Immunomodulatory effects of interferon-alpha on T cell subsets in Behcet's disease *ex vivo*, and the *in vitro* effects of treatment on healthy donor cells

David Shih-Fan Yang

Institute of Ophthalmology, University College London and

Moorfields Eye Hospital NHS Foundation Trust

Supervisors: Professor Susan Lightman and Doctor Virginia Calder

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Declaration

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, all material which is not my own has been properly acknowledged.

Abstract

Many patients with Behcet's disease (BD) have disease that is severe and do not respond to the conventional treatment of systemic corticosteroids and immunosuppressive agents. Recently, IFN-α therapy has shown promise as an effective treatment that may also improve long-term outcome. This study aims to provide an insight into the mechanisms of action and disease-modifying ability of IFN-α by focussing on the impact on T cell subsets.

In this study, I describe my investigation into the effects of an additional 6-month course of IFN-α therapy in modulating T cell subsets and their cytokine expression in BD patients *ex vivo* over the first 12 months. Investigation of T cell responses confirms previous findings on Th1 and Th2 cells and their associated cytokines following IFN-α treatment. Analysis of Th17 cells shows these cells are downregulated by IFN-α therapy in peripheral blood. On the other hand, $Foxp3^+$ T cells are upregulated by IFN-α therapy which is a novel mechanism that may contribute substantially towards the disease-modifying ability of IFN-α treatment. Notably, most effects of IFN-α on T cells, including Th17 and Treg cells, persist even after cessation of treatment.

Also, I report in details the responses of $CD4^+$, $CD8^+$ and γδ T cell subsets *in vitro* following treatment of healthy donor PBMCs with IFN-α. The findings are largely in agreement with the *ex vivo* study. Fopx3-expressing and IFN-γ-, IL-10-, and TGF-β-producing $CD4^+$ and $CD8^+$ T cells are all increased by IFN-α treatment. Whereas, IL-17-producing $CD4^+$, $CD8^+$ and γδ T cell are decreased following treatment with IFN-α.

Our data may provide new inroads into elucidating the immunomodulatory mechanisms involved in the disease-modifying ability of IFN-α therapy. Which of the above mechanisms plays the most important role in the observed beneficial effects of IFN-α in the treatment of BD remains to be elucidated.

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Abbreviation List

aa	amino acids
Ab	antibody
Ag	antigen
AHR	aryl hydrocarbon receptor
AICD	activation-induced cell death
ANA	anti-nuclear antibody
AP-1	activator protein-1
APC	antigen presenting cell
APC	allophycocyanin
ASCA	anti-Saccharomyces cerevisiae antibodies
aTreg	activated regulatory T cells
BAFF	B cell-activating factor
BATF	basic leucine zipper transcription factor, ATF-like
Bcl	B-cell lymphoma
Brn-3b	brain-specific homeobox/POU domain protein 3b
CIA	collagen-induced arthritis
c-Maf	cellular musculoaponeurotic fibrosarcoma
CNS	central nervous system
CRKL	CT10 regulator of kinase like proteins
CRP	C-reactive protein
CTLA-4	cytotoxic T lymphocyte antigen 4
DMSO	dimethyl sulfoxide
EAE	experimental autoimmune encephalomyelitis
EAU	experimental autoimmune uveitis
EIF4EBP1	eukaryotic translation-initiation factor 4E-binding protein 1
ELISA	enzyme linked immunosorbent assay
FACS	fluorescence activated cell sorting
FasL	Fas ligand
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
Foxp3	forkhead box protein 3
g	gravity (relative centrifugal force)

α-GalCer	glycolipid α-galactosylceramide
GAS	IFN-γ activated site
GATA-3	GATA-binding protein 3
GITR	glucocorticoid-induced tumor necrosis factor receptor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HLA	human leukocyte antigen
HSP	heat shock proteins
IBD	inflammatory bowel disease
ICAM	intercellular adhesion molecule
ICOS	inducible T cell co-stimulator
IFN	interferon
Ig	immunoglobulin
IgG	immunglobulin G
IL	interleukin
ILT	immunoglobulin-like transcript
IQR	interquartile range
IRBP	interphotoreceptor binding protein
IRF	interferon regulatory factor
ISG	IFN stimulated genes
ISGF3	IFN-stimulated gene factor 3
ISRE	IFN-stimulated response elements
iTreg	inducible regulatory T cells
Jak	janus kinase
kD	kiloDalton
LPS	lipopolysaccharide
M	molar
mAb	monoclonal antibody
MAD	mothers against decapentaplegic
ME	mercaptoethanol
µg	microgram
MAPK	mitogen-activated protein kinase
MHC	major histocompatability complex
MICA	MHC class I chain-related gene A
ml	millilitre

MMP	matrix metalloproteinases
mRNA	messenger ribose nucleic acid
MS	multiple sclerosis
NF-κB	nuclear factor-κB
NFAT	nuclear factor of activated T cell
NK	natural killer cells
NKT	natural killer T cells
nTreg	naturally occurring regulatory T cells
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PD1	programmed cell death 1
PE	phycoerythrin
PEG	polyethylene glycols
PerCP	peridinin chlorophyll protein
PHA	phytohemagglutinin
PI	propidium iodide
PI3K	phosphatidylinositol 3-kinase
PMA	phorbol myristate acetate
R	receptor
RA	rheumatoid arthritis
Rap1	Ras-related protein 1
RNA	ribose nucleic Acid
ROR	retinoic acid-related orphan receptor
RPE	retinal pigment epithelium
rpm	revolutions per minute
RPMI 1640	Roswell Park Memorial Institute 1640 culture medium
rTreg	resting regulatory T cells
Runx	Runt-related transcription factor
S-Ag	retinal soluble antigen
SD	standard deviation
SEM	standard error of the mean
sFasL	soluble Fas ligand
SLE	systemic lupus erythematosus
Smad	SMA and MAD related protein

SSH	suppression subtractive hybridisation
Stat	signal transducer and activator of transcription
T-bet	T-box-expressed-in-T-cells
Tc	cytotoxic T cell
TCR	T cell receptor
TGF-β	transforming growth factor-beta
Th	helper T cell
TLR	toll-like receptor
TNF-α	tumor necrosis factor-alpha
TNF-R	tumour necrosis factor receptor
Tr1	type 1 T regulatory cells
Treg	regulatory T cells
Tresp	responder T cells
VKH	Vogt-Koyanagi-Harada syndrome
vol/vol	volume to volume ratio

Chapter 1
Introduction

1.1 Overview of the immune system

The immune system is an elaborate and dynamic network of cells, tissues, and organs that work together to defend the body against a myriad of ever-evolving pathogens, including bacteria, viruses, and parasites, as well as disease. One critical feature of the immune system is its ability to distinguish self from non-self. It detects a wide variety of harmful stimuli and through a series of steps called immune response, the immune system seeks out and destroys disease-causing organisms or substance. In most cases, it does a great job of launching attacks that eliminate invading microbes, infected cells, and tumours while ignoring healthy tissues (Janeway et al. 2005). Importantly, the immune system normally does not mount a strong immune response against self antigens, a phenomenon called self-tolerance. When the immune system recognises a self antigen and mounts a strong response against it, autoimmune disease develops and may result in a serious threat to health. Therefore, it is imperative proper balance has to be maintained between preserving immune competence and preventing self-harm and autoimmunity (Jager & Kuchroo 2010).

The immune system is composed of two major subdivisions, the innate and the adaptive immune systems, and protects individuals with layered defences of increasing specificity. Both innate and adaptive immunity depend on the ability of the immune system to distinguish between self and non-self molecules, and achieving appropriate immune response against harmful stimuli requires coordination between the two types of immunity (Janeway et al. 2005). Although autoimmune diseases are mediated by the actions of adaptive immune cells targeting self-antigen in tissues, the innate immune system plays a key role in the initiation and propagation of tissue inflammation (Codarri, Fontana, & Becher 2010).

The innate immunity system is what we are born with and it is non-specific; all antigens are attacked pretty much equally. It is genetically based and we pass it on

to our offspring. This system does not confer long-standing immunity against a pathogen. It consists of external physical barriers such as the skin and mucous membranes and also cells like phagocytes (macrophages, neutrophils, and dendritic cells), mast cells, eosinophils, basophils and natural killer cells. The complement system participates in innate immunity as well. Once activated complement can lead to increased vascular permeability, recruitment of phagocytic cells, and lysis and opsonisation of pathogens. Innate immune responses are controlled by the recognition of conserved features of pathogens using invariant pattern recognition receptors such as toll-like receptors (TLRs) expressed mainly on antigen-presenting cells (APCs). However, this early phase of defence can be overcome by many pathogens (Janeway et al. 2005).

When an innate response fails to eliminate a pathogen, the more versatile adaptive immunity is initiated by the activation of T and B lymphocytes in secondary lymphoid tissues, where dendritic cells carry antigens collected in the periphery. Innate immunity is an essential prerequisite for the adaptive immune response, because the antigen-specific lymphocytes of the adaptive immune response are activated by co-stimulatory molecules that are induced on cells of the innate immune system during their interaction with foreign organisms or substance. The cytokines produced during these early phases also play a key role in stimulating the subsequent adaptive immune response and shaping its development (Janeway et al. 2005).

The adaptive immune system is mediated by two main cell types, T and B lymphocytes, and the response is closely regulated. B cells are produced in the stem cells of the bone marrow; they produce highly specific soluble receptors termed antibodies (Ab) and oversee humoral immunity. T cells are nonantibody-producing lymphocytes which are also produced in the bone marrow but sensitised in the thymus and constitute the basis of cell-mediated immunity. Many T cell effector functions are achieved through the production of pro-inflammatory, anti-inflammatory or regulatory cytokines, which help to amplify or modulate the immune response (Janeway et al. 2005).

1.2 B and T Lymphocytes

Lymphocytes comprise approximately 20-30% of leucocytes in the circulation. However, the relative proportion of cells populations in whole blood can vary greatly between individuals and with states of health and disease, and in response to stimuli. Within the lymphocyte subset, the relative proportion of T lymphocytes and B lymphocytes in healthy adults has been reported to range from 61-85% and 7-23% respectively (Junqueira, Carneiro, & Kelley 1992;Palmer et al. 2006). Each lymphocyte expresses cell surface receptors of a single specificity, generated by the random recombination of variable receptor gene segments and the pairing of distinct variable chains. The process enables lymphocytes to deal with virtually any antigens (Janeway et al.2005).

There are three sets of molecules responsible for the specificity of acquired immune response based on their capacity to bind foreign antigen. Lymphocyte antigen receptors, in the form of immunoglobulins (Ig) on B cells and T cell receptors (TCR) on T cells, are the means by which lymphocytes recognise antigens. Ig exist in both membrane-bound forms (B-cell receptors) and secreted forms (antibodies). On the other hand, TCR are membrane-bound proteins that are able to recognise short protein sequences (peptides) that are presented by APC. Major histocompatibility complex (MHC) molecules constitute a third class of antigen-binding molecules and consist of two types: class I and class II (Janeway et al. 2005).

In the early stages of Ig and TCR production, a mechanism of genetic recombination, known as VDJ recombination or somatic recombination, takes place. Antigen receptor gene rearrangement of variable (V), diversity (D) and joining (J) gene segments generates enormous repertoire of antigen receptors with different antibody specificities, providing the versatility that is essential to normal immune functioning. Also, antigen-dependent immunoglobulin gene diversification, via somatic hypermutation (SHM), and class-switch recombination (CSR) occur in mature B cells during the humoral immune response. SHM generates point mutations in the variable domains of the immunoglobulin heavy and light chains and CSR generates different antibody isotypes by recombination (Janeway et al. 2005).

In contrast to those of Ig and TCR, the binding groove of MHC is not a product of gene rearrangement. The function of MHC molecules is to present products of self or foreign proteins to T cells. The peptides that bind to class I molecules commonly derive from proteins synthesised intracellularly, whereas class II molecules usually bind peptides derived from proteins synthesised extracellularly. The two classes of MHC molecule are different in their structure and expression pattern on tissues of the body. Besides engagement by TCR, the two types of MHC molecules are recognised by different co-receptor molecules, CD8 and CD4, during antigen recognition and the binding is necessary for the T cell to make an effective response (Janeway et al. 2005).

For both B and T cells, signalling through antigen receptors of lymphocytes induces clonal expansion and differentiation of antigen-specific lymphocytes, provided that they receive a co-stimulatory signal. Antigens that stimulate B cells can be either in solution or fixed to a solid matrix and bind to membrane Ig. Whereas, T cells are generally stimulated by small peptides and TCR do not bind antigen in solution. After priming and clonal selection of T lymphocytes bearing antigen-specific receptors, activated T cells leave the lymphoid tissues and travel to the inflammation site, where they exert specific effector functions. To provide enhanced protection against reinfection, a subset of these proliferating lymphocytes differentiates into memory cells (Janeway et al. 2005). Priming, expansion and memory formation are controlled by APCs and evidence reveals that chemokine ligands and receptors control or modulate various phases of the adaptive immune response (Viola, Contento, & Molon 2006).

1.3 T cell development

1.3.1 Thymocytes and stages of their development

The earliest progenitors of T cells are produced in the bone marrow. T, in T cell, stands for thymus, since this is the principal organ responsible for the T cell's maturation. The thymus can be divided into an outer cortex and inner medulla. Within the thymus, T cell precursors develop into thymocytes and progress through a

series of phenotypically distinct stages. Immature thymocytes are present in the outer cortex of the thymus, and mature thymocytes are present in the inner medulla of the thymus. Distinct maturational phases are marked by changes in the status of T cell receptor genes and in the expression of the T cell receptor, and by changes in expression of cell surface proteins such as the CD3 complex and the co-receptor proteins CD4 and CD8. Particular combinations of cell surface proteins can thus be used as markers for T cells at different stages of differentiation. Thymocytes are subdivided roughly as being double negative (DN), double positive (DP), or single positive (SP), based on the expression of the CD4 and CD8 coreceptors (Janeway et al. 2005).

When precursor cells first enter the thymus from the bone marrow, they do not express TCR and lack most of the surface molecules characteristic of mature T cells such as CD4 and CD8. Such cells are called DN thymocytes. The DN stage is heterogeneous and can be subdivided into four distinct subsets in mice (DN I-IV) based on the expression of CD44 and CD25. On the other hand, immature thymocytes do not express the same surface markers in humans. The corresponding human DN subpopulations are characterised by the differential expression of CD34, CD38, and CD1a. Three distinct DN stages can be recognised: a $CD34^{+}CD38^{-}CD1a^{-}$ stage that represents the most immature thymic subset, followed by the $CD34^{+}CD38^{+}CD1a^{-}$, and $CD34^{+}CD38^{+}CD1a^{+}$ stages (Dik et al. 2005).

Following the acquisition of CD1a expression, human thymocytes mature via a CD4 immature single positive (ISP) intermediate in between the DN and DP stages. With regards to DP thymocytes, there are two discrete stages of development before surface expression of CD3. Firstly, the early DP (EDP) stage is characterised by the expression of CD8α, resulting in cells that are $CD3^{-}CD4^{+}CD8\alpha^{+}\beta^{-}$. Secondly, the DP blast stage results from the upregulation of CD8β, producing $CD3^{-}CD4^{+}CD8\alpha^{+}\beta^{+}$ cells. The next stage in maturation is the single positive stage, where cells lose expression of either CD4 or CD8, leaving them either $CD4^{+}CD8^{-}$ or $CD4^{-}CD8^{+}$. Single positive thymocytes migrate to the medulla of the thymus and remain there for 1-2 weeks. They are tested for interactions with self-MHC molecules and undergo final maturational changes before release to peripheral tissues (Joachims et al. 2006).

1.3.2 αβ and γδ lineage commitment

The thymus is able to generate distinct types of mature T cells that are differentiated for specific TCR recognition and effector functions. Multipotent precursor thymocytes rearrange and express the genes encoding TCR during development and mature as either αβ or γδ lineage T cells. αβ T cells represent the majority of thymocytes in the thymus and T cells in the periphery. On the other hand, γδ T cells have been reported to be prominent during embryonic thymic development, and represent a small minority population of T cells in the peripheral blood but a major subset in other anatomical locations. The development of γδ T cells may involve also extrathymic sites (Kreslavsky et al. 2010). Since αβ and γδ cells derive from a common precursor, the issue of when the two lineages diverge during development is of considerable interest.

Previous reports have indicated that human thymocytes first rearrange their TCRδ locus, followed closely by TCRγ locus. Progenitors that productively rearrange TCRγ and TCRδ loci express functional TCRγδ at the cell surface (Dik et al. 2005). Most that are successful early in development never attempt TCRβ gene rearrangements, and the majority of them become functionally mature without progression through the DP stage and egress to the periphery with a $CD4^-CD8^-$ (or, more rarely, with $CD4^-CD8^+$ or $CD4^+CD8^-$) phenotype (Janeway et al. 2005). However, γδ developmental potential persists into the later phases of human thymocyte development until at least the $CD3^-CD4^+CD8\alpha^+\beta^-$ (EDP) stage. Even though the majority of EDP cells express intracellular TCRβ ($TCR\beta^{ic}$), a small percentage of these cells still have the capacity to yield γδ cells. Nevertheless, the γδ potential in the next developmental stage (DP blasts) is drastically diminished. Thus, it is likely that γδ potential correlates inversely with the expression of $TCR\beta^{ic}$ in thymocyte populations (Joachims et al. 2006) (Fig 1.1).

The percentage of $TCR\beta^{ic+}$ cells continue to increase as the cells mature and reach a plateau at the EDP and DP blast stages (Joachims et al. 2006) (Fig 1.1). The process in which the TCRβ chain pairs with the pre-TCRα protein to produce a pre-TCR complex that signals survival, expansion, and allelic exclusion is referred to as β-selection. Expression of pre-TCR complex leads to a burst of proliferation,

upregulation of both CD4 and CD8 coreceptors, silencing of TCRγ expression, and initiation of TCRα rearrangement (which leads to the deletion of TCRδ found within TCRα locus) (Joachims et al. 2006). Hence, β-selection results in the expression of a complete TCRαβ. However, the point of β-selection in human thymocyte development is still controversial. Though one study indicated that β-selection occurs at the EDP to DP blast transition (Carrasco et al. 1999), other studies suggested it begins as early as the $CD34^{+}CD38^{+}CD1a^{+}$ (Dik et al. 2005) or CD4 ISP stages (Blom et al. 1999).

Over time it has become clear that correspondence between the type of TCR expressed and the developmental history of a cell is not always perfect. Therefore, it is of major interest to study whether the types of TCR play a role in αβ versus γδ lineage determination or only in the progression of already committed precursors. It has been reported that the majority of γδ thymocytes have variable gene segments of TCRβ in germline configuration, and a small percentage of γδ thymocytes have complete rearrangements in the TCRβ locus. However, the few complete TCRβ rearrangements are predominately out-of-frame. As a result, whether the few γδ cells with complete in-frame TCRβ rearrangements die by apoptosis or are diverted to the αβ lineage remains to be clarified (Joachims et al. 2006).

In addition, the majority of αβ thymocytes have been reported to have both their γ alleles rearranged, and one-third of the TCRγ rearrangements in αβ thymocytes are in-frame. The potential for a significant proportion of αβ cells to express a functional TCRγδ, therefore, could not be ruled out. However, data have demonstrated that αβ thymocytes are depleted of in-frame δ rearrangements, and productive TCRγ and TCRδ rearrangements are rare in αβ cells. Consequently, it has been suggested that vast majority of cells that develop into αβ thymocytes are those that could not express a functional TCRγδ (Joachims et al. 2006).

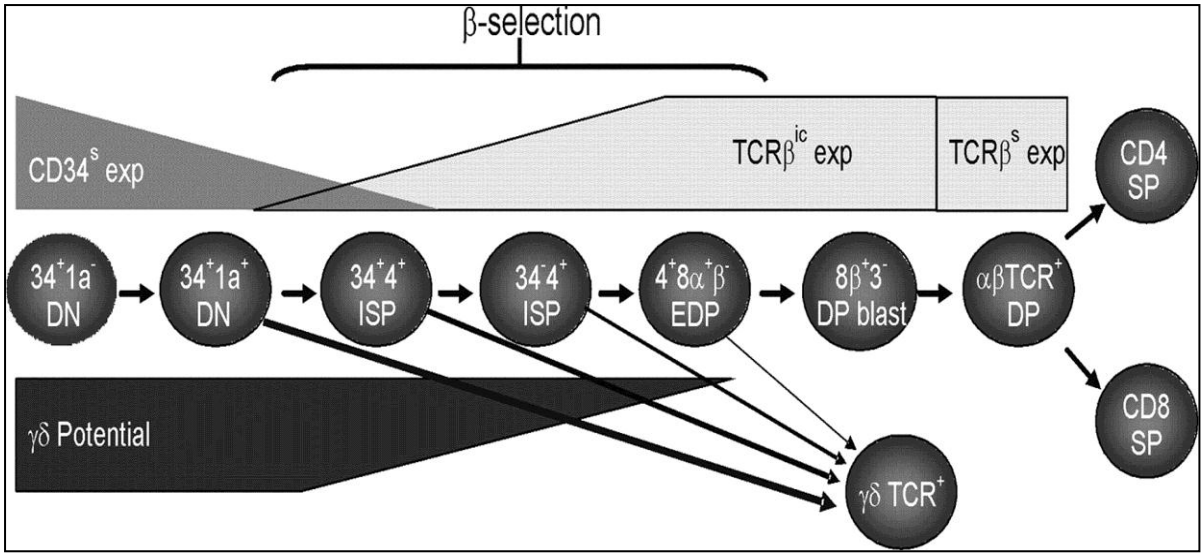


Fig 1.1: Human αβ and γδ thymocyte development

αβ and γδ thymocytes develop from a common precursor. γδ developmental potential decreases with increasing maturation, but is retained until at least the EDP stage. Decreasing γδ potential is paralleled by increasing proportions of cells expressing TCRβ^{ic}. Thus, cells undergo β-selection and commitment to the αβ lineage as they progress through several phenotypic stages (adapted from Joachims et al. 2006). TCRβ^{S} exp, surface expression of TCRβ.

Although human αβ vs γδ development is primarily influenced by the timing of TCR gene rearrangements and expression of functional TCR, other cellular factors and signalling pathways that regulate the accessibility of TCR loci and gene recombination can play a key role in lineage decisions. This is supported by reports that sustained Notch signalling efficiently skew the development of earliest thymic progenitors toward a γδ cell fate (De Smedt et al. 2002;Garcia-Peydro, de Yebenes, & Toribio 2003).

Intriguingly, in various murine transgenic and knockout systems, the TCR per se does not play a deterministic role in the lineage decision, leading to the development of γδ lineage cells driven by TCRαβ and vice versa. These puzzling observations were recently explained by the demonstration that TCR signal strength, rather than TCR type per se, instructs lineage fate, with stronger TCR signal favoring γδ and weaker signal favoring αβ lineage fates (Kreslavsky, Gleimer, & von Boehmer 2010). The potential relevance of the signal strength model of αβ/γδ lineage choice in humans is currently unknown and will require clarification by further investigation.

Collectively, development of thymocytes is a flexible and dynamic process during which CD4 and CD8 are successively upregulated. Also, αβ and γδ T cells share a large portion of their developmental paths and there is a prolonged window of development through which β-selection and γδ lineage commitment can occur simultaneously. As the development progresses through stages, the frequency of β-selected cells increases and γδ potential diminishes. Cells that survive the rigorous selective processes that operate in the central lymphoid organs are exported to the peripheral lymphoid organs before emerging as immunocompetent cells in the peripheral circulation. Upon entry to the peripheral bloodstream, the cells are considered mature T cells. With regards to effector functions, there are no major differences between γδ T cells and αβ T cells (Kreslavsky et al. 2010).

1.4 $CD4^+$ and $CD8^+$ T cells

Two different functional classes of αβ T cells are distinguished by the type of glycoprotein co-receptor (CD4 or CD8) expressed on the cell surface. $CD4^+$ and

$CD8^+$ T cells have distinct effector functions and recognise different class of MHC molecules. After engaging MHC class II molecules, $CD4^+$ T cells function to activate other effector cells of the immune system. MHC class II molecules are normally found on specialised APCs such as B lymphocytes, dendritic cells, and macrophages (Janeway et al.2005). When $CD4^+$ T cells recognise peptides bound to MHC class II molecules on B cells, they stimulate B cells to produce antibodies. In the absence of $CD4^+$ T cell help, although B cell responses can be initiated, the somatic hypermutation, isotype switching, and clonal selection necessary for production of high-affinity immunoglobulins are restricted (Castellino & Germain 2006). Moreover, $CD4^+$ T cells recognising peptides bound to MHC class II molecules on macrophages activate these cells to destroy foreign organisms or substances in their vesicles (Janeway et al. 2005).

By contrast, $CD8^+$ T cells predominantly recognise peptides presented by MHC class I molecules, which are expressed by almost all nucleated cells. $CD8^+$ T cells contribute to host defense during acute and chronic infection with viruses, intracellular bacteria, or single-cell as well as multi-cellular parasites, and they also participate in the elimination of transformed cells (Janeway et al. 2005). The differentiation of $CD8^+$ T cells is characterised by the acquisition of effector functions as well as modifications in adhesion molecule and chemokine receptor expression on the surface of the $CD8^+$ T cells. In comparison with $CD4^+$ T cells, the time required of antigen exposure to initiate proliferation is less for naive $CD8^+$ T cells, and they also divide sooner and more frequently (Seder & Ahmed 2003). Help by $CD4^+$ T cells is crucial in inducing $CD8^+$ memory, and a lack of $CD4^+$ T cell help will allow an acute but not a sustained $CD8^+$ T cell response (Castellino & Germain 2006).

In the course of immune responses, both MHC class I and MHC class II molecule expression is regulated by cytokines, in particular interferons. In the case of IFN-γ, it upregulates the expression of MHC class I and MHC class II molecules, and can promote the expression of MHC class II molecules on certain types of cells that do not normally express them. Interferons also augment the antigen presenting function of MHC class I molecules by facilitating the expression of intracellular components that allow peptides to be loaded onto MHC molecules (Janeway et al. 2005).

Ligation of the TCR and co-receptor is not enough to stimulate naive T cells to acquire effector capacity and proliferate. Co-stimulatory signal, delivered by the same APC is required as well. $CD8^+$ T cells have been suggested to need a stronger co-stimulatory signal than $CD4^+$ T cells. The two B7 glycoproteins, B7.1(CD80) and B7.2(CD86), are the best studied co-stimulatory molecules. CD28, a member of the immunoglobulin family, is the receptor for B7 molecules on the T cell. Ligation of CD28 by B7 molecules or by anti-CD28 antibodies is an absolute requirement for clonal expansion of naive T cells. After activation, naive T cells express an array of proteins that sustain or modify the co-stimulatory signal. One example is CD40 ligand (CD154), which transmits activating signals to the T cell and activates APCs to express B7 molecules after binding to CD40 on APCs. This enables further T cell proliferation as a result (Janeway et al. 2005).

1.5 Cytokines and chemokines

1.5.1 Cytokines

Cytokines are a family of pleiotropic host derived signalling proteins and peptides that provide a fundamental mechanism for cell-cell communication. They mediate and regulate immunity, inflammation, and haematopoiesis and link cells of the immune system to those in surrounding tissues. Actions of cytokines can contribute to clinical manifestations of diseases and complex interactions exist between cytokines, inflammation and the adaptive responses in maintaining homeostasis, health, and well-being (Elenkov et al. 2005). The names of the various cytokines reflect the context of their discovery, rather than a systematic naming strategy. For example, the term ‘interleukins’ was initially used as a naming system for proteins with the ability to act as communication signals between different populations of leucocytes, however, it then became recognised that the spectrum of action of interleukins was not confined to leucocytes (Janeway et al. 2005).

It is well appreciated that cytokines are produced by immunologically competent cells during host response to infection, invasion and inflammation. During development,

after injury or in tumour growth, cytokines can convey destructive or reparative signals to other cells (Stow et al. 2009). Notably, cytokines influence the quantitative, as well as the qualitative outcome of the immune response, i.e. they regulate the intensity of the inflammatory and specific immune reaction; and they determine whether the exposure to a given antigen will result in a state of specific tolerance. In addition, certain cytokines upregulate the effector function of self-aggressive cells, others participate in the suppression of such cells. Hence, cytokines are relevant to the regulation of self-tolerance (Janeway et al. 2005).

It is common for different cell types to secrete the same cytokine or for a single cytokine to produce multiple biologic effects on a variety of cells and tissues (pleiotropy). Cytokines are redundant in their activity, meaning similar functions can be stimulated by different cytokines. Cytokines can promote either induction or inhibition of the synthesis of other cytokines by their synergistic or antagonistic effects. They operate in both an autocrine and paracrine fashion to control the proliferation, differentiation, and activity of immune cells. Of note, significant quantity can be detected within the systemic circulation. This makes initiation of functional changes at tissue locations distant from the original site of synthesis possible, and thus, potentially all tissues are likely to be influenced by cytokines (Stow et al. 2009).

Cytokines typically act in cascades or networks and stimulate metabolic and biochemical changes in responding cells when present at low concentrations. They usually have a short duration of action and act over short distances, although some, such as colony stimulating factors, act at distant sites. Communication between cells can be mediated by cytokines through binding to specific cell surface receptors directly and activating intracellular second-messenger systems. Subsequent activation of protein kinases or phosphatases, in turn, regulates the activity of transcription factors that recognise specific sequences in the promoters of a variety of genes, including genes encoding message for cytokines. (Stow et al. 2009).

Depending on the cytokine, its extracellular abundance, the presence and availability of the matching receptor on the cell surface, and downstream signals activated by receptor binding, the effect of a particular cytokine on a given cell can vary. Responses to cytokines include increasing or decreasing expression of membrane

proteins (including cytokine receptors), proliferation, and secretion of effector molecules. Cytokine activity can be blocked by antagonists, molecules which bind cytokines or their receptors. Interestingly, a number of membrane-bound cytokine receptors also exist in a soluble form. Soluble cytokine receptors usually function as natural antagonists for the biological actions of the respective cytokines (Zidek, Anzenbacher, & Kmonickova 2009).

1.5.2 Chemokines

A special class of more than 50 cytokines has the generic term chemokines (derived from chemotactic cytokines) because they have a broad range of actions on the recruitment and function of specific populations of leukocytes at the sites of infection and inflammation. These proteins target many cell types and exert their biological effects by interacting with G protein-linked transmembrane receptors called chemokine receptors. Some chemokines are considered pro-inflammatory and can be induced during an immune response to recruit cells of the immune system to a site of infection, while others are considered homeostatic and are involved in controlling the migration of cells during normal processes of tissue maintenance or development (Janeway et al. 2005).

Chemokines are classified into four groups, depending on the relative position of the first N-terminal cystein residues. In the CC family (β-chemokines), the first two cysteins are adjacent; in the CXC family (α-chemokines), they are intervened by one amino acid. In the CX3C family (δ-chemokines), the first two cysteins are separated by three amino acids. The C family (γ-chemokines) contains only two of the four conserved cysteins. Chemokines are released by many different cell types and serve to guide cells of both innate immune system and adaptive immune system (Zidek, Anzenbacher, & Kmonickova 2009).

1.6 Helper T cells

The importance of distinct subsets of $CD4^+$ T lymphocytes in the aetiology of a variety of immune-mediated diseases has become clear in the last 20 years. They play a central role in immune protection and orchestrate diverse immune responses through production of cytokines and chemokines. $CD4^+$ helper T cells are important regulators of both humoral and cellular immunity, and can be differentiated from naive $CD4^+$ T cells upon antigen stimulation by professional APCs and the contextual micro-environment provided by the innate immune system (Murphy & Reiner 2002). Each subset of $CD4^+$ T lymphocytes expresses unique transcription factors which confer subset-specific expression profiles of cytokine production and effector function. $CD4^+$ T cells are capable of augmenting responses of $CD8^+$ T cells, helping B cells make antibodies, enhancing microbicidal activity in macrophages and recruiting neutrophils, eosinophils and basophils to sites of inflammation. Also, $CD4^+$ T cells are important mediators of immunologic memory (Zhu & Paul 2008).

1.6.1 Th1 and Th2 cells

In 1986, Mosmann and Coffman showed that the functional heterogeneity of murine $CD4^+$ T cells was due to their different profile of cytokine production and identified 2 main subsets of activated $CD4^+$ T cells, Th1 cells and Th2 cells (Mosmann et al. 1986). Th1 cells secrete interleukin IL-2, IFN-γ, and TNF-β, whereas Th2 cells secrete IL-4, IL-5, IL-6, and IL-10. Human $CD4^+$ T cell clones have similar, but not identical, restricted cytokine profiles. Human Th1 and Th2 subsets are usually defined according to IFN-γ/IL-4 production because the synthesis of IL-2, IL-6, and IL-10 is not stringently restricted to a single subset (Vukmanovic-Stejic et al. 2000).

Th1 cells are responsible for both phagocyte activation and the production of opsonising and complement-fixing antibodies, thus playing an important role in mediating immune responses against intracellular pathogens (Annunziato & Romagnani 2009). The principal cytokine products of Th1 cells include IFN-γ, lymphotoxin-α (TNF-β), and IL-2 (Wan 2010), and Th1 cells predominantly express chemokine receptors CXCR3 and CCR5 (Yamamoto et al. 2000). IL-12 has been

shown to play a central role in the differentiation of Th1 cells, and it is appreciated that IFN-γ also contribute to the induction of Th1 cells (Zhu, Yamane, & Paul 2010). Furthermore, stimulation of differentiated Th1 cells with IL-18 plus IL-12 induces TCR-independent IFN-γ production (Annunziato & Romagnani 2009).

IL-12, which is produced by activated APCs, activates the transcription factor, Stat4. In conjunction with TCR-dependent signals, Stat4-dependent signalling induces the expression of the transcription factor T-bet, also known as Tbx21 (Szabo et al. 2003). In addition, activation of Stat1 by IFN-γ has been shown to be important for the induction of T-bet during Th1 differentiation *in vitro* (Afkarian et al. 2002;Lighvani et al. 2001). Collectively, the outcomes of IFN-γ and IL-12 signalling are to solidify their Th1 function through promoting the expression of T-bet. T-bet belongs to the T-box family of transcription factors and is the only known T-box gene specifically expressed in the lymphoid system. It is rapidly and specifically induced in developing Th1 cells and is critical for initiating Th1 development. Thus, T-bet is recognised as a master regulator of Th1 differentiation (Szabo et al. 2000). T-bet also synergises with Stat4 to induce many Th1-specific genes, including IFN-γ, IL-12Rβ2, and IL-18R1 (Zhu, Yamane, & Paul 2010).

Conversely, Th2 cells mediate host defence against extracellular parasites including helminths and play an important role in the induction and persistence of allergic inflammatory diseases. The cytokines produced by Th2 cells include IL-4, IL-5, IL-9, IL-13, and IL-25 (Zhu & Paul 2008), and Th2 cells express chemokine receptors CCR3, CCR4, and CCR8 (Yamamoto et al. 2000). IL-4, produced by activated T cells and some innate immune cells, provides positive feedback for Th2 cell differentiation and activates Stat6, which upregulates the expression of the transcription factors GATA-3 and c-maf (Murphy & Reiner 2002). GATA-3 alone, however, is not sufficient to induce IL-4 production.

IL-2 is also important for initial IL-4 production, and Stat5 activation by IL-2 is critical for Th2 development (Annunziato & Romagnani 2009). Activated human basophils, eosinophils and mast cells express IL-25, which can upregulate GATA-3 expression in human memory Th2 cells (Wang et al. 2007). In addition, thymic stromal

lymphopoietin (TSLP) could induce naive $CD4^+$ T cells to differentiate into Th2 cells in the presence of allogeneic myeloid DCs (Lu et al. 2009).

Of note, T cells expressing both Th1 and Th2 cytokine patterns are designated Th0 and may be Th1/Th2 precursors (Miner & Croft 1998). There is a mutual regulation of Th1 and Th2 polarisation induced not only by IL-4 and IFN-γ, respectively, but also by the Th1-specific and Th2-specific transcription factors. GATA-3 has been revealed to downregulate Stat4 (Usui et al. 2003), and strong Stat5 activation suppresses T-bet expression (Zhu et al. 2003). In addition, T-bet can inhibit GATA-3 expression (Usui et al. 2006).

1.6.2 New fates of Helper T cells

In recent years, new fates of $CD4^+$ T cells have emerged. Each subset specialises in regulating different aspects of immunity and each has their own distinct cytokine products and biological functions. Some of these, such as naturally occurring regulatory T cells (nTreg) and natural killer T cells (NKT), are already distinct lineages when they leave the thymus. However, other than Th1 and Th2, naive $CD4^+$ T cells could differentiate into other distinct subsets including Th17, follicular helper T cells (Tfh), and ones that produce IL-9 (Th9) and IL-22 (Th22) only (Veldhoen 2009;Zhu & Paul 2008) (Fig 1.2).

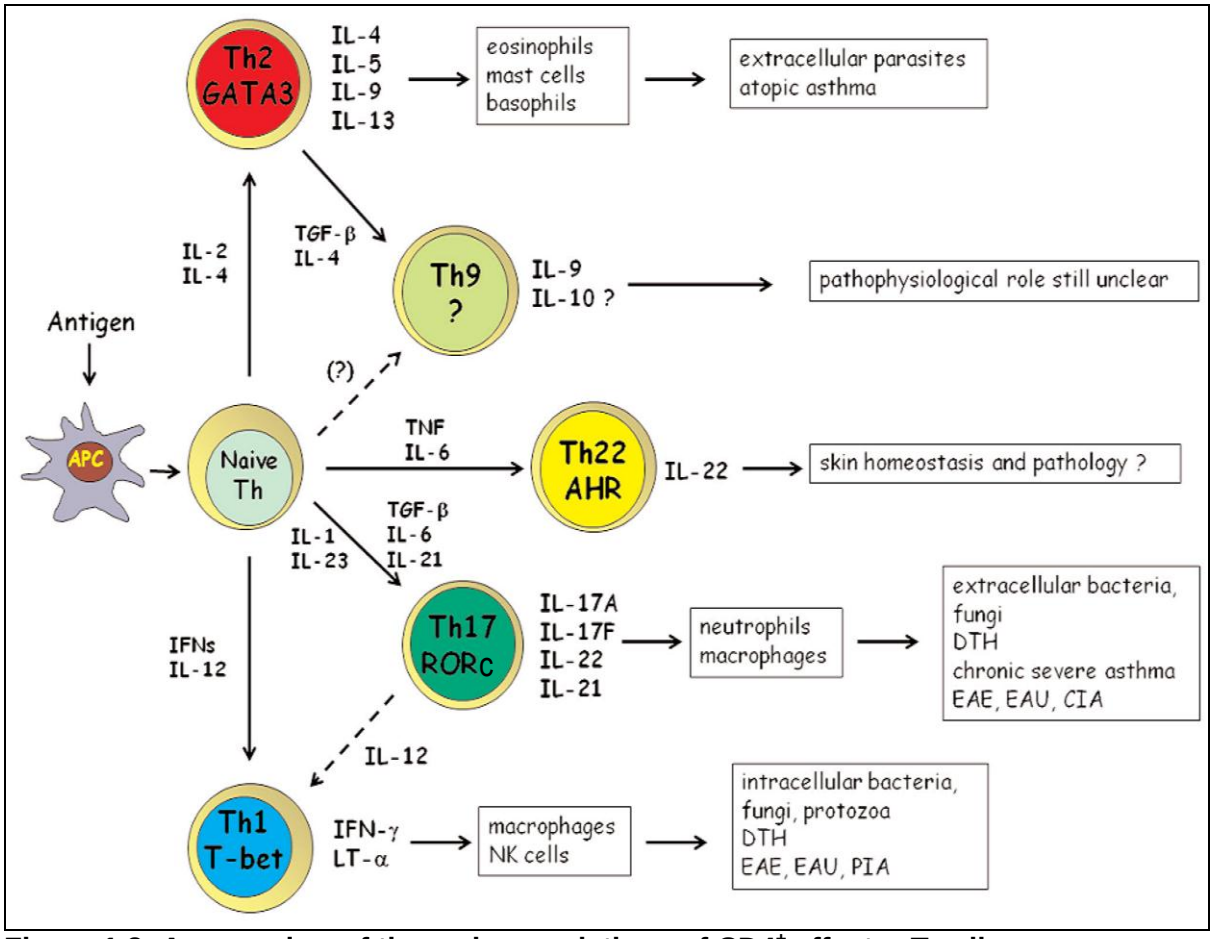


Figure 1.2: An overview of the main populations of CD4$^+$ effector T cells

When the naive CD4$^+$ T helper cell recognises a given antigen on the surface of the antigen presenting cell, the cytokines present in the microenvironment created by the response of the innate immunity play a critical role in dictating the type of effector cell that is subsequently induced (adapted from Annunziato & Romagnani 2009). DTH, delayed type hypersensitivity. EAE, experimental autoimmune encephalomyelitis. EAU, experimental autoimmune uveitis. PIA, peptoglycan-induced arthritis. CIA, collagen-induced arthritis.

Described since 2005, Th17 cells are characterised by IL-17 production and may have evolved for protection against microbes that Th1 or Th2 cells are not suited for (Harrington et al. 2005;Park et al. 2005). Although Th17 cells have been recognised as a unique subset only recently, IL-17 has been discovered for much longer. On the other hand, $CD4^+$ Tregs were originally identified by their constitutive expression of CD25 (IL-2 receptor-α) and have been referred to as $CD4^+CD25^+$ Tregs for many years (Sakaguchi et al. 1995). In 2003, forkhead box protein 3 (Foxp3) was reported to be the master transcriptional regulator for nTregs (Fontenot, Gavin, & Rudensky 2003;Hori, Nomura, & Sakaguchi 2003). As there have been substantial amount of discoveries on both Th17 and Treg due to intense interests recently, their development, function and molecular characterisation are described in more details in the following sections.

