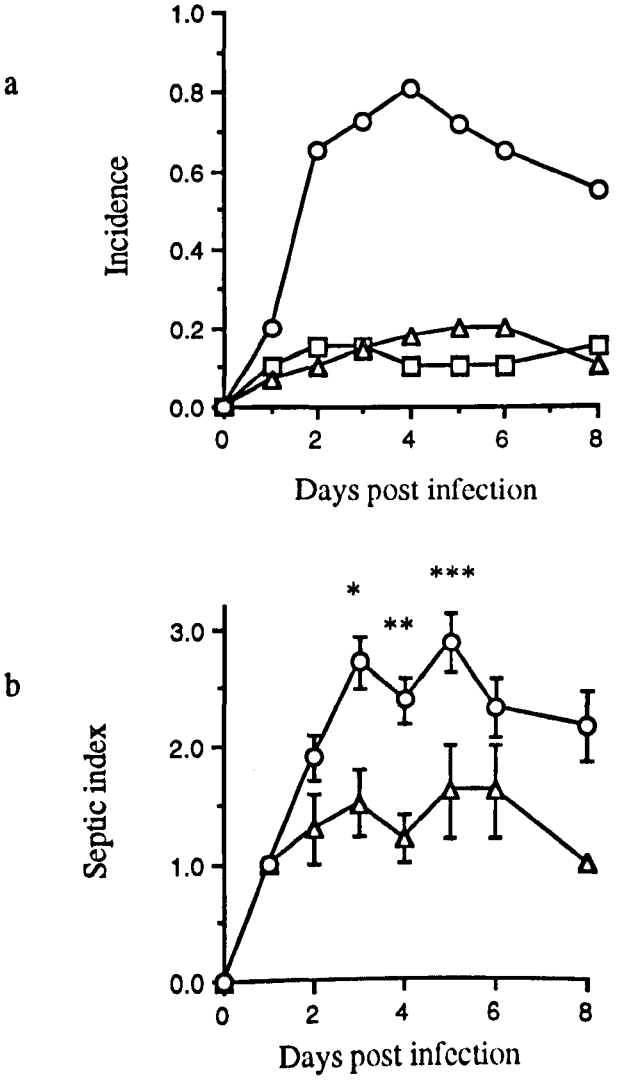
mice indicates that such Th1 bias is pro-inflammatory (Zhao, et al, 1995a; Zhao & Tarkowski, 1995b) and inhibition of T cell activity using anti-CD4 or anti-Vβ11 antibodies can significantly improve disease outcome (Abdelnour, et al, 1994b). These data indicate that immunological mechanisms constitute a major component of the observed articular pathology. Direct injection of bacterial superantigens, such as toxic shock syndrome toxin (TSST)-1 or SEB, leads to T cell mediated shock. In these circumstances, NO production is protective to the host (Florquin, et al, 1994). In contrast, the staphylococcal cell wall components, peptidoglycan and lipoteichoic acid, synergistically induce multiple organ failure in rats by an NO-dependent mechanism (De Kimpe, et al, 1995a; De Kimpe, et al, 1995b). The precise role of NO in gram-positive infectious arthritis and septicaemia is therefore currently unclear.

Wei, et al (1995) recently generated an iNOS gene targeted mouse, which facilitates investigation of the contribution of iNOS to inflammatory conditions in the context of normal constitutive eNOS and nNOS activity. This provides an ideal model in which to study the contribution of NO synthesis *in vivo* to both acute *Staphylococcus aureus* infection and its T cell-dependent pathological sequelae. By this means the relative contribution of microbicidal and immunomodulatory effects of NO in systemic and articular inflammation may be investigated.

6.1 Incidence and severity of staphylococcal septicaemia

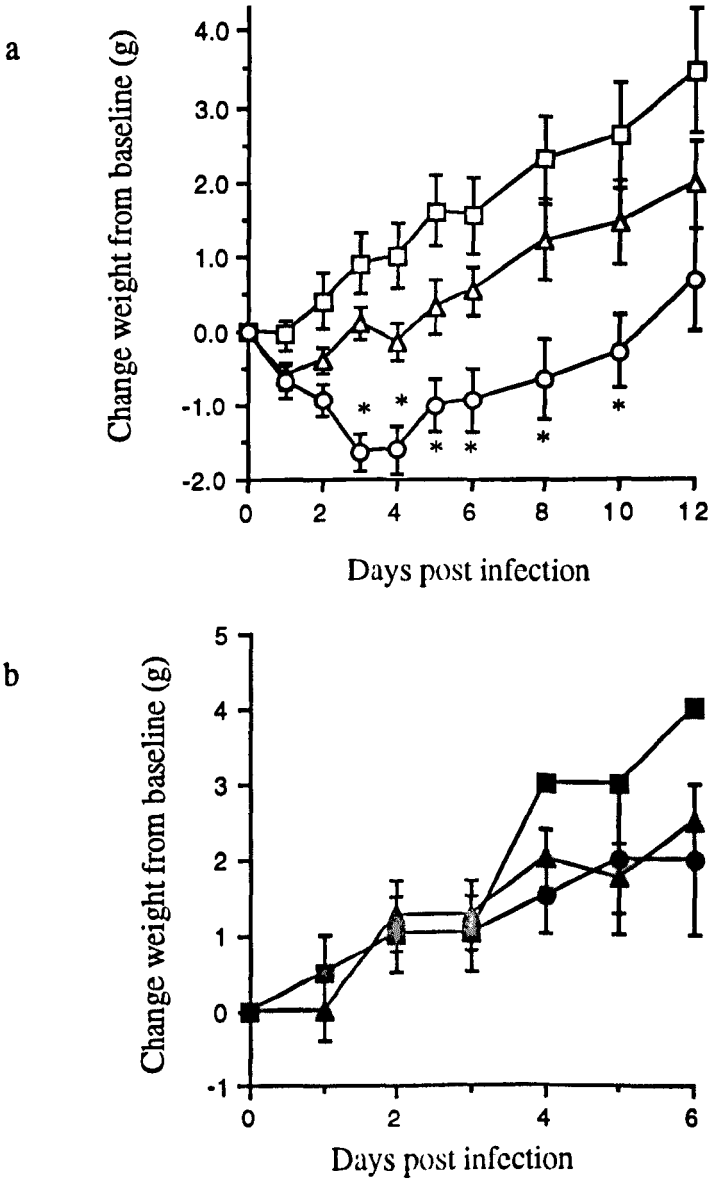
Although NO production has been firmly implicated in gram negative endotoxin-induced shock, its role in gram positive bacterial sepsis is less clear. Septicaemia is uncommon in wild-type Swiss mice following *Staphylococcus aureus* infection (Bremmell, et al, 1992). Initial experiments were thus performed to assess whether iNOS deficiency altered this normally resistant phenotype. Preliminary dose-ranging studies established that injection of $5x10^7$ CFU staphylococci /mouse led to onset of arthritis, but only rarely induced septicaemia in wild-type mice. In three separate experiments, iNOS deficient homozygous ($iNOS^{-/-}$; n=33), heterozygous ($iNOS^{+/-}$; n=28) or wild type ($iNOS^{+/+}$; n=20) mice therefore received $5x10^7$ CFU/ml of *Staphylococcus aureus* LS-1 strain by intravenous injection. 65% of $iNOS^{-/-}$ compared with 14% of $iNOS^{+/-}$ mice developed clinical evidence of sepsis within 48 hours, rising to 83% compared with 20% respectively after 4 days ($p<0.005$, Chi Square; Figure 6.1a). Disease severity in animals developing sepsis was significantly greater in $iNOS^{-/-}$ than $iNOS^{+/-}$ mice during the acute phase of disease up to day 6 ($p<0.001$ to $p<0.04$, Mann-Whitney; Figure 6.1b). These observations were reflected in significantly greater weight loss in $iNOS^{-/-}$ mice compared with either infected $iNOS^{+/-}$ or $iNOS^{+/+}$ controls ($p<0.01$, Mann-Whitney; Figure 6.2a), or with uninfected $iNOS^{-/-}$ littermates (Figure 6.2b). Increased mortality was observed in $iNOS^{-/-}$ mice, which reached 30% after 8 days ($p<0.005$ to $p<0.03$ compared with $iNOS^{+/-}$ or $iNOS^{+/+}$ mice, Log-rank; Figure 6.3). These data demonstrate that deficient iNOS expression is associated with increased incidence and severity of septicaemia and implicate NO *in vivo* in host defence to *Staphylococcus aureus* infection.

Figure 6.1 Incidence and severity of septicaemia



iNOS$^{-/-}$ mice developed higher incidence of septicaemia of greater severity than iNOS$^{+/-}$ or iNOS$^{+/+}$ littermates. iNOS$^{-/-}$ (○; n=33), iNOS$^{+/-}$ (△; n=28) or iNOS$^{+/+}$ (□; n=20) mice received 5x10^{7} CFU *staphylococcus aureus* i.v. The (a) presence and (b) severity of septicaemia were assessed daily by group-blinded observers. Incidence was higher from day 2 in iNOS$^{-/-}$ mice than in either control group ($p<0.005$, Chi-Square). Severity of septicaemic mice only in either group was compared and was greatest in iNOS$^{-/-}$ mice (*$p<0.02$, **$p<0.001$, ***$p<0.04$). Data are pooled from three separate experiments.

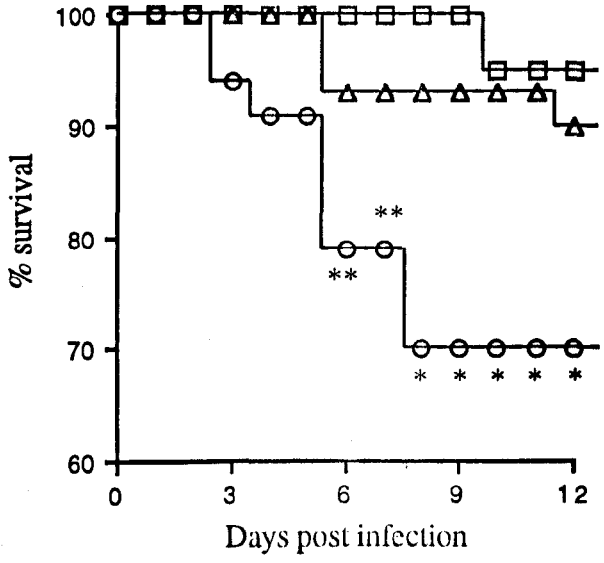
Figure 6.2 Weight change after staphylococcal infection



(a) Mice were weighed daily after i.v. infection with *staphylococcus aureus* and mean ± s.e.m. change in weight was calculated from baseline. $iNOS^{-/-}$ mice (O; n=33) lost more weight than $iNOS^{+/-}$ (Δ; n=28) or $iNOS^{+/+}$ (□; n=20) controls (*$p<0.01$, Mann-Whitney).

(b) Weight gain in uninfected controls (filled symbols) was identical in each group (n=6 /group).

Figure 6.3 Mortality following staphylococcal infection



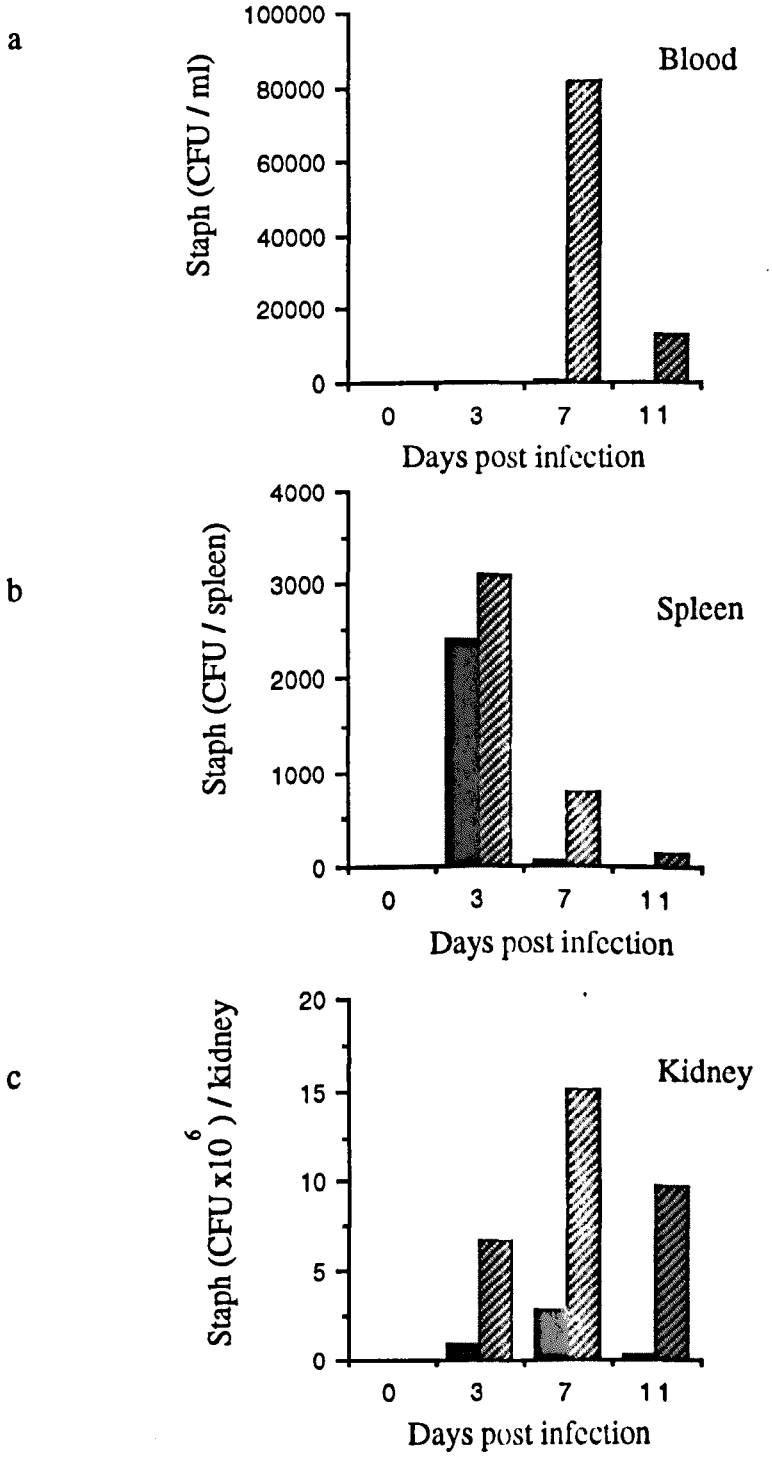
Mortality after *staphylococcus aureus* infection was higher in $iNOS^{-/-}$ (O) compared with $iNOS^{+/-}$ (△; **p<0.03, Log-rank) or with $iNOS^{+/+}$ mice (□; *p<0.02, **p<0.005). Wildtype and heterozygote mortality rates were not significantly different. Population numbers at outset were -/-n=33, +/-n=28 and +/+n=20.

It was next important to determine whether clinical septicaemia was reflected in altered bacterial distribution and viability *in vivo*. iNOS$^{-/-}$ and iNOS$^{+/-}$ mice were injected i.v. with 5x10^7 CFU *Staphylococcus aureus* as before. Ten-fold dilutions of blood and tissue homogenates were cultured on blood agar for 18 hours at 37°C to derive the number of viable CFU present at successive time intervals, up to day 11. Staphylococci were not detected in blood in either group at day 3, but were present in iNOS$^{-/-}$ mice at days 7 and 11. In contrast, iNOS$^{+/-}$ mice had few viable blood-borne bacteria even at day 7 (Figure 6.4a). High numbers of bacteria were present in the spleens of both groups after day 3, but persisted to days 7 and 11 only in iNOS$^{-/-}$ animals (Figure 6.4b). Together these data indicate that although both groups similarly localise staphylococci to spleen, their subsequent viability within, and dissemination from, the spleen is altered in iNOS$^{-/-}$ animals. That this was relevant to end organ infection was demonstrated by the greater number of viable staphylococcal CFU detected in kidneys of homozygous animals (Figure 6.4c). iNOS deficiency therefore impairs clearance of staphylococci, leading to widespread tissue deposition of bacteria and consequent pathology.

