Role of interleukin-15 and nitric oxide expression in chronic inflammatory disease

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To my parents & Sirene

Abstract

Interleukin-15 (IL-15) is a pleiotropic cytokine produced by macrophages and fibroblasts, which mediates biological activities primarily through binding to the β and γ components of the IL-2 receptor, together with its own unique α chain. IL-15 induces T cell migration and activation, NK cell activation, B cell maturation and antibody production and monocyte activation. IL-15 mRNA is widely expressed in both immune and non-immune tissues and cells lines. However, the role of IL-15 in the context of any pathological situation remains unclear. Therefore, the role of IL-15 in a T helper-1 (Th1) disease prototype, rheumatoid arthritis (RA), and a Th2 disease prototype, asthma, was investigated in the present study.

IL-15 was identified in RA synovial fluids. Following IL-15-mediated activation, peripheral blood (PB) T cells were capable of inducing TNF-α 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 synovial fluid (SF) T 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. Furthermore, a similar mechanism operated in regulating cell-contact-induced IL-15 production by monocytes. Thus, activated T cells appeared to be effective costimulators of TNF-α and IL-15 production by monocytes via cell-cell contact, thereby generating a positive feedback loop. IL-15 also modulated cytokine production and adhesion molecule expression by RA neutrophils. Finally, in a murine model, IL-15 blockade profoundly suppressed the development of collagen-induced arthritis (CIA). This was accompanied *in vitro* by marked reductions in antigen-specific proliferation and interferon-gamma (IFN-γ) synthesis by spleen cells from treated compared with control mice and *in vivo* by

significant reduction in serum anti-collagen antibody levels. Taken together, these data clearly demonstrated an important role for IL-15 in the development of inflammatory arthritis.

Parallel studies established that epithelial cells and inflammatory leukocytes from the respiratory tract of asthma patients expressed IL-15. Moreover, such cells also expressed high levels of inducible nitric oxide synthase and produced NO *in vitro*. Expression of both IL-15 and iNOS was significantly suppressed in patients receiving inhaled corticosteroid therapy. *In vitro* studies demonstrated synergistic upregulation of TNF-α by IL-15 and NO providing evidence for interactions between radical inflammatory mediators and cytokines in chronic inflammatory responses.

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Abbreviations

AA	adjuvant arthritis
ADCC	antibody dependent cellular cytotoxicity
APC	antigen presenting cell
BAL	bronchoalveolar lavage
BCG	Bucillus Calmette-Guerin
bp	base pairs
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
CD	cluster of differentiation antigens
CFA	complete Freund's adjuvant
CIA	collagen induced arthritis
Con A	concanavalin-A
CPR	cytochrome P450 reductase
EAE	experimental allergic encephalomyelitis
EBV	Epstein Barr Virus
EC	endothelial cells
ELISA	enzyme linked immunosorbent assay
FAD	flavin adenine dinucleotide
FCS	foetal calf serum
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
FMN	flavin mononucleotide
γc	common γ chain
GM-CSF	granulocyte macrophage - colony stimulating factor
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HSA	human serum albumin

HTLV	human T cell lymphotropic virus
ICAM	intercellular adhesion molecule
IDDM	insulin dependent diabetes mellitus
IFN	interferon
Ig	immunoglobulin
IL-	interleukin-
IRS	insulin receptor substrate
IU	international unit
JAK	janus-associated kinase
kD	kilo-Dalton
L-NMMA	N^{G}-monomethyl-L-arginine
LFA	lymphocyte function antigen
LPS	lipopolysaccharide
MCP	monocyte chemoattractant protein
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
NAP	N-acetyl penicillamine
NGS	normal goat serum
NK	natural killer
NO	nitric oxide
NOD	non obese diabetic
NOS	nitric oxide synthase
NRS	normal rabbit serum
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
s.d.	standard deviation
s.e.m.	standard error of the mean
SCID	severe combined immuno-deficiency
SE	staphylococcal enterotoxin
SF	synovial fluid
SFAC	synovial fluid adherent cells
SFMN	synovial fluid mononuclear cells
SFT	synovial fluid T cell enriched
SNAP	S-nitroso-acetyl penicillamine
STAT	signal transducers and activators of transcription
TBS	tris-buffered saline
TGF	transforming growth factor
TIMP	tissue inhibitors of metalloproteinase
TNF	tumour necrosis factor

TSST	toxic shock syndrome toxin
UTR	untranslated region
VCAM	vascular cell adhesion molecule
VLA	very late antigen

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 Interleukin-15 (IL-15)

Cytokines play an important role in regulating diverse biological processes, including proliferation and differentiation of hematopoietic cells (Arai, et al, 1990). Most cytokines exhibit a variety of activities on several target cells, and synergy or cross-talk are often observed among activities of multiple cytokines. In addition, many cytokines elicit similar and overlapping activities on target cells, suggesting that cytokine signaling pathways are non-linear and form a network with multiple potential cross-talk. The α-helical cytokines comprise a subset which includes more than 30 factors. These cytokines bind to a unique class of receptors and include interleukins, colony-stimulating factors, and hormones (Bazan, 1990). Receptor family can be further divided into subgroups based on the use of shared subunits (Taga and Kishimoto, 1995; Leonard, 1996). For instance, the common γ chain (γc) is a subunit of the interleukin-2 (IL-2), IL-4, IL-7, IL-9, and IL-15 receptors. This feature provides one molecular explanation for the biological redundancy occasionally described in this cytokine group.

Recently, the novel cytokine, IL-15 (synonym: IL-T), was simultaneously discovered in culture supernatants from two cell lines, the simian epithelial cell line, CV-1/EBNA, (Grabstein, et al, 1994) and the human T cell lymphotropic virus I (HTLV-I)-associated adult T-cell leukaemia line, HuT-102 (Burton, et al, 1994; Bamford, et al, 1994), which stimulated proliferation of the murine T-cell line CTLL-2. Many features are shared by IL-2 and IL-15, including the facts that both are members of the four α helix bundle cytokine family. In addition, both utilize the β and γc receptor subunits for their action in T and NK cells and consequently share many biological properties when added to T, NK, and B cells (Table 1.1; Grabstein, et al, 1994; Giri, et al, 1994; Bamford, et al, 1994). Initially, it was not clear why two such apparently similar cytokine activities are produced. However, further analysis revealed differences between the two cytokines in terms of their cellular sites of synthesis.

Table 1.1 Summary of Human Interleukin-15

Properties	Interleukin-15
Structure of cytokine	14 - 15 kDa, 114 aa Four helical bundle cytokine
Genomic structure	8 exon / 7 intron, chromosome 4q31
Cellular distribution of mRNA expression	Skeletal muscle, kidney, lung, fi broblasts, epithelial cells, and activated monocytes
Regulation of cytokine expression	Transcription, translation & intracellular trafficking Complex 5' UTR with 10 AUGs Unusually long (48 aa) signal peptide 3' End of mature protein coding sequence
Receptor in T & NK cells JAK/STAT signal transduction pathway in T cells	IL-15Rα, IL-2β, γ chain Jak1/Jak3 (Janus-associated kinases) Stat3/Stat5 (Signal Transducers and Activators of Transcription)
Receptor in mast cells JAK/STAT signal transduction pathway in mast cells	IL-15RX Jak2 Stat5

(after Tagaya, et al, 1996b)

Whereas IL-2 synthesis is primarily restricted to activated T cells, IL-15 mRNA is expressed in many non-lymphoid cells, including monocytes, fibroblasts, and endothelial cells and in normal human tissues, such as skeletal muscle, placenta and kidney (Grabstein, et al, 1994; Bamford; et al, 1994). Subsequent studies also revealed differences in post-transcriptional regulation and in the specific receptors and signaling pathways utilized by the two cytokines (Bamford, et al, 1996a, Tagaya, et al, 1996b).

1.1.1 The structure and Genomic Architecture of IL-15

The human IL-15 gene maps to chromosome 4q31, whereas the murine IL-15 gene is localized to the central region of chromosome 8 (Anderson, et al, 1995a). Human IL-15 mRNA contains at least a 316 nucleotide (nt) 5' untranslated region (UTR), a coding sequence of 486 nt and a 3' UTR of at least 400 nt (Grabstein, et al, 1994). The cDNA encodes a 162 amino acid (aa) peptide with a 48 aa leader sequence yielding a 114 aa mature protein (Bamford, et al, 1994). There is 97% sequence identity between human and simian IL-15 and 73% identity between human and murine IL-15. Recently, an alternative 21 aa leader sequence has been observed in many tissues, lung carcinoma cell line and T cell lines (Tagaya, et al, 1997; Meazza, et al, 1997; Onu, et al, 1997). The IL-15 protein is a 14-15 kDa member of the 4 α-helix-bundle cytokine family with two disulfide cross-links present at Cys42-Cys88 (homologous to IL-2) and Cys35-Cys91.

1.1.2 Regulation of IL-15 expression and production

The factors which upregulate IL-15 protein synthesis are poorly defined. IL-15 mRNA expression is observed in many tissues in contrast to limited protein expression. Using northern blot and RT-PCR analysis, upregulation of IL-15 mRNA levels in murine and human macrophages may be induced by lipopolysaccharide (LPS)

/ IFN-γ, *M. tuberculosis*, *T. gondii*, and *S. aureus* (Bamford, et al, 1996b; Doherty, et al, 1996; Chchimi, et al, 1997). Furthermore, in monocytes or macrophages from patients with a wide range of infectious and inflammatory diseases, IL-15 mRNA is readily detected (Agostini, et al, 1996; Jullien, et al, 1997; Kotake, et al, 1997). However, it remains difficult to demonstrate meaningful quantities of IL-15 protein in the supernatants of many of these cells (Jonuleit, et al, 1997; Chchimi, et al, 1997; Jullien, et al, 1997). The discrepancies between studies may result from differences in the sensitivity of the CTLL-2 cell clones and the ELISA systems employed. These observations suggest tight regulation of production of this potent cytokine activity.

IL-15 is predominantly regulated post-transcriptionally at the levels of protein translation and intracellular trafficking (Tagaya, et al, 1996b; Bamford, et al, 1997) rather than by transcriptional regulation, which is typical of many interleukins, including IL-2 (Paul & Seder, 1994; Kishimoto, et al, 1994). IL-15 translation is heavily down-regulated by a number of elements; in particular by the presence of multiple AUGs (5 in mice, 10 in humans) and the secondary structure in the 5' UTR (Bamford, et al, 1996b). In general, the 5' UTR of effectively translated messages are short, simple, and unencumbered by AUGs upstream of the initiation AUG. Kozak has emphasized that the presence of such AUGs in the 5' UTR of may dramatically reduce the efficiency of their translation (Kozak, 1987 & 1991). Fusion of the IL-15 mRNA with an HTLV-I R element in the HuT-102 cell line deleted the AUG rich 5' UTR sequence, leading to a marked increase in IL-15 mRNA transcription, translation and protein secretion (Bamford, et al, 1996b). Furthermore, the unusually long (48 aa) IL-15 signal peptide together with that for the mature protein of IL-15 impedes the translation of the protein such that the quantity of IL-15 generated increased approximately 20-fold when the IL-15 signal peptide was replaced by that of IL-2 (Bamford, et al, 1997). Recently, a shorter putative signal peptide (21 aa) was identified (Tagaya, et al, 1997) which shows a tissue distribution pattern that is distinct from that of the 48 aa signal peptide. This 21 aa signal peptide primarily controls the

intracellular trafficking of IL-15 to non-endoplasmic reticulum sites, as compared with the 48 aa, and predominantly regulates the rate of protein translation as a secretory signal peptide. Finally, an inhibitory *cis* element is present in the 3' coding sequence of IL-15, disruption of which leads to 5-10 fold increased protein expression (Bamford, et al, 1997). This stringent regulation of IL-15 production results in limited production of this cytokine under normal physiologic conditions, which will thus restrict its repertoire of functions while allowing efficient immune response against intracellular infection.

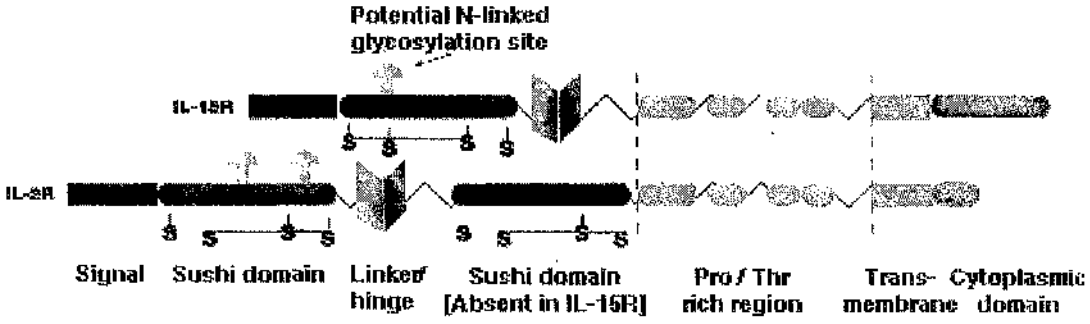
1.1.3 IL-15 receptor and signal transduction pathway

(a) The type I IL-15 receptor

The functional similarities between IL-2 and IL-15 are reflected in shared utilisation of the 70/75 kDa IL-2R β, and the 64 kDa γc receptor subunits. Thus, the effect of IL-15 on activated T cells can be inhibited by antibodies against IL-2 β 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 I membrane 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. Both receptors share a conserved motif known as the GP-1 motif or the "Sushi" domain (Figure 1.1; Giri, et al, 1995). IL-15Rα binds IL-15 with 1000-fold higher affinity than does IL-2Rα to IL-2, with no reported cross-reactivity (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.

Figure 1.1 Structural comparison of IL-15Rα and IL-2Rα chains



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). 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. The widespread distribution of IL-15Rα, IL-2Rβ, and γc elements of the IL-15R system is one of the factors that underlies the pleiotropy of IL-15.

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.

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, 1995). 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, 1995; Lin, et al, 1995). Other signalling events transduced by IL-15 include phosphorylation of $p56^{lck}$ and $p72^{syk}$ and induction of Bcl-2, with resultant protection or rescue from apoptosis (Miyazaki, et al, 1995; Akbar, et al, 1996).

(b) The type II IL-15 receptor

The observation that mast cells proliferate to IL-15 but not to IL-2, led to the discovery of a novel receptor for IL-15; designated IL-15RX (Tagaya, et al, 1996a). This 60-65 kDa IL-15 binding protein is different from all components (α, β, γc) of the IL-15R that are present in T cells. Whether a cofactor membrane protein exists, or indeed whether this receptor enjoys wider cellular distribution is not yet known. In addition, it utilises a JAK2 / Stat 5 dependent pathway in mast cells which is distinct from that in activated T cells (Tagaya, et al, 1996a). These data further demonstrate that the functional profiles of IL-2 and IL-15 are not identical.

1.1.4 Functions of IL-15

(a) T lymphocytes

IL-15 activates T cells as judged by several criteria. IL-15 induces proliferation of PHA activated $CD4^+$ and $CD8^+$ T cells, CD3 activated PB T cells, T cell clones and γδ T cells (Grabstein, et al, 1994; Nishimura, et al, 1996; Korholz, et al, 1997), with concomitant release of soluble IL-2 receptors (sIL-2R) (Treiber-Held, et al, 1996) and upregulation of CD25 expression (Korholz, et al, 1997). 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; Bamford, 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; Salmon, et al, 1997), 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). 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. Moreover, IL-15 may enhance production of IFN-γ, TNF-α and IL-10 from activated PB T cells (Korholz, et al, 1997). However, IL-15 also 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.

(b) 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. Furthermore, the failure to detect the IL-2 gene product within the bone marrow stroma and the presence of NK cells in IL-2-deficient mice leads to the suggestion that IL-15 may serve as a growth factor promoting NK cell differentiation and maturation from $CD34^+$ hematopoietic progenitor cells (Mrozek, et al, 1996; Cavazzana-Calvo, et al, 1996). Recently, IL-15Rα subunit has been described to cooperate with IL-2Rβγc to transduce an intracellular signal to picomolar concentrations of IL-15 which in the absence of serum, can sustain resting NK cell survival for up to 8 days in culture (Carson, et al, 1997). In contrast, apoptosis was observed in a $CD3^-CD56^+$ NK leukaemia cell line *in vitro* following costimulation with IL-15 and IL-12 (Ross & Caligiuri, 1997). Thus IL-15 may play an important role in regulating both NK cell development and its subsequent apoptosis.

(c) 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).

(d) Monocytes / Macrophages

IL-15 functions as an autocrine regulator of macrophage cytokine production, including IL-1, IL-6, IL-10 and TNF-α (Alleva, et al, 1997). IL-8 and monocyte chemotactic protein 1 (MCP-1) production is also upregulated from IL-15-activated monocytes, but is differentially regulated by IFN-γ and IL-4 (Badolato, et al, 1997). IFN-γ inhibits IL-15-induced IL-8 secretion, but synergizes with IL-15 to enhance MCP-1 production. IL-4 inhibits both IL-8 and MCP-1 induction by IL-15. These data suggest that in response to stimuli, monocytes / macrophages may produce IL-15 capable not only of inducing production of inflammatory cytokines, but also of promoting activation and infiltration of leukocytes.

(e) Neutrophils

IL-15, in contrast to IL-2, induces neutrophil morphological changes, enhanced phagocytosis and *de novo* RNA and protein synthesis (Girard, et al, 1996). In addition, IL-15 delays apoptosis of neutrophils more efficiently than IL-2, further demonstrating the differential effects of IL-2 and IL-15.

(f) 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, 1996a).

(g) 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.

(f) 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. Addition of IL-15 to a cultured myoblast cell line modifies parameters associated with skeletal muscle fibre hypertrophy, which suggests that IL-15 may be an anabolic agent that increases skeletal muscle mass (Quinn, et al, 1995). In addition, IL-15 induces activation of endothelial cells and stimulates angiogenesis *in vivo* (Angiolillo, et al, 1997).

Together, these properties reveal IL-15 to be a cytokine that is unique in terms of the complexity of the response it may elicit. It represents a mechanism whereby local tissue cells can contribute to the early "innate" phase of immunity, providing enhancement of NK cell, neutrophil, monocyte / macrophage and subsequently T cell responses, prior to optimal IL-2 production.

1.1.5 IL-15 in pathology

If production of IL-15 were not prevented by tight translational control, the widespread expression of IL-15 transcripts could lead to constitutive protein production, thereby disrupting immune homeostasis. That such IL-15 expression could lead to immunopathology forms a basic tenet of this thesis. Evidence for involvement of IL-15 in human pathology, however, is sparse (Table 1.2). 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). It is unclear what contribution such expression makes to dermal pathology.

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. Indeed, IL-15 is expressed by the synovial membrane, representing a pathway whereby T cells can be recruited and activated (McInnes, et al, 1996b). An 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. Furthermore, it may play an important role in activating neutrophils present adjacent to articular cartilage.

Table 1.2 Evidence for IL-15 Expression and Production

Setting	Organisms / Models	References
Infection	*Mycobacterium leprae*	Jullien, et al, 1997
	Mycobacterium tuberculosis	Doherty, et al, 1996
	Bacillus Calmette-Guerin (BCG)	
	Toxoplasma gondii	
	Salmonella choleraesuis	Nishimura, et al, 1996
	Staphylococcus aureus	Chehimi, et al, 1997
	Herpes virus-6 (HHV-6)	Flamand, et al, 1996
	Human immunodeficiency virus (HIV)	Agostini, et al, 1997; Kacani, et al, 1997
	Hepatitis C	Kakumu, et al, 1997
Inflammation	Rheumatoid Arthritis & Reactive Arthritis	McInnes, et al, 1996b; Thurkow, et al, 1997; Kotake, et al, 1997
	Sarcoidosis	Agostini, et al, 1996
	Ulcerative Colitis	Kirman & Nielsen, 1996
	Crohn's disease	
	Autoimmune thyroid diseases	Ajjan, et al, 1997
	Renal allograft rejection	Strehlau, et al, 1997

IL-15 has been localised 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 mediated alveolitis (Agostini, et al, 1996). IL-15 induces mast cell proliferation and supports T cell clone-derived IL-5 production (Tagaya, et al, 1996a; Mori, et al, 1996), these data together indicate that IL-15 might play a role in bronchial hyperreactivity although IL-15 has not yet been demonstrated in asthmatic bronchial samples. This thesis describes experiments designed to investigated the potential role of IL-15 in Th1 and Th2 mediated pathologies. RA was chosen to represent the former and asthma, the latter.

1.2 Rheumatoid Arthritis (RA)

1.2.1 Pathology of RA

RA is a chronic inflammatory disease, the aetiopathogenesis of which remains unclear. The clinical syndrome is characterised by remitting, relapsing inflammation within the synovial membrane, associated with progressive, erosive destruction of adjacent cartilage and bone. In RA marked synovial hyperplasia occurs. The membrane lining layer is increased, from 1-2 cells to 6-8 cells thickness, and contains increased numbers of type A macrophages as a result of increased extra-vascular migration (Edwards & Willoughby, 1982; Dreher, 1982). Local proliferation accounts for 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). In contrast to the acellular nature of normal synovial fluid (SF), inflammatory SF contains large number of neutrophils, along with T lymphocytes and macrophages. No basement membrane separates the membrane and fluid phase of synovium, indicating that egression 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, 1996). 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?

1.2.2 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). Similarly, 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 (reviewed by Mojcik & Shevach, 1997). 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.

Synovial lining layer cells and 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, et al, 1997). 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). 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 phenotype, 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, 1987). 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; Fox, 1997) and is listed below:

1. Abundance of T cells in synovial tissue and fluid.
2. Selective accumulation in the joint of specific T cell subsets defined by differentiation and activation markers.
3 Linkage of RA to specific class II MHC alleles.
4. Important role of T cells in animal models of inflammatory arthritis.
5. Improvement in RA with T cell-directed therapeutic approaches.
6. Presence of expanded oligoclonal T cell populations in RA.

Whether the T cell repertoire in RA displays a reproducible pattern of oligoclonality and whether synovial T cell responses to identifiable antigens are important in RA remain points of confusion and controversy. 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, 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.

1.2.3 Fibroblasts in RA

The contribution of the synoviocyte to RA has been subject to 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). Furthermore, 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). The identification of somatic mutations of p53 in RA synoviocytes further indicates that autonomous, anchorage independent behaviour may be of pathologic significance (Firestein, et al, 1997; Reme, et al, 1998).

1.2.4 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.

1.2.5 Neutrophils in RA

During active disease, SF is infiltrated by a variety of immune cells, of which neutrophils comprise between 60-90% of the total population (Zvaifler, 1997; Pillinger & Abramson, 1995). Neutrophils may be primed within the SF by a number of activation factors including immunoglobin aggregates and cytokines (Robinson, et al, 1993a; Watson, et al, 1993). Furthermore, activated neutrophils can secrete many of the cytokines and chemoattractants found in RA SF including IL-1β, IL-8, TNF-α, TGF-β and leukotriene B_4 (LTB_4) and possess a wide range of potent proteinases, hydrolases and MMP. In addition, their ability to generate a series of oxygen and nitrogen intermediates may also contribute to inflammation (reviewed by Edwards & Hallett, 1997). They are also found in the cartilage / pannus junction suggesting a direct role in tissue destruction.

1.2.6 Cytokine production in RA

Numerous cytokines have been detected in the SF and 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 (Buchan, et al, 1988; Simon, et al, 1994; Bucht, et al, 1996; Kotake, et al, 1997; Klimiuk, et al, 1997), immunohistochemical localisation of protein has proven difficult, demonstrating expression in small numbers

of cells only (Ulfgren, et al, 1995; Dolhain, et al, 1996). 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 and the presence of IL-13 remains controversial (Isomaki, et al, 1996, Kotake, et al, 1997). 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 (reviewed by Feldmann, et al, 1996) 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, 1995). 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 upregulation 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, 1996). 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, 1996). 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, transgenic mice which express the human TNFα gene, develop erosive polyarthritis characterised by marked synovial hyperplasia (Keffer, et al, 1991; Butler, et al, 1997). 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, Moreland, et al, 1997).

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 (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, 1996). 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'.

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, IL-15 has been shown to directly or indirectly stimulate the expression of a series of inflammatory cytokines, including IL-1, IL-6, IL-10 and TNF-α. IL-15 is expressed by the RA synovial membrane and serves as a T-cell chemoattractant and activator (McInnes, et al, 1996b, Al-Mughales, et al, 1996). This functional profile indicated that it might be highly relevant in the context of rheumatoid pathogenesis.

1.3 Asthma

1.3.1 Introduction

The aetiology of asthma remains unclear despite numerous efforts to determine those factors that lead to pulmonary damage and loss of function. The underlying inflammation leading to variable airflow limitation and airway hyperresponsiveness to various stimuli is often exacerbated by allergens (O'Byrne, 1988), respiratory tract infections (Empey, et al, 1976; Lemanske, et al, 1989), or environmental agents (e.g. air pollutants such as ozone or tobacco smoke) (McBride, et al, 1994). Localized cytokine and chemokine gene expression (Krug, et al, 1996), secretion of low molecular weight inflammatory mediators (Henderson, 1994), and the recruitment of specific leukocyte cell types (Bentley, et al, 1994; Poston, et al, 1992) have all been shown to be critical events. In addition, complex inheritance patterns attest to the polygenic nature of the disorder (Daniels, et al, 1996).

1.3.2 Pathology of Asthma

Asthma is characterized by reversible airway narrowing and inflammation of the airway wall, leading to epithelial cell damage, mucus plugging, stimulation of neural reflexes, and infiltration by inflammatory cells (reviewed by Bochner, et al, 1994). Structural changes to the airway wall, such as increased smooth muscle content and basement membrance thickening, associated with matrix deposition, are also present (Ebina, et al, 1993). In moderate-to-severe asthma examination of bronchoalveolar lavage (BAL) cytology consistently reveals increased numbers of mast cells, eosinophils, and epithelial cell, while total lymphocytes, macrophages, and neutrophils are usually not different from controls (Durham, et al, 1984; Tomioka, et al, 1984; Beasley, et al, 1989; Wilson, et al, 1992). Activation of T cells in BAL fluids from asthmatic subjects has been documented. Both early (CD25) and late (HLA-DR,

VLA-1) activation markers are increased on T cells in asthmatics compared to controls (Corrigan, et al, 1988; Wilson, et al, 1992; Robinson, et al, 1993b). Most of the T cells appear to be of the Th2 phenotype based on their pattern of expression of cytokine mRNA (Robinson, et al, 1992b). Macrophages from asthmatic subjects also appear to be activated (Cluzel, et al, 1987). In endobronchial biopsy studies in which lymphocytes were found to comprise the vast majority of mononuclear cells that infiltrate the asthmatic airway, increased numbers of macrophages expressing CD68 or HLA class II antigens (but not $CD14^+$) were identified also (Bradley, et al, 1991; Ollerenshaw & Woolcock, 1992; Ohashi, et al, 1992; Poston, et al, 1992). Most of the lymphocytes in the mucosa were T cells with increased CD25 expression (Bradley, et al, 1991; Bentley, et al, 1992). Increased numbers of eosinophils (Pin, et al, 1992; Fahy, et al, 1993) and basophils (Kimura, et al, 1975) have been found in induced sputum studies. Eosinophils from the sputum of asthmatic have a different surface phenotype than their counterparts in blood, expressing ICAM-1, HLA-DR, and different levels of other markers (Hansel & Walker, 1992). Airway smooth muscle is also capable of responding to cytokines and growth factors released from proinflammatory cells by undergoing mitogenesis (Hirst, 1996). Furthermore, smooth muscle cells may act as effectors in perpetuating airway inflammation by expressing and secreting chemokines (John, et al, 1997).

Many pathophysiological manifestations of asthma are associated with airway infiltration by leukocytes mediated by cytokines and chemokines. The extent of infiltration generally correlates with the severity of disease (Kelly, et al, 1989; Bousquet, et al, 1990; Walker, et al, 1991). In an antigen-induced mouse model, sensitized mice treated with aerosolized allergen develop leukocytic infiltrates of the airway lumen dominated by $CD4^+$ lymphocytes and eosinophils (Blyth, et al, 1996). SCID mice fail to develop either airway eosinophilia or hyperresponsiveness after ovalbumin sensitization (Corry, et al, 1996). Furthermore, the depletion of $CD4^+$ lymphocytes either by treatment with anti-CD4 antibodies or MHC class II gene

knockout eliminated both eosinophil airway infiltration and airway hyperresponsiveness in antigen-challenged mice (Gavett, et al, 1994). Together, these results imply $CD4^+$ T cells may play a critical role in asthmatic inflammatory response.

1.3.3 Cytokine production in Asthma

Cytokines, particularly those released by T cells, are pivotal in mediating many inflammatory responses in allergic diseases. Two types of T-helper cell are distinguished by the pattern of cytokines secreted on activation. Th1 cells release IL-2 and IFN-γ, whereas Th2 cells produce IL-4, IL-5, IL-10 (Mosmann, et al, 1986). Studies of T cell clones isolated in allergic diseases indicate that activated $CD4^+$ cells release the characteristic profile of Th2 cytokines (reviewed by Mosmann & Coffman, 1989; Kapsenberg, et al, 1991). Detectable levels of mRNA for TNF-α, IL-1, IL-4, IL-5, and GM-CSF have been reported in biopsies or BAL fluids, and a similar panel of cytokine proteins has been observed (reviewed by Bochner, et al, 1994). The source of these cytokines may include not only T cells but also macrophages, mast cells and eosinophils (Borish, et al, 1992; Broide, et al, 1992; Bradding, et al, 1992). In recent years, increasing evidence has suggested that activation of $CD4^+$ Th2 cells plays a central role in the pathogenesis of bronchial asthma through expression of IL-4, which is essential for B cell switching to IgE (Brestedt-Lindqvist, et al, 1988; Geha, 1992; Coyle, et al, 1995), and IL-5, which is required for the recruitment, activation and survival of eosinophils (reviewed by Gleich, 1990). Furthermore, the failure of both IL-4 and IL-5 deficient mice to develop eosinophil mediated airway infiltration or airway hyperresponsiveness following antigen challenge suggests that these cytokines are each important components of proinflammatory cascades that result in the pathophysiological changes characteristic of asthma (Brusselle, et al, 1995; Foster, et al, 1996). In addition, IL-5 transgenic mice developed pathophysiologic changes in airways characteristic of asthma, including eosinophil infiltration, epithelial

hypertrophy, goblet cell hyperplasia, increased mucus production and display airway hyperresponsiveness when challenged (Lee, et al, 1997).

Increased production of IL-1β and TNF-α may be observed from alveolar macrophages derived from asthmatic patients (Gosset, et al, 1991; Borish et al, 1992; Ackerman, et al, 1994) and TNF-α levels in sera obtained during bronchial asthma attacks are higher than those obtained in stable conditions (Kobayashi, et al, 1994). In asthma, IL-1β and TNF-α are cytokines with a wide spectrum of proinflammatory activities including endothelial cell activation, upregulation of soluble and surface expression of adhesion molecules, induction of bronchial hyperresponsiveness and potent NOS induction. Further biological effects described previously in section 1.2.6 may also be relevant (Walsh, et al, 1991; Dobrina, et al, 1991; Pigott, et al, 1992; Kips, et al, 1992; Liew, 1994). TNF-α increases chemokine RANTES production by human airway smooth muscle cells and bronchial epithelial cells in culture (Stellato, et al, 1995; John, et al, 1997). Together, these data demonstrate the complex network of cytokines likely involved in the pathogenesis of asthma. It has been suggested that abnormalities of IL-15 expression may participate in the pathogenesis of inflammatory disorders such as RA and sarcoidosis, however, its role in asthma has not yet been described.

1.4 Nitric oxide (NO) as a "non-cytokine" mediator in Asthma

NO has emerged as an important inflammatory mediator in asthma (reviewed by Barnes & Belvisi, 1993; Gaston, et al, 1994; Barnes & Liew, 1995). Increased exhaled NO has been demonstrated in asthmatic patients (Kharitonov, et al, 1994 & 1996). Expression of iNOS mRNA and protein can be induced within airway epithelial cells and NO is produced by bronchial epithelial cells in culture (Robbins, et al, 1994; Watkins, et al, 1997). The pathophysiological consequences of increased NO production in the respiratory tract are not yet known. NO itself is a

bronchodilator, and inhalation of high concentrations of NO results in a bronchodilatory response in asthmatic patients (Hogman, et al, 1993; Kacmarek, et al, 1996). This suggests that the increased level of NO may be beneficial in asthma by counteracting the bronchoconstriction produced by inflammatory mediators such as leukotriene D_4 and histamine (Kuo, et al, 1992). However, NO may also have deleterious effects, as it may increase bronchial blood flow and plasma exudation in the airways (Kuo, et al, 1992; Bernareggi, et al, 1997). It is also possible that the production of endogenous NO may be involved in the orchestration of eosinophilic inflammation that characterizes asthma. High local concentrations of NO may also have cytotoxic effects on airway epithelial cells (Heiss, et al, 1994), and thereby could contribute to the patchy epithelial shedding described in asthmatic airways.

NO has recently been found to play an important role in experimental models of other chronic inflammatory diseases, such as experimental arthritis, which has many analogies with asthma (Stefanovic-Racic, et al, 1993 & 1994; Evans & Ralston, 1996). NO may also play a critical role in immunological responses to foreign protein, and has evolved as a non-specific defence mechanism to protect against invasion by bacteria, parasites and viruses (reviewed by Lyons, 1995; MacMicking, et al, 1997; Nathan, 1997; Fang, 1997). Asthma may be considered to be a subversion of the inflammatory response mounted against parasites, and thus NO may be involved in amplifying the inflammatory response in asthma.

1.4.1 Regulation of NO synthesis

Three isoforms of NO synthase (NOS) have been identified and their enzymology extensively studied (Bredt & Snyder, 1994; Nathan & Xie, 1994; Michel & Feron, 1997). Constitutively expressed endothelial NOS (eNOS) and neuronal NOS (nNOS) 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 which requires novel protein synthesis, but thereafter, generates high concentrations of NO over prolonged periods. 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) and during activation, they form dimers in the presence of tetrahydrobiopterin, haem and L-arginine. However, unlike cNOS which is calcium dependent, calmodulin is tightly bound to a basic, hydrophobic site on iNOS, and enzyme activity is independent of ambient calcium concentration (Cho, et al, 1992).

iNOS was first cloned from murine macrophages and subsequently from human hepatocytes and chondrocytes (reviewed by Nathan & Xie, 1994; Geller, et al, 1993; Charles, et al, 1993), but not as yet 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, neutrophils and respiratory epithelial cells. Expression in human tumours has also been detected, including colorectal adenocarcinoma and glioblastoma.

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 (e.g. Denis, 1991; Dugas, et al, 1995; Burkrinsky, et al, 1995; Zembala, et al, 1994), others have been unable to detect any evidence of iNOS activity at all (Schneemann, et al, 1993 & 1997). The required stimuli for iNOS up regulation in human macrophages appear to differ 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. Whether this represents a functionally significant difference in the precise role of NO in the generation of

immune responses in rodents compared with humans remains unclear. Nevertheless, the widespread tissue distribution of iNOS confers upon host tissue cells of either species the ability to contribute to regulation of immune responses through high output NO generation.

iNOS has been suggested to be the predominant source of NO in inflammatory lesions. Given the ubiquitous effects of NO in inflammatory lesions it might be anticipated that many factors will control iNOS expression and activity. Thus, iNOS may be activated *in vitro* by 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, 1994; Lyons, 1995). Cytokines appear to 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 (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 (reviewed by Nathan & Xie, 1994; Liew, 1994; Lyons, 1995; MacMicking, et al, 1997).

Multiple inhibitory mediators have been described. 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 (reviewed by Nathan & Xie, 1994; Lyons, 1995; Liew, 1994). Species and tissue specificity appear important. TGFβ inhibits rodent macrophage and endothelial iNOS expression, but enhances NO production in Swiss 3T3 fibroblasts (Gilbert & Hershman, 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 (Dugas, et al, 1995). The temporal sequence of ligand binding appears important, since pre-exposure of macrophages to LPS suppresses subsequent IFN-γ induced NO production. 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 (reviewed by Nathan & Xie, 1994). 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 (Tao & Stout, 1993; Isobe & Nakashima, 1992). Similarly, activation of macrophage NO synthesis follows crosslinking of CD69 by antibody. 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 (Stout, et al, 1996). 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.

L-N$^{\omega}$-substituted arginines inhibit NOS activity in biological systems. L-N$^{\omega}$ methylarginine (L-NMMA) is commonly used when no isoform specificity is necessary. No isoform specific inhibitor has yet been identified, although L-N$^{\omega}$ nitroarginine (L-NNA) exhibits relative specificity for cNOS as does L-N$^{\omega}$ aminoarginine for iNOS. Aminoguanidine and N-iminoethyl-L-lysine are further 'iNOS specific' inhibitors often used in animal models. 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).