Th9 cells were proposed in 2008. TGF-β was shown to reprogram Th2 cells to lose their characteristic profile and switch to IL-9 secretion. Differentiation of these cells was able to be promoted in the presence of TGF-β and IL-4. They have not been shown to express any transcription factors related to other $CD4^+$ T cell subsets like T-bet, GATA3, RORγt and Foxp3, emphasising that Th9 cells are distinct from Th1, Th2, Th17 and $Foxp3^+$ iTreg populations (Veldhoen et al. 2008b).

Th22 cells became the latest addition to the $CD4^+$ T helper cell subsets in 2009 and they were hypothesised to be important in skin homeostasis and pathology. This distinct subset of human memory T cells was identified to express chemokine receptor CCR6, skin-homing receptors CCR4, and CCR10 and high levels of IL-22. Little IL-17 and IFN-γ were produced by these cells. Th22 cells could be induced by stimulation of naive T cells in the presence of IL-6 and TNF or by the presence of plasmacytoid dendritic cells. Compared with Th17 cells, they were less dependent on transcription factor, retinoic acid-related orphan receptor-γt (RORγt) for IL-22 production. Additionally, aryl hydrocarbon receptor (AHR) agonists were shown to promote Th22 cell differentiation and Th22 cells could be generated *in vitro* in the presence of IL-6 and TNF from naive $CD4^+$ T cells (Duhen et al. 2009;Trifari et al. 2009).

The term Tfh has been suggested for a subpopulation of B helper T cells that specifically localise to B cell areas of lymph nodes and spleen, and express CXCR5. The absence of a transcriptional master switch has long prevented the recognition of Tfh as a separate subset (Breitfeld et al. 2000;Schaerli et al. 2000). Tfh cells play a crucial role in orchestrating T cell-dependent B cell responses and can be regarded as a subset linking innate immunity and subsequent B cell responses (Reinhardt, Liang, & Locksley 2009). In peripheral blood, they were demonstrated to coexpress CD45RO and CCR7. However, CCR7 expression is lost in secondary lymphoid organs, which allows these cells to home to B-cell follicles and germinal centers where they express high levels of CD40 ligand and inducible T cell co-stimulator (ICOS). Tfh cells are also defined by enhanced expression of programmed cell death 1 (PD1), the transcriptional repressor B cell lymphoma 6 (Bcl-6) and cytokines IL-21 and IL-4, which stimulate B cell differentiation and antibody production (King 2009). Although the cytokine profile of these cells have been found to be heterogeneous, two cytokines are important in the development of Tfh: IL-6 and IL-21 (Vogelzang et al. 2008).

1.7 T helper 17 and IL-17-producing T cells

1.7.1 T helper 17 cells and associated cytokines

$CD4^+$ T cells are the best-characterised source of IL-17 during adaptive immune responses and perhaps during chronic inflammatory responses, but other cells such as neutrophils, $CD8^+$ T cells, invariant NKT cells and γδ T cells all were reported to express IL-17 (Ferretti et al. 2003;Lockhart, Green, & Flynn 2006;Michel et al. 2007;Shin et al. 1999). Human Th17 cells mediate immune responses against extracellular bacteria and fungi (Weaver et al. 2006) and produce IL-17A, IL-17F, IL-6, IL-21, IL-22, IL-26 and TNF-α, but not IFN-γ or IL-4 (Crome, Wang, & Levings 2010). IL-17A is now recognised as the founding member of a family of pro-inflammatory cytokines: IL-17A, IL-17B, IL-17C, IL-17D, IL-17E (also known as IL-25) and IL-17F (Kolls & Linden 2004). IL-17 has been known to play a role in inducing the activation of macrophages, fibroblasts, epithelial and endothelial cells and promoting the production of various inflammatory cytokines such as TNF-α, IL-1β,

and IL-6 as well as enhancing proliferation, maturation and recruitment of neutrophils (Kolls & Linden 2004;Witowski, Ksiazek, & Jorres 2004).

The inflammatory responses by IL-17 involve NF-κB and mitogen-activated protein kinase (MAPK) pathways (Schwandner, Yamaguchi, & Cao 2000;Shalom-Barak, Quach, & Lotz 1998), and most cells can potentially respond to this cytokine as the IL-17 receptor is expressed ubiquitously. Table 1.1 shows representative target cell types and the influence of cytokine IL-17. The target cell types best analysed are of non-immune origin, particularly epithelial and mesenchymal cells within diseased or inflamed tissues. IL-17-dependent activities have been revealed in immune cells as well, particularly B lymphocytes and APCs (Onishi & Gaffen 2010).

The receptor for IL-17 is a heterodimer made up of IL-17RA and IL-17RC. In contrast to IL-17RC, IL-17RA is expressed at particularly high levels in haematopoietic tissues. Both IL-17A and IL-17F use the IL-17RA chain for their signalling, but IL-17A binds to IL-17RA with much higher affinity. IL-17A and IL-17F are within close proximity on the chromosome and have a co-ordinated expression pattern, implying they have very similar functions and are likely to be regulated by similar mechanisms (Crome, Wang, & Levings 2010;Hymowitz et al. 2001). IL-21 promotes Th17 differentiation in an autocrine fashion (Nurieva et al. 2007) and serves as a positive feedback amplifier. IL-22, a member of the IL-10 family, is a multifunctional cytokine that has both pro-inflammatory and anti-inflammatory roles and can induce the expression of anti-microbial peptides (Hirota, Martin, & Veldhoen 2010). The expression of IL-22 is inhibited by TGF-β (McGeachy et al. 2007).

Table 1.1: Target cells and genes of IL-17.

IL-17 acts on a variety of cells due to its ubiquitous receptor. Shown are representative target cell types, the role of IL-17 (adverse or beneficial) and key target genes (Onishi & Gaffen 2010).

IL-17 Target Cells	Adverse condition	Beneficial condition	Target genes and note
Synoviocyte, chondrocytes 	Arthritis		MMP-1,2,3,9 and 13, PGE2,COX-2 IL-6, IL-8, TNFα, CXCL1, CXCL2 ,CCL20
Keratinocyte 	Psoriasis	Skin Infections	S100A8, S100A9 production. IL-19, 20 and 24 production increase proliferation. LL37 may interact with self DNA and activates DC. IL-23 mediated IL-22 induction induce skin thickness.
Lung/Gut Epithelial cells 		*K. pneumonia* infection in lung. Protection against *C. rodentium* .	G-SCF, CXCL1,CXCL2 to recruit neutrophil Anti-microbial peptides, 24p3,RegIIIY,β, S100A8 and S100A9 production.
Neutrophil 	Gastrointestinal fungal infection. Influenza infection.		Reduced fungicidal function, (anti-apoptosis?). Recruitment of neutrophil cause lung injury.
T cell 	$CD45RB^{hi}CD25^{-}CD4^{+}$ T-cell mediated colitis. Neuron degeneration in EAE.		Suppressing Th1 differentiation in Th0 to differentiate Th17. Brain-Blood Barrie disruption and neuronal cells degeneration by granzyme B.
B cell 	SLE	High Ig titer production.	Anti-apoptosis through bfl-1. Rgs13 and Rgs16 to stall GC formation.
DC 	Persistence of TMEV.	Vaccine against *M. uberculosis*	Inducing Bcl2 an BclX2 to prevent apoptosis. Modulating cytokine production to skew Th17. INFγ recall response.
Macrophage 		Protection against *F. tularensis* Clear *C. rodentium* infection(?)	Inducing IL-12 and IFNγ production to skew Th1 response. IL-1β,IL-9,GM-SCF, CCL3,CXCL3

1.7.2 Chemokine and other surface receptors for T helper 17 cells

Several studies have examined the pattern of chemokine and cytokine receptor expression on Th17 cells. CCR6 identifies almost all IL-17-producing T cells in human peripheral blood and $CD45RO^{+}CCR6^{+}$ peripheral blood T cells secrete 100-fold more IL-17 protein than do $CCR6^{-}$ T cells (Singh et al. 2008). However, $CCR6^{+}$ IL-17-secreting population is not homogeneous (Acosta-Rodriguez et al. 2007). Expression of CCR6 and CCR4 together identifies a homogeneous population of IL-17-secreting peripheral blood memory T cells. Whereas, CCR6 and CXCR3 together detects Th1 cells secreting IFN-γ only and $CD4^{+}$ T cells secreting both IFN-γ and IL-17. It is now generally accepted that Th17 cells express IL-23R in humans. Most recently, CD161, a C-type lectin, has been demonstrated to be a novel surface marker for human Th17 cells as well. It was observed that not only do human Th17 cells express CD161 during their life, but they also exclusively originate from a $CD161^{+}$ naive $CD4^{+}$ T cell precursor (Cosmi et al. 2008). AHR, a receptor which interacts with environmental toxins such as dioxin, is abundantly expressed in Th17 cells and is important in IL-22 expression (Veldhoen et al. 2008a). It remains to be determined whether human AHR polymorphisms exist that would link environmental pollutants with a genetic predisposition to the development of autoimmunity.

1.7.3 Transcription factors related to T helper 17 cells and associated cytokines

Stat3, the major signal transducer for IL-6, IL-21 and IL-23, is indispensable for IL-17 production and deletion of Stat3 results in the loss of IL-17 producing cells (Harris et al. 2007;Laurence et al. 2007;Mathur et al. 2007). Stat3 is also responsible for the induction of IL-23R and Foxp3 is upregulated in its absence. Like other T cell subsets, Th17 also have a lineage-specific transcription factor, namely retinoic acid-related orphan receptor gamma-t (RORγt) in mice or retinoic acid-related orphan receptor C isoform 2 (RORc2) in human (Ivanov et al. 2006). Cells deficient in these factors produce very little IL-17. Furthermore, over-expression of RORc2 induces many aspects of the Th17 cell phenotype, including expression of CCR6, CCR4, CD161 and induction of a Th17-associated cytokine profile (Crome et al. 2009). Another closely

related transcription factor, RORα, can also play a role in controlling IL-17A and IL-17F production (Yang et al. 2008c). Runx1 also interacts with RORγt and induces optimal RORγt expression and IL-17 production in Th17 cells (Zhang, Meng, & Strober 2008).

1.7.4 Positive and negative regulations of T helper 17 cells

The combination of cytokines that induce the differentiation of human Th17 cells is a subject of much debate. It is not easy to compare the results of experiments designed to investigate how human Th17 cells develop *in vitro,* because several different activation conditions, types of culture media and experimental readouts have been reported. Culturing human naive $CD4^+$ T cells from cord blood in serum-free medium revealed a need of TGF-β for Th17 differentiation (Manel, Unutmaz, & Littman 2008), but TGF-β appears to have dual effects on human Th17 differentiation in a dose-dependent manner.

While TGF-β is required for the expression of RORγt, it can inhibit the function of RORγt at high doses and favour inducible regulatory T cell differentiation (Manel, Unutmaz, & Littman 2008). However, inflammatory cytokines (IL-1β, IL-6, and IL-21 or IL-23) relieve the latter effect of TGF-β (Manel, Unutmaz, & Littman 2008). Therefore, inducible regulatory T cells and Th17 cells are reciprocally regulated during differentiation. A recent study has further explored the role of TGF-β in the development of human Th17 cells *in vitro*, and found the requirement of TGF-β is indirect, and related to suppression of Th1 development (Santarlasci et al. 2009). By using serum-free medium, it has been clarified that the optimum conditions for human Th17 differentiation are TGF-β, IL-1β, and IL-2 in combination with IL-6, and IL-21 or IL-23 (Amadi-Obi et al. 2007;Annunziato et al. 2007;Volpe et al. 2008;Yang et al. 2008a). Of the latter cytokines, IL-23 have been suggested to be the most effective (Chen et al. 2007b;Janeway et al. 2005;Manel, Unutmaz, & Littman 2008;Volpe et al. 2008;Wilson et al. 2007).

A key aspect of the control of Th17 proliferation is the regulation of IL-23R (Chen et al. 2007b). Although naive $CD4^+$ T cells express low levels of this receptor, the

combination of TGF-β, IL-1β, and IL-23 can induce IL-23R effectively (Chen et al. 2007b;Manel, Unutmaz, & Littman 2008). Thus, IL-23 promotes expression of its own receptor, enabling it to become an effective inducer of IL-17. In addition to cytokine-driven Th17 lineage commitment, it has been shown that exposure of memory $CD4^+$ T cells to prostaglandin E2 (PGE2), a mediator of tissue inflammation, results in populations that are enriched for IL-17-producing cells. PGE2, acting via the prostaglandin receptor EP2-mediated and EP4-mediated signalling and cAMP pathways, also induces upregulation of IL-23 and IL-1 receptor expression in human T cells (Boniface et al. 2009). Conversely, reduced IFN-γ production by freshly isolated memory T cells in the presence of PGE2 was observed. Therefore, PGE2 may affect the balance between the highly inflammatory cytokine IL-17 and the negative feedback loop exerted by IFN-γ considerably in chronically inflamed tissues (Napolitani et al. 2009).

Because of their potential for inducing immune-mediated damage to the host, it is perhaps not surprising that multiple mechanisms exist to inhibit Th17 production as demonstrated in murine studies. Both Th1-related IFN-γ and Th2-related IL-4 have been shown to downregulate Th17 differentiation (Harrington et al. 2005;Park et al. 2005). Recent studies have shown that IL-27, an IL-12 related cytokine, is able to inhibit Th17 development as well as induce IL-10 production from T cells (Yoshida, Nakaya, & Miyazaki 2009).

1.7.5 Co-expression of IL-17 and IFN-γ

Cells that co-express IFN-γ and IL-17 have been described in various papers and named Th17/Th1 (Acosta-Rodriguez et al. 2007; Annunziato et al. 2007). It is intriguing to note that both Th17 cells and these double-producer cells have been reported to co-express the lineage-specific transcription factors T-bet and RORC and express not only IL-23R but also IL-12Rβ2 chain. Stimulation of human Th17 clones in the presence of IL-12 can downregulate RORC and upregulate T-bet and enable these cells to produce IFN-γ in addition to IL-17A (Annunziato, Cosmi, & Romagnani 2010). Also, it has recently been demonstrated that human Th1 clones that express the Th17-associated molecule CD161, can express the transcription factor RORC

(Cosmi et al. 2010). Similar to cells that expressed IL-17 alone, these Th17/Th1 cells have been observed to be resistant to suppression of proliferation by regulatory T cells (Annunziato et al. 2007). These $CD4^+$ T cells that co-express IL-17 and IFN-γ may support the synergism between IL-17 and IFN-γ in producing tissue damage. Whether the cells producing both IL-17 and IFN-γ represent a stable population or a transitional phenotype is currently still unclear.

1.8 Regulatory T cells

Tregs play a key role in maintaining self-tolerance and in preventing immune pathologies by suppressing the strength and/or nature of the immune response to non-self antigens (Sakaguchi 2004;Shevach 2000). Treg cells were reported to represent ~5-10% of the peripheral $CD4^+$ T cells in humans (Dieckmann et al. 2001). In humans, several different types of Tregs have been described and these include specialised subsets of $CD4^+$, $CD8^+$, double-negative $CD3^+CD4^-CD8^-$, γδ T cells, and NKT cells (Allan et al. 2008b). It is likely that these Tregs interact with each other in a synergistic manner to maintain immune homeostasis and therapeutic research is most advanced for two main types of $CD4^+$ Tregs: $Foxp3^+$ and IL-10 producing type 1 T regulatory (Tr1) cells.

1.8.1 Mechanisms of action

The mechanisms of action of Tregs are complex and not well defined. These have included cell contact-mediated suppression and secretion of suppressor cytokines (IL-10, TGF-β, IL-35) (Brusko, Putnam, & Bluestone 2008;Collison et al. 2010). Treg cell-mediated suppression affects the function of APC as well. The interaction of CTLA-4 on Treg cells with CD80 and CD86 on DCs is an important pathway and inhibition of CD80 and CD86 expression by Treg cells limits the capacity of the DCs to stimulate naive T cells through CD28 resulting in immune suppression (Shevach 2009). Interactions between CTLA-4 and CD80 and CD86 can also lead to the expression of the regulatory molecule indoleamine 2,3-dioxygenase (IDO) which induces the catabolism of tryptophan into pro-apoptotic metabolites that result in the

suppression of effector T cell activation. IDO induction was found to depend on high expression of CTLA-4 on the Treg cells (Grohmann et al. 2002).

Another cell surface antigen that may play a role in Treg cell suppression of DC function is lymphocyte activation gene-3 (LAG-3), a CD4 homolog that binds MHC class II molecules with very high affinity. Binding of LAG-3 to MHC class II molecules expressed by immature DCs induces inhibition on DC maturation and immunostimulatory capacity. Due to capability of activated human T cells to express MHC class II molecules, Treg cell-mediated ligation of LAG-3 on effector cells might also result in suppression (Liang et al. 2008). One other anti-inflammatory mechanism that may be used by Treg cells is the catalytic inactivation of extracellular ATP by CD39. This allows Treg cells to quench ATP-driven pro-inflammatory processes on multiple cell types, particularly DCs (Borsellino et al. 2007).

Treg-mediated suppression can involve apoptosis and cytolysis of target cells by a variety of means. It has been reported that Treg cells can upregulate Fas and FasL expression following activation and suppress the proliferation of $CD8^+CD25^-$ cells by inducing Fas-mediated apoptosis (Strauss, Bergmann, & Whiteside 2009). Also, Human $CD4^+CD25^+Foxp3^+$ Treg cells can be activated by a combination of antibodies to CD3 and CD46 to express granzyme A and kill activated $CD4^+$ and $CD8^+$ T cells and other cell types in a perforin-dependent, Fas-FasL-independent manner (Grossman et al. 2004). Additionally, cytokine deprivation by Treg cells may contribute to their suppressive activity. It has been suggested that nTreg cells suppress conventional T cell activation through competition for IL-2 consumption, which may result in effector T cell death due to deprivation of growth factors (Shevach 2009;Wan & Flavell 2008). All these mechanisms are believed to operate collectively at the site of antigen presentation and a regulatory milieu that promotes bystander suppression and infectious tolerance is created.

The phenomenon of bystander suppression results when Tregs are first activated through their respective TCRs to induce suppressive activity, but once activated, the cells with one antigen specificity can suppress effector T cells with many other distinct antigen specificities in a non-specific manner. Infectious tolerance, on the

other hand, is a result of one population of Treg cells generating an immunoregulatory milieu that persists long after the removal of the eliciting cell population and promotes the outgrowth of a new population of Treg cells with antigen specificities distinct from those of the original Treg population (Tang & Bluestone 2008). This could explain how such a small population of cells can regulate a much larger population of responder cells *in vivo*, and the two mechanisms together allow Tregs of single specificity to establish a broad and stable immunoregulatory effects.

1.8.2 $CD4^+CD25^{+/high}Foxp3^+$ Treg cells

Human Treg cells were initially defined according to their high expression of CD25, based on the finding that murine $CD4^+CD25^+$ T cells are highly suppressive. In mice, $CD4^+CD25^+$ regulatory subset is isolated from all $CD25^+$ T cells regardless of their levels of CD25 expression. However, when similar criteria are followed to isolate these cells from human blood, the resulting $CD25^+$ cells did not consistently exhibit an anergic phenotype or significant suppressive function. This is likely due to the population being a mixture of activated effector T cells and Tregs. Further investigations led to the identification of $CD4^+CD25^{high}$ T cells which exhibited *in vitro* characteristics similar to those of the $CD4^+CD25^+$ murine regulatory cells (Baecher-Allan et al. 2001).

The $CD4^+CD25^{high}$ cells may exist in a semi-activated state *in vivo*, expressing a number of surface Ags that are usually associated with activated T cells, and these Treg cells may be primed for a fast response, or constantly turned out in order to engage in low level regulatory activity continuously. The suppressive function of these cells was reported to be cytokine-independent as the addition of blocking anti-TGF-β or anti-IL-10 Abs had no effect on the ability of $CD4^+CD25^{high}$ cells to suppress the proliferation of co-cultured $CD4^+CD25^-$ cells (Baecher-Allan et al. 2001).

More recently, different Treg populations have been described according to their origin, generation, and mechanism of actions and the Foxp3 transcription factor was identified as both a marker and a lineage commitment factor for $CD4^+CD25^+$ Tregs.

Foxp3$^+$ Treg cells exist in at least two forms. nTreg cells develop in the thymus and regulate self-reactive T cells in the periphery. Little is known about the requirements for thymic Treg cell development in humans. A second type, termed adaptive or induced regulatory T cells (iTreg), can differentiate from naive CD4$^+$CD25$^-$ T cell precursors in peripheral lymphoid organs after antigen stimulation (Bluestone & Abbas 2003).

It has been demonstrated that Foxp3$^+$ Tregs of both direct thymic and peripheral origin can specifically recognise a wide variety of Ags due to their broad αβ TCR specificity (Fazilleau et al. 2007a). nTreg and iTreg cells differ in their principal antigen specificities and strength of TCR stimulation needed for their generation. While nTregs are primarily generated in response to self-antigens expressed in the thymus, environmental antigens presented by dendritic cells in peripheral lymphoid organs are essential for iTreg development (Jordan et al. 2001). Compared with iTregs, the Foxp3 expression by nTregs are more stable, possibly due to these cells being continuously stimulated by self-antigens (Horwitz, Zheng, & Gray 2008).

In terms of the CD25 expression level, it is high in nTreg cells, but more variable in iTreg cells (Bluestone & Abbas 2003). For both types of Foxp3$^+$ Treg cells, IL-2 has important, non-redundant effects on their maintenance and survival. Evidence indicated that Stat5 activation, likely driven by IL-2 (and IL-15), is required for Foxp3 differentiation in Tregs (Burchill et al. 2008). As Tregs express little or no IL-7Rα (CD127), this in combination with high levels of CD25 expression was proposed as a way to identify Treg cells (Liu et al. 2006). Up to 85-95% of CD4$^+$CD25$^+$CD127^{lo} cells were reported to show Foxp3$^+$ expression after purification. However, like CD25, CD127 is not flawless as it is down-regulated early during T cell activation (Shevach 2006). Although other cell surface markers such as glucocorticoid-induced tumor necrosis factor receptor (GITR), CTLA-4, Neuropilin-1, and PD1 have all been described to be present on Treg cells, none of them are unique to Tregs (Brusko, Putnam, & Bluestone 2008). GITR and CTLA-4 can also be expressed on recently activated T cells.

Foxp3$^+$ cells in humans were recently reported to be a heterogeneous group in terms of their function and phenotype. They include not only suppressive T cells but also

non-suppressive ones that secrete pro-inflammatory cytokines. The main three subpopulations identified were $CD4^+CD45RA^-Foxp3^{high}$ activated Treg (aTreg) cells and $CD4^+CD45RA^+FoxP3^{low}$ resting Treg (rTreg) cells, both of which were potently suppressive *in vitro*, and non-suppressive $CD4^+CD45RA^-Foxp3^{low}$ non-Treg cells (Miyara et al. 2009).

Most of the $Foxp3^{high}$ aTreg cells were shown to originate from rTreg cells and these appeared to be terminally differentiated Treg cells. aTregs were indicated to be the main effectors of suppression which died rapidly after exertion of function. aTreg cells also suppressed the proliferation of rTreg cells in a negative feedback fashion. On the other hand, rTreg cells were demonstrated to be highly proliferative and upregulate Foxp3 expression and differentiate to aTreg cells upon stimulation (Miyara et al. 2009). Interestingly, activated and resting Tregs might operate on different suppressive mechanism as it was observed that aTreg cells were more active in IL-10 transcription but less active in TGF-β transcription than rTreg cells (Miyara et al. 2009).

1.8.3 The role of transcription factor Foxp3 in Treg cells

Foxp3 is still the best characterised Treg-specific marker, and controls some distinctive features of Tregs such as promoting the expression of CD25 (IL-2 receptor α chain), CTLA-4 and TGF-β receptor (Brusko, Putnam, & Bluestone 2008). The expression of Foxp3 has been demonstrated to correlate with suppressive ability (Yagi et al. 2004). Importantly, it is only when human cells express Foxp3 at high and constitutive levels, however, that they acquire suppressor function (Allan et al. 2008a). Although transient Foxp3 expression is also possible in $CD45RA^+$ cells with TGF-β stimulation *in vitro* following activation, its expression may not always correlate with suppressive capability (Tran, Ramsey, & Shevach 2007). Of note, not all $CD25^+$ cells are suppressive and not all $Foxp3^+$ cells are $CD25^+$ (Fontenot et al. 2005).

Foxp3 was originally suspected to be important for Treg functions in 2001 because its mutations were linked to the autoimmune scurfy mice and human

immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) (Brunkow et al. 2001;Patel 2001). Mice that lack Foxp3, either through spontaneous or targeted mutation, fail to develop Treg cells and succumb to fatal autoimmune lymphoproliferative disease. Mutations in the human Foxp3 gene, located on chromosome Xp11.23, result in IPEX syndrome, which is characterised by insulin-dependent diabetes, thyroiditis, massive T cell infiltration into the skin and gastrointestinal tract, high levels of serum autoantibodies, and chronic wasting. To date, about 20 different mutations in Foxp3 have been described in patients suffering from IPEX. Because Foxp3 is on the X chromosome, the affected population is mainly boys.

Foxp3 has three discernible functional domains, a carboxyl-termination forkhead domain (FKH; aa 338-421), a single Cys2His2 zinc finger (aa 200-223), and a leucine zipper-like motif (aa 240-261). There is also a domain at the amino terminal region that is reasonably proline-rich (aa 1-193). Mutations have been found throughout Foxp3 in IPEX patients, including in each of the domains described except for the zinc-finger, demonstrating that these regions of the protein are important for proper function (Buckner & Ziegler 2008). Moreover, Foxp3 has been shown to be part of a large macromolecular complex in Tregs. This complex includes both histone acetyltransferases (TIP-60) and histone deacetylaces (HDAC7 and HDAC). Interestingly, Foxp3 itself is also acetylated (Li et al. 2007).

In contrast to the mouse, there are two isoforms of human Foxp3, differing by the inclusion or exclusion of exon 2 (aa71-105). Although the expression levels of these two isoforms appear to be equivalent in human Treg cells, the isoform lacking exon 2, Foxp3B, is functionally different from the full-length one, Foxp3A, in interaction and suppression (Allan et al. 2005). The interaction between full-length Foxp3 and RORα inhibits transcriptional activation mediated by RORα, but not in the case of the isoform lacking exon 2 as the region of Foxp3 involved in the interaction is encoded by exon 2 (Du et al. 2008). However, either of the two isoforms can confer regulatory function when strongly overexpressed (Aarts-Riemens et al. 2008).

Despite the obvious importance of Foxp3 in the development and function of Treg cells, its mechanism of action needs to be investigated further. It has been shown so

far that Foxp3 can directly interact with NFAT, a key regulator of T cell activation and anergy, and the NF-κB subunit p65. NFAT forms complexes with the AP-1 family of transcription factors and regulates T cell activation-associated genes (Wu et al. 2006). Notably, many of the genes regulated by Foxp3 are also target genes for NFAT, including the CD25 and CTLA-4 genes which are promoted by both NFAT and Foxp3 (Hori, Nomura, & Sakaguchi 2003). Moreover, recent work has identified a crucial regulatory role for retinoic acid in promoting Foxp3 induction and inhibiting Th17 cell differentiation (Elias et al. 2008;Mucida et al. 2007).

1.8.4 IL-10 and IL-10-secreting Treg cells

IL-10 was originally identified by Mosmann and colleagues (Fiorentino, Bond, & Mosmann 1989;Moore et al. 1990). It has been known for many years that IL-10 is an immunosuppressive cytokine that can inhibit actions of many types of immune cells, suppress the progression of immune-mediated diseases and play a central role in maintaining tolerance (Moore et al. 2001). Apart from its immunosuppressive effects, IL-10 can also act as a growth factor for mast cells, promote B cell growth and differentiation, and stimulate $CD8^+$ T cell proliferation (Moore et al. 1993). IL-10 production can be promoted by IL-10 itself, IL-27 and TGF-β (Zhu & Paul 2008).

IL-10 is secreted by monocytes, stimulated macrophages, DCs, T cells, B cells, eosinophils, mast cells, keratinocytes, and epithelial cells and is normally produced late in the immune response compared with other cytokines to dampen the response by suppressing inflammatory cytokines (Moore et al. 1993;Mosser & Zhang 2008). $CD8^+$ T cells and all the $CD4^+$ T cell subsets including Th1, Th2, Th17, as well as Treg cells are capable of producing IL-10 under certain circumstances. In the case of IL-10 production by Th1 and Th17 cells, it may serve as a negative regulatory mechanism for limiting their own effector function to prevent host tissue damage (Jankovic & Trinchieri 2007). Of note, IL-10 production was reported to be independent of Foxp3 in Treg cells (Gavin et al. 2007).

The cytokine signals through a two-receptor complex consisting of two copies each of IL-10R1 and IL-10R2 (Moore et al.2001). IL-10R1 binds IL-10 with a relatively

high affinity and the recruitment of IL-10R2 to the receptor complex makes only a marginal contribution to ligand binding (Mosser & Zhang 2008). The binding of IL-10 to the receptor complex activates the Janus tyrosine kinases, JAK1 and Tyk2, associated with IL-10R1 and IL-10R2, respectively, to phosphorylate the cytoplasmic tails of the receptors. This results in the recruitment of Stat3 to the IL-10R1 (Donnelly et al. 2004). After homodimerisation of Stat3, the Stat homodimer is released from the receptor. This is followed by translocation into the nucleus and the binding of Stat 3 to Stat-binding elements in the promoters of various genes. IL-10 gene itself is upregulated by Stat3 (Staples et al. 2007). Stat3 also activates the suppressor of cytokine signaling 3 (SOCS3), which exerts negative regulatory effects on various cytokine genes (Murray 2007;O'shea & Murray 2008). Moreover, the phosphoinositide 3-kinase (PI3K) pathway is also activated by IL-10 (Mosser & Zhang 2008).

Most haematopoietic cells constitutively express low levels of IL-10R1, and receptor expression can often be considerably upregulated by various stimuli. Non-haematopoietic cells, such as fibroblasts and epithelial cells, can also upregulate IL-10R1 in response to stimuli. On the other hand, IL-10R2 is expressed on most cells, and thus a large number of diverse cells have the capacity to bind to and consume IL-10 (Mosser & Zhang 2008). Interestingly, activated T cells have been described to downregulate expression of IL-10R1 to overcome the suppressive effects of IL-10, whereas activated monocytes upregulate this molecule and become more prone to a negative feedback pathway (Liu et al. 1994;Moore et al. 2001).

Although many cell types produce IL-10, Tr1 cells characteristically produce high levels of IL-10 as well as moderate levels of IL-5, IFN-γ and TGF-β, and are one of the main T cell mediators of cytokine-dependent immune regulation (Roncarolo et al. 2006). Currently, neither a single cell surface marker nor a combination of markers could be used to track and purify Tr1 cells, thus it is difficult to isolate these cells *ex vivo* (Allan et al. 2008b). However, Tr1 cells have been reported to be induced from naive $CD4^+$ T cells activated by tolerogenic APCs in the periphery in the presence of IL-10 (Roncarolo et al. 2006). These Tr1 cells mediate suppressive effects by secreting IL-10 and TGF-β, and promote bystander suppression once activated via the TCR (Allan et al. 2008b). Although Tr1 cells from humans usually produce IFN-γ, the production levels

of the cytokine are at least 1 log lower than those produced by Th1 cells (Levings et al. 2001). Whether or not IFN-γ has any functional role remains to be determined. Apart from being able to regulate the responses of naive and memory T cells *in vitro* and *in vivo*, Tr1 cells also suppress the production of immunoglobulin by B cells (Kitani et al. 2000).

1.8.5 TGF-β and TGF-β-secreting Treg cells

TGF-β is another regulatory cytokine with a pivotal role in regulating immune responses and it has pleiotropic effects on proliferation, differentiation, migration and survival in a broad range of cell lineages. In mammals, three homologous TGF-β isoforms, TGF-β1, TGF-β2, TGF-β3, have been identified, and the effects of these three cytokines on immune cells are largely indistinguishable *in vitro*. TGF-β1 is the predominant form expressed in the immune system as lymphoid cells produce mostly TGF-β1 (Li & Flavell 2008). TGF-β is synthesised in an inactive form, pre-pro-TGF-β precursor, and additional stimuli are required to liberate active TGF-β. The active form of TGF-β can function in either a cell-surface bound form or a soluble form (Wan & Flavell 2008). TGF-β is operative as a surface bound protein on nTRegs, and blockade of cell-surface TGF-β disrupts the suppressive function of nTRegs (Abdulahad, Boots, & Kallenberg 2010).

By binding to TGF-β type I (TGF-βRI) and type II (TGF-βRII) receptors, both of which are serine/threonine kinases, TGF-β exerts its functions primarily through the Smad-dependent pathway. In addition, TGF-β transduces signals through Smad-independent pathways by activating phosphatidylinositol 3-kinase (PI3K) and various MAPKs (Li et al. 2006). TGF-β has strong anti-proliferative effects on $CD4^+$ T cells owing to its ability to inhibit IL-2 production and to upregulate cell cycle inhibitors (Gorelik & Flavell 2002). TGF-β also impedes Th1 and Th2 cell differentiation by suppressing the transcription factors required for expression of IFN-γ (T-bet) and IL-4 (GATA-3) respectively (Gorelik, Constant, & Flavell 2002;Heath et al. 2000). At the level of APCs, TGF-β can block the activation of macrophages and their ability to produce pro-inflammatory cytokines. In addition, it thwarts the maturation of DCs

and, like IL-10, inhibits upregulation of MHC class II surface expression (Gorelik & Flavell 2002).

TGF-β is produced by activated macrophages, but also by a wide variety of lymphoid and non-lymphoid cells (Wahl, McCartney-Francis, & Mergenhagen 1989). $Foxp3^+$ Treg cells also contributes significantly as a source of TGF-β and the production of this cytokine plays a crucial role in controlling autoimmune diseases (Li, Sanjabi, & Flavell 2006). Besides its well-known inhibitory activities on effector T cell differentiation and the acquisition of Th cell functions, TGF-β has an important role in the generation of Tregs. It has been shown that activation of $CD4^+$ cells in the presence of TGF-β promotes expression of CD25 and CTLA-4 (Zheng et al. 2002). Also, TGF-β activates Smad3 while TCR stimulation induces the activation of NFAT. Smad3 and NFAT collaborate to remodel the Foxp3 enhancer region and induce Foxp3 expression (Tone et al. 2008). As TGF-β induces the Foxp3 expressing $CD4^+CD25^+$ Tregs, it can therefore indirectly influence T cell activation.

T helper 3 (Th3) regulatory T cells, another subset of adaptive Tregs, express high levels of TGF-β, and are suggested to be antigen-induced regulatory T cells at the tissue site of inflammation (Chen et al. 1994). Surface marker CD25 cannot distinguish Th3 from thymic-derived Tregs as they can display this molecule after activation (Zheng et al. 2002).

1.8.6 $CD8^+$ regulatory T cells

In recent years, $CD4^+$ Treg function has become the object of intense study. Nevertheless, regulatory T cells are not strictly confined to the $CD4^+$ T cell compartment. In 1972, Gershon *et al.* published their landmark article about 'suppressor T cells' paving the way for the concept that T cells are not only able to augment but also to suppress immune response (Gershon et al. 1972). In those early years, the focus was in fact mainly on $CD8^+$ T cells. Although $CD8^+$ Tregs also contribute to immunoregulation, circulating $CD8^+Foxp3^+$ T cells constitute a minuscule fraction of peripheral $CD8^+$ T cells in humans. The relatively low frequency of $CD8^+$ Treg cells in the peripheral circulation in humans makes *in vivo*

identification and functional characterisation difficult. To date, knowledge on $CD8^+$ Tregs in general, remains scarce compared with $CD4^+$ Tregs. Research on $CD8^+$ Treg cells has also received considerably less attention (Gavin et al. 2006).

In parallel with naturally occurring and adaptive $CD4^+$ Treg cells, $CD8^+$ Treg cells may originate from both the thymus and the periphery, where they develop from naive or effector $CD8^+$ T cells (Aandahl, Torgersen & Tasken 2008). $CD8^+$ Treg cells generally express low levels of CD25 and Foxp3 and, as for $CD4^+$ Treg cells, no distinguishing marker has been identified that discriminates between naturally occurring and adaptive $CD8^+$ Treg cells (Aandahl, Torgersen & Tasken 2008).

Within $CD8^+$ T cell subset, $CD8^+CD25^+$ cells have been described to share similar phenotypic characteristics with $CD4^+CD25^+$ thymocytes, since they may be found in the same areas of human thymus and constitutively express Foxp3 and GITRmRNA. Following stimulation, $CD8^+CD25^+$ thymocytes express CTLA-4 and TGF-β1 on their surface but do not produce any cytokines or express much CD127 (Cosmi et al. 2003). Also, $CD8^+$ Treg cells have been reported to express TNFR2, and the combined expression of TNFR2 and CD25 can identify a potent subpopulation of $CD8^+$ Treg cells (Ablamunits, Bisikirska, & Herold 2010). Contact inhibition via CTLA-4 and TGF-β1 has been demonstrated to be the mechanism responsible for the suppressive capacity of $CD8^+CD25^+$ Treg cells. It has been suggested that $CD8^+CD25^+$ Treg cells may be responsible for the regulation of T cell responses directed against viruses and transformed cells, which are usually exploited by cytotoxic $CD8^+$ T lymphocytes (Cosmi et al. 2003).

Furthermore, two subsets of adaptive $CD8^+CD28^-$ Treg cells have been described to exist in humans. Antigen-specific $CD8^+CD28^-$ Treg are regulatory cells that differ from the non-antigen-specific $CD8^+CD28^-$ Treg subset by several aspects. In terms of phenotype, antigen-specific $CD8^+CD28^-$ Treg cells express both CTLA-4 and Foxp3 (Scotto et al. 2004). They act through cell-to-cell direct contact, promoting the upregulation of inhibitory ILT3 and ILT4 receptor molecules on APCs, which is essential to the tolerogenic phenotype acquired by APCs. Tolerogenic APCs show decreased expression of costimulatory molecules and induce antigen-specific unresponsiveness in $CD4^+$ T helper cells (Chang et al. 2002).

On the other hand, non-antigen-specific $CD8^+CD28^-$ Treg cells do not express Foxp3 and downregulate CD127 during their differentiation from circulating non-regulatory $CD8^+CD28^-$ T lymphocytes to Tregs. The generation process requires monocytes that secrete IL-10 after GM-CSF stimulation. GITR is involved in generation processes as well but not in suppressor function of these cells (Fenoglio et al. 2008). Their suppressive function, including one on the antigen-specific cytotoxic activity of cytotoxic T lymphocytes, is mediated by IL-10 (Filaci et al. 2004). Other types of $CD8^+$ regulatory T cells are less well characterised.

Like in CD4 T regulatory cells, CD8 Treg cells employ their suppressive function by releasing cytokines or by directly altering the functional status of T cells or APC. Secreted products and cell surface receptors facilitating suppression are only partially understood. Also, CD8 Treg cells are a heterogeneous group of subsets. Whether all these subsets derive from a common precursor or whether different types of CD8 T cells can differentiate into suppressor cells is not known.

1.9 Collaboration between transcription factors in fine-tuning T helper differentiation

The master transcription factors and the Stat proteins are indispensable for Th cell fate determination and cytokine production. Unlike the activities of master transcription factors which are mainly influenced by their expression levels, those of the Stats are controlled by cytokine-mediated post-translational modification, involving tyrosine and/or serine/threonine phosphorylation. Not only do the activated Stat proteins, in collaboration with master transcription factors, dictate the production of the key cytokines by Th cells, they also play crucial roles in the induction of the master transcription regulators (Zhu, Yamane, & Paul 2010). Other transcription factors, either constitutively expressed or induced by TCR and/or cytokine-mediated signalling, are also involved in attaining or modifying Th cell functions.

Runx proteins have been indicated to have important roles at multiple stages of T cell development and in the homeostasis of mature T cells. It was shown that Runx1

activates IL-2 and IFN-γ gene expression in conventional $CD4^+$ T cells by binding to their respective promoter. Furthermore, Runx1 interacts physically with Foxp3 protein, and it was demonstrated that this interaction might be responsible for the suppression of IL-2 and IFN-γ production and upregulation of Treg cell-associated molecules (Ono et al. 2007). Besides being important for Th2 cell differentiation, IRF4 is also indispensable for Th17 differentiation (Brustle et al. 2007). BATF, an AP-1 family transcription factor, plays a critical role in Th17 cell differentiation as it is required for RORγt induction (Schraml et al. 2009). Despite not being involved in determining T helper lineage fates, several transcription factors play a major role in cytokine production upon TCR and /or cytokine stimulation and these include members of the NFAT and Notch families.

T cells express NFAT1, NFAT2, and NFAT4. NFAT forms a complex with AP-1 and NFκB and regulates the expression of IL-2, IL-4, and IFN-γ in conventional T cells (Hu et al. 2007). NFAT proteins play key roles in modulating Th cell differentiation and are indispensable for the production of effector cytokine upon TCR activation in differentiated Th cells. TCR ligation increases intracellular Ca^{2+} concentration. Ca^{2+} binds to calmodulin, which in turn triggers the activation of calcineurin. Activated calcineurin dephosphorylates NFAT proteins, which results in the translocation of NFAT to the nucleus and subsequent induction of NFAT-dependent gene transcription (Macian 2005).

On the other hand, Notch signaling has an important co-stimulatory role in controlling optimal T cell activation as inhibition of Notch activation decreases the division of both $CD4^+$ and $CD8^+$ T cells considerably in response to TCR stimulation (Zhu, Yamane, & Paul 2010). Notch is also important in regulating differentiation of naive $CD4^+$ T cells into distinct Th lineages such as iTreg and Th17 cells. Collectively, all these molecules form a sophisticated transcription factor network, which is critical for Th cell fate determination, expansion, and function.