6.2 Incidence and severity of septic arthritis.

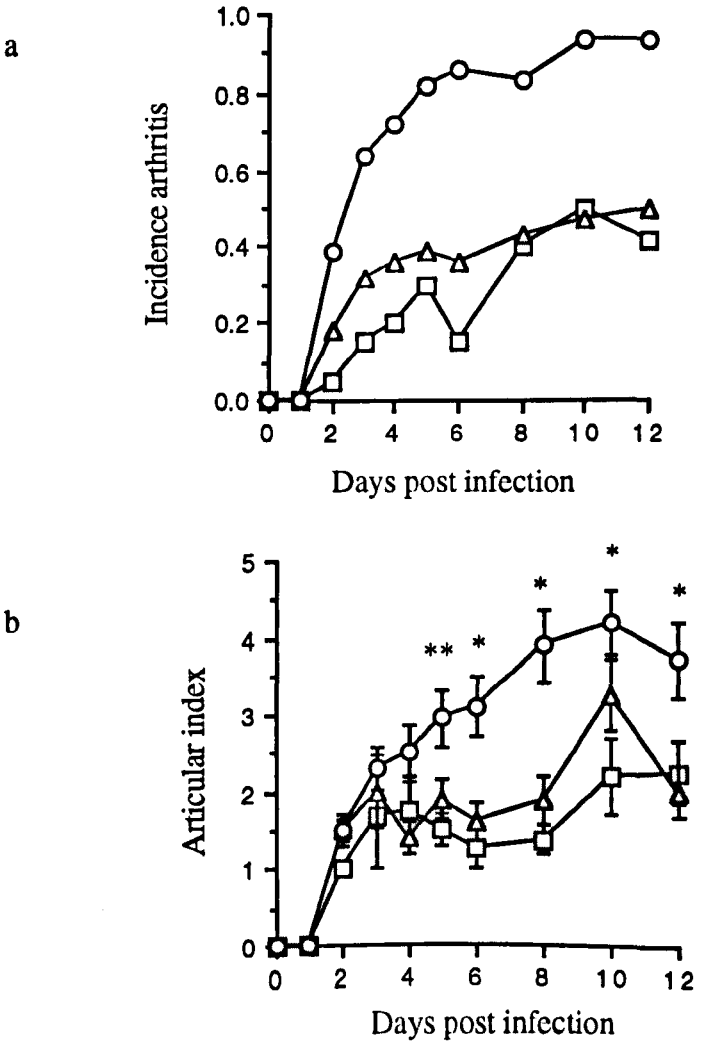
The above data clearly indicate the deleterious consequences of staphylococcal infection in iNOS$^{-/-}$ mice. Since NO has been implicated as an important pro-inflammatory mediator in rodent 'autoimmune' arthritis, it was therefore of interest to determine the net effect of iNOS deficiency on the development and severity of septic arthritis. iNOS$^{+/-}$ and iNOS$^{+/+}$ mice developed clinical signs of arthritis within 2 days of infection, which was evident in 50% of mice after 10 days (Figure 6.5a). No significant difference between these groups was observed. Individual animals presented a fluctuating course with minor daily variation in clinical severity. In

Figure 6.4 Tissue distribution of viable staphylococci after i.v. infection



Higher numbers of viable staphylococci were recovered from iNOS$^{-/-}$ mice (hatched bars) after i.v. *staphylococcus aureus* infection compared with iNOS$^{+/-}$ mice (solid bars). Tissues (n=3 mice/group) were harvested at time points indicated, diluted ten-fold (to n/10^6) in sterile PBS, and the number of viable CFU was assessed in duplicate cultures in blood agar over 18 hours.

Figure 6.5 Incidence and severity of septic arthritis

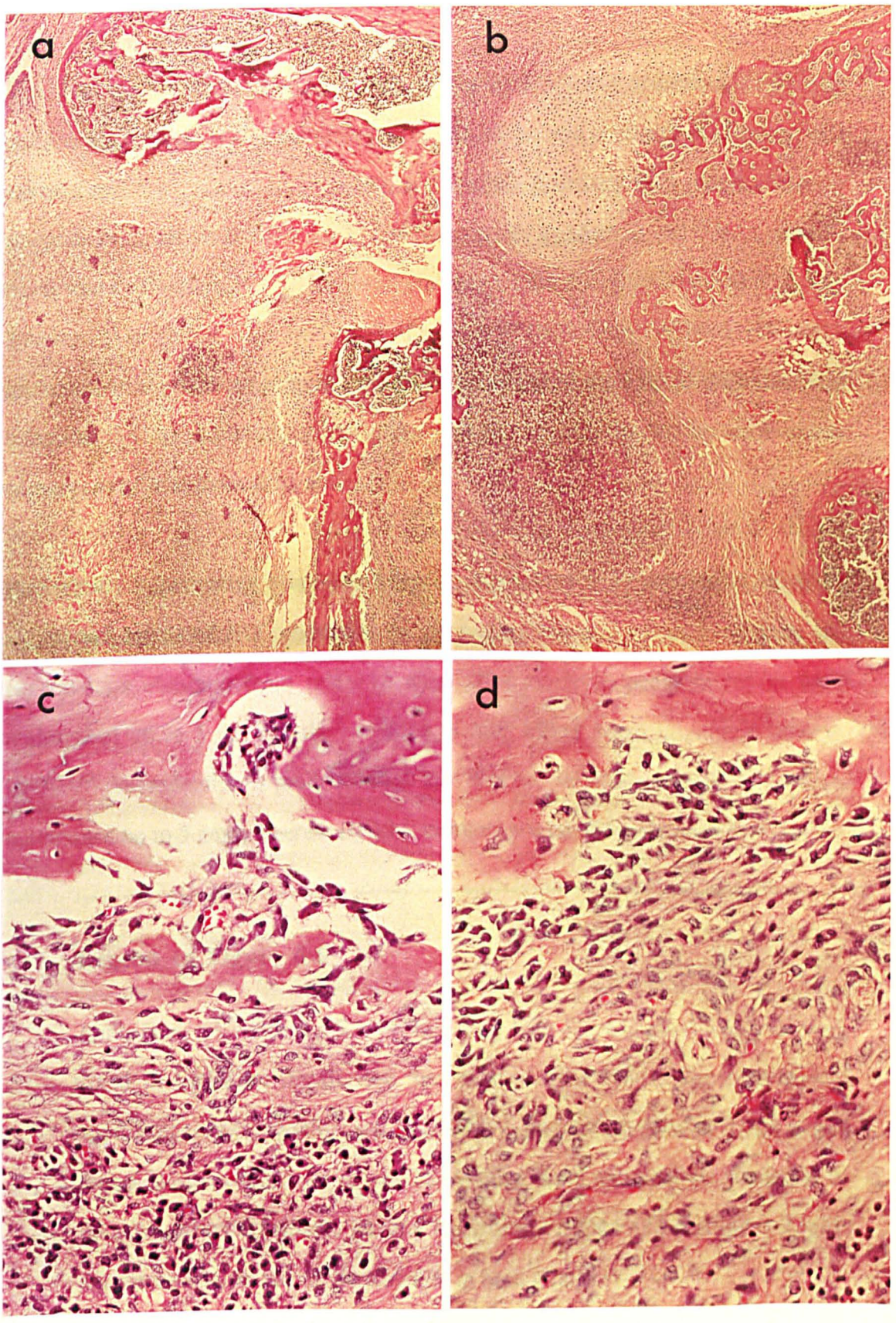


iNOS$^{-/-}$ (O; n=33) mice developed arthritis more frequently and with greater severity than did iNOS$^{+/-}$ (Δ; n=28) or iNOS$^{+/+}$ (□; n=20) littermates. The (a) incidence and (b) severity of arthritis following *staphylococcus aureus* infection was recorded daily by group-blinded observers. iNOS$^{-/-}$ mice exhibited significantly higher incidence of arthritis from day 2 ($p<0.003$) vs iNOS$^{+/+}$ and from day 3 ($p<0.003$, Chi Square) vs iNOS$^{+/-}$ groups. Comparison of severity of arthritic mice in each group demonstrated higher mean articular indices in iNOS$^{-/-}$ mice (*$p<0.01$, **$p<0.05$ vs iNOS$^{+/-}$ or iNOS$^{+/+}$ groups, Mann-Whitney).

contrast, iNOS$^{-/-}$ mice displayed disease onset at day 2, which was progressive and unremitting in up to 93% of animals by day 10 ($p<0.01$; Figure 6.5a). Thus, arthritis was more common in iNOS$^{-/-}$ mice.

The clinical severity of septic arthritis was compared in arthritic mice using an articular index determined daily by a 'cage-blinded' observer. The mean articular index was significantly higher in iNOS$^{-/-}$ than in iNOS$^{+/-}$ or iNOS$^{+/+}$ mice after day 5 ($p<0.05$ - $p<0.005$; Figure 6.5b). The peripheral distribution of arthritis was similar between groups, but iNOS$^{-/-}$ mice developed a higher incidence of paraspinal abscess formation, with resultant hind limb paralysis and urinary incontinence (iNOS$^{-/-}$ 39% v iNOS$^{+/-}$ 10.7%; $p<0.05$, Chi Square), perhaps reflecting the enhanced tissue distribution of viable staphylococci observed previously (Figure 6.4). The latter was often a pre-morbid presentation, but in 3 iNOS$^{-/-}$ mice was asymptomatic and found only at post mortem. The histological appearances of arthritic joints of equivalent clinical severity from 5 iNOS$^{-/-}$ and 5 iNOS$^{+/-}$ mice were compared, using H&E stained sections (Figures 6.6a - 6.6d). Large numbers of polymorphonuclear cells were evident in surrounding tissues. Hypertrophy of the synovial membrane was observed in all joints, in which a prominent mononuclear infiltrate was present. Invasive pannus eroding cartilage and bony surfaces was found in both groups, in which mononuclear cells and synoviocytes were detected, but not neutrophils (Figure 6.6c & 6.6d). No obvious difference in the overall appearances was observed between iNOS$^{-/-}$ and iNOS$^{+/-}$ joints. Taken together, these data indicate not only that NO protects against the development of septic arthritis, but that once established, the arthritic process might be modified by NO dependent pathways.

Figure 6.6 Histology of staphylococcal arthritis in iNOS$^{-/-}$ mice



Legend figure 6.6 next page

6.3 Effect of iNOS deficiency on immune responses in *Staphylococcus aureus* infected mice

The mechanisms whereby NO synthesis might modify septic arthritis are currently unknown. At least two possibilities arise, which are not mutually exclusive. NO exerts direct toxic effects on intracellular microbes, primarily through inhibition of enzymes essential for mitochondrial function, energy generation and DNA synthesis (reviewed by Stamler, 1994). Bacterial viability within the synovial membrane may therefore be enhanced in circumstances of relative NO deficiency. Alternatively, since immunological processes comprise the major pathological sequelae to staphylococcal infection, the immuno-modulatory functions of NO might influence the autoimmune component of host tissue destruction.

Preliminary experiments established that staphylococci were capable of inducing significant levels of NO production *in vitro* in spleen cell cultures. Spleen cell suspensions obtained from mice 12 days after staphylococcal infection were maintained up to 5 days in the presence of heat killed *Staphylococcus aureus*. $iNOS^{+/+}$ derived cultures produced high levels of nitrite within 3 days, which were maintained up to 5 days, whereas $iNOS^{-/-}$ cultures produced low levels of NO only,

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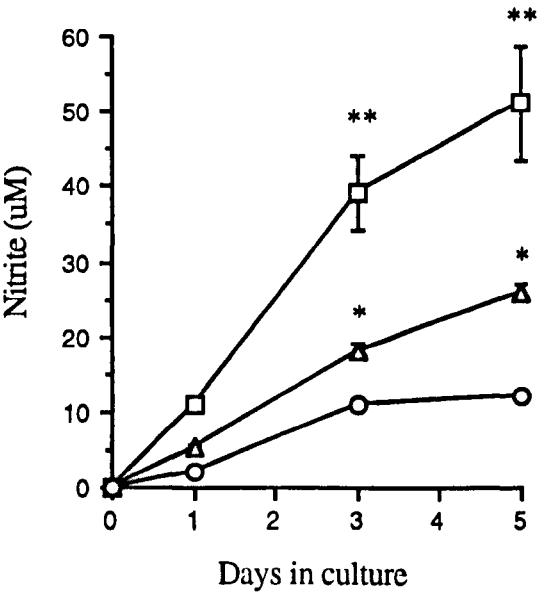
Legend to Figure 6.6

Joints from mice infected 5 days previously with *Staphylococcus aureus*, were fixed in formalin, decalcified then stained with H&E. The histological appearances in (a) iNOS-/- and (b) iNOS+/- joints were similar. High power views in (c) iNOS-/- or (d) iNOS+/- mice demonstrated the presence of invasive synovial pannus, leading to erosion of bone and cartilage. Light-microscopic appearances were similar in both groups.
(Magnification a & b x40, c & d x250).

even after 5 days ($p<0.001$; Figure 6.7). Low levels of NO production in $iNOS^{-/-}$ cultures have been detected previously and may result from partial induction of cNOS activity (Wei, et al, 1995; Amin, et al, 1995). $iNOS^{+/-}$ spleen cells exhibited intermediate NO production, which was significantly higher than in $iNOS^{-/-}$ cultures ($p<0.02$; Figure 6.7), perhaps indicating a gene dose effect as has been suggested elsewhere (MacMicking et al, 1995; Wei, et al, 1995). Spleen cell cultures derived from uninfected control mice produced no detectable nitrite after addition of heat-killed staphylococci, indicating that prior *in vivo* priming was necessary for optimal NO production.

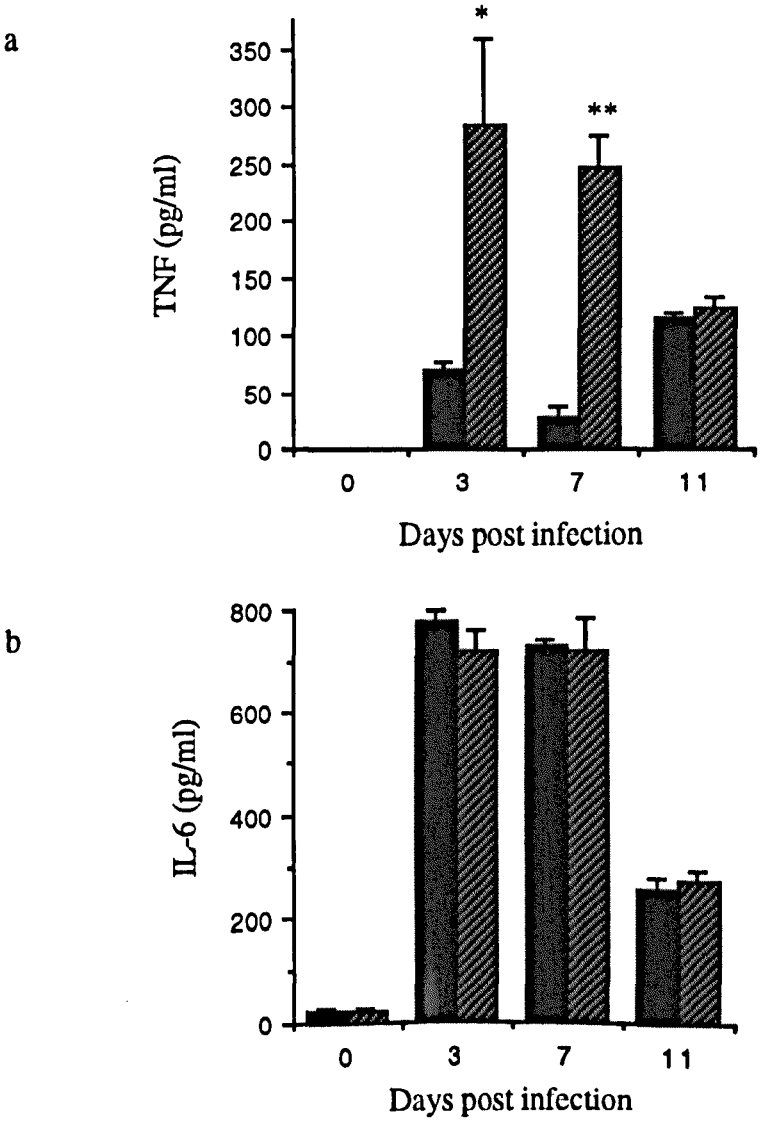
The production of pro-inflammatory cytokines during the course of infection was next investigated. TNFα has previously been implicated in pathogenesis of gram positive shock (Miethke, et al, 1992). Serum TNFα levels were significantly higher at day 3 ($p<0.05$) and day 7 ($p<0.01$) in $iNOS^{-/-}$ compared with $iNOS^{+/-}$ mice (Figure 6.8a). Although serum TNFα levels were similar in both groups by day 11, spleen cells from $iNOS^{-/-}$ donors cultured with heat-killed staphylococci *in vitro* at this time point generated significantly higher levels of TNFα ($p<0.01$, Figure 6.9) than those from similarly stimulated spleen cells from $iNOS^{+/-}$ or $iNOS^{+/+}$ mice. As expected, the elevated levels of serum TNFα correlated with the period of maximal clinical sepsis and weight loss (Figures 6.1b & 6.2a). Serum IFN-γ and IL-6 levels were also measured at similar time points. Whereas no IFN-γ was detected, high levels of IL-6 were present. In contrast to the above however, no significant difference was observed between $iNOS^{-/-}$ and $iNOS^{+/-}$ groups at any time point (Figure 6.8b). *In vitro*, NO differentially modulates LPS and IFN-γ induced IL-6 and TNFα release from J774 macrophages (Deakin, et al, 1995). These data indicate that the production of pro-inflammatory cytokines might similarly be differentially regulated *in vivo*.