1.4.2 Functional consequences of NO production in Asthma

(a) NO in acute inflammation

By virtue of its EDRF activity NO can induce vasodilatation through relaxation of vascular smooth muscle leading to erythema (reviewed by Schmidt & Walter, 1994). Data from murine dextran- and carrageenin-induced models of inflammation indicate that NO also induces clinically detectable oedema formation, through alteration of endothelial permeability (Wei, et al, 1995). Thus, two features of the classical inflammatory response are regulated by NO. A further level of complexity lies in the interaction of iNOS with constitutive and inducible isoforms of cycloxygenase and their product, the prostaglandins. This suggests that NO produced by airway epithelial and inflammatory cells may result in increased airway oedema, thus worsening asthmatic inflammation.

(b) T cell activation and cytokine synthesis

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 (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 (Park, et al, 1991). Of most interest however, has been the potential for

modulating effects on functional maturation of T lymphocytes. Recent studies in murine T cell clones have established that NO preferentially inhibits Th1 clonal proliferation to antigen, but had no effect on Th2 clones (Wei, et al, 1995; Liew, et al, 1991; Taylor-Robinson et al, 1994). Moreover, proliferative responses by spleen cells to mitogen and to staphylococcal superantigens are diminished in iNOS-deficient mice (McInnes, et al, 1998; Wei, et al, 1995). Thus, the local concentration of NO and the maturational phenotype together influence the modulatory effect of NO on T cells. Amplification of the cellular immune response may be further modified by NO through induction of apoptosis (Albina, et al, 1993), and through altered T cell recirculation and tissue ingress referred to previously. In particular, P-selectin, which is down-regulated by peroxynitrite *in vitro* has recently been shown to recruit Th1, rather than Th2 cells to inflamed skin or joints in murine inflammatory models (Lefer, et al, 1997). One can speculate that through such pathways, local NO synthesis may further modify the nature of the T cell compartment within an inflammatory lesion.

It has been suggested that NO may play an important role in amplifying and perpetuating the Th2 response in asthma (Barnes & Liew, 1995). iNOS may be induced in epithelial cells by exposure to proinflammatory cytokines such as TNF-α and IL-1β secreted by alveolar macrophages. It is possible that viral infections may also induce iNOS in airway epithelial cells, augmenting the secretion of NO during asthma exacerbations. IFN-γ has an inhibitory effect on cytokine expression by Th2 cells in the lung (Nakajims, et al, 1993). The large amounts of NO generated in the airway epithelium can result in suppression of Th1 cells with a concomitant reduction in the level of IFN-γ, leading to proliferation of Th2 cells. Th2 cells may then produce several cytokines including IL-4, IL-5 and IL-10, which further suppress Th1 cells.

(c) Monokine production

NO is also implicated in monokine production. Increased TNF-α production from PBMC exposed to NO-donors has been detected, although the cellular origin of TNF-α in this system was not specified (Lander, et al, 1993; Eigler, et al, 1993). Production of cytokines, including IL-6 and TNF-α, by purified blood monocyte/ macrophages or macrophage cell lines has been variously reported to be suppressed or enhanced in the presence of exogenous NO-donors (Deakin, et al, 1995; McInnes, et al, 1996a). Recently, enhanced TNF-α production by endogenous NO was reported from human U937 cells transfected with functional eNOS or iNOS (Yan, et al, 1997). The mechanism underlying these observations is unknown, but may reflect modification of transcription factors, such as NF-κB (Lander, et al, 1993) or function at least in part, through a cyclic adenosine monophosphate (cAMP)-dependent signalling mechanism (Wang, et al, 1997). NO has been demonstrated to induce TNFα synthesis by synovial tissues from rheumatoid arthritis patients indicating that such observations likely have pathological relevance (McInnes, et al, 1996a). Together these data indicate that complex feedback loops exist whereby the effector function of NO overlaps with its immunomodulatory role to 'fine-tune' ongoing immune responses.

1.5 Regulation of cytokines and iNOS by Glucocorticoids

Glucocorticoids, given either by inhalation or orally are the most effective form of therapy in asthma, such that inhaled steroids represent the mainstay of therapy. However, the actual mechanism by which glucocorticoids reduce airway inflammation in asthma remains poorly understood. Steroids have been shown to inhibit the transcription of most inflammatory cytokines and chemokines (reviewed by Barnes, 1996). Asthmatic patients respond to prednisolone therapy with a reduction in eosinophils as well as a decrease in activated T cells expressing IL-4 and IL-5 mRNA

transcripts but an increase in IFN-γ (Robinson, et al, 1993b). Furthermore, cell surface molecules mediating antigen presentation (Giuliani, et al, 1995) or cell adhesion (Van de Stolpe, et al, 1993; Brostjan, et al, 1997) are reportedly downregulated. Glucocorticoids are also effective in inhibiting iNOS (Di Rosa, et al, 1990), and have been shown to inhibit the expression of iNOS in epithelial cells in culture (Robbins, et al, 1994). 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-κB and not AP-1 binding in the iNOS promoter region is prevented (Kleinert, et al, 1996). Thus, a possible mechanism of action for glucocorticoids may be to inhibit NO production from epithelial and inflammatory cells, thereby increasing the proliferation of Th1 cells, which produce IFN-γ. This, in turn, may act on Th2 cells to suppress the production of IL-4 and IL-5. Ex vivo evidence for iNOS suppression by glucocorticoids treatment of patients has thus far been lacking.

1.6 Objectives

The pathological events in both RA and asthma encompass components of both humoral and cell-mediated arms of the immune system. Recent evidence suggests that IL-15 may play an important role in inflammatory responses. Since IL-15 mRNA is widespread, aberrant regulation of translation could amplify autoimmune responses in a wide variety of tissues. The present thesis describes experiments designed to more precisely understand the pathways whereby IL-15 may contribute to such host tissue damage. Moreover, the importance of interactions between cytokine networks and other inflammatory mediators, including nitric oxide, has only recently emerged, particularly in a pathological context. That IL-15- and NO-mediated events might be related was of particular interest to the author. Objectives of the present studies were therefore:

To investigate the role of IL-15 in RA pathogenesis, representing a Th1 disease model;

- by measuring modulation of pro-inflammatory cytokine synthesis, particularly TNF-α, since the latter has proven a most promising therapeutic biological target
- by investigating the ability of IL-15 to modify synovial neutrophil function
- using an IL-15 specific antagonist within the murine collagen arthritis model.

To investigate the role of IL-15 and NO in the pathogenesis of asthma, representing a Th2 disease model;

- by determining where and how NO and IL-15 are expressed / produced by human respiratory epithelial and immune cells
- by investigating the functional consequences of the presence of NO and IL-15 within the respiratory tract
- by investigating the impact of glucocorticoid therapy in modulating these responses.

Chapter 2

Materials and methods

2.1 Patients and clinical samples

Synovial fluid and peripheral blood samples were collected from RA patients attending the Centre for Rheumatic Diseases, Glasgow Royal Infirmary and the Rheumatology Department, Gartnavel General Hospital who satisfied the 1987 American College of Rheumatology diagnostic criteria (Arnett, et al, 1988). Buffy coats were provided by the Scottish Blood Transfusion Service (Law Hospital, Carluke, UK) within 2 hours of donation by normal volunteer donors.

Induced sputum samples were obtained from a total of 11 normals [all non-smokers, 8 males and 3 females, aged (mean ± SD, range): 33.4±8.5, 23-47 year] and 21 asthmatics [all non-smokers, 17 males and 4 females, aged: 42.4±13.1, 20-69 year]. All samples were collected at the respiratory research laboratory at Gartnavel General Hospital, Glasgow. Asthmatics were subdivided into those taking inhaled β-2 agonist alone (non steroid-treated, albuterol, 200 μg as required), and those on additional inhaled steroid therapy (steroid-treated, beclomethasone dipropionate, budesonide or fluticasone propionate, dose range 400-1000 μg daily). In the inhaled steroid group, 4 patients were also taking an inhaled long-acting β-2 agonist (salmeterol, 100 μg daily), 2 were taking oral theophyllines (400 mg daily), and 1 was taking inhaled ipratropium bromide (120 μg daily). Baseline lung function was measured using spirometry and forced expiratory volume in 1 second (FEV1) was found to be lower in the asthmatic group whether expressed (mean ± SD) as absolute values (3.03 ± 0.97 litres vs 3.82 ± 0.96 litres) or as percent of predicted values (82 ± 19% vs 97 ± 12%).

Sputum induction was performed as previously described (Pin, et al, 1992). Briefly, after volunteers were administered with albuterol 200 μg by metered-dose inhaler, and spirometry checked, sputum induction was started using hypertonic (3%) saline administered via an ultrasonic nebuliser (Schuco International Ltd, London, UK) over

a period of 20 minutes. The subjects were encouraged to expectorate at any time throughout the procedure, and in addition inhalation was stopped every 5 minutes to allow expectoration, and to allow spirometry to be carried out. The sample was collected in a sterile container and processed within 2 hours. Protocol dictates that the procedure be discontinued if FEV1 falls by more than 20%, although this fall was only observed in one patient at the end of the sputum induction and was completely reversed by albuterol.

2.2 Ethical considerations

Synovial fluid and blood samples were surplus to clinical requirements following routine therapeutic or diagnostic procedures and would otherwise have been discarded. These samples were collected only when clinically indicated, and informed consent was obtained from patients prior to research use of samples so obtained. Permission from the Ethical Committee in West Glasgow Hospitals University NHS Trust was applicable to the use of induced sputum samples for research purpose (Use of Induced Sputum to investigate lung disease including Asthma, Protocol No. 94/139; Dr. N.C. Thomson, Western Infirmary). All animal experimentation was performed under project licence 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 for the Collagen induced arthritis model in mice.

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 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 (Sigma Chemical, Poole, UK) and 2.5 µg/ml amphoteracin B. 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 50 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 25 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. Viability was assessed microscopically by trypan blue exclusion (0.1% trypan blue (Sigma), 0.1% acetic acid (BDH Lab. Supplies, Leics, UK), phosphate buffered saline[PBS]).

2.4.1 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. 5 ml diluted blood was layered over 5 ml Lymphoprep (Nycomed Pharma, Oslo, Norway) in a 13 ml 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).

2.4.2 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).

2.4.3 Isolation of neutrophils

Neutrophils were isolated from peripheral blood and synovial fluid using lymphoprep as previously described. Following centrifugation, the neutrophil rich pellet was harvested, contaminating erythrocytes were removed by adding equal volume of Tris-Ammonium chloride (0.21% Tris, 0.75% NH_4Cl in sterile distilled water) for 5 minutes and washed twice in complete Dulbecco's MEM. Adherent cells including monocytes / macrophages and lymphocytes were removed by two successive adherence steps at 37°C for 30 minutes. After purification, cells were washed twice in medium and resuspended in Dulbecco's MEM supplemented with 10% FCS. Cells viability and purity were determined by Trypan blue exclusion and FACS analysis respectively (section 2.5).

2.4.4 Induced Sputum culture preparation

Sputum-expectorated cells mixed in an equal volume of complete Dulbecco's MEM medium / 0.05% dithiotrietol (DTT, Sigma) were incubated at 37°C for 15 minutes with constant agitation, then passed through Nytex membrane (Cadisch & Son, London, UK). Samples were washed four times in complete Dulbecco's MEM medium to remove debris and salivary nitrite / nitrate, then resuspended in complete Dulbecco's MEM with 5 µg/ml amphotericin B and 5% fetal calf serum. Cell viability was determined by trypan blue exclusion.

Primary cultures were established as follows. 100 µl of heterogeneous sputum cells adjusted to $2x10^6$ cells/ml in complete Dulbecco's MEM, 5% FCS, were plated into 96 well flat-bottom culture plates (Nunclon, Denmark). After 1 hour, stimuli were added at different concentrations in a total volume of 100 µl in complete Dulbecco's MEM supplemented with 5% FCS, giving a final density of $1x10^6$ cells/ml in 200 µl (i.e. $2x10^5$ cells/well). Cultures were maintained from 24 to 96 hours. Stimuli included

staphylococcal enterotoxin B (SEB, stock 1 mg/ml, Sigma), lipopolysaccharide (LPS, stock 1 mg/ml, *Salmonella enteritidis*, Sigma), and recombinant human IL-15 (gift of Dr. D. Cosman, Immunex Corporation, Seattle, USA) at the concentrations indicated. 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). For cytokine study, sputum cultures or human monocyte cell lines (U937, THP-1; European Collection of Animal cell cultures, Salisbury, UK) were stimulated with nitric oxide donor S-nitroso-acetyl penicillamine (SNAP, Alexis Biochemicals, Nottingham, UK) and N-acetyl penicillamine (NAP, gift of Dr. I. Charles, The Cruciform Project, University College of London, UK) was used as a control. All chemicals were stored desiccated, at room temperature, and were dissolved immediately prior to use in appropriate medium, then filtered (Millex-GV 0.22 µm, Millipore, France). This solution was added to final culture. Culture supernatants were frozen at -20°C until assay for cytokine or nitrite production.

Cytospin preparations were made using a cytospin (Shandon, UK) after sputum cells been purified. Sputum samples were resuspended at $2x10^5$ cells/ml in PBS and spun at 500 rpm for 5 minutes. Cytopreps were fixed in cold acetone, rehydrated in PBS for 5 minutes and stained with May-Grünwald-Giemsa - 300 cells were counted per sample.

2.4.5 Proliferation and cytokine production by human cells

Proliferation assays were performed in triplicate in complete Dulbecco's MEM with 10% FCS. $2x10^5$ PBMC, PBTL or SFTL in 100 µl were incubated in U-bottom 96 well culture plates (Nunclon) 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 fibre 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:

$$\text{Stimulation index} = \frac{\text{(mean counts per minute test culture)}}{\text{(mean counts per minute medium alone)}}$$

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, recombinant interleukin-2 (IL-2, R&D Systems, Abingdon, 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).

2.4.6 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 $2x10^5$ cells/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, 1994).

2.4.7 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 triplicate 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 using supernatants derived from parallel triplicate cultures for various time in flat-bottom 96

well culture plates (Nunclon), 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. Bovine type II collagen was used during *in vitro* studies of Collagen Induced Arthritis (CIA) in male DBA/1 mice at concentrations indicated. Collagen was resuspended to 1 mg/ml in proliferation medium supplement supplemented 10% FCS before dilution and use in culture.

2.4.8 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) 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 complete RPMI supplemented with 10% FCS; 10% dimethyl sulfoxide (DMSO) in liquid nitrogen.

2.4.9 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 minutes 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 (pore size: 0.45 μm), 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 $4x10^6$ cells/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.

2.4.10 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 (Costar) 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.

2.4.11 Neutrophil culture and stimulation

Neutrophils were resuspended in complete Dulbecco's MEM supplement with 10% FCS. Cells were cultured in 96 well flat-bottom culture plates (Nunclon) at $2x10^5$ cells/well at 37°C in 5% CO_2 either with various concentrations of recombinant IL-15 or medium alone. At time intervals up to 48 hours, culture supernatants were removed and frozen at -70°C until assay for cytokine production by ELISA.

For receptor analysis, peripheral blood neutrophils were cultured at $4x10^6$ cells in 500 µl with complete Dulbecco's MEM, 10% FCS in 48 well culture plates. Cells were stimulated in the presence or absence of 100 ng/ml IL-15 or autologous cell-free synovial fluid (10% final volume). FACS analysis was performed at various time intervals 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).

2.5.1 Analysis of neutrophil receptor expression after stimulation

Purified synovial fluid and cultured peripheral blood neutrophils were washes twice in cold medium and suspended at $2x10^6$ cells/ml. Receptor expression was measured using antibodies to CD11b, CD11c (both PE labelled, Sigma), and in all experiment appropriate isotype control antibodies (Dual Taq, IgG1 FITC/PE, Sigma) were included. Cells were labelled as described above, washed twice in FACSFlow and fixed in 1% paraformaldehyde in PBS prior to analysis. A total of 10,000 cells were collected per sample.

2.5.3 Human cell subset analysis

Cell preparations from peripheral blood and synovial fluid were analysed using FACS to determine cellular purity. Single cell suspensions were adjusted to 10^6 cells/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) and (CD15 PE, CD56 PE; Sigma) 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. Lymphocyte, monocyte and neutrophil regions, determined by forward and side light scatter parameters, were examined to identify cell subsets present.

2.6 Immunohistochemistry

Freshly isolated and stimulated sputum cytospin preparations were made using a Shandon cytospin device. Sputum cells were cultured with 1 µg/ml SEB and LPS (Sigma) *in vitro* for 24 h. Cells were washed twice in PBS to remove debris and resuspended at $2x10^5$ cells/ml in PBS and spun at 500 rpm for 5 minutes. Freshly isolated sputum cells were prepared in a similar manner. Cytospins were air dried and immediately fixed by immersion in acetone (BDH) at 4°C for 15 minutes. Sections were air dried for 5 minutes and stored at -20°C in an air / moisture tight container before use. 300 cells were counted per sample and data are expressed as mean percentage ± SD.

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 100 µl 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 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). Cytospin 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). In order to remove the dark brown precipitates which might interfere with the labelling, the reaction solution

was briefly spun at 10,000g for 1 minute prior to addition to the samples. 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.

2.6.3 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).

Cytospin sections 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. Streptavidin conjugates, either alone, or in the presence of secondary antibodies, did not bind sputum cells 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).

2.6.4 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 Model of Inflammation

2.7.1 Induction of collagen induced arthritis (CIA) in mice

Collagen Induced Arthritis (CIA) is a widely used experimental model of polyarthritis. It can be induced in susceptible strains of mice which express the H-2^q haplotype by immunisation with type II collagen (CII) and has histopathologic features in common with rheumatoid arthritis (Trentham, et al, 1977). Male DBA/1 mice (6-8 weeks old, Harlan Olac, UK) were obtained for use in this study. Animals were housed in the Joint Animal Facility, University of Glasgow, in accordance with the UK Home Office animal care guidelines. Mice were quarantined for 7 - 10 days before the study began.

Bovine type II collagen (Sigma) was solubilised at 4 mg/ml in 0.01M acetic acid at 4°C overnight. Next day collagen was emulsified in an equal volume of freshly prepared Freund's complete adjuvant (Difco, Detroit, MI) supplemented with 5 mg/ml of heat killed *Mycobacterium tuberculosis*, H37RA (Difco). The DBA/1 mice were immunised intradermally by two injections at the base of the tail with a total of 100 µl of emulsion (CII; 200µg). At day 21 the animals were boosted with an intraperitoneal (i.p.) injection of 200 µg collagen in PBS. A gradual onset of arthritis was observed 7 - 10 days after the i.p. booster and animals were observed for up to 8 weeks for the development of arthritis.

2.7.2 Anti-cytokine treatment of CIA

To investigate the role of IL-15 in CIA, soluble IL-15 receptor alpha (sIL-15Rα) was administrated to collagen primed DBA/1 mice. sIL-15Rα was kindly provided by Mr. H. Ruchatz (Dept. of Immunology, University of Glasgow), it was expressed in *E. coli* M15 strain (Qiagen, Dorking, UK) and purified as 6x Histidine-tagged fusion protein using a Nickel agarose purification system (Qiagen). The protein consisted of 193 amino acid residues, 173 of which contained the "Sushi" domain, linker and Pro/Thr rich region of the native sIL-15Rα. The level of endotoxin contamination was less than 0.003 I.U. (< 6 pg/mg) of purified protein, as measured by *Limulus* amoebocyte lysate assay (E-toxate, Sigma). DBA/1 mice were injected intraperitoneally with (40 µg/animal, n = 60) of sIL-15Rα from day 22 for a period of 14 days. As control, the same amounts of human serum albumin (HSA, Sigma) were given in a parallel study (n = 30).

2.7.3 Monitor of progression of CIA

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)

2.7.4 Quantification of histology

At the end of the experiments 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, Dept. of Pathology, University of Glasgow). Sections were stained with haematoxylin and eosin using the standard method of the Dept. of Pathology, Western Infirmary, University of Glasgow. Quantification of arthritis was performed by two treatment-blinded observers (Dr. I. McInnes and the author) using scoring system described by (Joosten, et al, 1997). Briefly, the extent of inflammation, synovial hyperplasia and joint erosion were graded separately. For synovial infiltration and hyperplasia, a scale of 0-3 was established according to the amount of inflammatory cells in the synovial tissue. Joint destruction was scored on a scale of 0-3, ranging from area with dead chondrocytes to complete loss of the articular cartilage. Scores were pooled and means ± SD were obtained.

2.7.5 Measurement of serum anti-collagen antibodies

Serum anti-collagen II antibodies titres in pooled sera (N=5) obtained at the end of sIL-15Rα treatment were measured by ELISA. Briefly, 96-well micro-ELISA plates (Immulon 4, Dynatech, Succex, UK) were coated with 4 μg/ml bovine type II collagen in 0.1M NaH_2CO_3 overnight at 4°C, blocked with PBS/10% FCS and serial dilutions of sera were added. Bound total IgG was detected with HRP-conjugated goat anti-mouse IgG (Genzyme) or biotin-conjugated anti-mouse IgG1 or IgG2a (2 μg/ml, PharMingen, CA, USA) respectively, and developed as section 2.9.2. Plates were read at 630nm.

2.8 RT-PCR for mRNA expression in clinical samples

Induced sputum cells, synovial fluid T cells and neutrophils were pelleted by centrifugation at 450g for 5 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).

2.8.1 RNA extraction

Total mRNA was extracted using RNAzol™ (Biogenesis) as described (Chomczynski & Sacchi, 1987). Frozen cell pellets were disrupted in 400 μl RNAzol using a P1000 Gilson Pipette. To this was added 40 μl chloroform (Sigma) followed by shaking 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 RNasin RNase inhibitor, 2 µl containing 0.5 µg random primer, 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, 2 µl 10 mM DTT (all Promega, Southampton, UK) 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 (approximate 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 (Gibco BRL).

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). Oligonucleotide primers specific for human IL-15 receptor alpha chain (hIL-15Rα) were kindly provided by Dr. D.M. Anderson (Immunex Corp.). The two primers designed to amplify a 305 bp fragment that would include the entire transmembrane and cytoplasmic coding domain plus an addition 100 bp of 3' non-coding sequence. The sense primer - 5'-ACACCACTGTGGCTATCTC-3', which corresponds to nt 690-708 of GenBank U31628 and the anti-sense primer - 5'-GGTGAGCTTGCTCCTGGAG-3', corresponds to the complement of not 977-995 of GenBank U31628.

2.9 Cytokine assays

2.9.1 IL-15 ELISA

A commercial sandwich enzyme linked immunosorbent assay (ELISA) was employed to detect IL-15 in biological fluids (R&D Systems). 96-well micro-ELISA plates (Immulon 4, Dynatech) were coated with 2 μg/ml mouse monoclonal anti-human IL-15 (MAB647) in bicarbonate coating buffer (0.1M NaH_2CO_3, pH 8.2) overnight at 4°C, using a working volume of 100 μl. Plates were washed twice with PBS/0.05% Tween 20 (PBS/Tween) and blocked with 200 μl 10% heat-inactivated FCS in PBS

for 2 hours at room temperature (RT). After two washes with PBS/Tween, 100 µl aliquots of standard and samples (synovial fluid was diluted 1/4 in PBS/Tween) were added and incubated at RT for 2 hours. After four washes with PBS/Tween, bound IL-15 was detected using 100 µl biotinylated mouse anti-human IL-15 (BAM247) diluted to 200 ng/ml in PBS/10% FCS and incubated for 2 hours at RT. Plates were washed six times and 100 µl/well of peroxidase conjugated extravidin (Sigma), diluted 1:1000 in PBS/10% FCS was added and incubated for further 30 minutes at RT. After eight final washes, plates were developed with 100 µl TMB Microwell Peroxidase Substrate (Kirkegaard & Perry Laboratories, MA, USA) and the optical density read at 630 nm in an automated plate reader (MR5000 ELISA reader, Dynatech). The lower limit of detection of this assay was 1.5 pg/ml (Fig. 3.1a). Similar results were obtained by replacing the primary antibody MAB647 with sIL-15Rα (2 µg/ml) as a source of "capture" anti-cytokine. Lower level of sensitivity was 5 pg/ml (Fig. 3.1b).

2.9.2 Removal of Rheumatoid factors

Although IL-15 can be detected within the synovial lining layers in rheumatoid patients (McInnes, et al, 1996b; Thurkow, et al, 1997), the precise levels presence within synovial fluid remains controversial. It has been suggested that the contaminating rheumatoid factors (RF) could interfer with the quantification of IL-15 within synovial fluids (Carson, 1997). RF are antibodies directed against the charged tertiary structure of the IgG Fc fragment. They occur in all immunoglobulin classes including IgM, IgG and IgA. Thus, RF can potentially "cross-link" the primary antibody with the detecting antibody, giving rise to false positive results. In order to eliminate this possibility, RF were removed from synovial fluid using γ-globulin coated polystyrene beads (RapiTex RF, Behring, Milton Keynes, UK). Briefly, 100 µl of synovial fluid was incubated with equal volume of γ-globulin coated polystyrene beads at RT for at least 30 minutes in an eppendorf tube with constant shaking. After centrifugation (10 min, 13,000 g)

the clarified supernatant was collected, diluted with equal volume of PBS/Tween and IL-15 was assayed as described above. The author observed removal of RF did not significantly alter the results obtained by the above ELISA system, thus, levels of IL-15 detected within synovial fluid in this study are unlikely due to RF interference.

2.9.3 General ELISA protocol

Human TNFα and murine TNFα, IFN-γ, IL-4, IL-6 and IL-10 were detected in culture supernatants or biological fluids 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 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. Modifications for the other cytokines: human IL-1α and IL-8, coating: 2 μg/ml murine monoclonal antibodies (Genzyme); second antibodies: polyclonal rabbit anti-human IL-1α or IL-8 (both at 1 μg/ml); and peroxidase-conjugated donkey anti-rabbit IgG (1:1000, Jackson Immuno-research Lab, West Grove, PA). The lower limit of detection in each assay was as follows:-

mTNFα	10 pg/ml	hTNFα	10 pg/ml
mIFN-γ	80 pg/ml	hIL-1α	5 pg/ml
mIL-4	20 pg/ml	hIL-8	40 pg/ml
mIL-6	20 pg/ml		
mIL-10	80 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 µl of 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 0.5M 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%.

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

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. Spearman rank correlation test was used to correlate cytokine levels in synovial fluid. Significance was accepted at $p<0.05$.

Table 2.1 Primary antibodies used for immunohistochemistry

Antibody	Specificity¶	Host Species	Type	Presentation	Dilution/ concentration	Source
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+
MAB647	hIL-15	Mouse	IgG1	supernatant	10 μg/ml	R&D Systems

¶ 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

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

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

Introduction

Interleukin-15 (IL-15) is a novel cytokine with biological functions similar to those of IL-2 but with no significant amino acid sequence homology. IL-15 is derived from several cell types, including macrophages and fibroblasts (Grabstein, et al, 1994; Burton, et al, 1994). It uses the IL-2 receptor β and common γ chain subunits, together with an unique α-chain, IL-15Rα (Giri, et al, 1995), through which it induces T cell chemotaxis and activation together with B cell maturation and isotype switching (Wilkinson & Liew, 1995; Armitage, et al, 1995). It enhances NK cell cytotoxicity and cytokine production, activates neutrophils and modifies monokine secretion (Carson, et al, 1995; Girard, et al, 1996; Alleva, et al, 1997). Thus IL-15 potentially mediates diverse effects at multiple stages of the immune response.

Rheumatoid arthritis (RA) is a chronic, presumed autoimmune disease characterised by synovitis associated with adjacent cartilage and bone destruction. The pathologic lesion consists of extensive infiltration of the synovial membrane by T lymphocytes, which are mainly of the $CD45RO^+$ "memory" phenotype, plasma cells and macrophages, together with the presence of activated fibroblast-like synoviocytes (Duke, et al, 1982). Within the synovial membrane, high levels of pro-inflammatory cytokines are detectable, including TNF-α, IL-1, IL-6, IL-8 and GM-CSF (Feldmann, et al, 1996), leading to the suggestion that RA is predominantly a "macrophage-led" process (Firestein & Zvaifler, 1991). T cell-derived cytokines such as IFN-γ, IL-2 and IL-4 may be detected at the mRNA and protein level but are only found with low cellular frequency (Simon, et al, 1994; Dolhain, et al, 1996, Kotake, et al, 1997). Together, these observations have raised questions as to the functional contribution of T lymphocytes in RA.

Recent studies in our laboratory have revealed several functional properties of IL-15 with potential relevance to rheumatoid pathogenesis. These include promotion of

inflammatory cell recruitment *in vivo* following footpad injection in mice primed with either *C. Parvum* or type II bovine collagen and in chemotactic assays *in vitro*, induction of human T cell polarization and invasion into collagen gels (Wilkinson & Liew, 1995; McInnes, et al, 1996b). 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 and RA synovial fluid (SF) contains potent chemotactic activity attributable, at least in part, to the presence of IL-15 (McInnes, et al, 1996b; Al-Mughales, et al, 1996). Moreover, RA peripheral blood T cells exhibit enhanced proliferative responses to rIL-15 compared with T cells from normal controls. These data strongly suggest 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 shown that macrophage-derived TNF-α is a key factor in the inflammatory processes occurring in RA (Feldmann, et al, 1996). 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). These observations formed the basis for successful clinical trials of anti-TNF-α antibody in RA, thereby confirming the importance of TNF-α (Elliot, et al, 1994; Rankin, et al, 1995; Moreland, et al, 1997). Those mechanisms promoting monocyte / macrophage production of TNF-α in RA joints are not yet understood, but will likely provide rich therapeutic potential. Following mitogen stimulation *in vitro*, T lymphocytes can induce macrophage production of cytokines and MMP by cell-contact (Vey, et al, 1992; Lacraz, et al, 1994). The identification of IL-15 in RA where it apparently serves as a T-cell activation factor, provided the opportunity to test the hypothesis that a similar T cell-contact-mediated macrophage activation pathway might operate in the context of RA.

The current studies were performed to address the possibility that IL-15-activated T cells might regulate monocyte TNF-α production. Furthermore, as human and murine monocytes have been shown to express IL-15 following activation with conventional stimuli (Carson, et al, 1995; Doherty, et al, 1995), the ability of T cells to costimulate monocyte IL-15 production via cell-contact was investigated. These studies suggested that IL-15-activated synovial T cells, can exert pro-inflammatory effects through a cell-contact dependent mechanism, in the absence of local antigen recognition or cytokine secretion. They further raise the possibility that a positive autoregulatory loop exists involving both TNF-α and IL-15.

3.1 Assays employed for IL-15 detection and quantification in biological fluids

A commercial sandwich ELISA was employed to detect IL-15 in biological solutions, using a murine monoclonal anti-human IL-15 antibody (MAB647) for capture and a biotinylated mouse anti-human IL-15 (BAM247) for detection. The lower limit of detection of this assay was 1.5 pg/ml (Figure 3.1a). To further investigate the specificity of "IL-15" detection in RA synovial fluid (SF) and serum samples, the primary "capture" antibody was subsequently replaced by murine soluble IL-15 receptor alpha (sIL-15Rα, kindly provided by Mr. Holger Ruchatz, Department of Immunology, University of Glasgow). This assay demonstrated specific binding of recombinant human IL-15 with a lower detection limit of 5 pg/ml (Figure 3.1b). Recombinant human IL-2, IL-1, TNFα, IFN-γ and purified IgG were not detected in the latter ELISA system.

3.2 Detection of IL-15 in SF

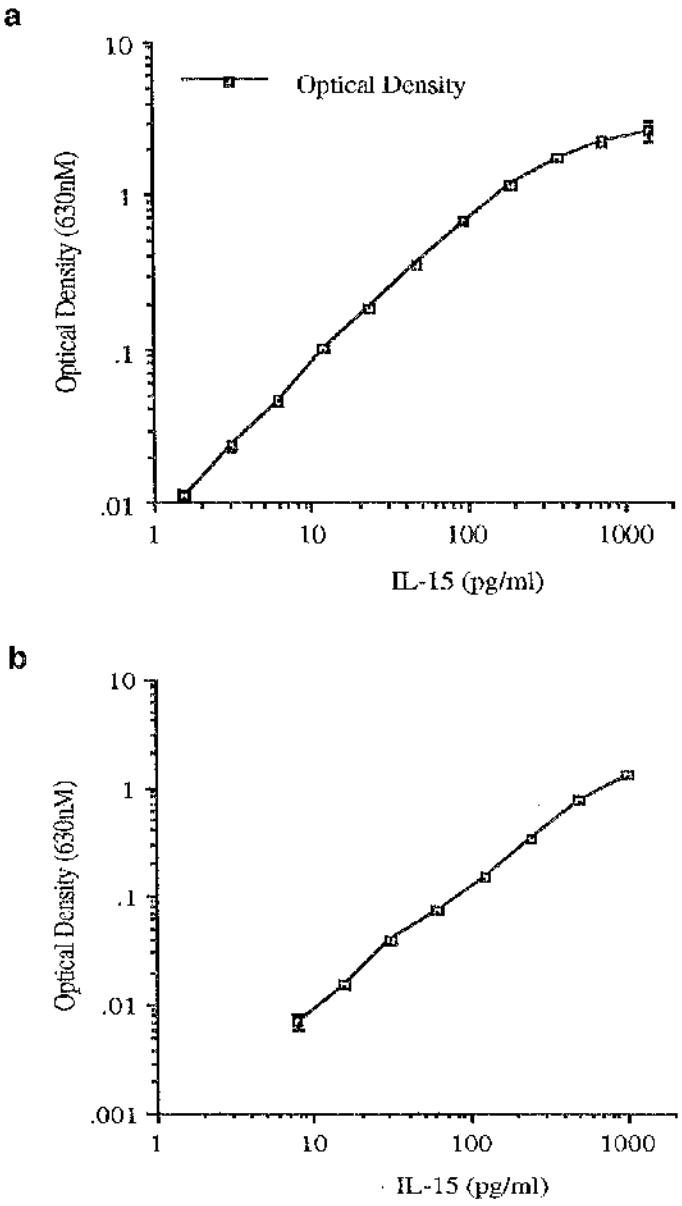
Although IL-15 has been reproducibly detected immunochemically within the synovial membrane of RA patients (McInnes, 1996b; Thurkow, et al, 1997), the precise levels present within SF remains controversial. Synovial fluids were therefore collected from 53 RA patients. IL-15 was detected in 30/53 RA SFs in the range 10-1128 pg/ml (median of positive samples 198 pg/ml) by ELISA using paired murine monoclonal antibodies (BAM247 & MAB647). Next, matched synovial fluid and serum samples consecutively acquired in the clinic from 18 RA patients were compared. SF was found to contain significantly higher levels of IL-15 than serum in matched samples (185.6 ± 43.4 vs 86.8 ± 26.1, $p<0.005$, Figure 3.2a), although it was of interest that IL-15 was detected even in some sera. Since TNF-α has been reported to be present in large quantity in SF of RA patients (Feldmann, et al, 1996), levels of non-receptor bound TNF-α were determined in these samples. SF contained

significantly higher concentrations of TNF-α than serum samples (260 ± 65.5 vs 127.7 ± 42.9, $p<0.005$). Moreover, Figure 3.2b shows that the concentration of IL-15 and TNF-α in SF, was significant correlated ($p<0.005$, Spearman's rank test), suggesting a possible link between the two cytokines.

It was possible that rheumatoid factor (RF) could render the quantification of IL-15 within SF inaccurate (Carson, 1997). RF are antibodies directed against the charged tertiary structure of the IgG Fc fragment and may be of any immunoglobulin class including IgM, IgG and IgA. Thus, RF could potentially "cross-link" the primary antibody with the detecting antibody, giving rise to false positive results in ELISAs. It was important to investigate this possibility prior to seeking IL-15 in routinely acquired SF. RF were therefore removed from SF from 29 RA patients and IL-15 levels were compared prior to and after RF removal using both ELISA systems. Figure 3.3 shows that the concentration of IL-15 present within SF was similar in the presence or absence of RF (Pearson Correlation test, $p<0.001$).