1.10 Plasticity of T helper subsets

$CD4^+$ T cell lineage commitment was considered as a unidirectional process in early studies. However, the discovery of new $CD4^+$ T cell fates and observation of cells with mixed phenotypes have raised the questions about the stability of $CD4^+$ T cell subsets and relationship among lineages. Recent findings suggest that $CD4^+$ T cells frequently have the capacity to redirect their programs as to achieve balance between regulatory and effector functions. This requires plasticity in the regulation of transcriptional targets and the process allows the host to shift resources as appropriate to eliminate offending pathogens and limit potential collateral tissue damage (Veldhoen 2009).

Th1 and Th2 cells antagonise each other's development and have not been observed to be converted into Th17 cells (Veldhoen 2009). However, Th17 clones were described to express T-bet, the Th1 transcription factor, and they could be shifted to Th1 by the addtion of IL-12, an effect that was partially antagonised by IL-23. This suggested a flexibility of human Th17 cells and their possible common developmental origin with Th1 cells (Annunziato et al. 2007). Also, *in vitro* generated mouse Th17 cells were able to be converted into Th2 cells in response to IL-4 (Lexberg et al. 2008). However, this has not been verified in humans.

In addition, $Foxp3^+$ Treg cells capable of IL-17 production have been identified and these cells co-express transcription factors RORc2 and Foxp3 (Koenen et al. 2008;Voo et al. 2009). Inflammatory cytokines including IL-21, IL-23 and especially IL-1β promote IL-17 production by Tregs (Koenen et al. 2008;Voo et al. 2009). Conversion of Tregs into IL-17-producing cells can be facilitated by the stimulation of APCs, especially monocytes, and IL-2 as well (Deknuydt et al. 2009;Koenen, Smeets, Vink, van Rijssen, Boots, & Joosten 2008). However, the addition of TGF-β and IL-6 has no effect (Koenen et al. 2008). The IL-17-producing $Foxp3^+$ Treg cells preferentially express CCR6 and these cells inhibit the proliferation of $CD4^+$ responder T cells *in vitro*, albeit to a lesser extent than freshly isolated Tregs (Koenen et al. 2008; Voo et al. 2009). Notably, unlike conventional Th17 cells, these $IL\text{-}17^+FoxP3^+$ T cells have not been detected to express CD161, IL-22 or TNF-α (Ayyoub et al. 2009).

After incubation with low numbers of CD40-activated allogeneic B cells, naive human $CD4^+$ T cells have been shown to preferentially differentiate into alloantigen-specific $CD4^{high}CD25^{high}$ Treg cells. These differentiated cells potently suppress effector T cell responses and express T-bet, IFN-γ, and CXCR3, the features of Th1 effector cells. As CXCR3 confers the preferential trafficking of T cells to tissue sites of IFN-γ, these Th1-like Treg cells have been suggested to be useful for regulating pathological Th1 effector responses (Zheng et al. 2010).

As yet, the functional significance of this plasticity between Th17, Th1 and $Foxp3^+$ Treg cells in humans is poorly understood. It has been suggested by promoting Th17 cells during the initial response, Treg-mediated suppression could be undermined via production of inflammatory cytokines. On the other hand, persisting inflammation might drive cells into Th1-like phenotype and increase their susceptibility to suppression by Treg cells (O'Connor, Taams, & Anderton 2009). Reprogramming of T helper cell effector functions may be critical for host defense to specific microbial challenge.

1.11 Subsets of $CD8^+$ effector T cells

Historically, $CD8^+$ T cells have been regarded as a homogeneous population of cytotoxic cells producing a limited number of cytokines. Indeed, some of the $CD8^+$ T cells can become directly cytotoxic via perforin and granzyme B secretion (Kaech & Wherry 2007). More recently, it has become clear that $CD8^+$ T cells have the potential to produce a much wider array of cytokines, and the existence of distinct subsets of $CD8^+$ T cells that are similar to their $CD4^+$ counterparts has been established (Vukmanovic-Stejic et al.2000). $CD8^+$ effector T cells designated Tc1 or Tc2, based on their production of IFN-γ or IL-4, can be generated *in vitro* with the addition of IL-12 and IL-4 respectively (Seder & Ahmed 2003). Tc1 clones have been shown to favour the development of $CD4^+$ effectors that are Th1-biased. Whereas Tc2 clones not only can promote Th2 effectors but also can efficiently suppress the development of Th1 cells (Vukmanovic-Stejic et al. 2000). With regards to *in vitro* cytolytic activity and the level of FasL expression, however, no

difference has been reported between Tc1 and Tc2 cells (Vukmanovic-Stejic et al. 2000).

Two of the best-described regulators of effector $CD8^+$ T cell differentiation are a pair of highly homologous T-box transcription factors, Eomesodermin and T-bet. Studies have indicated that Eomesodermin is responsible for regulating expression of granzyme B, perforin, and cytotoxicity (Pearce et al. 2003). T-bet, the main transcription factor controlling Th1 differentiation, have been reported to be rapidly induced in activated $CD8^+$ T cells downstream of TCR, IFN-γ and IL-12 signalling (Rutishauser & Kaech 2010). The introduction of T-bet into polarised Tc2 cells can result in the conversion of these cells into Tc1 cells with production of IFN-γ and repression of IL-4 production (Szabo et al. 2002). Also, GATA-3 has been demonstrated to be negatively correlated with the proportion of Tc1 cells and positively correlated with the percentage of Tc2 cells in peripheral blood mononuclear cells (Tan et al. 2007).

Recently, IL-17 producing $CD8^+$ T cells (Tc17) have been identified in $CD27^{+/-}$ $CD28^+CD45RA^-$ memory subsets and reported to differentiate from the same precursors as IFN-γ-producing $CD8^+$ T cells. These cells could produce IL-17 alone or in combination with IFN-γ (Kondo et al. 2009). Tc17 cells are capable of secreting high levels of IL-22 and they express cytokine receptor IL-23R (Billerbeck et al. 2010). CD161 are a relevant marker for these human IL-17 producing cells as well. In terms of chemokine receptors, they have been shown to express CCR6 and a high level of CCR5 (Kondo et al. 2009). Also, these cells are associated with transcription factors consistent with type 17 differentiation, including RORγt (RORc) and RORα (Billerbeck et al. 2010). By culturing naive $CD8^+$ T cells stimulated with anti-CD3 and anti-CD28 mAbs in the medium with IL-1β, IL-6, IL-23, and TGF-β, Tc17 cells can be induced (Kondon et al. 2009).

1.12 γδ T cells

Patients with bacterial, parasitic and viral infections often have increased numbers of γδ T cells in the peripheral blood and in autoimmune disease patients, γδ T cells are

often noted for being overrepresented among the lymphocytes that infiltrate target tissues (Chien, Jores, & Crowley 1996;Konigshofer & Chien 2006). γδ T cells not only support regeneration of epithelium but also attract neutrophils just after tissue injury so as to remove necrotic epithelial cells (Kuhl et al. 2009). Like αβ T cells, they develop in the thymus before entering the periphery. They make up a minor part of human thymocytes and T cells in the spleen, lymph nodes, and peripheral blood after birth, but are greatly enriched in epithelial tissues, such as the epidermis and mucosa of the digestive and reproductive tracts (Bonneville, O'Brien, & Born 2010). They play a major role in mucosal immunity and are induced in the early response just after innate but well ahead of the conventional adaptive immune response (Deknuydt, Scotet, & Bonneville 2009). Several γδ T cell subsets have a memory and pre-activated phenotype in naive individuals that can respond to a given challenge and enable responses to occur rapidly, without the need for prior selection and expansion. In fact, antigen-naive γδ T cells are known to produce large amount of effector cytokines within hours upon infection (Roark et al. 2008).

In support of their roles in early responses to microbial infections, studies have revealed that some human γδ T cells express functional TLRs such as TLR2 (Deetz et al. 2006;Martin et al. 2009). Unlike αβ T cells, the detection of stress-induced molecules by γδ T cells is achieved by both TCR and non-TCR molecules, such as TLRs and NK receptors that act separately, synergistically or additively to activate particular γδ T cell effector functions (Bonneville, O'Brien, & Born 2010). Of note, γδ T cells frequently express invariant or closely related γδ TCRs in a given tissue site, which differ from one tissue to another, reflecting homogeneous, but distinct, antigen recognition repertoires. γδ T cells express mostly Vγ9Vδ2 (Vγ2Vδ2 in the American nomenclature) TCR in peripheral blood but mostly Vδ1 and Vδ3 TCR in spleen and gut respectively (Deknuydt, Scotet, & Bonneville 2009). The repertoire of γδ TCRs is very limited but they directly recognise poorly defined polymorphic structures without the requirement for antigen-processing and presentation by specialised APCs. Unlike αβ T cells, human $V\delta2^+$ T cells do not recognise peptides presented by classical or non-classical MHC molecules. Instead $V\delta2^+$ T cells recognise a group of non-peptidic prenyl pyrophosphate antigens known as phosphoantigens (Sireci et al. 2001).

It has been demonstrated that γδ T cells are able to produce a range of cytokines similar to those of αβ T cells (Konigshofer & Chien 2006). Apart from the production of soluble mediators, γδ T cells usually display potent cytotoxic effector activity. Also, these cells can kill susceptible target cells by the secretory pathway through the production of perforin and granzymes, but can also make use of the receptor-ligand such as Fas/FasL pathway (Dalton et al. 2004;Narazaki et al. 2003). Vδ2 T cells generally have high cytotoxic activity and display a predominant Th1-like pattern of cytokine production as they release massive amounts of TNF-α and IFN-γ. However, certain subsets of γδ T cells, like $V\delta1^+$ T cells, have lower cytotoxic potential and produce either Th1, Th2 cytokines or IL-17 (Bonneville, O'Brien, & Born 2010;Deknuydt, Scotet, & Bonneville 2009). In contrast to the slow adaptive Th17 response, γδ T cells along with NKT cells are potent innate IL-17 producers which contribute to the initial defence against pathogens (Michel et al. 2007;Roark et al. 2008).

Several transcription factors have been described in relation to γδ T cell effector function. IFN-γ production is regulated by the transcription factors T-bet and eomesodermin (Chen et al. 2007a), IL-4 production correlates with GATA3 expression (Qi et al. 2009), and IL-17 production is controlled by RORγt (Fenoglio et al. 2009;O'Brien et al. 2010). Besides, potent regulatory properties have been reported in γδ T cells as deficiency in these cells exacerbated colitis in animal models. In one recent study, human γδ T cells with negative Foxp3 expression were shown to suppress T helper cell growth at least to the same degree as $CD4^+CD25^+$ T cells in an IL-2 independent manner and they responded poorly to CD3/CD28 stimulation. The mechanism of suppression was suggested to be related to strong TGF-β secretion, especially by the Vδ1 subset as they were found to be the main TGF-β producer compared to Vδ2 T cells (Kuhl et al. 2009). In a separate study, Vδ2 Treg cells which expressed Foxp3, was induced *in vitro* with the combination of TGF-β1, IL-15 and TCR stimulation. These $Foxp3^+$ Vδ2 T cells displayed higher levels of activated phenotype ($CD69^+$, $CD25^+$, and $HLA\text{-}DR^+$) and an upregulated expression of CTLA-4 molecules compared with the $Foxp3^-$ Vδ2 T cells. Similar to nTreg cells, cell contact appeared to be central to the inhibitory activity in these cells (Casetti et al. 2009).

1.13 NKT cells

NKT cells are a unique and relatively small subset of T cells that were originally defined to share properties of both conventional T and NK cells. They are involved in immediate immune responses, tumor rejection, immune surveillance and control of autoimmune diseases. They make up <0.1% of peripheral blood and are distinguished from NK cells by the expression of the CD3/TCR complex as well as NK cell markers, such as CD161c and CD56 (Hammond & Godfrey 2002). They are a heterogeneous population of lymphocytes and are currently divided into three categories according to their TCR α-chain diversity, CD1d dependency, and reactivity to the glycolipid α-galactosylceramide (α-GalCer). Type I classical "invariant" NKT (iNKT) cells possess invariant TCR α-chains and react to α-GalCer in a CD1d-dependent manner. Type II cells show diverse TCR α-chains but are also CD1d dependent. Type III cells are CD1d independent and possess diverse TCR α-chains. Both types II and III NKT cells do not react to α-GalCer (Rachitskaya et al. 2008).

Human iNKT cells express a Vα24-Jα18 TCR α-chain coupled with a Vβ11 β-chain and can be further allocated as $CD4^+$ iNKT, $CD8^+$ iNKT or double-negative iNKT cells (Gabriel, Morley, & Rogers 2009). Due to the constitutive expression of IFN-γ and IL-4mRNA, iNKT cells were demonstrated to produce great amounts of both Th1- or Th2-type cytokines within hours of TCR stimulation (Godfrey & Kronenberg 2004). Interestingly, innate production of IL-17 by this group of cells was discovered recently in human as well. IL-17 production was detected after purified NKT cells from healthy donors were stimulated with anti-CD3 Ab and IL-23 for 24 hours, independent of exogenous IL-6 (Rachitskaya et al. 2008). Although NKT cells potentially can act as effector cells, it is likely that regulatory function reflects their true physiologic role which is more consistent with their small numbers. Similar to $CD4^+CD25^+$ Tregs, iNKT cells have been identified to express CD25 and regulate a range of immune responses. Therefore, it was suggested that some activity attributed to $CD4^+CD25^+$ Tregs could actually be related to $CD4^+CD25^+$ NKT cells (Godfrey & Kronenberg 2004). Aberrant number and/or function of iNKT cells has been reported for various organ-specific and systemic autoimmune conditions such as RA, MS and SLE (Hammond & Godfrey 2002).

1.14 Central and peripheral tolerance

The goal of the immune system is to protect individuals from foreign agents while avoiding pathology or undesirable outcomes to self during such useful responses or owing to undesirable reactivity to self. The immune system relies on continuous generation of lymphocytes in primary lymphoid organs and hence, tolerance induction to self must be a continuous process throughout life. Because the receptors of immune cells are generated in a random process, ones with self-reactive receptors need to be eliminated (Daniel, Nolting, & von Boehmer 2009).

Self-tolerance is achieved via multiple checkpoints operating on both precursor lymphocytes during their development in primary lymphoid tissues (central tolerance) and on mature cells in secondary lymphoid tissues as well as parenchymal sites (peripheral tolerance). Self-reactive immune cells that manage to escape these checkpoints can pose a serious threat to health, as they can lead to the development of autoimmunity (Castellino & Germain 2006).

To ensure the usefulness and safety of mature T cells, developing T cells within the thymus undergo rigorous selection. During development, most of the thymocytes undergo 'death by neglect' as most TCRs cannot recognise any self-peptide/MHC molecules. If their TCRs have low affinity for self-peptide/MHC molecules, the thymocytes stay alive and expand. This positive selection ensures that mature T cells have the potential to recognise foreign peptide in association with self-MHC. At the late stage of positive selection, CD4/CD8 lineage commitment occurs and thymocytes differentiate into either single $CD4^+$ or single $CD8^+$ T cells (Daniel, Nolting, & von Boehmer 2009).

Positively selected $CD4^+$ and $CD8^+$ thymocytes test their TCRs against a broad array of self-MHC and self-peptide combinations for self-reactivity. If TCRs have a high affinity for self-peptide/MHC molecules, negative selection (deletion) occurs as the cells are induced to upregulate Bim, a protein which drives cell death by apoptosis. Negative selection removes potentially dangerous T cells that recognise self-peptides, and dendritic cells are important for the negative selection of T cells in the thymus. However, such negative selection is not a foolproof mechanism, and some

T cells capable of damaging autoimmune reactivity escape to the periphery (Daniel, Nolting, & von Boehmer 2009). Despite the processes of positive and negative selection in the thymus, all individuals still harbor a subset of cells that have TCRs directed toward self-antigens in peripheral tissues and organs. Yet, most individuals never develop autoimmune disease.

To further limit effector responses to both self as well as harmless environmental antigens, multiple mechanisms in the immune system function to control unwanted immunity. To activate mature T lymphocytes, the dose and affinity of the antigen required is higher than for immature thymocytes, which leads to activation-related ignorance of many self-peptides presented in the periphery. Also, T cell responses are regulated by requirements for simultaneous or coordinated signalling. Antigen presenting cells, in particular DCs, require stimulation through innate receptors, such as TLRs, to be highly effective in triggering naive T cells and to induce production of mediators that facilitate T cell clonal expansion, viability, and development of effector function. A requirement for multiple antigen-specific lymphocytes to interact, either directly with each other or through the intermediation of DCs, further decreases the risk of inappropriate responses to harmless antigens and limit the capacity of potentially autoaggressive cells (Castellino & Germain 2006). Another pathway involved is activation-induced cell death (Bach 2002).

In addition, some thymocytes are selected to become regulatory T cells, which are usually cells which have an intermediate affinity for self-peptide (Hogquist, Baldwin, & Jameson 2005). Peripheral tolerance mechanisms involving regulatory T cells are essential in dealing with low-affinity, self-reactive cells and keep them in check (Daniel, Nolting, & von Boehmer 2009). It is now well established that, in addition to central tolerance established as a result of induced apoptosis of strongly self-reactive cells in the thymus, peripheral tolerance exists with significant contribution coming from Treg cell populations (Littman & Rudensky 2010).

1.15 Autoimmunity

Many autoimmune diseases are driven by self-reactive T helper cells. Until recently, organ-specific autoimmune diseases were primarily associated with Th1 cells but not Th2 cells. However, the discovery of a number of new effector T cell subsets, like Th17 and Th9 cells, and regulatory T cells, like Tregs and Tr1 cells, has changed the way we view and understand autoimmunity at cellular and molecular levels. In recent years, IL-17-producing Th17 cells have emerged as major players in autoimmunity. The complicated relationship between Th1 and Th17 cells, as well as the intricate balance between Tregs and Th17 cells, provides a basis for understanding the immunological mechanisms that induce and regulate autoimmunity. Here, we give an overview of the interplay between different effector T cell subsets and regulatory T cell subsets, and how they contribute to the development of autoimmunity and tissue inflammation (Jager & Kuchroo 2010).

1.16 Autoimmune diseases and the roles of Th1/Th2 subsets

The term 'autoimmunity' denotes an adaptive immune response directed against self-antigen. Autoimmune diseases form a spectrum ranging from organ-specific conditions to systemic disorders in which the pathology is distributed throughout the body. It is estimated that 5-10% of the world population is affected by autoimmune diseases (Shoenfeld et al. 2008). The ability to discriminate between self and non-self antigens is vital to the functioning of the immune system and failure in self-tolerance can result in pathological autoimmune states. An effector phase typified by chronic inflammation and tissue damage subsequent to autoimmunity against self-antigens inevitably leads to significant morbidity and mortality. Many autoimmune diseases including RA, MS and IBD are characterised by the presence of macrophages with an "M1" classically activated phenotype at sites of inflammation. These macrophages are believed to be key mediators in immunopathology through the production of cytokines such as TNF, IL-1, and IL-6 (Mosser & Edwards 2008).

In the development of autoimmune diseases, a strong maybe deregulated Th1 response has been implicated. This is owing to the observations that the adoptive

transfer of Th1 clones and treatment with IL-12 induced or aggravated experimental autoimmune encephalomyelitis (EAE) and collagen-induced arthritis (CIA) respectively (Dardalhon et al. 2008). Also, at the peak of EAE, Th1 cytokines such as IFN-γ were readily detectable in central nervous system (CNS) inflammatory lesions but decrease during remission (Renno et al. 1995). However, it is now clear that Th1 cells are not solely responsible for driving autoimmune tissue damage in EAE and CIA. This is because exacerbated disease in the absence of IFN-γ signalling was associated with massive neutrophil and macrophage infiltrates in the CNS in EAE (Ferber et al. 1996) and a reduced IFN-γ signalling resulted in more severe disease in CIA (Matthys et al. 1998). These observations question the classic pro-inflammatory role of IFN-γ but also the Th1/Th2 paradigm as a basis for explaining the regulation of autoimmune diseases. Current evidence suggests that IFN-γ may act as a double-edged sword in immune responses and inflammatory processes as it can either promote or contain autoimmunity depending on the specific disease and the timing, location, and intensity of IFN-γ action (Kelchtermans, Billiau, & Matthys 2008).

Classically, IFN-γ has been treated as a pro-inflammatory cytokine as they play a key role in macrophage activation, inflammation and immunity. Recently, it is recognised as a pleiotropic cytokine (Figure 1.3) which has physiological importance in controlling immune and inflammatory responses. IFN-γ induces the production of cytokines, upregulates the expression of class I and II MHC antigens, Fc receptor and leukocyte adhesion molecules on a variety of cells. On the one hand, it promotes a Th1 response, not only in the effector phase but also as one of the autocrine factors favouring Th1-directed differentiation. The functional activity of macrophages is enhanced by the presence of IFN-γ and IFN-γ is known to influence isotype switching and potentiate the secretion of immunoglobulins by B cells. Moreover, it augments Th cell expansion and promotes T cell migration to the site of inflammation (Kelchtermans, Billiau, & Matthys 2008).

On the other hand, the intensity of Th1-induced inflammation potentially triggers self-regulating mechanisms in response to overt inflammation. IFN-γ has been shown to reduce tissue destruction by attenuating tissue infiltration by neutrophils and monocytes and suppressing genes responsible for tissue-destructive enzymes such

as matrix metalloproteinases (MMPs), serine proteases and enzymes related to prostaglandin metabolism (Hu & Ivashkiv 2009). Another important way is attributed to the ability of IFN-γ to downregulate IL-17 secretion (Meyers et al. 2006). Moreover, IFN-γ treatment was shown to promote negative feedback with differentiation of $CD4^+$ Treg cells as characterised by increased expression of Foxp3, from $CD4^+CD25^-$ T cells in a recent murine study. These converted cells acquired marked regulatory properties after *in vitro* treatment and showed a significant increase in the ability to inhibit autologous T cell proliferation induced by anti-CD3 and anti-CD28 antibodies (Wang et al. 2006).

Th2 cells provide optimal help for humoral immunity, especially in B cell activation and immunoglobulin isotype switching and mucosal immunity, through induction of mast cell and eosinophil differentiation and expansion. They evoke especially strong antibody responses but relatively weak cellular activity (Janeway et al. 2005). Th2-derived cytokines suppress several macrophage functions. Studies have shown that IL-4 impedes macrophage secretion of IL-1β, TNF-α, and IL-6 (Schulze-Koops & Kalden 2001). IL-10, although not an unique cytokine to Th2 cells, can also inhibit monocyte/macrophage function by potently suppressing the production of pro-inflammatory cytokines, including IL-12 and TNF-α, and downregulating the surface expression of MHC class II, which profoundly affects the ability of APCs to activate T cells (Akdis & Blaser 2001;Mosser & Zhang 2008). It has thus been suggested that skewing immune responses towards a Th2 phenotype could protect from or reduce severity of an autoimmune disease.

Nevertheless, IL-4 has no known immunosuppressive effect on either naive or resting memory Th cells. In fact, Th2-cytokines may participate in autoimmune diseases with a predominant humoral component. Th2 cytokines are implicated in the development of SLE, a human autoimmune disease characterised by T cell abnormalities and polyclonal B cell activation, as an upregulation of Th2 cytokine gene expression in this disease was described (Richaud-Patin, Alcocer-Varela, & Llorente 1995). In accordance with this finding, another study has reported that $IL\text{-}4^+$ and $IL\text{-}10^+$ cells are present more frequently in SLE patients than in normal subjects. Whereas, the proportions of $IFN\text{-}\gamma^+$ and $IL\text{-}2^+$ cells are lower in both $CD4^+$ and $CD4^-$ cells in these patients. Thus, it has been suggested that deviation of immune

responses towards a Th2 phenotype may have an important role in the induction of the polyclonal B cell activation in SLE patients (Funauchi et al. 1998).

Although other cell types could trigger auto-aggressive responses independently of $CD4^+$ T cells, a lack of cooperation from these cells would limit the damage to the host due to the reduced duration of such responses as a result. Indeed, a sustained memory $CD8^+$ T cell response and high-affinity immunoglobulin production by B cells are restricted without $CD4^+$ T cell help (Castellino & Germain 2006).

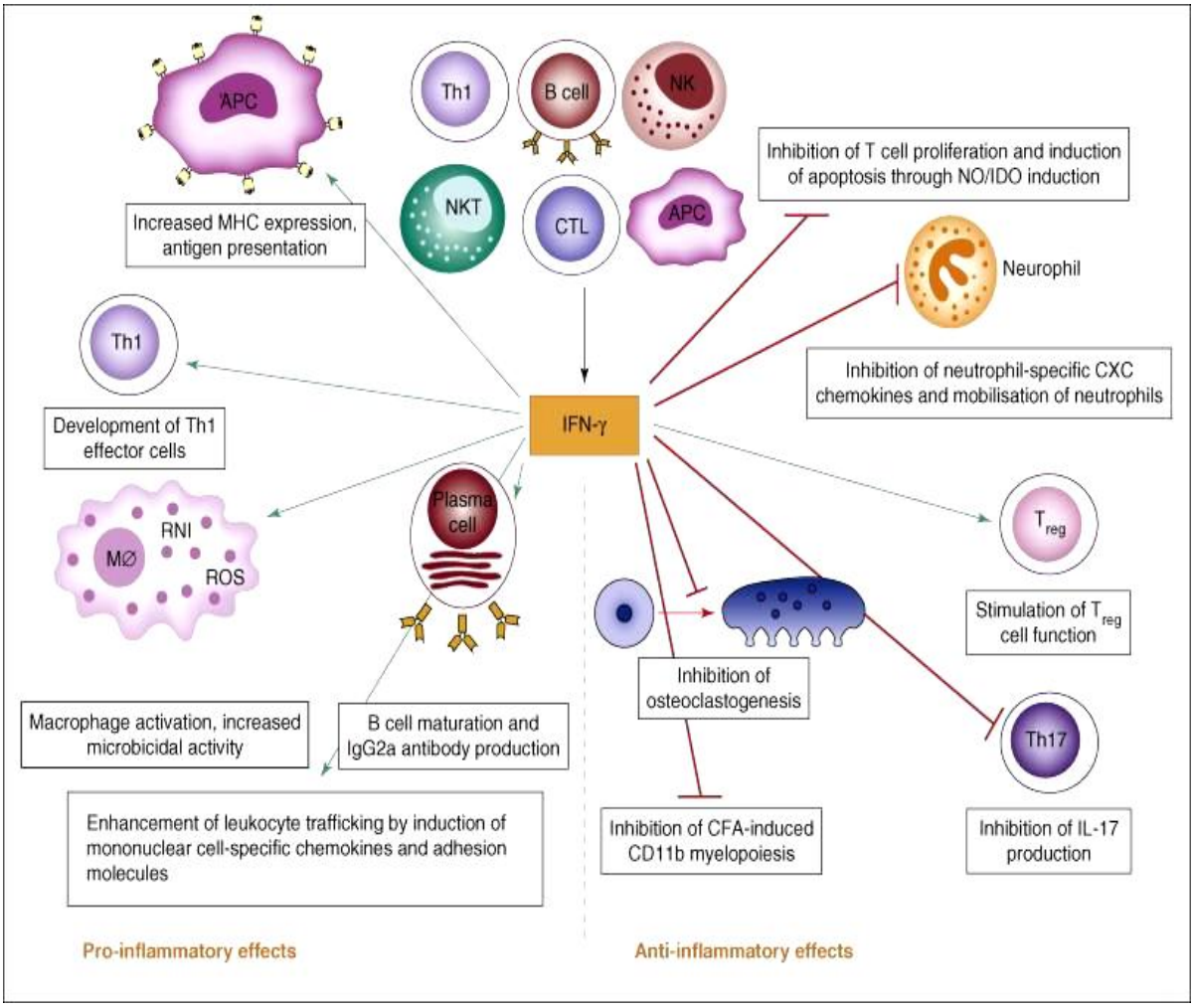


Figure 1.3: An overview of the pro- and anti-inflammatory properties of IFN-γ.

This figure summarises how IFN-**γ** can function both as an inducer and a regulator of immune responses. Red and green arrows represent, respectively, inhibitory and stimulatory actions of IFN-**γ**. Please see text for more descriptions. APC, antigen-presenting cell (including macrophages and dendritic cells); RNI, reactive nitrogen intermediates; ROS, reactive oxygen species (adapted from Kelchtermans, Billiau & Matthys 2008).

1.17 IL-23/IL-17 axis in autoimmune and inflammatory diseases

Before the discovery of Th17 subset as a distinct $CD4^+$ effector population, it was considered that Th1, Th2 and B cells were the main mediators of pathology in autoimmunity. Recent discoveries have forced immunologists to revisit and reinterpreting existing models of autoimmunity. A more balanced role for Th1 cells and IFN-γ in autoimmune diseases is also emerging as autoimmune pathology has been demonstrated to develop in the context of either a Th17 or a Th1 effector response (Luger et al. 2008). Factors that may drive the dominant effector phenotype could include quality/quantity of TLR and other innate signals, as well as the identity and diversity of cells functioning as APCs at the site of initial Ag exposure. Studies on IL-23p19-deficient mice and the anti-IL-23p19 antibody have revealed IL-23, a new IL-12 family member, as the culprit in autoimmunity (Cua et al. 2003;Hue et al. 2006;Yen et al. 2006).

IL-23 shares with IL-12 the p40 subunit - the heterodimer of IL-12 being made up of p40 and p35, and that of IL-23 being made up of p40 and p19 (Oppmann et al. 2000). Interestingly, the IL-23 receptor (IL-23R) shares with IL-12 a chain of its receptor as well - the IL-12 receptor being consisted of IL-12Rβ1 and IL-12Rβ2 chains, and that of IL-23 being consisted of IL-12Rβ1 and IL-23R chains (Parham et al. 2002). IL-23 has been shown to induce the proliferation of antigen-experienced T cells, and the resulting IL-23R-expressing Th17 cells are highly pathogenic in causing autoimmunity (Langrish et al. 2005). A not very well studied function of IL-23 is its role in promoting IFN-γ-producing Th1 cells. Current published data suggest that IL-23 affects the Th1 response, as its genetic lack or neutralisation by antibodies reduces not only the IL-17, but also the IFN-γ response to Ag (Luger et al. 2008). Therefore, IL-23 could be required to promote Th1 as well as Th17 effector responses. Furthermore, IL-10 production is counter-acted by IL-23, which helps explain the essential role of IL-23 in mediating autoimmunity despite the fact that it is dispensable for Th17 differentiation (McGeachy et al. 2007). Therefore, IL-23 is implicated as a crucial factor to inflammation and autoimmune diseases.

As IL-17 has profound pro-inflammatory effects and induces tissue damage during the course of various immune-mediated diseases, Th17 cells are increasingly being

recognised to occupy a central position in autoimmune and inflammatory diseases. After stimulation by IL-23, Th17 cells produce a variety of inflammatory mediators such as TNF-α, IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF), CXCL1 and CCL20 (Kunz & Ibrahim 2009). Th17 cells have been shown to be involved in autoimmune pathology in studies using murine models of EAE and CIA, two experimental autoimmune diseases that had been previously attributed to unchecked Th1 responses, as IL-17A-deficient mice have reduced incidence and severity of CIA and EAE (Koenders & van den Berg 2010). There have been reports of a relationship between Th17 cells and autoimmune uveitis as well (Amadi-Obi et al. 2007;Chi et al. 2007). Moreover, aberrant IL-17 expression has been described to be associated with human diseases including RA, IBD, SLE and psoriasis (Annunziato et al. 2007).

Compared to Th1 disease with predominant macrophage infiltrates, Th17 cell-mediated disease was characterised by heavy infiltrates of neutrophils (Hu & Ivashkiv 2009). It is now widely believed that the interactions between environmental exposures and genetic predisposition contribute to the onset of autoimmune disease. In genetically susceptible individuals, disease may develop after contact with environmental factors. Recently, with the discovery of the AHR expression in Th17 cells, a link between Th17 cells and the environmental theory of autoimmunity has been suggested. AHR is a transcription factor involved in direct recognition of aromatic hydrocarbons found in cigarette smokes, grilled food and industrial pollutants. Interestingly, AHR was also found in Treg, albeit at a much lower level than Th17 cells (Veldhoen et al. 2008a).

Autoreactive B and T cells are eliminated by apoptosis. As a result of defects in apoptotic signalling, autoimmune diseases can develop from persistence of self-reactive lymphocytes. IL-17, alone and especially in synergy with the B cell-activating factor (BAFF), was shown to protect B cells from apoptosis and in so doing, the proliferation, survival, and differentiation of B cells into plasma cells were promoted (Doreau et al. 2009). Like Th1 and Th2 cells, Th17 cells were found to modulate B cell function and promote production of IgM, IgG and IgA, but not IgE (Annunziato et al. 2007). Whether the ability of Th17 cells to modify B cell function directly contributes to autoimmune pathologies is to be further clarified. While most

studies on IL-17 production focused on $CD4^+$ αβ T cells, γδ T cells have also been shown to be a potent source of IL-17. Studies have reported that many γδ T cells already exist as effector memory cells, ready to mount strong IL-17 responses quickly.(Peng et al. 2008;Roark et al. 2008). In CIA, a mouse model that shares many hallmarks with human rheumatoid arthritis, the $Vγ4Vδ4^+$ subset of γδ T cells was shown to expand and produce IL-17. Depletion of these γδ T cells resulted in less severe disease (Roark et al. 2008). All these findings make a strong case for a role of IL-23/IL-17 axis in human autoimmune diseases.

1.18 Regulatory T cells and autoimmune disease

Treg cells serve to restrain overactivation of effector T cells, control the reactivity of self-aggressive T cells not eliminated in the thymus and maintain the immune system in homeostatic balance. Immune responses against pathogens must be regulated to prevent bystander immune pathology, and these mechanisms of regulation may also promote remission from autoimmune inflammatory response and prevent excessive tissue damage (Allan et al. 2008b).

The importance of T cell regulation is highlighted by autoimmunity and massive immunopathology that occur in disease states in human and mouse. In scurfy mutant mice, a frameshift mutation in Foxp3 transmitted by X-lined inheritance results in production of non-functional product and give rise to a fatal syndrome of extensive immune activation, oversecretion of cytokines and multi-organ inflammatory infiltration (Brunkow et al. 2001). In humans, IPEX is resulted from mutations in Foxp3 gene located on chromosome Xp11.23 and the rare disease is usually fatal due to poor efficacy of immunosuppressive treatment. Its reported manifestations include refractory and life-threatening diarrhoea, type1 diabetes, hypothyroidism, haemolytic anaemia, thrombocytopaenia and eczema (Bacchetta et al. 2006).

To address the question of whether immune suppression by Treg cells is impaired in the setting of human autoimmune disease, it is crucial to understand the potential means by which a defect may arise. As shown in Fig 1.4, defects in the number and

function of Treg cells, as well as a resistance of effector T cells to Treg cell-mediated suppression, could each contribute to unsuccessful T cell regulation.

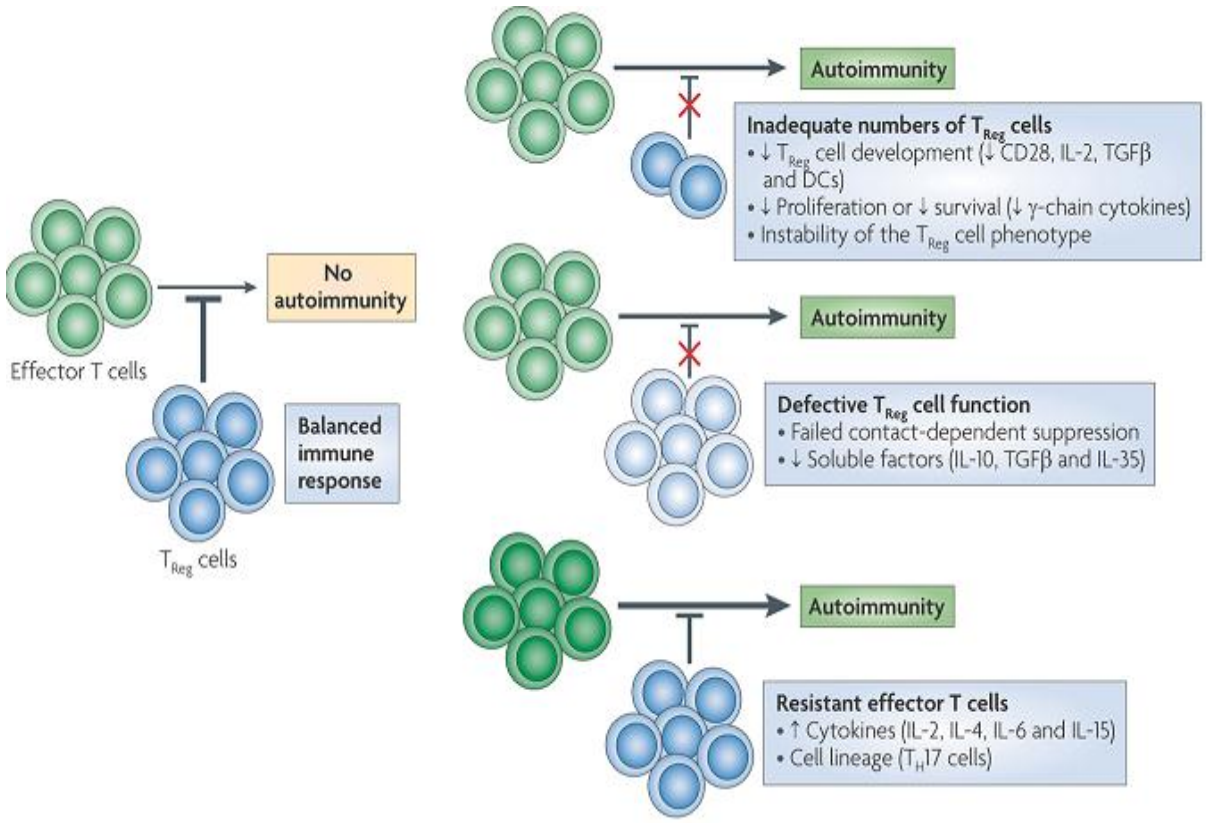


Figure 1.4: Causes of impaired Treg cell-mediated suppression in autoimmunity. Autoimmunity can result from a loss of regulation of autoreactive T cells. Failures of Treg-mediated regulation include: inadequate numbers of Treg cells; defects in Treg cell function; and resistance of pathogenic effector T cells to suppression by Treg cells owing to factors that are intrinsic to the effector cells or factors that are present in the inflammatory milieu and that support effector T cell resistance (adapted from Buckner J 2010).

The number of Treg cells found in individuals with autoimmune disease is dictated by Treg cell development, proliferation, and survival in the periphery and homing to the site of inflammation. Genetic factors are likely to have the strongest impact on thymic output of Treg cells. On the other hand, maintenance of Treg cells in the periphery is a dynamic process, influenced in part by conditions that promote Treg cell induction in the periphery and assist their proliferation and survival. Factors that favour the thymic development, peripheral growth and persistence of Treg cells, including CD28, TGF-β, dendritic cells and the common cytokine receptor γ-chain cytokines (IL-2, IL-4, IL-7 and IL-15), have also been shown to have an effect on Foxp3 expression (Buckner 2010).

Treg cell dysfunction in autoimmune disease may result from a defect in one of the many mechanisms through which Treg cells function. This could happen through diminished expression of cell surface molecules that are known to be involved in contact-dependent suppression, such as CTLA-4, CD39, lymphocyte activation gene 3 (LAG3), granzyme A and FAS or as a result of a failure to produce soluble factors, including TGF-β, IL-10 and IL-35, that are involved in aspects of suppression. Underlying genetic factors may have an impact on these mechanisms. In addition, the composition of the local milieu, such as the types of APCs and cytokines (including TNF, IL-4, IL-6, IL-12, IL-7, IL-15 and IL-21), can call influence Treg cell function (Buckner 2010).

Either reduced frequency or impaired function of $CD4^{+}CD25^{high}$ Treg cells has been reported in patients with a number of autoimmune diseases. In SLE patients, a significant drop in Treg cell numbers in untreated patients and a reduction in Foxp3 expression in patients with or without treatment were reported. Impaired Treg function in SLE patients was also revealed compared to healthy donors (Lyssuk et al. 2007). Similarly, Treg cells from peripheral blood of patients with MS showed defects in Treg function and clonal expansion. A decrease in Foxp3 levels in the Tregs were detected in MS patients as well and this correlated with deterioration of Treg function (Huan et al. 2005).

$CD4^+CD25^{high}$ Treg cells derived from active RA patients, although still anergic, displayed compromised function as they were not able to inhibit pro-inflammatory cytokine secretion from effector T cells and monocytes, or convey a suppressive phenotype to effector $CD4^+CD25^-$ T cells (Ehrenstein et al. 2004). Interestingly, higher frequencies of Treg cells were found in synovial fluid than blood in patients and these synovial cells displayed an enhanced suppressive capacity compared with cells from blood (Lawson et al. 2006;van Amelsfort et al. 2004). Diminished suppressor activity in both blood and target tissue Treg cells was demonstrated in psoriasis (Sugiyama et al. 2005). Moreover, decreased frequency and diminished function of peripheral blood $CD4^+CD25^{high}$ Treg cells were reported to be associated with active uveitis seen in Vogt-Koyanagi-Harada (VKH) syndrome (Chen et al. 2008).

Multiple mechanisms by which effector T cells resist Treg cells have been proposed. Cell-intrinsic resistance to suppression has been reported to occur in Th17 cells (Annunziato et al. 2007; Crome et al. 2009). The cytokines IL-2, IL-4, IL-7 and IL-15 support the proliferation of effector T cells in the presence of Treg cells, indicating that despite the favourable roles of these cytokines in Treg cell homeostasis, the presence of these cytokines in the short term allows effector T cells to bypass suppression by Treg cells (Yates et al. 2007). In addition, it has been suggested that Treg cells may not be able to suppress strongly activated responder T cells (Tresp) in highly inflammatory environments (Costantino, Baecher-Allan, & Hafler 2008). This has important implications in patients with autoimmune disease as autoreactive T cells are readily activated in these patients compared to healthy individuals (Baecher-Allan & Hafler 2006;Scholz et al. 1998). The report of the loss of IL-6 during autoimmune responses and the resultant significant increase in numbers of Foxp3 expressing regulatory T cells has given further supports to the theory (Korn et al. 2007).