Figure 6.7 Nitrite production by spleen cell cultures stimulated with heat-killed staphylococci



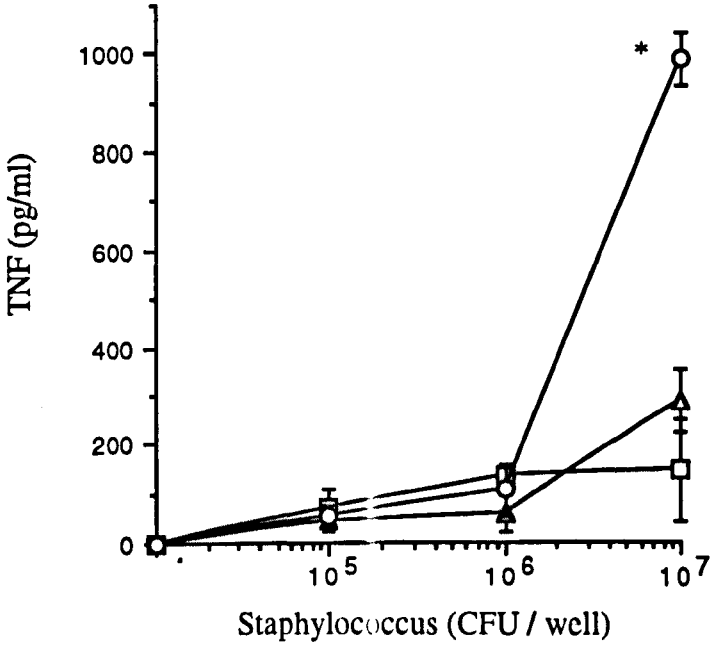
NO production was significantly reduced in iNOS$^{-/-}$ cultures stimulated by staphylococcal cell wall. Pooled spleen cell cultures were established from iNOS$^{-/-}$ (O), iNOS$^{+/-}$ (Δ), or iNOS$^{+/+}$ (□) mice (n=4 mice /group) to which were added 1x10^{7} CFU /ml of heat-killed *Staphylococcus aureus*. Supernatants were harvested at the time points indicated and the nitrite concentration measured by the griess reaction (2.10.1). Data are mean ± s.d. of duplicate assay of triplicate cultures. *p<0.02 - heterozygote vs homozygote; **p<0.001 - wild type vs homozygote; **p<0.01 wild type vs heterozygote.

Figure 6.8 Serum cytokine levels after staphylococcal infection



iNOS$^{-/-}$ mice (hatched bars) exhibited significantly higher serum TNFα levels than iNOS$^{+/-}$ mice (solid bars) after 3 and 7 days (*$p<0.05$, **$p<0.009$, Mann-Whitney). No significant difference in serum IL-6 levels was detected. Serum from individual mice was collected at time points indicated (n=3 / time point) and frozen until triplicate assay for cytokine concentration by ELISA (section 2.9.2). Data are mean ± s.e.m.

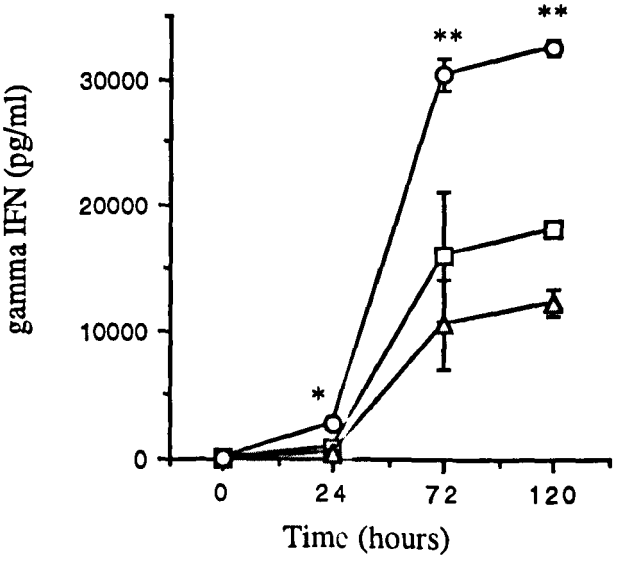
Figure 6.9 Production of TNFα by spleen cell cultures from *Staphylococcus aureus* infected mice



TNFα production from iNOS$^{-/-}$ (O) pooled splenic cultures exceeded that from iNOS$^{+/-}$ (Δ) or iNOS$^{+/+}$ (□) controls ($p<0.005$, Mann-Whitney). Spleens (n=4 mice /group) were removed 12 days after i.v. staphylococcal infection and cultured in the presence or absence of heat-killed *Staphylococcus aureus* LS-1 at the concentrations shown for 24 hours. TNFα production was measured by ELISA. Data are mean ± s.e.m. of duplicate assay of triplicate culture, representative of two separate experiments. Similar relative production was observed after 48 and 72 hours culture.

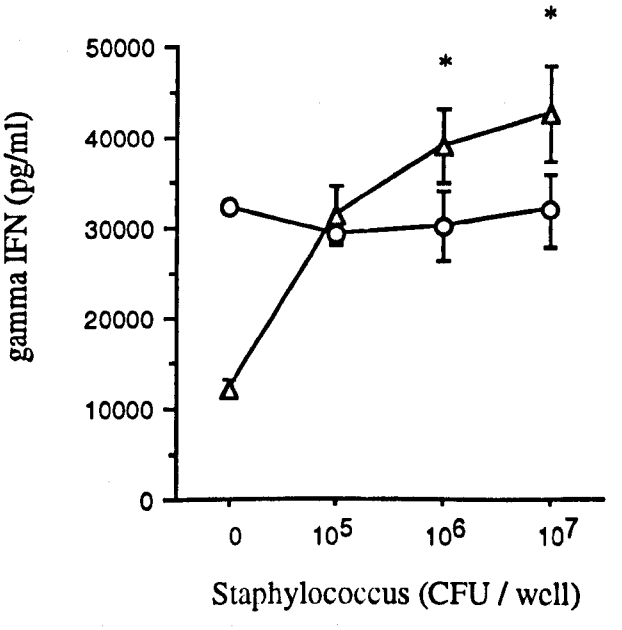
High concentrations of NO-donors *in vitro* inhibit the proliferation and cytokine secretion of Th1 cell clones to antigen (Taylor-Robinson, et al, 1994; Huang, F.P. personal communication) and enhanced antigen-specific Th1 responses in $iNOS^{-/-}$ mice have been reported following *Leishmania major* or *Listeria monocytogenes* infection (Wei, et al, 1995; MacMicking, et al, 1995). Thus, the possibility that deregulated Th1/Th2 balance might underlie the adverse outcome in $iNOS^{-/-}$ mice following staphylococcal infection was investigated. The proliferative response and production of IFN-γ and IL-4 was compared in pooled spleen cell suspensions obtained from mice 12 days post-infection. Cultures were maintained in the presence or absence of heat-killed *Staphylococcus aureus* (10^4 to 10^7 CFU/well) for up to 5 days and cytokine production was estimated by ELISA. Peak ^{3}H-thymidine uptake occurred after 72 hours, but no significant difference in proliferation was observed between the dose responses of $iNOS^{-/-}$ and $iNOS^{+/-}$ derived cultures up to 5 days. In contrast, spontaneous IFN-γ production was significantly higher in $iNOS^{-/-}$ cultures ($p<0.005$, Mann-Whitney; Figure 6.10) at each time point. Addition of heat-killed staphylococci led to significant up-regulation of IFN-γ synthesis in $iNOS^{+/-}$ and $iNOS^{+/+}$ cultures, to levels similar to those spontaneously produced by $iNOS^{-/-}$ spleen cells (Figure 6.11). This shows that the diminished spontaneous IFN-γ production observed was not due to inherent inability to produce IFN-γ in heterozygote or wild type mice. However, no further up-regulation of IFN-γ synthesis in $iNOS^{-/-}$ cultures was observed, indicating that maximal induction of IFN-γ production had been achieved *in vivo* (Figure 6.11). These data suggest that IFN-γ synthesis was differentially upregulated *in vivo* in infected $iNOS^{-/-}$ and $iNOS^{+/-}$ mice. No IL-4 was produced by any group, even after staphylococcal stimulation. Together, these observations are consistent with the presence of a dominant Th1 response which is enhanced in $iNOS^{-/-}$ mice.

Figure 6.10 Spontaneous production of IFN-γ by spleen cell cultures post-staphylococcal infection



iNOS$^{-/-}$ (O) spleen cell cultures produced significantly higher levels of IFN-γ than iNOS$^{+/-}$ (Δ) or iNOS$^{+/+}$ (□) controls. Pooled spleen cell cultures, prepared from animals (n=4 mice /group) sacrificed 12 days after i.v. staphylococcal infection, were maintained for up to 5 days *in vitro* and IFN-γ produced was assayed by ELISA (2.9.2). Data are mean ± s.e.m. of duplicate assay of triplicate culture (*$p<0.005$,**$p<0.001$, Mann-Whitney).

Figure 6.11 Effect of heat-killed staphylococci on IFN-γ production by spleen cell cultures



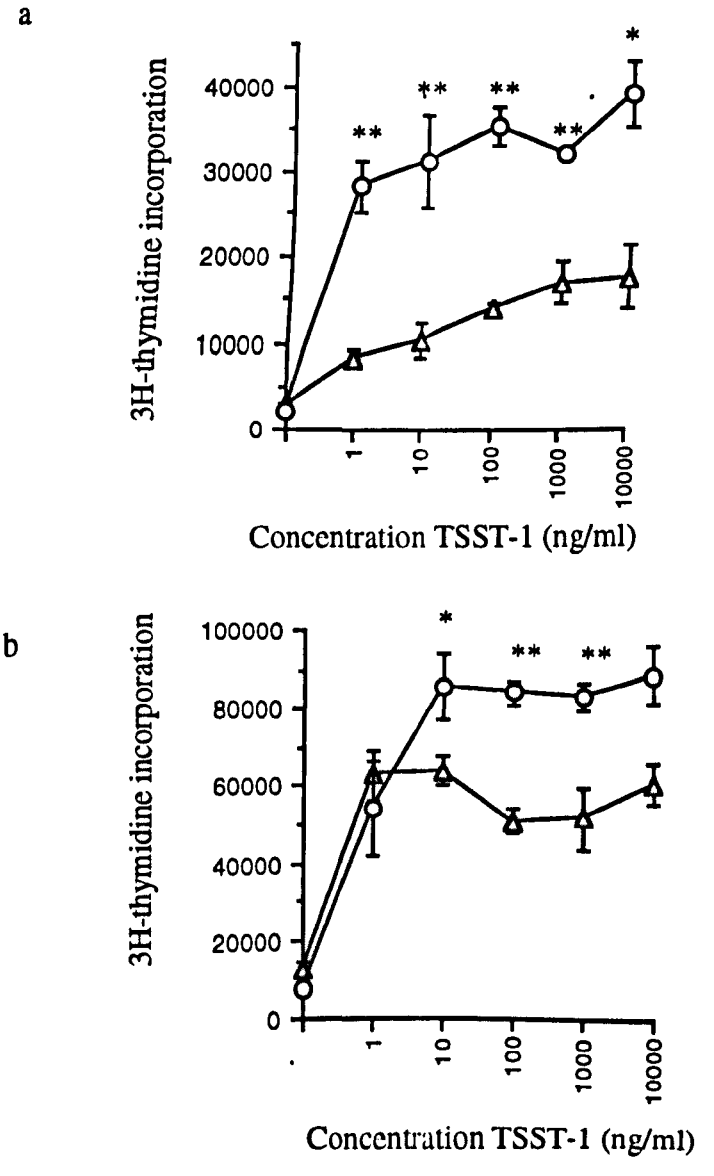
Spleen cell cultures from iNOS$^{-/-}$ (O) or iNOS$^{+/-}$ (Δ) mice infected 12 days previously with *Staphylococcus aureus*, were maintained with concentrations shown of heat-killed staphylococci for 5 days. Significant enhancement of IFN-γ production was observed in iNOS$^{+/-}$ cultures (*$p<0.01$ vs iNOS$^{+/-}$ baseline without staphylococci). Peak production was not significantly different between groups (Mann-Whitney). IFN-γ produced was measured by ELISA. Data are mean ± s.e.m. of duplicate assay of triplicate culture.

6.4 Superantigen responses in mice lacking iNOS

Vβ11 transgenic mice develop severe septic arthritis with associated mortality when infected with *Staphylococcus aureus* LS-1, which secretes TSST-1 (Abdelnour, et al, 1994b). Together with studies demonstrating the toxic consequences of direct injection of bacterial toxins, such as SEB (Florquin, et al, 1994), these data indicate a critical role for superantigen production in disease outcome following staphylococcal infection. Studies were therefore undertaken to determine whether lack of iNOS altered spleen cell proliferation and cytokine production to different superantigens in the absence of intact bacteria.