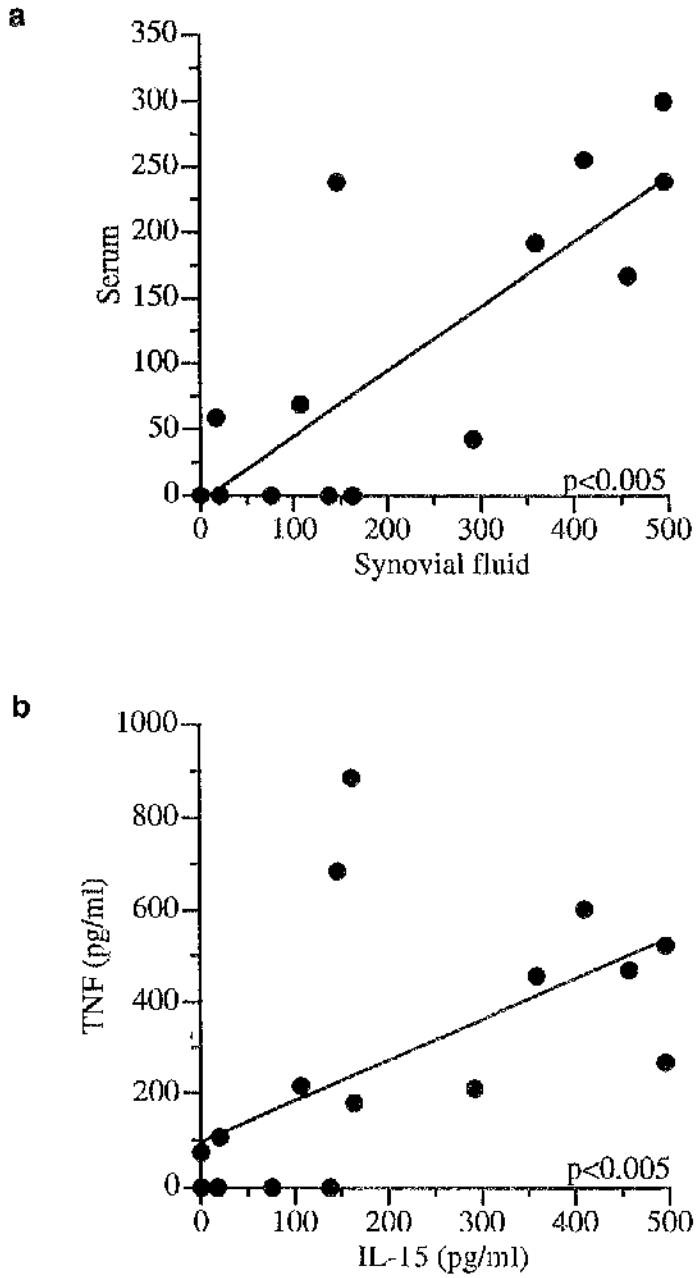
Moreover, SF samples with intermediate to high levels of RF (n=12) were artificially "spiked" with recombinant IL-15, then submitted to the commercial ELISA - the recovery rate observed was between 90 - 102% (Table 3.1). Together, these results demonstrate not only the specificity of the IL-15 detection system, but clearly indicate that IL-15 is detectable in RA SF.

Figure 3.1 Standard curves for IL-15 ELISA



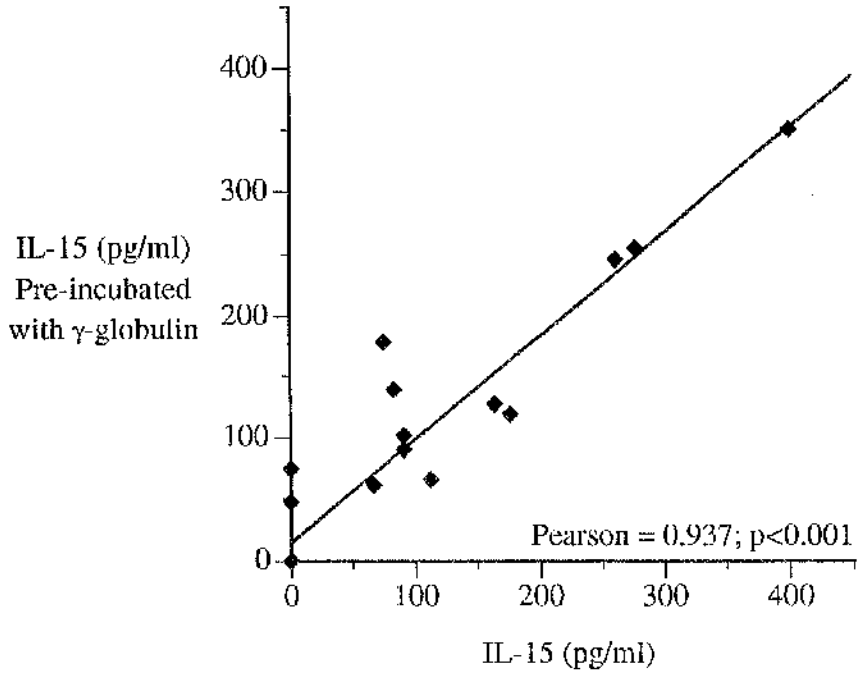
rIL-15 was diluted two-fold in PBS / 10% FCS and submitted to assay with (a) R&D IL-15 sandwich ELISA as described in section 2.9.1. Serum and synovial fluids were diluted in PBS / Tween then assayed as for the standard. (b) Murine sIL-15Rα is employed as a "capture" antibody to confirm the specificity of IL-15 detection as previously described. Sensitivity was typically 1.5 pg/ml and 5 pg/ml respectively.

Figure 3.2 Detection of IL-15 & TNF-α in RA paired samples



Serum and synovial fluids from RA patients were collected (n=18), and assayed for IL-15 presence by ELISA. (a) Levels in SF were significantly higher than in serum ($p<0.001$, paired t-test), a significant correlation was also observed between detectable levels of IL-15 in serum and SF ($p<0.005$). (b) IL-15 and TNF-α levels in RA SF were significantly correlated ($p<0.005$).

Figure 3.3 Rheumatoid factor did not affect IL-15 quantification in RA synovial fluids



Rheumatoid factors were removed from synovial fluids using γ-globulin coated polystyrene beads as described in section 2.9.2. IL-15 levels in SF were then compared prior to (x-axis) and after (y-axis) RF removal and found to be similar (Pearson Correlation test, $p<0.001$).

Table 3.1 Recovery of IL-15 from IL-15 "spiked" RA synovial fluids

Patient No.	Original IL-15 (pg/ml)	Spiked IL-15 (pg/ml)	Recovered IL-15 (pg/ml)	% Recovery
1	53	250	243	97.2
2	350	750	720	96.0
3	1128	1500	1396	93.1
4	16	100	90	90
5	50	250	232	92.8
6	15	100	96	96
7	210	500	454	90.8
8	110	250	238	95.2
9	27	100	93	93
10	482	750	713	95.1
11	526	750	683	91.1
12	127	250	255	102

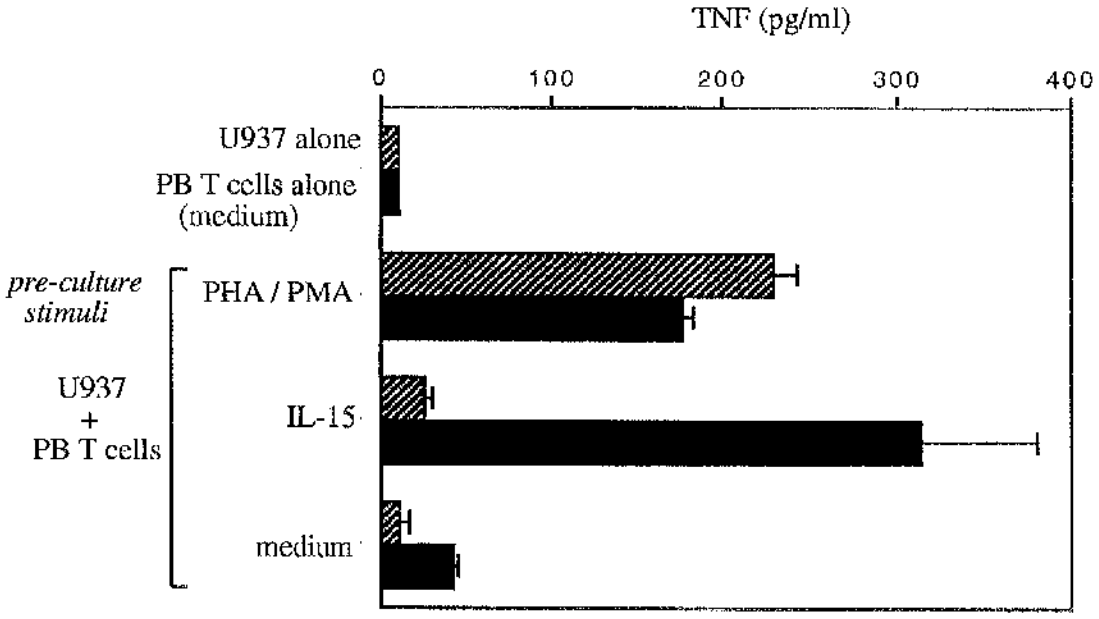
RA synovial fluids were spiked with recombinant human IL-15 at concentration indicated above and assayed using IL-15 ELISA as before. Recovered concentrations were compared to expected concentrations and expressed as percent recovery.

3.3 IL-15-dependent up-regulation of TNFα production from macrophages

The functional consequences of IL-15 expression in the synovial compartment were next explored. Previous studies have established that peripheral blood (PB) and SF T cells from RA patients exhibit brisk proliferative responses to IL-15 in excess of those to mitogens e.g. PHA. Moreover, IL-15 directly induces TNF-α production by SF T cells *in vitro* under these conditions but not from PB T cells (McInnes, et al, 1997). However, macrophages are the major source of TNF-α in RA synovitis (Chu, et al, 1991b), and so the effect of IL-15 on TNF-α 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 primary effects on macrophages via indirect pathways. Within the RA synovial membrane, T cells and macrophages are found in close proximity, and exhibit reciprocal adhesion molecule expression. Therefore, the possibility that IL-15-activated T cells could induce TNF-α synthesis by monocytes / macrophages via cell-contact was addressed. PB T cells from normal donors were stimulated with IL-15 or mitogen (PHA / PMA) for 72 hours, then co-cultured in double-chamber wells, either in contact with, or physically separated by a semi-permeable membrane from U937 cells. TNF-α production was observed only in cultures in which IL-15-stimulated T cells and macrophages were in direct contact (Figure 3.4). As expected, PHA / PMA-stimulated T cells induced TNFα production in the presence or absence of co-culture (Figure 3.4). These data indicated that a cell-contact event between IL-15-activated T cells and macrophages was capable of inducing TNF-α production.

Figure 3.4 Double chamber culture of PB T and U937 cells

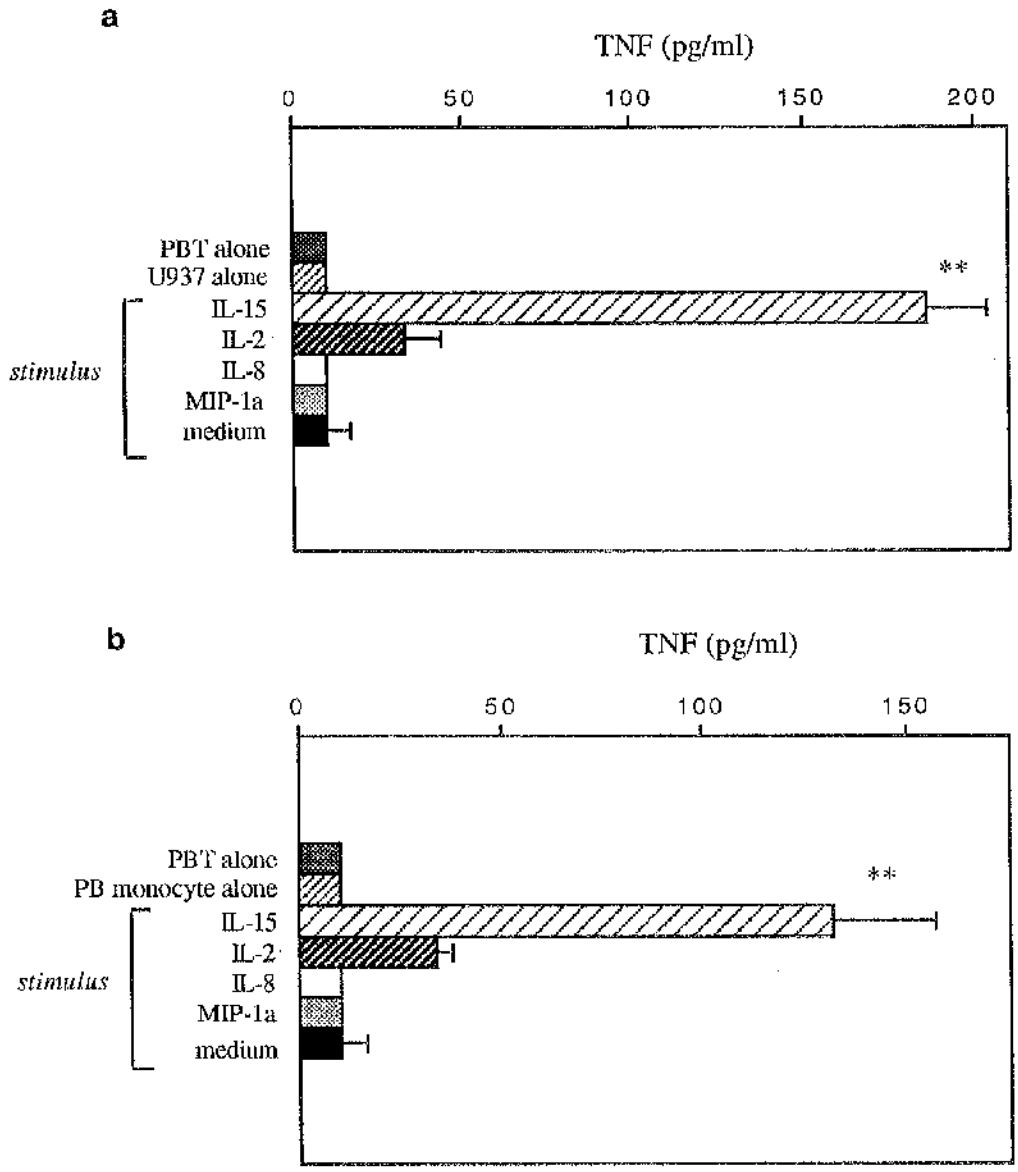


T cell / macrophage cell-contact is required for IL-15-induced TNF-α production. PHA (1 μg/ml) / PMA (1 nM) or IL-15 (100 ng/ml) activated PB T cells were cultured in contact with (solid bars), or separated by semi-permeable membrane from (hatched bars), unprimed U937 cells. IL-15-activated T cells required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

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, thus 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. They were subsequently fixed in 1% PFA, then co-cultured with U937 cells for a further 48 hours. Preliminary experiments have 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 (McInnes, et al, 1997).

As previously reported, PHA / PMA activated T cells consistently induced macrophage TNF-α synthesis in this system (Lacraz, et al, 1994). Similarly, PFA fixed-T cells, previously stimulated with IL-15 (100 ng/ml) induced production of significant concentrations of TNF-α by U937 cells (Figure 3.5a). In contrast, prior activation of T cells with IL-2 (100 ng/ml) was effective in inducing TNF-α production only in some donors, and did so at lower levels (Figure 3.5a, $p<0.003$ vs IL-15-activated PB T cells, Wilcoxon). This was 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 and polarization (Wilkinson & Liew, 1995; McInnes, et al, 1997). Identical results were obtained when highly purified autologous peripheral blood-derived monocytes (>92% $CD14^+$, <2% $CD3^+$ by FACS analysis) were used instead of U937 cells (Figure 3.5b), demonstrating that these results are unlikely to be due to an allogeneic effect between T cells and macrophages. Finally, TNF-α production could be significantly reduced by pre-treating PB monocytes and U937 cells with cycloheximide or

Figure 3.5 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) autologous 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.

actinomycin D, inhibitors of protein synthesis and mRNA translation respectively (Figure 3.6 a&b), thereby confirming that macrophages were the primary source of cytokine synthesis in co-cultures.

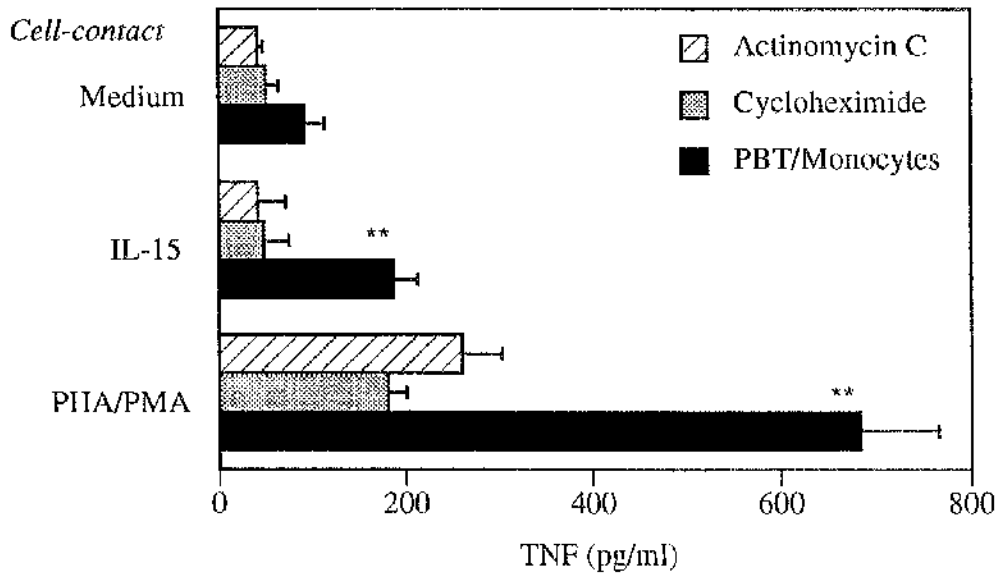
3.4 Induction of TNF-α production by cells of synovial origin

To determine whether a similar mechanism might operate *in vivo* in RA, freshly isolated SF mononuclear cells from RA patients (n=8) were immediately fixed with 1% PFA without prior stimulation and then added directly to U937 cells. Significant TNF-α production was observed after 48 hours in all co-culture supernatants (Figure 3.7a), demonstrating that synovial T cells activated *in vivo* can induce TNF-α production by macrophages through cell-contact. It was of interest to determine whether IL-15 was required to maintain this property of synovial T cells *ex vivo*. SF T cells were therefore cultured for 24 hours with medium alone, IL-15 or IL-2, then fixed with PFA, before co-culture with U937 cell as before. TNF-α 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 3.7b).

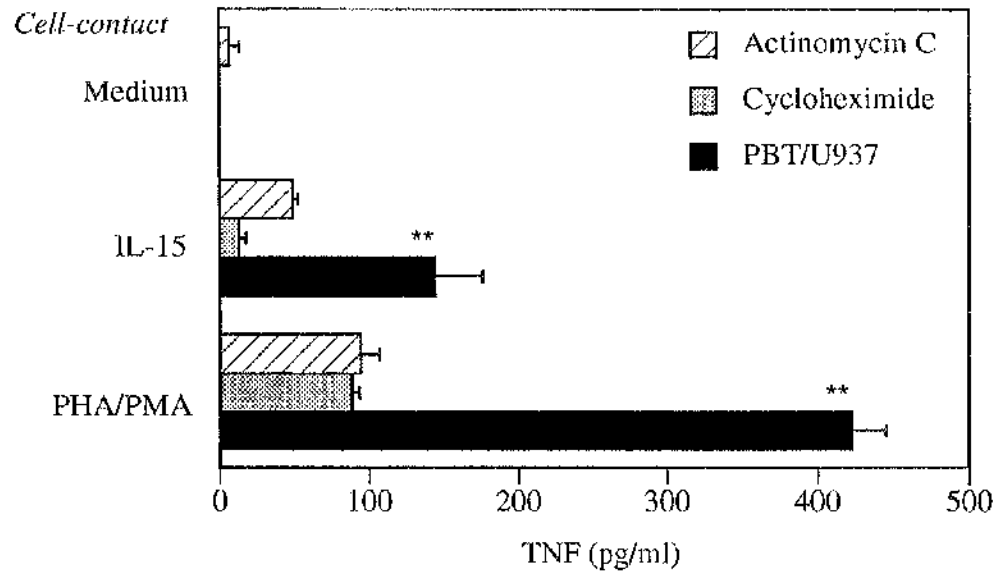
Similar results were obtained using autologous peripheral blood monocytes (instead of U937 cells) which were obtained from each RA patient at the time of joint aspiration (Figure 3.8a). To confirm that synovial macrophage TNF-α synthesis could be enhanced by this pathway, PFA-fixed PB T cells from 4 RA patients were added to autologous synovial macrophage / synoviocyte co-cultures (>75% $CD68^+$, <3% $CD3^+$ by cytoprep analysis). TNF-α synthesis was observed only with prior activation of PB T cells by IL-15 (Figure 3.8b). Together, these data demonstrated that synovial T cell-mediated upregulation of TNF-α production by macrophages through cell-contact could occur in RA, and indicated that IL-15 within the synovial membrane enhanced this activity.

Figure 3.6 Transcription and translation is required for cell-contact TNF-α production by monocytes and U937

a

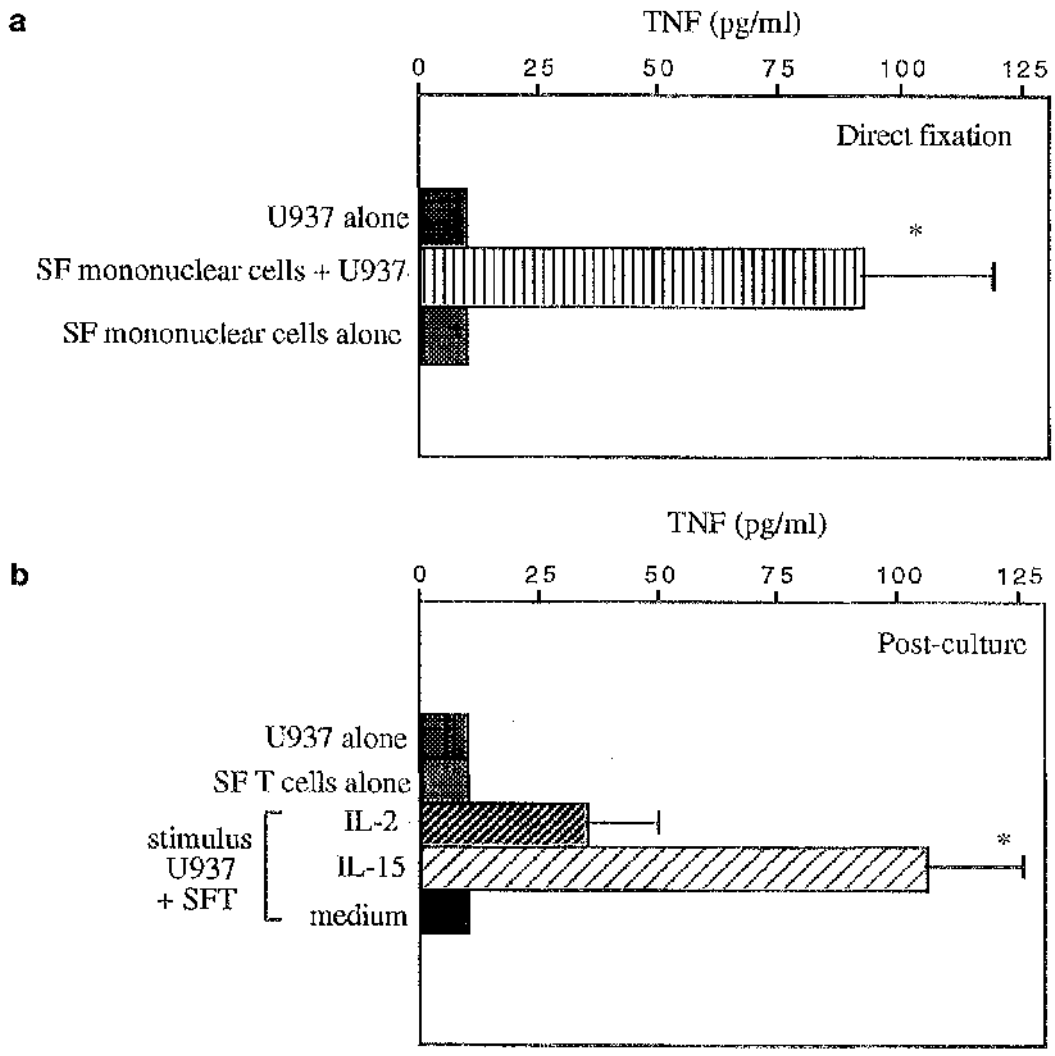


b



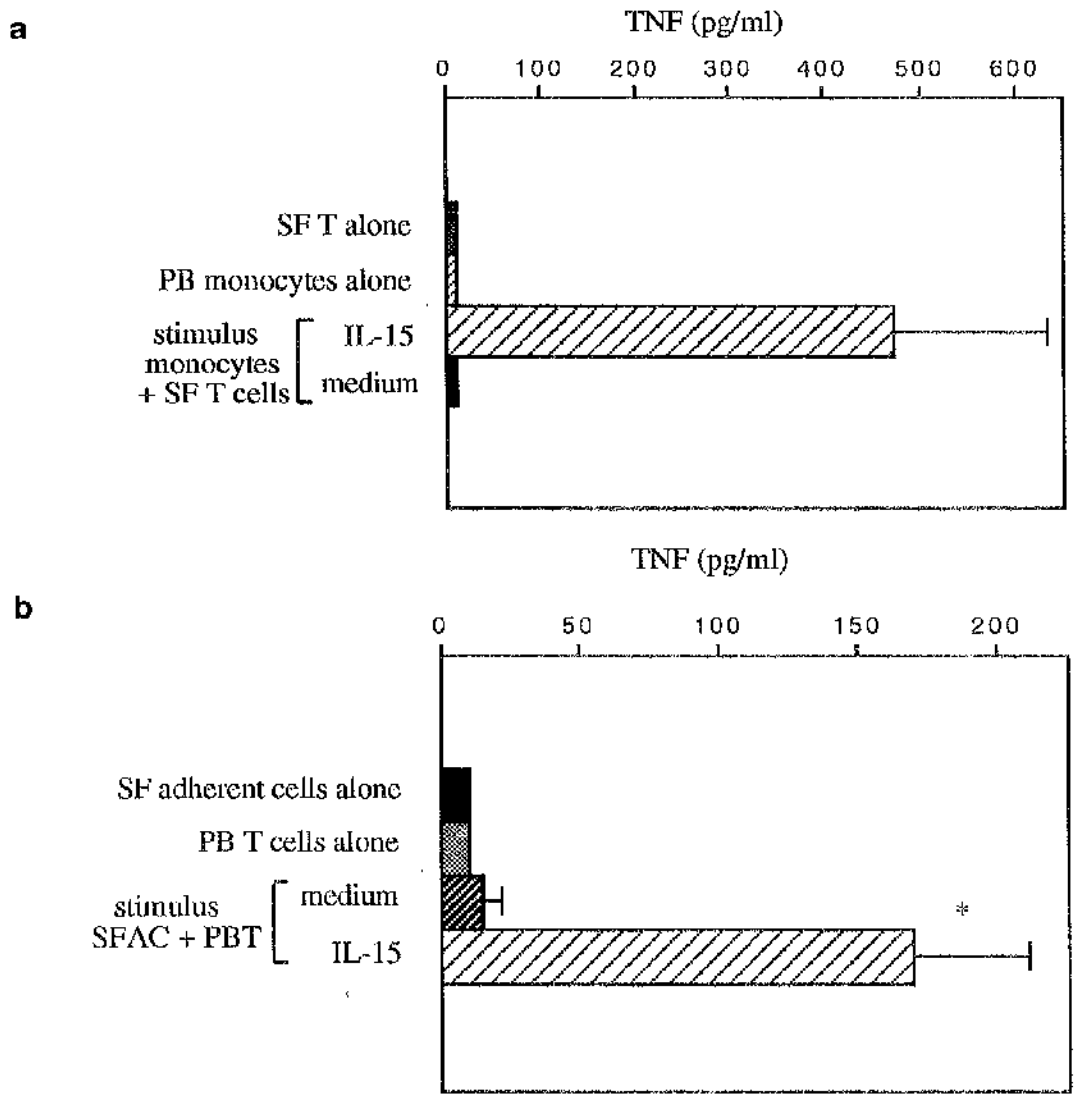
PFA-fixed activated PB T cells from normal donors were cultured with (a) autologous PB monocytes (n=3), or (b) U937 cells (n=3) in the absence (solid bar) or presence of 2 μg/ml cycloheximide (dotted bar) or 2 μg/ml actinomycin D (hatched bar). Supernatants were collected after 48hr and TNF-α was determined by ELISA. Data are mean ± s.e.m, **p<0.005.

Figure 3.7 Synovial T cells induce TNF-α production via cell contact



IL-15 is required to sustain synovial T cell-mediated cell-contact induction of TNF-α synthesis. PFA fixed RA SF T cells induced TNF-α 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 3.8 Cell contact regulates TNF-α 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.

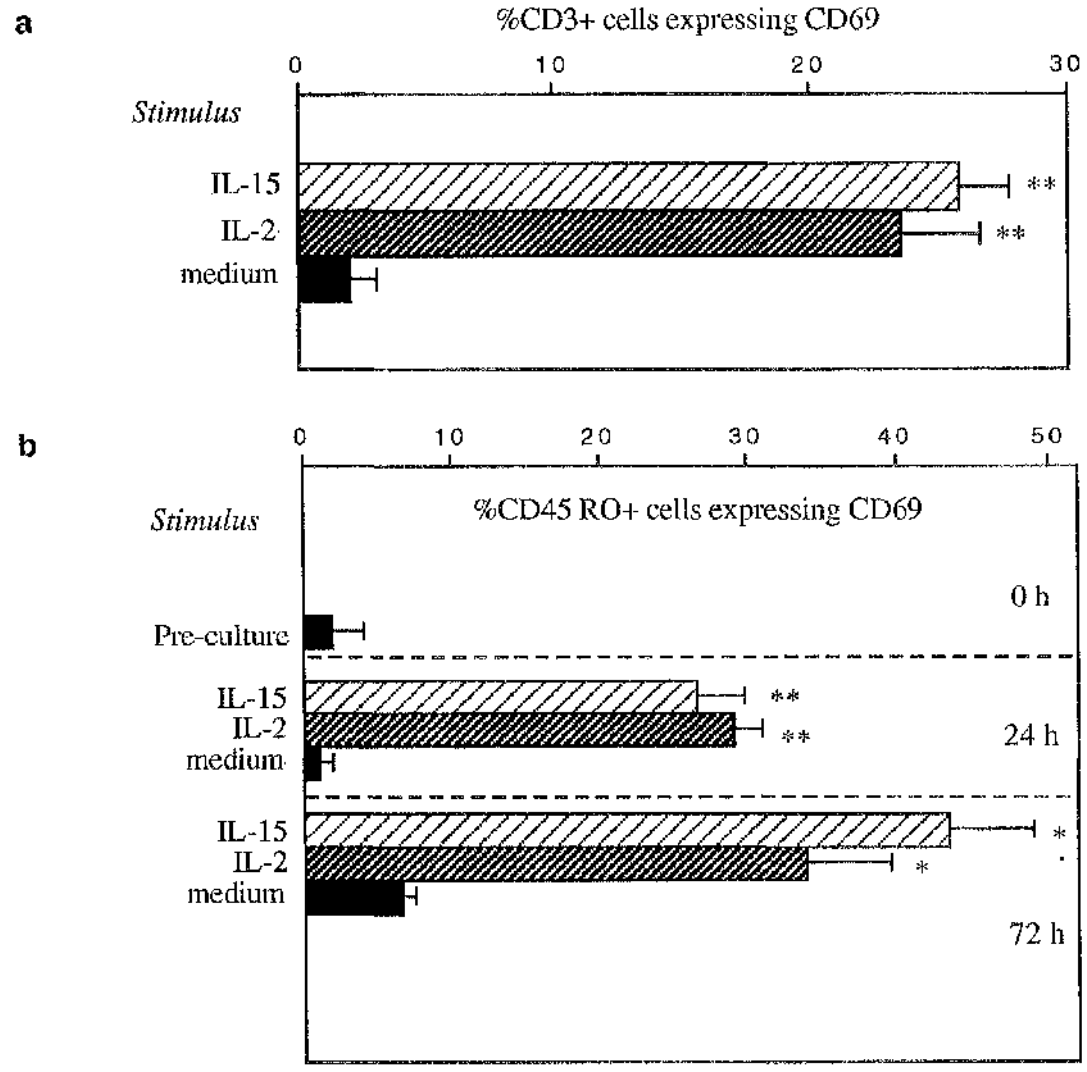
3.5 Effect of IL-15 on PB lymphocyte phenotype

The ability of single cytokines to induce cell division and to sustain cell-contact mediated macrophage activation suggested that those responding cells were of an *in vivo* 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 within 16 hours and remained present up to 72 hours ($p<0.005$, Figure 3.9a). 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 16 hours, and enhanced seven-fold in $CD45RO^+$ PB T cells by 72 hours after addition of IL-15 *in vitro* ($p<0.02$, Figures 3.9b).

3.6 Molecules involved in T lymphocyte / macrophage cell-contact

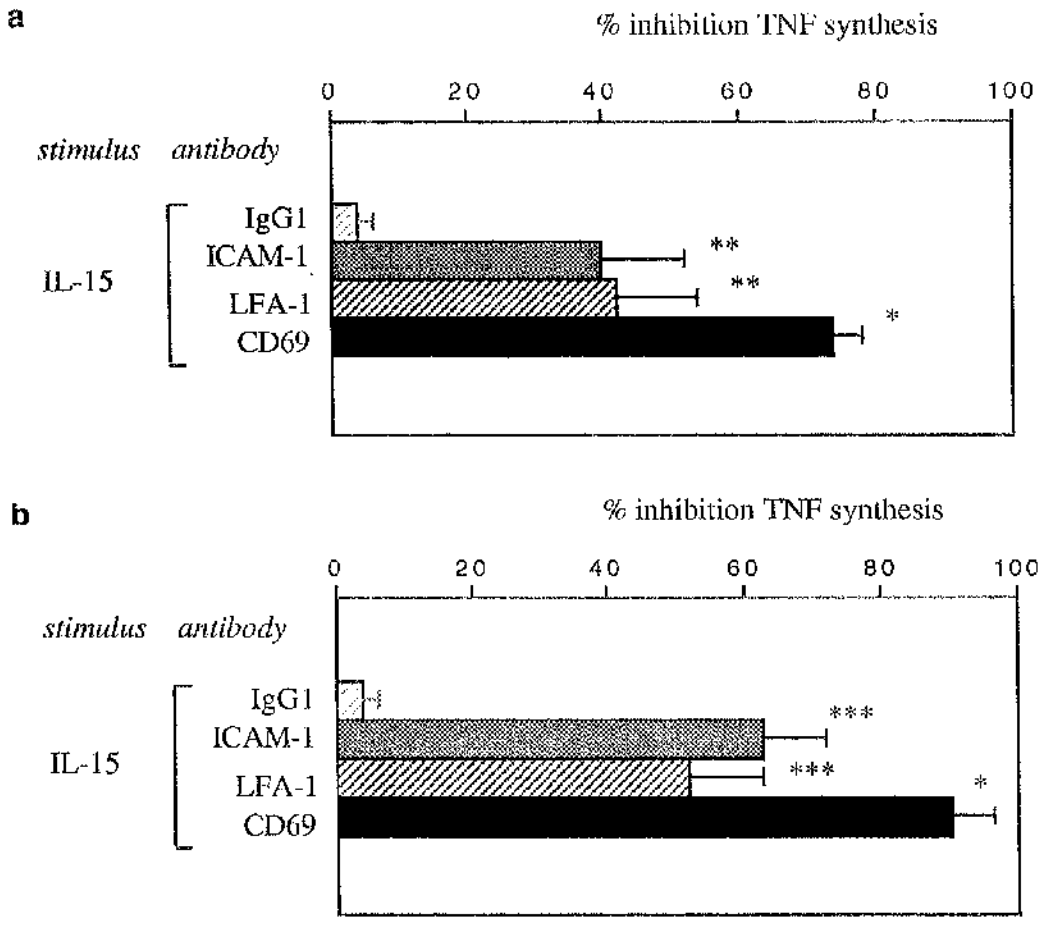
Many surface receptors have been implicated in T cell / macrophage membrane contact, including the LFA-1 and CD69 (Vey, et al, 1992; Isler, et al, 1993). The contribution of these markers to the production of TNF-α by monocytes after contact with IL-15-activated T cells was therefore investigated using neutralising monoclonal antibodies. TNF-α production by PB T cell / U937 co-cultures ($n=4$) was significantly reduced by neutralization 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 3.10a). 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 TNF-α by blood-derived monocytes induced by IL-15-activated T cells (Figure 3.10b). In contrast an isotype-matched murine monoclonal antibody of irrelevant specificity at identical

Figure 3.9 FACS analysis of PB T cells 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 3.10 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 (*$p<0.001$, **$p<0.03$, ***$p<0.01$). Polyclonal human IgG (5 μg/ml) was present in all cultures to block Fc binding. Data are mean ± s.e.m. from four separate experiments.