Although adoptive transfer of Tregs has been shown to be effective in preventing or even reversing disease in animal models, there have been no clinical studies of adoptive immunotherapy with Tregs in human autoimmune diseases (Brusko, Putnam, & Bluestone 2008). An alternative to adoptive transfer of Tregs is the

promotion of de novo Treg development *in vivo*. Glatiramer acetate (GA), a drug commonly used in MS patients, has been shown to induce generation of $Foxp3^+$ Treg cells. These $Foxp3^+$ Tregs induced by GA either *in vitro* or *in vivo* show suppressive function, and GA treatment is correlated with disease improvement in patients with MS (Hong et al. 2005). Another common treatment used in MS patients is IFN-β therapy, which, in relapsing-remitting MS patients can also lead to an increase in the percentage of functional $Foxp3^+$ Tregs in the peripheral blood (de Andres et al. 2007). Type 1 diabetes is a hallmark manifestation of IPEX syndrome. The use of rapamycin can promote expansion of functional $CD4^+CD25^+Foxp3^+$ Tregs in type 1 diabetic patients, in whom an intrinsic defect in $CD4^+CD25^+$ Tregs has been reported (Monti et al. 2008).

1.19 Behcet's disease

1.19.1 Overview

The first description of Behcet's disease was attributed to the Turkish dermatologist Hulusi Behçet in 1937 who described the classical signs of the disease: oral ulcers, genital ulcers and uveitis (Bonfioli & Orefice 2005). However, it was originally described by Hippocrates 2500 years ago. BD is a chronic, multisystem immune-mediated disorder that is heterogeneous in onset and has variable organ involvement. The underlying pathological process of BD is a systemic, immune-mediated occlusive vasculitis involving small and large arteries and veins (George et al. 1997;Kalayciyan & Zouboulis 2007). It is primarily characterised by recurrent oral aphthous ulcers, genital ulcers, skin lesions, and uveitis (Sakane et al. 1999). In addition, BD patients may suffer from non-deforming arthritis, gastrointestinal tract lesions, and vascular and central nervous system problems in severe cases. Eye involvement occurs in more than 50% of the patients with BD and is the presenting symptoms in 20% of cases (Evereklioglu 2005).

The clinical course of BD is characterised by exacerbation and remission of unpredictable duration and frequency (Evereklioglu 2005). The disease can result in considerable morbidity and increased mortality. The prognosis of ocular involvement

has always been considered to be poor and it is especially true if the posterior segment is affected. Despite vigorous treatment, development of recurrent, sight-threatening ocular disease is still unpredictable, and blindness may ensue in 25% of BD patients (Houman & Hamzaoui 2006). This, along with neurological involvement, represents one of the most devastating and, for the patient, most worrying manifestations of BD. Thrombosis of the major vessels and gastrointestinal perforation also lead to poor prognosis (Evereklioglu 2005). Overall, more disease severity is linked to male patients of younger age (Zakka et al. 2009).

There are no laboratory findings specific for BD. There may be an increase in inflammatory parameters, such as C-reactive protein, erythrocyte sedimentation rate, peripheral leukocytes and platelet counts during the active phase of the disease (Evereklioglu 2005). The diagnosis of BD is based on clinical findings. Two separate diagnostic systems currently exist. One is by the International Study Group for Behcet's disease in 1990 (Table 1.2) and the other is by Behcet's Research Committee of Japan in 1974 (Table 1.3).

Table 1.2: International Study Group Criteria

(International Study Group for Behcet's Disease 1990)

Recurrent oral ulcers:

Minor aphthous, major aphthous or herpetiform ulceration observed by a physician or reported reliably by the patient that is recurrent at least 3 times in one 12-month period

Plus 2 of the following

1. Recurrent genital ulcers

   Aphthous or scarring ulceration, especially males, observed by a physician or reliably reported by the patient

2. Eye lesions

   Anterior/posterior uveitis, cells in vitreous on slit lamp, or retinal vasculitis observed by ophthalmologists

3. Skin lesions

Erythema nodosum-like lesions observed by a physician or reliably reported by the patient; pseudo-folliculitis, papulopustular or acneiform nodules observed by a physician in post-adolescent patients not receiving corticosteroids.

4. Positive pathergy test

   Read by a physician at 24-48h after a prick to the forearm by a sterile needle (the development of an aseptic non-specific hyperreactivity, which varies from an indurated erythema to pustular formation at the prick site)

Table 1.3: Revised diagnostic criteria proposed by the Behcet's Disease Research Committee of Japan in 2003

(Kurokawa & Suzuki 2004)

Main Points:

- Main symptoms:
  - Recurrent aphthous ulcers on oral mucosa
  - Skin lesions (erythema nodosum, subcutaneous thrombophlebitis, follicular papules, acneiform papules, skin hypersensitivity)
  - Ocular lesions (anterior and/or posterior uveitis)
  - Genital ulcers
- Additional symptoms:
  - Arthritis without deformity or sclerosis
  - Epididymitis
  - Gastrointestinal lesion (Ileocecal ulceration)
  - Vascular lesions
  - Central nervous system lesions
- Criteria for diagnosis of disease types:
  - Complete type:
    - Four main symptoms
  - Incomplete type
    - Three of the main symptoms, or two main symptoms and two additional symptoms
    - Typical ocular lesion and another main symptom, or two additional

symptoms

BD suspected

Some main symptoms appear, but the case does not meet the criteria for the incomplete type

Typical additional symptom is recurrent or becomes more severe

Special lesions

Gastrointestinal lesions (abdominal pain and occult blood)

Vascular lesions (vasculitis of aorta, artery, large or small veins)

Neuronal lesions (headache, paresis, lesions of brain and spinal cord, mental symptoms)

- Laboratory data

Negative or positive pathergy test

Negative or positive prick test for vaccine for streptococci

Inflammatory responses (increase of erythrocyte sedimentation rate, C-reactive protein positive, neutrophilia, increase in complement activity)

Positive for HLA-B51

Pathological findings

1.19.2 Epidemiology

BD is most commonly seen in those countries that extend from the Mediterranean to the Far East along the ancient trading route known as the ‘silk road’. It has the highest incidence in the Middle East, the Mediterranean basin and the Far East regions, but it is rare in North America and Western Europe. The prevalence is 10/100,000 in Japan and 8–38/100,000 in Turkey. However, there are only approximately 2000 patients estimated to be present in the UK (Haskard 2006). The usual onset of the disease is in the third decade. Although patients between 25 and 35 years old were the most common, BD have been reported in all ages (Zakka, et al. 2009). The complete type of Behcet’s disease has been suggested to be more common in males, whereas there is a more equal distribution between sexes for the incomplete type (Zakka et al. 2009).

1.19.3 Mucocutaneous manifestations

Almost all patients present with recurrent aphthous ulcerations of the oral mucosa. This is frequently the earliest and universal sign of the disease and can precede the other manifestations of the syndrome by many years. Minor aphthous ulcers (<10 mm in diameter) are the most common type (85%); major or herpetiform ulcers are less frequent. Although aphthae are usually multiple and occur more frequently in BD, they are indistinguishable from those of recurrent oral ulcers due to other causes (Yurdakul & Yazici 2008).

Genital ulcers are the second most commonly observed onset manifestation. They are larger and deeper than mouth lesions and have a predilection for the scrotum in the male, or the vulva in the female. Furthermore, urethritis or dysuria is not a feature of BD, unlike Reiter's syndrome and venereal disease. As genital ulcers are usually deep and painful, they affect the quality of life. The ulcers usually heal in 2-4 weeks; large ulcers frequently leave a scar whereas small ulcers and those on the minor labia heal without leaving a mark (Mat et al. 2006).

Various kinds of skin lesion occur in about 80% of patients with BD during the course of the disease. Nodular lesions are observed in 50% of patients, usually confined to the lower limbs in the form of either erythema nodosum-like lesions or superficial thrombophlebitis. Acne-like lesions or papulo-pustular lesions can also develop and these are mainly of cosmetic concern. These lesions are seen frequently not only at the usual acne sites of the face, upper chest and upper back, but also at uncommon sites such as the legs and arms. They are not considered specific for BD (Yurdakul & Yazici 2008).

The pathergy reaction is the excessive subacute inflammatory reaction of the skin to non-specific trauma such as a needle prick. In a positive reaction, a papule or pustule typically forms in 24-48 hours after insertion of a needle into the forearm skin - although whether or not pathergy occurs with formal testing depends on the size of needle and the number of sites tested. The formal pathergy test involves the introduction of a 20-gauge needle 5mm obliquely into the patient's flexor aspect of the forearm skin under sterile conditions without injection of saline. Pathergy

positivity is fairly specific to BD patients; however, its sensitivity varies in different countries. Whereas 60-70% of patients in Turkey and Japan have a positive pathergy test, it is infrequently observed in patients with BD from Northern Europe and North America (Yurdakul & Yazici 2008). In the UK, it has been seen in less than 10% of the 'definite' and 'probable' patient groups (Pickering & Haskard 2000). An alternative to pathergy test is to inject monosodium urate crystals into the forearm skin and observe the inflammatory response, which tends to be significantly prolonged in patients with BD (Pickering & Haskard 2000).

1.19.4 Ocular manifestations

Eye disease is seen in half of all patients but is more frequent and more severe among the male. It typically occurs within 3 years of disease onset. In general, initial exacerbations tend to be more anterior and unilateral, whereas subsequent attacks tend to involve the vitreous and posterior segment of the eye, becoming bilateral (Evereklioglu 2005). Anterior uveitis with hypopyon, in which the inflammatory exudates forms a visible layer of cells in the anterior chamber, is a characteristic sign of ocular BD but is only observed in one-third of patients. Common complications of the recurrent anterior segment involvement include anterior and posterior synechiae, which may lead to secondary glaucoma and cataract formation. Conjunctivitis, sicca syndrome, episcleritis, scleritis, keratitis with or without corneal ulcerations and lid lesions present rarely in BD patients (Deuter et al. 2008).

The most common and universal posterior segment findings are persistent vitritis and retinal perivasculitis that usually involve the veins (periphlebitis) and less frequently the arteries (periarteritis). Retinitis is another common finding. During the active phase of the disease, vascular leakage with diffuse retinal or optic disc oedema, venous engorgement, and intraretinal haemorrhages may be present (Evereklioglu 2005). Recurrent inflammation in the posterior eye segment may result in retinal oedema, especially macular oedema, and retinal atrophy with vascular occlusion, the main cause of loss of useful vision (Deuter et al. 2008). In advanced cases, periphlebitis may also lead to gliotic inflammatory vessel sheathing, macular

degeneration with pigment epithelial changes, scarring and epiretinal membrane formation, optic disc swelling, neovascularisation of the iris, retina and/or optic disc and tractional retinal detachment (Evereklioglu 2005). Importantly, severity and number of repeated inflammatory exacerbations of the posterior segment determine the extent of permanent ocular structural changes and, therefore, the resultant rate of irreversible visual loss.

1.19.5 Musculoskeletal manifestations

Joint disease is observed in around 50% of patients in the form of synovitis, arthritis, and/or arthralgia. It is mostly oligoarticular and asymmetrical. Joint disease resolves in a few weeks and it seldom results in deformity and radiological erosions. Chronic arthritis and osteonecrosis are seen occasionally. Arthritis in BD affects most commonly the medium and large joints. Knees are most frequently involved, followed in frequency by ankles, wrists, and elbows. Involvement of the spine and sacro-iliac joints is uncommon, and back pain is quite rare. Also, myositis can be seen in local or generalised forms (Yurdakul & Yazici 2008).

1.19.6 Cardiovascular manifestations

Vascular involvement can involve both the venous and arterial sides and is more common in men than in women. One-third of patients have thrombophlebitis of either the deep or the superficial veins, usually of the lower extremities. It is generally migratory in nature, appearing simultaneously at several locations (Yates & Michelson 2006). Importantly, BD can lead to thrombotic occlusion of the venous system of the upper limbs, the inferior and superior vena cava, the dural sinuses and the hepatic (Budd-Chiari syndrome) and renal veins (Pickering & Haskard 2000). Despite the high frequency of thrombophlebitis, thromboembolism is rare, most probably due to adherence of thrombi to the diseased veins.

Arterial disease is less frequent but is a serious cause of morbidity and mortality, especially when it involves the pulmonary arteries. Arterial involvement predisposes

to aneurysm formation or less commonly arterial thrombosis and it can involve the entire arterial tree. The most frequently affected vessels, in decreasing order, are the pulmonary, femoral, popliteal, subclavian and carotid arteries (Al Otaibi, Porter, & Poate 2005). Thrombosis has been suggested to relate to vascular inflammation and the resulting endothelial dysfunction (Deuter et al. 2008).

Overall, cardiac involvement is rare. However, sporadic cases have been reported with pericarditis, valvular lesions, myocarditis, intracardiac thrombosis, endomyocardial fibrosis, coronary artery involvement and ventricular aneurysms (Marshall 2004). Available data currently show that atherosclerosis is probably not increased in BD (Yurdakul & Yazici 2008).

1.19.7 Central nervous system manifestations

The neurological complications of BD predominantly involve the CNS, although, rarely, there may be involvement of muscle and peripheral nerves (Al Otaibi, Porter, & Poate 2005). Headaches are common and often migrainous in nature (Pickering & Haskard 2000). CNS involvement occurs in 5-10% of patients and is one of the most serious manifestations of BD, causing both increased morbidity and mortality (Sakane et al. 1999). The natural history is extremely variable and characterised by fluctuations that may mimic multiple sclerosis. The outcome varies from full recovery to a slowly progressive, intermittent course resulting in paralysis and death. Pathologic examination can show softening of both white and gray matter with foci of demyelination in the brain and nerves (Yates & Michelson 2006).

CNS disease of BD can be divided into two categories, namely, parenchymal and non-parenchymal (vascular). Most patients (80%) have parenchymal brain involvement, which mainly affects the brainstem, manifested by pyramidal, followed by cerebellar and sensory symptoms and signs, sphincter disturbances and behavioural changes. Non-parenchymal disease (20%) takes the form of intracranial hypertension due to dural sinus thrombosis manifested by headaches and papilloedema. Simultaneous involvement of the dural sinuses and brain parenchyma

is unusual. Dural sinus thrombosis has a relatively benign prognosis in comparison to parenchymal involvement (Yurdakul & Yazici 2008).

Psychological disturbances can be present at some point in at least half of BD patients. Changes include memory impairment, dementia, irritability, hallucination, delusions, anxiety, depression, and insomnia. These disturbances sometimes lead to suicide attempts (Yates & Michelson 2006).

1.19.8 Gastrointestinal manifestations

The involvement of the gastrointestinal tract is very variable in different populations and can be present in up to 30% of BD patients. BD shares many of the features of the inflammatory bowel disease and differentiation is difficult (Deuter et al. 2008). The symptoms include anorexia, vomiting, dyspepsia, diarrhoea and abdominal pain. Mucosal ulcerations can occur at any point along the gastrointestinal tract and they are most commonly seen in the ileum, followed by the caecum and other parts of the colon. The ileocaecal ulcers have a distinct tendency to perforate (Yurdakul & Yazici 2008).

1.19.9 Renal manifestations

Several renal disorders have been associated with BD and can be divided into five groups, namely, (1) glomerulonephritis, (2) amyloidosis, (3) renal vascular involvement, (4) interstitial nephritis, and (5) other problems such as complications of drug therapy or genitor-urinary system abnormalities (Akpolat et al. 2002).

1.20 Aetiopathogenesis of Behcet's disease

Despite research in the last decades, the aetiology and pathogenesis of BD remains unclear. A complex genetic background leading to a pro-inflammatory, innate immune system-derived activation, perpetuated by the adaptive immune response of

T cells against infectious- and/or auto-antigens has been suggested in BD pathogenesis (Direskeneli 2006).

1.20.1 Autoimmune features of Behcet's disease

BD does not present like a classical autoimmune disease with anti-nuclear antibody (ANA) positivity, female dominance or association with other autoimmune diseases (Yazici 1997). In contrast to most classical autoimmune disorders which have an MHC class II association, a class I antigen, HLA-B51, is associated with BD (Direskeneli 2001). However, there are features that suggest BD as being 'autoimmune'. A general B cell activation and autoantibodies against cell surface antigens such as anti-endothelial cell (AECA) and anti-lymphocyte antibodies have been demonstrated (Direskeneli et al. 1995;Eksioglu-Demiralp et al. 1999b). Interestingly, PBMC from BD patients with active disease were shown to be highly sensitive to spontaneous and Fas-mediated AICD after PHA activation, which indicate chronic *in vivo* activation (Frassanito et al. 1999).

1.20.2 Genetic background

Among various genetic markers, the association of BD with HLA-B5 and its subclass B51 allele on chromosome 6p21, provides the strongest evidence supporting the involvement of genetic factors in BD pathogenesis in high prevalence countries. The HLA-B51 association is most pronounced in patients from the Middle and the Far East (Verity et al. 1999). Of these, HLA-B5101 and HLA-B5108 are particularly associated with increased risk of developing BD (Deuter et al. 2008). Recently, the first genome-wide association study of BD results clearly showed a dual, independent contribution of two HLA alleles to BD's pathogenesis: HLA-B5101 and HLA-A26 (Meguro et al. 2010). Additionally, HLA-B5701 has been identified to be associated with disease susceptibility in Caucasian patients in the United Kingdom, carrying a relative risk of disease equivalent to that of HLA-B51 (Ahmad et al. 2003). However, the direct role of HLA-B5 in BD development remains unclear.

Another important finding of the recent genome-wide association studies was discovery of the IL-10 gene association with BD. One study found two single nucleotide polymorphisms (rs1800871 and rs1800872) in the promoter region of the IL-10 gene giving the strongest association result in Japanese patients (Mizuki et al. 2010). Another study identified an intronic polymorphism in a high linkage disequilibrium block of the IL-10 gene region (rs1518111). This variation was associated with lower mRNA epression as well as decreased production of IL-10 from mononuclear cells/monocytes following stimulation with LPS or muramyl dipeptide+PAM_3Cys, respectively (Remmers et al. 2010). The association between IL-10 polymorphism and BD helps at least partially explaining the dysregulated/exaggerated inflammatory response observed in BD patients as IL-10 is an essential cytokine in the regulation of both innate and adaptive immune responses.

Another shared genetic association in Turkish and Japanese BD patients was located within an intergenic region between the IL-23R and IL-12RB2 genes, which was demonstrated to be in linkage disequilibrium block of the IL-23R gene (Mizuki et al. 2010; Remmers et al. 2010). IL-23 has been shown to stimulate Th17 cell proliferation and increase the production of inflammatory cytokines including IL-1, IL-6, IL-17 and TNF-α (Langrish et al. 2005). On the other hand, IL-12 plays a key role in Th1 responses, T-cell and NK-cell cytotoxicity, and IFN-γ production by T cells and NK cells (Annunziato & Romagnani 2009).

Gene in linkage disequilibrium with HLA-B51, such as MHC class I chain-related gene A (MICA), has been indicated to contribute to pathogenesis of BD (Mizuki et al. 2000). The link between BD and TNF-α gene, which is closely linked to the HLA-B51 gene, has also been investigated recently and TNF-α-1031C allele was reported to be associated with susceptibility to BD (Akman et al. 2008). Moreover, IL-1, IL-12, IFN-γ and TGF-β1 gene polymorphisms were all found to be increased in BD patients and these could contribute to host susceptibility to BD (Alayli et al. 2007;Coskun et al. 2005;Dilek et al. 2009).

Similar to other complex disorders, BD is unlikely to be a single-gene disease. Genome-wide association studies and other approaches would help to elucidate the

inflammatory pathways further as well as yet unknown networks between the associated genes and/or pathways. The demonstration of new loci of susceptibility to BD is of great significance in the pathogenesis of the disease, detecting patients negative to HLA-B5. Each disease associated functional polymorphism may have a distinct role within the context of other associated genes, all interacting in a network (Gul 2011). Their interaction with yet uncertain environmental factors may contribute to the complex nature of the disease.

1.20.3 Infectious agents

Various microorganisms such as streptococci and herpes simplex virus have been implicated in the aetiopathogenesis of BD (Evereklioglu 2005). No reliable animal model, however, has been described for BD and the model with the highest clinical resemblance to the human disease is that induced by inoculation with herpes simplex virus in which animals show several symptoms similar to BD (Deuter et al. 2008).

Due to an immune hyper-reactivity in patients, a role of streptococci in BD has been suggested. KTH-1, an extract of S. sanguis SSH-83 and encoded by BES-1 gene, was shown to promote IL-6 and IFN-γ secretion by peripheral blood T cells in BD patients (Hirohata, Oka, & Mizushima 1992) and the immunogenic antigen was observed to upregulate γδ T cells from patients with BD, which secrete the pro-inflammatory mediators IL-6, IL-8 and TNF-α (Mochizuki et al. 1994). The similarities of the KTH-1 protein amino acid sequence encoded by BES-1 gene with human intraocular peptide Brn-3b have also made this protein a candidate for auto-reactive response in BD (Yoshikawa et al. 1998). In addition to streptococcal antigens, Escherichia coli and Staphylococcus aureus have been reported to activate lymphocytes from BD patients to release increased amount of IL-6 and IFN-γ (Hirohata & Hashimoto 1998). Therefore, these bacteria seem to participate and, at least, act as triggers during the course of BD.

Furthermore, serum titres of antibodies against Saccharomyces cerevisiae (ASCA) has been found to be increased in BD patients with gastrointestinal involvement (Fresko et al. 2005), although the clinical relevance of this finding is uncertain.

1.20.4 Heat shock proteins

HSPs are highly conserved molecules induced by variable stress factors. The presence of molecular mimicry and possible cross-reactivity between microbial and human HSPs have been implicated as the pathogenetic mechanism in BD (Direskeneli 2001). Lymphoproliferative responses to the 65 kDa mycobacterial and 60 kDa human HSP derived peptides were shown to be significantly higher in patients with BD compared to healthy controls. Also, stimulation of peripheral blood lymphocytes in patients with BD by specific epitopes of human HSP60, especially peptide 336-351, was found to result in excess amounts of Th1 cytokine production (Direskeneli et al. 2000;Kaneko et al. 1997).

Moreover, it was observed that the expression of HSP60 in mucocutaneous lesions was increased in patients with BD (Ergun et al. 2001). For intestinal BD, HSP60 was expressed in peripheral blood lymphocytes and intestinal tissues of BD in the active state of the disease (Imamura et al. 2005). Also, dominant infiltration of TLR-2 and TLR-4 expressing cells was recently revealed in the intestinal lesions of BD. The TLR-2 expressing cells produced IL-12 and could contribute to the induction of Th1-dominant immune responses in intestinal BD. As it has been shown that HSP60/65 is a ligand for TLR-2 and TLR-4, TLR/HSP60 interaction may be important for the pathogenesis and progression of intestinal inflammation of BD (Nara et al. 2008).

1.20.5 BD-related autoantigens

A well-recognised immunological abnormality in BD is the hyperresponsiveness of T cells to a variety of antigens. Attempts to identify the putative antigens have produced candidates such as retinal soluble antigen (S-Ag), a potent uveitic

autoantigen which is localised in the photoreceptor area of the retina. S-Ag specific T cells, despite being at a low frequency, were found in patients with active disease. In response to S-Ag challenge, PBMCs from BD patients with active uveitis produced IFN-γ and TNF-α but not IL-2, IL-4, or IL-17 (Zhao et al. 2008). In other studies, cellular proliferative response to S-Ag peptides in BD patients were described (de Smet et al. 2001;Yamamoto et al. 1993). This finding is similar to the sensitisation against HSPs.

Besides S-Ag, immune responses to interphotoreceptor retinoid binding protein (IRBP), another retina-specific autoantigen, has been suggested to be involved in the pathogenesis of human uveitis, including BD (Takeuchi et al. 2010). It was reported that IRBP-stimulated IFN-γ and IL-17 production by PBMCs was significantly higher in BD patients than in healthy controls, indicating Th1 and Th17 polarisation. In particular, IFN-γ produced by IRBP-specific T cells was significantly increased in BD patients during the active phase of uveitis.

A significant increase in lymphoproliferative response to α-tropomyosin as well as autoantibodies to tropomyosin have been observed in BD patients with posterior uveitis (Mahesh et al. 2005). Also, α-enolase protein has been found to be the target protein of serum anti-endothelial cell antibodies (AECA) in BD patients (Lee et al. 2003). Approximately 20% of the BD patients with uveitis has been identified to be positive for anti-selenium-binding protein antibody and these patients have more frequent ocular inflammation (Okunuki et al. 2007). Moreover, detection of serum kinectin autoantibody has been demonstrated in Chinese patients with BD (Lu et al. 2005).

1.20.6 Immunological abnormalities

In an early study, it was shown that ocular exacerbations were associated with a corresponding reduction in serum IgA immune complexes and an elevation in concentration of IgM and IgG immune complexes (Levinsky, Paganelli, & Lehner 1979). Although the number of total B lymphocytes was found to be normal, they were functionally activated during the course of BD and expressed CD13, CD33 and

CD80 with an increased immunoglobulin secretion. An expansion of memory B cell subsets was also observed (Eksioglu-Demiralp et al. 1999).

Additionally, T lymphocyte has been implicated to be the predominant cell type in the immunopathogenesis of BD (Zakka et al. 2009). In patients with active disease, a significant increase of peripheral Th1 cells was detected compared with those in remission and healthy controls (Frassanito et al. 1999). The finding of upregulated T-bet expression in active BD patients further substantiated the preferential skewing of the immune response toward the Th1 pathway (Li et al. 2003). Surprisingly, the serum levels of IFN-γ in patients with BD were demonstrated to be significantly higher than in normal controls but the titre of IFN-γ correlated inversely with disease activity in an early study (Ohno et al. 1982).

Various pro-inflammatory cytokines, including IL-1, IL-6, IL-8, IL-18, TNF-α, and soluble IL-2 receptor (sIL-2R), have been reported to be elevated in the sera of BD patients (Evereklioglu et al. 2002;Oztas et al. 2005;Yosipovitch et al. 1995). Although many immunologically active cells and neutrophils may produce these cytokines following stimulation, one of the major sources is monocytes and may play a part in the pathogenesis of chronic inflammation in this disorder. Although NK cell activity in the peripheral blood of patients in the clinically active stage of Behcet's disease is significantly lower than that of patients in the inactive stage and normal controls, the actual number of NK cells is markedly increased in the peripheral blood of patients with active disease (Kaneko et al. 1985). In accordance with this early study, an abnormal killer inhibitory receptor (KIR) expression on NK cells of BD patients has recently been reported (Takeno et al. 2004). On the other hand, neutrophils are hyperactive in BD, with increased chemotaxis, phagocytosis, superoxide production and myeloperoxidase expression and produce several cytokines such as IL-12 (Verity et al. 2003;Zierhut et al. 2003).

1.20.7 γδ T cells

Besides, various studies have reported the presence of elevated γδ T cells in BD patients. An increased number of Vγ9Vδ2 subsets of T cells was demonstrated in

the intraocular fluid of BD patients with uveitis (Verjans et al. 2002). Recently, the diversity of γδ T cells of BD patients in the peripheral blood and in oral mucosa was investigated and polyclonal activation was revealed (Freysdottir et al. 2006). Studies also demonstrated a specific increase in CD8 expression by γδ T cells, and in absolute numbers of $CD8^+$ γδ T cells. Most of these $CD8^+$ γδ T cells were revealed to be high IFN-γ, but poor IL-2, IL-4, IL-10, and TGF-β producing cells (Clemente et al. 2010;Freysdottir, Lau, & Fortune 1999).

Notably, γδ T cells from inflammatory sites were reported to exhibit a higher cytotoxic activity (Hamzaoui et al. 1994). In the presence of bacterial supernatants, $Vδ2^+$ cells from BD patients were demonstrated to expand considerably more than normal after a short time in culture (Bank, Duvdevani, & Livneh 2003). Thus, it was suggested that γδ T cells in BD patients had previously been primed to respond to bacterial antigens, and a second encounter with bacterial products or cross-reactive autoantigens could lead to inappropriate activation of these cells and result in an autoaggressive attack against host cells.

1.20.8 Immunohistopathology of Behcet's disease lesions

The pathology of BD varies according to the site of lesions. The hallmark of BD features perivascular lymphocytic and monocytic cellular infiltrates, with or without fibrin deposition in the vessel wall, especially venules, swelling and surrounding tissue necrosis. Also, it was reported that neutrophils and $CD4^+$ T lymphocytes accumulated around the vasa vasorum and perivascular area, which expressed IL-1α, TNF-β, IFN-γ, and IL-2 receptors (Evereklioglu 2005).

A recent histopathological and immunofluorescence study of papulopustular lesions detected an immune complex-mediated leukocytoclastic vasculitis as compared with non-lesional skin in patients with BD. The vessels of the lesional skin demonstrated a higher IgM deposition than non-lesional skin (Alpsoy et al. 2003). In line with this finding, immunoglobulin and complement depositions were identified in the biopsies from mucocutaneous ulcers and papulopustular skin lesions in earlier studies (Onder & Gurer 1999;Onder & Gurer 2001). Also, erythema nodosum-like lesions were

reported to be characterised by phleblitis and arteriolitis, demonstrating variable numbers of neutrophils, lymphocytes, and histiocytes with expressions of adhesion molecules such as E- and L-selectins (Kim & LeBoit 2000;Senturk et al. 2003).

In biopsies of oral ulcers, it was found the majority of lymphocytes present in the oral ulcers were T cells, with no observed difference in the proportion of $CD4^+$ or $CD8^+$ cells in patients with BD. The Th1-related cytokines IL-12, IFN-γ, and TNF-α and the Th1-associated chemokine receptors CCR5 and CXCR3 were increased as compared to normal controls. However, low levels of the Th2 cytokine IL-4 was detected in oral ulcers of BD patients. It was thus suggested that the complex mixture of responses in the BD patients may be due to a mixture of antigenic stimuli (Dalghous, Freysdottir, & Fortune 2006). Likewise, intestinal lesions of BD expressed IFN-γ, TNF-α and IL-12 mRNA, and mRNA of Txk, a Tec family tyrosine kinase specific to Th1 cells, was also expressed in the lesions. These indicated Th1-dominant responses *in vivo* (Imamura et al. 2005).

Throughout the courses of neuro-Behcet's disease, lesions are typified by perivascular cuffing of T lymphocytes and monocytes (Hirohata 2008). In the brain, demyelination is another common finding (Zakka, Chang, Giuliari, & Foster 2009).

Ocularly, diffuse infiltration of predominantly neutrophils in the active stage, followed by lymphocytes and plasma cells during remission have been found in the anterior chamber, iris, ciliary body and choroids. When the disease becomes chronic and recurrent, collagen fibres proliferate which can lead to iris atrophy, cyclitic membrane formation, choroidal thickening and eventually hypotonia and phthisis bulbi (Hegab & Al Mutawa 2000). Retinal vasculitis, which involves veins more than arteries, is characterised by infiltration of leukocytes and plasma cells. The photoreceptor layers in the retina are often damaged and optic neuritis, ischemia, and even atrophy may result in more advanced cases. Recently, mural IgG, IgA, and C_3 in both episcleral and choroidal vessels, along with increased serum concentrations, have also been observed in patients with BD (Bardak & Aridogan 2004). Moreover, intraocular infiltration of $CD8^{bright}CD56^+$ T cells in BD patients has been reported and these cells are characterised as cytotoxic effector cells with functional NK receptors

that kill through both Fas ligand-dependent and perforin-dependent pathways (Ahn et al. 2005).

1.21 Treatment in Behcet's disease

Treatment of various manifestations in BD is empirical due to the paucity of adequately powered, randomised controlled trials and the poor understanding of the aetiopathogenesis. The conventional regimen was based on inducing severe immunosuppression in patients by targeting broad pathways of inflammation of the disease in a non-specific manner, and the regimen comprises systemic corticosteroids, colchicine, and immunosuppressive agents such as azathioprine, cyclosporine, mycophenolate mofetil and methotrexate in cases proven to be highly steroid dependent. These have been tried with some success, and anti-inflammatory drugs including NSAIDs may also prove useful.

Corticosteroids produce a broad and non-selective inhibition of the immune system by suppressing cyclo-oxygenase and lipo-oxygenase pathways. Therefore, the formation of prostaglandins, leukotrienes, and thromboxane is reduced. Also, it decreases lymphocyte migration and chemotaxis, circulating monocytes, macrophage activity, as well as the levels of complement and interleukins. Although topical and systemic corticosteroids are commonly used for the treatment of vast majority of BD manifestations, controlled studies are lacking and their long-term use is to be avoided if possible because of significant adverse effects. Systemic corticosteroid treatment is started with a high dose with subsequent tapering over several weeks as quickly as clinical manifestations allow. Despite successfully suppressing acute inflammation, corticosteroids alone often fail to prevent relapses, so they are frequently used in combination with other medications (Evereklioglu 2005).

Cyclosporine is a calcineurin inhibitor with more specific effects on the immune system than corticosteroid and cytotoxic drugs. It is a cytostatic agent and its primary effect is to inhibit T cell activation and recruitment (Evereklioglu 2005). Because of its rapid action, cyclosporine is usually the treatment of choice for sight-

threatening and progressive uveitis, especially with retinal vasculitis (Yurdakul & Yazici 2008). However, the long-term use of cyclosporine is limited by the development of neurological side effects, hirtutism, hyperglycaemia, hepatotoxicity, nephrotoxicity and hypertension (Evereklioglu 2005).

Azathioprine and methotrexate are anti-metabolite agents. The action of azathioprine depends on the depletion of cellular purine stores, thus suppressing both DNA and RNA synthesis (Al Mutawa & Hegab 2004). Patients on azathioprine should be followed for gastrointestinal disturbances and be monitored by a complete blood count every month for bone marrow suppression and liver function test every three months for hepatotoxicity (Evereklioglu 2005).

Methotrexate is a cyclo-oxygenase inhibitor and suppresses the production of thymidylate and de novo purine synthesis. It has multiple *in vivo* and *in vitro* effects, including inhibitory effects on B and T cell lymphocyte proliferation, endothelial cell growth, chemotaxis, neovascularisation, and generation of inflammatory mediators, such as lipo-oxygenase products. Methotrexate also inhibits dihydrofolate reductase, which is an enzyme responsible for the reduction of folic acid. Therefore, use of folic acid or folinic acid therapy can reduce toxicity without affecting the efficacy of methotrexate (Al Mutawa & Hegab 2004). Patients on methotrexate should be monitored for gastrointestinal side effects, hepatotoxicity, renal toxicity, and bone marrow suppression (Evereklioglu 2005).

Mycophenolate mofetil (MMF) is a relatively new immunosuppressive drug and is a specific inhibitor of B and T lymphocytes. Since its efficacy has been well established in the management of various autoimmune diseases as well as in different forms of non-infectious uveitis, it may have a role to play in the treatment of BD (Marshall 2004). Also, colchicine is an anti-inflammatory plant alkaloid and inhibits neutrophil chemotaxis by accumulating in these cells and suppressing the microtubule function (Evereklioglu 2005).

Combined treatment is also often used in order to diminish corticosteroid dose. Drug combinations are often preferred and many patients progress through various drug combinations with time, usually due to adverse effects. Medications must be given

under expert specialist supervision and need regular monitoring to detect potential side effects and determine response. Relief of symptoms, control of active inflammation, and prevention of recurrences and hence of irreversible end-organ damage and functional disability are the main goals (Evereklioglu 2005).

Although the prognosis has improved with early and aggressive immunosuppressive treatment, many patients have refractory disease. Over the past decade, biological agents that target one cytokine or inflammatory mediator have emerged as powerful therapies for a number of inflammatory disorders. They are also proving to be most useful in BD and can result in dramatic reduction of disease burden and improvement of long-term outcome. Small case series have demonstrated the value of biological therapies such as IFN-α and TNF-α antagonists, particularly infliximab, even in patients resistant to conventional treatment. Both IFN-α and infliximab have proved capable of rapidly inducing remission in active disease. If an adequate initial dose is used, IFN-α is capable of triggering a dramatic improvement within a few days (Deuter et al. 2008). For infliximab, the benefit is usually temporary and infusions are required every 4-8 weeks to sustain remission (Imrie & Dick 2007); very few cases have been described in which treatment could be stopped without relapses within a few months. In contrast, 50-60% of patients treated with IFN-α do not relapse one year after cessation of therapy (Deuter et al. 2008).

In order to develop evidence-based recommendations for the management of BD, the European League Against Rheumatism (EULAR) combined current evidence from clinical trials with expert opinion (Hatemi et al. 2008). Recommendations related to the eye, mucocutaneous disease and arthritis are mainly evidence based, but recommendations on vascular disease, neurological and gastrointestinal involvement are based largely on expert opinion and uncontrolled evidence from open trials and observational studies (Table 1.4).

Table 1.4: EULAR recommendations for treatment of BD 2008

(Hatemi et al. 2008;Mendes et al. 2009)

Eye disease:

- Azathioprine and local and systemic corticosteroids

Refractory eye involvement:

- Cyclosporine A or infliximab in combination with azathioprine and corticosteroids
- IFN-α alone or with corticosteroids

Major vessel disease:

- Acute deep vein thrombosis: corticosteroids, azathioprine, cyclophosphamide or cyclosporine A
- Thrombosis of the vena cava and Budd-Chiari syndrome: cyclophosphamide
- Pulmonary and peripheral arterial aneurysms: cyclophosphamide and corticosteroids; surgery
- Anticoagulants, antiplatelet and antifibrinolytic agents are not recommended (pulmonary embolism is rare and there is the risk of major bleeding in case there are concomitant pulmonary aneurysms)

Gastrointestinal involvement:

- Sulfasalazine, corticosteroids, azathioprine, TNF-α antagonists or thalidomide; surgery

Articular involvement:

- Colchicine; IFN-α, azathioprine, TNF-α antagonists in resistant cases

Neurological involvement:

- Parenchymal disease: corticosteroids, IFN-α, azathioprine, cyclophosphamide, methotrexate, TNF-α antagonists
- Dural sinus thrombosis: corticosteroids
- Cyclosporine should be avoided in case of neurological involvement due to neurotoxicity

Mucocutaneous involvement (oral, genital and skin lesions)

- Topical measures: corticosteroids preparations, lidocaine gel, chlorhexidine, sucralfate suspension
- Erythema nodosum: colchicines
- In resistant cases: azathioprine, IFN-α, TNF-α antagonists

1.22 Interferon-alpha as a treatment option for Behcet's disease

IFN-α was the first cytokine identified and is of major clinical value in various diseases (Isaacs & Lindenmann 1957). Interferons have well documented efficacy in the treatment of haematological and solid tumours, viral hepatitis, and disorders of presumed autoimmune aetiology (Pfeffer et al. 1998). Upon viral infection, type I IFNs are the first cytokine mediators induced in huge quantities and they orchestrate the immediate innate response of the host toward viral infection. In 1986, recombinant leukocyte IFN-α was first introduced for the treatment of BD by Tsambaos et al. based on the putative association between BD and viral infection (Tsambaos, Eichelberg, & Goos 1986). Today, there are two different human recombinant IFN-αs commercially available, IFN-α2a and IFN-α2b and they differ only in one amino acid in position 23 (Garcia-Garcia et al. 2010). It is of note that defects detected in BD patients including decreased percentage of pDCs and reduced serum levels of IFN-β can be corrected with recombinant IFN-α therapy (Pay et al. 2007).

Surprisingly, however, there are reports that BD patients have higher levels of IFN-α compared to healthy control subjects and serum levels are more elevated in patients with active disease as compared to patients in remission (Kotter et al. 2005;Pay et al. 2007). One possible explanation for the increased production of IFN-α in BD is that the cytokine is not active enough to eliminate a persistent pathogen which induces genes encoding IFN-α. Alternatively, there might be a diminished responsiveness to IFN-α signalling in BD patients, which can be rectified by exogenous IFN-α (Kotter et

al. 2005). The latter explanation is supported by polymorphisms recently demonstrated in the receptor for the type 1 IFNs in patients with BD (Pay et al. 2009).

Recent studies have focused on IFN-α2a, either used alone or in association with steroids and/or immunosuppressive agents. Due to the immunomodulatory rather than immunosuppressive nature, it is reasoned that the use of interferons needs an unsuppressed immune system (Onal et al. 2009). Therefore, the use of IFN-α combined with immunosuppressants remains a matter of debate and it has been suggested by some that concurrent use of immunosuppressives is to be avoided and corticosteroids should be used in low doses (Mackensen, Max, & Becker 2006). Treatment modalities of IFN-α vary greatly, with doses ranging from an injection of 3 to 9 million units 3-7 times a week and treatment duration between 11 days to 64 months. Optimal treatment duration is not clear but intermediate to high IFN-α dosages were shown to be more effective than low-dose regimens. Also, long-term remissions were associated with higher dosages, but no benefit was noticed with longer therapy in terms of efficacy or duration of induced remission (Kotter et al. 2004a;Zouboulis & Orfanos 1998).

Indeed, the risk of developing binding and neutralising antibodies increases with longer treatment, possibly decreasing its beneficial effects (Antonelli et al. 1991;Zouboulis & Orfanos 1998). Recombinant IFN-α2a induces neutralising antibodies in about 20% of patients, in contrast to only about 6% of those treated with recombinant IFN-α2b (Antonelli et al. 1991). Autoantibodies can be detectable three to twelve months after initiation of IFN-α therapy and their production or increase in titre has been observed in up to 50% of patients treated. Thyroid autoantibodies have been reported to be the most frequent. However, manifestations of overt autoimmune disease have been observed in only 1-2% of patients.(Hauschild et al. 2008;Pellicano et al. 2005) Overall, a meta-analysis of 144 BD patients from 22 reports showed that despite different therapeutic regimens employed in individual studies, overall efficacy of IFN-α therapy was 74% for mucocutaneous manifestations, 93% for arthritis and 95% for uveitis (Zouboulis & Orfanos 1998). Regarding time to response to IFN-α therapy, remission in ocular manifestations usually occurred in 2 to 4 weeks and nonocular manifestations remitted in 4-6 weeks (Kotter et al. 2004a).