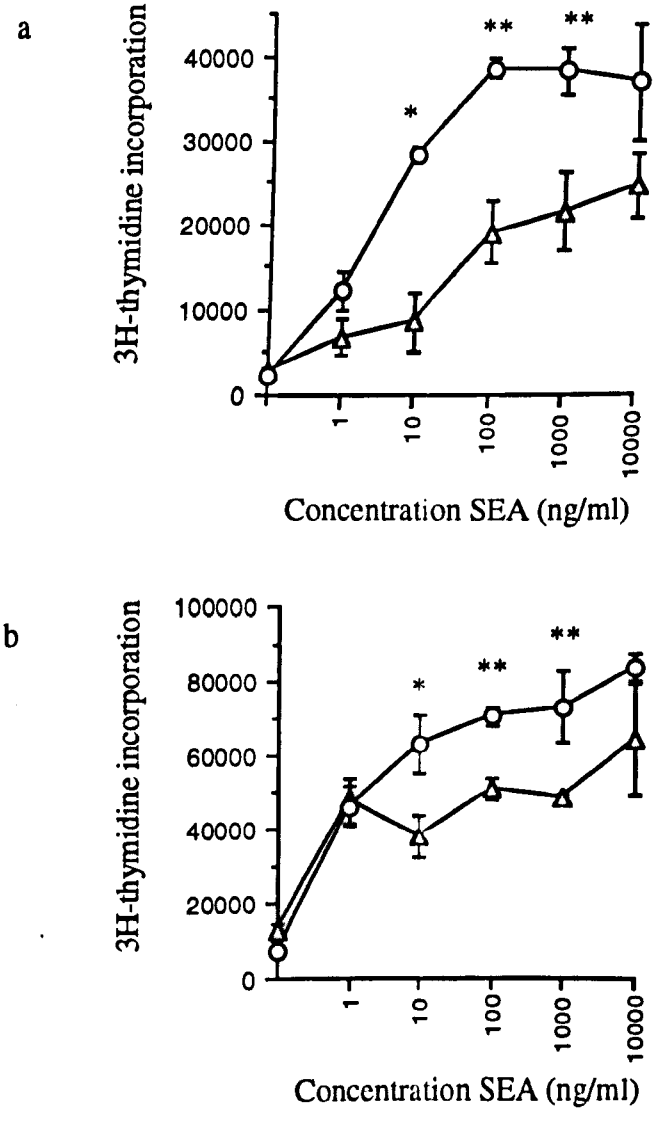
Spleen cells from uninfected $iNOS^{-/-}$ or $iNOS^{+/-}$ mice were cultured with increasing concentrations of TSST-1 or SEA. Significantly enhanced proliferation was observed to TSST-1 and SEA in $iNOS^{-/-}$ cultures after 48 and 96 hours ($p<0.01$ to $p<0.05$; Figures 6.12 & 6.13). IFN-γ production in these cultures was similarly increased compared to that in $iNOS^{+/-}$ derived spleen cell cultures ($p<0.001$ to $p<0.01$; Figures 6.14 & 6.15). Low levels of IL-4 were detected in cultures at 96 hours, but in contrast to the situation with IFN-γ, no significant difference was found between $iNOS^{-/-}$ and $iNOS^{+/-}$ groups (Figures 6.14 & 6.15). FACS analysis was performed after 48 hours, which demonstrated the presence of similar $CD4^+$ and $CD8^+$ populations in both $iNOS^{+/-}$ (19.67 ± 2.5% $CD4^+$, 7 ± 0.7% $CD8^+$) and $iNOS^{-/-}$ (21 ± 2% $CD4^+$, 7.5 ± 0.5% $CD8^+$) spleen cell cultures (mean ± s.d. of three individual experiments), suggesting that the enhanced IFN-γ production was not due to different T cell subset proportions *in vitro*. Nitrite production was sought in all cultures using the Griess reaction, but none was detected (sensitivity <2 μM), similar to previous observations using heat-killed bacteria in spleen cultures from uninfected mice (section 6.3).

Figure 6.12 Spleen cell culture proliferation to TSST-1



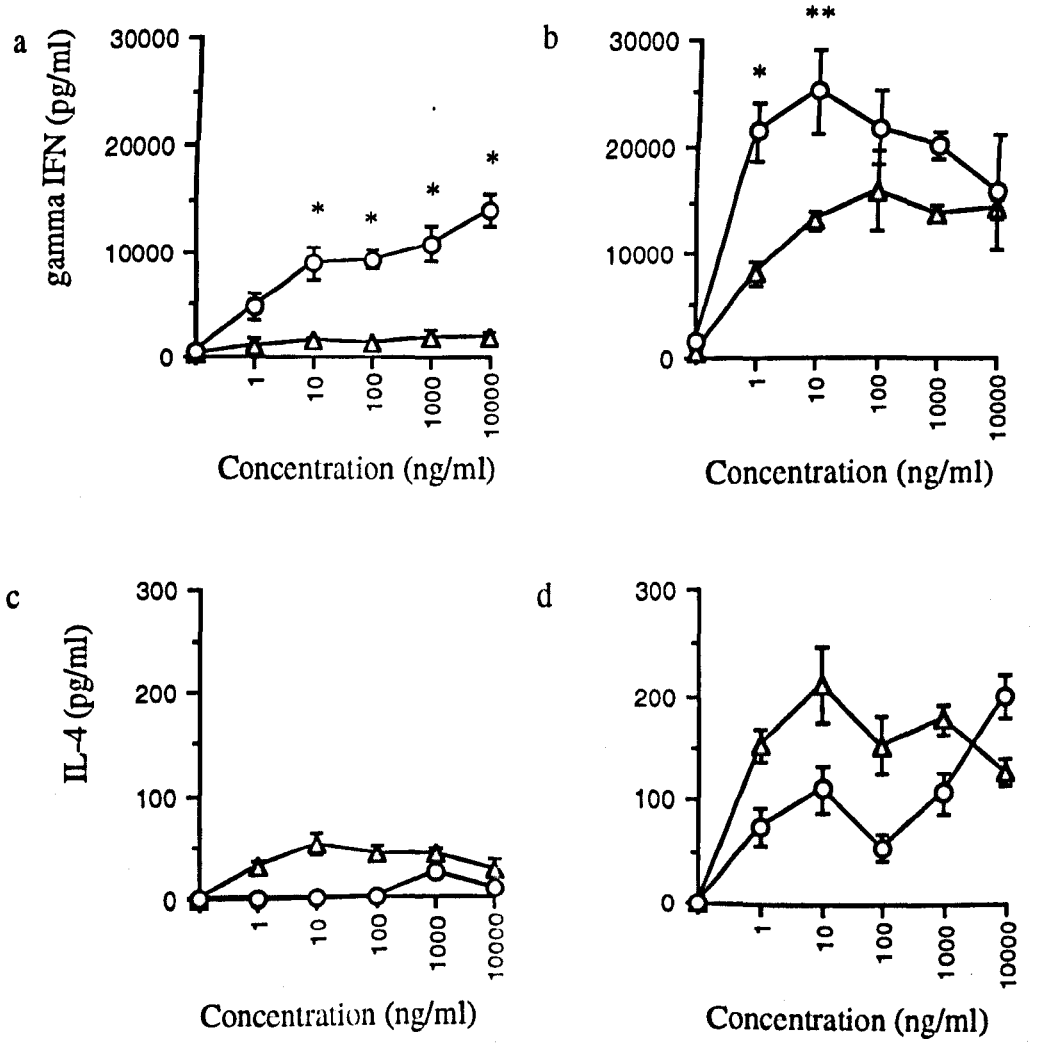
Pooled spleen cell cultures from uninfected mice were maintained for (a) 48 or (b) 96 hours after addition of increasing concentrations of TSST-1. ^{3}H-thymidine incorporation was measured during the final 6 hours of culture. Data are mean ± s.e.m. of quadruplicate cultures, representative of two similar experiments. iNOS$^{-/-}$ (O) cultures proliferated significantly more than those derived from iNOS$^{+/-}$ (Δ) mice (*$p<0.05$, **$p<0.01$, Mann-Whitney).

Figure 6.13 Spleen cell culture proliferation to SEA



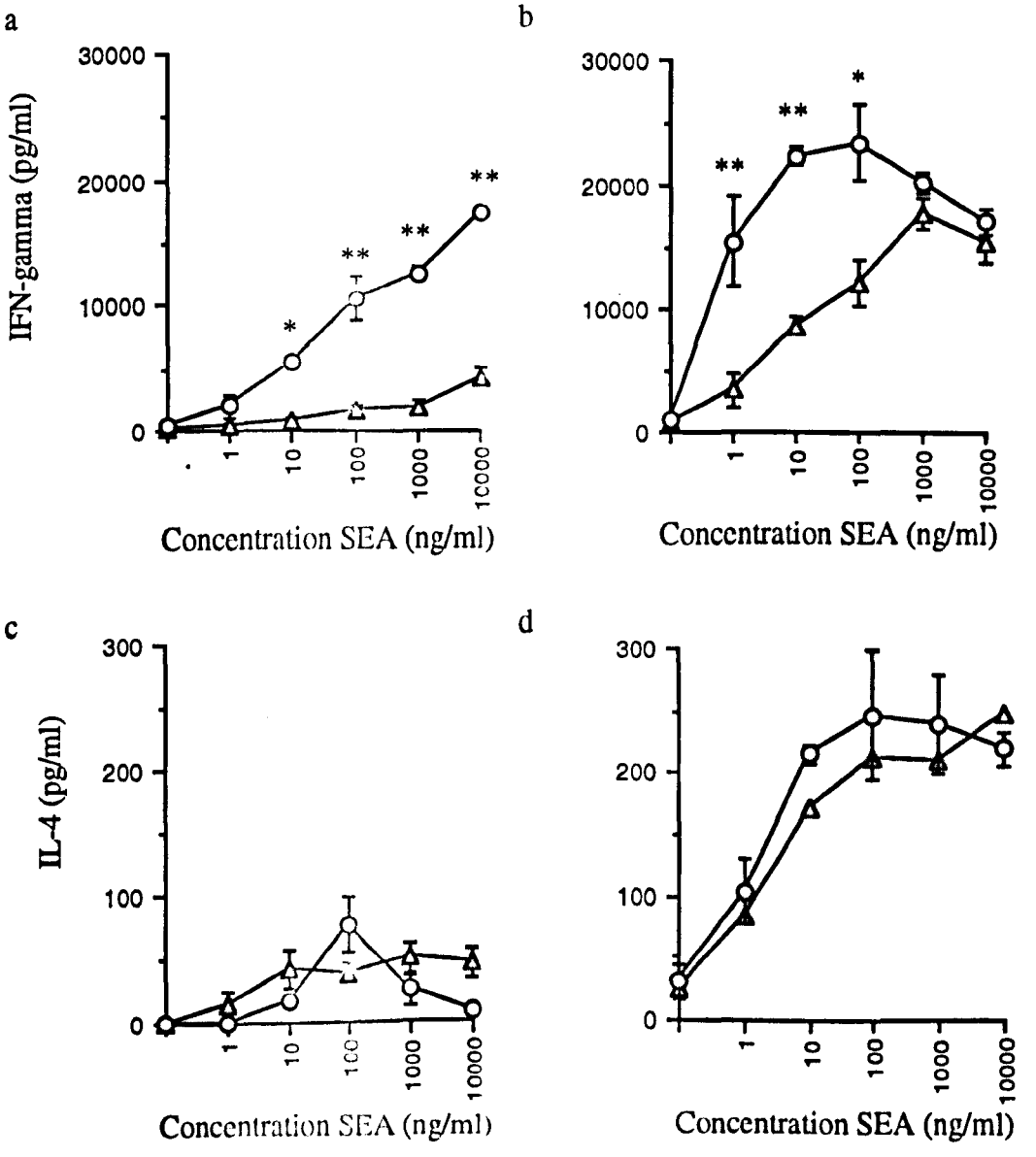
Pooled spleen cell cultures from uninfected mice were maintained for (a) 48 or (b) 96 hours after addition of increasing concentrations of SEA. ^{3}H-thymidine incorporation was measured during the final 6 hours of culture. Data are mean ± s.e.m. of quadruplicate cultures, representative of two similar experiments. iNOS$^{-/-}$ (O) cultures proliferated significantly more than those derived from iNOS$^{+/-}$ (Δ) mice (*$p<0.02$, **$p<0.05$, Mann-Whitney).

Figure 6.14 IFN-γ and IL-4 production by spleen cell cultures to Toxic shock syndrome toxin-1



Pooled splenic cultures from uninfected iNOS$^{-/-}$ (O) or iNOS$^{+/-}$ (Δ) mice were stimulated with TSST-1 at the concentrations shown for (a,c) 48 hours or (b,d) 96 hours and the production of IFN-γ (a,b) and IL-4 (c,d) was measured by ELISA. iNOS$^{-/-}$ cultures generated significantly higher IFN-γ concentrations at both time points than iNOS$^{+/-}$ cultures (*$p<0.001$, **$p<0.01$, Mann-Whitney). IL-4 synthesis was similar in both groups. Data are mean ± s.e.m. of quadruplicate cultures.

Figure 6.15 IFN-γ and IL-4 production by splenic cultures to Staphylococcal enterotoxin A.



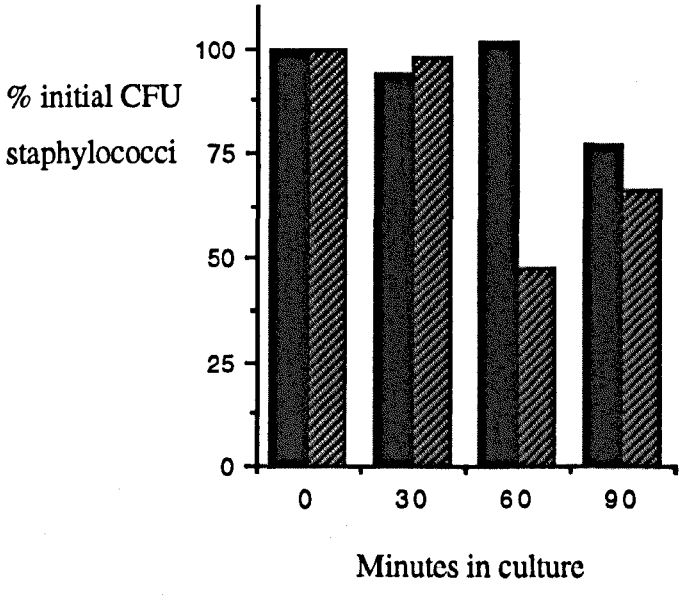
Pooled spleen cell cultures from uninfected iNOS$^{-/-}$ (O) or iNOS$^{+/-}$ (Δ) mice were stimulated with SEA at the concentrations shown for (a,c) 48 hours or (b,d) 96 hours and the production of IFN-γ (a,b) and IL-4 (c,d) was measured by ELISA. iNOS$^{-/-}$ cultures generated significantly higher IFN-γ concentrations at both time points than iNOS$^{+/-}$ cultures (*$p<0.01$, **$p<0.005$, Mann-Whitney). IL-4 synthesis was similar in both groups. Data are mean ± s.e.m. of quadruplicate cultures.

Thus, iNOS$^{-/-}$ spleen cells exhibit significantly enhanced proliferation and IFN-γ production in response to superantigens. These data indicate that following *Staphylococcus aureus* LS-1 infection *in vivo*, TSST-1 production can induce an exaggerated Th1 response, which could in turn lead to increased pathology.

6.5 Effect of iNOS deficiency on bacterial viability

The above data strongly suggest that immunomodulatory defects in the iNOS$^{-/-}$ mice contribute to poor outcome following staphylococcal infection Investigations were performed to address the possibility that direct bacterial killing was also impaired in these mice. *Staphylococcus aureus* LS-1 were opsonised, then cultured in whole blood from uninfected iNOS$^{-/-}$ and iNOS$^{+/-}$ mice for up to 90 minutes, in two separate experiments. Bacterial killing was similar in both groups (Figure 6.16) suggesting that iNOS expression was not critical for blood-borne neutrophil mediated bactericidal effects. These observations were in accord with previous reports which showed that immediate killing by neutrophils was ROI dependent, relative to RNI (Kaplan, et al, 1996). Moreover, the delay associated with iNOS expression implies that it is unlikely to occupy a critical position in immediate bacterial killing activity.

Figure 6.16 Staphylococcal killing by murine blood cultures *in vitro*



No difference in viability was observed in staphylococci incubated with whole blood cultures from iNOS$^{-/-}$ (hatched bar) or iNOS$^{+/-}$ (solid bar) mice. 100 µl of 10^7 CFU/ml freshly opsonised live *Staphylococcus aureus* were added to 100 µl heparinised blood, pooled from 4 mice from each group, for the times indicated. Cells were then lysed with ice cold water and the number of viable bacterial CFU remaining was estimated by culture on blood agar for 18 hours after ten-fold dilutions in PBS. Data are mean of duplicate cultures, representative of two similar experiments.

Discussion

Bacterial septic arthritis leads to severe articular destruction with significant associated morbidity and mortality (Goldenberg, 1989). The incidence is higher in patients with underlying inflammatory arthritis than in the general population, and is likely to increase further with wider therapeutic use of immunosuppressive drugs, total joint arthroplasty and the emergence of antibiotic resistant bacterial species (Goldenberg, 1989; Östensson & Geborek, 1991). Mechanisms underlying joint destruction are not properly understood, but appear to depend on both host- and bacteria-derived products. The rapid onset, time course and histopathological appearances of murine staphylococcal infection closely resemble those seen in human septic arthritis. It is therefore an ideal model in which to study the net effects of the microbicidal and immunomodulatory functions of NO in articular inflammation. Data presented here clearly demonstrate increased incidence and severity of septicaemia and arthritis in mice lacking the capacity for high output of NO. Thus, in contrast to the situation in 'autoimmune' inflammatory models in rodents, in which the generation of NO is associated with aggravation of pathology, NO production in the context of staphylococcal septicaemia and subsequent T cell mediated articular pathology appears to play a protective role.