% inhibition was calculated as follows:

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

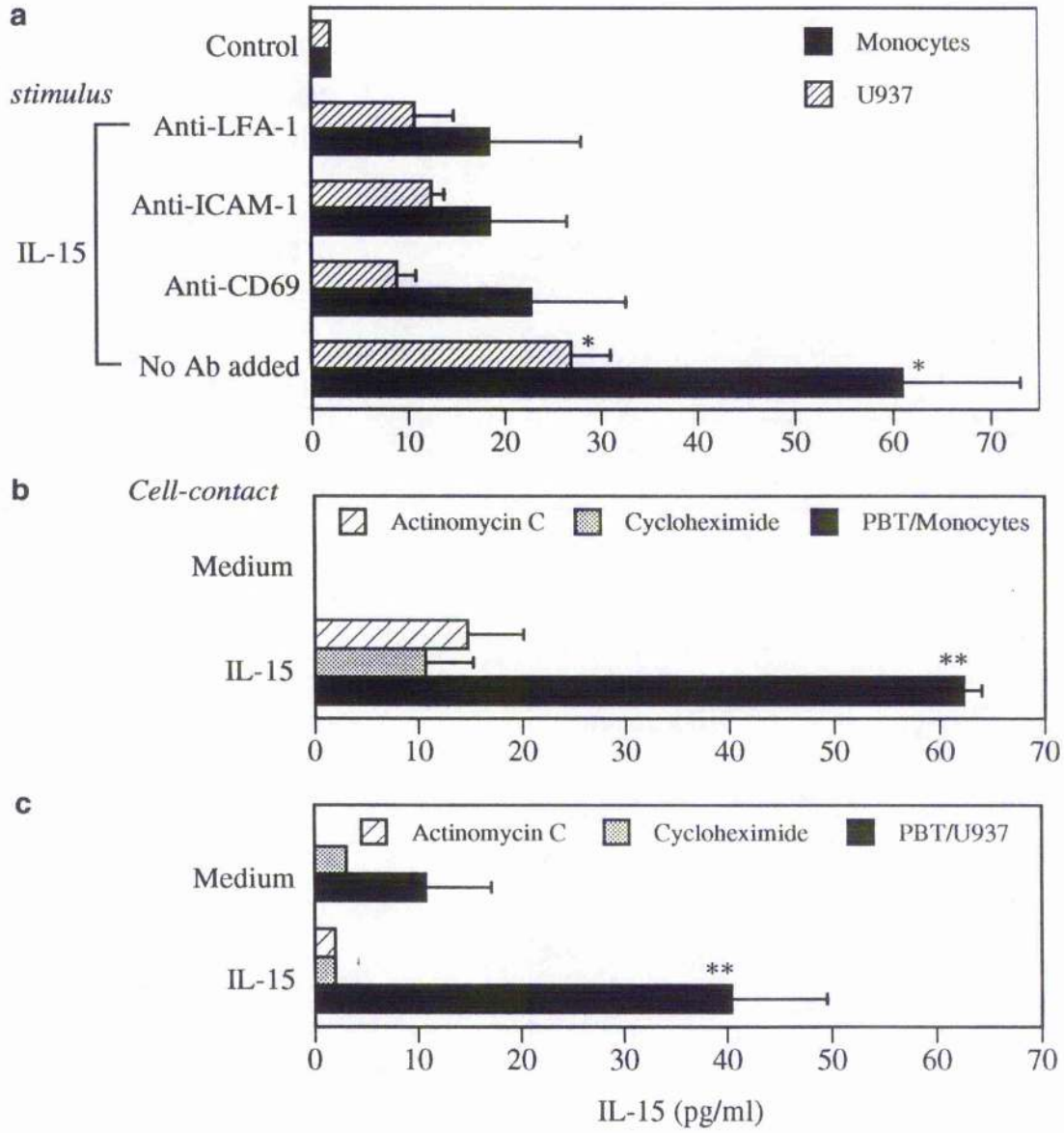
concentration failed to block the effects of IL-15 on cell-contact mediated TNF-α production. Thus, at least CD69 and the LFA-1 / ICAM-1 pathway were implicated in IL-15-activated T cell-mediated macrophage activation.

3.7 T cells stimulated with IL-15 induce monocyte production of IL-15

Human and murine monocytes have been shown to express IL-15 in response to LPS, mycobacteria, or *Toxoplasma gondi* (Carson, et al, 1995; Doherty, et al, 1995). However, the mechanisms involved in inducing monocyte production of IL-15 in RA synovium are not yet understood. The ability of T cells to costimulate monocyte IL-15 production was therefore investigated.

PB T cells purified from normal donors were stimulated with IL-15 as described above, then fixed in 1% PFA and co-cultured with either autologous PB monocytes obtained from the same donor or U937 cells. Whereas unstimulated T cells did not induce monocyte IL-15 production, T cells pre-stimulated with IL-15 (100 ng/ml) induced significant IL-15 production by macrophages after 48 hours culture in three of eight experiments (Figure 3.11a). This IL-15 production by PB T-cell / monocyte or U937 co-cultures (n=3) could be significantly reduced by neutralisation of LFA-1, ICAM-1 or CD69 ($p<0.05$), suggesting that T cells costimulation of IL-15 is in part mediated via these molecules. In addition, IL-15 production could be significantly inhibited by pre-treating monocytes or U937 cells with actinomycin D or cycloheximide (Figure 3.11 b&c), thereby excluding the possibility that shedding of previously added rIL-15 could explain the observations. Moreover, IL-15 synthesis was also induced by PHA / PMA activated T cells in which no rIL-15 was added.

Figure 3.11 Activated PB T cells induce IL-15 production by monocytes and U937 cells

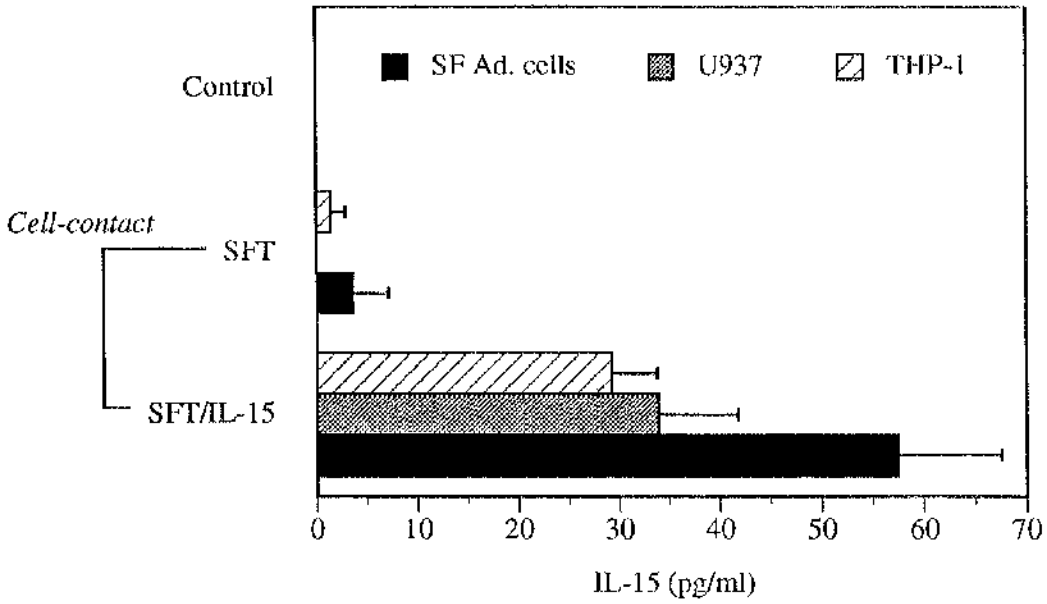


(a) IL-15 activated PFA-fixed PB T cells induced IL-15 synthesis by U937 cells or monocytes from the same donor (n=3). Pre-incubation of the fixed T cells with neutralising antibodies to CD69, LFA-1 or ICAM-1 significantly inhibited IL-15 synthesis. IL-15 synthesis was significantly reduced by cycloheximide or actinomycin D (n=2) when PFA-fixed IL-15-activated PB T cells were cultured with (b) PB monocytes, or (c) U937 cells, indicating that transcription and translation was required. Data are mean ± s.e.m., *$p<0.05$, **, $p<0.005$.

3.8 Induction of IL-15 production by cells of synovial origin

To assess whether a similar mechanism might operate in RA, SF T cells from RA patients (n=5) were cultured either in medium alone or with IL-15 (100 ng/ml) for 48 hours, then fixed in PFA before addition to autologous synovial monocyte / fibroblast co-cultures. IL-15 synthesis was induced only by IL-15-activated synovial T cells but not by unstimulated controls. This confirmed that stimulated SF T cells expressed cell surface molecules which could trigger SF adherent cells to produce IL-15 (Figure 3.12) and indicated that IL-15 could thereby participate in its own up-regulation through this cell contact dependent pathway leading to a positive feedback loop.

Figure 3.12 Synovial T cells induce IL-15 production via cell contact



IL-15 activated, PFA-fixed RA SF T cells induced IL-15 synthesis by SF adherent cells from the same donor (n=5). Lower levels of production were observed with U937 and THP-1 cocultures (n=5). Data are mean ± s.e.m.

Discussion

The inflammatory cytokines TNF-α, IL-1β, IL-6, IL-8 and GM-CSF are considered major participants in the cytokine network in RA (Feldmann, et al, 1996). Data from collagen-induced animal arthritis models (Williams, et al, 1992) and from recent clinical trials in RA patients (Elliott, et al, 1994; Rankin, et al, 1995; Morcland, et al, 1997) indicate that TNF-α occupies a pivotal position in the regulation of synovial inflammation. However, the factors which up regulate TNF-α production in RA remain unclear. Data presented here demonstrate that IL-15 is critical in this process.

IL-15 was detected by ELISA in RA synovial fluids at significantly higher levels of than in serum in matched samples. This activity correlated with SF TNF-α concentrations and remained after removal of rheumatoid factor. IL-15-induced proliferation and direct TNF-α production in T cells derived from synovial fluid is enhanced compared to those from blood, showing that T cell responsiveness to IL-15 was upregulated *in vivo* (McInnes, et al, 1997). However, T cells represent only a minor source of TNFα compared with macrophages in RA synovium (Chu, et al, 1991b). A number of studies (Vey, et al, 1992; Isler, et al, 1993; Lacraz, et al, 1994), have shown that monocytic cells secrete IL-1β, TNF-α and MMPs in response to signals mediated by contact with activated T cells. Moreover, it has been suggested that cell-contact mediated activation of T helper cell subsets as they transmigrate may be a step in the priming of T cells for the effects of cytokines and growth factors within inflammed tissue (St Louis, et al, 1993).

IL-15-activated PB T cells induced significant TNF-α production from either unprimed U937 cells, autologous blood-derived monocytes, or RA synovial macrophage / synoviocyte cultures, by a cell-contact dependent mechanism, which could be inhibited by pre-treatment with actinomycin D or cycloheximide. Thus, freshly recruited IL-15-activated T cells may contribute to TNF-α 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 likely operates *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. Thus, synovial T cells under the control of IL-15 are clearly able to activate monocytes to produce TNF-α. Significantly, recent recruited $CD14^+$ monocyte / macrophage constitute the major source of TNF-α in the synovial membrane (Chu, et al, 1991b).

The next critical question was to determine which factorsregulate IL-15 synthesis. Despite the widespread expression of IL-15 mRNA in a variety of cell and tissue types, including placenta, kidney, lung, liver, fibroblasts, and activated monocytes (Grabstein, et al 1994), it has been difficult to demonstrate meaningful quantities of IL-15 protein in the culture supernatants of most cells and cell lines. IL-15 is predominantly regulated post-transcriptionally at the levels of translation and intracellular trafficking (Bamford, et al, 1997). In humans, IL-15 mRNA consists of a long 5' untranslated region (UTR) including 10 multiple upstream AUGs, which may dramatically reduce the efficiency of its translation (Bamford, et al, 1996). Furthermore, the IL-15 signal peptide is usually long (48 ααs), and together these factors contribute to the inefficiency of IL-15 protein release (Bamford, et al 1997, Onu, et al, 1997). When monocytes were incubated with fixed, IL-15-stimulated SF T cells, significant levels of IL-15 production were detected in these supernatants. Thus, activated T cells appear to be effective costimulators of TNF-α and IL-15 production by monocytes via direct cell-cell contact. Actinomycin D inhibition experiments had further confirmed the absolute requirement of mRNA translation in IL-15 synthesis, suggesting that the IL-15 mRNA pool store may not be sufficient for IL-15 synthesis. IL-15 production could also be significantly reduced by cycloheximide, thus excluding the possibility that it was derived either from intracellular pool of inactive protein present within the monocytes or shedding of rIL-15 previously fixed onto T cells. The reasons for the variable induction of monocyte

IL-15 production by stimulated PB T cells and SF T cells are not yet clear. As discussed below, it has been shown that SF T cells isolated from matched samples were enriched with activated ($CD69^+$) memory ($CD45RO^+$, Rb^{dim}) subset when compared with PB T cells (Kohem, et al, 1996). It is possible that the variation in IL-15 production may be explained partly by such T cell phenotype variation, as T cells in different states of activation may respond differently to the exogenous stimuli.

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 (Burmester, et al, 1987; Pitzalis, et al, 1987a; 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). 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 majority of T cells at sites of inflammatory lesions need not be antigen-specific. In leprosy skin lesions and reactive arthritis SF, limiting dilution analysis has shown that only 0.1-2% of T cells are specific for the relevant bacterial protein (Modlin, et al, 1984; Sieper, et al, 1993). A similar situation may exist in RA synovial membrane.

The cell-contact pathway for macrophage activation by T cells in synovium is likely to utilise multiple cell surface molecules. T cell surface molecules CD69, CD11a (LFA-

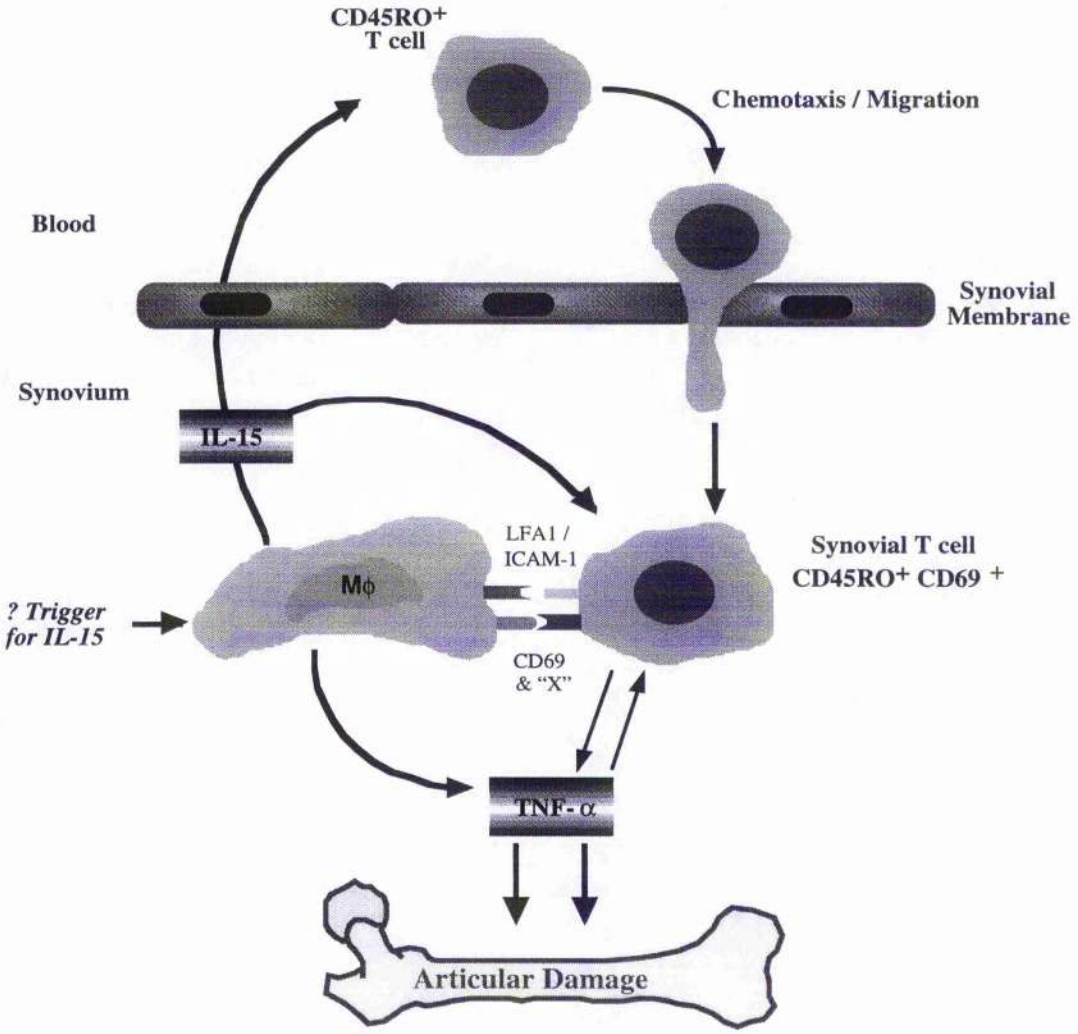
1), CD2 and CD40L have been suggested to regulate production of several monokines including IL-1β, TNF-α and IL-12 (Manie, et al, 1993; Isler, et al, 1993; Wagner, et al, 1994; Shu, et al, 1995). As expected, the LFA-1 / ICAM-1 pathway was implicated by the present study. Following chemokine-induced 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). 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). The CD69 activation pathway is linked to the TCR / CD3 receptor complex (Rincon, et al, 1989). The large cytoplasmic domain of CD69 is probably involved in signal transduction (Testi, et al, 1989). 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

In combination with the divergent effects of IL-15 and IL-2 in generating PB and SFT 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. Alternatively, IL-15RX expression may be of relevance (Tagaya, et al, 1996a).

Thus, it is attractive to hypothesise that 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 (Figure 3.13). Therefore, IL-15 or IL-15Rα antagonists represent potential approaches to attenuating RA and perhaps other inflammatory diseases. Further efforts are required to determine the identity of other 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.

Figure 3.13 IL-15 induced amplification of rheumatoid arthritis synovitis



Synovial macrophage - or fibroblast - derived IL-15 mediates recruitment and activation of T lymphocytes and confers upon them the capacity for macrophage activation / TNF-α production through cell-contact, in the absence of significant IFN-γ synthesis. Such cell-cell interactions are likely modified by local cytokines e.g. IL-1, IL-6 and TNF-α (Sebbag, et al, 1997).

Chapter 4

IL-15 induces activation in RA synovial neutrophils

Introduction

Rheumatoid arthritis (RA) is characterized by chronic infiltration of the synovial membrane by T lymphocytes, plasma cells and macrophages. Together with activated fibroblast-like synoviocytes, this constitutes pannus, an invasive tissue capable of eroding into adjacent cartilage and bone. While disease pathogenesis clearly involves the multiple interaction of these cell types (Arend & Dayer, 1995; Feldmann, et al, 1996; David, 1997), increasing evidence suggests that infiltration of the synovium by activated neutrophils may also contribute to the inflammatory process and subsequent pathology.

During inflammation, the normally acellular synovial fluid (SF) is infiltrated by a variety of immune cells, of which neutrophils comprise between 60-90% of the total population (Zvaifler, 1973; Pillinger & Abramson, 1995). Similarly, neutrophils represent the majority (>70%) of inflammatory cells in the SF of rats with adjuvant arthritis (Santos & Tipping, 1994), and administration of neutrophil depleting antibodies in the same model leads to significant improvement in joint inflammation (Santos, et al, 1997). Accumulation of neutrophils and destruction of cartilage are also a prominent feature of bacterial septic arthritis (Goldenberg and Cohen, 1976). Activated neutrophils can secrete many of the cytokines that are detectable within RA SF including IL-1, IL-8 and TNF-α, together with a wide range of potent proteinases, hydrolases and matrix metalloproteinases. Their ability to generate a series of reactive oxygen and nitrogen intermediates may also contribute to inflammation (Kitsis & Weissmann, 1991; Edwards & Hallett, 1997). Neutrophils require sequential priming then activation. In RA SF neutrophils may be primed within the inflammed joint by a number of activation factors including immunoglobulin aggregates and cytokines (Robinson, et al, 1993a; Watson, et al, 1993).

IL-15, an IL-2-like cytokine has been identified within the synovial membrane of RA patients (McInnes, et al, 1996b; Thurkow, et al, 1997). IL-15 is a pleiotropic cytokine derived from several cell types including macrophages and fibroblasts, which mediates its activity through a heterotrimeric receptor consisting of a unique IL-15Rα chain, in combination with the β and γ chains of the IL-2 receptor (Tagaya, et al, 1996b). IL-15 can exert a variety of immunological effects and may play an important role in the pathogenesis of RA as described in chapter 3.

Human neutrophils have been shown to express both the β and the γ chains of the IL-2 receptor, whereas the IL-2Rα is undetectable (Liu, et al, 1994; Nakarai, et al, 1994). Recently, IL-15 has been described to modulate phagocytosis, cytoskeletal rearrangement and apoptosis in blood neutrophils (Girard, et al, 1997). However, the role of IL-15 in cytokine synthesis and adhesion molecule expression by neutrophils is currently unclear. The present studies were undertaken to determine whether, in addition to its effects on T cells, IL-15 might also promote neutrophil recruitment and activation through enhancement of adhesion molecule and cytokine / chemokine expression respectively.

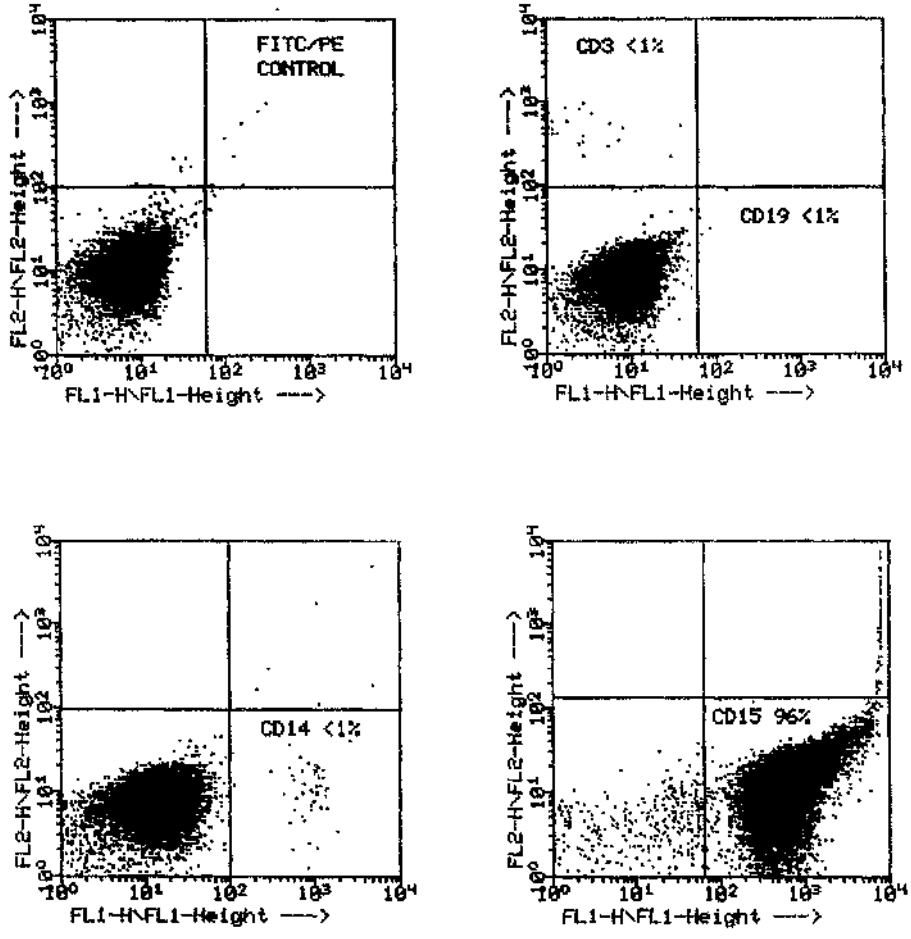
4.1 Preparation of Neutrophils

Neutrophils were isolated from peripheral blood (PB) and SF of RA patients by centrifugation over lymphoprep as described in Section 2.4.3. Adherent cells including monocytes, fibroblasts and lymphocytes were removed by two successive adherence steps at 37°C for 30 min. The purity of the neutrophil preparations thus obtained was 94 ± 5% (mean ± sem, Figure 4.1) judged by morphology on Giemsa-stained cytopreps and FACS using analysis using the neutrophil marker CD15. Remaining cells were B lymphocytes (CD19, <3%), T lymphocytes (CD3, <2%) and monocytes (CD14, <1%). Neutrophils were used within one hour of preparation.

4.2 Expression of IL-15Rα mRNA in RA neutrophils

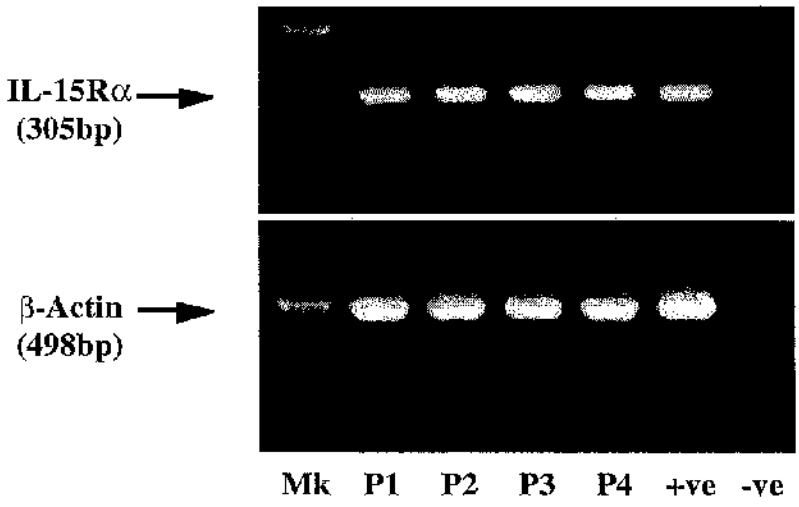
IL-15 mediates its effects through the IL-2Rβ and IL-2Rγ chains, both of which are known to be present on neutrophils (Liu, et al, 1994; Nakarai, et al, 1994). However, it remained unclear whether IL15Rα, the third component of the IL-15 heterotrimeric receptor was present on neutrophils. In the absence of available reagents at the protein level, IL-15Rα expression in RA SF neutrophils was sought at the mRNA level. RT-PCR was performed using primers specific for human IL-15Rα (Anderson, et al, 1995b) which were designed to amplify the entire transmembrane and cytoplasmic coding domain plus an additional 100 bp of 3' non-coding sequence (kindly provided by Dr. D. Anderson, Immunex Corp.). RT-PCR of preparations from 4 RA SF neutrophil samples generated the expected 305 bp fragment (Figure 4.2). For control, β-actin was amplified, as was a positive control derived from a plasmid containing human IL-15Rα cDNA (Figure 4.2). RA PB neutrophils also spontaneously expressed IL-15Rα mRNA, consistent with a previous report that IL-15 could induce PB neutrophil activation (Girard, et al, 1996).

Figure 4.1 Analysis by FACS of purified neutrophils



A representative analysis is shown for neutrophil which were isolated from PB and SF of RA patients as described in section 2.4.3.

Figure 4.2 RT-PCR for IL-15Rα in RA synovial fluid neutrophils

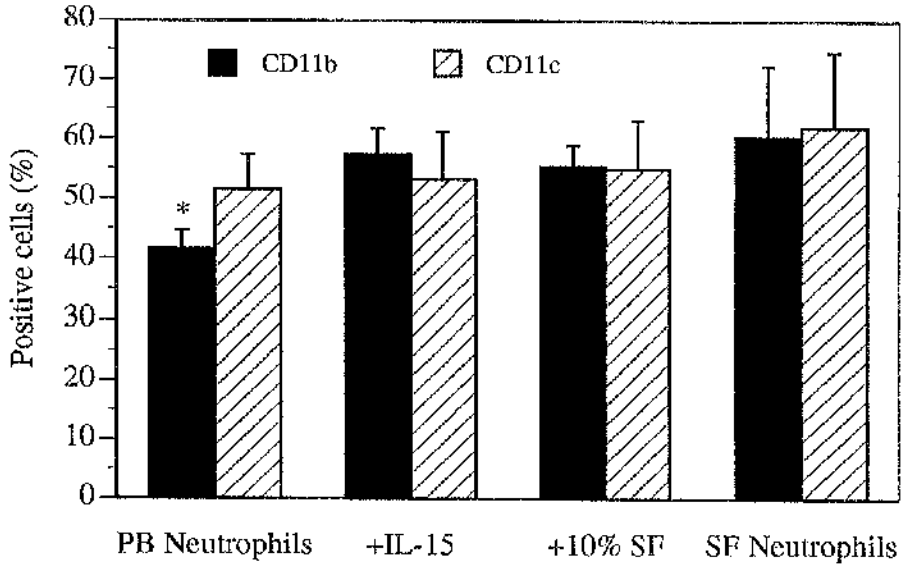


RT/PCR was performed to detect human IL-15Rα mRNA expression in freshly isolated RA SF neutrophils. Mk - 1 kb marker, P1-4 - representative RA patients, +ve - positive control (human IL-15Rα cDNA), -ve - negative control (dH_2O & RT/PCR reagents). β-actin was amplified for control.

4.3 IL-15 induced expression of CD11b on human neutrophils

The ability of IL-15 to modify adhesion molecule expression was of interest. In PB T cells, IL-15 induces uropod formation and redistribution of cell adhesion molecules including ICAM-1, -2, -3, CD43 and CD44, thereby modulating the adhesive properties of T lymphocytes (Nieto, et al, 1996). Similar morphological changes were observed in IL-15 activated PB neutrophils (Girard, et al, 1996) although the precise adhesion molecules involved were not clarified. Since CD11b and CD11c are crucial for neutrophil transmigration, these markers were studied. Neutrophils were purified from PB and SF from 5 RA patients and expression of CD11b and CD11c was determined by FACS analysis. In paired samples, PB neutrophils from RA patients expressed surface levels of CD11c that were comparable to those expressed on the surface of SF neutrophils. However, expression of CD11b was significantly increased in the SF neutrophils compared to those of PB ($p<0.01$, Figure 4.3), indicating up-regulation during recruitment to the synovial compartment. To determine whether factors present within SF were capable of up-regulating expression of CD11b, PB neutrophils isolated from above patients were incubated with 10% cell-free SF and expression of both CD11b and CD11c was measured after 3 hours incubation. This increased CD11b cell surface expression in all patient samples ($p<0.01$, Figure 4.3) while CD11c expression remained unaltered. The percentage of CD11b positive cells post-incubation was similar to that observed in freshly isolated SF neutrophils. Similar data were obtained by stimulating PB neutrophils directly with recombinant IL-15. PB neutrophils cultured with 100 ng/ml of IL-15 for 4 hours expressed significantly higher levels of CD11b than unstimulated cells ($p<0.01$, Figure 4.3).

Figure 4.3 FACS analysis of RA PB neutrophils following IL-15 activation



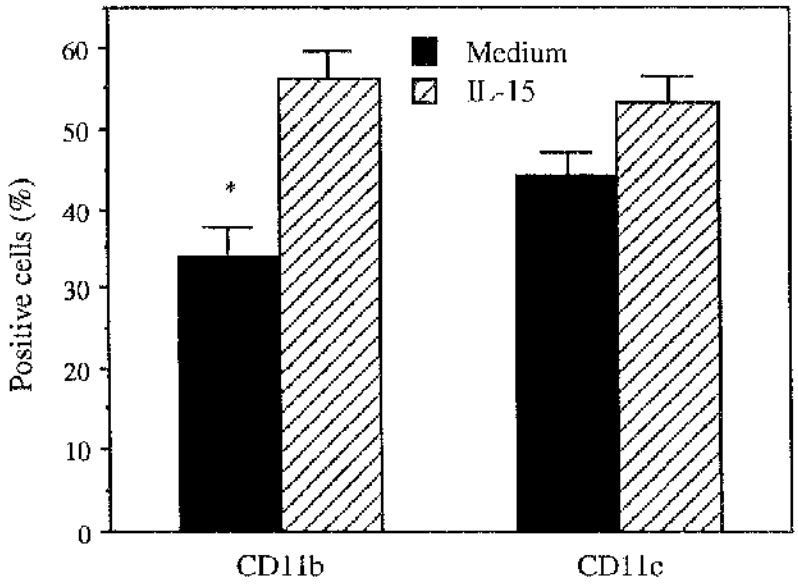
IL-15 and 10% cell-free synovial fluid upregulated expression of CD11b on RA PB neutrophils. Purified RA PB neutrophils (n=5) were cultured for 3 hours in the presence of medium alone, IL-15 (100 ng/ml) or 10% cell-free SF. FACS analysis was performed at the end of the culture period to determine the percentage of $CD11b^+$ and $CD11c^+$ cells. CD11b expression was enhanced by addition of IL-15 or SF (*$p<0.01$, paired-T test). No significant difference in CD11c expression was detected. Data are mean ± s.e.m.

To confirm and extend the above findings, PB neutrophils were isolated from 12 RA patients and cultured either in the presence or absence of IL-15 (100 ng/ml) for 3 hours. Figure 4.4 shows that cells cultured with IL-15 expressed significantly higher levels of CD11b than unstimulated cells. By comparison, CD11c expression were similar in both control and stimulated cells. These data demonstrate that IL-15 may be added to the group of cytokines in SF capable of neutrophil activation which includes IL-1α, TNF-α, GM-CSF and IL-8.

4.4 Induction of cytokine production by IL-15 activated Neutrophils

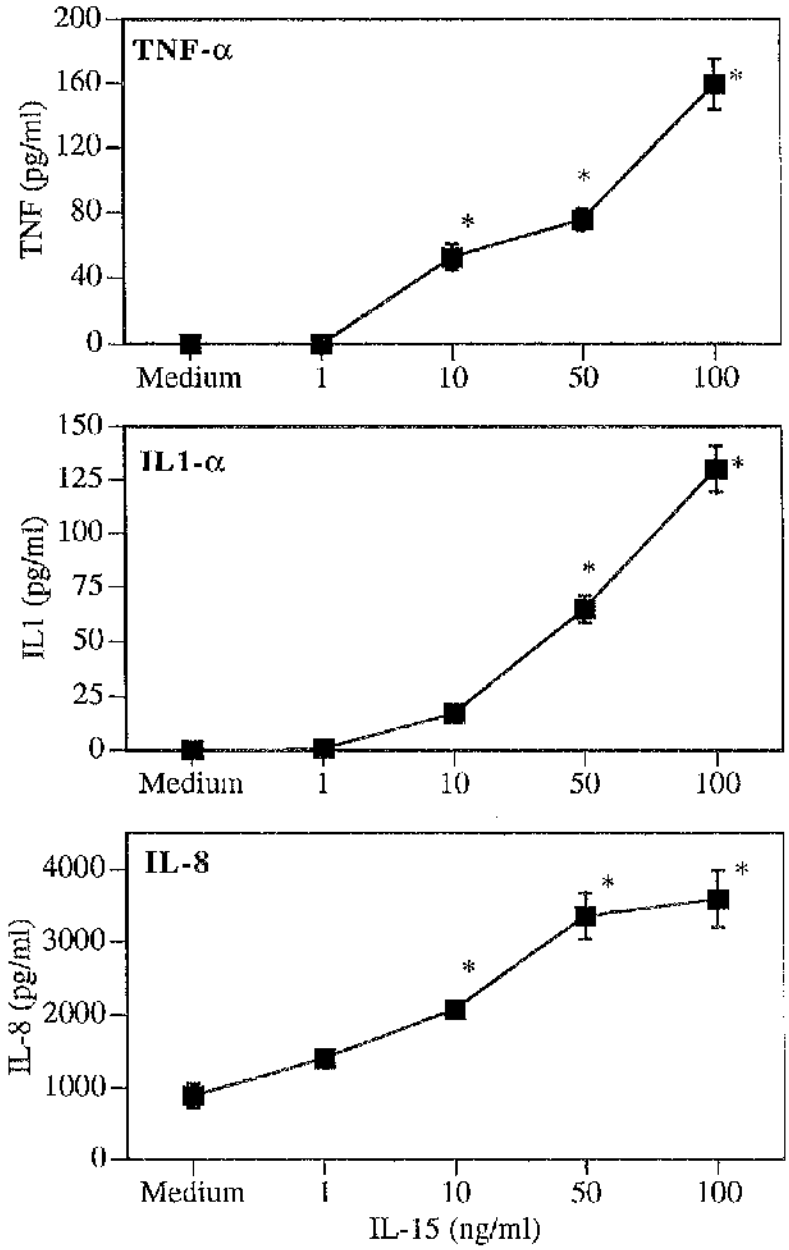
Neutrophils, like monocytes / macrophages, have the ability to produce cytokines which participate in the inflammatory reaction. Activated neutrophils secrete many of those cytokines which are detectable within RA SF including IL-1, IL-8 and TNF-α (Edwards & Hallett, 1997). Although IL-15 has been shown to regulate cytokine production in a variety of cells including T lymphocytes, NK cells and monocytes, its effect on neutrophil cytokine regulation has not been described. To investigate whether IL-15 might induce production of pro-inflammatory cytokines, SF neutrophils derived from RA patients (n=10) were stimulated with increasing concentrations of IL-15 and the levels of IL-1α, IL-8 and TNF-α in culture supernatants were determined by ELISA. IL-15 induced dose dependent production of IL-1α, IL-8 and TNF-α in SF neutrophil enriched cultures (Figure 4.5). The kinetics of cytokine production varied: for IL-1α and TNF-α, the maximal response was observed at 12 hours while that of IL-8, was noted at 24 hours (Figure 4.6). All culture supernatants were therefore compared for peak cytokine production at 100 ng/ml IL-15 after either 12 hours (IL-1α and TNF-α) or 24 hours (IL-8). The addition of IL-15 induced significant cytokine production in all 10 RA SF neutrophil cultures (Figure 4.7). In medium control cultures, only IL-8 synthesis was consistently detected (2552 ± 1084 pg/ml, Table 4.1).