The side effect profile of IFN-α is generally less severe than other systemic immunosuppressants. Common side effects of IFN-α include injection site reactions, flu-like symptoms (fever, headache, myalgia, arthralgia, sweating and fatigue), mild leukopaenia and alopecia. The flu-like symptoms could be alleviated by concomitant administration of paracetamol and represent a good prognostic marker for a response to IFN-α treatment as this may indicate the absence of pre-existing anti-IFN-α autoantibodies (Deuter et al. 2008;Mackensen, Max, & Becker 2006). Even without pre-existing psychiatric disease, depression and suicidal ideations can occur during therapy. An asymptomatic increase in liver enzymes has been reported, especially ones who receive high-dose IFN-α. In case of an greater than five times elevation of liver enzymes, a therapy pause followed by a dose reduction has been suggested (Hauschild et al. 2008). IFN-associated retinopathy with features such as cotton wool spots, arteriolar occlusions, retinal oedema and haemorrhages has been described in hepatitis C and melanoma patients treated with IFN-α but so far this has not been confirmed in BD patients. Risk factors reported include arterial hypertension, diabetes mellitus and age above 45 years (Deuter et al. 2008). Gastrointestinal side effects such as dyspepsia, diarrhoea, loss of appetite and weight loss can occur, as can other less common side effects which include thrombocytopaenia, hypertriglyceridaemia, reduced libido, hypotension and cardiac disturbances (Hauschild et al. 2008).

The two IFN-α isotypes have a comparable adverse effect profile, although flu-like symptoms and mild leukopaenia are more commonly reported with IFN-α2a regimens and mild alopecia more commonly with IFN-α2b treatment (Zouboulis & Orfanos 1998). Fortunately, nearly all of the side effects are temporary, dose-dependent and reversible, and discontinuation of IFN-α therapy due to adverse effects has been reported to be rare as a result (Deuter et al. 2008). Of note, type 1 diabetes occasionally develops during IFN-α treatment (Devendra & Eisenbarth 2004). Interestingly, disorders such as systemic lupus erythematosus, sarcoidosis, rheumatoid arthritis, autoimmune hepatitis, epilepsy and psoriasis may present for the first time during IFN-α therapy. These conditions are contraindications for IFN-α treatment and treatment should be individually evaluated with the risk-benefit profile taken into careful consideration (Deuter et al. 2008;Hauschild et al. 2008;Selmi et al.

2006). Unlike anti-TNF therapy, INF-α treatment is not associated with an increased risk of infection (Imrie & Dick 2007).

1.23 Clinical trials on interferon-α in Behcet's disease

Despite a lack of data from randomised controlled trials, the results of studies performed so far with IFN-α therapy have been promising especially for those patients with disease resistant to conventional immunosuppressive treatments. In particular, the clinical efficacy of IFN-α in the treatment of BD associated uveoretinitis appears fairly convincing.

1.23.1 The beneficial effects of IFN-α on ocular manifestations

In one open-label, prospective series involving 50 BD patients with sight-threatening refractory uveitis, a 92% response rate was demonstrated with recombinant human IFN-α2a at initial doses of six million units subcutaneously daily. Retinal infiltrates resolved after 2-3 weeks in all patients, macular oedema resolved, and visual acuity improved or remained stable in 97% of affected eyes (Kotter et al. 2004b). The IFN-α dose was subsequently adjusted according to the clinical response, and was discontinued whenever possible. Importantly, 40% of patients remained in remission for a mean of 29.5 months after cessation of treatment. IFN-α treatment was also beneficial for extraocular problems including arthritis, genital ulcers, and cutaneous and gastrointestinal manifestations as well, but less so for oral ulcerations.

In a retrospective analysis carried out on 44 BD patients with uveitis unresponsive to conventional immunosuppressants, IFN-α2a was given at an initial dose of 3-6 million units daily for a mean duration of 12.4 months and led to a partial or complete response in 91% of patients (Tugal-Tutkun, Guney-Tefekli, & Urgancioglu 2006). Visual acuity significantly improved and this benefit was preserved in 95% of patients throughout follow-up. Interestingly, patients who received IFN-α treatment at the time of a relapse did not behave differently compared with those whose treatment was started during remission. Another retrospective study by the same group found

that treatment with IFN-α2a resulted in resolution of neovascularisation of the optic disc, whether inflammation- or ischaemia-induced (Tugal-Tutkun et al. 2006).

An open, prospective study also evaluated the intermediate-term efficacy of IFN-α2a in patients with active Behcet's uveitis who failed to respond to conventional treatments, and 53 patients (106 eyes) were assessed. Complete remission (no relapse within the first year of follow-up) was obtained in 38 patients (71.6%). This was achieved even in patients with chronic inflammation associated with retinal and optic nerve vasculitis, neovascularisation of the optic disc, and cystoid macular oedema, which were unresponsive to previous conventional treatments. The mean ocular attack rate of 3.6 per year decreased to 0.56 per year at one year follow-up. Moreover, fifteen patients (28.3%) were off treatment for all the medications and disease free for 28 months. However, reinstitution of IFN-α2a treatment was required at a median follow-up of 65 months (Sobaci et al. 2010).

Recent studies have provided valuable information concerning the beneficial effect of IFN-α on long-term visual prognosis in BD. A retrospective study of 32 patients with severe refractory uveitis who were treated with IFN-α2a at a dose of 3-6 million units three times a week in combination with initial high doses of steroids demonstrated promising results, with uveitis being controlled in 28 patients (87.5%) and 19 patients (59.4%) being able to stop IFN-α after a mean treatment period of 32 months. Of the 28 responders, the mean follow-up period was 70.6 months. 13 patients (68%) were free of recurrences, and visual acuity was stabilised or improved in 87.5% of eyes (Gueudry et al. 2008). In another study of 45 patients who received IFN-α for ocular BD, patients were followed up for a mean of 6.67 years. Nine patients (20%) were able to stop IFN-α after a mean treatment period of 33 months. Those patients remained relapse free for a mean of 37 months. Importantly, 92% of the affected eyes showed improvement in or stabilisation of visual acuity during follow-up (Krause et al. 2008).

These results on favourable long-term visual prognosis are supported by finding of a retrospective study of 53 BD patients (96 eyes) with sight-threatening uveitis. Higher initial dosage of 6 million IU every day was used and visual acuity of the affected eyes in the series improved or remained unchanged in 91 eyes (94.8%). IFN-α was

able to be discontinued in 47 patients (88.7%), but twenty of these 47 (42.6%) needed a second treatment course during a median follow-up of 6.0 years. Nevertheless, ocular disease remained in remission in 50% of the patients 45.9 months after cessation of the first IFN-α course (Deuter et al. 2010).

1.23.2 The beneficial effects of IFN-α on non-ocular manifestations

The beneficial effects of IFN-α for the non-ocular manifestations of BD have also been demonstrated. A randomised, placebo-controlled, double-blind trial showed that both pain and duration of oral ulcers were reduced in 23 patients treated with IFN-α2a at a dosage of six million units subcutaneously three times a week for 3 months (Alpsoy et al. 2002). IFN-α decreased the frequency of both genital ulcers and papulopustular lesions. Additionally, in five of six patients, the severity and frequency of ocular attacks was noted to improve. However, all manifestations had the tendency to return to pre-treatment levels after cessation of therapy.

In an open, self-controlled trial of 20 patients treated with IFN-α2b for 16 weeks and followed for 48 weeks, the frequency and duration of arthritic attacks has been demonstrated to reduce after treatment with IFN-α2b. The mean number of mucocutaneous lesions also showed a tendency to decrease. However, except for the duration of arthritis, the mean number and frequency of all symptoms tended to return to pretreatment levels in the post-treatment phase (Hamuryudan et al. 1994). Currently, there is little information concerning the efficacy of IFN-α therapy for involvement of gastrointestinal tract, central nervous system and large vessels, although one small study demonstrated remission in 9 of 10 patients with vascular disease and 4 of 4 with neurological disease after a mean duration of 22.2 months of IFN-α treatment (Calguneri et al. 2003).

1.23.3 The combined use of azathioprine and IFN-α

The efficacy of the combined use of azathioprine and IFN-α was examined in a small open study of ten male BD patients with retinal involvement. For 24 weeks, they were treated with azathioprine and IFN-α2b five million units three times a week. The IFN-α was stopped after 24 weeks and the patients continued to take azathioprine or switched to cyclosporin A as indicated. The mean visual acuity of either eye improved significantly during treatment and throughout the follow-up. However, frequent but reversible myelosuppression, mainly leukopaenia, was observed and short withdrawals of either or both drugs were necessary (Hamuryudan et al. 2002).

In one long-term retrospective analysis, experience of IFN-α treatment on seven paediatric BD patients with severe uveitis was described (Guillaume-Czitrom et al. 2007). IFN-α2a was given at a dosage of 1.5 or 3 million units thrice a week in children weighing 20-30kg or 30-50kg, respectively. The treatment was combined with azathioprine for two of the children. The results are encouraging due to rapid steroid-sparing effect in five patients and sustained remission was achieved in four children. The results, together with the above study, suggest the combination of IFN-α and azathioprine might be an option in the management of severe refractory BD uveitis.

The details of these trials are summarised in Table 1.5.

Table 1.5: Significant clinical studies reporting the use of interferon-alpha in Behcet's disease

Reference	No. of patients on IFN	Type of IFN	Concurrent therapy	Manifestation(s) studied	Study design
Hamuryndan et al. (1994)	20	α2b	Not reported	Mucocutaneous, joint	Open, self-controlled
Alpsoy et al. (2002)	23	α2a	None	Mucocutaneous, joint, oral and genital ulcers, ocular	Randomised, placebo-controlled, double-blind
Hamuryndan et al. (2002)	10	α2b	Azathioprine	Ocular	Open
Calguneri et al. (2003)	29	NR	Colchicine, Benzathine penicillin (cyclophosphamide in 1 patient)	Ocular, joint, vascular, neurological	Open, prospective
Kotter et al. (2004)	50 (79 eyes)	α2a	Prednisolone ≤ 10mg/day, immuno-suppressives stopped	Ocular, joint, mucocutaneous, oral and genital ulcers, vascular, gastrointestinal	Open, non-randomised, uncontrolled, prospective
Tugal-tutkun et al. (2006a)	44	α2a	Prednisolone ≤ 10mg/day, immuno-suppressives stopped	Ocular	Retrospective
Tugal-tutkun et al. (2006b)	26 (38 eyes)	α2a	Prednisolone ≤ 10mg/day, immuno-suppressives stopped	Ocular (neo-vascularisation of the optic disc)	Retrospective
Guillaume-Czitrom et al. (2007)	7	α2a	Corticosteroids, Azathioprine in 2	Ocular	Retrospective
Gueudry et al. (2008)	32 (60 eyes)	α2a	IV methyl-prednisolone then prednisolone 1mg/kg/day	Ocular	Retrospective
Krause et al. (2008)	45 (79 eyes)	α2a	Prednisolone tapered from 100mg/day to 10mg/day in 2 weeks	Ocular	Retrospective
Sobaci et al. (2010)	53 (106 eyes)	α2a	Prednisolone tapered from 1-2mg/kg/day to 10mg/day in 4-6 weeks before starting IFN-α therapy	Ocular	Open, non-randomised, uncontrolled, prospective
Deuter et al.(2010)	53 (96 eyes)	α2a	Prednisolone ≤ 10mg/day, immuno-suppressives stopped	Ocular	Retrospective

1.24 Pegylated interferon-alpha

The clinical use of recombinant IFN-α has been restricted due to its short circulating half-life, which makes frequent dosing over an extended period necessary. The frequently used three times weekly administration schedule of IFN-α produces peak drug concentration/trough concentration cycles which compromise the tolerability of the drug because of the high incidence of peak concentration-related adverse effects (Perry & Jarvis 2001). This problem has been overcome with the introduction of pegylated interferons as they have a covalently attached polyethylene glycol chain which helps to reduce immunogenicity and clearance, and improve compliance and efficacy (Foster 2004). Polyethylene glycols (PEG) are biologically inert molecules that can be attached to proteins to reduce their degradation and the molecular weight and conformation of PEG chains can have an impact on the conjugate's properties (Foster 2004).

Two products are now in widespread clinical use. The 40 kDa PEG-IFN-α2a (F. Hoffmann-La Roche Ltd, Switzerland) is linked to a branch chain, 40 kDa PEG moiety and the 12 kDa PEG-IFN-α2b (Schering-Plough, USA) consists of IFN-α2b linked to a linear PEG chain. The two PEG-IFN-α drugs differ in the way that the PEG chain is cross-linked to the IFN-α. The PEG molecule in PEG-IFN-α2b is attached via a urethane bond. This urethane linkage is unstable and susceptible to hydrolysis such that, once injected, native interferon-α2b is released and circulates in the body. Thus, PEG-IFN-α2b is probably best regarded as a pro-drug. On the other hand, the PEG polymer in PEG-IFN-α2a is attached to a lysine residue within the IFN-α2a molecule via a hydrolytically stable amide bond. Consequently, the entire pegylated molecule circulates intact and interacts with the cell surface receptor (Foster 2004).

Conventional IFN-α has an absorption half-life of 2.3 hours and 7 to 12 hours after subcutaneous administration conventional IFN-α reaches peak serum levels. It is rapidly eliminated with a clearance of 6000ml/h and has an elimination half-life of 4 to 16 hours. Twenty-four hours after administration there is almost no measurable IFN in the serum (Chatelut et al. 1999;Wills 1990). PEG-IFN-α2b has a relatively rapid absorption (absorption half life of 4.6h), a wide volume of distribution

(approximately 0.99L/kg), and reduced clearance (725ml/h) (Glue et al. 2000). Maximum concentrations are achieved between 15 and 44 hours post-dosing and are sustained for 48-72 hours (Foster 2004). Whereas, the 40 kDa PEG-IFN-α2a is absorbed more slowly (absorption half life of 50h), has a more limited volume of distribution (8L, suggests distribution predominantly into the intravascular compartment), and a noticeably diminished rate of clearance (60ml/h) (Bailon et al. 2001). Maximum concentrations occur approximately 80 hours after dose administration and are sustained up to 168 hours (Foster 2004).

The bioactivity of PEG-IFN-α2b has been shown to be at least comparable to that of the frequently used thrice weekly administration of non-pegylated IFN-α2b (Jen et al. 2001) (Fig 1.5). The dosage of PEG-IFN-α2b must be adjusted for body weight to overcome the effect of extensive tissue distribution and metabolism of the drug. Whereas, PEG-IFN-α2a can be given as a single fixed dose (Reddy 2004). Of note, increased serum concentrations after several weeks of dosing have been reported for both pegylated and conventional interferons (Jen et al. 2001).

PEG-IFN-α2a is cleared by both the liver and kidney and the liver plays an important role in the metabolism of the drug. On the other hand, PEG-IFN-α2b releases free IFN-α2b soon after administration, and this is renally excreted (Foster 2010). From the clinical experience, the relatively rapid absorption of PEG-IFN-α2b leads to swift onset of side effects and a fall in neutrophil and platelet counts could take place. On the other hand, the side effects of PEG-IFN-α2a gradually increase over the first few weeks and neutrophil and platelet counts might not plummet until the second month of therapy (Foster 2004).

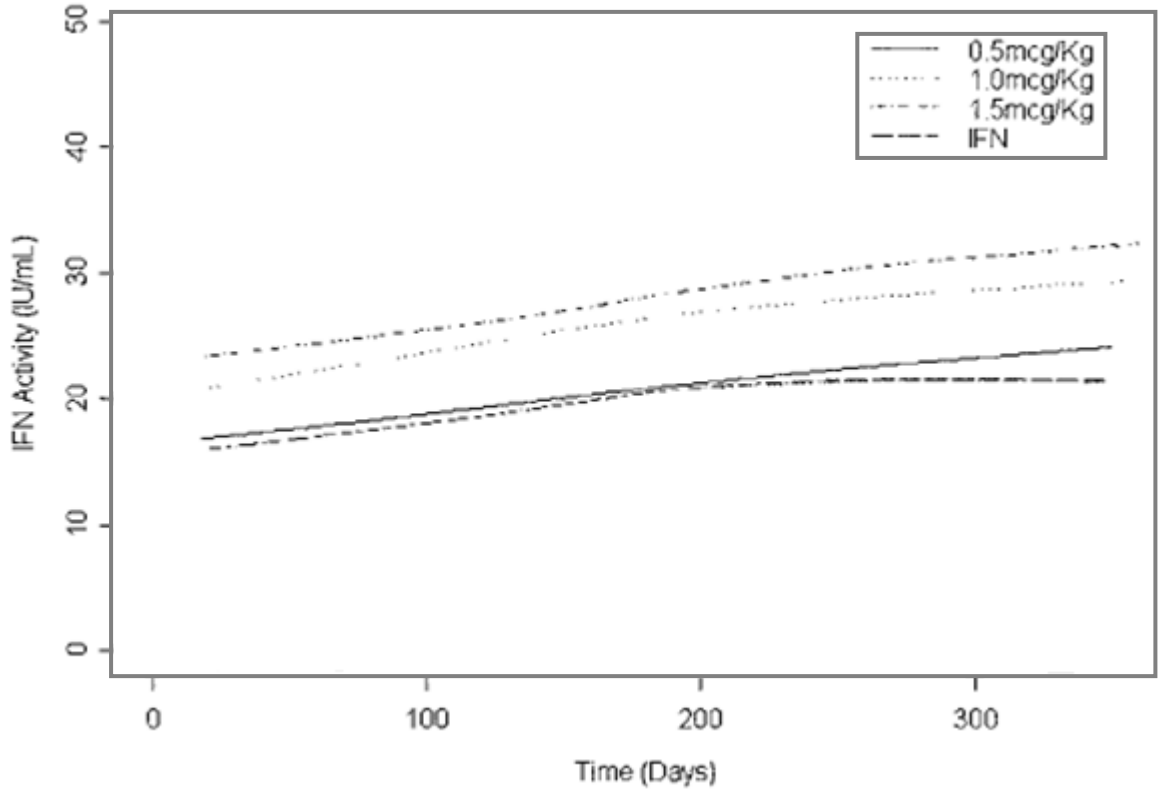


Figure 1.5: Plot of the time trend of activity for different PEG-IFN-α2b dose and conventional PEG-IFN-α2b groups.

The average interferon activities were estimated to be between 19.0 and 25.2 IU/mL for patients treated with PEG-IFN-α2b at doses from 0.5 to 1.5 μg/kg. The increase in the interferon activity was dose related. The average interferon activity for patients treated with non-pegylated IFN-α2b (IFN) at $3x10^6$ IU 3 times a week was 17.5 IU/mL, which was lower than that of the lowest PEG-IFN-α2b dose. Interferon activity increased as treatment continued (adapted from Jen et al. 2001).

1.25 Biological effects of interferon-alpha and possible mechanisms of action

IFN-α belongs to the type 1 interferon family and consists of at least 13 different isotypes in man (Table 1.6) (Pestka 1997a). The biological significance of the expression of several closely similar IFN-α proteins is not known. IFN-α proteins are encoded by a multigene family comprising 13 genes clustered on human chromosome 9 (Pestka, Krause, & Walter 2004). The similarity between the other IFN-α subtypes is between 78-95% at the protein level, and 79 of the 166 amino acids in the IFN-α family are conserved (Kontsek 1994). Macrophages and other APCs are the main cell types that contribute to the early production of IFN-α, but IFN-α can be produced by virtually all somatic cells after viral infection (Imrie & Dick 2007). Its effects are highly pleiotropic as it has antiviral, anti-proliferative, anti-angiogenetic, and immunomodulatory properties (Pfeffer et al. 1998).

Table 1.6: Classification of human IFNs

IFNs have been classified in three types based on their receptor usage. In humans, type I IFNs contain 13 IFN-α, 1 IFN-β, 1 IFN-ω, 1 IFN-ε and 1 IFN-κ. Type II IFN includes a single IFN-γ, while type III IFNs consist of IFN-λ1, IFN-λ2, and IFN-λ3 (Pestka 1997a)

Type I	Type II	Type III
Thirteen subtypes of IFN-α: α1, α2, α4, α5, α6, α7, α8, α10, α13, α14, α16, α17 and α21	IFN-γ	Three subtypes of IFN-λ: λ1, λ2, and λ3
IFN-β		
IFN-κ		
IFN-ε		
IFN-ω		

The receptor for the Type 1 IFNs consists of two chains, IFN-αR1 and IFN-αR2c (Pestka, Krause, & Walter 2004), which are encoded by two different genes located on chromosome 21q (Lutfalla et al. 1992). The receptor is expressed by nearly all cell types and tissues, including immune cells (Prinz et al. 2008). By interacting with their specific receptor, IFN-α activates Stat complexes which are a family of transcription factors that regulate the expression of certain immune system genes. Stat activation initiates the most well-defined cell signalling pathway for all IFNs, the classical Jak-Stat signalling pathway (Pestka 1997b;Stark et al. 1998). In this pathway (Figure 1.6), Jaks associate with IFN receptors and, following receptor engagement with IFN, phosphorylate both Stat1 and Stat2. As a result, an IFN-stimulated gene factor 3 (ISGF3) complex forms - this contains Stat1, Stat2 and a transcription factor called interferon regulatory factor 9 (IRF9) - and moves into the cell nucleus. Inside the nucleus, the ISGF3 complex binds to specific nucleotide sequences called IFN-stimulated response elements (ISRE) in the promoters of certain genes, known as IFN stimulated genes (ISG). Binding of ISGF3 and other trasncriptional complexes induces transcription of these genes (Platanias 2005).

In addition, Stat homodimers or heterodimers form from different combinations of Stat-1, -3, -4, -5, or -6 during IFN signalling; these dimers initiate gene transcription by binding to IFN-γ activated site (GAS) elements in gene promoters (Platanias 2005). The signalling by IFN-α is predominantly through Stat1 and Stat2, however, the pathways can involve Stat3, Stat4, Stat5, and IFN regulatory factors in various cells and under diverse conditions (Su & David 2000;Ziegler-Heitbrock et al. 2003). Type I IFNs can induce expression of genes with either ISRE or GAS elements, but gene induction by type II IFN can occur only in the presence of a GAS element (Platanias 2005).

Besides the JAK-STAT pathway, IFNs can activate several other signalling cascades. Both type I and type II IFNs activate a member of the CRK family of adaptor proteins called CT10 regulator of kinase like (CRKL), a nuclear adaptor for Stat5 that also regulates signalling through the C3G/Rap1 pathway (Platanias 2005). Type I IFNs further activate p38 MAPK to induce gene transcription. Anti-viral and anti-proliferative properties of IFN-α are resulted from p38 MAPK signalling (Platanias 2005). Another pathway, the PI3K signalling pathway, is also regulated

by type I IFNs. PI3K activates P70-S6 Kinase 1, an enzyme that increases protein synthesis and cell proliferation; phosphorylates of ribosomal protein s6, which is involved in protein synthesis; and phosphorylates a translational repressor protein called eukaryotic translation-initiation factor 4E-binding protein 1 (EIF4EBP1) in order to deactivate it (Platanias 2005).

Interferons have diverse effects, influencing both innate and adaptive immune responses and form a network of complex interactions with other cytokines (Baccala, Kono, & Theofilopoulos 2005). As well as augmenting the number of B cells and monocytes (Corssmit et al. 1997;Wejstal et al. 1992), IFN-α has been found to promote $CD4^+$ T cells (Simon et al. 2003). Furthermore, it was reported to enhance human leukocyte antigen-1(HLA-1) expression on monocytes in BD patients (Piazzolla et al. 1996) and improve the activity of natural killer cells and cytotoxic T cells (Arnaud 2002). Under IFN-α therapy, the number of circulating NK cell, $CD8^+$ γδ T cells, $CD3^+$ γδ T cells and leukocytes, especially neutrophils were all reported to be substantially lowered (Simon et al. 2003;Treusch et al. 2004)

By interacting with the cytokine cascade, IFN-α has displayed anti-inflammatory properties including abilities to inhibit IL-8 expression (Aman et al. 1993), increase IL-10 production (Aman et al. 1996;Kaser, Molnar, & Tilg 1998) and induce IL-1 receptor antagonist (Tilg et al. 1993) and soluble TNFR-55 and TNFR-77 (Corssmit et al. 1997;Tilg, Vogel, & Dinarello 1995). Also, it has been shown that IFN-α suppressed IL-1- and PMA-induced IL-1 synthesis by PBMCs (Schindler, Ghezzi, & Dinarello 1990). However, the production of IL-4 was not always reported to be augmented by IFN-α (Brinkmann et al. 1993;Kaser, Molnar, & Tilg 1998). On the other hand, the production of another Th2 cytokine, IL-5, as well as IL-13, were shown to be downregulated by IFN-α (Kaser, Molnar, & Tilg 1998;Schandene et al. 1996).

Surprisingly, although IFN-α was described to suppress TNF-α gene expression and protein synthesis *in vitro* and *in vivo* (Abu-Khabar, Armstrong, & Ho 1992;Larrea et al. 1996), it was demonstrated to enhance IFN-γ production by $CD4^+$ T cells (Brinkmann et al. 1993;Simon et al. 2003). However, IFN-γ has been suggested more recently to be not only a mediator of disease but also as a protective factor in

models of autoimmunity (O'shea, Ma, & Lipsky 2002). Furthermore, it has been revealed that IFN-α can restore the impaired intracellular IL-2 production up to normal levels by promoting Th1 differentiation. IL-2, along with B7 co-stimulators, has been demonstrated to promote the development of Treg cells or apoptosis of activated T cells. Thus, termination of immune responses and maintenance of self tolerance can be achieved (Abbas 2003;Amberger et al. 2007). Other actions demonstrated by IFN-α involve abilities to suppress T cell binding to endothelial cells and inhibit intercellular adhesion molecule-1 (ICAM-1) expression on endothelial cells stimulated by IFN-γ (Eguchi et al. 1992). The capacity of IFN-α to inhibit T cell adhesion to endothelial cells can conceivably impede with the transmigration of inflammatory cells and reduce tissue injury as a result.

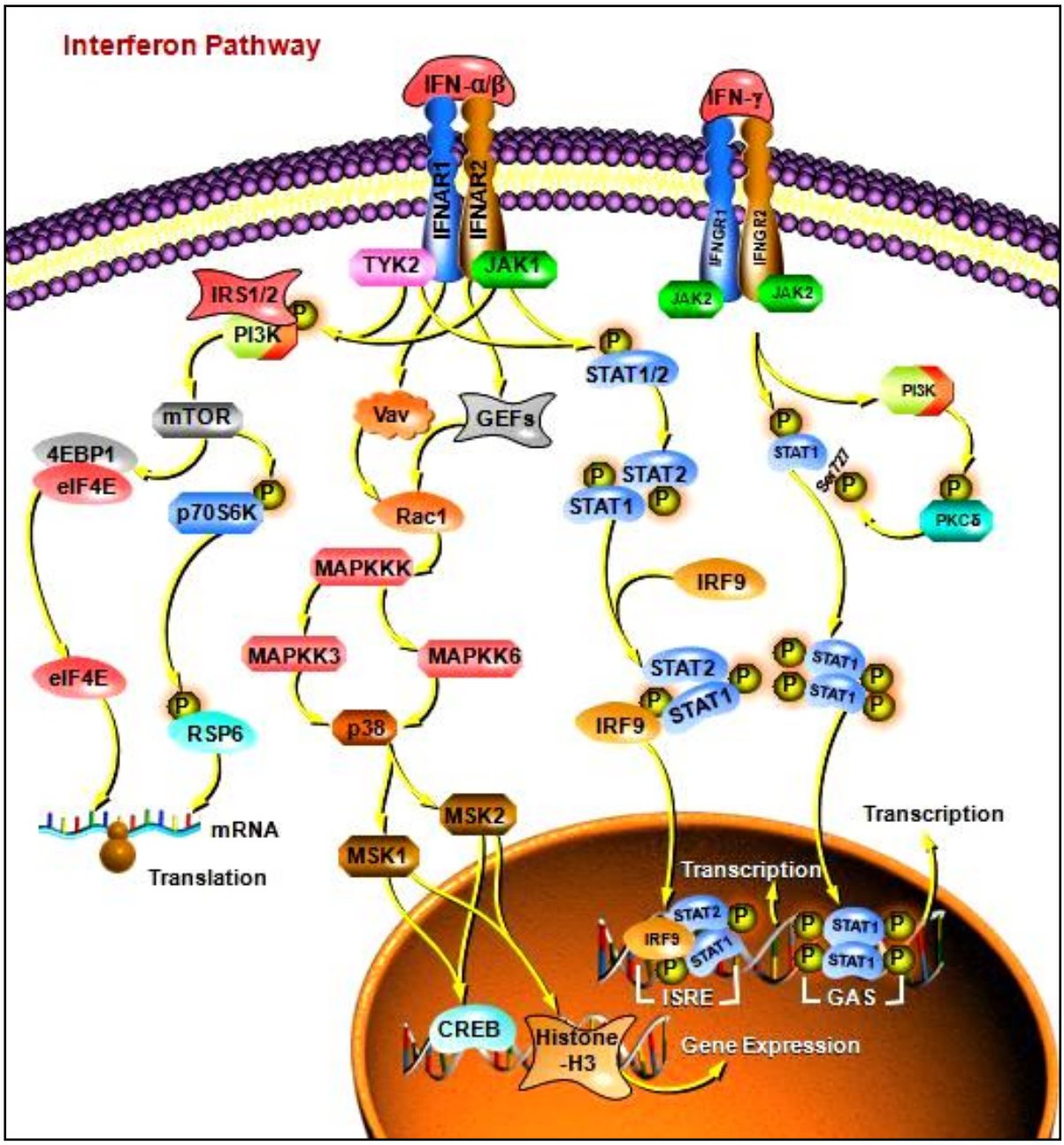


Figure 1.6: Interferon pathway.

Interferon-α signalling takes place through an IFN receptor complex consisting of two alpha chains (Type I receptor) complexed with Jak1 and Tyk2. Activation of these kinases results in tyrosine phosphorylation of Stat1 and Stat2; this leads to the formation of Stat1-Stat2-IRF9 complexes, which are known as ISGF3 complexes. These complexes translocate to the nucleus and bind ISREs in DNA to initiate gene transcription. Type I IFNs also induce the formation of Stat1-Stat1 homodimers that translocate to the nucleus and bind GAS elements that are present in the promoter of certain ISGs, thereby initiating the transcription of these genes (modified from www.sabiosciences.com).

1.26 Aims of the project

The overarching aim of this project is to examine further the immunomodulatory effects of recombinant IFN-α, with special reference to its role as a disease-modifying therapy in Behcet's disease, an immune-mediated disorder. In order to achieve this, aspects of the influence of IFN-α will be assessed in several different populations of T cells. The first major area to be studied is the conventional T helper subsets, Th1 and Th2, and expression of their related cytokines in response to a 6-month course of subcutaneous IFN-α2b therapy in BD patients *ex vivo*. In the first results chapter (Chapter 3), the influence of subcutaneous IFN-α2b therapy on the relative proportions of $CD4^+$, $CD8^+$, and $TCR\alpha\beta^+$ T cells and pattern of cytokine release by $CD3^+$ T cells, including IFN-γ, TNF-α, IL-2, and IL-4, in BD patients are assessed *ex vivo*. The longitudinal effect of the IFN-α is of particular interest, and the data from before, during and after IFN-α therapy are compared.

In the second results chapter (Chapter 4), this analysis is extended to Th17 and Treg cells, exploring the influence of a 6-month course of subcutaneous IFN-α2b therapy on these cell populations and the expression of IL-17, IL-10, TGF-β, and Foxp3 by T cells in BD patients *ex vivo*. Again, the longitudinal effect of the IFN-α is of particular interest, as this is crucial, along with longitudinal data from Chapter 3, to help gain insights into the disease-modifying nature of the IFN-α therapy and its potential to induce long-term remission. The effect of subcutaneous IFN-α2b therapy on the relative proportion of $CD56^+$ NKT cells is examined as well.

In Chapters 5-7, preliminary tests are first performed using healthy donor PBMC to establish *in vitro* models in order to test the immunomodulatory effects of IFN-α subsequently. In the third results chapter (Chapter 5), the modulating action of both forms of recombinant IFN-α (2a and 2b) on $CD4^+$ and $CD8^+$ T cells producing IFN-γ and IL-17 cytokines are assessed. In the fourth results chapter (Chapter 6), the objective is to investigate whether recombinant IFN-α2a and IFN-α2b induce expansion of regulatory T cell subsets and expression of immunoregulatory cytokines, IL-10 and TGF-β, as well as Foxp3. In the final results chapter (Chapter 7), the influence of a 6-month course of subcutaneous IFN-α2b therapy on the relative proportions of $TCR\gamma\delta^+$ T cells in BD patients was analysed *ex vivo*. This is

of particular interest, as γδ T cells have been implicated to play key roles in the pathogenesis of BD. The *ex vivo* experiments are followed by assessment of the changes in the proportion of γδ T cell within lymphocytes and their production of cytokines, including IFN-γ and IL-17, under the influence of recombinant IFN-α2a and IFN-α2b *in vitro*. Finally, the kinetics of cytokine production by T cells are explored by the introduction of IFN-α2a at different time-points during *in vitro* assays and the resultant changes in cytokine levels expressed by T cells are analysed for Foxp3 and all the cytokines examined in Chapter 5-7.

Chapter 2

Materials and Methods

2.1 Materials

Product	Product Code	Supplier
2-Mercaptoethanol	M6250	Sigma Laboratories UK
All-trans retinoic acid	554720	Merck Chemicals UK
Bovine serum albumin	A4503	Sigma Aldrich UK
Brefeldin A	B7651	Sigma Aldrich UK
Candida albicans	1200	Allergopharma
Cytofix/Cytoperm	554772	BD Biosciences UK
DMSO	038K0742	Sigma Aldrich UK
FACS Lysing Solution	349202	Becton Dickinson
Gentamicin	15710	Invitrogen UK
Heat inactivated foetal calf serum	4-101-500	LabTech International UK
Histopaque	1077	Sigma Aldrich UK
IL-2, recombinant, human	200-02	PeproTech UK
IMDM	I3390	Sigma Aldrich UK
Ionomycin	10634	Sigma Aldrich UK
LPS	L2630	Sigma Aldrich UK
Non essential amino acids	11140	Invitrogen UK
PDS-Ag (aa341-354)	800371	Biotrend
Perm/Wash buffer	554723	BD Pharmingen UK
PHA	L8754	Sigma Aldrich UK
PBS	P4417	Sigma Aldrich UK
PMA	P1585	Sigma Aldrich UK
Recombinant human IFN-α2a	11100-1	PBL Interferon Source
Recombinant human IFN-α2b	11105-1	PBL Interferon Source
RPMI-1640 Dutch Modification	22409031	Invitrogen UK
Sodium pyruvate	11360	Invitrogen UK
Tropomyosin	T2400	Sigma Aldrich UK
Zymosan	Z4250	Sigma Aldrich UK

2.2 Antibodies

Table 2.1: Primary antibodies used for flow cytometry

Antibody	Clone	Host	Isotype	Source
Anti-human CD3,purified	HIT3a	Mouse	IgG_{2a}	BD
Anti-human CD3 FITC	UCHT1	Mouse	IgG_1	BD
Anti-human CD3 PerCP	SK7	Mouse	IgG_1	BD
Anti-human CD4 PE	RPA-T4	Mouse	IgG_1	BD
Anti-human CD4 PerCP	SK3	Mouse	IgG_1	BD
Anti-human CD8 FITC	RPA-T8	Mouse	IgG_1	BD
Anti-human CD8 PerCP	SK1	Mouse	IgG_1	BD
Anti-human CD25 APC	2A3	Mouse	IgG_1	BD
Anti-human CD28, purified	CD28.2	Mouse	IgG_1	BD
Anti-human CD56 APC	B159	Mouse	IgG_1	BD
Anti-human Foxp3 Alexa 488	259D/C7	Mouse	IgG_1	BD
Anti-human Foxp3 PE	259D/C7	Mouse	IgG_1	BD
Anti-human IFN-γ FITC	25723.11	Mouse	IgG_{2b}	BD
Anti-human IFN-γ APC	25723.11	Mouse	IgG_{2b}	BD
Anti-human IgG APC	G18-145	Mouse	IgG_1	BD
Anti-human IgG FITC	X40	Mouse	IgG_1	BD
Anti-human IgG PE	G18-145	Mouse	IgG_1	BD
Anti-human IgG PerCP	X40	Mouse	IgG_1	BD
Anti-human IL-2 PE	MQ1-17H12	Rat	IgG_{2a}	BD
Anti-human IL-4 FITC	MP4-25D2	Rat	IgG_1	BD
Anti-human IL-10 APC	JES3-19F1	Rat	IgG_{2a}	BD
Anti-human IL-10 PE	JES3-19F1	Rat	IgG_{2a}	BD
Anti-human IL-17 PE	eBio64DEC17	Mouse	IgG_1	eBioscience
Anti-human TCRαβ FITC	T10B9.1A-31	Mouse	IgM	BD
Anti-human TCRγδ AP	B1	Mouse	IgG_1	BD
Anti-human TCRγδ FITC	11F2	Mouse	IgG_1	BD
Anti-human TCRγδ PE	B1	Mouse	IgG_1	BD
Anti-human TGF-β PE	TB21	Mouse	IgG_1	IQ Products
Anti-human TNF-α PE	MAb11	Mouse	IgG_1	BD

2.3 Preparation and storing of stock solutions and reagents

A number of reagents were prepared, aliquoted and stored at optimum temperature in order to prevent degradation of the reagents as a result of multiple freeze thaw cycles and to protect stock solutions from contamination.

2.3.1 2-Mercaptoethanol

2-mercaptoethanol (2ME; molecular weight 78.13) was purchased as a 14.3M stock solution. 14.3M 2-ME was diluted in PBS [1/14.3 vol/vol] in the laminar flow hood. This was then sterilised by passing through a 0.22μM filter and aliquoted in 500μl volumes and stored at -20°C until use. When 2-ME was required to be used, it was thawed and further diluted [1/50000 vol/vol] in culture medium to give a final concentration of $2x10^{-5}$ M.

2.3.2 All-trans retinoic acid (ATRA)

A stock solution was made up to 25mg/ml by adding DMSO to stock powder. It was stored at 4°C in the fridge and diluted in culture medium if necessary before use to achieve intended final concentrations.

2.3.3 Bovine serum albumin (BSA)

In order to make up a stock solution of 10% BSA, 1g BSA lyophilised powder was added to 9ml of sterile PBS. To make a buffer solution containing 0.1% BSA to dilute IFN-α2a, 100μl of 10% BSA was added to 9.9ml of sterile PBS.

2.3.4 Brefeldin A (BFA)

A stock solution of BFA was made up to 5mg/ml by adding 1ml of DMSO to the powder in the laminar flow hood. This was aliquoted and stored at -20°C in the freezer until used. During cell culture, BFA was diluted 1:10 in RPMI before appropriate amount was added to provide a final concentration of 10µg/ml.

2.3.5 Candida antigens

A stock solution was made up to 2mg/ml by adding sterile PBS to stock powder. It was stored at 4°C in the fridge and diluted in culture medium if necessary before use to achieve desired final concentrations.

2.3.6 FACS lysing solution

This reagent was provided as a 10 x concentrate and therefore was diluted 1:10 in deionised water and stored at room temperature before use. The prepared solution is stable for up to 1 month when stored at room temperature (20° to 25°C).

2.3.7 Foetal calf serum (FCS)

After complete thawing of heat-inactivated FCS, it was aliquoted in 50ml volumes under aseptic condition. FCS was then stored at -20°C until use and thawed in a 37°C water bath when required for tissue culture.

2.3.8 IFN-α2a

To make a stock solution of 10^6U/ml, each 5µl of IFN-α2a [5.4 x 10^7 U/ml] was diluted with 265µl of sterile PBS containing 0.1% BSA under sterile conditions in

accordance to the manufacturer's instructions. This was then aliquoted and stored at -70°C in the freezer. When IFN-α2a was required to be used, it was thawed at room temperature and diluted in culture medium if necessary to achieve desired final concentrations.

2.3.9 IFN-α2b

A stock solution [10^6U/ml] was aliquoted without dilution and stored at -70°C in the freezer. When it was required to be used, it was thawed at room temperature and diluted in culture medium if necessary to achieve desired final concentrations.

2.3.10 Non-essential amino acids (NEAA)

Sterile NEAA solution [100x stock] was stored at 4°C in the fridge.

2.3.11 Ionomycin

A stock solution of 0.5mg/ml was made up by adding ethanol to ionomycin powder in the laminar flow hood. This was then aliquoted and stored at -20°C in the freezer until use. When it was required to be used, it was thawed at room temperature and diluted 1:10 in RPMI 1640 Dutch Modification. Appropriate amount was then added to provide a final concentration of 1µg/ml.

2.3.12 L-Glutamine

Glutamine stock solution [200mM] was thawed in a 37°C water bath and aliquoted in 5ml volumes under aseptic condition. Aliquots were then refrozen and stored at -20°C in the freezer. When it was required to be used, it was thawed in a 37°C water bath in order to be used in tissue culture media at a final concentration of 2mM.

2.3.13 Lipopolysaccharides (LPS)

A stock solution of 1mg/ml was made up by adding sterile PBS to stock powder and aliquoted under aseptic conditions. Aliquots were stored at -20°C in the freezer. When it was required to be used, it was thawed at room temperature and diluted in culture medium if necessary to achieve desired final concentrations.