By virtue of its diverse roles in maintenance of vascular tone, major organ perfusion, leukocyte adhesion, and platelet aggregation, NO has been implicated in gram negative bacterial shock, in which LPS mediates induction of high levels of iNOS expression (Kilbourn, et al, 1990; Wei, et al, 1995; reviewed by Lyons, 1995; Gross, et al, 1996). Whether NO similarly modifies gram positive bacterial pathology, in the absence of LPS, has remained controversial. The staphylococcal cell wall component, lipoteichoic acid (LTA), induces delayed circulatory failure in rats by an NO-dependent mechanism (De Kimpe, et al, 1995a), and in synergy with peptidoglycan (PG), LTA promotes multiple organ failure in anaesthetised rats

through induction of high levels of TNFα, IFN-γ and NO production (De Kimpe, et al, 1995b). However, discrepancies in the effects of NO production in anaesthetised compared to alert mice in gram negative sepsis have been reported (MacMicking, et al, 1995) and it is possible that such experiments do not accurately reflect the physiological circumstances which accompany infection. In contrast, SEB-induced T cell mediated shock in mice induces high levels of NO production, which are protective through down regulation of TNFα and IFN-γ production, with consequent reduction of mortality (Florquin, et al, 1994). However, the NOS inhibitor employed in these studies was not isoform specific, raising the possibility of complicating cardiovascular effects. Moreover, staphylococcal infection *in vivo* will reflect the combined effect of cell-wall component and superantigen-driven responses, such that data derived from use of individual components, including LTA or SEB, require cautious interpretation.

The $iNOS^{-/-}$ septic arthritis model described here avoids such criticisms, since cNOS dependent cardiovascular and neuronal functions are intact, and the TSST-1 secreting, *Staphylococcus aureus* strain, LS-1 was employed. The incidence and severity of septicaemia in $iNOS^{-/-}$ mice was significantly higher than that observed in $iNOS^{+/-}$ or $iNOS^{+/+}$ controls. This was reflected in increased weight loss, mortality and bacterial dissemination to tissues, and by elevation of serum TNFα levels, which correlated with the period of maximal clinical sepsis. Increased IFN-γ production in $iNOS^{-/-}$ mice compared with controls was observed, consistent with the suggestion that Th1 cytokine production mediates late septicaemic complications of staphylococcal infection (Zhao & Tarkowski, 1995a). These data clearly indicate that NO is a critical mediator in gram positive shock and establish a net protective effect for high output of NO following staphylococcal infection.

Low levels of NO production in response to staphylococcal challenge were detected in $iNOS^{-/-}$ spleen cells *in vitro*, suggesting that NO synthesis was not completely

abrogated in these mice. It is attractive to hypothesise that the precise level of NO production within inflammatory lesions may therefore be crucial to outcome. Low concentrations of NO enhance cytokine production and proliferation by Th1 clones *in vitro*, in contrast to the inhibitory effect of NO at higher concentrations (Taylor-Robinson, et al, 1994; Huang, F.P. personal communication). Low levels of NO production in $iNOS^{-/-}$ mice may therefore have been sufficient to enhance harmful immune responses, but were apparently inadequate to confer protection upon the host. In contrast to $iNOS^{+/-}$ controls, $iNOS^{-/-}$ mice thus fail to extract the advantage of staphylococcal clearance and immunological down regulation afforded by high levels of NO production.

T cells lie central to pathogenesis of staphylococcal infection. They are detected in synovial membrane within 48 hours of infection and anti-CD4-mediated T cell depletion ameliorates subsequent septic arthritis (Abdelnour, et al, 1994a). Non-exotoxin producing mutant staphylococci exhibit impaired pathogenicity (Bremmell & Tarkowski, 1995) and clonal expansion by superantigens is required for arthritis development (Abdelnour, et al, 1994b), such that TSST-1 producing staphylococcal arthritis is attenuated by prior deletion of Vβ11 TCR expressing T lymphocytes. Moreover, Vβ3 TCR transgenic mice show increased mortality associated with enhanced IFN-γ production after infection with SEA-producing *Staphylococcus aureus* (Zhao, et al, 1995b) These data indicate an important role for superantigen secretion, mediated primarily through their effect on T cell activation and Th1 cytokine production. The present data demonstrate that NO is involved in regulating these processes. Naive $iNOS^{-/-}$ spleen cells proliferated more and produced IFN-γ at higher levels than did $iNOS^{+/-}$ controls, in response to either TSST-1 or SEA stimulation. IL-4 production, in contrast, was of low level and was identical in the two groups. These data suggest that NO normally restricts Th1 responses induced by superantigens, analogous to the NO-dependent suppression of antigen specific Th1 responses in murine leishmanial infection *in vivo* (Wei, et al, 1995) and of Th1 clones

in vitro (Taylor-Robinson, et al, 1994; Huang, F.P. personal communication). Since IFN-γ is an important iNOS activator, this provides an important negative feedback loop *in vivo*.

It therefore seems likely that the inability to generate high levels of NO following staphylococcal infection in $iNOS^{-/-}$ mice led to an exaggerated superantigen-driven Th1 response. The mechanism whereby NO mediates such effects is unclear. Direct regulation of cytokine production may be exerted by NO at the transcriptional level, through alteration of NF-κB binding, or by mRNA stabilisation (Matthews, et al, 1996; Huang, F.P. unpublished observations). However, given its previously reported role in induction of apoptosis (Albina, et al, 1993; Messmer, et al, 1993; Fehsel, et al, 1995), it is also possible that NO enhances the apoptosis which is the normal consequence of superantigen-mediated activation for the majority of T cells (Kawabe & Ochi, 1991). Other regulatory pathways which have been established in staphylococcal superantigen-driven shock include increased endogenous glucocorticoid production and synthesis of anti-inflammatory cytokines, such as IL-10 and G-CSF (Florquin & Goldman, 1996). The interaction of NO with these pathways is currently unclear.

Staphylococcal arthritis was observed with increased frequency and severity in $iNOS^{-/-}$ mice. However, the role of Th1/Th2 imbalance in determining the outcome of septic arthritis is less well defined, reflecting doubt as to the precise role of IFN-γ in the pathogenesis of murine arthritis. Whereas IFN-γ up-regulates MHC class II expression and pro-inflammatory monokine production, including IL-1β and TNFα (Farrar & Schreiber, 1993; Verhoef & Mattsson, 1995), it can also inhibit fibroblast proliferation, collagen, prostaglandin and MMP secretion and can oppose IL-1β mediated bone resorption and cartilage degradation (Freundlich et al, 1986; Gowen, et al, 1986; Seitz, et al, 1994). Direct intra-articular injection of IFN-γ exacerbates collagen-induced arthritis (CIA), and increased levels of IFN-γ mRNA are detectable

in the synovial membrane following staphylococcal infection, suggesting that high levels of local IFN-γ production are arthritogenic (Mauritz, et al, 1988; Zhao, et al, 1996). However, systemic administration of IFN-γ inhibits CIA (Nakajima, et al, 1990; Williams, et al, 1993), and IFN-γ receptor (IFNγR) knockout mice develop milder septic arthritis than do $IFN\gamma R^{+/+}$ controls following staphylococcal infection (Zhao, et al, 1995b). Recent studies in Xid mice also determined that high levels of IFN-γ production were arthroprotective in septic arthritis (Zhao, et al, 1995c). *In vitro* data in this study indicate that $iNOS^{-/-}$ mice generated higher levels of IFN-γ than $iNOS^{+/-}$ controls, but developed more frequent and severe arthritis. It is therefore possible that the protective effects of IFN-γ observed in other models at articular inflammatory sites are NO dependent. Further studies designed to quantify the level of IFN-γ expression in $iNOS^{-/-}$ and $iNOS^{+/-}$ synovia during infection are now required to confirm that local IFN-γ expression is enhanced. Moreover, since the light microscopic histological appearances were similar in the two groups, it will also be important to characterise the cell subsets present, given the recognised ability of NO to modify cell adhesion and migration (Kubes, et al, 1993; Xin, et al, 1993).

NO has also been implicated in direct killing of gram positive bacteria and it seems likely that this will contribute to the clinical consequences of staphylococcal infection in $iNOS^{-/-}$ mice. Cytokineplasts from human neutrophils kill staphylococci by an NO-dependent mechanism (Malawista, et al, 1992) but these pathways appear secondary to ROI in importance for immediate killing. In cell free systems, NO enhances staphylococcal killing over longer periods, suggesting that it might play a role if ROI production were compromised (Kaplan, et al, 1996). Consistent with this, neutrophils from $iNOS^{-/-}$ and $iNOS^{+/-}$ mice exhibited similar killing activity *in vitro* in a short term bactericidal assay. However, the increased dissemination of viable staphylococci to blood and kidneys in $iNOS^{-/-}$ mice after initial localisation to the spleen, provided circumstantial evidence that bacteria preferentially survived in the $iNOS^{-/-}$ splenic environment. It is thus possible that NO-dependent killing will be

observed only in longer term assays using spleen-derived macrophages. Recently, human neutrophil phagocytosis of fluorescein-labelled fixed staphylococci was shown to be augmented by addition of L-arginine and abrogated by NOS inhibitors (Moffat, et al, 1996). Further studies using more sensitive bactericidal assays are therefore required to address the specific role of NO in bacterial phagocytosis and subsequent killing in $iNOS^{-/-}$ compared with $iNOS^{+/-}$ derived polymorphonuclear cells and macrophages. The interaction with ROI in these circumstances also deserves further investigation. Several recent reports have identified a key protective role for NO by diverting ROI from causing intracellular damage (Wink, et al, 1993; Wink, et al, 1996) and transient inhibition of the effects of ROI on staphylococcal killing has been observed after addition of NO-donors (Kaplan, et al, 1996). Another possibility is that NO mediated effects are mediated not through immediate viability, but rather by modifying bacterial adhesion and tissue localisation. Expression of cellular adhesion factors, such as collagen adhesin, are important virulence factors which offer enticing targets for nitrosylation, functional modification and thus, host defence (Patti, et al, 1994).

Several clinical and therapeutic implications arise. These data provide the first evidence for a protective role for NO in rodent articular pathology *in vivo*. The use of specific iNOS inhibitors in treatment of human inflammatory arthritis has been proposed on the basis of their inhibitory effects in animal inflammatory models. The incidence of septic arthritis is already increased in such patients and removal of a major natural anti-bacterial mediator may further increase this risk. Since NO exerts influence *in vitro* on diverse pro- and anti-inflammatory pathways which are present *in vivo* in the synovial membrane, it seems unlikely that such an approach will afford significant clinical improvement. Indeed, the potentially harmful removal of a major component of non-specific synovial defence against infection may outweigh any putative benefits. Human synovial membrane is capable of NO production in response to bacterial superantigen and these observations should provoke a search for

direct evidence for NO synthesis in human septic arthritis. NO production in that context may constitute an important pathway whereby bacterial killing is optimised and T cell activation is opposed. Together with reported amelioration of septic arthritis after CD4 and Vβ11 T cell depletion (Abdelnour, et al, 1994b), this raises the challenging proposal that therapeutic T cell suppression might be desirable in treating such patients.

Superantigens have been implicated in induction of autoimmunity by mechanisms which include activation of quiescent auto-reactive T cells, partial activation of auto-reactive B cells through class II recognition, or induction of aberrant cognate help for B cells by T cells by superantigen-mediated crosslinking (Friedman, et al, 1991; Goodacre, et al, 1994). Through suppression of superantigen responses, NO may reduce the tendency for auto-reactivity following staphylococcal infection, whereas impaired NO production may result in the opposite effect. Moreover, inadequate NO production at an initial inflammatory site might precipitate enhanced Th1 cytokine production, with increased levels of tissue MHC class II expression. Aberrant class II expression has been implicated previously in thyroid and pancreatic autoimmunity (Bottazzo, et al, 1983). Further investigations are required to address such novel disease mechanisms.

Chapter 7

General Discussion

Neither the factors which account for onset of RA, nor those which mediate chronicity have been defined. Moreover, it should not be assumed that these two processes are identical. Nevertheless, the synovial immune response offers an attractive therapeutic target, although it is currently unclear which pathways offer the most potential clinical benefit. Controversy surrounding the nature of the critical regulatory cells within synovium, therefore lies central to rational choice in further development of biological therapies (Firestein & Zvaifler, 1990; Panayi, et al, 1992; Kingsley, et al, 1996). In normal circumstances, the interaction of T lymphocytes and macrophages is critical to generate cytokines from both cells, which together regulate the nature and outcome of the immune response. Since outcome encompasses a spectrum from complete resolution to chronicity, this relationship is likely to be critical in RA. Perhaps the most puzzling feature of RA synovial immuno-biology, therefore, has been the presence of a large population of T cells of activated phenotype, in the context of relatively low levels of T cell activation cytokines, either those capable of autocrine stimulation of T cells, e.g. IL-2, or those providing evidence for T cell effector function, e.g. IFN-γ In contrast, widespread evidence exists of macrophage activation and of monokine synthesis. The identification of IL-15, a cytokine of macrophage or fibroblast derivation, which is capable of T cell recruitment and activation in the synovial membrane, therefore provides a novel pathway, whereby co-ordinate T cell and macrophage function can occur in RA.

IL-15 expression was detected in RA synovial lining layer and within T lymphocyte aggregates. Parallel staining in the former indicated that mostly $CD68^+$ cells were IL-15 positive. Extensive staining in T cell rich areas was observed, raising the possibility that synovial T cells might contribute to their own activation through autocrine production of IL-15, although this appearance might also represent membrane bound cytokine, or macrophages which are also present in these areas. IL-15 expression has now been confirmed immunohistochemically in RA synovial

membrane, including biopsy material from early RA patients (Tak, P.P, personal communication). IL-15 was also detected to a significantly lesser extent in reactive arthritis synovial membrane (Thurkow, et al, 1996). Interestingly, double staining with T cell markers in the latter investigation confirmed that synovial T cells expressed IL-15. Future *in situ* hybridisation studies will be required to properly characterise the cellular location of IL-15 synthesis in synovial membrane.

Several mechanisms have been proposed to explain synovial T cell activation in the relative absence of IL-2. Synovial T cells are of mature $CD45RO^{+}$, RB^{dim}, $CD29^{+}$, $CD27^{-}$ phenotype and simultaneously express activation and adhesion markers, characteristic of various stages of the 'normal' T cell activation pathway. This phenotype almost certainly reflects preferential recruitment of specific T cell subsets from peripheral blood, which possess intrinsic migratory potential (Pitzalis, et al, 1991; Iannone, et al, 1994; Kohem, et al, 1996). Activation is not, however, a prerequisite for such migration and other factors presumably operate to induce the observed phenotype. These include activation by adhesion molecule-ligand interactions following endothelial cell contact during extravasation and, since β1 and β2 integrin receptors also bind to components of the extra-cellular matrix, further interactions in the synovial membrane itself (e.g. Damle & Aruffo, 1991; Gilat, et al, 1996). In this context, IL-15 provides an important additional pathway whereby T cell activation can be initiated and maintained. IL-15 readily induces proliferation of PHA-T cell blasts (Grabstein, et al, 1994; Tagaya, et al, 1996a) and of a subset of circulating RA PB T cells. The latter likely represent previously primed $CD45RO^{+}$ T cells, since these cells preferentially express CD69, an early marker of T cell activation, within 24 hours of IL-15 addition *in vitro*. Moreover, T cells from the RA synovial compartment exhibit enhanced responsiveness to IL-15, assessed either by proliferation or by cytokine production, compared with PB T cells.