Figure 4.4 IL-15 upregulated CD11b expression on RA PB neutrophils



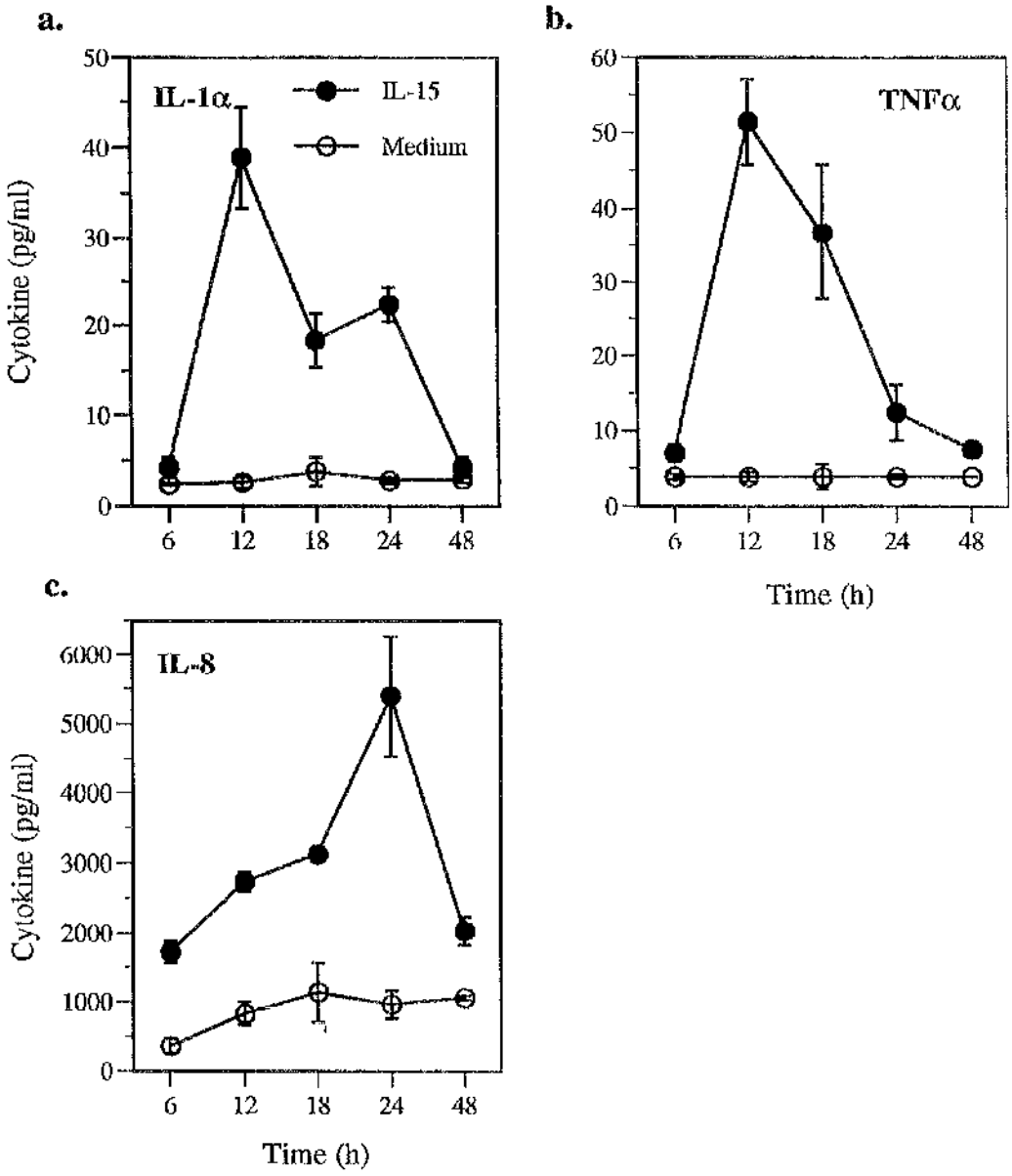
IL-15 upregulated expression of CD11b on PB neutrophils. CD11b expression on purified RA PB neutrophils was enhanced by stimulation with IL-15 (100 ng/ml) for 3 hours (n=12, *$p<0.01$). No significant difference in CD11c expression was detected. Data are mean ± s.e.m.

Figure 4.5 IL-15 induced cytokine production by synovial fluid neutrophils



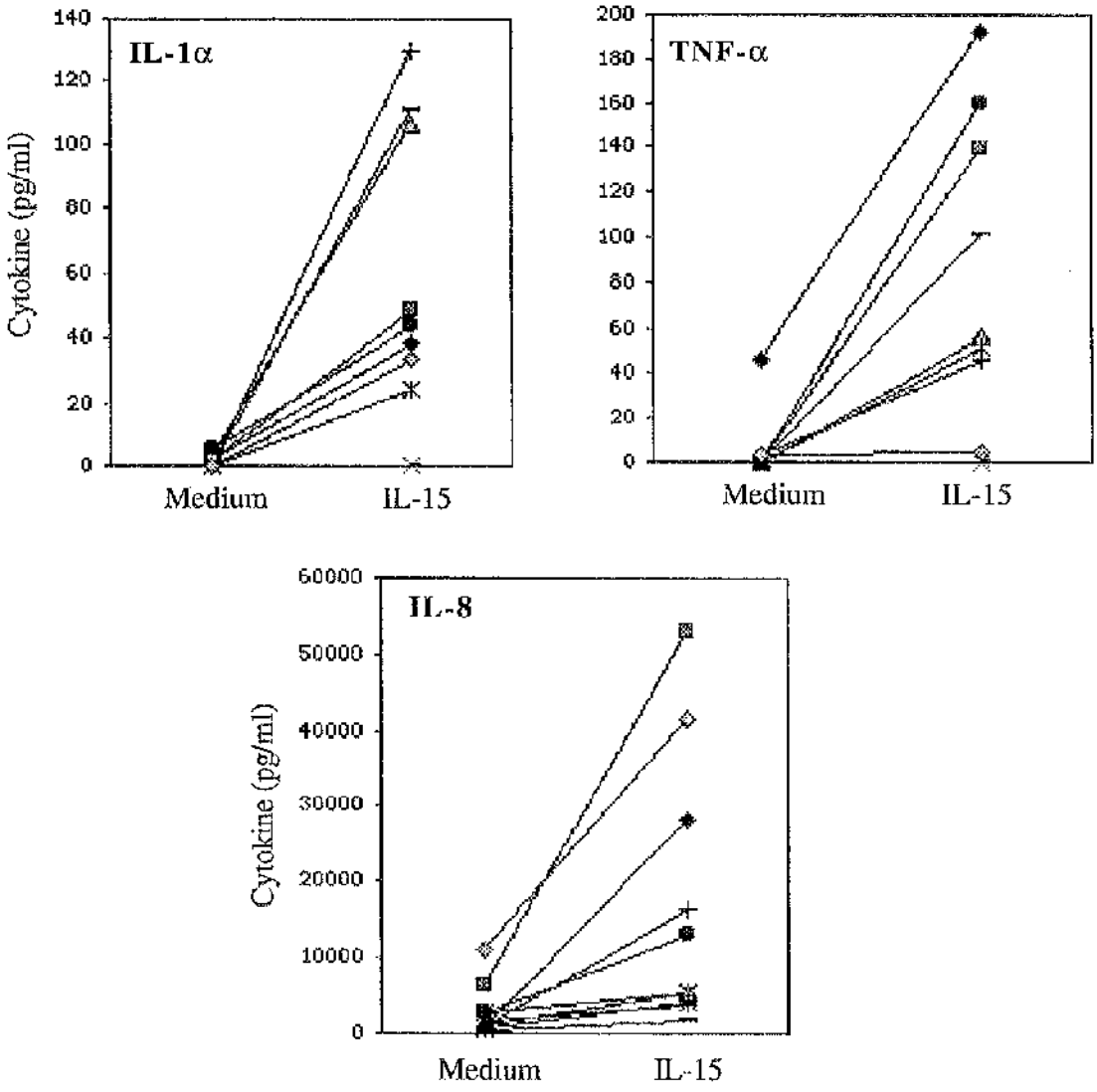
Dose response for IL-15 induced TNF-α, IL-1α and IL-8 production, by synovial fluid neutrophils from a RA patient (*$p<0.01$ compared with medium alone). Data are mean ± s.e.m., representative of 10 similar experiments.

Figure 4.6 Production of cytokines by IL-15 activated synovial fluid neutrophils



Time course of cytokine production by synovial neutrophils from a RA patient. Parallel cultures were established and harvested at the time points indicated, then stored at -20°C prior to ELISA. For TNF-α and IL-1α, production with IL-15 was significantly higher than basal levels ($p<0.001$, Mann-Whitney) at all time point between 12-48 hours. For IL-8, production was significantly enhanced at all time points ($p<0.001$) with maximal production observed at 24 hours. Data are mean ± s.e.m., representing 6 similar experiments.

Figure 4.7 Cytokine production by synovial fluid neutrophils stimulated *in vitro* with IL-15



IL-1α, IL-8 and TNF-α production by SF neutrophils was significantly enhanced by addition of IL-15 (100 ng/ml). Supernatants from individual cultures were collected at the optimal time point for cytokine production and frozen until triplicate assay for cytokine concentration by ELISA. Data are mean of individual patients (n=10).

Table 4.1 IL-15 stimulated the production of cytokines by purified SF neutrophils

IL-15 (ng/ml)	Cytokine levels (pg/ml) (n=10)		
	IL-1α	IL-8	TNFα
0	1.4 ± 0.6	2552.6 ± 1084.8	5.4 ± 4.5
1	2.5 ± 1.2	5717.5 ± 2741.2	5.5 ± 4.6
10	13.3± 3.4*	8471.5 ± 3447.1	20.3 ± 6.0*
50	36.2 ± 9.4**	12,782.5 ± 4811.7**	53.2 ± 16.1*
100	53.7 ± 14.6**	17,203.1 ± 5661.8**	75.0 ± 22.0*

Purified neutrophils were cultured in RPMI medium containing 10% FCS and increasing concentrations of recombinant IL-15. Cytokine levels in supernatants were determined by ELISA after 12hr (IL-1α and TNFα) or 24hr (IL-8). Data are expressed as mean ± s.e.m. of 10 RA patients. *$p<0.05$, **$p<0.01$ as compared with medium control.

4.5 Induction of *de novo* protein synthesis by IL-15

It was next important to determine the ability of IL-15 to induce *de novo* cytokine synthesis in neutrophils since it has been suggested that cytokine secretion in neutrophils requires active gene expression (Edwards & Hallett, 1997). Moreover, it was necessary to rule out the possibility of cytokine "leakage" as a result of apoptosis. Freshly isolated SF neutrophils from RA patients (n=4) were pre-treated with cycloheximide or actinomycin D (2 μg/ml) for 30 min before addition of an optimal dose of IL-15. As shown in Figure 4.8, cycloheximide significantly inhibited the induction of IL-1α, IL-8 and TNF-α production by IL-15 ($p<0.005$), suggesting that these cytokines were newly synthesized rather than derived from pre-formed intracellular pools. Similar results were obtained with actinomycin D, demonstrating that transcription of mRNA was required. Finally, basal IL-8 production was sensitive to both inhibitors; neutrophils pre-treated with actinomycin D exhibited marked reduction of IL-8 synthesis ($p<0.03$) and similar inhibition was observed with cycloheximide ($p<0.05$).

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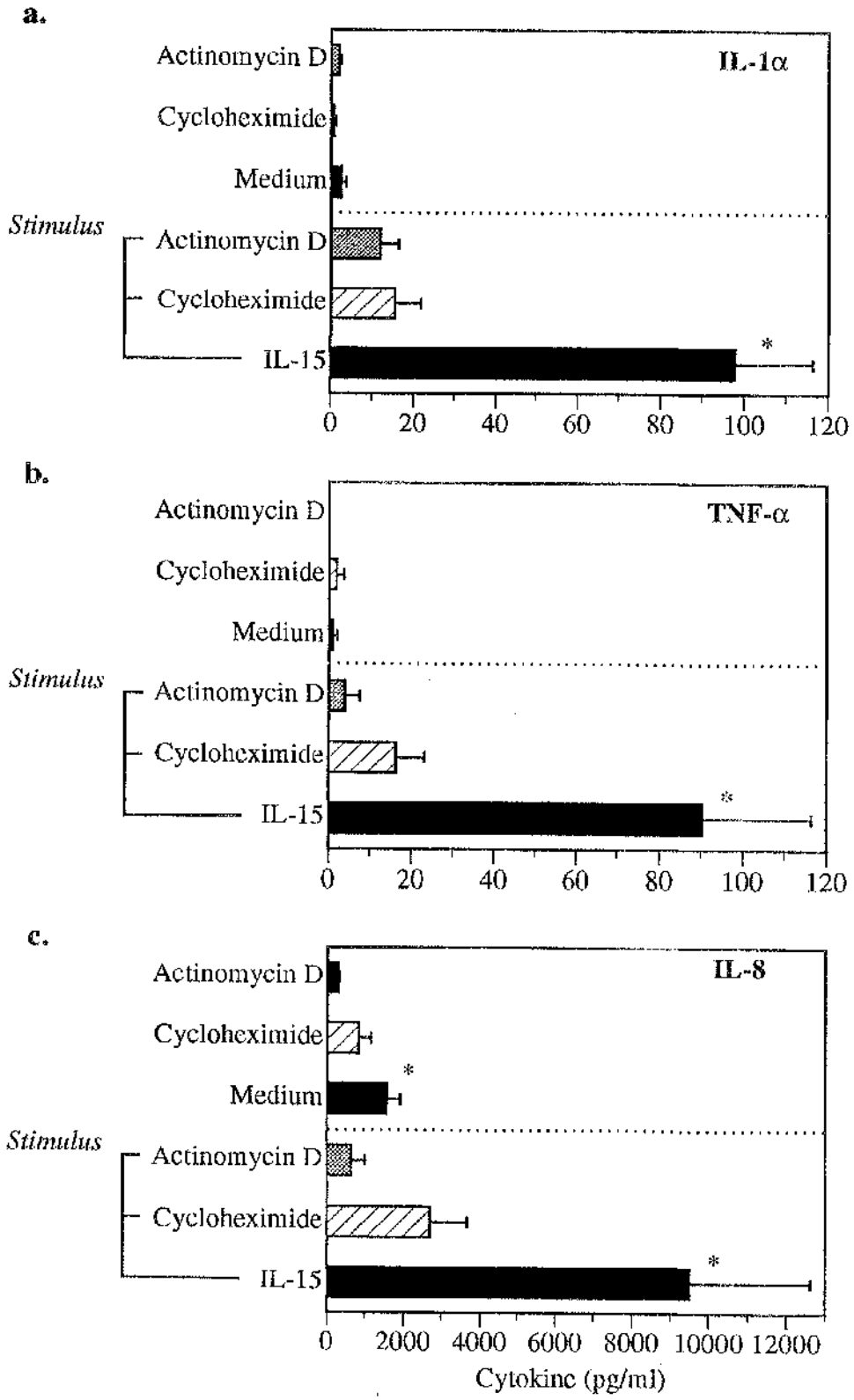
Transcription and translation was required for cytokine production by IL-15 stimulated RA SF neutrophils. Supernatants from individual cultures (n=4) were collected at optimal time points and cytokine concentrations were estimated by ELISA. The synthesis of IL-1α, TNF-α and IL-8 was increased by IL-15 (100 ng/ml, solid bar) and could be successfully inhibited by actinomycin D (dotted bar, $p<0.005$) or cycloheximide (hatched bar, $p<0.005$). Furthermore, basal IL-8 production was sensitive to both actinomycin D ($p<0.03$) and cycloheximide ($p<0.05$). Date are mean ± s.e.m.

Figure 4.8 De novo synthesis of cytokines by neutrophils in response to IL-15



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Discussion

Neutrophils play an important role in host defence which likely extends beyond that of phagocytosis and release of cytotoxic compounds. In particular, they are considered to be capable of synthesizing *de novo* proteins such as cytokines with the potential to function in the afferent and efferent limbs of the immune response (Lloyd & Oppenheim, 1992; Cassatella, 1995). It is also clear the cytokines and other pro-inflammatory stimuli are capable of modulating a number of neutrophil responses.

IL-15 mediates its activity through a heterotrimeric receptor consisting of a unique IL-15Rα chain, in combination with the β and γ chains of the IL-2 receptor (Tagaya, et al, 1996b). Recently, both of these components were shown to be present on human neutrophils (Liu, et al, 1994; Nakarai, et al, 1994). However, the IL-2α chain is absent on neutrophils and seems to be non-inducible, either at the mRNA or protein level (Giri, et al, 1994, Djeu, et al, 1993). However, by using RT-PCR IL-15Rα mRNA was detected in freshly isolated RA PB and SF neutrophils. That this was associated with the presence of a functional receptor complex for IL-15 binding was suggested by the ability of IL-15 to induce adhesion molecule (CD11b) expression and *de novo* cytokine production. Formal proof of protein expression awaits the availability of appropriate reagents capable of specificially binding human IL-15Rα chain, since it is possible that IL-15 can bind to and mediate activity through βγ dimers. Also, the IL-15RX is described on mast cells and may so present on neutrophils (Tagaya, et al, 1996a).

In RA, the full spectrum of cellular and humoral elements of the immune system are activated and likely contribute to disease pathology. RA has usually been considered a T-cell, monocyte / macrophage, or fibroblast driven disease (Fox, 1997; Feldmann, et al, 1996) despite considerable evidence for neutrophil involvement in synovial inflammation (Edwards & Hallett, 1997). SF from RA patients is heavily infiltrated

with neutrophils, in addition to high concentrations of immune complexes and cytokines. Neutrophils isolated from the RA SF exhibit biochemical properties characteristic of primed and activated cells (Nurcombe, et al, 1991; Robinson, et al, 1993a). Furthermore, many of the cytokines found within SF including IL-1, IL-8, TNF-α and TGF-β may be secreted by neutrophils (Beaulieu & McColl, 1994; Cassatella, 1995).

Data presented here suggest that once recruited into the synovium, IL-15 may modulate cytokine production by activated neutrophils. Dose-dependent enhancement of IL-1α, IL-8 and TNF-α production was observed. Inhibition studies with actinomycin D and cycloheximide confirmed that this was not secondary to preformed cytokine release. However, although neutrophils have the ability to produce cytokines that participate in the inflammatory reaction, their quantity is generally less than that produced by monocyte / macrophages, leading to the suggestion that the contribution of neutrophils to the total pool of cytokines may be limited (Lloyd & Oppenheim, 1992). Activated neutrophils can produce large amounts of IL-8, reaching at least 10% of that produced by mononuclear phagocytes (Bazzoni, et al, 1991). Since the majority of cells present in the SF are neutrophils, it is likely that IL-8 derived from neutrophils represents an important intra-articular cytokine source. IL-8 in turn has various biologic effects on neutrophils *in vitro*, including induction of directional migration, release of storage enzymes, oxygen metabolite synthesis, expression of adhesion molecules, and transendothelial migration (Baggiolini, et al, 1994). Moreover, it is a potent T cell chemokine (Wilkinson & Newman, 1992; Al-Mughales, et al, 1996). In rats, subcutaneous injection of IL-8 has been shown to induce neutrophil accumulation *in vivo* (Kudo, et al, 1991). *In vitro* studies have demonstrated that IL-8 produced by cytokine-activated endothelial cells (EC) has chemotactic activity for neutrophils. This is associated with decreased expression of L-selectin and concomitant increased expression of CD11b/CD18, thereby strengthening neutrophil interactions with EC and subsequent transmigration (Huber,

et al, 1991). IL-1α and TNF-α mediate numerous proinflammatory effects in the synovium (Arend & Dayer, 1995). Of particular relevance in this context may be their ability to increase the expression of endothelial adhesion molecules involved in leukocyte recruitment, such as ICAM-1 (Mojcik & Shevach, 1997). Thus, it is clear that neutrophils can generate mediators capable of inducing an inflammatory response within the joint and that IL-15 produced within the synovium, may potentiate this process. It is uncertain whether IL-15 can modulate adhesion molecule and cytokine expression on EC. EC were recently shown to express the IL-15Rα chain and the IL-2/15R β and γ chains. Furthermore, IL-15 may induce rapid tyrosine phosphorylation of proteins in EC and functions as a stimulator of angiogenesis *in vivo* (Angiolillo, et al, 1997). Thus, effects on both blood leukocytes and the EC path of entry may be hypothesised for IL-15.

Neutrophil transmigration from the circulation into synovium requires expression of the adhesion molecule CD11b/CD18 (Mac-1) which interacts with ICAM-1 on endothelial cells (Mojcik & Shevach, 1997). Consistent with previous results, SF neutrophils showed enhanced expression of CD11b when compared to PB neutrophils (Watson, et al, 1993). A short incubation of "unprimed" PB neutrophils with cell-free SF or IL-15 could induce similar patterns of receptor expression. Although IL-15 has no direct effect on neutrophil chemotaxis (Wilkinson & Liew, 1995), the above observations may provide an alternative whereby it can contribute to the large influx of neutrophils present within SF. It is interesting that the levels of IL-15 detectable in SF were significantly higher than those found in paired serum samples (Figure 3.3a).

Activated neutrophils also possess a range of potent serine proteinases, hydrolases and matrix metalloproteinases which have direct effects on cartilage degradation (Kitsis & Weissmann, 1991; Edwards & Hallett, 1997). IgG aggregates in SF may activate neutrophils to release superoxide radicals (Robinson, et al, 1992a). Furthermore, they can secrete IL-1 receptor antagonist (IL-1ra), which blocks IL-1 receptor function and

can promote an anti-inflammatory response (McColl, et al, 1992; Beaulieu & McColl, 1994). It remains to be determined whether IL-15 can also modulate these properties of neutrophils. Thus, these data extend the functional role of IL-15 in RA synovitis beyond that of T cell and macrophage activation, to include polymorphonuclear cell activation. They also provide further evidence for a crucial role for IL-15 during early stages of innate immune responses.

Chapter 5

sIL-15Rα administration prevents murine collagen-induced arthritis

Introduction

The foregoing data clearly indicate a potential role for IL-15 in inflammatory arthritis. This was next investigated *in vivo*. Numerous experimental models which closely imitate human RA, have been characterized as a means to understanding disease pathophysiology and identifying new strategies for treatment. The two animal models of RA that have been studied in greatest detail are the collagen-induced arthritis (CIA) model in mice and adjuvant arthritis (AA) model in rats. CIA is induced by the intradermal injection of native type II collagen in complete Freund's adjuvant (CFA) into susceptible strains of mice (Trentham, et al, 1977; Staines & Wooley, 1994). This elicits an inflammatory polyarthritis, characterized by hyperplasia of the synovium with infiltration of polymorphonuclear and mononuclear cells into the synovial tissue, which is followed by destruction of joint cartilage and osteolysis (Stuart, et al, 1988). The genetics of CIA have been extensively studied, with susceptibility linked to the H-2^q haplotype (Wooley, et al, 1985). Both B and T lymphocytes are required for the full development of pathology. The generation of antibodies to type II collagen represents one similarity with clinical RA. The involvement of T cells, particularly of the $CD4^+$ subset, is evidenced by the protection obtained following T cell depletion *in vivo* (Osman, et al, 1993). Thus an advantage of CIA over AA is the presence of an autoimmune response to a major component of articular cartilage rather than to a bacterial antigen in the former. The CIA model has been widely used for the evaluation of potential anti-rheumatic agents. For instance, CIA in DBA/1 mice can successfully be treated with antibodies to TNF-α (reviewed by Feldmann, et al, 1996), IL-1α/β (Joosten, et al, 1996) and CD4 (Williams, et al, 1994) and have provided the rationale for initiating clinical trials with anti-TNF-α antibody therapy in RA patients (Elliot, et al, 1994; Rankin, et al, 1995; Moreland, et al, 1997).

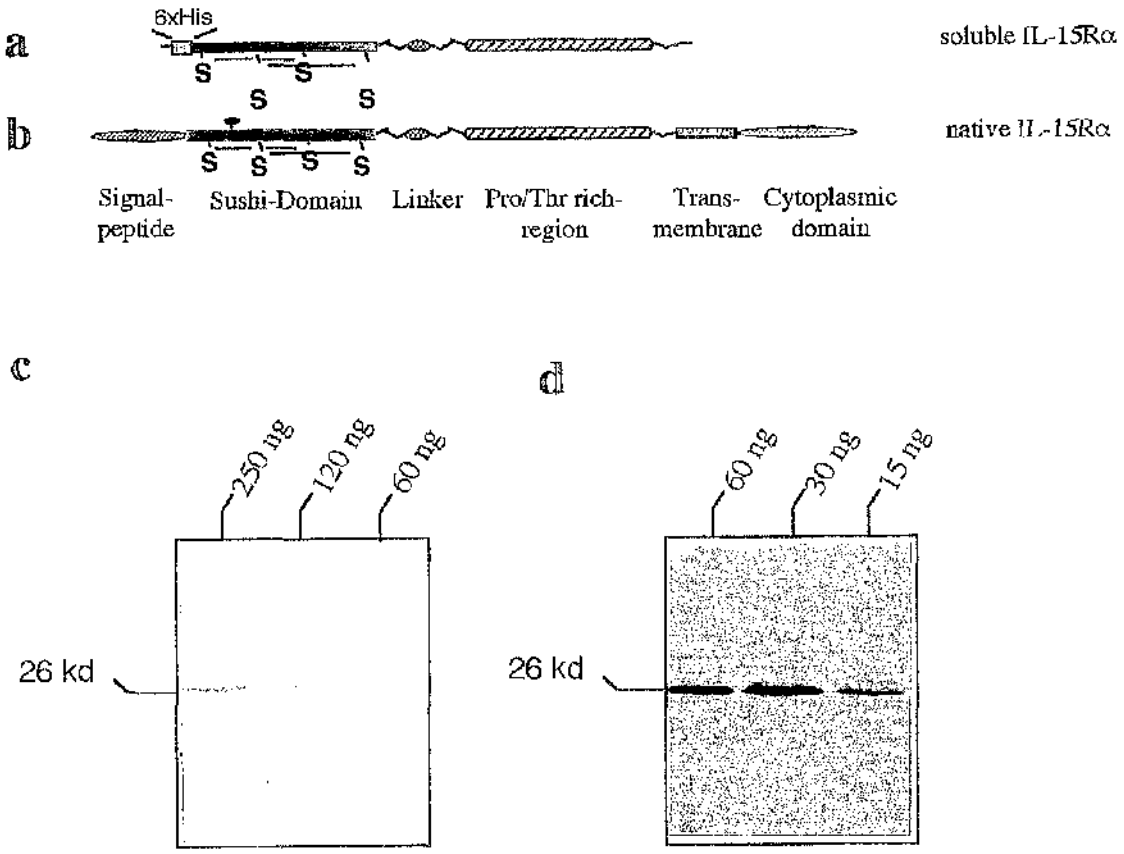
In order to investigate the role of IL-15 in the evolution of immune responses in CIA, a soluble fragment of the murine IL-15 receptor α chain (sIL-15Rα) was generated. The sIL-15Rα offers the following advantages: (i) specificity, (no antibody to murine IL-15 was available during this study), (ii) high binding affinity approximating to $1x10^{11}M^{-1}$, (1,000-fold higher affinity than that of IL-2Rα for IL-2 (Giri, et al, 1995)), thus allowing the receptor to work at extremely low concentration, and (iii), potential avoidance of neutralizing anti-globulin response as previously observed in the anti-TNF-α antibody study (William, et al, 1994).

5.1 Bioactivity of soluble IL-15Rα chain *in vitro*

A soluble IL-15Rα fragment was generated by Mr. Holger Ruchatz (Dept. of Immunology, University of Glasgow) using a sequence corresponding to the extracellular domain of murine IL-15Rα (Figure 5.1 a & b). mRNA was prepared from an IFN-γ activated murine macrophage cell line (J774) by nested PCR and reversed transcribed to cDNA which was inserted into an expression vector and expressed in *E. coli*. The soluble protein was then extracted and purified to homogeneity as a 6x Histidine tagged fusion protein using a nickel tagged agarose purification system. The purified sIL-15Rα was analysed on SDS-PAGE stained for total protein, or by Western blot analysis against anti-IL-15 antibody following binding with recombinant simian IL-15. A single band at 26 kDa molecular weight was observed in both blots (Figure 5.1 c & d).

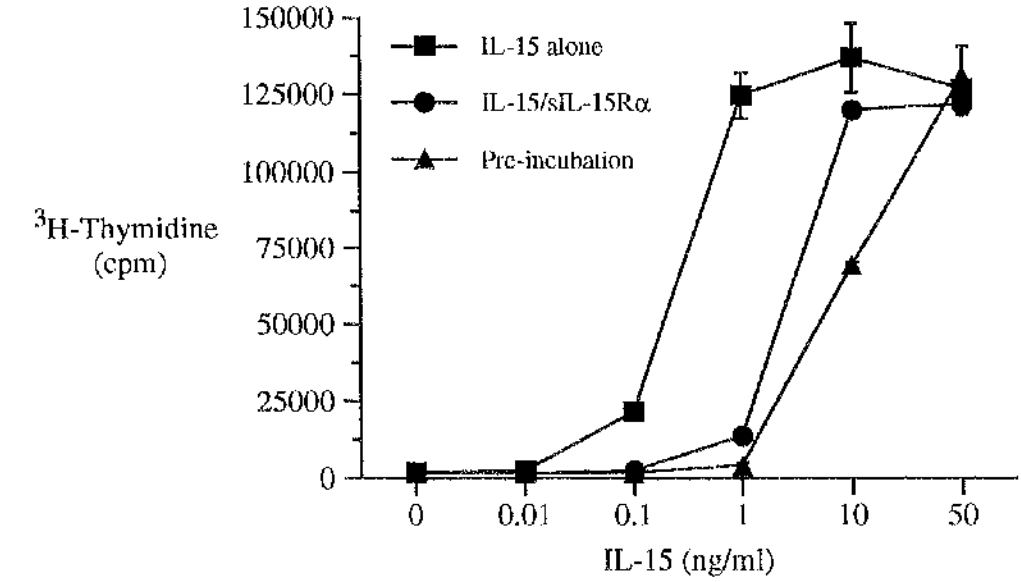
To determine the neutralising activity of sIL-15Rα. Murine $CD4^+$ T cell line (D10) was exposed to sIL-15Rα (200 ng/ml) and increasing concentrations of recombinant IL-15 (0.01 - 50 ng/ml). As shown in Figure 5.2, T cells proliferated vigorously to rIL-15 in a dose-dependent manner. Addition of sIL-15Rα resulted in inhibition of proliferation, which was most marked when sIL-15Rα was pre-incubated with IL-15 prior to addition to cells. Similar results were obtained using the murine $CD8^+$ CTLL-2 T cell line. This inhibition was unlikely to be due to cytotoxicity as cell viability remained unaffected throughout culture as assessed by trypan blue. Furthermore, it could be successfully overcome by addition of excess IL-15 (Figure 5.2). To verify the specificity of neutralization, another member of the short chain four α helix bundle cytokine family was used, namely IL-2. In the presence of sIL-15Rα, IL-2 induced T cell clone proliferation remained unaffected. These data demonstrated the specificity and bio-activity of sIL-15Rα, and indicated that it represented an ideal reagent with which to explore the role of IL-15 synthesis in CIA.

Figure 5.1 Cloning and expression of sIL-15Rα



Schematic representation of sIL-15Rα (a) and native full length of IL-15Rα (b). The purified sIL-15Rα was analysed on SDS-PAGE stained for total protein with coomassie blue (c), or by Western blot and developed with ECL (Amersham International, UK) (d). Western blot was probed with recombinant simian IL-15 and was detected with anti-IL-15 monoclonal antibody. Figures kindly provided by Mr. Holger Ruchatz, Dept. of Immunology, University of Glasgow.

Figure 5.2 Neutralising activity of sIL-15Rα



Murine CD4$^+$ T cell line (D10) was exposed to sIL-15Rα (200 ng/ml) and increasing concentrations of recombinant human IL-15 (0.01 - 50 ng/ml). rIL-15 induced T cell proliferation in a dose-dependent manner which could be inhibited by the addition of sIL-15Rα. This was most marked when sIL-15Rα was pre-incubated with rIL-15 prior to addition to cells. The inhibition was reversible by addition of excess rIL-15. Data are mean ± s.e.m. of triplicate cultures.

5.2 sIL-15Rα administration prevents development of CIA

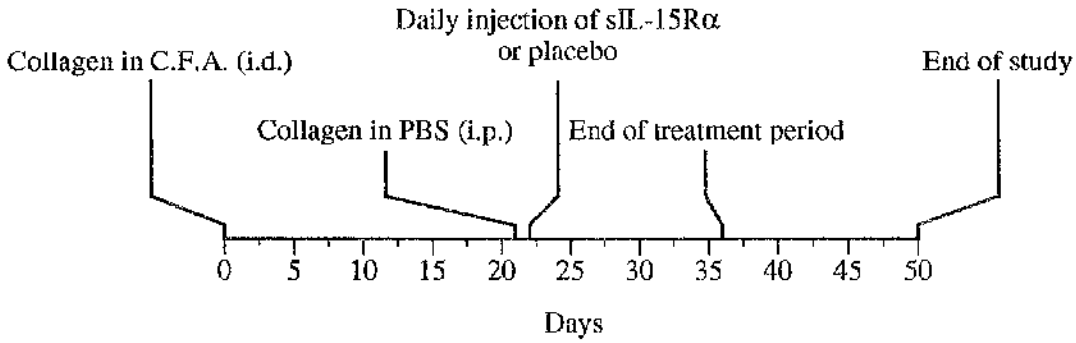
Susceptible DBA/1 mice injected intradermally with type II collagen in CFA developed severe arthritis when challenged intraperitoneally 21 days later with collagen. Animals were divided randomly into those receiving daily sIL-15Rα (40 μg/animal) beginning the day after collagen challenge (day 22) for a duration of 14 days or controls which received human serum albumin (HSA, Figure 5.3). Mice were monitored daily for signs of arthritis as described in section 2.7.3 and results are presented in Figure 5.4 & 5.5.

Collagen primed mice which received sIL-15Rα proved less susceptible to induction of arthritis, since the incidence rate was significantly less than in HSA control mice (Figure 5.4a, $p<0.005$). The mean clinical score in sIL-15Rα treated mice was significantly reduced ($p<0.001$, Figure 5.4b). In addition, paw-swelling was markedly reduced in the sIL-15Rα treated group relative to HSA controls ($p<0.002$, Figure 5.5a). In CIA, new arthritic paw involvement is an important indicator of the progression of disease. In control mice there was a progressive increase in paw involvement, which reached approximately 75% by the end of the treatment period (d36). In contrast, less than 10% of paws were involved in mice which received daily sIL-15Rα ($p<0.002$, Figure 5.5b).