2.3.14 Phosphate buffered saline (PBS)

PBS was prepared following the manufacturer's instructions. Each PBS tablet was dissolved in 200ml of deionised water with a magnetic stirrer. Sterile PBS was prepared in aseptic conditions by placing PBS in a 25ml syringe and filtering through a 0.22μM filter.

2.3.15 PDSAg

A stock solution of 1mg/ml peptide was made up by adding sterile PBS to stock powder and aliquoted under aseptic conditions. Aliquots were stored at -20°C in the freezer. When it was required to be used, it was thawed at room temperature and diluted in culture medium if necessary to achieve desired final concentrations.

2.3.16 Permwash buffer

Concentrated Permwash buffer was diluted 1:10 in deionised water before use. Once diluted, the solution was stored at 4°C and used within 7 days.

2.3.17 Phorbol myristate acetate (PMA)

A stock solution of 1mg/ml was made up by dissolving PMA powder in DMSO under aseptic condition. This was then aliquoted and stored at -20°C in the freezer until use. When it was required to be used, it was thawed at room temperature and diluted 1:1000 in RPMI 1640 Dutch Modification. Appropriate amount was then added to achieve a final concentration of 25ng/ml.

2.3.18 Phytohemagglutinin (PHA)

A stock solution was made up to 1mg/ml by adding sterile PBS to powder under aseptic condition and aliquoted. Aliquots of PHA were stored at -20°C in the freezer until use. When it was required to be used, it was thawed at room temperature and diluted 1:10 in RPMI Dutch Modification. Appropriate amount was then added to provide a final concentration of 1µg/ml.

2.3.19 Purified mouse anti-human CD3 antibody

Stock solution [0.5mg/ml] was stored at 4°C in the fridge. During cell culture, this was diluted 1:1000 in RPMI 1640 Dutch Modification and appropriate amount was added to achieve a final concentration of 5ng/ml.

2.3.20 Purified mouse anti-human CD28 antibody

Stock solution [0.5mg/ml] was stored at 4°C in the fridge. During cell culture, this was diluted 1:10 in RPMI 1640 Dutch Modification and appropriate amount was added to achieve a final concentration of 1µg/ml.

2.3.21 Sodium pyruvate

Sterile sodium pyruvate solution was stored at 4°C in the fridge.

2.3.22 T cell medium

T cell medium was prepared using RPMI 1640 Dutch Modification or in the case of Th1/Th17 *in vitro* experiments, Iscove's modified Dulbecco's medium (IMDM). In aseptic conditions 67.5ml of medium was removed from a 500ml bottle of medium. The bottle of medium was then supplemented with FCS [10% v/v], 1% sodium pyruvate [1mM], 1% non-essential amino acids [1mM], L-glutamine [2mM], gentamicin [50μg/ml] and 2-mercaptoethanol [$2x10^{-5}$ M], and stored at 4°C until use. Once the medium was made, it was used within 14 days.

2.3.23 Tropomyosin

A stock solution of 5mg/ml was made up by adding sterile RPMI to lyophilised powder under aseptic conditions and stored at 4°C in the fridge until use. When it was required to be used, it was then diluted in culture medium if necessary and appropriate amount was added in order to achieve desired final concentrations.

2.3.24 Zymosan

In order to make a stock solution of 1mg/ml, 5ml of ethanol was added to 50mg of zymosan powder in a 50ml container under aseptic conditions. The mixture was vortexed to homogenise before the addition of 45mls of sterile endotoxin-free water. This was then stored at 4°C in the fridge until use. When it was required to be used, it was then diluted in culture medium if necessary and appropriate amount was added in order to achieve desired final concentrations.

2.4 Subjects and blood samples for *ex vivo* studies

Patients with systemic and/or ocular features of BD, who required systemic treatment with steroids and a second line agent, or second line agent(s) alone, were recruited at several hospitals including Moorfields Eye Hospital. The diagnosis of BD was based on clinical criteria of the International Study Group for BD. On enrolment to the trial, all patients have consented to their blood being used for immune studies and this therefore provides valuable resources to investigate the mechanisms involved in inducing disease remission. The trial has already completed recruitment and ethic's permission was obtained for blood samples to be collected as part of the trial. Only patients recruited at Moorfields Eye Hospital were included in this study.

Patients were randomised to receive IFN-α2b therapy or stay on the conventional treatment alone. For the treatment group, long-acting once weekly pegylated IFN-α2b (Schering-Plough, USA) was given subcutaneously for 6 months, in addition to the drug regimen that would normally be used to control patients' disease activity. The dosage of the IFN-α therapy is shown in Table 2.2. Attempts were made in both groups to reduce systemic steroid requirement to ≤10mg daily gradually over the first 3-6 months and to stop the second line immunosuppressive agents. The daily prednisolone dosage and the combinations of immunosuppressive treatment for the two groups of BD patients on the IFN-α trial at baseline visits are shown in Table 2.3 and Table 2.4.

Table 2.2 IFN-α2b treatment dose for trial patients with Behcet's disease

Dosage table of weekly administration of subcutaneous pegylated IFN-α2b. The dose is adjusted according to body weight.

Patient weight (kg)	Dosage of weekly pegylated IFN-α2b (μg)
50-100	30
101-116	35
117-133	40
134-149	45
150-165	50

Table 2.3 Prednisolone dosage for the two groups of trial patients at baseline

Dosage table of the daily intake of oral prednisolone for patients in the conventional treatment group and the IFN-α treated group.

Treatment	Standard treatment group Mean (range) of dosage in mg	IFN-α therapy group Mean (range) of dosage in mg
Prednisolone	11.6 (0-60)	9.9 (0-25)

Abbreviation: mg=milligrams

Table 2.4 Immunosuppressive treatment for the two groups of trial patients at baseline

Treatment	Standard treatment group No. (%) of patients	IFN-α therapy group No. (%) of patients
Pred + CSA + MMF	2 (9.5)	2 (9.5)
Pred + AZA + CSA	2 (9.5)	2 (9.5)
Pred + CSA	1 (4.8)	3 (14.3)
Pred + MMF	4 (19.0)	3 (14.3)
Pred + AZA	4 (19.0)	1 (4.8)
Pred + MTX	2 (9.5)	0
Pred	2 (9.5)	6 (28.6)
AZT	2 (9.5)	0
MMF	0	1 (4.8)
Etanercept	1 (4.8)	0
None	1 (4.8)	3 (14.3)

Abbreviations: Pred=prednisolone; CSA = cyclosporin A; MMF=mycophenolate mofetil; MTX=methotrexate; AZA=azathioprine

Whenever ocular or systemic relapse occurred, the patients were assessed by the trial doctors and medications, other than IFN-α2b, were adjusted accordingly. To take rapid control over the intraocular or systemic inflammation during a relapse, topical or systemic corticosteroids were utilised. Following control of intraocular inflammation, dose of systemic corticosteroids was rapidly tapered to a maximum of 10mg per day prednisone equivalent or discontinued. Occasionally, introduction of another second line agent was required in order to contain repeated relapses, especially in patients on conventional treatment alone.

IFN-α2b therapy was discontinued after 6 months and the follow-up period is 3 years for each trial patient. The first blood samples were obtained at baseline visits and in the case of IFN-α2b treatment group, before the commencement of IFN-α2b therapy. After that, serial blood samples were taken from both groups at formal assessment visits at 3, 6, 12 and 24 month follow-ups. As I am not involved in the long-term follow-ups of these patients, my data are focused solely on detecting the changes in the first 12 months.

2.5 Sample preparation

2.5.1 Study on patients with BD

Bloods were taken from patients by venous puncture into both heparinised and non-heparinised tubes. Apart from using whole blood for various immunoassays, serum, PBMC, RNA, DNA and cell culture supernatants were collected for future studies. To collect serum, non-heparinised tubes containing venous blood from patients were centrifuged at 3000rpm for 10 minutes. Each 500μl of serum was placed in 1.8ml cryotubes and stored at -70°C in the freezer. With regards to samples in heparinised tubes, a small aliquot of blood was firstly spotted onto FTA Elute Cards (Whatman plc, UK) to store DNA for each patient. The amount of whole blood required for phenotyping, intracellular cytokine staining and PCNA proliferation assays were set aside, and the remaining BD patient blood was processed to collect PBMC.

PBMCs were isolated by differential density centrifugation. 15ml of Histopaque-1077 solution was placed in a 50ml Leucosep tube (Greiner Bio-one, Germany) and centrifuged for 90 seconds at 1000g to allow the histopaque to go beneath the filters in the tubes. After that, the blood was diluted 1:1 with RPMI 1640 Dutch Modification before being added onto the filters within the Leucosep tubes under aseptic conditions. These tubes were then centrifuged for 12 minutes at 1000g (Fig 2.1). After centrifugation, mononuclear cells were retrieved from the enriched cell fraction above the porous barrier by means of a Pasteur pipette. The porous barrier is for avoiding recontamination with pelleted erythrocytes and granulocytes.

The cells were then washed with 10ml of sterile RPMI and centrifuged twice at 400g. 1 ml of RPMI 1640 Dutch Modification was added to the cell pellet to resuspend the cells before the number of cells was counted in a haemocytometer. Cell viability was determined by Trypan Blue exclusion. $1x10^6$ cells were taken from each patient visit and placed in a 1.5ml microcentrifuge tubes with 300µl of RNAprotect cell reagent (Qiagen, UK) for immediate RNA stabilisation. These were stored at -70°C for future RNA studies. Those PBMC which were not used were put in two 1.8ml cryotubes for each patient and pre-cooled before mixing 1:1 with DMSO buffered with 20% FCS under aseptic conditions. Cryotubes were immediately wrapped in paper to insulate and placed in the freezer at -70°C for 1-2 days. Afterwards, PBMC were transferred to liquid nitrogen tanks for long term storage.

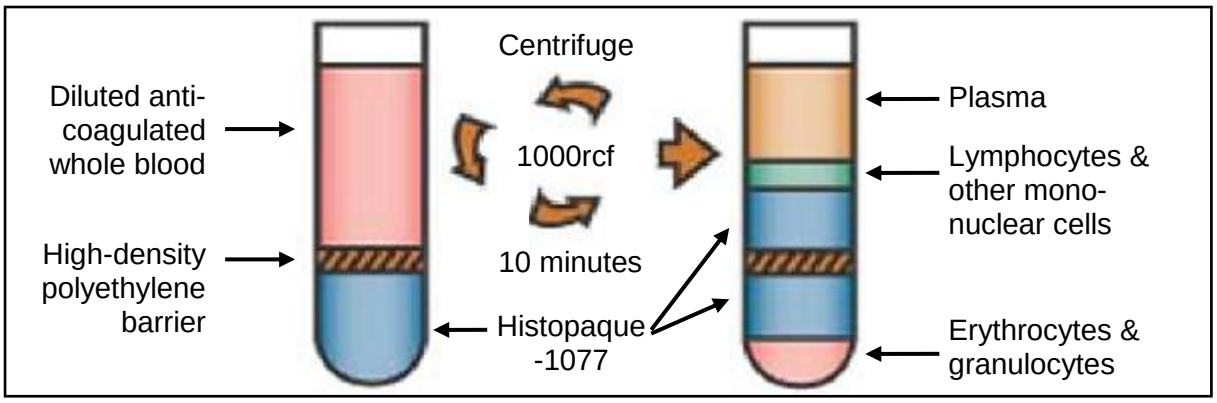


Fig 2.1: Schematic figure of a density gradient centrifugation

Anticoagulated venous blood is layered onto Histopaque-1077. During centrifugation, erythrocytes and granulocytes are aggregated by polysucrose and rapidly sediment; whereas, lymphocytes and other mononuclear cells remain at the plasma-Histopaque-1077 interface. Erythrocyte contamination is negligible (Sigma-Aldrich product information, 2009).

2.5.2 PBMC preparation for *in vitro* studies

PBMCs were isolated by differential density centrifugation. 15ml of Histopaque-1077 solution was placed in 50ml Leucosep tubes and centrifuged for 90 seconds at 1000g. Buffy coats obtained from normal healthy adult donors (North London Blood Transfusion Service) were diluted 1:5 with RPMI 1640 Dutch Modification under sterile conditions. After that, diluted buffy coats were added directly onto the filters within the Leucosep tubes. These tubes were then centrifuged for 12 minutes at 1000g. After centrifugation, mononuclear cells were retrieved from the enriched cell fraction above the porous barrier by means of a Pasteur pipette.

The cells were then washed with 10ml of sterile RPMI 1640 Dutch Modification and centrifuged twice at 400g. 10ml of RPMI 1640 Dutch Modification was added to the cell pellet to resuspend the cells before the number of cells was counted in a haemocytometer. Cell viability was determined by Trypan Blue exclusion. In each 1.8ml cryotube, $1x10^7$ pre-cooled cells suspended in 500µl of RPMI 1640 Dutch Modification were mixed 1:1 with DMSO buffered with 20% FCS. Cryotubes were immediately wrapped in paper to insulate and placed in the freezer at -70°C for 1-2 days. Afterwards, PBMCs were transferred and stored at -196ºC in liquid nitrogen tanks. Healthy donor PBMCs obtained this way were used in all *in vitro* experiments, except in some preliminary experiments testing the polarising effects of tropomyosin and PDSAg, where PBMCs from BD patients were used instead.

2.5.3 Thawing of PBMCs

20ml of RPMI 1640 Dutch Modification with 10% FCS was prepared for each tube of cryo-preserved PBMCs and the medium was warmed in a 37°C water bath until use. Sufficient amount of cryotubes containing cryo-preserved PBMCs required for experiments were taken out of storage in liquid nitrogen tank and thawed quickly in a 37°C water bath. The tubes were swirled around to facilitate thawing of cells. As soon as PBMCs were defrosted, they were added drop-wise into the warm medium under aseptic conditions. These were then centrifuged for 10 minutes at 400g. Supernatants were decanted and pellets of cells were resuspended. Cells were

washed again with warm RPMI 1640 Dutch Modification containing 10% FCS to remove DMSO. Cells were counted in a haemocytometer and viable cells were determined by Trypan Blue exclusion. Appropriate amount of T cell medium, either RPMI 1640 Dutch Modification- or IMDM-based, was then added to achieve 1 x 10^6 live PBMC in each 750µl of medium. During *in vitro* experiments, these were further diluted with the addition of various reagents and T cell medium to reach a final concentration of 1 x 10^6 cells/ml.

2.6 Experimental assays

2.6.1 Cell surface phenotyping in patients with BD *ex vivo*

Whole blood studies were performed so that drugs were still present in the blood. Changes in cell surface phenotype were evaluated using fluorochrome-conjugated antibodies and flow cytometry. 100µl aliquots of whole blood were placed in 1.5ml microcentrifuge tubes and stained with 5µl of anti-surface marker antibodies and the surface markers examined included CD3, CD4, CD8, CD25, CD56, TCRαβ and TCRγδ. These were left in the dark at room temperature for 30 minutes before red cell lysis was carried out with the addition of 1ml 1x FACS lysing solution according to manufacturer's instructions and left in the dark for 10 minutes. Afterwards, samples were centrifuged at 4000rpm for 4 minutes. Supernatants were decanted and washing was carried out using 1ml of PBS. These samples were stored in 300µl of PBS at 4ºC until ready for flow cytometry.

In addition, intracellular expression of Foxp3 was examined in these experiments. After red cell lysis and decanting of supernatants, cells were fixed and permeabilised with 100µl of Cytofix/Cytoperm in keeping with manufacturer's instructions for 20 minutes at 4°C. 1ml of Permwash was then added to microcentrifuge tubes to keep cells permeabilised and to serve as an antibody diluent and cell wash buffer. Subsequently, samples were incubated with 5µl of anti-Foxp3 antibodies for 30 minutes at 4°C. 1ml of Permwash was applied again before finally keeping the cells in 200µl of PBS at 4ºC until ready for flow cytometry.

2.6.2 Intracellular cytokine staining in patients with BD

Whole blood was diluted 1:1 with RPMI 1640 Dutch Modification medium and aliquoted, 200µl/well, in 96 well flat bottom cell culture plates. Cell cultures were unstimulated, stimulated with anti-CD3 [5ng/ml] and anti-CD28 [1µg/ml] monoclonal Abs, or a combination of PMA [25ng] and ionomycin [1µg/ml] for 24 hours. BFA [10µg/ml] was added to all cell cultures to prevent protein secretion and allow accumulation of cytokines within cells. After incubating overnight (approximately 22 hours) at 37°C in a humidified 5% CO_2 atmosphere, cells were transferred to 1.5ml microcentrifuge tubes before staining. Staining of surface markers was performed with 5µl of fluorochrome-conjugated anti-CD3, CD4 and CD25 Abs or matched isotype control mAbs for 30 minutes at room temperature in the dark. Red cell lysis was carried out with 1ml 1x FACS lysing solution as described in Section 2.6.1. Subsequently, cells were fixed and permeabilised with 100µl of Cytofix/Cytoperm and incubated at 4°C for 20 minutes.

Cells in each tube were washed with 1ml 1x Permwash before staining with fluorochrome-conjugated anti-cytokine antibodies including IFN-γ^{FITC} (5µl), IL-2^{PE} (5µl), IL-4^{FITC} (50µl of 1:150 dilution with 1x Permwash) , IL-10^{PE} (50µl of 1:50 dilution with 1x Permwash), IL-10^{APC} (25µl of 1:250 dilution with 1x Permwash), TNF-α (5µl), IL-17 (5µl), and TGF-β_1 (5µl), as well as anti-Foxp3 Ab (5µl) and matched isotype controls (5µl of 1:50 dilution with 1x Permwash) for 30 minutes. Incubations were carried out in the dark in order to prevent fluorochrome from photobleaching. Finally, washing in Permwash was carried out and samples were stored in 200µl of PBS at 4ºC until acquisition of data with flow cytometry.

2.6.3 PCNA proliferation assay in patients with BD

Whole blood was diluted 1:5 with RPMI 1640 Dutch Modification and aliquoted, 250µl/well, in 24 well flat bottom cell culture plates. Cells were stimulated with anti-CD3 [5ng/ml] and anti-CD28 [1µg/ml] mAbs, PHA [1µg/ml], and a selection of antigens, including recall Ag tuberculin purified protein [0.1, 0.5, 1, 5, and 10µg/ml] (Statens Seruminstitut, Denmark), and putative Behcet's Ags such as PDSAg [0.5, 1,

2.5, and 5µg/ml], α-tropomyosin [0.01, 0.1, 1, 10µg/ml], and heat shock protein 60 [5, 10, 50µg/ml] (HSP60, aa336-351; Biotrend, Germany). In each well, the volume was made up to 500µl/well with RPMI 1640 Dutch Modification and the plates were then incubated in an incubator with 5% CO_2 at 37°C for 5 days. Aliquots of supernatants from these cultures were taken at 24hours under aseptic conditions and stored at -70ºC for future studies as well.

After 5 days, content of the wells was transferred to 5ml polystyrene round bottom test tubes and red cell lysis was performed with 3.5ml 1x FACS lysing solution. After 10 minutes in the dark at room temperature, cells were washed with 4ml of PBS and centrifuged at 300rcf for 5 minutes. Supernatants were decanted and 1ml of methanol was added to each tube. The content of each tube was mixed well by vortexing gently and cells were then incubated at 4°C for 30 minutes. Afterwards, each tube of cells were washed with 3ml of PBS before adding 100µl of Cytofix/Cytoperm. Again, cells were incubated at 4°C for 30 minutes. Subsequently, cells were washed wtih 1x Permwash followed by adding 2.5µl of $PCNA^{FITC}$ (Abd Serotec, UK) to each tube. The tubes were incubated overnight at 4°C. On final day, each tube of cells were washed with 2ml 1x Permwash before adding 200µl to resuspend the cells. Immediately before aquisition of data with flow cytometry, 2µl propidium iodide [1mg/ml] (Sigma-Aldrich, UK) was added to each tube.

Although I have performed these proliferation assays and acquired some of the data in collaboration with my colleagues, this area was not the focus of my research and therefore I did not carry out analysis of data obtained from these assays.

2.6.4 Preliminary tests *in vitro*

2.6.4.1 PBMC stimulation with antigens and polyclonal stimuli

In order to establish *in vitro* models to investigate the effects of IFN-α on T cells, required amount of frozen PBMCs were thawed as described in Section 2.5.3. 300µl ($4x10^5$ cells) of healthy donor PBMCs were placed in each well of 48 well flat bottom culture plates and these cells were untreated or stimulated with polyclonal stimuli,

including anti-CD3 [5ng/ml] and anti-CD28 [1µg/ml] Abs, and PMA [25ng/ml] and ionomycin [1µg/ml], or with Ags such as LPS; zymosan, Candida albicans, and ATRA. In the case of BD-related antigens, such as PDSAg and α-tropomyosin, PBMCs from BD patients were used as a trial instead of healthy donor cells. Appropriate amount of T cell medium was then added to make up to 400µl for each well. Cells were cultured in an incubator with 5% CO_2 at 37°C for either 24 or 39 hours. BFA was added straight after the introduction of stimuli in cultures incubated over 24 hours. In the case of 39 hour cultures, BFA was added after 15 hours of incubation. At the end of cell culture, intracellular cytokine staining was carried out as described in Section 2.6.7. Staining and data analysis were performed to examine IFN-γ and IL-17 producing $CD4^+$ and γδ T cells, as well as $CD4^+$ T cells expressing IL-10 and Foxp3.

2.6.4.2 Tests of the influence of 1 day resting on PBMC cultures *in vitro*

It was examined whether resting frozen healthy donor PBMCs for one day in culture medium in the presence or absence of IL-2 after thawing could alter cytokine production by these cells. On day 1, required amount of frozen healthy donor PBMCs were thawed as described in Section 2.5.3, and resuspended in IMDM-based T cell medium. 600µl ($8x10^5$ cells) of PBMCs were placed in wells of 24 well flat bottom culture plates under aseptic conditions, except the wells designated for tests without resting the cells. For half of these wells, suboptimal IL-2 [0.5U/ml] was added before T cell medium was added to make up to 800µl per well. After 24 hours of incubation with 5% CO_2 at 37°C, 600µl ($8x10^5$ cells) of freshly thawed frozen healthy donor PBMC were placed in the wells earmarked for tests without resting.

Straight after, cells defrosted on both day 1 and day 2 were activated with polyclonal stimuli, including anti-CD3 [5ng/ml] and anti-CD28 [1µg/ml] Abs, and PMA [25ng/ml] and ionomycin [1µg/ml], or left untreated. PHA [1µg/ml] was used as a trial stimulus as well. BFA was added 2 hours after the introduction of stimuli to all wells, and cells were further incubated for 22 hours in an incubator with 5% CO_2 at 37°C. At the end of culture on day 3, intracellular cytokine staining was performed as described in

Section 2.6.7. Staining and data analysis were carried out to examine IFN-γ and IL-17 producing $CD4^+$ and γδ T cell populations only.

2.6.4.3 Tests of the effects of medium choice on PBMC cultures *in vitro*

On day 1, required amount of frozen healthy donor PBMCs were thawed as described in Section 2.5.3, and resuspended in either RPMI 1640 Dutch Modification- or IMDM-based T cell medium. 600µl ($8x10^5$ cells) of healthy donor PBMCs were placed in wells of 24 well flat bottom culture plates under aseptic conditions. After that, cells were activated with polyclonal stimuli, including anti-CD3 [5ng/ml] and anti-CD28 [1µg/ml] Abs, and PMA [25ng/ml] and ionomycin [1µg/ml], or left untreated. T cell medium was then added to make up to 800µl per well. BFA was added 2 hours after the introduction of stimuli to all wells, and cells were further incubated for 22 hours in an incubator with 5% CO_2 at 37°C. At the end of culture on day 2, intracellular cytokine staining was performed as described in Section 2.6.7. Staining and data analysis were carried out to examine IFN-γ and IL-17 producing $CD4^+$ and γδ T cell populations only.

2.6.4.4 Tests of the influence of longer incubation time on PBMC cultures *in vitro*

On day 1, required amount of frozen healthy donor PBMCs were thawed as described in Section 2.5.3, and resuspended in IMDM-based T cell medium. 600µl ($8x10^5$ cells) of healthy donor PBMCs were placed in wells of 24 well flat bottom culture plates under aseptic conditions. After that, cells were activated with polyclonal stimuli, including anti-CD3 [5ng/ml] and anti-CD28 [1µg/ml] Abs, and PMA [25ng/ml] and ionomycin [1µg/ml], or left untreated. T cell medium was then added to make up to 800µl per well. Cells were incubated for 24 and 39 hours in an incubator with 5% CO_2 at 37°C, and BFA was added in the last 22 hours of incubation for all wells. At the end of culture, intracellular cytokine staining was performed as described in Section 2.6.7. Staining and data analysis were carried out to examine IFN-γ and IL-17 producing $CD4^+$ and γδ T cell populations only.

2.6.5 Tests of the effects of recombinant IFN-α on PBMC cultures *in vitro*

Required amount of frozen healthy donor PBMCs were thawed as described in Section 2.5.3, and resuspended in T cell medium. 600µl ($8x10^5$ cells) of healthy donor PBMCs were placed in wells of 24 well flat bottom culture plates under aseptic conditions. After that, cells were treated with IFN-α2a (10^1-10^5 U/ml) or IFN-α2b (10^1-10^4 U/ml), or left untreated. This was then followed by activation with polyclonal stimuli, including anti-CD3 [5ng/ml] and anti-CD28 [1µg/ml] Abs, and PMA [25ng/ml] and ionomycin [1µg/ml], or left alone without stimulation. T cell medium was then added to make up to 800µl per well. Cells were incubated for 24 hours, and in some earlier experiments 39 hours, in an incubator with 5% CO_2 at 37°C, and BFA was added in the last 22 hours of incubation for all wells. At the end of culture, intracellular cytokine staining was performed as described in Section 2.6.7.

2.6.6 Kinetics of the influence of IFN-α on PBMC cultures *in vitro*

On day 1, required amount of frozen healthy donor PBMCs were thawed as described in Section 2.5.3, and resuspended in T cell medium. 600µl ($8x10^5$ cells) of healthy donor PBMCs were placed in wells of 24 well flat bottom culture plates under aseptic conditions. One-fifth of the wells were treated with 10^4 U/ml IFN-α2a straight away before incubation in an incubator with 5% CO_2 at 37°C. 24 hours later on day 2, half of the wells were stimulated with PMA [25ng/ml] in combination with ionomycin [1µg/ml], and the other half was left alone without stimulation. In addition, 10^4 U/ml IFN-α2a was added to one-fifth of the wells for each of the 3 time-points at 4 hours before, the same time as, or 4 hours after, the introduction of PMA/ionomycin on day 2. T cell medium was added to make up to 800µl per well, and BFA was added in the last 22 hours of incubation for all wells. At the end of culture, intracellular cytokine staining was performed as described in Section 2.6.7.

2.6.7 Intracellular cytokine staining for *in vitro* cell cultures

At the end of cell culture at 37°C in a humidified 5% CO_2 atmosphere, cells were transferred from cell culture plates to 1.5ml microcentrifuge tubes. Samples were centrifuged at 4000rpm for 4 minutes in order to take off supernatants. Dispersed cells were then stained for surface markers with 5µl of fluorochrome-conjugated anti-CD3, CD4, CD8, CD25 and TCRγδ antibodies (BD Biosciences) or matched isotype control mAb and cells were incubated in the dark for 30 minutes at room temperature. After that, each tube of cells was washed with 1ml of PBS and then fixed and permeabilised with 100µl of Cytofix/Cytoperm according to manufacturer's instructions. After 20 minutes of incubation at 4°C, each tube of cells was washed with 1ml 1x Permwash. Subsequently, intracellular staining was carried out with fluorochrome-conjugated anti-cytokine antibodies including IFN-γ^{FITC} (5µl), IFN-γ^{APC} (5µl), IL-10^{PE} (50µl of 1:50 dilution with 1x Permwash), IL-10^{APC} (25µl of 1:250 dilution with 1x Permwash), IL-17^{PE} (5µl), and TGF-$\beta_1{}^{PE}$ (5µl), as well as anti-$Foxp3^{AlexaFluor488}$ (5µl), anti-$Foxp3^{PE}$ (5µl) and matched isotype controls (5µl of 1:50 dilution with 1x Permwash) for 30 minutes at 4°C. Cells were washed once more with 1x Permwash before 200µl PBS was added to each tube. Samples were stored at 4ºC until ready for acquisition with flow cytometry.

2.7 Flow cytometry and analysis

To quantify the populations of T cell subsets, acquisition was carried out using four-colour FACSCalibur and CellQuest software (BD Biosciences). Data analysis was performed using Winlist Version 3.0 (Verity Software House, USA). At least 10,000 events were acquired for all experiments in *ex vivo* studies, and in the case of samples stained with Foxp3, at least 25,000 events were attained. For *in vitro* studies, at least 25,000 events were acquired for all samples except for preliminary experiments. Viable lymphocytes were gated according to forward and side scatters, and careful attention was paid to avoid inclusion of debris and dead cells from analysis. Flow cytometric determination of cytokines in the cytoplasm of peripheral $CD3^+$, $CD4^+$ and $CD8^+$ T cells was performed. Cytokine-positive T cells were defined by setting regions with the lower-limits for cytokine positivity determined from

unstimulated cultures. Results were expressed as the percentages of cytokine-producing cells in $CD3^+$, $CD4^+$ or $CD8^+$ T cell populations. For Treg cell analysis, $CD4^+CD25^{high}$ T cells were defined by selecting those $CD4^+$ lymphocytes whose CD25 expression exceeded the level of CD25 positivity seen on the $CD4^-$ population (usually more than 10^2 of fluorescence intensity) (Fig 4.2). Also, unreactive isotype-matched Abs were used as negative controls and to verify the amount of background staining, and as a guide for setting markers to delineate "positive" and "negative" populations.

For data analysis in Chapters 3 and 4, data from follow-up assessment visits were compared to data from initial visits for BD patients. The aims are to identify changes in immune cell and cytokine profile in IFN-α treated patients throughout the 12 month observation period and whether these alterations are distinct features when compared to those receiving conventional immunosuppressive therapy alone. Patients were analysed only when paired data were available. That is, patients were not included in the analysis if either baseline or follow-up visits were missing. Due to this same reason, 12 month visits were examined separately from 3 and 6 month ones as not all patients who have been through 3 and 6 months visits have data from 12 month assessment visits. This way, maximal numbers of patients could be included in the analysis of the 3 and 6 month data, and the disease modifying ability of IFN-α, even when patients were off treatment, could be examined separately. Also, some samples were omitted from the *ex vivo* experiments due to insufficient cell numbers, and these data were not included in the analysis. In addition, less patient numbers were available for Th17 cell analysis in *ex vivo* intracellular cytokine staining experiments as these staining combinations were included after half way through the trial and baseline data were not available for more than half of BD patients.

2.8 Statistical analysis

Statistical calculations were performed using Prism 5 (Graphpad Software, Inc, USA) for *ex vivo* BD patient data and Excel 2007 (Microsoft Corporation, USA) for *in vitro* data. Wilcoxon signed-rank test and Student's t-test were used for statistical

analysis of *ex vivo* and *in vitro* data respectively. A p-value of <0.05 was taken to be significant and marked with '*' on the figures ('**' if p-value was <0.01), unless otherwise stated in the figures. Boxplots show the median and interquartile range (IQR) and the ends of the whiskers illustrate the minimum and maximum of all the data for individual variables.

Chapter 3

Immunomodulatory effects of subcutaneous interferon-alpha 2b on conventional T helper subsets and their cytokines in patients with Behcet's disease *ex vivo*

3.1 Introduction

T helper (Th) cells are a heterogeneous population of lymphocytes that are involved in activating and directing other immune cells. Mature Th cells are described to always express the surface protein CD4 (Zhu & Paul 2008). Th cells play a central role in immune protection and orchestrate the magnitude and persistence of diverse immune responses through production of cytokines and chemokines (Zhu & Paul 2008). They are essential in helping B cells to produce antibodies in the primary response, in the activation and growth of cytotoxic T cells, and in maximising bactericidal activity of phagocytes such as macrophages. Th cells are neither phagocytic nor cytotoxic (Zhu, Yamane, & Paul 2010).

The reason for the heterogeneity of Th cells is mainly related to their protective function, because it enables the best type of response to combat pathogens and maintain immune homeostasis. It is the diversity in function and their role in influencing other cells that gives T helper cells their name (Annunziato & Romagnani 2009). $CD4^+$ T cells are generally treated as having a pre-defined role as helper T cells within the immune system, although there are known rare exceptions. For example, there are sub-groups of regulatory T cells, natural killer T cells, and cytotoxic T cells that are known to express CD4 (although cytotoxic examples have been observed in extremely low numbers in specific disease states, they are usually considered non-existent) (Gabriel, Morley, & Rogers 2009;van de Berg et al. 2008). All of the latter $CD4^+$ T cell groups are not considered T helper cells.

The initial understanding of the existence of distinctive populations of differentiated $CD4^+$ T cells came from the analysis of mouse $CD4^+$ T cell clones that were shown to be divisible into two major groups, designated Th1 and Th2 cells by Mosmann & Coffman (Mosmann & Coffman 1989). Subsequently, distinct subpopulations of Th

cells similar to those first identified by Mosmann et al in mice are found also in humans (Del Prete et al. 1991). Th1 and Th2 clones could be distinguished mainly by the cytokines produced by the cells, and through the expression of different patterns of cell surface molecules. Establishment of a Th1/Th2 balance plays an important regulatory role in the immune system and imbalances of cytokine production contribute to immunopathologic conditions (Jager & Kuchroo 2010).

With regard to cytokine expression, Th1 cells make IFN-γ as their signature cytokine and also uniquely produce lymphotoxin (TNF-β). Th1 cells tend to be good IL-2 producers, and many make TNF-α as well. The most important function of Th1 cells is to promote cell-mediated immunity as they are crucial for activating macrophages and induce proliferation of cytotoxic $CD8^+$ T cells. Over-exuberant pro-inflammatory activities of Th1 cells can cause tissue damage and elicit unwanted inflammatory response. By contrast, Th2 cells fail to produce IFN-γ or lymphotoxin. Their signature cytokines are IL-4, IL-5, and IL-13. They also make TNF-α, and some produce IL-9. Although initially thought to be unable to make IL-2, later results indicated that Th2 cells could often produce relatively modest amounts of IL-2 (Zhu, Yamane, & Paul 2010). Th2 cells promote allergic reactions, and were viewed as the primary helpers of B cell responses and, therefore, autoantibody production (Palmer & Weaver 2010). Both Th1 and Th2 cells also produce IL-10 (O'Garra & Vieira 2007).

Behcet's disease is a chronic, relapsing vasculitis of uncertain aetiology and immunopathogenesis which may produce distressing symptoms with the combinations of variable systemic involvements (Yates & Michelson 2006). It is characterised by exacerbations and remissions with varying healing time among patients (Evereklioglu 2005). There is strong evidence for generalized aberrant T cell responses in BD. The percentage of $CD4^+$ cells has been reported to be significantly lower in patients with BD as compared to healthy controls. This is especially in the $CD4^+CD45RA^+$ subset and when patients are in the active phase of the disease. On the other hand, increase in $CD8^+$ cells have been observed among BD patients (Kahan, Hamzaoui, & Ayed 1992).

The examination of mucocutaneous biopsies of BD patients has revealed enhanced levels of IL-8, IL-10, monocyte chemoattractant protein 1 (MCP-1), IFN-γ and IL-12 mRNA. The parallel increase in IL-12 and IFN-γ expression within mucocutaneous lesions, associated with the absence of Th2-related cytokines IL-4 and IL-13, points to a strong polarised Th1 immune response (Ben Ahmed et al. 2004). Observation of increased serum IL-18, a factor capable of inducing IFN-γ production (Gracie, Robertson, & McInnes 2003), and TNF-α levels in BD patients further supports Th1 predominance (Oztas et al. 2005). As IL-8 is a strong activator of neutrophil function, the upregulation of its level may support the implication of polynuclear cell hyperfunction in the pathogenesis of BD lesions (Ben Ahmed et al. 2004).

BD patients on immunosuppressive treatment were recruited at various sites but blood samples were only collected from Moorfields Eye Hospital patients (more details in Chapter 2). Patients were randomised into two groups with one group receiving additional 6 months of IFN-α therapy. As Th1 predominance has been reported in BD patients and IFN-α treatment has been suggested to induce Th1-related cytokines, especially IFN-γ (Simon et al. 2003;Takaoka et al. 2000). The influence of subcutaneous IFN-α2b therapy was examined first on conventional αβ T cell subsets and related cytokines. Given the significant role of γδ T cells in BD, its response following IFN-α treatment was examined as well and results were shown in a separate chapter (Chapter 7).

3.2 Aims

IFN-α is known to exert pleiotropic effects and we aim to verify whether previously reported effects of IFN-α including the induction of Th1-related cytokines can be demonstrated following IFN-α treatment in BD patients. More specifically, the influence of a 6-month course of subcutaneous pegylated IFN-α2b therapy on modulating conventional Th1 and Th2 subsets and the production of IFN-γ, TNF-α, IL-2 and IL-4 by peripheral blood lymphocytes before, during and after cessation of therapy are examined.

Another aim is to investigate the effect of pegylated IFN-α2b therapy on surface phenotype of T cells, such as CD4, CD8 and TCRαβ expression, in whole blood samples of BD patients.

3.3 Results

The experiments presented here were performed in conjunction with Mr A Nouraeinejad and Ms G Galatowicz. Approximately 70% of the samples used for data analysis in this section were processed by me, and all data analysis is my own work.

3.3.1 IFN-α therapy does not induce any changes in the surface CD4, CD8, and TCRαβ expression in T lymphocytes in BD patients

To examine the influence of IFN-α therapy on CD4 and CD8 expression by peripheral blood T lymphocytes in BD patients and the proportions of TCRαβ-expressing subpopulations within the $CD4^+$ and $CD8^+$ subsets, serial whole blood samples from BD patients receiving conventional immunosuppressive therapy with or without recombinant IFN-α2b treatment were phenotyped. The CD4/CD8 phenotyping results were obtained after gating on the $CD3^+$ lymphocytes during analysis. As shown in Figure 3.1, there was no significant alteration of the levels of either CD4 or CD8 surface expression on peripheral blood T lymphocytes in BD patients whether they were on IFN-α or not.

As for the assessment of TCRαβ expression, presence of CD8 surface protein was used to discriminate between $CD4^+$ and $CD8^+$ T cell population after gating on lymphocytes. Like the individuals on conventional immunosuppressive treatment, no significant effect on the TCRαβ expression was consistently displayed by the patients on IFN-α therapy (Figure 3.2).

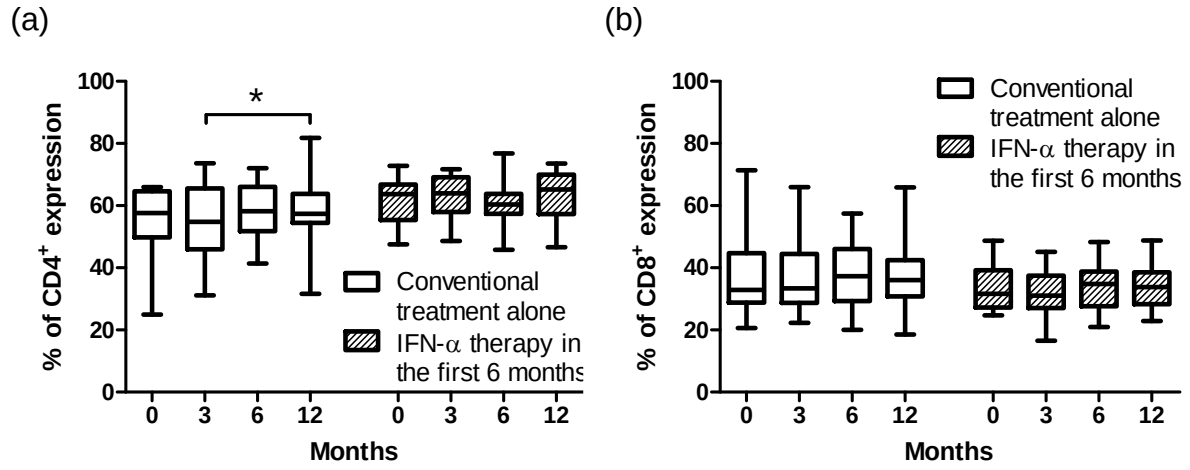


Fig 3.1: IFN-α therapy does not change the percentage of $CD4^+$ or $CD8^+$ lymphocytes
Whole blood from BD patients receiving either the conventional treatment alone (n=16) or with IFN-α2b therapy (n=15) were obtained at 0, 3, 6 and 12 month assessment visits. These samples were stained with anti-CD3, anti-CD4, and anti-CD8 antibodies. (a), The graph shows percentages of the $CD4^+$ fraction. (b), The graph displays the proportions of $CD8^+$ T cells. The results are presented as boxplots.