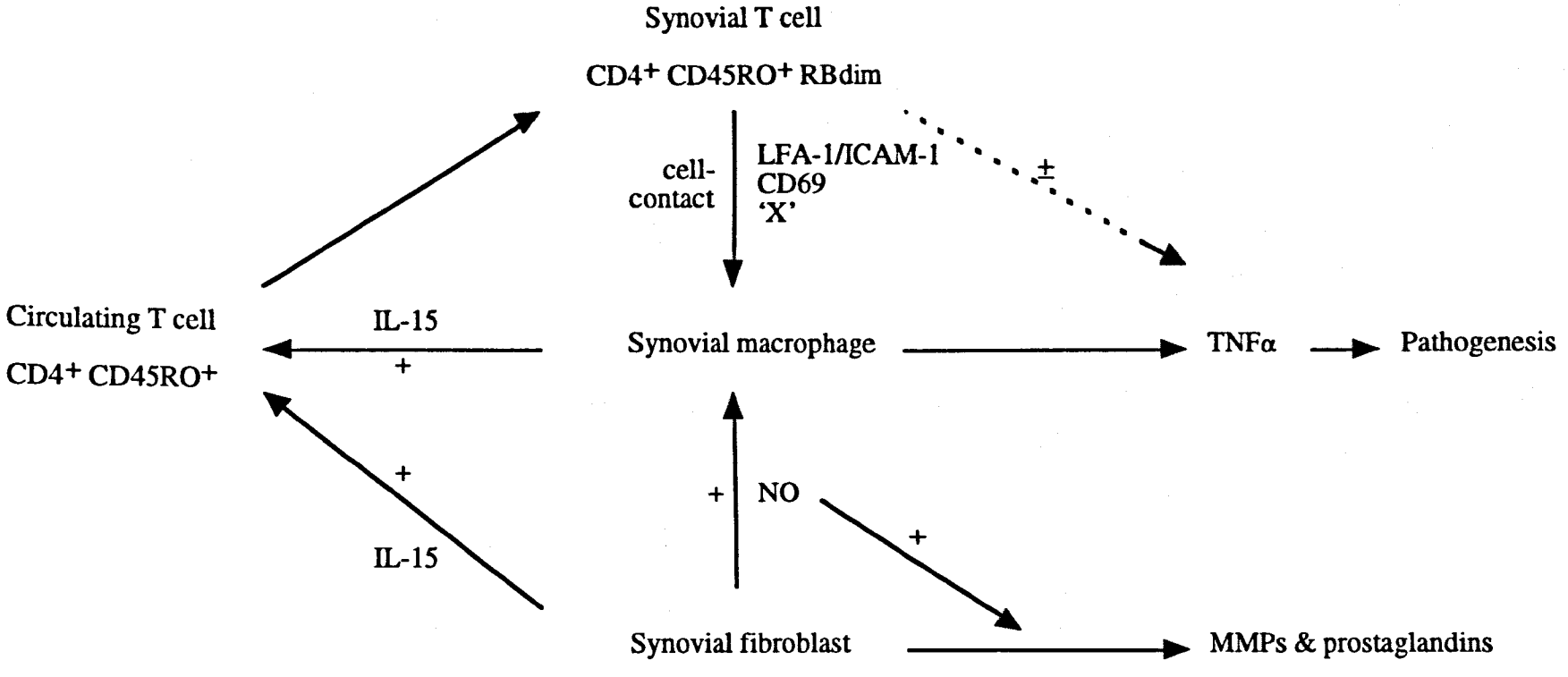
The clinical effects of T cell modulation in RA are generally favourable, indicating some regulatory role. This may represent inhibition of specific arthritogenic clones, or perhaps reflect general inhibition of polyclonal T cells. Clinical efficacy of anti-CD4 therapy is related to the extent of synovial T cell binding by therapeutic antibody, indicating that the majority of synovial T cells are indeed contributing to pathogenesis (Choi, et al, 1996). However, this effector pathway does not seem to include cytokine production by the majority of T cells within the synovial membrane. IFN-γ and IL-5 production have been detected following IL-15 stimulation of T cells (Seder. et al, 1995; Seder, 1996; Mori, et al, 1996), and modest TNFα production was detected following IL-15 stimulation of synovial T cells *in vitro* (Chapter 4). Nevertheless, it appears that *in vivo*, the presence of IL-15 alone is not sufficient to induce significant IFN-γ or IL-2 production by synovial T cells. It was therefore of interest to investigate novel mechanisms whereby IL-15 activated T cells might contribute to inflammation in RA.

Studies in animal models and in clinical trials in RA using neutralising monoclonal antibodies against TNFα have indicated that the latter cytokine occupies a central position in the regulatory cytokine network in synovial membrane (Williams, et al, 1992; Maini, et al, 1995). TNFα synthesis is therefore a biologically relevant indicator of macrophage activation *in vitro*. T cells and macrophages lie in juxtaposition in lymphocyte aggregates, in which IL-15 expression was observed, raising the possibility that cell-contact mediated communication could occur. Previous studies have established that T cells or T cell clones, after activation by mitogen, can induce cytokine and MMP production by macrophages and fibroblasts through cell contact (Vey, et al, 1992; Isler, et al, 1993; Lacraz, et al, 1994). The present study demonstrated that IL-15-mediated activation conferred similar properties on PB T cells. Furthermore, synovial T cells exhibited this property *ex vivo*, which was IL-15 dependent. Thus, polyclonal synovial T cells can induce macrophage activation and TNFα production, with pro-inflammatory consequences.

It is attractive to hypothesise that IL-15 may also be produced by similarly activated macrophages, providing a positive feedback loop leading to further T cell recruitment and activation (Figure 7.1). This hypothesis therefore predicts firstly, that IL-15 activated T cells will induce production of IL-15 by synovial macrophages or fibroblasts. Secondly, interruption of IL-15 mediated T cell activation using neutralising antibodies against IL-15, or soluble IL-15Rα chain, will down regulate TNFα production, either in animal models or in synovial membrane culture systems. Thirdly, effective anti-T cell therapies will exhibit efficacy in proportion to the extent of synovial T cell binding or inhibition of T cell adhesion (Kavanaugh, et al, 1995; Choi, et al, 1996). Finally, T cells need have no absolute requirement for T cell cytokine production, nor for the recognition of local antigen. Further investigations are now required to test at least the first two predictions.

IL-15 may contribute to RA synovitis by several means other than T cell activation and cell-contact. Effects of IL-15 on adhesion molecule redistribution on T cells have been reported (Nieto, et al, 1996). Furthermore, IL-15 is also a potent chemotactic factor for T lymphocytes. Evidence *in vitro* (Wilkinson & Liew, 1995; Al-Mughales, et al, 1996) and *in vivo* in the current study indicates that IL-15 can induce significant T cell recruitment. The combination of effects on adhesion and locomotion imply an important role for IL-15 in regulating T cell extravasation and simultaneous endothelial contact events, which mediate lymphocyte migration to the synovial compartment. In addition, the ability to activate B cells and to induce isotype switching indicates a possible role in rheumatoid factor production in the synovial membrane. Significant numbers of plasma cells synthesising rheumatoid factor are present in synovial membrane, often surrounding T cell aggregates (Otten, et al, 1993; Brown, et al, 1995), but their pathological role is unclear. Immune complexes formed from local auto-antibody production can contribute to synovial inflammation, through complement fixation, and up regulation of cytokine

Figure 7.1 IL-15 mediated T cell activation leads to up regulation of TNFα production



production (Chantry, et al, 1989; Plater-Zyberk, et al, 1992). Whether IL-15 is involved in these processes remains unclear. Finally, IL-15Rα chain expression has been detected in activated macrophages (Giri, et al, 1995; Anderson, et al, 1995b), and it remains possible that IL-15 can exert direct effects on macrophages in an autocrine fashion. Taken together, these data indicate a crucial role for IL-15 in RA pathogenesis.

The present study represents the first demonstration of IL-15 expression in a pathological setting. Preliminary experiments have now identified the presence of IL-15 by immunohistochemistry in alveolar macrophages from asthmatic patients (Leung, B.P. & McInnes, I.B. unpublished observations). Moreover, IL-15 increases IL-5 production by human Th2 cell clones (Mori, et al, 1996), and mast cells proliferate and mature through IL-15 mediated binding to its own IL-15X receptor (Tagaya, et al, 1996b), indicating a possible role in type I bronchial hypersensitivity. IL-15 has also now been identified in alveolar macrophages from patients with pulmonary sarcoidosis (Agostini, et al, 1996). It is intriguing that sarcoid, like RA, is associated with macrophage activation, and has possible aetiological associations with mycobacterial species. Furthermore, *M. tuberculosis* and B.C.G. are inducers of IL-15 expression, at least in murine macrophages (Doherty, et al, 1996). IL-15 likely constitutes an important component of early host defence to infection. However, tight control of IL-15 mRNA translation has been reported (Bamford, et al, 1996a; Bamford, et al, 1996b) and rapid down regulation of IL-15Rα chain follows T cell activation, in comparison to IL-2Rα (Kumaki, et al, 1996), indicating that its functional role is short lived in the normal event. Such close control of protein synthesis implies that excess production comprises a danger to the host. It is therefore attractive to hypothesise that RA could result from failure to properly regulate IL-15 expression following an infective insult, leading to T cell recruitment and activation and subsequent chronicity. Further investigation of the factors which up regulate IL-15 production

by human synovial macrophages and synoviocytes is required to clarify the significance of such observations.

The recent recognition that NO was a potent immunoregulatory molecule prompted investigation of its presence and functional contribution in RA synovial membrane. The present study has characterised the expression of iNOS *ex vivo* in human synovial membrane. NO production in the articular environment has now been detected in cartilage, bone and synovial membrane (Stadler, et al, 1992; Charles, et al, 1993; Rediske, et al, 1994; Ralston, et al, 1995) in both inflammatory and degenerative arthropathies. Prior to the present study, the net effect of such NO generation *in vivo* in arthritis models has been detrimental (Ialenti, et al, 1993; Stefanovic-Racic, et al, 1994a; MacCartney-Francis, et al, 1994). Whereas the staphylococcal infectious arthritis model is not directly comparable to other auto-immune inflammatory diseases in rodents, it does provide an opportunity to study the mechanisms whereby T cell mediated articular destruction can occur. In this context, failure to generate high output NO synthesis was clearly detrimental to the host, manifest as increased incidence and severity of septicaemia and of arthritis. Poor clinical outcome was associated with enhanced Th1 responses in $iNOS^{-/-}$ mice. Although specific cytokine neutralisation experiments are required to confirm that this relationship is causal, the evidence from SEB-induced T cell mediated shock suggests that this is likely to be the case (Florquin, et al, 1994). It can be inferred from these observations that NO functions normally to regulate Th1 responses *in vivo* providing sufficient host defence at minimum cost. Aberrant regulation of NO synthesis might therefore prejudice immune responses to host destruction in certain circumstances.

NO exhibits pleiotropic effects which might influence at least three areas of articular physiology - homeostasis, immunoregulation and synovial defence.

(i) *Homeostasis* - It is clear from studies in bovine, rodent and recently human bone and cartilage, that NO production is implicated in cytokine mediated effects on matrix biosynthesis and degradation (Hauselmann, et al, 1994; Jarvinen, et al, 1995; Fukuda, et al, 1995). Remodelling is essential for normal tissue integrity, indicating that alterations in local NO synthesis can have significant consequences. At high concentrations NO down regulates osteoclast activity and can mediate cytokine-induced osteoblast activation. In contrast, at lower concentrations, NO potentiates bone resorption (Evans, & Ralston, 1996). These data implicate NO in erosion formation, and perhaps also in the local and systemic osteoporosis characteristic of RA patients. Similarly, NO exerts precise effects on chondrocyte function which may depend on the time course of inflammation, e.g. IL-1β induced catabolism of bovine cartilage is inhibited by NO in the acute phase, but is enhanced in the chronic phase *in vitro* (Stefanovic-Racic, et al, 1996).

NO may exert a vascular regulatory role in synovial membrane. High levels of endothelial iNOS expression were detected in the current study. NO has been shown to promote neovascularisation and was originally recognised as a vasodilator (Furchgott & Zawadski, 1980; Ignarro, et al, 1987; Leibovich, et al, 1994; Tamura, et al, 1996). It may therefore oppose the activities of vasoconstrictor substances, such as endothelin-1, which can also be detected in inflammatory synovium (Miyasaka, et al, 1992). Synovial hypoxia has been implicated as pro-inflammatory factor (Blake, et al, 1989). Thus, NO appears integral to several homeostatic processes, reducing its ultimate value as a specific therapeutic target.

(ii) *Immunoregulation* - Data in the present study and elsewhere (Ialenti, et al, 1993; Oyanagui, 1994) indicate that immunoregulatory activities of NO can significantly alter the outcome of articular inflammation. NO derived from

endothelial cells and synovial fibroblasts might therefore influence the behaviour of neighbouring macrophages and T lymphocytes within the synovial compartment. These effects need not all be pro-inflammatory. Thus, in the present study, NO-donors increased TNFα production *in vitro* from synovial macrophages / fibroblasts, and macrophage cell lines, and enhanced MMP and prostaglandin production by NO have also been reported (Salvemini, et al, 1993; Murrell, et al, 1995; Manfield, et al, 1996). However, synovial NO levels were sufficient to suppress T cell responsiveness *in vitro* (Merryman, et al, 1993). Moreover, observations in the T cell-driven staphylococcal model indicate that NO mediated effects on T cells may indeed have physiological relevance in articular pathology. Further investigations are now required to determine whether NO regulates IL-15 production in a similar manner to TNFα. The possibility that a reciprocal relationship exists whereby NO production by synovial fibroblasts is, in turn, induced by IL-15 activated T cells through cell-contact is intriguing.

(iii) *Microbicidal* - NO is implicated in host defence to numerous protozoans, fungi, viruses and bacteria and in many systems, the role of NO is crucial to successful outcome. A similar role in synovial defence is strongly indicated by the increased virulence of staphylococcal infection in $iNOS^{-/-}$ mice.

Therapeutic intervention with iNOS inhibitors awaits the development of isoform specific agents. The above however indicates that no clear protective effect can be predicted. The production and regulation of NO in human and rodents is not synonymous, with high output of NO apparently delegated to different cell-types in each species. It is not known whether this has clinically significant and biological consequences, although it highlights that cautious interpretation of data from short term animal models is required.

It is not clear whether currently available anti-rheumatic drugs inhibit either iNOS activity or the effects of NO in tissue. Methotrexate, D-penicillamine, 5-amino-salicylate, and indomethacin have been reported to inhibit NO synthesis *in vitro* or to protect against peroxynitrite production (Amin, et al, 1995; Murrell, et al, 1996; Whiteman, et al, 1996). However, many commonly used drugs show little such activity (Whiteman, et al, 1996), yet are of apparently similar efficacy *in vivo* and further investigation is required. Interestingly, tetracycline based antibiotics exhibit potent protection against nitrosylation of tyrosine residues *in vitro*, and have been implicated in treatment of RA, perhaps indicating a possible disease modifying mechanism (Kloppenberg, et al, 1994; Tilley, et al, 1995). Recently, NO has been conjugated to NSAIDs to produce agents with anti-inflammatory activity in acute murine inflammation models, but which are associated with significantly reduced gastrointestinal toxicity, apparently through local release of NO (Del Soldato, et al, 1996). Whether the latter also has protective activity at the site of inflammation is unclear.

Conclusions

The present study was concerned with immunoregulation in RA synovial membrane (section 1.6).

1. It set out to address mechanisms of synovial T cell activation in RA and thereafter to implicate polyclonal T cells in pro-inflammatory pathways in the synovial membrane. The identification of IL-15 in synovial membrane and the demonstration that IL-15 can activate synovial T cells, induce T cell migration *in vivo* and, through T cell-contact with macrophages, up regulate TNFα production, provides a novel pro-inflammatory pathway with potential for therapeutic intervention.

2. Definitive evidence for NO production by human synovial membrane has been obtained and the distribution of iNOS in inflammatory and degenerative synovium has been characterised. NO was shown to increase TNFα production by synovial macrophage / fibroblast cultures, providing a further mechanism for TNFα up regulation. This provides a precedent for modulation of synovial cytokine production by NO. The *in vivo* activity of NO in a model of septic arthritis suggested a protective role, at least in part through regulation of T cell activity. This again provided evidence for immunoregulatory modification of articular pathology by NO. These observations have implications for therapeutic manipulation of NO synthesis in human arthritis.

Future Studies

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

- *IL-15 receptor expression* - its distribution and regulation of expression in synovial membrane.
- *IL-15 in arthritis models* - Development of appropriate murine reagents to allow investigation of the role of IL-15 in animal models of articular inflammation.
- *Regulation of IL-15 production* - identification of the factors which up regulate IL-15 production in synovial membrane.
- *Regulation of NO production* - investigation of the role of cytokines and of T cell contact in activation of fibroblast iNOS in the synovial membrane.
- *NO in cytokine regulation* - what is the role of NO in regulation of other pro-inflammatory cytokines in synovial membrane, including IL-15?