5.3 Histological analysis of joint destruction

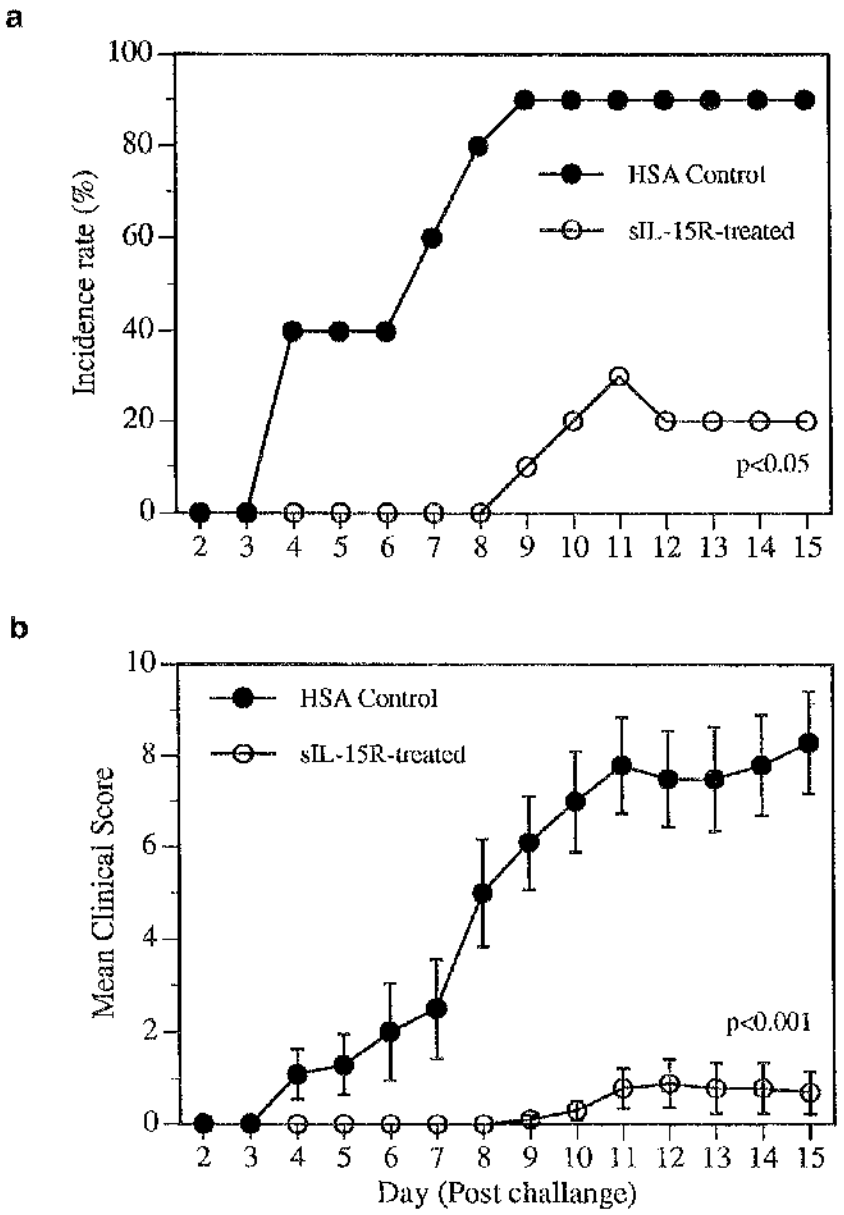
After establishing that sIL-15Rα was effective in inhibiting the onset of detectable inflammatory synovitis, histological examination was carried out to determine whether erosion of cartilage and bone was also prevented. At day 36, hind limbs from both groups (n=5) were evaluated and histological scores were derived (Figure 5.6).

Figure 5.3 Induction and sIL-15Rα treatment of collagen induced arthritis in mice



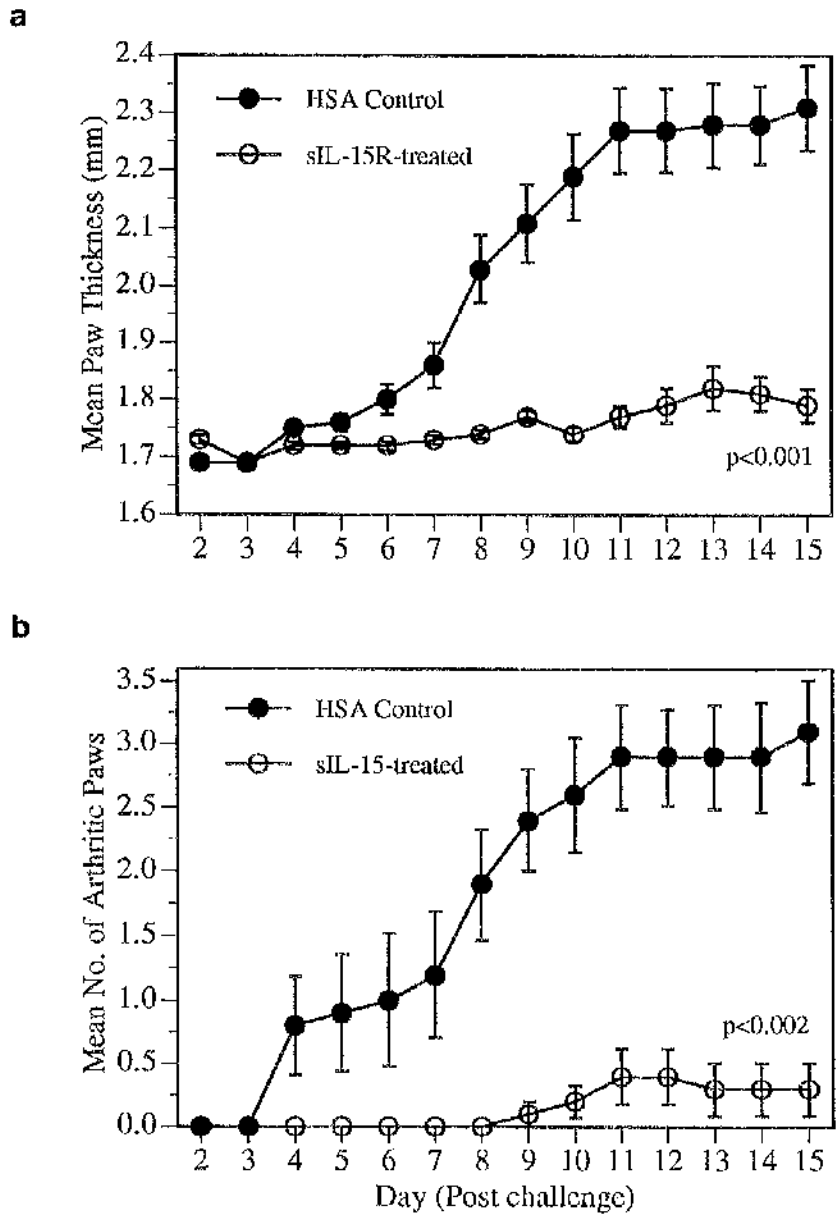
At day 0, male DBA/1 mice (6-8 weeks old) received 200 μg of bovine type II collagen in Freund's complete adjuvant (CFA) by intradermal injection (i.d.). Collagen (200 μg in PBS) was given again on d21 by intraperitoneal (i.p.) injection. Daily injections of sIL-15Rα (40 μg/animal) or HSA (40 μg/animal) were administered i.p. from d22 for two weeks. In order to determine the duration of biological effects of sIL-15Rα, animals were observed for up to 14 days post-treatment (d50).

Figure 5.4 Recombinant sIL-15Rα inhibited development of CIA



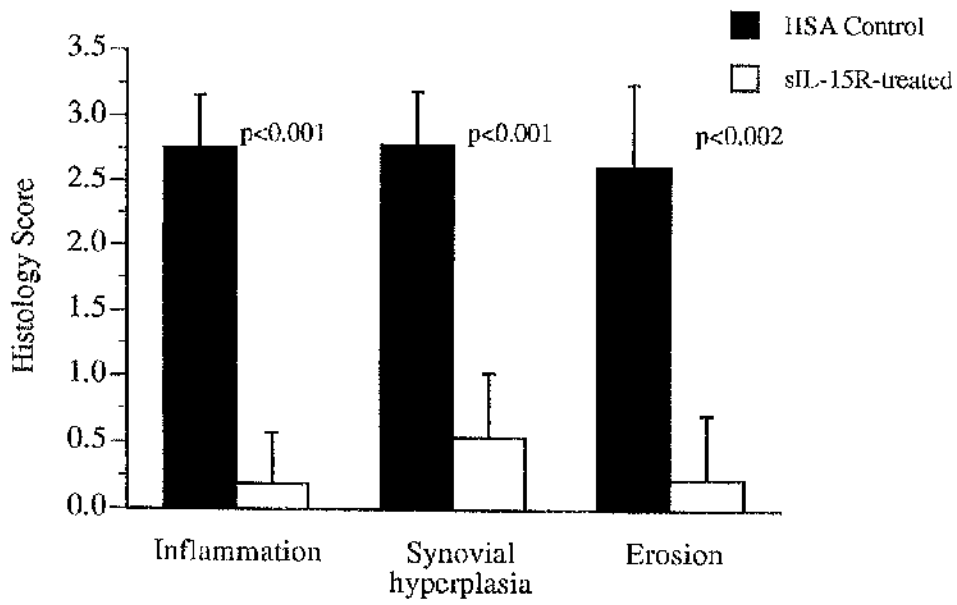
Collagen-primed DBA/1 mice were divided into groups of 10, challenged on day 21, and received 14 daily i.p. injections of 40 μg of sIL-15Rα (O) or 40 μg of HSA (●), starting from d22. Mice were monitored daily for disease progression quantified as incidence rate (a) or mean clinical score (b). At the end of treatment (d36), 20% of mice developed mild CIA in the sIL-15Rα-treated group compared with 90% which developed severe CIA in the HSA control group ($p<0.05$, Mann-Whitney). Data are mean ± s.e.m.

Figure 5.5 sIL-15Rα administration prevented development of CIA



Comparison of sIL-15Rα (40 µg/animal) vs. the same dose of irrelevant protein control (HSA, 40 µg/animal). Mice were treated as in Figure 5.4. (a) Paw-swelling was markedly reduced in the sIL-15Rα treated group compared to the HSA control ($p<0.002$, Mann-Whitney). (b) Mean number of affected joints for each treatment group; significant reduction was observed in mice which received sIL-15Rα ($p<0.002$, Mann-Whitney). There were 10 mice per group, data are mean ± s.e.m.

Figure 5.6 Histological assessment of the effect of sIL-15Rα on CIA



sIL-15Rα significantly reduced histological parameters of articular inflammation and destruction. Immediately following 14 days of sIL-15Rα administration, hindlimbs (5 mice / group) were removed, formalin-fixed and decalcified and H&E sections were prepared (d36). Histologic appearances in knee, carpus and interphalangeal joints in parallel sections (HSA n=17, sIL-15Rα n=13) were scored independently (0-3) by two treatment-blinded histologists. Extensive erosion ($p<0.002$), inflammatory infiltration ($p<0.001$) and synovial hyperplasia ($p<0.001$) were observed in HSA treated animals. Data are mean ± s.e.m., the Wilcoxon rank test was used for statistical comparisons.

Representative images of the ankle joints of a sIL-15Rα treated and control mice are shown in Figure 5.7. Extensive inflammatory changes were observed in the joints of control mice, with hyperplasia of the synovial membrane, and heavy infiltration of mononuclear and polymorphonuclear cells, together with the presence of pannus formation. These features were markedly reduced in the sIL-15Rα treated mice.

5.4 Decreased humoral response to collagen in sIL-15Rα treated mice

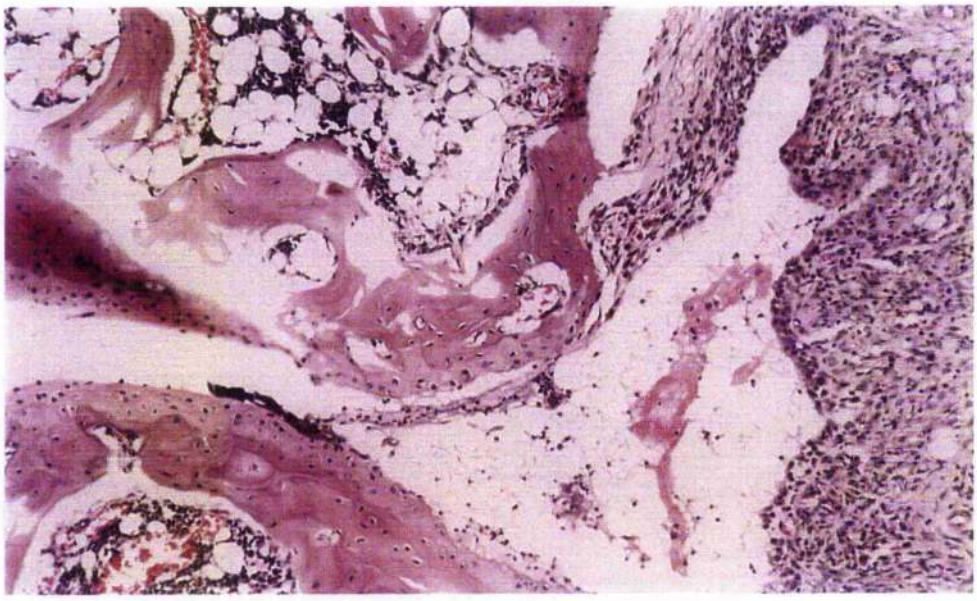
Murine susceptibility to CIA is associated with high anti-collagen antibody responses (Seki, et al, 1988). To determine whether the neutralization of IL-15 could modify the humoral response to collagen, pooled serum samples were collected at the end of treatment (d36). Figure 5.8 shows the total IgG and isotype profile of anti-collagen Abs in the two groups of mice. Humoral responses, including total IgG, as well as the IgG2a and IgG1, were significantly reduced in recipients ($p<0.01$).

5.5 Reduction of serum cytokine levels in sIL-15Rα treated mice

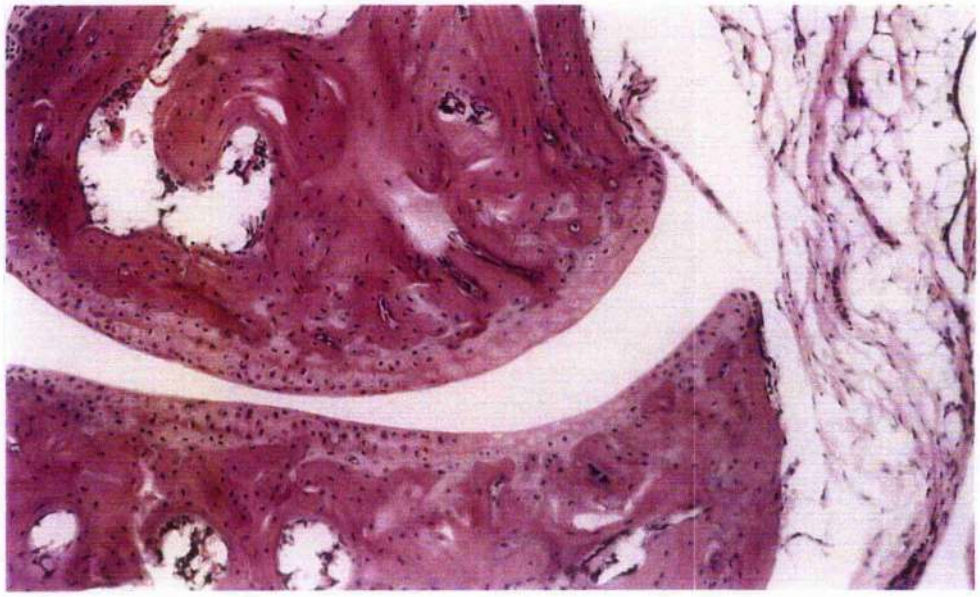
Previous studies have established a role for Th1 cells and several pro-inflammatory cytokines, including TNF-α and IL-1β (Mauri, et al, 1996; Joosten, et al, 1996) in CIA. To study the influence of IL-15 on cytokine modulation, serum samples were analyzed for the presence of IL-4, IL-5, IL-6, IL-10, TNF-α and IFN-γ at day 36. TNF-α levels were low and comparable in each group, compatible with previous reports of only transient early up-regulation of serum TNF-α in CIA. In contrast, IL-6 was easily detected and was significantly reduced in IL-15Rα treated animals (267 ± 5 vs 193 ± 15, $p<0.05$). Similar significant reduction of IFN-γ and IL-10 levels were observed in actively treated mice (Figure 5.9). No detectable of IL-4 and IL-5 could be measured in either group of mice.

Figure 5.7 Reduction in knee joint destruction in sIL-15Rα treated mice

a

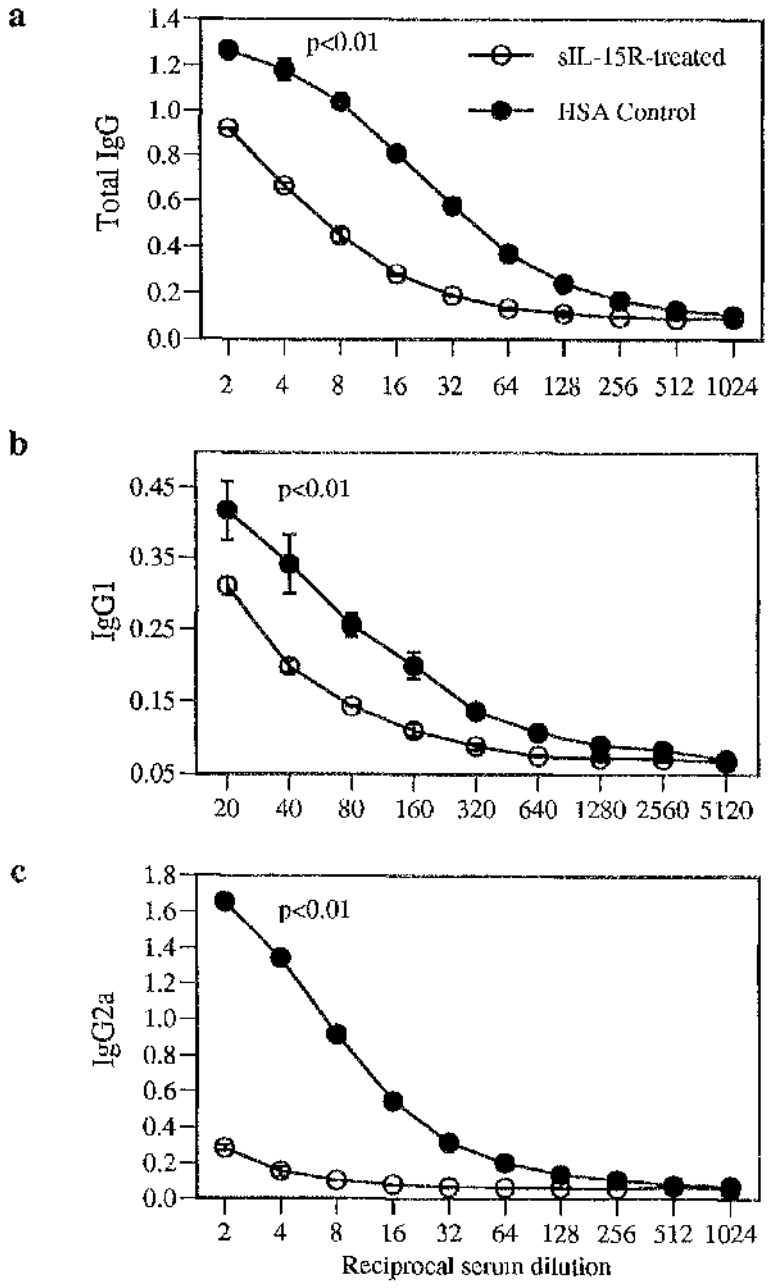


b



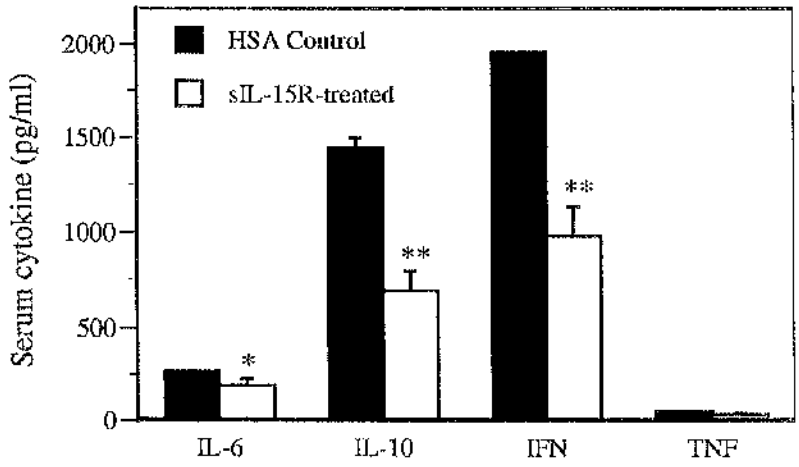
H&E sections of knee joints on day 36 of CIA. (a) Arthritic knee joint of a mouse treated with HSA control and (b) knee joint of a mouse treated with sIL-15Rα. Reduction of both mono- and polymorphonuclear cells infiltration in the synovial membrane, together with reduction in pannus formation and joint erosion can be seen in the sIL-15Rα treated animal.

Figure 5.8 Attenuation of serum anti-collagen antibody response in sIL-15Rα-treated mice



Anti-collagen antibody isotype distribution in sIL-15Rα (O) and HSA (●) treated mice. Anti-collagen antibody titres, total IgG (a), IgG1 (b) and IgG2a (c), were measured at the end of treatment (day 36) by ELISA. Data are from pooled serum (5 mice per group), expressed as mean absorbance (O.D. 630 nm) from doubling dilutions ± s.e.m. The Wilcoxon rank test was used for statistical comparisons.

Figure 5.9 Effect of sIL-15Rα treatment on serum cytokine levels

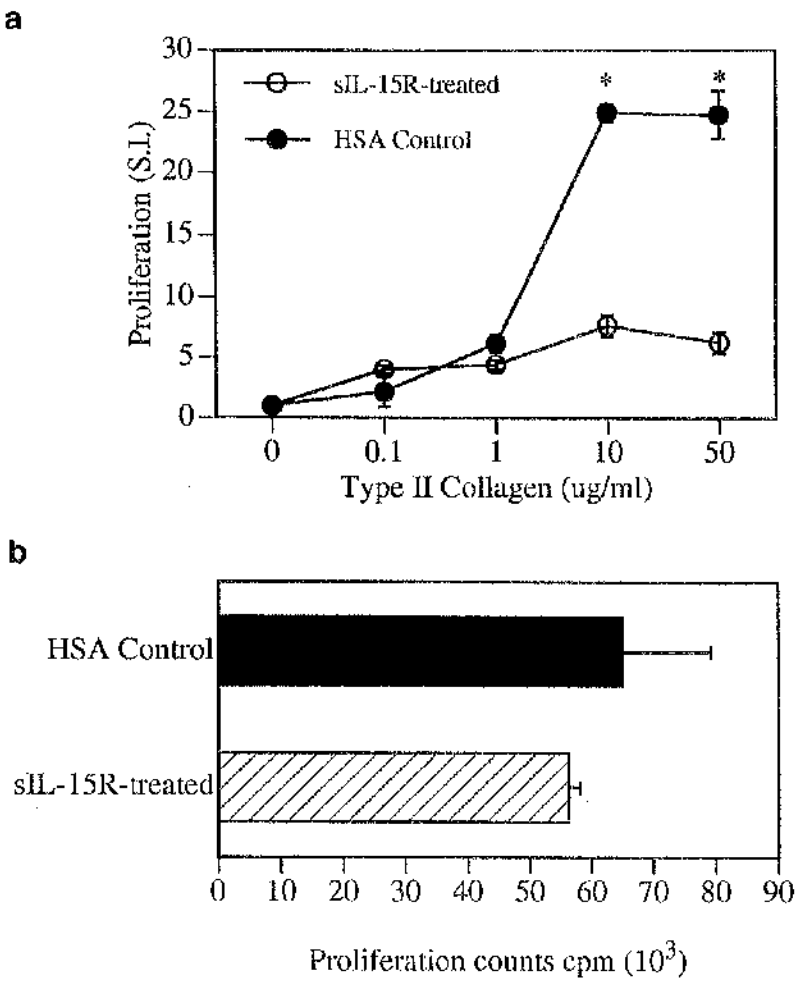


Serum cytokine levels in sIL-15Rα and HSA treated mice immunised with collagen. Pooled sera (n=5) were collected at the end of treatment (d35) and serum cytokine concentrations were analysed by ELISA. Data are mean ± s.e.m., *$p<0.05$, **$p<0.005$, Mann-Whitney test.

5.6 *In vitro* immunologic consequences of *in vivo* sIL-15Rα administration

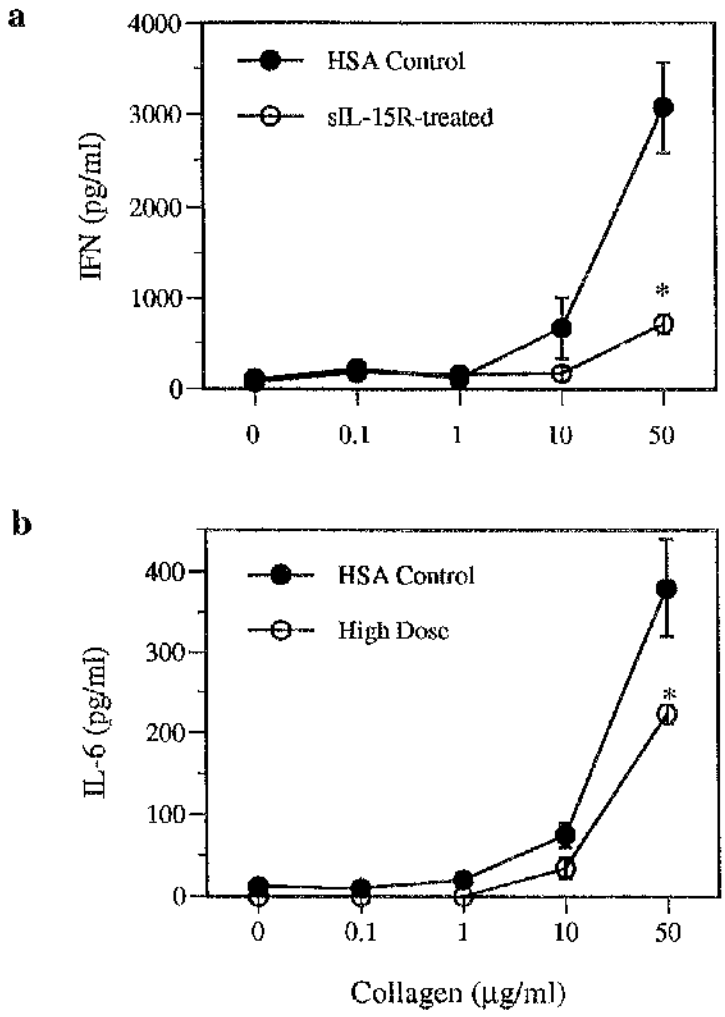
To further explore the effect of sIL-15Rα on immunological responses, spleen cells were harvested from mice (n=5) after two weeks of treatment with sIL-15Rα, or control HSA and cultured with type II collagen *in vitro*. Cells from arthritic mice which received control protein, proliferated vigorously in response to collagen in a dose-dependent manner. In contrast proliferation to collagen was significantly reduced in sIL-15Rα-treated mice-derived cultures (Figure 5.10a, $p<0.001$). The suppression of the immune response was not universal, as the T cell mitogen, concanavalin A (Con A) stimulated equally high levels of proliferation by spleen cells from both group of mice (Figure 5.10b). To determine whether the altered disease phenotype in sIL-15Rα treated mice *in vivo* was due to the modification of Th1 or Th2 responses, or of both, parallel cultures were established and antigen specific cytokine production was measured by ELISA. Spleen cells from the sIL-15Rα-treated mice produced less IFN-γ (Figure 5.11a, $p<0.05$) than control mice in response to collagen, indicating suppression of Th1 responses. Moreover, IL-4 was undetectable and IL-10 was found only at low levels (100-120 pg/ml) which were indistinguishable between both groups, suggesting that the mechanism of disease suppression by sIL-15Rα was not preferential enhancement of Th2 cells. IL-6 production was significantly reduced in sIL-15Rα-treated animals (Figure 5.11b, $p<0.05$). TNF-α synthesis however was not detected, consistent with previous observations (Mauri, et al, 1996).

Figure 5.10 Effect of sIL-15Rα treatment on antigen specific T cell proliferation



Suppression of proliferative responses *in vitro* to collagen by sIL-15Rα-treated mice. Spleen cells (pooled from 5 mice per group) were collected from sIL-15Rα-treated or HSA-treated mice at the end of the two week treatment phase (d36) and (a) cultured with graded concentrations of collagen or (b) with Con A for 96 hours. T cell proliferation, determined by [^{3}H] thymidine uptake, is expressed as mean stimulation index (S.I.) ± s.e.m. of triplicate cultures (medium control ranged from 3500-5000 cpm) or for Con A, cpm ± s.e.m. *p<0.001, Mann-Whitney test.

Figure 5.11 Effect of sIL-15Rα treatment on antigen specific cytokine production

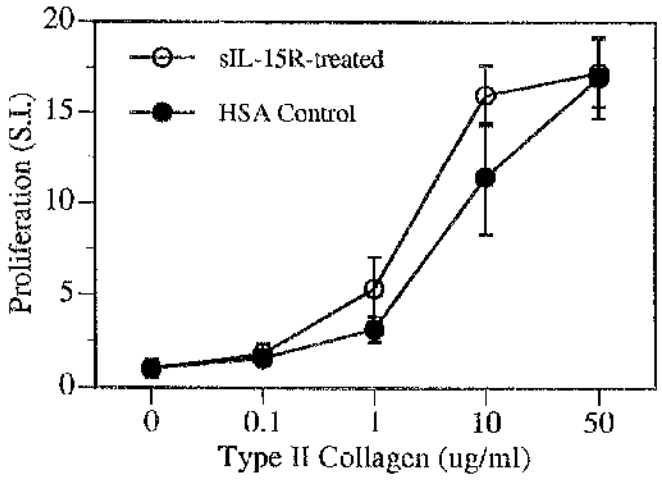


Assessment of *in vitro* cytokine production to collagen by spleen cells from mice treated with sIL-15Rα or HSA. Supernatants from parallel cultures prepared as described in figure 5.10 were collected after 72 h and IFN-γ (a) and IL-6 (b) concentrations determined by ELISA and expressed as mean ± s.e.m. *$p<0.05$, Mann-Whitney test.

5.7 Discontinuation of sIL-15Rα administration facilitates disease expression

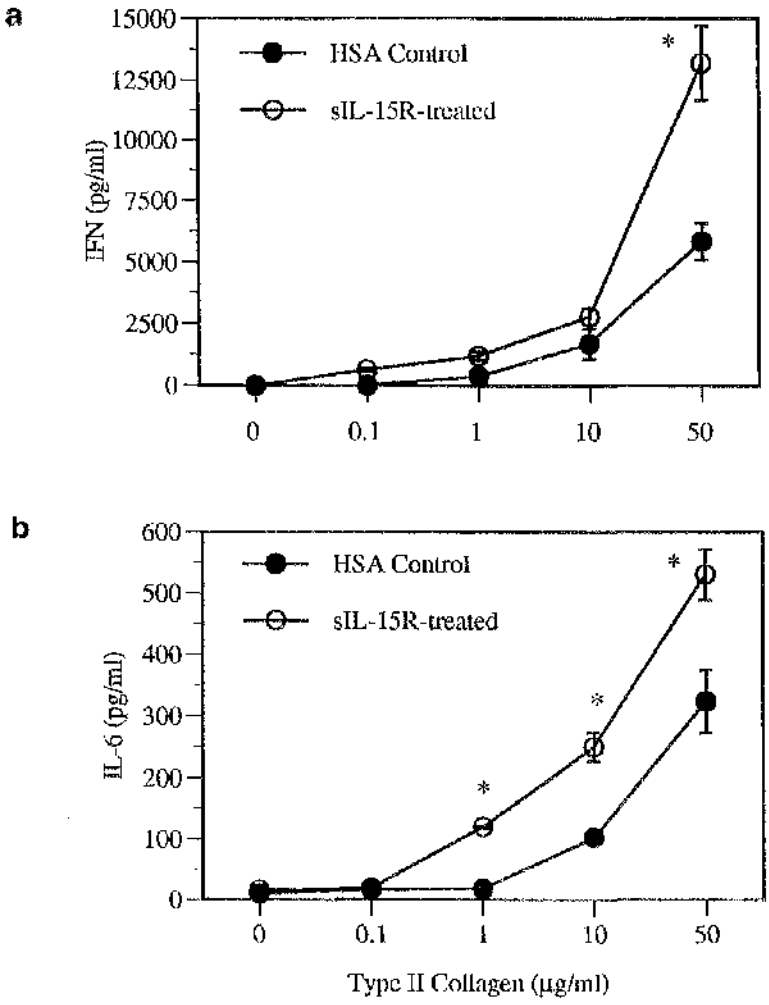
To determine the duration of disease suppression, sIL-15Rα administration was withdrawn after 14 days. Onset of CIA was observed clinically 5-7 days after cessation of treatment and after 10 days, 90% of previous sIL-15Rα-recipients developed arthritis indistinguishable from the control animals. Thus, treated mice developed acute CIA soon after discontinuation of sIL-15Rα which was reflected in immune responsiveness *in vitro*. Two weeks after cessation of treatment, spleen cells from sIL-15Rα-treated mice proliferated with the same magnitude in response to collagen as control spleen cells (Figure 5.12). Furthermore, higher concentrations of IL-6 and IFN-γ were found in these cultures when compared either with parallel cultures from HSA controls (Figure 5.13, $p<0.05$), which were by then in the chronic phase of CIA, or with spleen cell responses obtained earlier from littermates at the end of sIL-15Rα treatment. IL-4 remained undetected and levels of IL-10 were similar in both groups.

Figure 5.12 Discontinuation of sIL-15Rα administration facilitated antigen specific T cell responses



Loss of suppression of collagen-specific T cell responsiveness following sIL-15Rα withdrawal. Two weeks after cessation of treatment (d50), spleen cells (pooled from 3 mice per group) from previously sIL-15Rα- or HSA-treated mice were cultured with collagen as before. T cell proliferation was measured as previously described and found to be similar in both groups. Data are expressed as mean stimulation index (S.I.) ± s.e.m. of triplicate cultures (medium control ranged from 3000-4500 cpm)

Figure 5.13 Cytokine production by spleen cells following sIL-15Rα withdrawal



Parallel culture supernatants prepared as described previously in figure 5.12 were collected at 72 hours and IFN-γ (a) and IL-6 (b) synthesis was determined by ELISA. Data are means of triplicate cultures, *$p<0.05$, Mann-Whitney test.

Discussion

Interleukin-15 mediates pleiotropic effects on a variety of immune cells (Tagaya, et al, 1996). However, unlike IL-2, with which it shares partial functional homology, IL-15 may be generated not only by cells within the immune system, but also by cells belonging to other tissues, including keratinocytes and synoviocytes (Mohamadzadeh, et al, 1995; McInnes, et al, 1996b; Thurkow, et al, 1997). By this means, host tissues may contribute significantly to the regulation of protective, or autoimmune responses. Functional effects of IL-15 demonstrated thus far include activation of NK cells and neutrophils, and autocrine regulation of macrophages, and an important role for IL-15 in early, innate defence has been proposed (Carson, et al, 1995; Girard, et al, 1996; Alleva, et al, 1997). However, IL-15 also induces activation of T cell blasts and will support maturation and isotype switching of B lymphocytes. Thus, a role in development of antigen-specific responses might be predicted, although formal demonstration of this *in vivo* has until now been lacking.

IL-15 expression has been detected in several human diseases including RA, pulmonary sarcoidosis, inflammatory bowel disease and chronic active hepatitis, providing circumstantial evidence for a role in chronic immunopathology (Chapter 3; McInnes, et al, 1996b; Thurkow, et al, 1997; Agostini, et al, 1996; Kirman & Nielsen, et al, 1996; Kakumu, et al, 1997). The mechanisms whereby it could modify such inflammatory tissue destruction have not been clearly defined. The present data indicate that IL-15 expression is required for the induction of erosive inflammatory arthritis following challenge of collagen-primed DBA/1 mice. Moreover, the altered serum immunoglobulin levels detected, and *in vitro* evidence for suppressed spleen cell proliferation and cytokine production indicate that the collagen-specific response has been significantly modified. Since IL-15 induces T cell chemotaxis *in vivo* and *in vitro* (McInnes, et al, 1996b; Wilkinson & Liew, 1995), and proliferation, adhesion molecule expression and cytokine production *in vitro* (McInnes, et al, 1997; Kanegane

& Tosato, 1996), it is therefore possible that IL-15 mediates effects in inflammatory arthritis, at least in part, through its activities on antigen-specific T cells. Several recent data have provided suggestive evidence for antigen-driven T cell clonal expansion in patients with long-standing RA (Schmidt, et al, 1996; Scotet, et al, 1996). Further possible explanations for these observations include alteration of antigen-presentation or subsequent T cell co-stimulation, since both peripheral blood- and skin-derived dendritic cells are known to express IL-15 (Jonuleit, et al, 1997), or modification of adjuvant activity, since IL-15 up-regulation has been detected during mycobacterial infection (Jullien, et al, 1997).