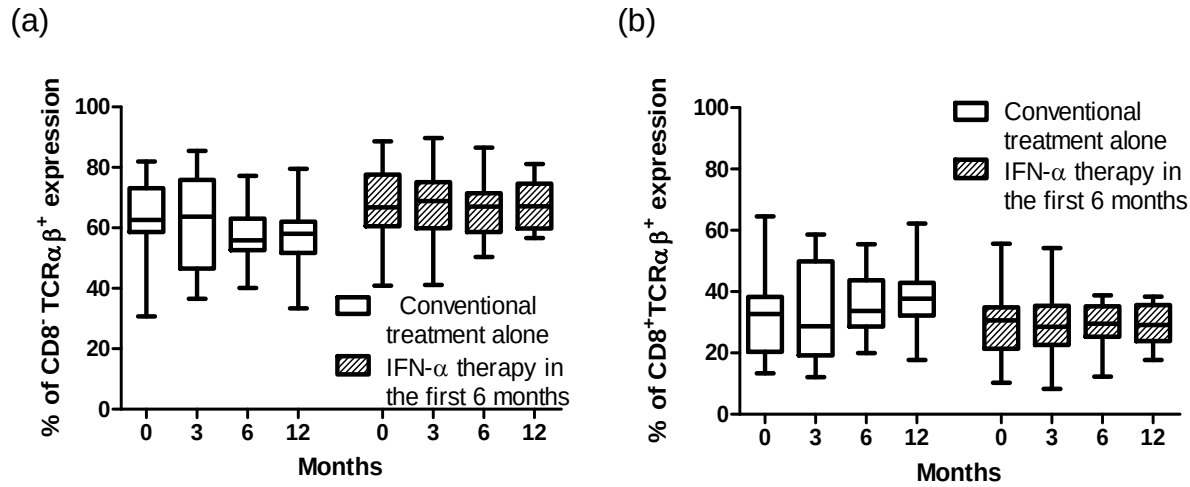


Fig 3.2: IFN-α therapy does not change the percentage of TCRαβ expression in either $CD4^+$ or $CD8^+$ lymphocytes

Whole blood from BD patients receiving either the conventional treatment alone (n=13) or with IFN-α2b therapy (n=12) were obtained at 0, 3, 6 and 12 month assessment visits. These samples were stained with anti-CD8, and anti-TCRαβ antibodies. (a), The graph shows percentages of the TCRαβ expression in the CD4 T cells. (b), The graph displays the proportions of the TCRαβ expression in the CD8 T cells. The results are presented as boxplots.

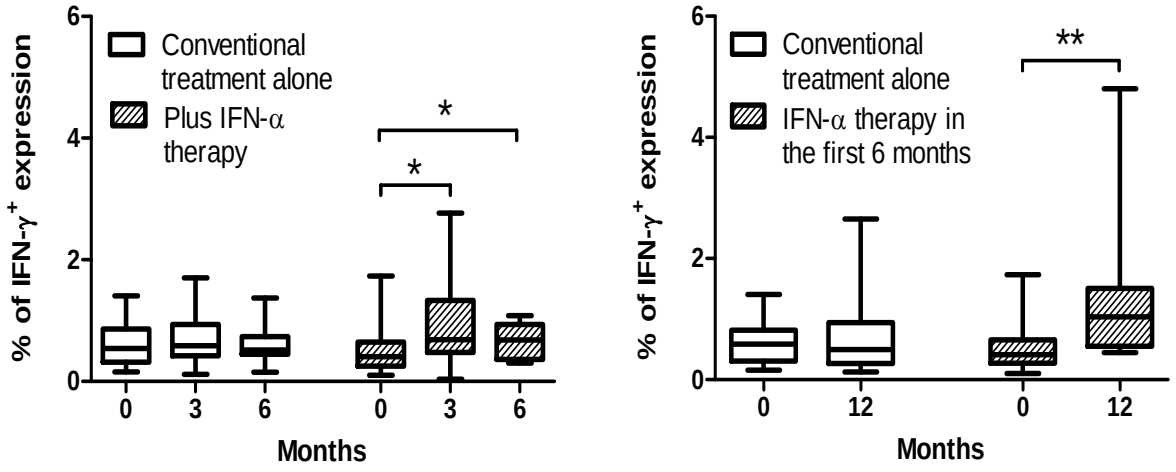
3.3.2 IFN-α2b therapy upregulates IFN-γ expression by T cells in BD patients

To investigate the effects of IFN-α on signature Th1 cytokine, IFN-γ, serial whole blood samples from BD patients on conventional immunosuppressive therapy with or without additional IFN-α2b treatment were studied using intracellular cytokine staining method and flow cytometry. Cell cultures were either untreated or treated with anti-CD3/CD28 Abs or PMA/ionomycin for 24 hours before staining. Cytokine production was analysed by gating on either $CD3^+$ or $CD4^+$ lymphocyte populations.

In terms of IFN-γ^+ production by $CD3^+$ T cells in untreated cultures (Fig 3.3a), a significant increase was detected in the IFN-α2b-treated group at 3 months after initiation of therapy (median 0.7% and IQR 0.5-1.3%; $p<0.05$) as compared to data from baseline visits (median 0.4% and IQR 0.3-0.7%). This upregulation of IFN-γ level was maintained at 6 month visits (median 0.7% and IQR 0.4-0.9%; $p<0.05$) and even after cessation of therapy at 12 months (median 1.0% and IQR 0.6-1.5%; $p<0.01$) in the IFN-α treated patients. In cultures stimulated with PMA/ionomycin (Fig 3.3b), such enhancement of IFN-γ-expressing cells was only observed at 12 month follow-ups (median 9.0% and IQR 6.5-11.0%; $p<0.05$) as compared to initial visits (median 4.6% and IQR 3.3-7.9%). In CD3/CD28 stimulated cultures, no significant change in levels was detected in either group of BD patients (data not shown). Similarly, a tendency to increase in IFN-γ expression was evident in $CD4^+$ T cells (Fig 3.4) from patients with IFN-α treatment, however, none of the results was statistically significant.

No change in IFN-γ production by either $CD3^+$ or $CD4^+$ T cells was identified in the individuals on conventional immunosuppressants alone. However, a significantly decreased expression by $CD4^+$ T cells was found in 12 month visit samples after activation with PMA/ionomycin (median 6.8% and IQR 3.3-10.4%; $p<0.05$) as compared to baseline visit ones (median 4.0% and IQR 1.4-6.7%) (Fig 3.4b).

(a) Untreated



(b) PMA/ionomycin stimulation

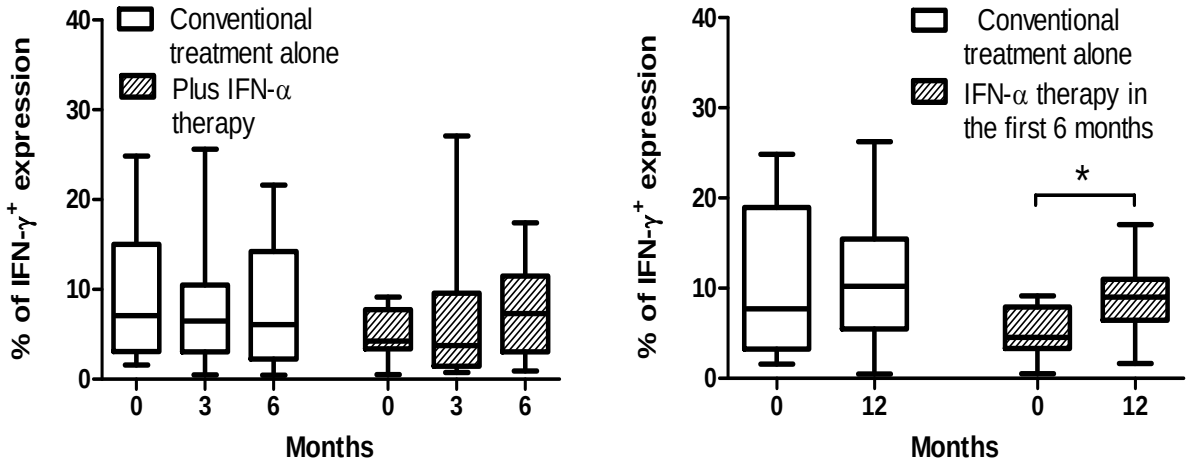
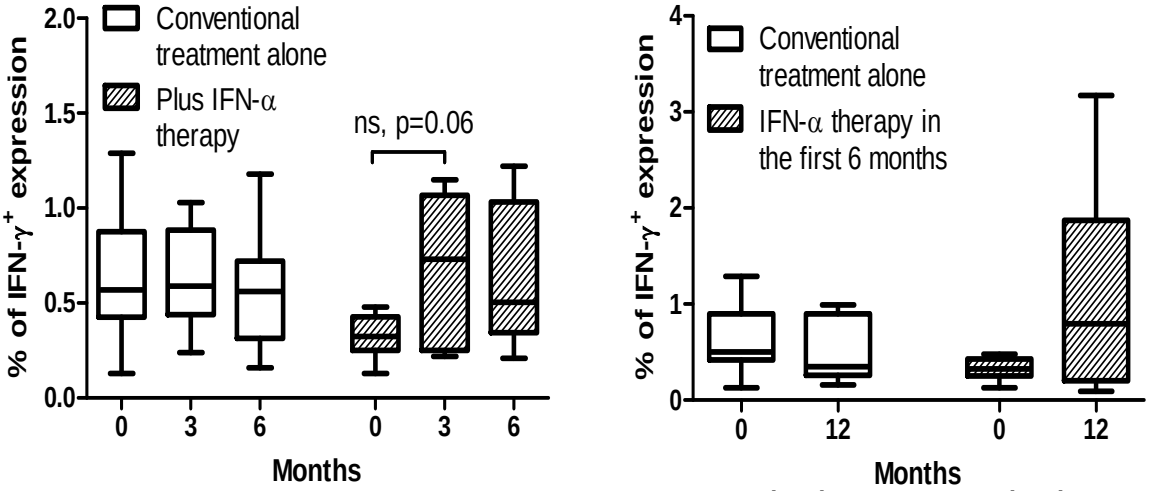


Fig 3.3: Upregulation of IFN-γ expression with IFN-α therapy in CD3$^+$ T cells

Whole blood samples from BD patients were obtained at baseline visits and follow-ups at 3, 6, and 12 months. These were stained with anti-CD3 surface antibodies before intracellularly with anti-IFN-γ antibodies. The data are shown as percentages of IFN-γ$^+$ cells among CD3$^+$ lymphocytes. Of the patients presented here, 17 (14) BD patients were on conventional treatment alone and 15 (14) were on IFN-α2b therapy as well. The number in brackets denotes the number of patients represented in the 12 month data. In (a), results were from untreated cell cultures; in (b), results were from cell cultures stimulated with PMA and ionomycin. All results are presented as boxplots.

(a) Untreated



(b) PMA/ionomycin stimulation

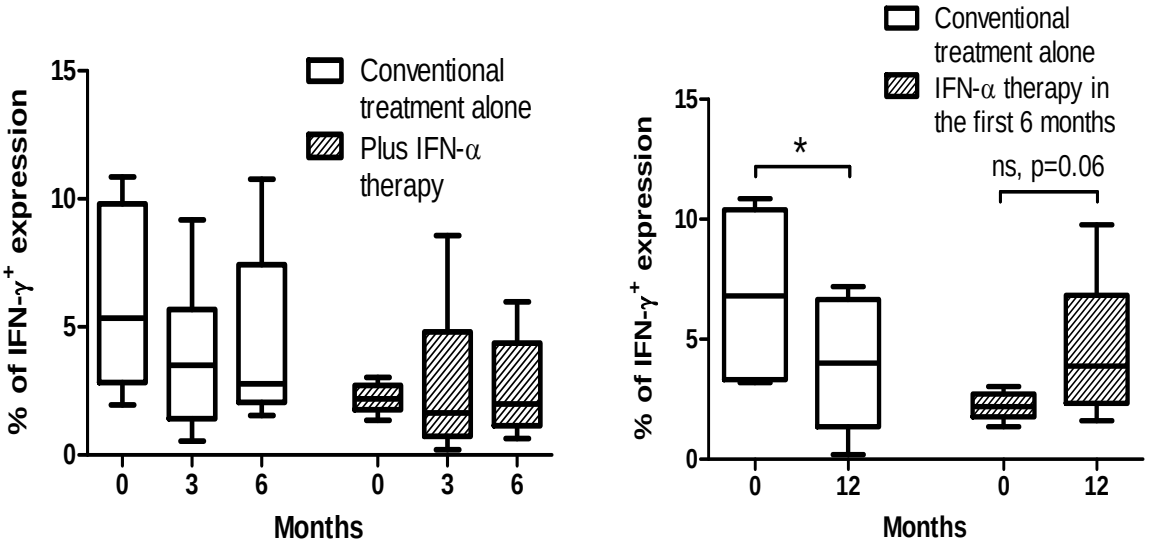


Fig 3.4: Tendency of IFN-γ expression to upregulate with IFN-α therapy in CD4$^+$ T cells
Whole blood samples from BD patients were obtained at baseline visits and follow-ups at 3, 6, and 12 months. These were stained with anti-CD4 surface antibodies before intracellularly with anti-IFN-γ antibodies. The data are shown as percentages of IFN-γ$^+$ cells among CD4$^+$ lymphocytes. Of the patients presented here, 9 (7) BD patients were on conventional treatment alone and 6 (6) were on IFN-α2b therapy as well. The number in brackets denotes the number of patients represented in the 12 month data. In (a), results were from untreated cell cultures; in (b), results were from cell cultures stimulated with PMA and ionomycin. All results are presented as boxplots. ns=not significant.

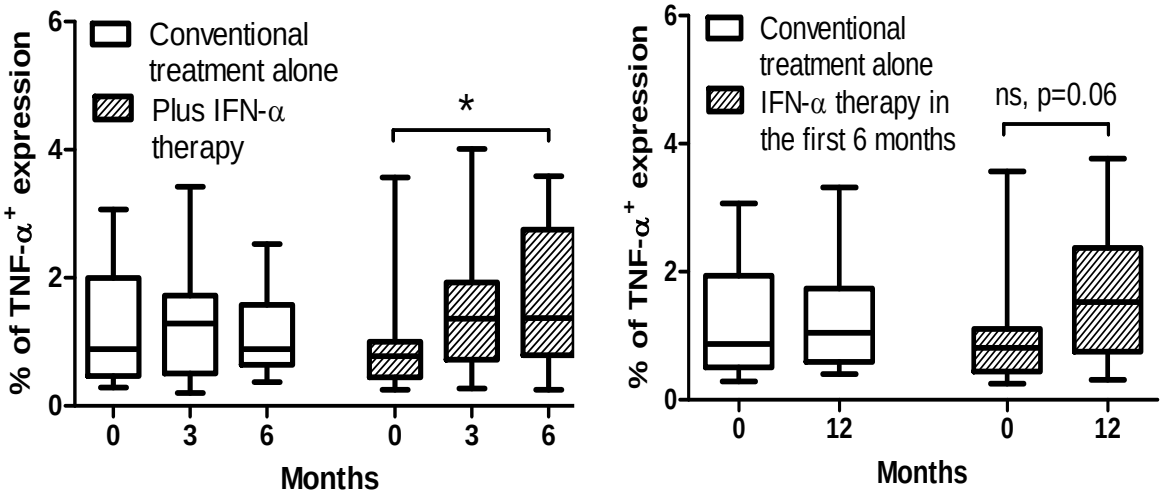
3.3.3 IFN-α2b therapy enhances TNF-α expression by T cells in BD patients

Using intracellular cytokine staining method as described in Materials and Methods, the influence of IFN-α on TNF-α expression in T cells were examined. Cell cultures were either untreated or activated with anti-CD3/CD28 Abs or PMA/ionomycin for 24 hours before staining. Cytokine expression was analysed by gating on $CD3^+$ lymphocytes.

Overall, no significant alteration in the amount of TNF-α produced by $CD3^+$ T cells was demonstrated consistently in either groups of BD patients. In the group on conventional treatment alone, no significant variation in TNF-α expression levels by $CD3^+$ T cells was detected.

In the IFN-α treatment group, there appeared to be a trend for heightened TNF-α levels. In CD3/CD28 stimulated cultures (median 1.4% and IQR 0.8-2.8%; $p<0.05$) (Fig 3.5a), an upregulation of TNF-α production was noticeable after 6 months of treatment, when compared to values before initiation of IFN-α therapy (median 0.8% and IQR 0.5-1.0%). The trend of increased expression was also evident in the 12 month data, as the median levels were augmented from 3.2% (IQR 1.3-5.4%) at baseline visits to 10.0% (IQR 6.7-13.6%; $p<0.01$) in cultures stimulated with PMA/ionomycin (Fig 3.5b). Additionally, significant upregulation was observed in the 12 month untreated samples (median 0.8% and IQR 0.6-1.2%; $p<0.05$) when evaluating against the numbers obtained from earliest visits (median 0.5% and IQR 0.4-0.8%) (data not shown).

(a) CD3/CD28 stimulation



(b) PMA/ionomycin stimulation

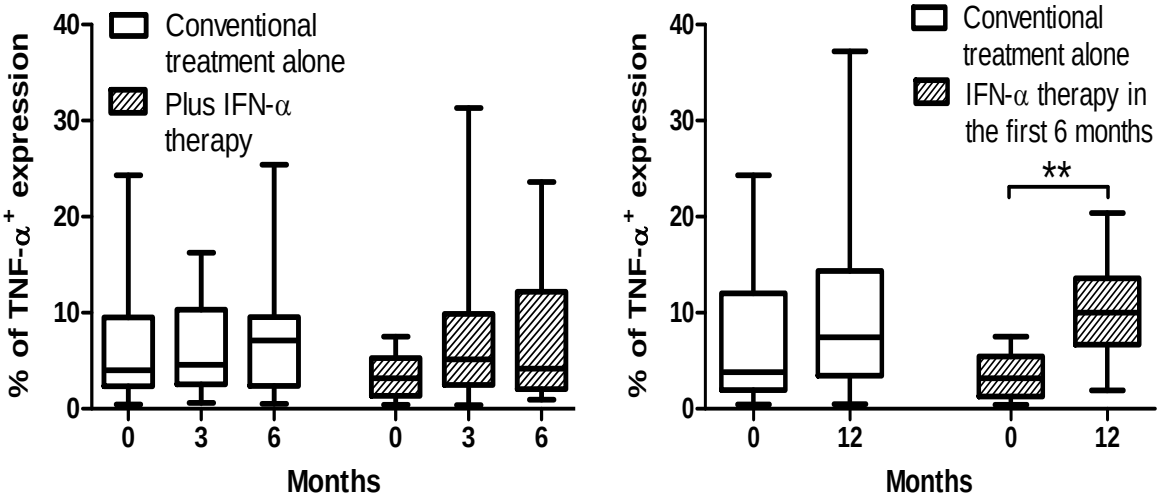


Fig 3.5: Enhancement of TNF-α expression with IFN-α therapy in CD3$^+$ T cells

Whole blood samples from BD patients were obtained at baseline visits and follow-ups at 3, 6, and 12 months. These were stained with anti-CD3 surface antibodies before intracellularly with anti-TNF-α antibodies. The data are shown as percentages of TNF-α$^+$ cells among CD3$^+$ lymphocytes. Of the patients presented here, 17 (14) BD patients were on conventional treatment alone and 15 (14) were on IFN-α2b therapy as well. The number in brackets denotes the number of patients represented in the 12 month data. In (a), data were from cell cultures with CD3/CD28 stimulation; in (b), data were from cell cultures stimulated with PMA and ionomycin. All results are presented as boxplots. ns=not significant.

3.3.4 IFN-α2b therapy increases IL-2 expression by T cells in BD patients

Analysis of the effects of IFN-α on IL-2 production by $CD3^+$ T cells was approached by using intracellular cytokine staining on whole blood samples as described in Materials and Methods. Cell cultures were either untreated or activated with anti-CD3/CD28 Abs or PMA/ionomycin for 24 hours before staining. Cytokine expression was analysed by gating on $CD3^+$ lymphocytes.

From our data, there was a global enhancement of IL-2 production by $CD3^+$ T cells in the IFN-α treatment group. In CD3/CD28 stimulated cultures (Fig 3.6a), the levels were significantly increased at 3 months (median 0.6% and IQR 0.2-1.4%; $p<0.05$) and 6 months (median 0.5% and IQR 0.3-1.1%; $P<0.05$) when compared to the data from initial visits (median 0.2% and IQR 0.2-0.6%). This upregulation was still evident when evaluating 12 month data (median 0.7% and IQR 0.5-1.6%; $p<0.01$) against baseline ones (median 0.3% and IQR 0.2-0.6%). Similar results were observed in cultures stimulated with PMA and ionomycin as well (Fig 3.6b). When compared to data from initial ones (median 1.8% and IQR 0.8-6.7%), the proportion of $CD3^+$ T cells expressing IL-2 was increased as early as at 3 months (median 7.4% and IQR 1.3-29.9%; $p<0.05$) following initiation of IFN-α therapy. Consistently, the median levels were enhanced from 2.2% (IQR 0.7-7.7%) before initiation of IFN-α therapy to 9.4% (IQR 5.1-14.3%; $p<0.05$) at 12 months. Furthermore, similar results of IL-2 expression upregulation were found with data acquired from untreated samples (data not shown).

In the group on conventional treatment alone, no significant alteration in the expression of IL-2 by $CD3^+$ T cells was detected with the exception of a decrease observed in CD3/28 stimulated samples when comparing 3 month values (median 0.6% and IQR 0.3-1.3%) with the 6 month ones (median 0.4% and IQR 0.2-0.5%; $p<0.05$) (Fig 3.6a).

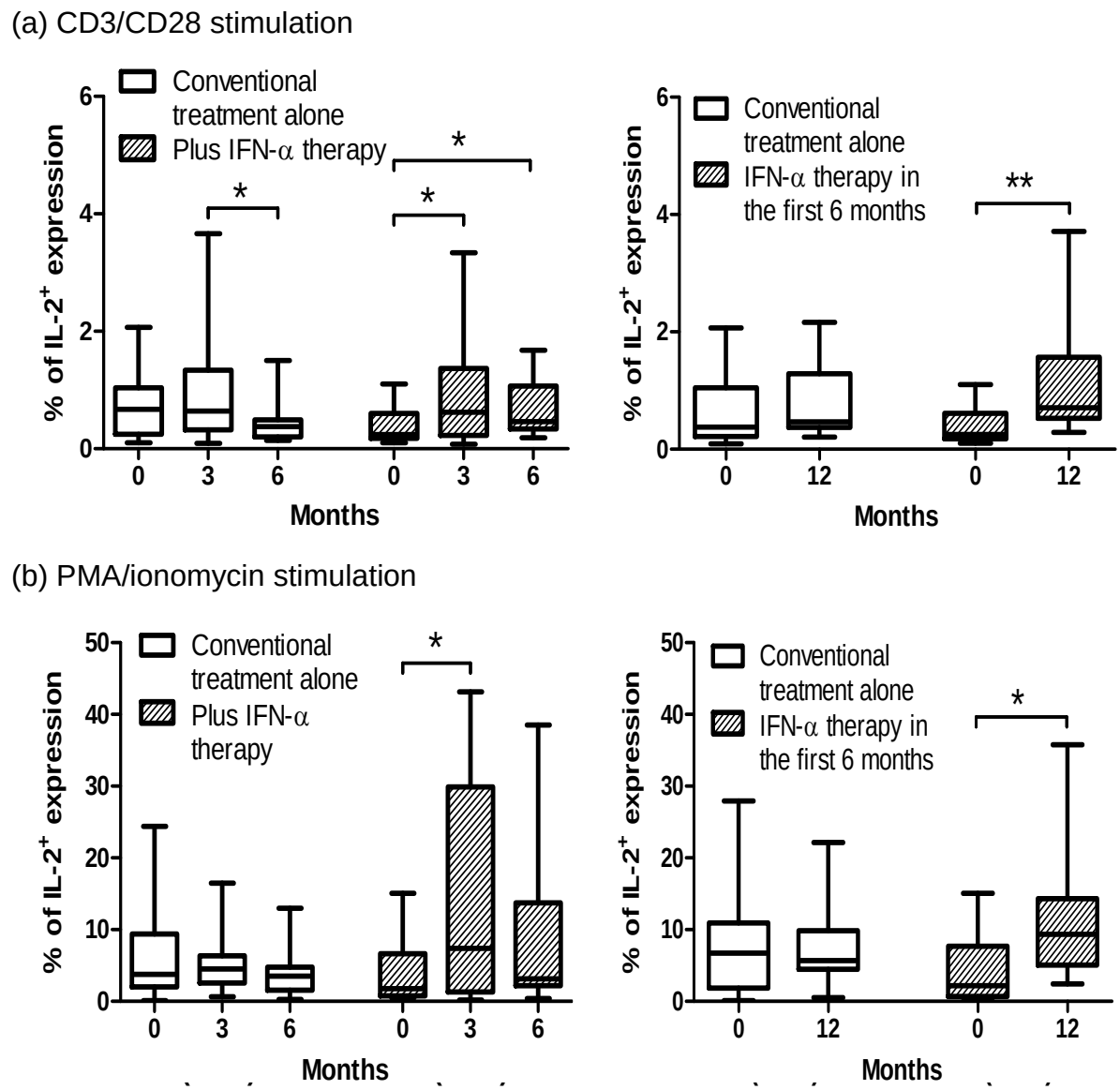


Fig 3.6: Upregulation of IL-2 expression with IFN-α therapy in $CD3^+$ T cells

Whole blood samples from BD patients were obtained at baseline visits and follow-ups at 3, 6, and 12 months. These were stained with anti-CD3 surface Ab before intracellularly with anti-IL-2 Ab. The data are shown as percentages of $IL\text{-}2^+$ cells among $CD3^+$ lymphocytes. Of the patients presented here, 16 (15) BD patients were on conventional treatment alone and 14 (13) were on IFN-α2b therapy as well. The number in brackets denotes the number of patients represented in the 12 month data. In (a), data were from cell cultures with CD3/CD28 stimulation; in (b), data were from cell cultures stimulated with PMA and ionomycin. All results are presented as boxplots.

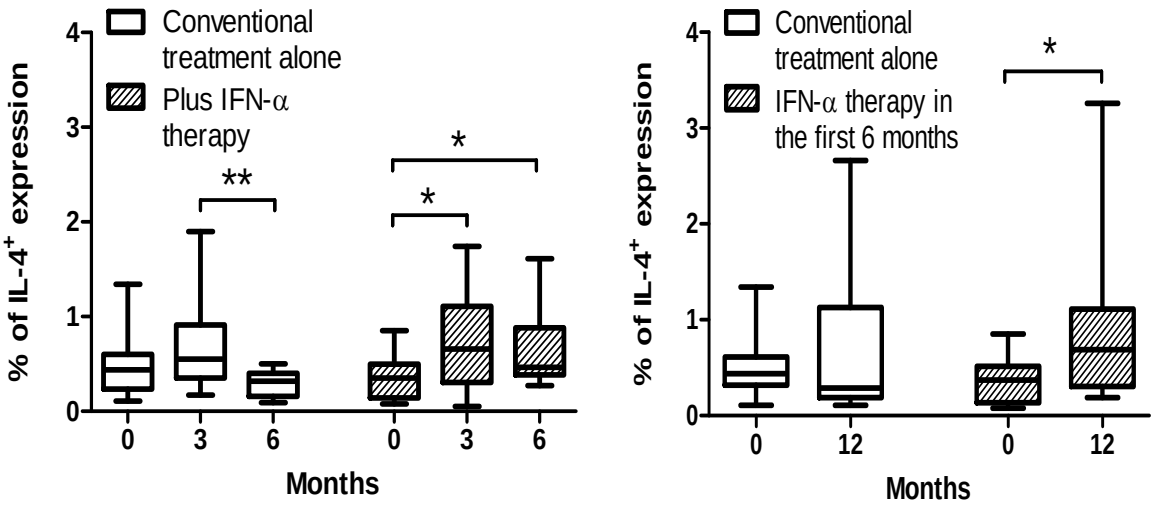
3.3.5 IFN-α2b therapy upregulates IL-4 expression by T cells in BD patients

To investigate the effects of IFN-α on signature Th2 cytokine, IL-4, serial whole blood samples from BD patients were studied using intracellular cytokine staining method as described in Materials and Methods. Cell cultures were either untreated or activated with anti-CD3/CD28 Abs or PMA/ionomycin for 24 hours before staining. Cytokine expression was analysed by gating on $CD3^+$ lymphocytes.

Similar to IL-2 data, there was a generalised upregulation of IL-4 production by $CD3^+$ T cells in the IFN-α treatment group throughout the 12 month observation period. In CD3/CD28 stimulated cultures (Fig 3.7a), the levels of IL-4 expression by $CD3^+$ T cells were significantly elevated at 3 months (median 0.7% and IQR 0.3-1.1%; $p<0.05$) and 6 months (median 0.5% and IQR 0.4-0.9%; $p<0.05$) when evaluated against the levels at initial visits (median 0.4% and IQR 0.1-0.5%). This augmentation of IL-4 expression was still evident in data obtained from the 12 month visit samples (median 0.7% and IQR 0.3-1.1%; $p<0.05$) when compared to initial assessment visits (median 0.4% and IQR 0.1-0.5%). Similarly, in cultures stimulated with PMA and ionomycin (Fig 3.7b), the proportion of $CD3^+$ T cells producing IL-4 was upregulated when comparing 3 month data (median 1.3% and IQR 0.8-2.6%; $p<0.01$) with initial ones (median 0.7% and IQR 0.4-1.0%). This increase in IL-4 expression could still be demonstrated at 12 month assessments as the median levels of IL-4 were upregulated from 0.7% (IQR 0.4-0.8%) at baseline to 1.5% (IQR 1.2-2.4%; $p<0.01$) at 12 months. Consistently, augmentation of IL-4 expression was also observed in the untreated samples at 3, 6 and 12 months (data not shown).

In the group on conventional treatment alone, no significant change in the production of IL-4 by $CD3^+$ T cells was shown when comparing follow-up visit data to the initial visit ones. However, in CD3/28 stimulated samples, the levels of IL-4 expression was downregulated at 6 months (median 0.3 and IQR 0.2-0.4%; $p<0.01$) when compared to 3 month values (median 0.6% and IQR 0.4-0.9%) (Fig 3.7a). Likewise, in cultures stimulated with PMA/ionomycin, there was significant reduction of the proportions of $CD3^+$ T cells-expressing IL-4 at 6 months (median 0.8% and IQR 0.4-0.9%; $p<0.01$) when evaluated against 3 month data (median 1.2% and IQR 0.9-2.8%) (Fig 3.7b).

(a) CD3/CD28 stimulation



(b) PMA/ionomycin stimulation

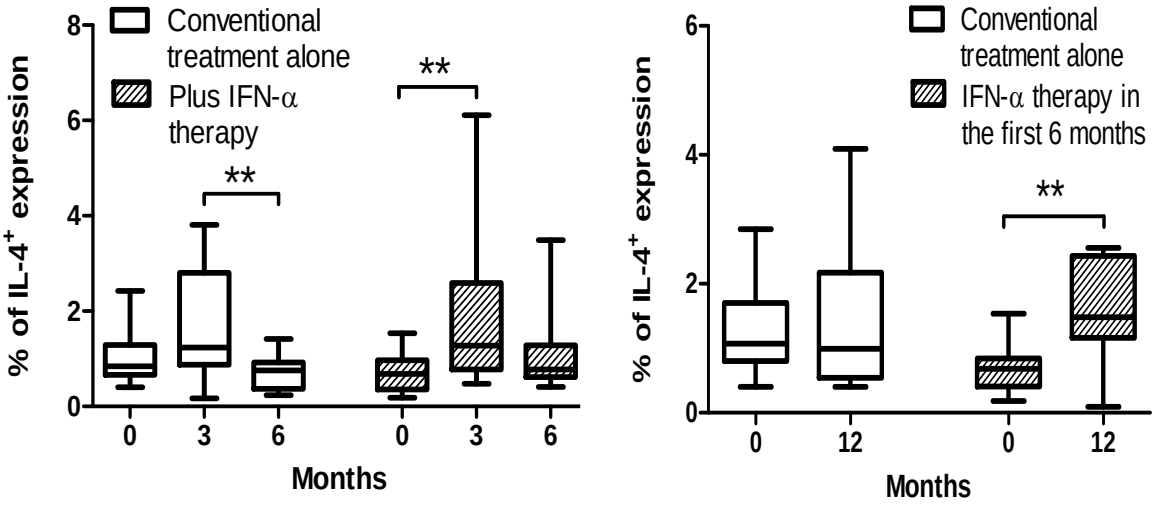


Fig 3.7: Augmentation of IL-4 expression with IFN-α therapy in $CD3^+$ T cells

Whole blood samples from BD patients were obtained at baseline visits and follow-ups at 3, 6, and 12 months. These were stained with anti-CD3 surface Ab before intracellularly with anti-IL-4 Ab. The data are shown as percentages of $IL\text{-}4^+$ cells among $CD3^+$ lymphocytes. Of the patients presented here, 16 (15) BD patients were on conventional treatment alone and 14 (13) were on IFN-α2b therapy as well. The number in brackets denotes the number of patients represented in the 12 month data. In (a), data were from cell cultures with CD3/CD28 stimulation; in (b), data were from cell cultures stimulated with PMA and ionomycin. All results are presented as boxplots.

3.4 Discussion

IFN-α has wide-ranging immunomodulatory properties and it has differential influences on cells of the immune system. In an early study on the short-term effects of IFN-α, a single subcutaneous injection IFN-α2b induced noticeable changes in peripheral blood leukocyte numbers as well as activation of neutrophils and monocytes in healthy individuals. IFN-α administration resulted in an increase in neutrophil counts and provoked sustained decrease in the number of lymphocytes, basophils and eosinophils (Corssmit et al. 1997).

3.4.1 Influence of IFN-α on peripheral blood lymphocytes

IFN-α treatment was reported to induce expansion of peripheral blood lymphocytes and monocytes in patients with severe steroid-induced asthma. This is in contrast to the number of neutrophils which was substantially reduced under the influence of IFN-α therapy. Although no alteration was detected in the number of $CD8^+$ T cells, a relative increase in $CD4^+$ T cells was observed (Simon et al. 2003). In our experiments, there was no significant change in the expression levels of CD4, CD8 or TCRαβ by peripheral blood T lymphocytes in BD patients whether they were on IFN-α or not at any time points.

3.4.2.1 Biology of IFN-γ

IFN-γ is a 17kD protein produced mainly by NK cells and NKT cells as part of the innate immune response, and by lymphocytes expressing the surface antigens CD4 and CD8 once antigen-specific immunity develops. Within the $CD4^+$ T cell population, IFN-γ is produced predominantly by the Th1 subset (Billiau & Matthys 2009). The IFN-γ receptor consists of two subunits, IFN-γ receptor 1 (IFNGR1) and IFNGR2, and each molecule interacts with a member of the JAK family, which are non-receptor protein tyrosine kinases. JAKs phosphorylate receptors and transcriptional coactivators known as Stats. Thus, the binding of IFN-γ to its receptor activates the JAK-Stat pathway (Saha et al. 2010).

IFN-γ has physiological importance in regulating immune responses and inflammatory events and its effects include the ability to enhance the functional activity of macrophages, promotion of T and B cell development, and modulation of both class I and II MHC antigen expression on a various cell types (Billiau & Matthys 2009). By induction of the transcription factor T-bet, IFN-γ enables B cells to undergo class switching more efficiently (Xu & Zhang 2005). IFN-γ activates NK cells, enhancing their cytotoxicity and cell-mediated immune responses. IFN-γ also plays an important role in chemokine-controlled processes. Generally, induction of neutrophil chemotactic chemokines is inhibited, but induction of mononuclear cell-chemotactic chemokines is stimulated by IFN-γ (Billiau & Matthys 2009).

IFN-γ plays important roles in modulating T cell activation by two main pathways: by direct effects of IFN-γ on T cells or via modulation of APC function. During generation of a primary Th1 response, IFN-γ acts as a positive regulator by selectively inducing Th1 differentiation. Traditionally, IFN-γ was seen as a pro-inflammatory cytokine, but it should be recognised that IFN-γ has important anti-inflammatory potential as well. In recent years, a more balanced role for Th1 cells and IFN-γ in autoimmune inflammation has become apparent. The intensity of Th1-induced inflammation can trigger a self-regulating mechanism in response to overt inflammation. In the early stages of $CD4^+$ T cell activation, IFN-γ regulates T cell proliferation by inducing caspase and ensuing apoptosis. Once Th1 cells become committed, they lose their capacity to be directly affected. However, IFN-γ counter-regulatory circuits can still contribute by promoting Th1 cell death indirectly through induction of numerous factors, such as nitric oxide, indoleamine 2,3 dioxygenase (IDO), and TNF-α receptors (Kelchtermans, Billiau, & Matthys 2008).

3.4.2.2 Th1 cells, IFN-γ and Behcet's disease

An early study has indicated that BD is characterised by an increased percentage of peripheral IL-2- and IFN-γ-producing T cells in peripheral blood lymphocytes during active phase of disease, indicating a strong, polarised Th1 immune response *in vivo*

(Frassanito et al. 1999). The disease activity in patients with BD is also correlated with serum IL-12 levels. IL-12 rescues Th1 lymphocytes from spontaneous and Fas-mediated apoptosis and initiates production of Th1 cytokine IFN-γ from Th precursor cells. Moreover, IL-12 promotes differentiation of precursor cells along the Th1 pathway and subsequent proliferation (Frassanito et al. 1999). It is difficult at the present time to understand the exact biological role of IFN-γ in BD and issues, such as whether the increased IFN-γ is the cause or the result of developing this disease, need to be investigated further in order to elucidate the pro- and anti-inflammatory roles of IFN-γ in BD.

3.4.2.3 The immunomodulatory effect of IFN-α on IFN-γ

From our BD patient data, an upregulation or a tendency to upregulate IFN-γ-expressing cells was detected in both $CD3^+$ and $CD4^+$ T cell populations during IFN-α2b therapy and even 6 months after the termination of treatment. This is in contrast to the patients on conventional treatment alone, where even a significant downregulation of IFN-γ expression in $CD4^+$ lymphocytes was sporadically observed. The findings are in accordance with results from another study, which showed that $CD4^+$ T cells producing IFN-γ were upregulated further in BD patients under IFN-α treatment (Amberger et al. 2007). Consistently, in a highly efficacious trial of IFN-α treatment in patients with corticosteroid-unresponsive asthma, the therapy dramatically increased the capacity of peripheral blood T cells to generate IFN-γ (Simon et al. 2003).

Interestingly, it has been revealed in an very early study that the serum levels of IFN-γ in patients with BD were significantly higher than in normal controls but the titre of IFN-γ correlated inversely with disease activity (Ohno et al. 1982). Although IFN-γ can mediate disease, its anti-inflammatory properties can serve as a protective factor in models of autoimmunity (Kelchtermans, Billiau, & Matthys 2008). Hence, enhancement of $CD4^+$ T cells producing IFN-γ are not necessarily counterintuitive to the effectiveness of IFN-α as a treatment for BD. It has been suggested that type I IFNs, IFN-α/β, augment IFN-γ signalling via association of type I and type II IFN receptor subunits (Takaoka et al. 2000). Also, the type of adaptive immune

response is conditioned by the type of preceding innate immunity. IFN-α produced by dendritic cells and/or IFN-γ released by NK cells upon stimulation by IL-12 activate Stat1 in the naive $CD4^+$ T cells. Activated Stat1 upregulates T-bet expression in T cells and subsequently promotes early T cell IFN-γ production and IL-12Rβ2 expression. T cells with IL-12Rβ2 expression can then respond to IL-12 directly and induce high IFN-γ production and sustain the expression of IL-12Rβ2 through activation of Stat4 (Szabo et al. 1997). These interactions could offer one explanation why Th1 polarisation was observed in our patients after treatment with exogenous IFN-α. Moreover, it has been reported that IFN-α2a induces phenotypic maturation of dendritic cells and enhances their capacity to stimulate T cells. In CD40-stimulated DCs, IFN-α2a augments the production of IL-12, which contributes to Th1 polarisation and IFN-γ secretion (Tamir et al. 2005).

3.4.3.1 Biology of TNF-α

TNF-α, a 17 kD peptide, is a multi-functional inflammatory cytokine, which plays a fundamental role in the establishment and maintenance of the inflammatory response. It is produced predominantly as a secreted protein by several types of cells including T and B lymphocytes, mast cells, NK cells, neutrophils, endothelial cells, smooth and cardiac muscle cells, fibroblasts and osteoclasts, but especially by activated macrophages (Bradley 2008). Cells expressing CD4 secrete TNF-α while $CD8^+$ cells secrete little or no TNF-α.

The effects of TNF-α are mediated by binding of TNF-α to distinct TNF receptor type 1 (TNFR1) and type 2 (TNFR2). The two TNF-α receptors have similar affinities, and they are found on many cell types, including activated T and B cells, macrophages, and neutrophils (Benveniste & Benos 1995). Apart from mediating cell survival and pro-inflammatory signals through NF-κB and AP-1, TNFR1 can initiate cell death signalling pathways. Ligation of TNFR1 is both necessary and sufficient to induce cytotoxic and pro-inflammatory TNF-α responses. However, the signalling pathways initiated by TNFR2 are less clearly defined, and TNFR2 appears to signal both shared and opposing effects to TNFR1. TNFR2 may promote cell activation, migration or proliferation (Bradley 2008).

TNF-α is produced early and rapidly during inflammation and its release stimulates numerous other pro-inflammatory mediators such as IL-1, IL-6, IL-8, and TNF-α itself, which can further influence the course and outcome of an inflammatory response and subsequent pathologic consequences (Duzgun et al. 2005). At sites of inflammation, chronic TNF-α signals can contribute to the survival of effector T cells (Clark et al. 2005). The cytokine has been described to modulate immunological responses by enhancing the expression of MHC class I and class II molecules (Ma 2001). Also, TNF-α is a major inducer of endothelial adhesion molecules and chemokines. Hence, the upregulation of TNF-α may have a major effect on the recruitment of leukocytes to active inflammatory sites (Duzgun et al. 2005).

TNF-α possesses immunosuppressive effects as well. In an early murine study, it has been demonstrated that chronic exposure to TNF-α suppresses the response of both Th1 and Th2 subsets and attenuates T cell receptor signaling *in vivo* (Cope et al. 1997). Upon macrophage activation, TNF-α signalling can selectively inhibit IL-12 gene expression as part of the scheme of cytokine feedback and self-limiting modulations (Ma et al. 2000). Moreover, TNF-α can promote lymphoid T cell apoptosis, inhibit dendritic cell co-stimulation and induce other cytokines that can inhibit cell-mediated immunity (O'shea, Ma, & Lipsky 2002). Recently, it was reported that the suppressive function of Treg cells was regulated by TNF-α through its receptor TNFR2, constitutively expressed on human Tregs and particularly at higher levels in the $CD45RA^-$ subset. TNF-α/TNFR2 signalling was demonstrated to inhibit the suppressive function of $CD4^+CD25^{high}$ Tregs via activation of the NF-κB cascade (Nagar et al. 2010) and pre-treatment (up to 48 hours) of Treg cells with TNF-α caused a marked downregulation of Foxp3 mRNA and protein expression (Valencia et al. 2006).