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Appendix I

Buffers

1. PBS (x10 stock)

80g NaCl
11.6g NaH_2PO_4
2g KCl
2g KH_2PO_4

2. PBS / Tween

0.5 ml Tween 20
1000 ml PBS (x1)

3. Coating buffer (ELISA)

0.1M $NaHCO_3$, pH 8.2

4. Vector Kit buffer

0.1M Tris-HCl, pH 8.2

5. TBS (ICC Wash buffer)

900 ml 0.9% NaCl
100 ml 50mM Tris-HCl, pH 3.6
50 mM Tris-HCl
- Add conc HCl to 25 ml 0.2M tris-HCl to pH 7.36
Make up to 100 ml with dH_2O

6. Paraformaldehyde

1g PFA
100 ml PBS
100 µl 2M NaOH
Heat to 60°C then gently mix until solution clears.

7. Fast red solution

A 100 mg naphthol-AS-MX phosphate
5 ml N.N.-dimethylformamide
100 ml TBS, pH8.2

B 0.1M levamisole in dH_2O

8. NSE stain phosphate buffers

A 9.08 g/l KH_2PO_4 (0.067M)

B 11.9 g/l Na_2HPO_4, $2H_2O$ (0.067M)
Add 98.5 ml of A to 1.5 ml of B.

9. Griess Reaction

A 0.1% α-naphthyl-amine in dH_2O

B 1% sulfanilamide in 5% phosphoric acid
Mix equal volumes for Griess reagent
Store away from light

Appendix II

Awards arising

1. Young Investigator Award
   XVI European Workshop for Rheumatology Research

   McInnes, I.B., Al-Mughales, J., Leung, B.P., Sturrock. R.D., Huang. F-P., Wilkinson, P.C., Field, M., Liew, F.Y. (1996) Interleukin-15 may potentiate inflammation in rheumatoid arthritis synovitis.

2. Scottish Society for Experimental Medicine
   Research Prize 1996

   McInnes, I.B., Leung, B.P., Al-Mughales, J., Wilkinson, P.C., Field, M., Sturrock, R.D., Liew, F.Y. (1996) Role for interleukin 15 in pathogenesis of rheumatoid arthritis.

Appendix III

Publications

Original Papers

1. McInnes, I.B., Leung, B.P., Field, M., Sturrock, R.D., Liew, F.Y. (1996) Interleukin 15 mediates T cell dependent regulation of TNFα production in rheumatoid arthritis. *Nature (Medicine)* (in press)

2. McInnes, I.B., Leung, B.P., Field, M., Wei, X.Q., Huang, F-P., Sturrock, R.D., Kinninmonth, A., Mumford, R.A. & Liew, F.Y. (1996) Production of nitric oxide in the synovial membrane of rheumatoid and osteoarthritis patients. *J. Exp. Med.* **184**, 1519-1525.

3. McInnes, I.B., Al-Mughales, J., Field, M., Leung, B.P., Huang, F-P., Dixon, R., Wilkinson, P.C., Sturrock, R.D. & Liew, F.Y. (1996) The role of Interleukin 15 in T cell migration and activation in rheumatoid arthritis. Nature (Medicine) **2**, 151-159.

Clinical and related papers published in parallel studies

4. Porter, D.P., Capell, H.A., McInnes, I.B., Hunter, J,. Madhok, R., Munro, R., Thomson,. E.A.. (1996) Is RA becoming a milder disease? Or are we starting second line treatment earlier? *Brit. J. Rheumatol.* (in press)

5. McInnes, I.B., Porter, D., Thomson, E.A., Pullar, T., Hunter, J., Madhok, R., Capell, H.A. (1996) Sulphasalazine desensitisation does not reduce the incidence of adverse effects in rheumatoid arthritis. *Ann. Rheum. Dis.* **55**, 328-330.

6. Crilly, A., McInnes, I.B., MacDonald, A., Capell, H.A., Madhok, R. (1995) Interleukin 6 and soluble interleukin 2 receptor levels during methotrexate therapy in rheumatoid arthritis. *J. Rheumatol.* **22**, 224-226.

7. Neilly, B., McInnes, I.B., Kelly, I., Field, M., Gray, H. (1995) Scintigraphic findings in synovial chondromatosis at presentation. *Brit J Rheumatol* **34**, 687-689.

8. McInnes, I.B., Capell, H.C. (1995) Management of rheumatoid arthritis *Scot. Med. J.* **40**, 35-36.

9. Crilly, A., McInnes, I.B., Capell, H.A., Madhok, R. (1995) The effect of azathioprine on serum levels of interleukin 6 and soluble interleukin 2 receptor. *Scand J Rheumatol* **23**, 87-89.

10. Porter, D.R., McInnes, I.B., Hunter, J., Capell, H. (1994) Outcome of second line therapy in rheumatoid arthritis *Ann Rheum Dis* **53**, 812-815.

11. McInnes, I.B. (1993) Down's syndrome, inflammatory arthritis and thyrotoxicosis. *Rheumatol. Review* **2**, 51-54.

Original Papers - Manuscripts submitted or in preparation

12. McInnes, I.B., Leung, B.P., Wei, X.Q., Gemmell, C.G. & Liew, F.Y. (1996) *Staphylococcus aureus* arthritis in mice lacking inducible nitric oxide synthase. (in preparation)

13. Leung, B.P., Thomson, J,. McInnes, I.B., McSharry, C., Thomson, N. & Liew, F.Y. (1996) Nitric oxide and cytokine production in induced sputum from asthmatic patients. (in preparation)

Communications published in abstract form

14. Clinton, L., McInnes, I.B., Sturrock, R.D., Field, M. (1993) Enhanced GM-CSF receptor-expression in peripheral blood monocytes in patients with rheumatoid arthritis. *Clin Rheumatol* **12**, 20.

15. McInnes, I.B., Field, M., Armstrong, S., Sturrock, R.D. (1993) The use of single dose infusion of the monoclonal antibody Campath-1H in treatment of intractable rheumatoid arthritis. *Clin Exp Rheumatol* **8**, S190.

16. Clinton, L., McInnes, I.B., Gemmell, C., Sturrock, R.D. (1994) Decreased polymorphonuclear Fcγ III receptor intensity in rheumatoid arthritis *Clin Rheumatol* **13**, 25

17. McInnes, I.B., Field, M., Kinninmonth, A., Telfer, J., Sturrock, R.D., Liew, F.Y. (1995) Nitric oxide production by primary synovial cultures from rheumatoid and osteoarthritis patients. *Brit. J. Rheumatol.* **34**, S1 64.

18. McInnes, I.B., Field, M., Sturrock, R.D., Liew, F.Y. (1995) Clinical effects of a nitric oxide inhibitor on developing and established collagen induced arthritis in DBA/1 mice. *Brit. J. Rheumatol.* **34**, S1 44.

19. McInnes, I.B., Liew, F.Y., Field. M., Sturrock, R.D. (1995) Investigation of a role for nitric oxide in pathogenesis of inflammatory and degenerative arthropathies *Clin. Rheumatol.* **14**, 254.

20. McInnes, I.B., Al-Mughales, J., Leung, B., Huang, F-P., Field, M., Sturrock, R.D., Liew, F.Y. (1995) A role for interleukin 15 in T cell migration and activation in rheumatoid arthritis. *Arthritis Rheum.* **38**, S235.

21. McInnes, I.B., Al-Mughales, J., Leung, B,P., Sturrock. R.D., Huang. F-P., Wilkinson, P.C., Field, M., Liew, F.Y. (1996) Interleukin-15 may potentiate inflammation in rheumatoid arthritis synovitis. 16th European Workshop for Rheumatology Research, Stockholm.

22. McInnes, I.B., Al-Mughales, J., Leung, B.P., Sturrock, R.D., Huang, F-P., Wilkinson, P.C., Field, M., Liew, F.Y. (1996) Pro-inflammatory effects of IL-15 in rheumatoid arthritis. *Brit. J. Rheumatol.* **35**, S1.

23. McInnes, I.B., Leung, B.P., Wei, X.Q., Gemmell, C.G. & Liew, F.Y. (1996) *Staphylococcus arthritis* in mice lacking inducible nitric oxide synthase. *Arthritis Rheum.* **39**, Supplement 1.

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Brief Definitive Report

Production of Nitric Oxide in the Synovial Membrane of Rheumatoid and Osteoarthritis Patients

By Iain B. McInnes,*‡ Bernard P. Leung,* Max Field,‡ Xiao Q. Wei,* Fang-Ping Huang,* Roger D. Sturrock,‡ Andrew Kinninmonth,§ Jeffrey Weidner,‖ Richard Mumford,‖ and Foo Y. Liew*

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Summary

We have demonstrated spontaneous nitric oxide (NO) production by primary synovial cultures from rheumatoid (RA) and osteoarthritis patients. Increased NO production followed addition of staphylococcal enterotoxin B. Immunochemical double staining with specific anti–human inducible NO synthase (iNOS) and nonspecific esterase (NSE), or anti-CD68 (markers for tissue macrophages) showed that although many lining layer cells in RA synovium expressed iNOS, most (~90%) were NSE^- and $CD68^-$, with only a minor population (~10%) which were $iNOS^+$, $CD68^+/NSE^+$. These data demonstrate the capacity for high output of NO by human synovial tissue and show that, although human macrophages can express high levels of iNOS, the majority of cells expressing iNOS are fibroblasts. We also report that synoviocytes, and macrophage cell lines, cultured with the NO donor, *S*-nitroso-acetyl penicillamine, produced high concentrations of tumor necrosis factor (TNF)-α. These results suggest that NO may mediate pathology in RA through the induction of TNF-α production.

Rheumatoid arthritis (RA) is characterized by chronic inflammatory infiltration of the synovium, with destruction of cartilage and underlying bone, mediated by cytokines, metalloproteinases and superoxide radicals (1). Nitric Oxide is produced constitutively by endothelial (eNOS) or neuronal NO synthases, or in higher concentrations by inducible NO synthase (iNOS) after stimulation by bacterial products and cytokines, including IFN-γ, TNF-α, and IL-1 (2, 3). As such, it has emerged as an important regulatory and effector mediator in several models of inflammation (3), including streptococcal cell wall and adjuvant arthritis in the rat (4, 5) and renal and articular pathology in MRL-*lpr/lpr* mice (6, 7). iNOS activity has been demonstrated in rodent synoviocytes and macrophages, and in rodent and human neutrophils, chondrocytes and mast cells (2, 8–10). Evidence for NO production in human synovium is provided by elevation of nitrite levels in synovial fluid relative to serum in RA patients (11) and by demonstration of elevated urinary nitrate/creatinine ratios in RA (12). Furthermore, a recent report has suggested the presence of iNOS in human synovium and implied that macrophages are the major source of synovial NO (13).

We now provide direct evidence for spontaneous NO production by human synovial tissue from RA and osteoarthritis (OA) patients, which may be further upregulated by bacterial superantigen. We have shown that the predominant cellular source of NO is the synovial fibroblast. Furthermore, NO activated synovial cells produced TNF-α, a critical proinflammatory cytokine in RA synovitis (1).

Materials and Methods

Patients/Cell Culture. Synovial tissue was obtained at arthroplasty from 25 patients satisfying American College of Rheumatology criteria for RA (14), and 7 with clinical and radiological features of OA (mean age 62, range 28–83 yr). Single-cell suspensions were prepared from 13 RA and 7 OA tissues as previously described (15). Cell subsets assessed by FACS® analysis fell within ranges previously reported (15). 10^6 cells/ml were cultured in triplicate in complete Dulbecco's MEM, 10% FCS (GIBCO BRL, Paisley, UK) for 72 h unless indicated. LPS, stephylococcal enterotoxin B (SEB) (Sigma Chemical Co., Poole, UK), N^G-monomethyl-L-arginine (L-NMMA) (provided by Dr. H. Hodson, Wellcome, Beckenham, UK). L-NMMA did not reduce cell viability or proliferation by synovial cultures to SEB (data not shown).

NO Measurement. NO production was measured as its oxidative product, nitrite, using the Griess reaction as described (7). Sensitivity was 2 μM.

Immunohistochemistry. Acetone fixed frozen sections (3–6 μm) from 10 RA synovia, blocked with goat/human serum, were incubated with rabbit antiserum against a human iNOS peptide (NO53), (16) then with biotin goat anti–rabbit Ig (DAKO, High Wycombe, UK), streptavidin-alkaline phosphatase (DAKO), fast red (Vector, Peterborough, UK) and hemotoxylin for light mi-

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Table 1. *Production of NO by Primary RA and OA Synovial Cultures*

Patients	Duration	Drug Therapy	Unstimulated		SEB*	
RA	*yr*		*Nitrite μM*			
RA1	14	H	9.3	(3.0)	33	(7)*
RA2	20	M	13	(1.7)	34	(0.5)
RA3	5	M + H + P	51	(2.7)	131	(6)
RA4	13	G	<2		6	(2.2)
RA5	9	M	10.5	(1.3)	16.2	(0.5)
RA6	19	A + P	<2		11.7	(3.2)‡
RA7	22	S + P	10.1	(0.3)	32	(5.9)
RA8	20	—	<2		<2	
RA9	15	D	10.4	(2)	8.8	(1.0)‡
RA10	20	—	5.2	(1.2)	6.7	(2.8)‡
RA11	10	—	3.2	(0.5)	44	(8)
RA12	10	M	4.3	(0.5)	41	(3.9)
RA13	15	—	<2		19	(2.5)
OA						
OA1	10		15	(0.5)	32	(3.6)
OA2	7		<2		3.3	(1.0)‡
OA3	5		<2		3.2	(1.0)‡
OA4	10		22	(2.5)	57	(8)
OA5	3		128	(26)	98	(8)
OA6	5		10	(3.3)	24	(3)
OA7	8		76	(9)	112	(8)

SEB-stimulated RA synovial tissues produce enhanced nitrite levels (mean ± SD, $P < 0.01$).
*Optimal response to SEB (range 1–10 μg/ml) at 72 h for each patient. ‡1 μg/ml LPS present. G, IM gold; S, sulphasalazine; M, methotrexate; H, hydroxychloroquine; D, penicillamine; A, azathioprine; and P, prednisolone.

croscopy. For double immunofluorescence, murine anti-CD3 or anti-CD68 was followed by FITC Fab_2 goat anti–mouse Ig (DAKO), and anti-iNOS antiserum by biotin goat anti–rabbit Ig and PE-streptavidin (DAKO). Positive staining was acquired by confocal microscopy (Nikon Optiphot-2; Bio-Rad, Hertfordshire, UK). Normal rabbit serum (NRS) or murine IgG1 (DAKO) of irrelevant specificity served as controls and were negative even after coincubation with anti-CD68 or NRS, respectively (data not shown). For neutralization experiments, the immunizing iNOS peptide YRASLEMSAL, rat iNOS peptide YEEPKATRL (COOH terminus rat iNOS), or recombinant human eNOS (gift of Dr. I. Charles, The Cruciform Project, University College London, UK) at 50 μg/ml were incubated overnight at 4°C with anti-iNOS before staining. Nonspecific esterase (NSE) was detected as described (17) on sections stained with anti-iNOS developed with fast blue (Vector) to contrast with NSE. Staining was assessed by two histologists counting >500 cells in more than three high power fields per section (×400). The percentage of iNOS positive cells is expressed as (positive cells)/(total number cells in field) ×100.

RT-PCR. RT-PCR was performed as previously described (8). Primers for human iNOS were a gift from Dr. I. Charles: -5′-GCCTCGCTCTGGAAAG-3′ and 5′-TCCATGCAGCAA-CCTT-3′. Human chondrocyte iNOS cDNA and β-actin primers 5′-CCACACTGTGCCCATCTACGAGGGGT-3′ and 5′-AGG-GCAGTGATCTCCTTCTGCATCCT-3′ (Genosys, Cambridge, UK) served as internal controls. The PCR product was sequenced (Amersham Life Science, Buckinghamshire, UK) and was identical to that predicted from human chondrocyte iNOS (8).