The CIA model provides an opportunity to study the relative contribution of immune pathways to the development of inflammatory arthritis. Several groups have shown that CIA can be treated effectively with anti-TNFα antibody or other TNFα inhibitors. Monoclonal antibodies to TNFα ameliorated CIA when administered prior to disease onset (Thorbecke, et al, 1992; Piguet, et al, 1992). In addition, anti-TNFα monoclonal antibody treatment was used successfully after disease onset (Williams, et al, 1994) and analogous results have been obtained using IgG-TNF-receptor fusion protein as an inhibitor of TNFα activity (Wooley, et al, 1993). Subsequent clinical trials with neutralising antibodies against TNFα and soluble TNFα receptors have demonstrated efficacy in human RA (Elliott, et al, 1994; Rankin, et al, 1995; Morcland, et al, 1997), vindicating the use of the CIA as a surrogate for determining the role of cytokines in RA.

The evidence in the literature regarding the deleterious role of IFN-γ in CIA, a Th1 cytokine is controversial. IFN-γ induced IgG2a anti-collagen antibody can elicit mild arthritis when passively transferred to normal DBA/1 recipients (Watson, et al, 1985; Terato, et al, 1992) and administration of IFN-γ to collagen primed mice exacerbates the disease (Cooper, et al, 1988). However, it has been suggested that the outcome of anti-IFN-γ treatment depends on the time of antibody administration, with early

treatment at the time of immunization being associated with reduced severity of arthritis and late treatment being associated with aggravation of disease (Boisser, et al, 1995; Williams, et al, 1993). Similar contradictory findings have been reported in AA (Jacob, et al, 1989; Wiesenberg, et al, 1989). Moreover, IFN-γ receptor knockout mice developed CIA more readily than their wild-type counterparts (Vermeire, et al, 1997; Manoury-Schwartz, et al, 1997). Interestingly, IL-15 blockade also profoundly suppressed serum IL-10 levels. Although many properties of IL-10 are compatible with anti-inflammatory and immunoregulatory effects, the reduction of IL-10 may be beneficial to the host as its B cell stimulatory effects may be important in the production of rheumatoid factors (Rousset, et al, 1992). Recently, enhanced IL-10 production by IL-15 activated T cells has been reported (Korholz, et al, 1997). Thus the efficacy and effect of IL-15Rα in CIA are potentially of great interest, in particular the apparent capacity to down-regulate Th1 responses as evidenced by the reduction of IFN-γ and IgG2a anti-collagen antibody.

Besides its postulated function in innate immunity (Carson, et al, 1995; Girard, et al, 1996), a critical role in modulation of acquired immunity is suggested for IL-15. IL-15Rα administration effectively suppressed collagen-specific responses measured *in vivo* by serum immunoglobulin, and *in vitro* by spleen cell responses. This was unexpected since the animals were IL-2 replete. IL-15 and IL-2 share occupancy of the IL-2/15R β chain and of the common γ chain, and transduce similar JAK-1/JAK-3, STAT-3/STAT-5 dependent pathways thereafter (Johnston, et al, 1995b), leading to the suggestion that some functional redundancy might exist. However, soluble IL-15Rα chain exhibited no binding to IL-2 *in vitro*, nor did it inhibit IL-2-mediated murine T cell proliferation (D10 and CTLL-2), making it unlikely that cross-reactivity could explain these observations. Rather, it is likely that early IL-15 production during antigen-challenge is necessary for normal development of specific immune responses. Availability of soluble IL-15Rα will facilitate future studies to investigate the precise relationship and functional crossover, if any, between IL-2 and IL-15.

Thus, soluble IL-15Rα profoundly suppressed the development of CIA and markedly inhibited the onset of the humoral and Th1 cell-mediated anti-collagen responses. These results provide *in vivo* data to show a role for IL-15 in inflammatory arthritis and suggest that antagonists to this cytokine could be of therapeutic benefit. Wider application to other chronic inflammatory conditions in which IL-15 expression has been localised should also be considered.

Chapter 6

Nitric oxide and Cytokine synthesis in Asthma

Introduction

Nitric oxide (NO) has emerged as an important mediator in several inflammatory diseases (Moncada & Higgs, 1993; Nathan, 1997). NO is formed when L-arginine is enzymatically converted to L-citrulline by nitric oxide synthase (NOS). Several isoforms of NOS have been described including endothelial NOS (eNOS) and neuronal NOS (nNOS), which constitutively produce small amounts of NO, and an inducible NOS (iNOS). The latter is upregulated in response to bacterial products, such as LPS and SEB, or by inflammatory cytokines, including IFN-γ, TNF-α and IL1-β, resulting in the formation of large amounts of NO (Bredt & Synder, 1994; Nathan & Xie, 1994; Michel & Feron, 1997; McInnes & Liew, 1998).

NO is an important regulator to pulmonary physiology. Effects include modifying airway tone, regulating pulmonary vascular tone, stimulating mucin secretion, modulating mucociliary clearance through effects on ciliary beat frequency, and immune surveillance, including tumoricidal and bactericidal effects (Schmidt & Walter, 1994). NO has also emerged as an important pathological mediator in asthma (Gaston, et al, 1994; Barnes & Liew, 1995). Increased exhaled NO has been demonstrated in asthmatic patients (Kharitonov, et al, 1994 & 1996). Expression of iNOS mRNA and protein can be induced in airway epithelial cells and NO is produced by bronchial epithelial cells in culture derived from normals and asthmatic patients (Robbins, et al, 1994; Watkins, et al, 1997). In addition to this, a role for NO as a regulator of inflammatory responses is suggested by studies showing that NO modulates cytokine synthesis in a variety of cells (Deakin, et al, 1995; McInnes, et al, 1996a; Yan, et al, 1997), raising the possibility that a similar role may exist in pulmonary inflammation. Local production of cytokines such as IL-1, IL-5, IL-8 and TNF-α has been demonstrated in asthmatic patients (Gelder, et al, 1995) and is thought to contribute to pathogenesis, through promotion of chemotaxis, and through direct effects within the bronchial mucosa (Till, et al, 1995). Recently, expression of

the novel macrophage-derived cytokine, IL-15, has been described in chronic inflammatory conditions including sarcoidosis, rheumatoid arthritis, and inflammatory bowel disease (Agostini, et al, 1996; McInnes, et al, 1996b; Kirman & Nielsen, 1996). However, a role for IL-15 in asthma has not previously been described.

The experiments described in the present chapter set out to establish whether human respiratory cells were indeed capable of significant NO production, and if so to determine the cellular location of such synthesis. Thereafter, the effect of corticosteroid therapy on NO and inflammatory cytokine expression was explored. Finally a potential link between NO, and cytokine synthesis, namely TNF-α and IL-15, was investigated.

6.1 Assay for nitrite in biological fluids

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

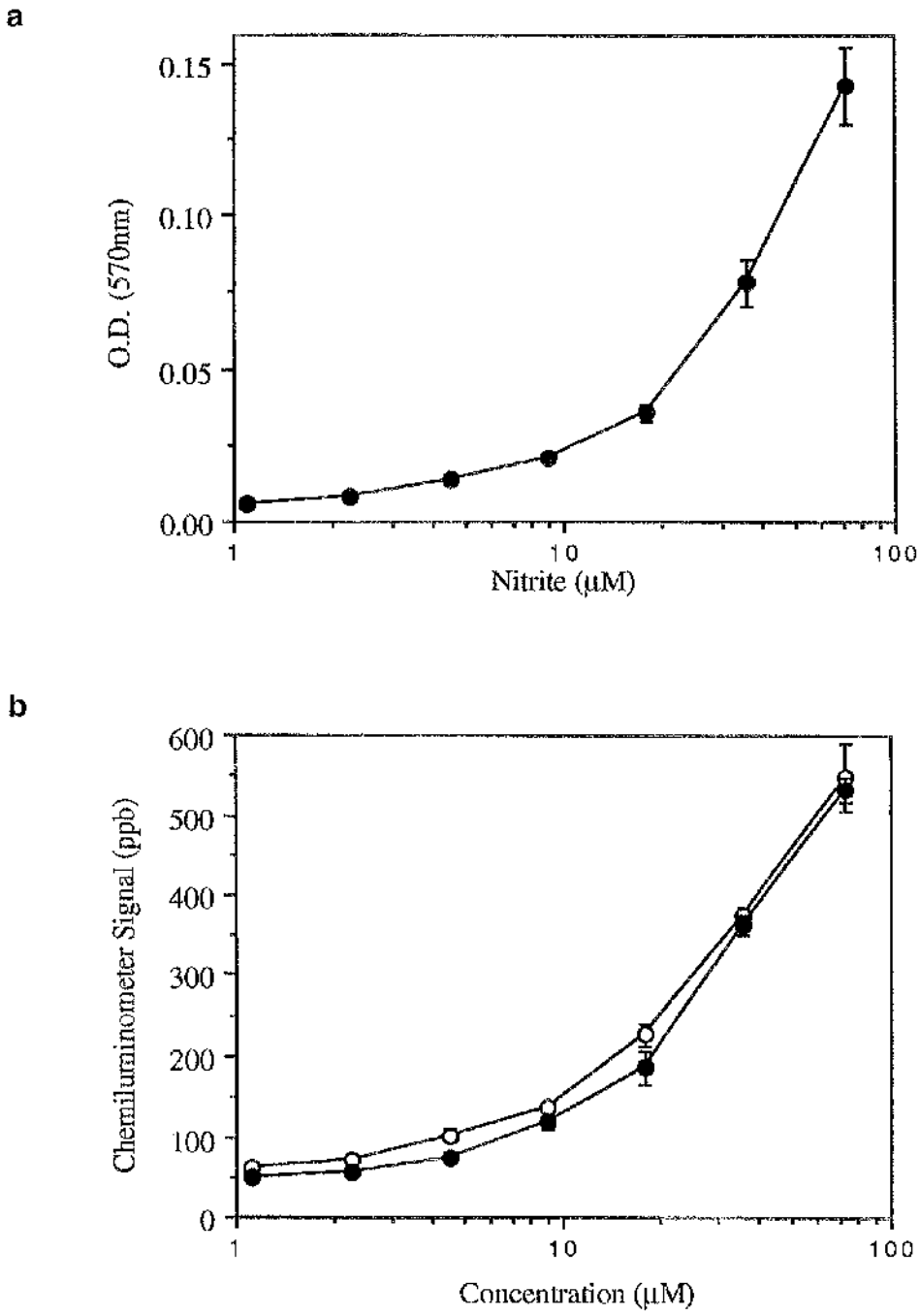
(a) The Griess reaction was performed to detect nitrite in either fresh, or previously frozen (-20°C) culture supernatants, with typical sensitivity of 2μM (Figure 6.1a).

(b) The Griess reaction develops at acid pH raising the possibility of protein precipitation in test samples. Total serum nitrite levels were therefore measured by chemiluminescence, after reduction of nitrate to nitrite using nitrate reductase. A typical standard curve is shown in Figure 6.1b, demonstrating sensitivity of 2μM.

6.2 Nitrite / nitrate in serum from asthmatic patients

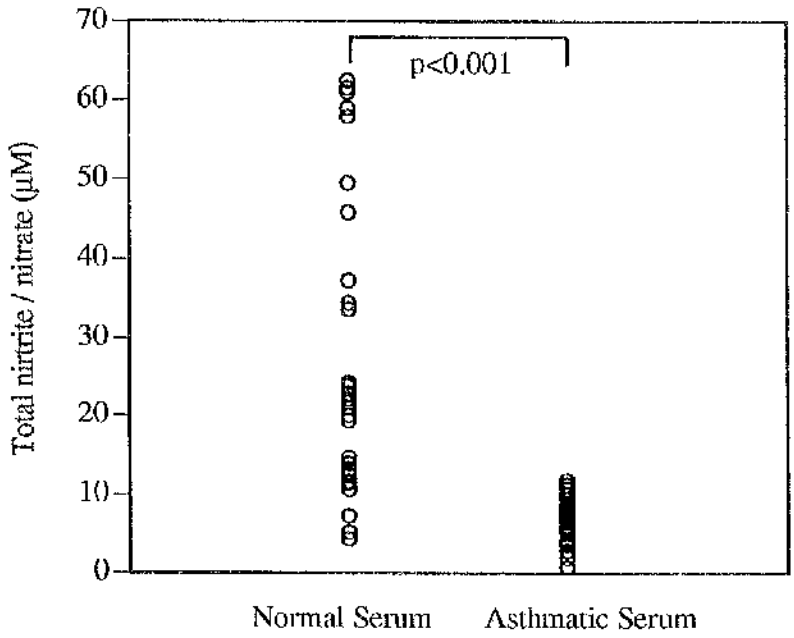
Serum samples were collected from 32 steroid-treated asthmatic patients and stored at -20°C prior to assay for nitrite / nitrate concentration using chemiluminescence. Figure 6.2 shows that the mean concentration of nitrite, representing total nitrite / nitrate levels after enzymatic nitrate reduction, was significantly reduced when compared with a similar number (n=32) of age and gender matched normal controls ($p<0.001$; Mann-Whitney). However, serum samples from non-steroid treated patient were not available during the present study, thus it was not possible to compare the effects of steroid within asthmatic patients. These data are compatible with those observed in patients receiving either oral (Massaro, et al, 1995) or inhaled steroid therapy (Kharitonov, et al, 1996) in which significant reduction in exhaled NO was noted.

Figure 6.1 Standard curves for nitrite /nitrate estimation



(a) Standard curve derived from triplicate sodium nitrite dilutions in Dulbecco's MEM estimated by Griess reaction as described in section 2.10.1. (b) Standard curves derived from dilutions of sodium nitrite and (O) and sodium nitrate (●) in Dulbecco's MEM estimated by chemiluminescence as described in section 2.10.2.

Figure 6.2 Nitrate concentration in serum obtained from asthmatic patients

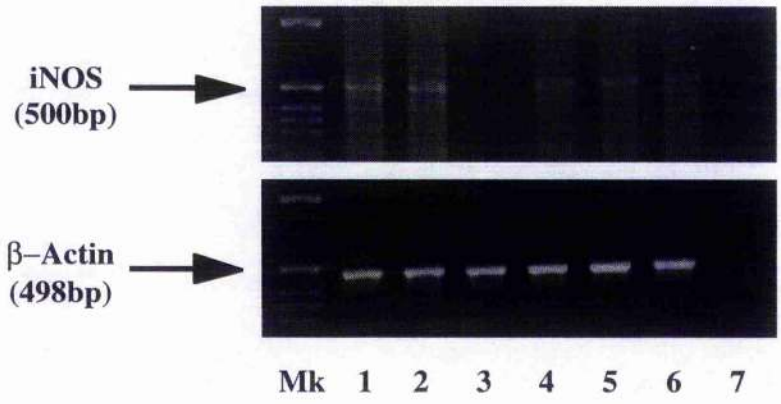


Total nitrite / nitrate levels were assayed using chemiluminescence (section 2.10.2). Nitrite / nitrate levels were compared in serum from asthmatic patients (n=32) and healthy normal controls (n=32). Significant reduction of total nitrite / nitrate levels were observed in asthmatic patients (mean ± s.e.m., 6.5±0.5 vs 29.1±3.6 μM, $p<0.001$).

6.3 Expression of iNOS mRNA in induced sputum samples

Inducible NOS expression in human respiratory tract samples obtained in induced sputum was determined first at the mRNA level. RT/PCR was performed using primers specific for human chondrocyte iNOS (Charles, et al, 1993). Induced sputum samples from 5 patients were collected and RT/PCR was performed. In 4/5 samples, the PCR generated the expected 500 bp product, consistent with that predicted from human chondrocyte iNOS (Figure 6.3). 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 and found to be similarly expressed among all 5 samples.

Figure 6.3 RT-PCR for iNOS in human respiratory samples



RT/PCR was performed to detect human iNOS mRNA expression in freshly isolated sputum samples. Lane 1-5 - representative asthmatic patients, lane 6 - positive control (human iNOS cDNA), lane 7 - negative control (dH_20 + RT/PCR reagents). Subsequent sequencing confirmed that the PCR product was identical to human iNOS. β-actin mRNA was amplified for control.

6.4 Nitric oxide production in induced sputum cells

Since the principal objective of this study was to obtain direct evidence for NO production by human respiratory cells, primary heterogeneous cell cultures were established from samples obtained from induced sputum. The induction of sputum from asthmatic patients allowed the identification of pro-inflammatory factors *ex-vivo* which could be readily compared with normal control subjects. Induced sputum samples from a total of 8 normal volunteers [all non-smokers, 6 males, 2 females, aged (mean±SD, range): 33.4±8.5, 23-47 year] and 17 asthmatic patients [all non-smokers, 13 males and 4 females, aged: 42.4±13.1, 20-69 year] were included in this study. Asthmatics were subdivided into those taking inhaled β-2 agonist alone (non steroid-treated, n=8), and those on additional inhaled steroid therapy (steroid-treated, n=9). Corticosteroids are the most effective form of therapy in asthma (Laitinen, et al, 1992). They inhibit the transcription of most pro-inflammatory cytokines in asthma (Barnes, 1996), probably via an indirect effect on critical transcription factors, and may inhibit iNOS expression by inhibiting NF-κB binding (Xie, et al, 1994). While there are increasing data on the influence of steroids on regulation of many genes relevant to asthma, there is a relative lack of clinical data to support proposed mechanisms of action of corticosteroids in asthmatic cells or tissues. Differential cell counts for both patient treatment groups and normal controls are listed at table 6.1; no significant difference was observed. Spontaneous nitrite generation *in vitro* by sputum cells as determined by the Greiss reaction was measured after 48 hours (Table 6.2) and found to be extremely low and identical in each study population. These data indicated that NO production was tightly regulated and indicated that iNOS expression might require constant stimulation.

Table 6.1 Distribution of cells in induced sputum samples

Study Subjects	Cell type (%) mean ± S.D.		
	Epithelial	Macrophages	Polymorphs
Normal Controls (n = 8)	60.2 ± 9.0	27.4 ± 8.8	12.5 ± 7.8
Steroid-treated (n = 8)	64.2 ± 19.6	25.5 ± 13.7	9.2 ± 7.2
Non-steroid treated (n = 9)	47.8 ± 22.0	25.7 ± 14.3	24.3 ± 24.0

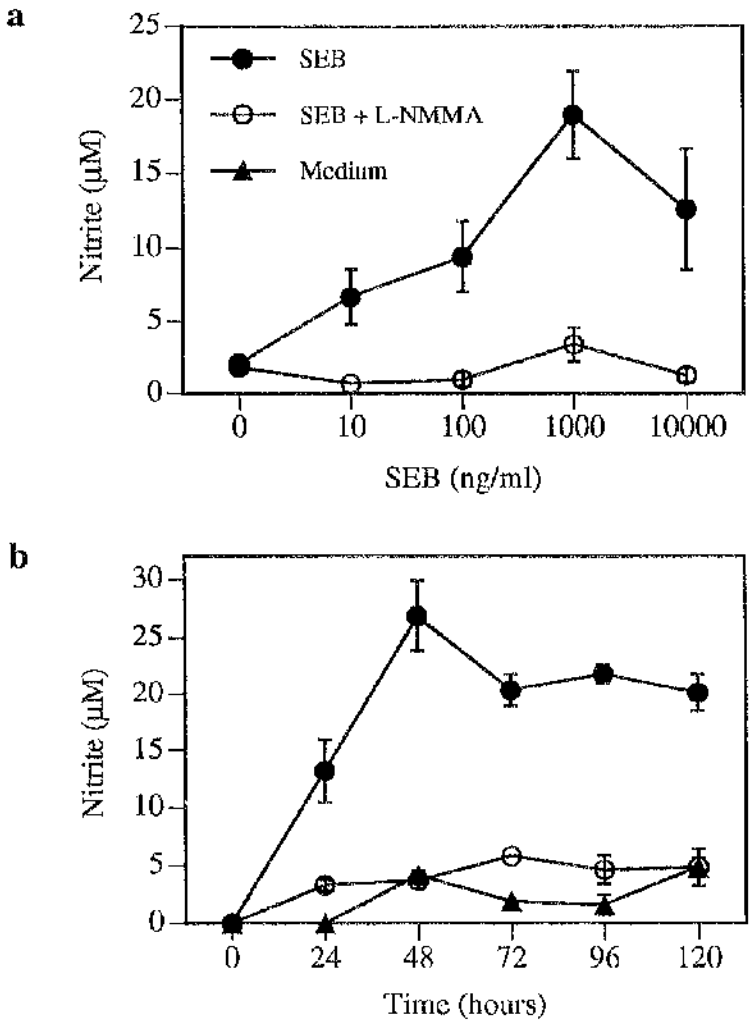
Sputum cells were isolated as described in section 2.4.4, cytospin preparations were made and at least 300 cells were counted per sample. No significant differences between asthmatic patients and normal controls was observed (Mann-Whitney test). Data are mean ± s.d.

6.5 Induction of NO production *in vitro* in induced sputum cultures

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 production 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). SEB stimulates NO production by murine macrophages in the presence of T lymphocytes (Isobe & Nakashima, 1992) as well as in human macrophages and fibroblasts (McInnes, et al, 1996a). SEB was therefore chosen as a stimulus for sputum cell NOS induction. Parallel experiments also utilised LPS, a potent iNOS upregulator in rodents (Liew & Cox, 1991).

Sputum cells from normal donors cultured with SEB exhibited dose- and time-dependent upregulation of NO production (Figure 6.4 a & b). A similar pattern was observed in samples derived from non-steroid treated asthmatic patients. Thus, all cultures were compared for peak nitrite production using 1 μg/ml SEB stimulation for 48 hours. The addition of SEB induced significant nitrite production in both non-steroid treated asthmatic patients ($p<0.01$, Mann-Whitney) and normal controls ($p<0.05$, Mann-Whitney, Table 6.2). However, sputum cells derived from patients treated with corticosteroids exhibited significantly less enhancement of NO synthesis to SEB *in vitro* compared with either normals or non-steroid treated patients ($p<0.05$). Initial experiments determined that LPS alone (1 μg/ml) was less effective than SEB in inducing nitrite production. Furthermore, when the two agents were combined, addition of LPS reduced SEB-mediated enhancement of NO synthesis in both controls and non-steroid treated patients (Table 6.2). This may reflect the local synthesis of anti-inflammatory cytokines, such as IL-10 or TGF-β. Production of nitrite *in vitro*

Figure 6.4 Production of NO by induced sputum cultures



(a) Dose response for SEB-induced (●) nitrite production by heterogeneous sputum cells from a representative asthmatic patient. Production of nitrite was significantly inhibited by L-NMMA (○), $p<0.01$, Mann-Whitney. (b) Time course of nitrite production by sputum cells. Parallel cultures were established and harvested at the time points indicated, then stored prior to nitrite assay. Production with SEB was significantly greater than basal levels (▲) ($p<0.01$, Mann-Whitney) at all time points from 24 hours. Data are mean ± s.e.m., representing 3 similar experiments.

Table 6.2 Nitrite production by induced sputum cultures

Patients		Nitrite (μM) mean ± SEM		
	(n)	SEB	SEB + LPS	Medium
Control	8	19.9* ± 7.6	13.9* ± 4.8	2.6 ± 0.8
Steroid-treated	8	5.6 ± 2.8	3.3 ± 0.8	2.6 ± 0.8
Non steroid -treated	9	35.1** ± 14.3	16.2* ± 4.0	2.2 ± 0.4

Sputum cells were cultured either in medium alone or stimulated with SEB ± LPS (both at 1 μg/ml). Supernatants were harvested after 48 h and nitrite levels determined by the Griess reaction. Significant (*$p<0.05$, **$p<0.01$) amounts of nitrite were detected when compared with medium controls after culture with SEB or SEB together with LPS. Furthermore, sputum cells derived from non steroid-treated asthmatic patients produced significantly more ($p<0.05$) nitrite upon stimulation with SEB ± LPS when compared with steroid-treated patients. Nitrite production could be completely inhibited by L-NMMA (5 mM).

was completely inhibited by addition of 5 mM L-NMMA (Figure 6.4b), confirming the specificity of the system. Sputum cell viability was not affected by L-NMMA throughout culture as assessed by trypan blue exclusion.

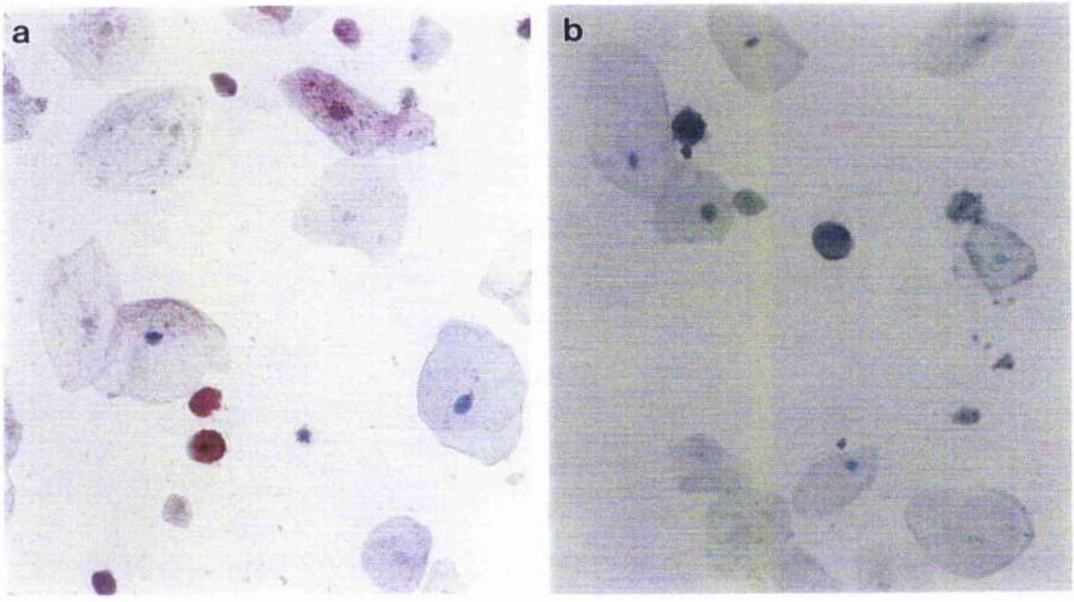
Together these data clearly demonstrated that high levels of NO synthesis by sputum cells was possible. Moreover, they strongly suggested that one effect of steroids may be to suppress the ability of respiratory mucosal cells to generate NO in response to local stimuli. It was next of interest to determine the precise cell of origin.

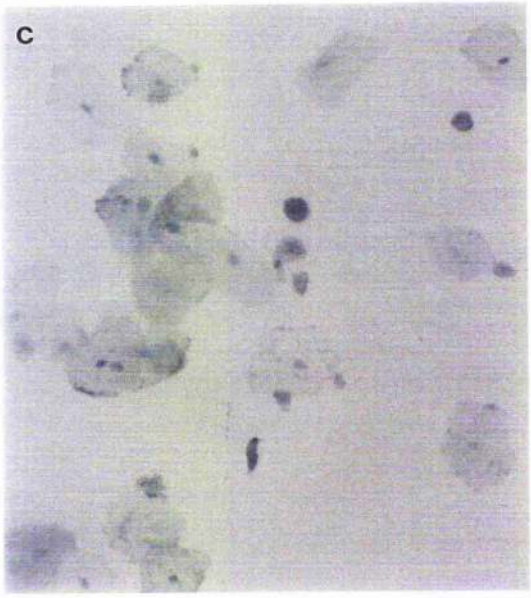
6.6 Cellular localisation of iNOS in induced sputum cells

Initial efforts to localise iNOS in human respiratory 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 (Hamid, et al, 1993). Those antibodies investigated in the present study are listed in table 2.1. Several monoclonal and polyclonal anti-murine iNOS antibodies exhibited extensive staining which was not readily neutralized by recombinant iNOS protein, thus considerable doubt remained as to the specificity of these reagents.

To overcome this problem, a rabbit antiserum raised specifically against the C-terminal 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 and has been shown to specifically bind to human iNOS in tissues cryosections (Nicholson, et al, 1996; McInnes, et al, 1996a). Following stimulation of sputum cells with SEB for 48 hours, cytopreps were made. Staining was observed in cells from all non steroid-treated asthmatic patients (Figure 6.5a). In contrast, the frequency of staining was markedly reduced in preparations from patients treated with corticosteroids ($p<0.05$, Table 6.3). The percentage of positive cells varied

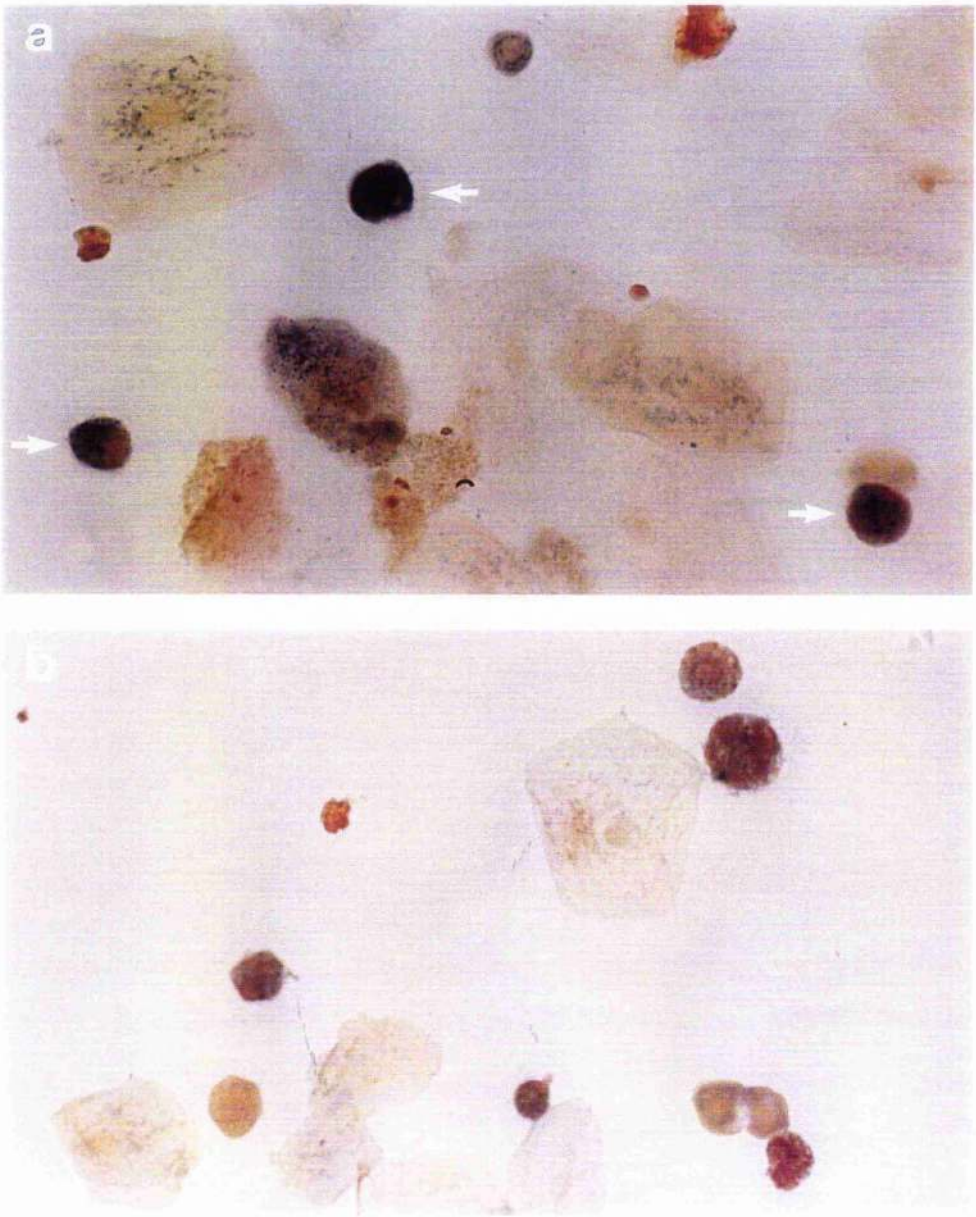
Figure 6.5 Immunohistochemical identification of iNOS in induced sputum cells





Immunocytochemistry of iNOS in sputum cells from a representative (n=6) non steroid-treated asthmatic patient. Sputum cells were stimulated with SEB+LPS *in vitro* for 24 h and cytospin preparations were stained with (a) anti-human iNOS antiserum (fast red). (b) anti-iNOS serum after pre-incubation with human iNOS peptide, (c) normal rabbit IgG. No staining was observed in unstimulated sputum cells cultured in medium alone. (Magnification x200)

Figure 6.6 Co-localisation of non-specific esterase and iNOS in induced sputum cells



To identify iNOS expression in macrophages, (a) sputum cells were double stained (indicated with arrow) with anti-human iNOS (fast blue), then with NSE (red brown). No nuclear counterstain was used. (b) Specificity of staining was demonstrated by pre-incubation with human iNOS peptide. (Magnification x400)

Table 6.3 Immunohistochemical localisation of iNOS in sputum cells

	Positive cells (%) mean ± s.d.		
Study Subjects	Total	Macrophages	Epithelial cells
Normal controls (n=5)	10.6 ± 3.1	2.9 ± 1.1	7.7 ± 2.5
Steroid-treated (n=4)	3.7 ± 1.8*	0.6 ± 0.6*	2.1 ± 1.3*
Non-steroid treated (n=5)	15.6 ± 7.1	6.3 ± 4.4	9.3 ± 6.8

Sputum cells from normal controls and asthmatic patients were stimulated with SEB+LPS *in vitro* and stained with a polyclonal anti-iNOS antibody as described (section 2.6). iNOS expression was greater in non-steroid treated asthmatic patients when compared with the steroid-treated group ($p<0.05$, Mann-Whitney). No difference was observed between normal controls and non-steroid treated patients. At least 300 cells were counted per sample, data are mean (%) ± s.d.

considerably between different patients, as predicted by the heterogeneity of nitrite production in *in vitro* culture. Staining revealed intracellular localisation, and was neutralised by pre-incubation of the anti-serum against immunising human iNOS peptide (NO54, Figure 6.5c).

The identity of iNOS positive cells was next sought. Consistent with previously published data (Robbins, et al, 1994; Watkins, et al, 1997), epithelial cells, identified by distinctive morphology, from the non steroid-treated patients were found to be iNOS positive (Figure 6.5a). By double staining with NSE and anti-iNOS antibody, alveolar macrophages in this cell population expressing iNOS protein were also clearly identified (Figure 6.6a).

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

The ability of human monocytes / macrophages to produce NO remains controversial (Schneemann, et al, 1993, Dugas, et al, 1995). Light microscopic data presented here clearly indicated that human macrophages activated in an inflammed environment are capable of expressing iNOS. Similar results have been reported in monocytes / macrophages collected from other chronic inflammatory conditions including alcoholic hepatitis, endemic malaria, RA, osteoarthritis (reviewed by MacMicking, et al, 1997), as well as in patients with hepatitis A under treatment with IFN-α (Sharara, et al, 1997). However, when similar culture conditions as induced NO synthesis by sputum cells were applied to either purified blood monocytes or the monocytic cell lines THP-1 and U937, no combination of reagents or *in vitro* conditions was capable of inducing consistent NO production as measured by the Griess reaction. Murine macrophage, J774 cells, served as a positive control and reproducibly generated NO *in vitro*.

6.8 Cytokine expression in Sputum derived leukocytes

NO is a labile molecule which may perform important biological roles both within cells in which it is synthesized, as well as in interactions with nearby cells and molecules (Stamler, 1994). iNOS appears to play both regulatory and effector roles (Schmidt & Walter, 1994). However, the cytokines that regulate iNOS expression have been only partially characterised in asthma (Guo, et al, 1997). It was therefore decided to investigate cytokine synthesis in the present culture system.

(a) *In vitro* TNF-α synthesis

TNF-α is a pleiotropic cytokine produced mainly by activated monocytes / macrophages, but also by other cells including T lymphocytes and NK cells. It has a wide range of biological effects including induction of adhesion molecules such as E-selectin, intracellular adhesion molecule (ICAM-1) and vascular cell adhesion molecule (VCAM-1) which promote cell migration and adhesion, and induction of inflammatory and tissue-damaging molecules, such as prostaglandins. Moreover, it is a recognised stimulator of iNOS expression (Nathan & Xie, 1994) in rodents and in human airway epithelial cells (Watkins, et al, 1997). Sputum cells from normal controls and steroid-treated patients cultured with medium alone produced low concentration of TNF-α whereas non-steroid treated sputum cells produced significantly higher amounts of TNF-α spontaneously (Table 6.4, $p<0.05$). This synthesis was further augmented by the presence of SEB and LPS.

Table 6.4 Production of TNF-α by cultured sputum cells

	Patients (n)	TNF-α (pg/ml) mean ± SEM Medium	SEB & LPS
Normal Controls	6	31 ± 31	107 ± 64
Steroid-treated	7	148 ± 88	237 ± 150
Non steroid-treated	7	650 ± 308	1265 ± 436*

Sputum cells were cultured either alone or with SEB + LPS at 1 μg/ml for 48h. Supernatants were harvested and TNF-α was determined by ELISA. Non-steroid treated asthmatic patients produced significantly more TNF-α either spontaneously or after stimulation when compared with normal controls ($p<0.02$, Mann-Whitney) or steroid-treated patients ($p<0.05$). *Significant upregulation of TNF-α post-stimulation when compared with baseline levels ($p<0.05$, paired t-test).

(b) Cellular localisation of IL-15 expression

Previous chapters described data linking TNF-α with the synthesis of IL-15. In order to test whether IL-15 was present in asthmatic bronchial tissues, the distribution of IL-15 in freshly isolated sputum cells was investigated by immunohistochemistry, using monoclonal anti-human IL-15 antibody (M111, Immunex and MAB647, R&D System). IL-15 expression was detected mostly in epithelial cells, and to a lesser extent in NSE positive alveolar macrophages (Figure 6.7 a&d). To confirm the specificity of the binding, neutralisation was carried out by pre-incubating the detecting antibody with recombinant human IL-15, which abolished the IL-15 staining (Figure 6.7 b). Interestingly, cells from non steroid-treated asthmatic patients showed strongly positive staining compared to those from steroid-treated patients which showed weak and inconsistent staining ($p<0.001$, Figure 6.8), thus suggesting increased expression of IL-15 in asthmatic patients airways which can be reduced by the administration of steroid. Normal controls exhibited low and inconsistent staining, suggesting that IL-15 may not consitutively present at the protein level.

Legend to Figure 6.7

Immunocytochemistry for IL-15 expression in sputum cells isolated from a representative (of 5) non steroid-treated asthmatic patient. Freshly isolated sputum cells were stained with anti-human IL-15 mAb (fast red) (a). Both epithelial cells (large) and macrophages were positively stained. Specificity of staining was demonstrated by pre-incubation with rIL-15 (b), or normal murine IgG1 (c). For localisation of IL-15 in macrophages, sputum cells were double stained (indicated by arrow) with anti-human IL-15 (fast blue) and NSE (brown) (d). (Magnification x200)

Figure 6.7 Immunohistochemical localisation of IL-15 in induced sputum cells

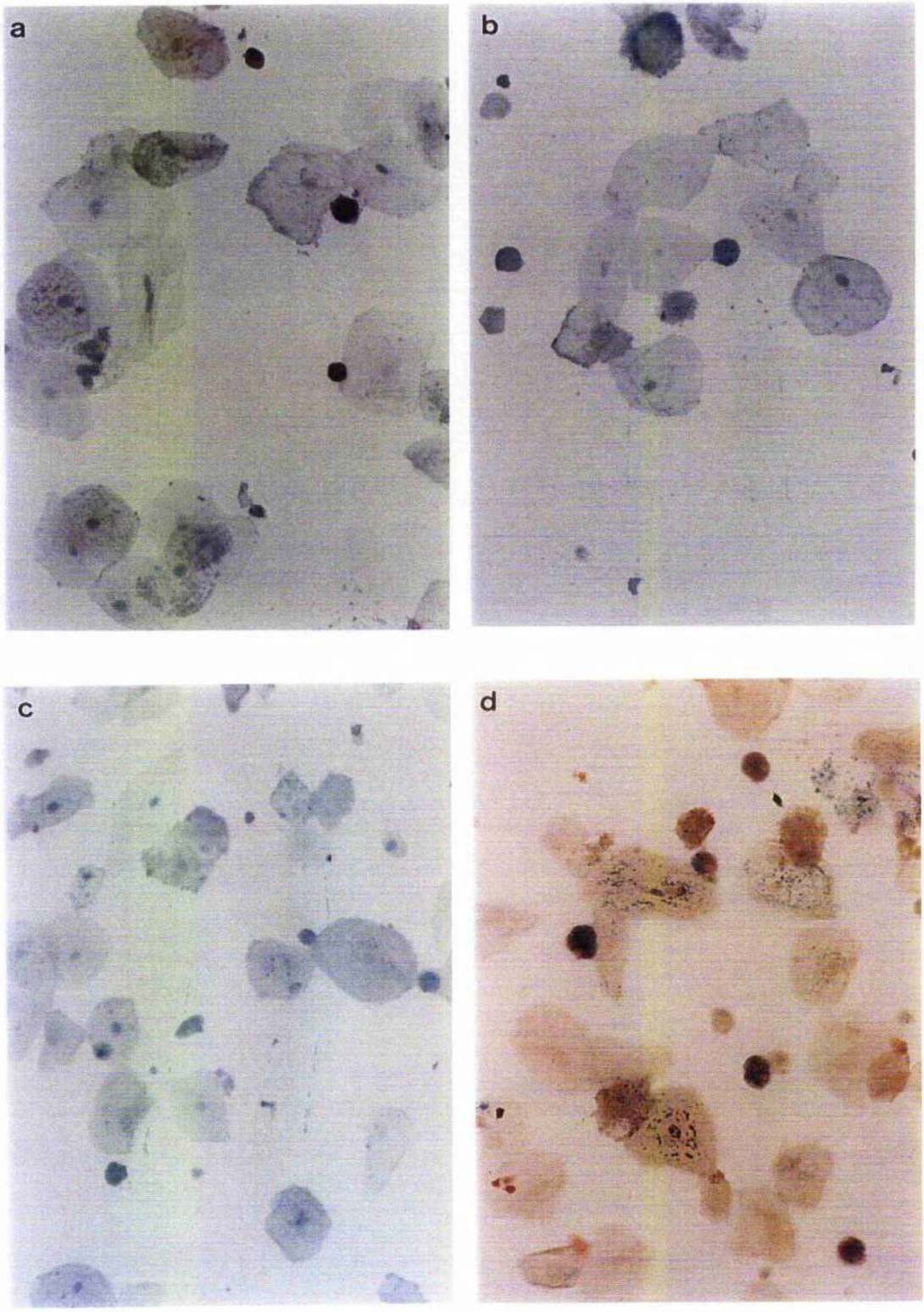
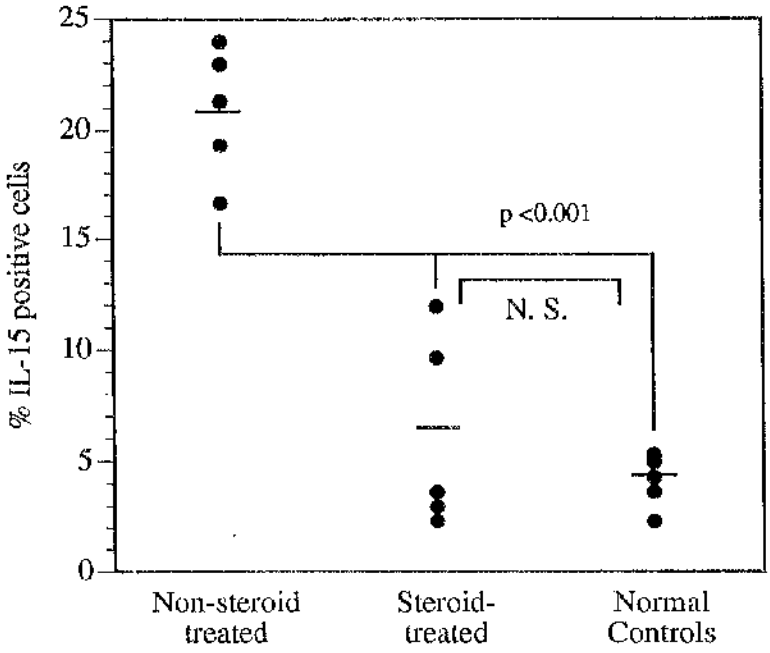


Figure 6.8 Percentage distribution of IL-15 expression in induced sputum cells



Freshly isolated sputum cells were stained with anti-human IL-15 antibody and the positively stained cells counted by light microscopy (%). At least 300 cells were counted per sample. Asthmatic patients were subdivided into those who were taking inhaled steroid (n=5) and those who were not (n=5). Cells from normal controls (n=5) were also included. $p<0.001$, Mann-Whitney test; N.S., not significant.

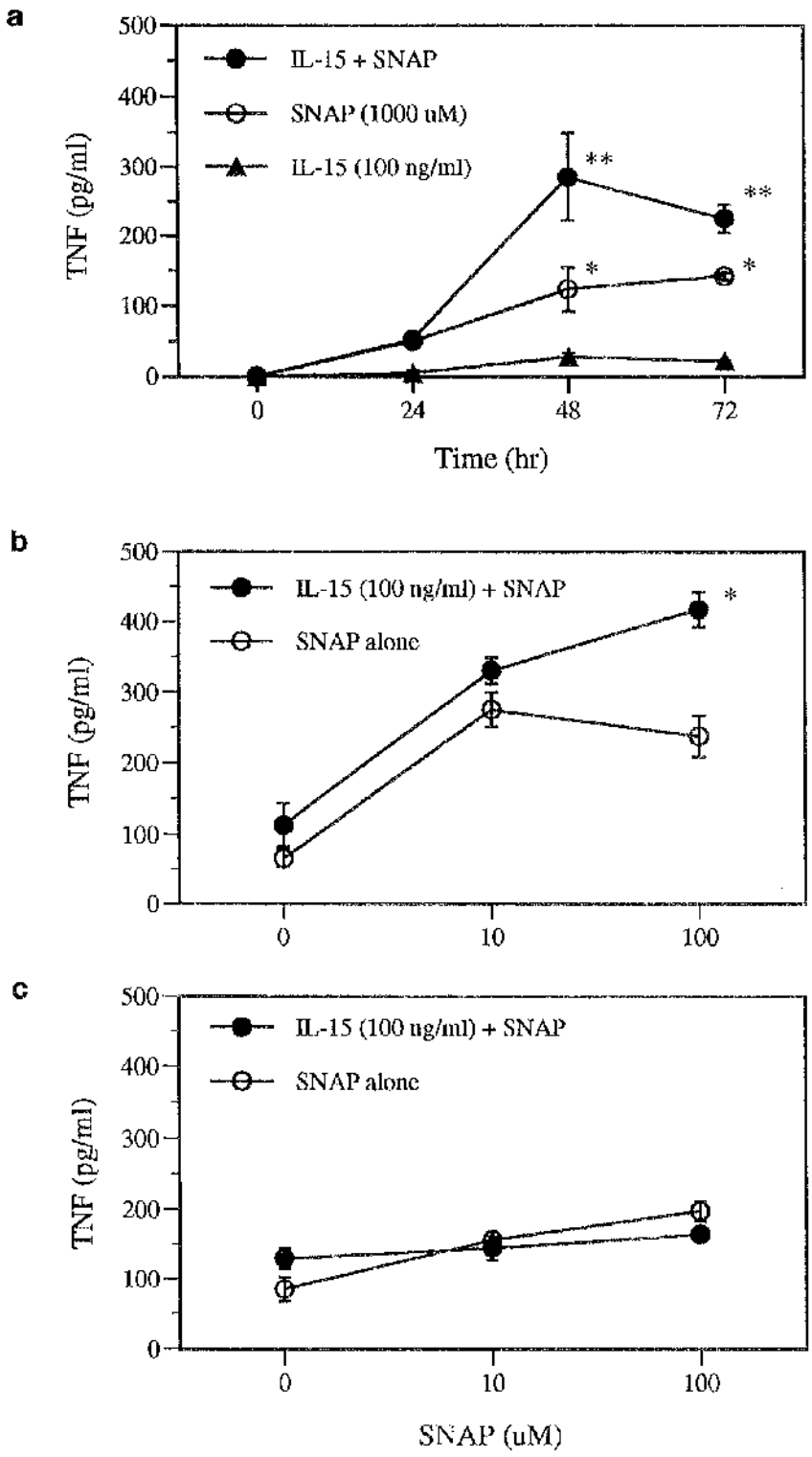
6.9 NO and IL-15 dependent regulation of TNF-α production

NO and IL-15 can independently induce TNF-α synthesis in human inflammatory conditions such as rheumatoid arthritis (McInnes, et al, 1996a & 1997). Moreover, direct autocrine regulation of macrophage function by IL-15 has been reported (Alleva, et al, 1997). Thus the possibility that NO and IL-15 might modulate TNF-α synthesis by sputum cells was investigated. Initially, the human monocytic cell line THP-1 was used to established the kinetics of TNF-α synthesis. Cells were primed with IFN-γ (50 IU/ml) and SEB (1 μg/ml) for 16 hours, then cultured at 2 x 10^6 / ml in the presence of the NO donor, S-nitroso-N-acetylpenicillamine (SNAP, 1mM) and IL-15 (100 ng/ml), and the levels of TNF-α were determined by ELISA. Cells cultured with either IL-15 or SNAP alone produced low to intermediate levels of TNF-α. Levels were markedly enhanced by the presence of both stimuli, resulting in high levels of TNF-α synthesis in a time-dependent manner (Figure 6.9a). This was reproduced in sputum cell cultures derived from normal donors, but not in those from patients treated with inhaled steroid (Figure 6.9 b&c). The analogue N-acetylpenicillamine (NAP) which does not release NO, failed to induce TNF-α synthesis. These data suggest the presence of complex regulatory feedback loops involving NO and cytokine synthesis in asthma.

Legend to Figure 6.9

TNF-α production in response to IL-15 and SNAP by (a) pre-activated THP-1 cells (representative of 3 experiments), or (b) sputum cells from a representative (of 4) normal donor, or (c) sputum cells from a representative (of 3) steroid-treated asthmatic patient. Cells were cultured with IL-15 (100 ng/ml) and SNAP (1 mM) for up to 72 h (a), or graded concentrations of SNAP for 48 h (b, c). In all cases, the control compound NAP was without effect. Data are mean ± s.d. of triplicate cultures, *$p<0.05$, **$p<0.01$.

Figure 6.9 Induction of TNF-α synthesis by IL-15 and NO



Discussion

The pathophysiological consequences of increased NO production in the respiratory tract are not yet known. iNOS activity has been detected in a variety of cells including alveolar macrophages from tuberculosis patients, epithelial cells and activated eosinophils (Nicholson, et al, 1996; Watkins, et al, 1997; Del Pozo, et al, 1997). Using RT/PCR, freshly isolated sputum samples from asthmatic patients were iNOS positive. However, when cultured *in vitro*, sputum cells failed to generate NO spontaneously. Stimulation with SEB induced dose- and time- dependent upregulation of NO production. It has been suggested that normal human large-airway epithelium is iNOS positive (Kobzik, et al, 1993, Guo, et al, 1995), probably as a result of constant exposure to inductive stimuli including ozone, LPS and airborne pathogens. Interestingly, culture of airway epithelial cells *ex vivo* without stimulation leads to loss of iNOS gene expression, thus, it is difficult to conclude whether iNOS expression in airway epithelium is "constitutive" or is continually "induced". Recently, the same group suggested that a combination of IFN-γ / IL-4, which is present in lung epithelial lining fluid, leads to maintenance of iNOS expression in human airway epithelium through production of "soluble mediators" and stabilization of mRNA (Guo, et al, 1997). The present study therefore suggests that confirmed stimulation is a pre-requisite for iNOS expression and NO synthesis.

Immunohistochemical localisation identified iNOS expression in epithelial cells and macrophages from asthmatic patients as well as normal controls. The anti-iNOS antibody employed here also detected alveolar macrophages expressing iNOS in pulmonary tuberculosis patients (Nicholson, et al, 1996). Neutralisation studies confirmed that it did not cross-react with either rodent iNOS or human eNOS. The iNOS staining pattern observed contrasted with that reported using antibody raised against murine iNOS, which suggested more extensive iNOS expression (Hamid, et

al, 1993). The latter may reflect some cross reactivity characteristic of anti-rodent iNOS antisera.

Controversy over the issue of NO production by human macrophages has been intense (Denis, 1991, Dugas, et al, 1995, Schneemann, et al, 1993 & 1997). iNOS has been difficult to demonstrate in human macrophages derived *in vitro* from normal donor monocytes. However, when monocytes / macrophages are taken from patients with inflammatory or infectious diseases and studied either directly or following stimulation *in vitro* with cytokines, results now emerging seem consistently positive (reviewed by MacMicking, et al, 1997). Thus, the problems encountered *in vitro* are more appropriately viewed as a deficiency in culture techniques and immunologic knowledge than as an inadequacy of the cell.

NO is likely to influence numerous immunoregulatory pathways in asthma. Airway inflammation is characterized by activation of mast cells and macrophages, and infiltration of eosinophils. Moreover, there is clear evidence suggesting that T-helper (Th) cells are activated in the airway mucosa, with preferential proliferation of Th2 cells and expression of IL-5 mRNA (Hamid, et al, 1991). NO can down regulate the Th1 cell response (Wei, et al, 1995; McInnes, et al, 1998), thus enhancing the differentiation of Th2 cells to produce IL-4, and IL-5 which is important for recruitment of eosinophils into the airway. This process could be further augmented by IL-15 which can both induce IL-5 production from human Th2 cell clones (Mori, et al, 1996) and serving as a T cell recruitment factor (Wilkinson & Liew, 1995). Recently, IL-15 has been shown to enhanced pro-inflammatory cytokine production including TNF-α, IL-1 and IL-6 in activated macrophages (Alleva, et al, 1997), suggesting an important role in monokine regulation. Moreover, a novel IL-15 receptor has been reported on mast cells (Tagaya, et al, 1996), raising the possibility that IL-15 may contribute to mast cell degranulation in asthma. Thus, the finding of

IL-15 in the airway of asthmatics, and together with the presence of NO, provides an important new pathway through which inflammation may be perpetuated.

TNF-α has a postulated role in the pathogenesis of asthma. Cells from bronchoalveolar lavage (BAL) fluid from stable atopic asthmatics express more TNF-α mRNA when compared with normals (Ying, et al, 1991), and marked elevation of TNF-α levels has been reported in BAL fluid from patients with symptomatic asthma (Broide, et al, 1992). Immunohistochemical techniques show a similar pattern, with bronchial biopsies showing an increase in TNF-α positive cells in symptomatic subjects compared with asymptomatic and control subjects (Ackerman, et al, 1994). TNF-α has been shown to induce bronchial hyperresponsiveness in rats (Kips, et al, 1992) and is a recognised inducer of iNOS expression in animal systems (Nathan & Xie, 1994; Liew, 1994). The mechanism by which NO in turn regulates TNF-α synthesis remains unclear. In peripheral blood mononuclear cells, the upregulation of TNF-α by NO has been associated with changes in the binding activity of NF-kB, a transcriptional factor that increases TNF-α synthesis at the promoter level (Lander, et al, 1993). The present study clearly demonstrates the existence of complex feedback loops involving both proinflammatory cytokines and NO. The therapeutic potential of such observations remains unclear.

Corticosteroids, given either by inhalation or orally are the most effective form of therapy in asthma, with inhaled administration representing the mainstay of therapy. There is clear evidence of active inflammation in the airways of even newly diagnosed asthmatics (Laitinen, et al, 1993), and corticosteroids can be shown to reduce such inflammation (Laitinen, et al, 1992), with discontinuation of treatment often leading to exacerbation of disease (Haahtehla, et al, 1994). Corticosteroids inhibit the transcription of most inflammatory cytokines present in asthma, including IL-1β, IL-3, IL-4, IL-5, IL-6, TNF-α, GM-CSF and the chemokines IL-8, and RANTES along with monocyte and macrophage chemoattractants (Barnes, 1996), probably via

indirect effect on critical transcription factors. Furthermore, they inhibit iNOS expression by inhibiting NF-kB binding (Xie, et al, 1994). Although the effect of corticosteroids on IL-15 remains unclear, dexamethasone is known to down regulate peripheral blood mononuclear cell responses to IL-15 by inhibiting IL-15Rα expression, thus blocking the high affinity IL-15 to IL-15Rα interaction (Chae, et al, 1996). These results also offer another mechanistic explanation for the efficacy of inhaled glucocorticoid treatment in asthma.

In conclusion, these results demonstrated the expression of significant levels of NO and IL-15 in the airways of asthmatic patients. Such expression was downregulated in steroid treated patients. NO and IL-15 are therefore potential important targets for effective therapeutic approaches in asthma.

Chapter 7

General Discussion

7.1 Rheumatoid arthritis

Neither the initiating events, nor the perpetuating factors in the pathogenesis of RA are well understood. The cellular components present within the synovium comprise T lymphocytes, monocyte / macrophages, mast cells, neutrophils, and plasma cells, together with an expanded population of activated synovial fibroblasts (synoviocytes). No consensus exists as to which is the principal regulatory cell, representing the optimal therapeutic target, nor indeed, whether such regulatory contribution is stable with disease progression (Firestein & Zvaifler, 1990; Panayi, et al, 1992; Kingsley, et al, 1996).

Given the postulated autoimmune basis for RA, most debate surrounds the role of T lymphocytes in pathogenesis (reviewed by Fox, 1997). Several factors suggest a pro-inflammatory role for T cells in RA, including their critical role in animal arthritis models, the partial efficacy of T cell directed therapies in clinical studies and, most persuasively, the disease association with specific HLA-DR alleles. However, T cell derived cytokines are detected only at low levels in synovial membrane and bone / cartilage destruction does not necessarily correlate with the presence of synovial inflammation. The majority of synovial T cells are $CD45RB^{dim}$, RO^{+}, $CD27^{-}$, representing a mature, memory T cell phenotype, and express a complex combination of early and late activation markers, including multiple adhesion molecules. However, although extensive receptor analyses have identified oligoclonality in the synovial T cell repertoire, as yet there are no consistent data to indicate a single antigen-driven process. 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 for T cells in contributing to the inflammatory process in RA.

IL-15 exhibits a high degree of redundancy and pleiotropy controlling a wide range of functions within the immune system. This redundancy is explained in part by the sharing of common receptor subunits if IL-2R β and γc chains. In addition, a novel 60-65 kDa receptor for IL-15 has been identified on mast cells, designated IL-15RX, which requires neither IL-2Rβ nor γ chain for signalling. This receptor recruits distinct signalling pathways. Whereas IL-15αβγ complex signals through JAK 1/3 and STAT 3/5, IL-15RX utilises JAK 2 and STAT 5. IL-15 mRNA is detected in RA synovial membrane and its relative expression varies with prior immunosuppressive therapy (Kotake, et al, 1997). IL-15 was detected by ELISA in 30/53 RA synovial fluids in the range 10-1128 pg/ml (median of positive samples 198 pg/ml). Interestingly, this activity correlated with SF TNF-α concentrations and remained after removal of rheumatoid factor, which might have interfered with earlier assay systems. IL-15 can also be detected immunohistochemically in the RA synovial membrane, using several monoclonal antibodies, where it is localised to macrophages and fibroblasts in the synovial lining layer and within lymphocytic aggregates (McInnes, et al, 1996b). Subsequent histologic studies have confirmed the presence of IL-15 in RA and also in synovial biopsies from reactive arthritis patients, in which synovial T cells also express IL-15 (Thurkow, et al, 1997). It remains unclear whether this represents membrane bound or synthesised cytokine and *in situ* mRNA studies are awaited. Together these data clearly demonstrate that IL-15 can be expressed at the mRNA and protein level during synovial inflammation.

In normal circumstances, the interaction of T lymphocytes and macrophages is critical to generate cytokines from either cell type, 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. Data from animal arthritis models, *in vitro* synovial cultures and from recent clinical trials in RA patients indicate that TNF-α occupies a pivotal position in the regulation of synovial inflammation (Feldmann, et al, 1996). However, less is known about those

factors which in turn up-regulate TNF-α synthesis, particularly in the relative absence of IFN-γ. Although IL-15 induced TNF-α production by SF T cells *in vitro*, it appeared not to have a direct effect on macrophages, which represent the predominant source of TNF-α *in vivo*. Immunohistochemical studies clearly demonstrate juxtaposition of T cells and macrophages in RA synovial membrane, with concomitant reciprocal adhesion molecule expression, suggestive of regulatory 'cross-talk' (McInnes, et al, 1996b). Therefore, the possibility that IL-15 might induce macrophage activation through cognate interactions with activated T cells was explored. Previous studies have established that PFA-fixed T cells and T cell clones, stimulated with non-physiological mitogens (PHA, PMA, OKT3) induce proinflammatory cytokine and MMP^3 production by macrophages and fibroblasts through cell-contact (Vey, et al, 1992; Isler, et al, 1993; Lacraz, et al, 1994). Using a similar assay system in the present study, freshly isolated synovial T cells induced TNF-α synthesis by blood- or synovial-derived macrophages *ex vivo* through cell-membrane contact, with no requirement for secretory factors. This activity was maintained *in vitro* by addition of rIL-15 but not of rIL-2. Moreover, rIL-15 conferred similar properties upon $CD45RO^+$ PB T cells, such that rIL-15-activated PB T cells from RA patients induced TNF-α synthesis in synovial macrophage / synoviocyte co-cultures. Neutralisation studies implicated at least CD69, LFA-1 and ICAM-1 in this pathway. Furthermore, IL-15 was also produced by similarly activated macrophages via cell-contact.

Thus, it is attractive to hypothesise that under the continuing influence of IL-15, newly arrived T cells within the synovial membrane can up regulate TNF-α 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 TNF-α, which can further activate macrophages to produce IL-15 (Figure 3.13). In addition, T cells need have no absolute requirement for T cell cytokine production, nor for the recognition of local antigen.

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. Recently, neutrophil activation, cytoskeletal rearrangement and protection from apoptosis by IL-15 has been reported (Girard, et al, 1996). The present study demonstrated that IL-15 may also promote neutrophil recruitment and activation through enhancement of adhesion molecule (CD11b) and cytokine production. 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, up regulation of cytokine production, and neutrophil activation (Chantry, et al, 1989; Plater-Zyberk, et al, 1992; Robinson, et al, 1992a). 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 (Alleva, et al, 1997).

The CIA model provides an opportunity to study the relative contribution of immune pathways to the development of inflammatory arthritis. Using this approach, previous studies have demonstrated a role for Th1 cells and several pro-inflammatory cytokines, including TNF-α and IL-1β (Arend & Dayer, 1995; Mauri, et al, 1996). Subsequent clinical trials with neutralising antibodies against TNF-α and soluble TNF-α receptors

have demonstrated efficacy in human RA (Elliott, et al, 1994; Rankin, et al, 1995; Moreland, et al, 1997). The present data indicate that IL-15 expression is required for the induction of erosive inflammatory arthritis following challenge of collagen primed DBA/1 mice as administration of sIL-15Rα profoundly suppressed the development of CIA in these animals. Moreover, the altered serum immunoglobulin levels detected, and *in vitro* evidence for reduced spleen cell proliferation and cytokine production indicate that the Th1 collagen-specific response has been significantly modified. Taken together, these results provide *in vitro* and *in vivo* data which suggest a crucial role for IL-15 in RA pathogenesis and suggest that antagonists to this cytokine could be of therapeutic benefit.

7.2 Asthma

The present study represents the first demonstration of IL-15 expression in asthma. IL-15 staining is increased in macrophages and airway epithelial cells from steroid-naive asthmatics compared to normals. This increased expression is abolished in asthmatics receiving inhaled corticosteroids. 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, 1996a), indicating a possible role in type I bronchial hypersensitivity. In addition, IL-15 has been identified in alveolar macrophages from patients with pulmonary sarcoidosis (Agostini, et al, 1996). Moreover, it induces proinflammatory cytokine production including IL-1, IL-6 and TNF-α from activated macrophages (Alleva, et al, 1997). IL-15 production is strictly controlled (Bamford, et al, 1996b, Tagaya, et al, 1997) 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. It is therefore attractive to hypothesise, given the widespread expression of IL-15 mRNA, that failure to properly regulate IL-15 production

following an initial immunologic challenge could lead to constitutive expression, thereby disrupting immune homeostasis in the bronchial lining tissues.

NO has been implicated in physiologic regulation and in inflammation in the lung (Barnes & Belvisi, 1993; Gaston, et al, 1994). Staining for iNOS was increased in cells from steroid-naive asthmatics, and inhaled steroid therapy resulted in decreased production of NO from stimulated cells from asthmatics. There is clear evidence suggesting that T-helper cells are activated in the airway mucosa, with proliferation of Th2 cells and expression of IL-5 mRNA (Hamid, et al, 1991). NO can down regulate the Th1 cell response (Wei, et al, 1995, McInnes, et al, 1998), thus enhancing the differentiation of Th2 cells to produce IL-4, and IL-5 which are important for recruiting eosinophils into the airway. Furthermore, NO induces TNF-α synthesis by synovial tissues from RA patients, indicating that such observations likely have pathological relevance (McInnes, et al, 1996a). The observation that IL-15 and the NO donor SNAP together increase TNF-α production is of further interest raising the possibility that IL-15 and NO may together enhance Th2 responses in asthma.

NO likely has activities beyond effects on T cell differentiation. Endothelium-derived NO may be important in regulating airway blood flow and, indirectly, plasma exudation. NO is the neurotransmitter of bronchodilator nerves in human airways and counteracts the bronchoconstriction due to cholinergic neural mechanisms. iNOS is expressed in human epithelial cells in response to pro-inflammatory cytokines and oxidants, probably via activation of the transcription factor NF-kB. This is increased expression of iNOS in the airway epithelial cells and alveolar macrophages may account for the increased concentration of NO in the exhaled air of patients with inflammatory airways disease. Increased NO production in the airways may result in hyperaemia, plasma exudation, mucus secretion and indirectly in increased proliferation of Th2 lymphocytes responsible for eosinophilic inflammation. Glucocorticoids inhibit the induction of iNOS in epithelial cells and reduce the elevated

exhaled NO to normal values (Robbins, et al, 1994; Kharitonov, et al, 1996). The present study provides further evidence for direct effect of inhaled steroids on iNOS expression. Moreover, glucocorticoids inhibit the transcription of most inflammatory cytokines and chemokines in asthma. Interestingly, the increased expression of IL-15 in asthmatic cells observed in the current study was abolished in patients receiving inhaled glucocorticoids, and although the mechanism of this effect is not known, dexamethasone is known to downregulate PBMC response to IL-15 by inhibiting IL-15Rα expression, thereby blocking the high affinity IL-15 to IL-15Rα interaction (Chae, et al, 1996). This may offer another possible mechanism for the action of inhaled glucocorticoids treatment in asthma.

Conclusions

The present study investigated the role of IL-15 in chronic inflammatory responses. The principal conclusions are as follows:

Through its effects on T lymphocytes via cell-contact with monocytes and fibroblasts, IL-15 upregulated TNF-α and its own production within the rheumatoid synovial membrane. IL-15 was also capable of upregulation of adhesion molecule expression and cytokine / chemokine production by neutrophils. Finally, IL-15 blockade was effective in preventing the onset of collagen-induced arthritis, in part through modification of collagen specific immune responses. Together these data indicate an important role for IL-15 in articular inflammation.

IL-15 was expressed by epithelial and inflammatory cells induced from the human respiratory tract of asthmatic patients. Similarly iNOS expression and NO production was demonstrated from the same cell population. NO and IL-15 were synergistically capable of inducing TNF-α production in respiratory cells *in vitro*, suggesting complex interactions between NO and cytokine production in human inflammatory tissues.

Future Studies

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

- Regulation of IL-15 production - identification of molecules involved in T cell / macrophage contact leading to IL-15 secretion.

- IL-15Rα expression - to determine its distribution and regulation of its expression in chronic inflammatory sites.

- Cartilage degradation - to study whether IL-15 can modulate production of MMP, proteinases and hydrolases by activated T cells and neutrophils.

- NO in cytokine regulation - what is the role of NO in regulation of production of other proinflammatory cytokines in asthma and RA, including IL-15 and TNF-α?

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Appendix 1

Buffers

1. PBS (x10 stock)

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

2. PBS / Tween (0.05%)

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
50 µl 1M 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

Publications

1. McInnes, I.B., B.P. Leung, M. Field, X.Q. Wei, F.P. Huang, R.D. Sturrock, A. Kinninmonth, J. Weidner, R. Mumford and F.Y. Liew. (1996) Production of nitric oxide in the synovial membrane of rheumatoid and osteoarthritis patients. *J. Exp. Med.* 184:1519-1524.

2. McInnes, I.B., J. al-Mughales, M. Field, B.P. Leung, F.P. Huang, R. Dixon, R.D. Sturrock, P.C. Wilkinson and F.Y. Liew. (1996) The role of interleukin-15 in T-cell migration and activation in rheumatoid arthritis. *Nat. Med.* 2:175-182.

3. McInnes, I.B., B.P. Leung, R.D. Sturrock, M. Field and F.Y. Liew. (1997) Interleukin-15 mediates T cell-dependent regulation of tumor necrosis factor-alpha production in rheumatoid arthritis. *Nat. Med.* 3:189-195.

4. Wilson, R., I. McInnes, B. Leung, J.H. McKillop and J.J. Walker. (1997) Altered interleukin 12 and nitric oxide levels in recurrent miscarriage. *Eur. J. Obstet. Gynecol. Reprod. Biol.* 75:211-214.

5. McInnes, I.B., B. Leung, X.Q. Wei, C.C. Gemmell and F.Y. Liew. (1998) Septic arthritis following Staphylococcus aureus infection in mice lacking inducible nitric oxide synthase. *J. Immunol.* 160:308-315.

6. Xu, D., W.L. Chan, B.P. Leung, F. Huang, R. Wheeler, D. Piedrafita, J.H. Robinson and F.Y. Liew. (1998) Selective expression of a stable cell surface molecule on type 2 but not type 1 helper T cells. *J. Exp. Med.* 187:787-794.

7. Ruchatz, H., B.P. Leung, X.Q. Wei, I.B. McInnes and F.Y. Liew. (1998) Soluble interleukin-15 (IL-15) receptor α chain administration prevents murine collagen-induced arthritis - a role for IL-15 in development of antigen-induced immunopathology. *J. Immunol.* (in press)

8. Leung, B.P., L.J. Thomson, I.B. McInnes, C. McSharry, X.Q. Wei, G.W. Chalmers, N.C. Thomson and F.Y. Liew. (1998) Interleukin-15 in asthma and its effect with nitric oxide on the induction of tumour necrosis factor-α production in sputum cells. (in preparation)

9. Leung, B.P., W.A. Sands, I.B. McInnes and F.Y. Liew. (1998) Interleukin-15 induces T cell activation by a phosphatidylinositol 3-kinase dependant pathway. (in preparation)

10. Leung, B.P., K. Chaudhuri, R.J. Forsey, F.Y. Liew and I.B. McInnes. (1998) Interleukin-15 (IL-15) induces cytokine production by rheumatoid arthritis (RA) synovial neutrophils. (in preparation)

Communications published in abstract form

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

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

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

14. McInnes, I.B., B.P. Leung, R.D. Sturrock, M. Field and F.Y. Liew. (1997) Interleukin-15 mediates T cell-dependent regulation of tumor necrosis factor-alpha production in rheumatoid arthritis. *Brit. J. Rheumatol.* 36, S212.

15. Leung, B.P., K. Chaudhuri, R.J. Forsey, F.Y. Liew and I.B. McInnes. (1997) Interleukin-15 (IL-15) induces cytokine production by rheumatoid arthritis (RA) synovial neutrophils. *Arthritis Rheum.* 40:1457-1457.

16. McInnes, I.B., B.P. Leung, R.J. Forsey, E. Hack, F.Y. Liew and R.P. Tak. (1997) Interleukin-15 (IL-15) induces granzyme synthesis by rheumatoid arthritis (RA) synovial T cells. *Arthritis Rheum.* 40:1458-1458.

17. Leung, B.P., K. Chaudhuri, R.J. Forsey, F.Y. Liew and I.B. McInnes. (1998) Interleukin-15 (IL-15) induces cytokine production by rheumatoid arthritis (RA) synovial neutrophils. *Clin. Exp. Rheumatol.* 16, S16.

18. Ruchatz, H., B.P. Leung, X.Q. Wei, I.B. McInnes and F.Y. Liew. (1998) Effect of soluble Interleukin-15 receptor alpha in collagen induced arthritis. *Clin. Exp. Rheumatol.* 16, S84.