Induction of TNF-α Synthesis by NO. Synoviocyte/macrophage cultures were prepared using adherent cells after 16 h culture of primary synovial tissue digests, or synovial fluid mononuclear cells obtained using lymphoprep (Nycomed Pharma, Oslo, Norway). U937 cells were matured for 24 h with 10 nM PMA (Sigma). Cells at 2×10^6/ml were stimulated with *S*-nitroso-acetyl penicillamine (SNAP) or *N*-acetyl penicillamine (NAP, gifts from Dr. I. Charles) for 48 h and TNF-α concentrations determined by ELISA using paired antibodies (MAb1, MAb11, sensitivity <10 pg/ml; PharMingen, San Diego, CA). Cell viability was >90% by trypan blue exclusion. U937 cells cultured with 1 mM SNAP for 48 h, then pulsed with 1 μCi [^{3}H]thymidine for 6 h, show similar uptake to controls (data not shown).

Statistical Analysis. Comparison between groups was by Mann-Whitney test. Paired samples were compared using a *t* test or Wilcoxon matched pairs signed rank sum test.

Results and Discussion

Nitrite production by primary synovial cultures from 9/13 RA patients and 5/7 OA patients was detected in the absence of exogenous stimulation (Table 1). Together with the demonstration of iNOS mRNA expression in synovial tissue (Fig. 1), these observations show that NO generation has been initiated in vivo in both RA and OA synovium. No difference was observed between nitrite produced by synovial cultures from RA and OA tissues, nor was serum acute phase response correlated with nitrite levels (data not shown). The superantigen SEB induces T cell proliferation, cytokine secretion, and upregulates mononuclear cell cytokine expression after MHC class II binding (18, 19). Addition of SEB to synovial cultures from RA and OA patients clearly induced increased NO synthesis in a dose-

Figure 1. RT-PCR for human iNOS mRNA in snap-frozen synovium. Lanes *1–4*, representative RA patients; lane *5*, representative OA patient; lane *6*, human iNOS cDNA; lane *7*, negative control.

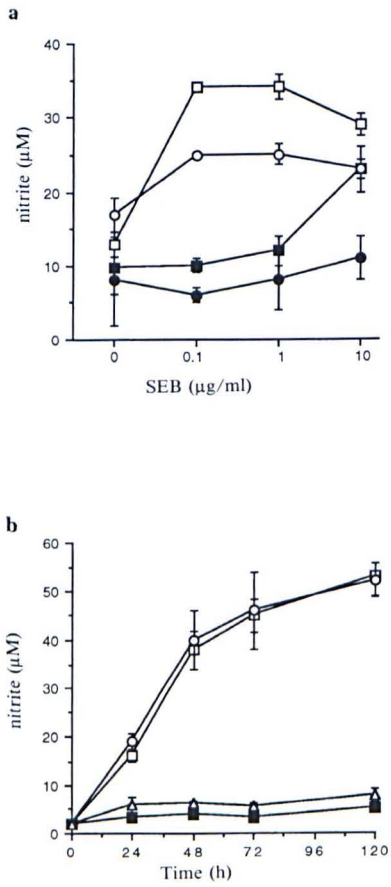

Figure 2. (*a*) SEB-induced nitrite production by synovial cultures from representative patients RA12 (*open*) and OA06 (*closed*). □, SEB 1 μg/ml; ○, SEB (1 μg/ml) + LPS (1 μg/ml). (*b*) Nitrite production by RA synovial tissue over time (representing four similar experiments). Supernatants were harvested at times indicated and kept for simultaneous assay. □, SEB (1 μg/ml); ○, SEB (1 μg/ml) + LPS (1 μg/ml); staphylococcal exterotoxin, SEB (1 μg/ml) + L-NMMA (1 mM); △, LPS (1 μg/ml).

and time-dependent manner (Fig. 2, Table 1). When LPS (0.1–10 μg/ml) was added with SEB, no significant increase in nitrite concentration was observed. Production of nitrite was inhibited by addition of L-NMMA (1 mM), a competitive iNOS inhibitor (Fig. 2 *b*). Thus, in addition to cartilage (20), human synovial membrane itself has the potential for significant NO generation.

Previous efforts to localize iNOS in human synovium used cross-reacting antibodies to rodent iNOS (13). We used rabbit antiserum raised specifically against a peptide from human iNOS (17), which recognized neither human eNOS nor rodent iNOS. Intracytoplasmic staining (Fig. 3 *a*) was observed in 10 RA synovia in the following distribution (mean ± SD [range]): lining layer 19 ± 13.1 [2–56], interstitium 5 ± 5.6 [0–20], aggregates 7 ± 7.8 [0–37] ($P < 0.001$ LL vs Is or Agg). Variable iNOS staining between patients reflected the heterogeneity of spontaneous nitrite production in primary culture. iNOS$^+$ smooth muscle and endothelial cells were identified around blood vessels in 8/10 RA tissues. Staining was abolished by preincubation with human iNOS peptide (Fig. 3 *b*), but not with either rodent iNOS peptide or recombinant human eNOS (Fig. 3 *c*), demonstrating specificity for human iNOS.

The RA synovial lining layer consists primarily of activated macrophages and fibroblasts (1). Double staining with NSE and anti-iNOS (Fig. 3 *d*) revealed that the majority of iNOS$^+$ cells (89 ± 5%) in the lining layer, or interstitium, were NSE$^-$ and therefore unlikely to be macrophages (Ta-

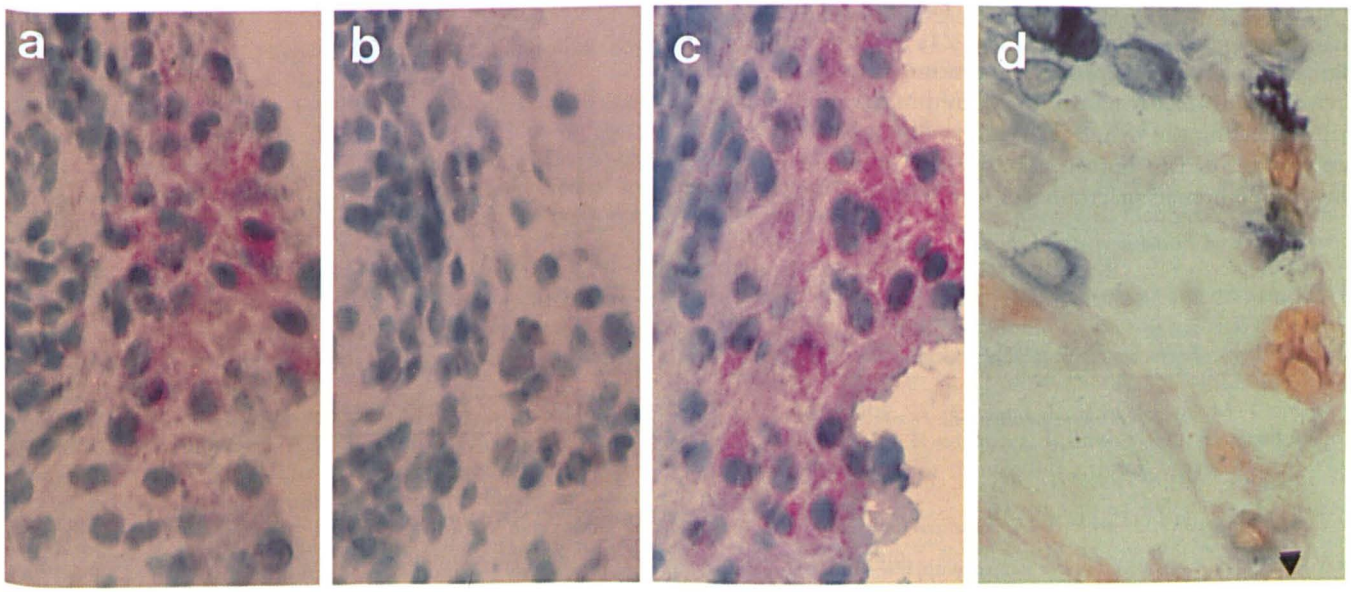

Figure 3. RA synovial lining layer stained with (*a*) anti–human iNOS antiserum (fast red), preincubated with (*b*) human iNOS peptide, or (*c*) anti–rodent iNOS peptide. Preincubation with human eNOS was similar to (*c*). (*d*) Double stain with anti-human iNOS (fast blue) and NSE (red-brown): arrow shows a double-labeled cell (×300).

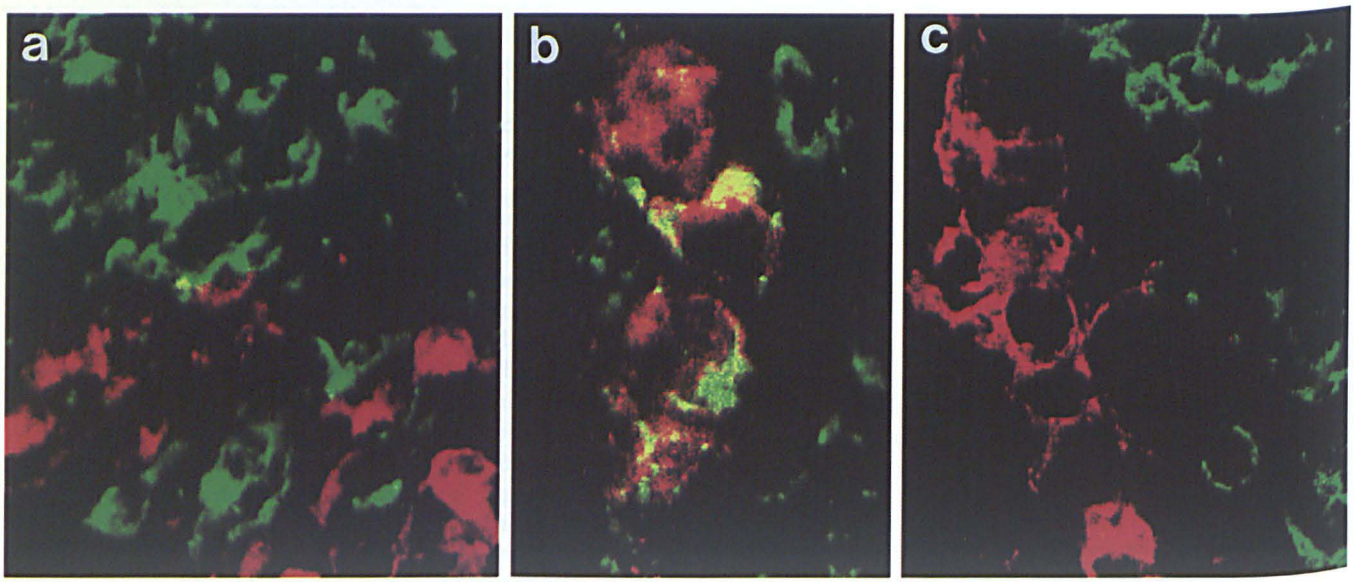

Figure 4. Confocal images of RA synovial sections double-stained with anti–human iNOS (red) and (*a*) anti-CD68 (green), showing that most iNOS-positive cells are $CD68^-$ (×250); (*b*) anti-CD68 at higher magnification, showing that $CD68^+/iNOS^+$ cells are present (double stain appears yellow [×500]); or (*c*) anti-CD3 (green), showing that T cells are $iNOS^-$ (×400).

ble 2). Confocal microscopy also demonstrated that most $iNOS^+$ cells were $CD68^-$ (Fig. 4 *a*), although $iNOS^+/CD68^+$ cells were occasionally observed (Fig. 4 *b*). Thus, although synovial macrophages possess the capability of NO generation, fibroblastlike synoviocytes are the predominant source of NO in vivo. Moreover, a majority of NSE^+ and $CD68^+$ cells in the lining layer were $iNOS^-$, indicating that most macrophages are not directly involved in NO production (Table 2). $CD3^+$ synovial T cells were all $iNOS^-$ (Fig. 4 *c*). The shared species and isotype of anti-CD3 with anti-CD68 antibody provided an additional specificity control for the double staining observed with anti-iNOS and anti-CD68.

There is currently controversy as to the capacity of human macrophages to produce NO (21, 22). Optimal conditions for iNOS expression in murine macrophages are apparently distinct from those required in the human, perhaps reflecting the tissue of origin, since blood monocytes are mainly used in human experiments. Where NO production by human macrophages was detectable (22), levels

Table 2. *Colocalization of iNOS with NSE in RA Synovium*

$NSE^+/iNOS^-$	$NSE^-/iNOS^+$	$NSE^+/iNOS^+$
	Percent positive cells	
49 ± 24	45 ± 23	5 ± 3.1
(0–80)	(16–93)	(0–12.5)

RA sections (n = 4) were double stained with anti–human iNOS and NSE. Greater than 250 lining layer and interstitial area cells positive for NSE and/or iNOS were counted per section and the number of single or double positive cells expressed as a percentage of the total number of stained cells counted (mean ± SD [range]).

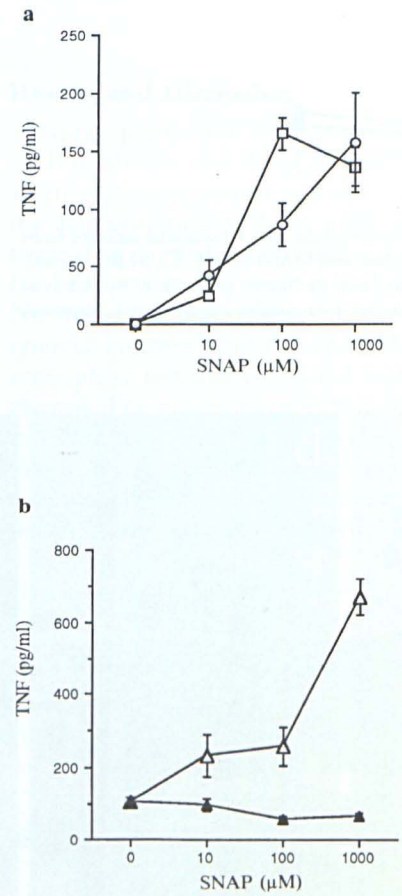

Figure 5. TNF-α production (mean ± SEM) in response to SNAP by (*a*) adherent cells from RA synovial fluid (SFAC) (○, n = 4), or RA synovial tissue (□, n = 2); or (*b*) PMA-matured U937 cells (△, SNAP; ▲, NAP: representative of three similar experiments). NAP did not induce TNF-α production from SFAC (n = 3, data not shown). 2 mM SNAP contained <0.0015 IU LPS by limulus amebocyte lysate assay (E-toxate; Sigma).

were an order of magnitude lower than those from murine macrophages. Our study demonstrates clearly that some synovial macrophages in RA can express high levels of iNOS. The reason why only a limited number of macrophages is activated to produce NO is at present unclear.

We next investigated a potential role for NO in the synovial membrane. U937 cells or synovial mononuclear cells cultured with the NO donor, SNAP, produced TNFα in a dose-dependent manner (Fig. 5). RA synovial macrophages are activated, producing proinflammatory cytokines such as IL-1, TNF-α (1), and IL-15 (23) and clinical trials using monoclonal anti-TNF-α therapy indicate that such cytokine generation is critical in ongoing synovitis (24). However, factors responsible for TNF-α upregulation remain unclear. Our data clearly show that synovial macrophages may express iNOS, but are unlikely to be the principle producers of NO. A reciprocal pathway may exist whereby NO from synovial fibroblasts enhances proinflammatory cytokine production by macrophages, which in turn may upregulate iNOS expression (2, 3), thereby generating a positive feedback loop. NO also upregulates MMP production (25) and is implicated in IL-1β mediated inhibition of proteoglycan synthesis (26), suggesting a proinflammatory role for NO. However, NO levels in our synovial cultures are sufficient to suppress T cell proliferation and may contribute to the hyporesponsiveness of synovial T lymphocytes (27). In addition, an acute chondroprotective role for endogenous NO in bovine cartilage has recently been proposed (28). Thus, in contrast to the situation in animal models in which NO is usually detrimental (4, 5), the net effect of NO production in human arthritis remains unclear.

Both RA and OA synovia produced NO. Although some OA synovial tissue contains an inflammatory infiltrate, the etiology of OA is not considered to be inflammatory. NO production is therefore unlikely to be a unique feature of primary inflammatory arthritides, but may reflect a nonspecific synovial response to injury or inflammation with potential protective or pathologic consequences.

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