Interesting findings on Treg cells were demonstrated in RA patients treated with an anti-TNF-α agent, infliximab, as well. $CD4^+CD25^{high}$ T cells isolated from patients with active RA showed inability to suppress pro-inflammatory cytokines released by effector T cells and monocytes. After treatment with infliximab, the capacity of Treg cells to suppress cytokine production was restored to the level found in healthy individuals, and a regulatory phenotype was able to be conveyed to effector T cells

(Ehrenstein et al. 2004). A subsequent study by the same group revealed that the restoration of function of the $CD4^{+}CD25^{high}$ population after infliximab therapy was most likely to be due to the suppressive effects of the newly differentiated $Foxp3^{+}CD25^{high}CD62L^{-}$ Treg cells which suppressed via TGF-β- and IL-10-dependent mechanisms. Of note, the natural $CD62L^{+}$ Treg cells remained defective in infliximab-treated patients (Nadkarni, Mauri, & Ehrenstein 2007).

These findings suggest a mechanism by which TNF-α can modulate Treg cells. It has been postulated that at the start of an inflammatory process, intense TNF-α production may debilitate the activity of Treg cells and support induction of immune reactivity and the effector phase of lymphocyte responses. During recovery, a reduction in TNF-α level may lead to enhanced Treg function that keeps immune reactivity in check and avoids triggering immune responses to autologous tissue antigens revealed during inflammation (Valencia et al. 2006).

3.4.3.2 TNF-α and Behcet's disease

TNF-α has been considered to be associated with the pathogenesis and activity of BD as higher levels of TNF-α have been found in the aqueous humour and serum of BD patients with uveitis (Dilek et al. 2009;Santos et al. 2001). This is in accordance with another study which showed a significant increase of both serum IL-18 and TNF-α in BD patients with active disease when compared with healthy volunteers (Oztas et al. 2005). Increased levels of IL-18 may support Th1 predominance in Behcet's disease. In addition, the number of TNF-α producing cells was reported to be increased in the active phases of the disease (Misumi et al. 2003).

TNF-blocking agents such as infliximab and etanercept have been reported to induce rapid and effective suppression of almost all manifestations of systemic and ocular BD, at least in the short term (Sfikakis et al. 2007). The most commonly used anti-TNF agent has been infliximab, and it has been shown to promote a complete and rapid remission of sight-threatening ocular inflammation and improvements of visual acuities (Sfikakis et al. 2004). A single infusion of infliximab (5 mg/kg) can lead to complete response of vitritis and retinitis in about half of the cases. Cystoid

macular oedema, the most-therapy resistant manifestation of ocular BD, resolve in 90% of patients by day 28. The initial response is fast, e.g. within 24 hours, but relapses can necessitate continuous treatment. The recent recommendations are to include it as an add-on immunosuppressive therapy for selected patients with BD, who are refractory or intolerant to traditional immunosuppressives (Sfikakis et al. 2007). Moreover, in the only placebo-controlled study of any anti-TNF agent in BD, etanercept was demonstrated to significantly reduce the mean numbers of oral ulcers, nodular and papulopustular lesions (Melikoglu et al. 2005).

3.4.3.3 The immunomodulatory effect of IFN-α on TNF-α

With the exception of data analysed using 6 month CD3/CD28 stimulated samples, our data did not reveal any meaningful upregulation in the proportion of $CD3^{+}TNF\text{-}\alpha^{+}$ T cells in BD patients in the first 6 months while they were on IFN-α2b therapy. However, after 6 months of IFN-α2b treatment and especially after cessation of therapy, significantly elevated TNF-α expression levels were sporadically observed in the blood samples of these patients. On the other hand, increased TNF-α production was not detected in the group of patients on conventional treatment alone.

In a previous study, a significant increase in TNF-α level, reflecting activation of macrophages, in the serum of BD patients during treatment with IFN-α2a was reported (Kotter et al. 2005). This is in line with an early study which demonstrated IFN-γ augmentation in IL-2-, mitogen-, or PMA-induced production of both TNF-α and TNF-β by PBMCs (Nedwin et al. 1985). Therefore, the observed upregulation of IFN-γ in our experiments could contribute to the sporadic increase in TNF-α production levels.

The proportions of $CD3^{+}$ T cells producing TNF-α were low in our experiments on BD patients as the mean levels were less than 2% with CD3/CD28 stimulation and less than 10% with PMA/ionomycin stimulation. The data is comparable to an early study where healthy donor PBMCs were stimulated with PMA/ionomycin for 24 hours. The proportion of $CD3^{+}$ cells producing TNF-α was found to be less than 5% (Mascher, Schlenke, & Seyfarth 1999).

3.4.4.1 Biology of IL-2

IL-2, a 15kD cytokine, is historically known as T cell growth factor. It is a cytokine that is produced primarily by newly activated $CD4^+$ T cells, although $CD8^+$ T cells, B cells, NK cells, NKT cells, and dendritic cells activated by microbial stimuli have also been reported to produce IL-2 in low levels (Gaffen 2001;Letourneau et al. 2009). Three distinct receptor chains have been identified as components of the IL-2 receptor (IL-2R): the common γ chain (γc) or CD132, which is shared by the common γ chain cytokine family, comprising IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21; the β chain or CD122, which is shared by both IL-2R and the IL-15 receptor; and the α chain or CD25 (Kovanen & Leonard 2004;Letourneau et al. 2009).

Two functional IL-2 receptor forms have been described: the dimeric low-affinity receptor, which is composed solely of CD122 and γc, and the trimeric high-affinity receptor, which is composed of CD25, CD122, and γc. The low-affinity receptor is found at low levels on naive $CD4^+$ T cells, at intermediate levels on naive $CD8^+$ T cells and memory $CD4^+$ T cells, and at high levels on memory $CD8^+$ T cells and NK cells. In contrast, the high-affinity receptor is essentially expressed on $CD4^+CD25^+Foxp3^+$ Treg cells and activated T cells (Letourneau et al. 2009).

Early in an immune response, IL-2 maintains T cell survival and supports the growth and expansion of T cells. If the response is enduring, as in the case of self-antigens, the prolonged IL-2 exposure makes T cells sensitive to Fas-mediated apoptosis and terminates the response (Abbas 2003). IL-2 has initially been reported to inhibit the differentiation of Th17 cells (Laurence et al. 2007). However, this effect is abolished in the presence of IL-1β (Kryczek et al. 2007) and under these conditions, IL-2 has instead a positive effect on IL-17 production by human $CD4^+$ T cells (Manel, Unutmaz, & Littman 2008).

Recent studies demonstrated that IL-2, along with B7 co-stimulators, promoted the development of Treg cells. Thus, along with the effect on apoptosis of activated T cells, termination of immune responses and maintenance of self tolerance can be achieved (Abbas 2003;Amberger et al. 2007). IL-2 is indispensable for the induction of Tregs as IL-2 signalling is required for thymic development, peripheral expansion

and suppressive activity of Treg cells (Shevach et al. 2006). In line with previous studies, treatment of cancer patients with exogenous IL-2 has been correlated with an increase in the frequency of $Foxp3^+$ Tregs. These cells generated by IL-2 therapy expressed similar levels of Foxp3 and had similar potency for suppression compared to Treg cells present in healthy controls (Ahmadzadeh & Rosenberg 2006;Zhang et al. 2005). Although regulatory $CD4^+CD25^{high}$ cells express high levels of IL-2 receptors, the suppressive ability of these cells is inhibited with the addition of exogenous IL-2 to co-cultures of $CD4^+CD25^{high}$ cells and $CD4^+CD25^-$ responder cells *in vitro*. It has thus been suggested that suppression by Treg cells at the initiation of significant inflammatory responses *in vivo* would be kept in check by the secretion of IL-2 by Ag-responsive T cells (Baecher-Allan et al. 2001).

3.4.4.2 IL-2 and Behcet's disease

Early studies revealed that serum level of IL-2 in BD patients is comparable to healthy controls (Kosar et al. 1999;Sayinalp et al. 1996). However, a more recent study showed that the fraction of $CD3^+$ and $CD4^+$ T cells producing IL 2 was substantially lower in untreated BD patients compared to healthy controls. On the other hand, there was no difference for the $CD8^+$ subsets between patients with BD and healthy individuals (Amberger et al. 2007).

3.4.4.3 The immunomodulatory effect of IFN-α on IL-2

From our data, there was an upregulated expression of IL-2 by $CD3^+$ T cells noted in BD patients who received IFN-α2b therapy. This augmentation effect was observed even after cessation of IFN-α2b therapy. This result is supported by previous findings that IFN-α2a therapy enhanced $CD3^+$ T cells producing IL-2 in BD patients, which may be explained by the promotion of Th1 differentiation (Amberger et al. 2007). Increased IL-2 levels in myeloma patients treated with IFN-α2a have been demonstrated as well (Sonmez et al. 2004). It is possible that the upregulation of IL-2 may impede autoimmune inflammatory responses through induction of Treg cells. By this means, patients may regain self-tolerance and clinical remission is seen.

3.4.5.1 Biology of IL-4

IL-4, a 20kD glycoprotein, is a pleiotropic common gamma chain cytokine best known for its ability to direct antigen-activated T cells to differentiate into Th2 and Tc2 effectors and its action on activated B cells to undergo IgE isotype switch (Annunziato & Romagnani 2009). Apart from T cells, numerous sources of IL-4 have been identified, including γδ T cells, eosinophils, basophils, NKT cells and mast cells (Min 2010). Stat5 activation induced by IL-2 is crucial for Th2 cell differentiation *in vitro* (Paul & Zhu 2010). IL-4 exerts its biological effects by binding to the IL-4 receptor α-chain, a component of both the type 1 and type 2 IL-4 receptors (IL-4R). In the type 2 IL-4R, IL-4Rα is paired with IL-13Rα1 rather than the common γ chain, which also binds IL-13 (Nelms et al. 1999). These receptors are widely expressed throughout the body. IL-4 modulates T cell activation, mast cell proliferation, and haematopoiesis. It is an important growth factor for T and B cells and increases the survival of cultured human lymphocytes. Overproduction of IL-4 has been associated with elevated IgE production and allergic diseases *in vivo* (Schulze-Koops & Kalden 2001).

Of importance in regulating immune responses is the ability of IL-4 to downregulate the activation and the inflammatory functions of monocytes and macrophages. IL-4 enhances the expression of MHC class II molecules and of several cytokine inhibitors such as IL-1 receptor antagonist (IL-1Ra), soluble IL-1 receptor type II and TNF receptors while inhibiting the secretion of pro-inflammatory cytokines, including IL-1β, TNF-α, IL-6, IL-8 and IL-12 (Schulze-Koops & Kalden 2001). The ability of Th2 effectors to control Th1-mediated inflammatory responses has been attributed largely to these anti-inflammatory effects of IL-4. Consequently, IL-4 has been used *in vivo* as a successful treatment for a number of experimental autoimmune diseases that are caused by activated Th1 cells in animals, such as experimental allergic encephalomyelitis. Moreover, IL-4 downregulates metalloproteinase production *in vitro* and stimulates tissue inhibitor of metalloproteinases 1 (TIMP1) production, suggesting a protective effect of IL-4 towards extracellular matrix degradation (Schulze-Koops & Kalden 2001). In mice, IL-4 has also been shown to downregulate Th17 differentiation (Harrington et al. 2005;Park et al. 2005).

More recently, IL-4 was demonstrated to induce the development of $CD4^+CD25^+$ T cells with regulatory capacity from peripheral naive $CD4^+CD25^-$ precursor cells. The IL-4-induced generation of peripheral Tregs was independent of the presence of TGF-β or IL-10, but was dependent on Ag-specific stimulation and B7 costimulation. Also, endogenous IL-2 was demonstrated to have a role as a growth factor for $CD25^+$ Treg cells. IL-4-induced $CD25^+$ Tregs phenotypically and functionally resembled naturally occurring $CD25^+$ Treg cells. IL-13, the other IL-4R α-chain-binding cytokine, was identified to have similar effects on the induction of $CD25^+$ Treg cells (Skapenko et al. 2005). Interestingly, it has been shown in a recent murine study that Treg cells with attenuated Foxp3 expression preferentially convert into IL-4-producing Th2 effector cells even in a Th1-polarising environment (Wan & Flavell 2007).

3.4.5.2 IL-4 and Behcet's disease

Like in healthy control subjects, it has been described that the amount of IL-4-producing cells is low in patients with BD. Previous studies showed that there was no significant difference in the frequency of IL-4-producing $CD3^+$, $CD4^+$ and $CD8^+$ cells in patients with active BD, BD patients in complete remission, and healthy control subjects (Frassanito et al. 1999;Sugi-Ikai et al. 1998). These findings suggested that resolution of active BD did not involve a Th2 immune response.

3.4.5.3 The immunomodulatory effect of IFN-α on IL-4

Analysing our data, IL-4 upregulation was detected in T cells as early as 3 months after initiation of IFN-α therapy in BD patients and this trend was evident at 6 month assessments as well. At 12 months, this increase in $CD3^+$ T cells expressing IL-4 was still maintained despite cessation of IFN-α therapy in the treatment group. In accordance to our observation, it was demonstrated that at the protein level, IFN-α increased IL-4 synthesis dose-dependently in PBMCs from healthy donors, especially after stimulation with Concanavalin A or anti-CD28/PMA (Kaser, Molnar, & Tilg 1998). Also, in the presence of IFN-α, $CD4^+$ T cells stimulated with PMA and

anti-CD28 Ab were shown to increase in IL-4 mRNA accumulation after 24 hours of culture (Schandene et al. 1996).

As IL-2 has been shown to be necessary for T cells to acquire IL-4 producing capacity (Paul & Zhu 2010), the enhanced amounts of IL-2 expression detected in our experiments could contribute to IL-4 augmentation. This is further supported by the finding in a murine study which have shown that CD25 (IL-2Rα) expression is higher in developing Th2 cells than in developing Th1 cells, possibly due to the action of transcription factor c-Maf. Hyperresponsiveness of IL-2 may be conferred as a result of such higher expression of CD25 (Hwang, White, & Ho 2002).

Nevertheless, the production of IL-4 has not always been reported to be augmented by IFN-α. In a study on freshly isolated, purified human $CD4^+$ and $CD8^+$ T cells stimulated for 24 hours via TCR/CD3 complex, it was demonstrated that IFN-α did not increase the frequency of IL-4-producing cells and it antagonised the suppressive effect of IL-4 on IFN-γ production (Brinkmann et al. 1993). The molecular basis for these variable effects of IFN-α on IL-4 production remains to be specified.

In our experiments on BD patients, the mean proportions of $CD3^+$ T cells producing IL-4 were up to 2% after cells were stimulated with PMA/ionomycin overnight. These levels are similar to a study on healthy subjects where the mean percentages of $CD3^+$ T cells and subpopulations of $CD4^+$ T cells producing IL-4 after 6 hours of stimulation with PMA/ionomycin were shown to be 2.1% and 5.1% respectively (Baran et al. 2001). In another study, the proportions of IL-4 producing $CD4^+$ T cells from patients with BD and normal subjects were shown to be around 5% after PBMCs were cultured with Concanavalin A (ConA) followed by PMA/ionomycin (Koarada et al. 2004).

3.4.6 The potential influence of systemic steroids and immunosuppressants

The findings from experiments were not always consistent with previous studies, and this could be explained in part because patient blood samples were collected in

different disease phases and in part due to ongoing immunosuppressive therapies. Unfortunately, the influence of subcutaneous IFN-α therapy could not be tested in the absence of some degree of immunosuppression in our patients. As a result, the variability observed in phenotype and cytokine profile could partly be explained by individual treatment history of each patient.

Glucocorticoids (GCs), such as prednisolone, are among the most potent immunosuppressive and anti-inflammatory drugs available and widely used as therapeutics for inflammatory and autoimmune disorders. GCs were shown to suppress the production by APCs of IL-12, the main inducer of Th1 responses. Since IL-12 is extremely potent in enhancing IFN-γ and inhibiting IL-4 synthesis by T cells, the effect on IL-12 was found to be associated with decreased IFN-γ but increased production of IL-4 by T cells. GCs were also reported to have a direct effect on Th2 cells by upregulating their IL-4 and IL-13 production (Elenkov 2004). In a recent study on MS patients, TNF-α production by peripheral blood cells was observed to be suppressed following a short course of IV methylprednisolone. The inhibitory effect of dexamethasone on LPS-stimulated TNF-α production by peripheral blood cells *in vitro* was demonstrated as well (van Winsen et al. 2010). With regards to IL-2, GCs were shown to block its signaling via the Jak-Stat pathway (Bianchi, Meng, & Ivashkiv 2000).

Cyclosporine A was found to significantly block IFN-γ production by polyclonally stimulated PBMCs as well as $CD4^+$ and $CD8^+$ T cells from both BD patients and normal controls. The *ex vivo* production of IFN-γ by PBMCs was also significantly inhibited in BD patients with active uveitis after 1 and 3 months of cyclosporine A treatment in ELISA experiments (Chi et al. 2010). With regards to TNF-α, cyclosporine A was shown to inhibit its production in 24 hour co-cultures of activated peripheral blood lymphocytes and THP-1 cells from a monocytic leukaemia cell line (Gonzalez-Alvaro et al. 2009). In terms of IL-2 protein secretion, it was demonstrated to be significantly inhibited in anti-CD3/CD28 stimulated whole blood cultures *in vitro* when cyclosporine A was added (Hartel et al. 2003).

It was shown that methotrexate inhibited anti-CD3/CD28 stimulated IFN-γ, TNF-α and IL-4 cytokine production by whole blood, PBMCs and T cells from healthy

donors *in vitro*, and oral intake of 10 mg methotrexate by rheumatoid arthritis patients led to marked inhibition of cytokine production in blood drawn after 2 hours (Gerards et al. 2003). In the presence of mycophenolic acid throughout the culture period, the production of IL-2 by polyclonally activated peripheral blood lymphocytes was elevated after 24 hours and the mechanism was indicated to be a decrease in IL-2 consumption due to blocking of the T cell proliferation. In the same system, however, the production of IFN-γ was suppressed (Izeradjene et al. 2001). Also, it was shown that mycophenolic acid did not have any influence on the production of TNF-α (Gonzalez-Alvaro et al. 2009).

Due to these findings on the influence of immunosuppressants, it was considered to divide the patients into smaller subgroups to examine our results further according to patients' individual treatment in order to minimise biases. However, this approach would have required a much larger sample size before meaningful results could be achieved.

3.5 Summary of findings

We did not detect any dissimilarity in terms of the number of $CD4^+$, $CD8^+$ and αβ T cells before and after the initiation of IFN-α2b therapy. Six months of subcutaneous pegylated IFN-α2b therapy showed the tendency to enhance T cell production of IFN-γ but not TNF-α on a regular basis in BD patients. Under the influence of IFN-α2b therapy, upregulation of IL-2 and IL-4 expression by $CD3^+$ T cells was also consistently observed. All the positive effects of IFN-α described here were clearly enduring as they were evident even after the termination of IFN-α2b treatment. One must be aware that although Th1 and Th2 cells are the major sources of their respective cytokines such as IFN-γ and IL-4, many others cells within and outside the immune system also produce these. However, it wasn't in the scope of this study to explore this further.

Chapter 4

The effects of subcutaneous interferon-alpha 2b on Th17 and regulatory T cells and their cytokines in patients with Behcet's disease *ex vivo*

4.1 Introduction

Th17 cells are currently defined as $CD4^+$ cells that produce IL-17A and F, but not IFN-γ or IL-4 (Crome, Wang, & Levings 2010). IL-17 has been known to promote the production of various pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 as well as enhancing proliferation, maturation and recruitment of neutrophils. Based on evidence that Th17 cells can mediate inflammation and tissue destruction, there has been intense interest in defining their origins, functions and developing strategies to block their pathological effects (Kolls & Linden 2004;Witowski, Ksiazek, & Jorres 2004).

In BD patients, apart from the previously known pathogenic cytokines, increased serum IL-17 levels have been demonstrated as compared to that of healthy controls (Hamzaoui et al. 2002). It was also reported that IL-17 serum levels in active BD patients were enhanced to a higher degree than that of patients in remission, and a possible role of IL-17 in the pathogenesis of BD was thus suggested. A recent study investigated the role of IL-23 and IL-17 in BD and identified increased expression of IL-23 and IL-17 in patients with active uveitis compared to normal controls and patients in remission (Chi et al. 2008). It was shown that IL-17 was predominantly produced by $CD45RO^+$ memory T cells and the percentage of $CD4^+$ T cells producing IL-17 was significantly higher than $CD8^+$ T cells producing IL-17. In addition, it has been revealed that IL-17A has an important role in acute attacks of not only eye disease but also oral ulcers, genital ulcers, and articular manifestations in BD patients (Ekinci et al. 2010). Moreover, it has been reported that IL-17 gene single nucleotide polymorphisms (SNPs) may influence the susceptibility of BD (Jang et al. 2008).

Treg subsets and their associated cytokines, including IL-10 and TGF-β, play a crucial role in maintaining self-tolerance and in preventing immune pathologies by

regulating the strength and/or nature of the immune response to non-self antigens (Sakaguchi 2004;Shevach 2000). It was reported recently that although the percentages of $CD4^+CD25^{high}$ Treg cells among $CD4^+$ T cells in BD patients with inactive disease were normal, the levels decreased significantly before ocular attacks (Nanke et al. 2008). This is in contrast to an earlier report that the frequency of Tregs increased significantly in a transient fashion during inflammatory manifestations and recovered to normal values during the remission phase (Hamzaoui, Hamzaoui, & Houman 2006).

In addition, *in vitro* experiments have demonstrated that the suppressive capacity of $CD4^+CD25^{high}$ Treg cells in BD patients with active disease may be reduced, comparing with Treg cells from healthy controls. Also, it is possible that the most potent $CD4^+CD25^{high}$ T cells are not found in the peripheral blood but exert their suppressive activity in the target organs (Shevach 2004). In CSF, $CD25^{high}$ and Foxp3-expressing CD4 cells have been demonstrated to be significantly increased in BD patients when compared with non-inflammatory neurological control patients (Hamzaoui, Houman, & Hamzaoui 2007).

Surprisingly, the serum levels of IL-10 were found to be significantly higher in BD patients compared to healthy controls and it may elevate even further during active disease (Hamzaoui et al. 2002). Regarding the serum levels of TGF-β in BD patients, they were demonstrated to be comparable to those in both rheumatoid arthritis and osteoarthritis patients (Ertenli et al. 2001). Unfortunately, the serum TGF-β levels were not compared to the ones in healthy individuals in the study.

4.2 Aims

Having observed the positive influence of IFN-α on Th1 and Th2 cytokines, the aim of this chapter is to investigate the effect of a 6-month course of subcutaneous IFN-α2b therapy on modulating the non-conventional T cell subsets associated with immune-mediated disorders. In particular, changes to the proportions of peripheral blood Th17 and $Foxp3^+$ Treg subsets and their cytokine profile before, during and after cessation of therapy are examined.

Another aim is to study the effect of pegylated IFN-α2b therapy on the relative amount of $CD3^+CD56^+$ NK T cells in whole blood samples of BD patients by phenotyping, as these cells have been shown to have the capacity to secrete IL-17 or exhibit regulatory function.

4.3 Results

The experiments presented here were performed in conjunction with Mr A Nouraeinejad and Ms G Galatowicz. Approximately 70% of the samples used for data analysis in this section were processed by me, and all data analysis is my own work.

4.3.1 IFN-α2b therapy suppresses IL-17 expression by $CD4^+$ T cells in BD patients

To investigate the effects of IFN-α on Th17 cell population, serial whole blood samples from BD patients on conventional immunosuppressive therapy with or without IFN-α2b treatment were studied using intracellular cytokine staining method and flow cytometry. Cell cultures were either untreated or treated with anti-CD3/CD28 Abs or PMA/ionomycin for 24 hours before staining. Cytokine expression was analysed by gating on either $CD4^+$ lymphocyte populations.

Although, no significant change was detected in the proportions of Th17 cells in the untreated cultures and cultures with CD3/CD28 stimulation (data not shown), a significant downregulation of Th17 cells was found in the IFN-α2b-treated group over the 12 month observation period in cultures stimulated with PMA/ionomycin (Fig 4.1a). As early as 3 months after initiation of IFN-α therapy, the percentage of $IL\text{-}17^+$ expression by $CD4^+$ T cells in our patients was observed to be reduced (median 0.8% and IQR 0.3-1.9%; $p<0.05$) compared to the initial values from baseline visits (median 2.0% and IQR 1.7-2.7%). Although no further decrease was seen at follow-ups afterwards, the levels of Th17 cells remained low at 6 months (median 0.8% and

IQR 0.7-1.2%; $p<0.05$) with IFN-α therapy and even after the end of treamtent period (median 1.0% and IQR 0.7-1.8%; $p<0.05$). The suppressive effect of IFN-α treatment on Th17 was not detected in the individuals on conventional treatment alone.

As $CD4^+$ T cells producing both IL-17 and IFN-γ has been reported (Annunziato et al. 2007), this cell population was included in our data analysis as well. Unlike patients treated with IFN-α, there was a significant decrease in the levels of $CD4^+$ T cells expressing both IL-17 and IFN-γ comparing 12 month values (median 0.1% and IQR 0.0-0.2%; $p<0.05$) with the ones from initial visits (median 0.4% and IQR 0.4-0.8%) in individuals on conventional treatment alone (Fig 4.1b). This was only demonstrated in data with PMA/ionomycin. Thus, this is unlikely to be a consistent trend as it was not observed in other culture conditions or at other time points.

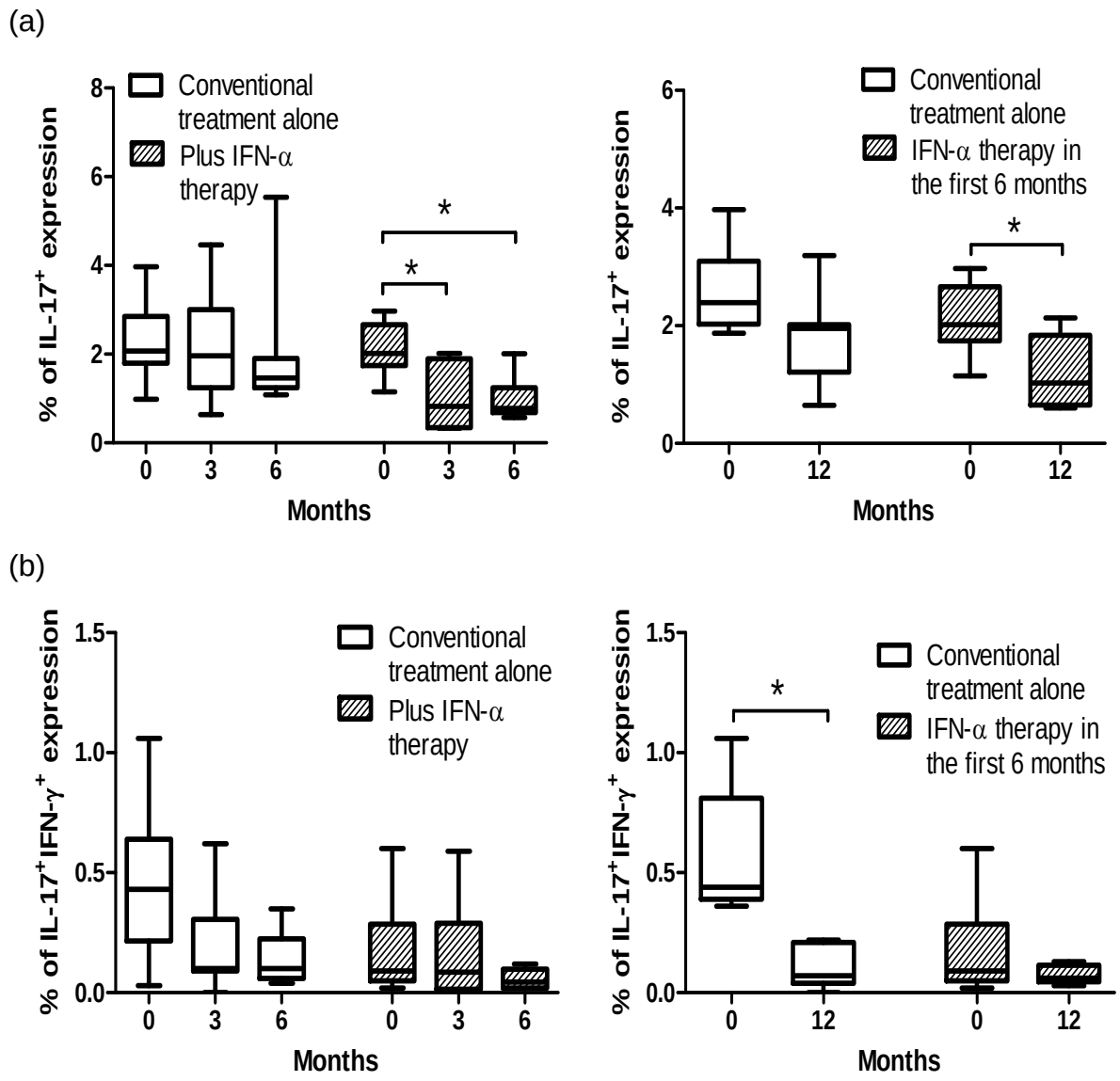


Fig 4.1: Downregulation of IL-17 expression with IFN-α therapy in CD4$^+$ T cells

Whole blood samples from BD patients were obtained at baseline visits and follow-ups at 3, 6, and 12 months. These were stained with anti-CD4 surface antibodies before intracellularly with anti-IFN-γ and anti-IL-17 antibodies. The data are shown as percentages of IL-17$^+$ and IL-17$^+$IFN-γ$^+$ cells among CD4$^+$ lymphocytes. Of the patients presented here, 9 (7) BD patients were on conventional treatment alone and 6 (6) were on IFN-α2b therapy as well. The number in brackets denotes the number of patients represented in the 12 month data. In (a), results showed the changes in the IL-17$^+$ cells; in (b), results showed the changes in the IL-17$^+$IFN-γ$^+$ cells. All data were obtained from cultures stimulated with PMA and ionomycin. The results are presented as boxplots.

4.3.2 IFN-α2b therapy potently enhances Foxp3 expression by $CD4^+$ T cells in BD patients

To examine the influence of IFN-α administration on cell populations positive for Foxp3, the marker's expression levels in the peripheral blood of each BD patient treated with or without IFN-α2b therapy were established using intracellular cytokine staining method and flow cytometric analysis. Cell cultures were either untreated or treated with anti-CD3/CD28 Abs or PMA/ionomycin for 24 hours before staining. Foxp3 expression pattern was analysed by gating on either $CD4^+$, $CD4^+CD25^+$, or $CD4^+CD25^{high}$ lymphocyte populations. An example of the gates for lymphocytes, $CD4^+CD25^+$, and $CD4^+CD25^{high}$ cell populations is shown in Fig 4.2. In Fig 4.3, representative flow cytometry data on $CD4^+$ T cells expressing Foxp3 from a BD patient who was treated with a 6 month course of IFN-α2b therapy are displayed. In Fig 4.4, the influence of IFN-α on Foxp3 expression is further illustrated in both $CD25^+$ and $CD25^-$ cell populations.

With regards to the Foxp3 expression within $CD4^+$ T cells, it was clear that an upregulation of Foxp3-expressing cells could be seen in all 3 culture conditions in the group on IFN-α therapy. In unstimulated cultures (Fig 4.5a), the proportions of $Foxp3^+$ cells were observed to be increased at 3 months (median 5.7% and IQR 2.5-6.8%; $p<0.01$) and at 6 months (median 5.0% and IQR 3.2-8.7%; $p<0.01$) from baseline levels (median 2.4% and IQR 1.5-3.4%). At 12 months, this enhancement was maintained (median 5.0% and IQR 3.5-7.4%; $p<0.01$) when compared to initial visits (median 2.3% and IQR 1.5-3.5%). In cultures stimulated with PMA/ionomycin (Fig 4.5b), similar augmentation of Foxp3 expression could be observed as the levels were raised comparing follow-up visits at 3 months (median 12.0% and IQR 6.8-14.3%; $p<0.01$) and 6 months (median 9.4% and 5.7-14.0%; $P<0.05$) to the ones before initiation of treatment (median 6.3% and IQR 3.9-7.8%). The data from 12 month visits were found to be in agreement as well (median 9.8% and IQR 8.0-12.0%; $p<0.01$). In addition, enhancement of Foxp3 expression was consistently identified when examining results from cultures activated with anti-CD3/CD28 Abs over the 12 month assessment.

Human Treg cells were initially defined according to their high expression of CD25, based on the finding that murine $CD4^+CD25^+$ T cells are highly suppressive (Baecher-Allan et al. 2001;Sakaguchi et al. 1995). Therefore, Foxp3-expressing cells within the $CD4^+CD25^+$ and $CD4^+CD25^{high}$ cell populations were explored. Within $CD4^+CD25^{high}$ cell populations, the percentage of $Foxp3^+$ cells was upregulated under all 3 cultures conditions. Without cell activation (Fig 4.7a), the proportion of Foxp3 expression was enhanced at 3 (median 34.6% and IQR 22.9-58.8%; $p<0.01$) and 6 months (median 44.2% and IQR 17.0-63.7%; $p<0.01$) after commencing IFN-α treatment when evaluated against initial levels (median 14.0% and IQR 9.1-33.6%). Similarly, the percentage was augmented from baseline values (median 25.0% and IQR 18.8-42.2%) to heightened levels at 3 (median 48.6% and IQR 33.3-67.7%; $p<0.01$) and 6 months (median 59.0% and IQR 30.5-66.7%; $p<0.01$) with PMA/ionomycin stimulation (Fig 4.7b). Reliably, the levels were upregulated at 12 months with both forms of polyclonal stimulation, and this change was evident in the cultures stimulated witih anti-CD3/CD28 Abs as well (data not shown).

$CD4^+CD25^+$ T cells expressing Foxp3 were studied as well and their relative percentage was found to be increased at follow-ups in the IFN-α treatment group. The trend was observed on data obtained from unstimulated cultures (Fig 4.6a) as the levels were upregulated at 3 months (median 9.8% and IQR 5.0-12.8%; $p<0.01$) and at 6 month visits (median 7.5% and IQR 5.3-14.1%; $p<0.01$) when compared to the numbers before treatment (median 4.1% and IQR 2.8-5.9%). After the cessation of therapy, the proportion of Foxp3-expressing cells was still high (median 7.6% and IQR 6.4-11.9%; $p<0.01$) relative to initial values (median 4.1% and IQR 2.8-5.2%). With PMA/ionomycin stimulation (Fig 4.6b), tendency of Foxp3 augmentation was apparent as well. After 3 months of IFN-α therapy, the proportions of T cells expressing Foxp3 was raised (median 15.9% and IQR 9.9-21.1%; $p<0.01$) in comparison to baseline (median 8.6% and IQR 6.6-8.8%). At 12 months, the levels are still maintained (median 15.2% and IQR11.9-18.7%; $p<0.01$).

The results on the individuals receiving conventional treatment alone, in contrast, did not show any upregulation of Foxp3 expression. In some cases, even a significant downregulation was revealed. In $CD4^+$ T cells, the proportion of Foxp3-expressing cells was decreased in data obtained from cultures activated with PMA/ionomycin at

6 month assessment (median 4.3% and IQR 3.2-7.2%; $p<0.05$) relative to baseline values (median 6.8% and IQR 4.7-9.2%) (Fig 4.5b). Likewise, with PMA/ionomycin stimulation, the percentage of $CD4^+CD25^+$ T cells expressing Foxp3 were downregulated at 6 months (median 9.2% and IQR 6.1-10.4%; $p<0.05$) from baseline results (median 10.2% and IQR 8.4-13.1%) (Fig 4.6b). Similar trends were observed at 12 months visits as well.

To verify the results from intracellular cytokine staining experiments, whole blood samples from BD patients were phenotyped without cells being activated or cultured to investigate the influence of IFN-α2b therapy on Foxp3 expression by peripheral blood $CD4^+$ T lymphocytes. Within $CD4^+$ T cells (Fig 4.8a), there were certainly increases after starting IFN-α therapy at 3 (median 4.3% and IQR 3.1-7.0%; $p<0.01$), 6 (median 5.2% and IQR 3.4-7.7%; $p<0.01$), and 12 month (median 3.5% and IQR 3.0-6.2%; $p<0.01$) follow-up visits, when evaluated against initial visit data (median 2.1% and IQR 1.2-3.2%). Within $CD4^+CD25^+$ T cells (Fig 4.8b), there was enhancement of Foxp3 levels with IFN-α therapy at 3 (median 5.9% and IQR 5.1-10.8%; $p<0.01$), 6 (median 8.0% and IQR 5.2-12.4%; $p<0.01$), and 12 month (median 6.3% and IQR 4.2-8.7%; $p<0.01$) assessments, when compared to baseline values (median 3.1% and IQR 2.1-5.0%). Likewise, impressive results were obtained from data on $CD4^+CD25^{high}$ T cells (Fig 4.8c) as the levels were raised from low levels before IFN-α treatment (median 16.6% and IQR 8.1-19.5%) to substantially elevated ones at 3 (median 34.0% and IQR 18.8-58.6%; $p<0.01$), 6 (median 31.8% and IQR 21.0-60.6%; $p<0.01$) and 12 (median 33.3% and IQR 22.7-45.3%; $p<0.01$) month assessments.

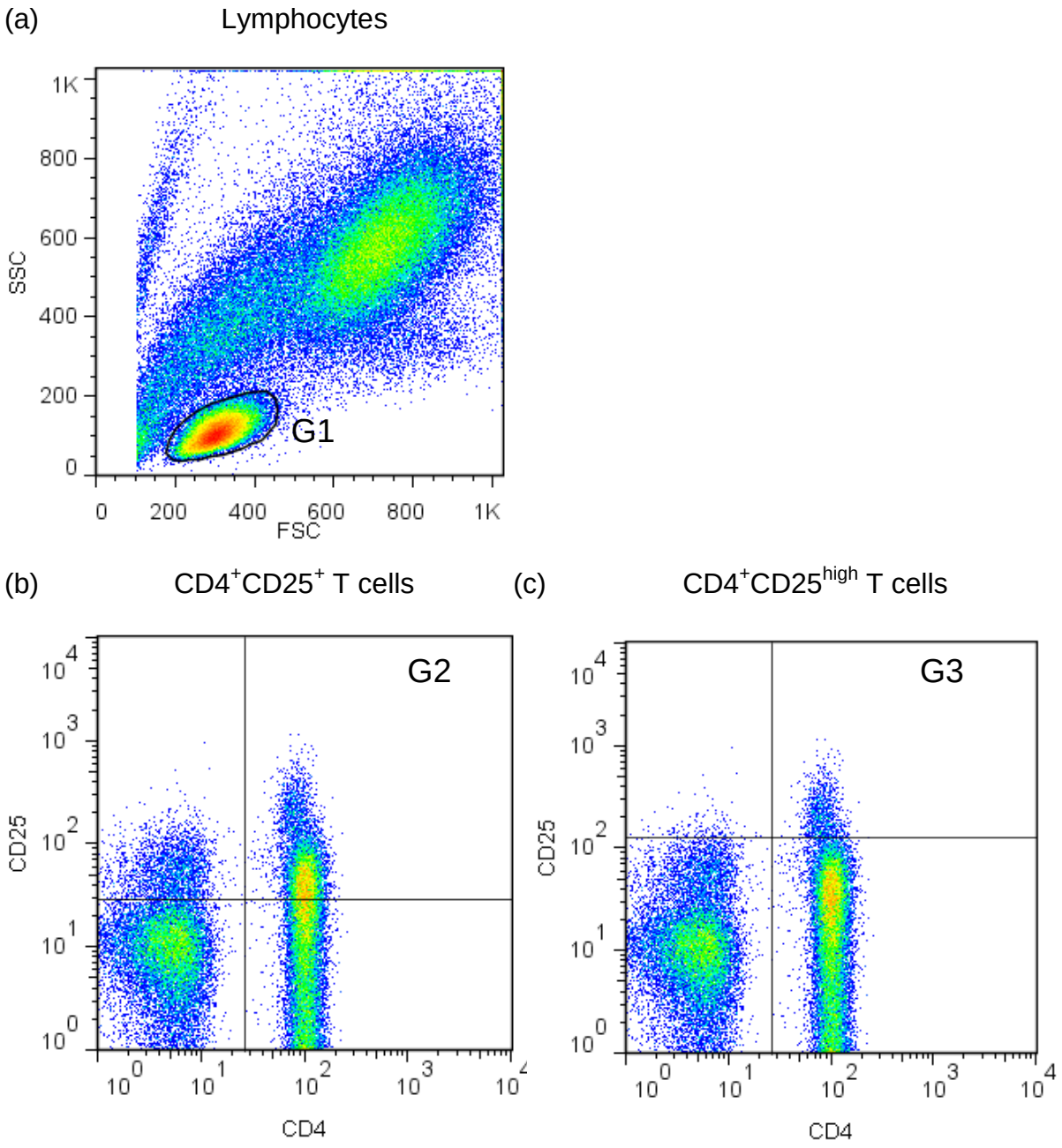


Fig 4.2: Flow cytometry: CD25 gating for identifying Treg subsets

Flow cytometric gating strategies to identify Treg cells in patients with BD. (a) Dot plot of forward scatter (FSC) versus side scatter (SSC) for all events showing all peripheral blood cell populations and G1 gate indicates lymphocyte population. (b) Lymphocytes were analysed on the basis of surface markers CD4 and CD25; the G2 gate identifies the percentage of $CD4^+CD25^+$ T cells. (c) The G3 gate identifies the percentage of $CD4^+CD25^{high}$ T cells.

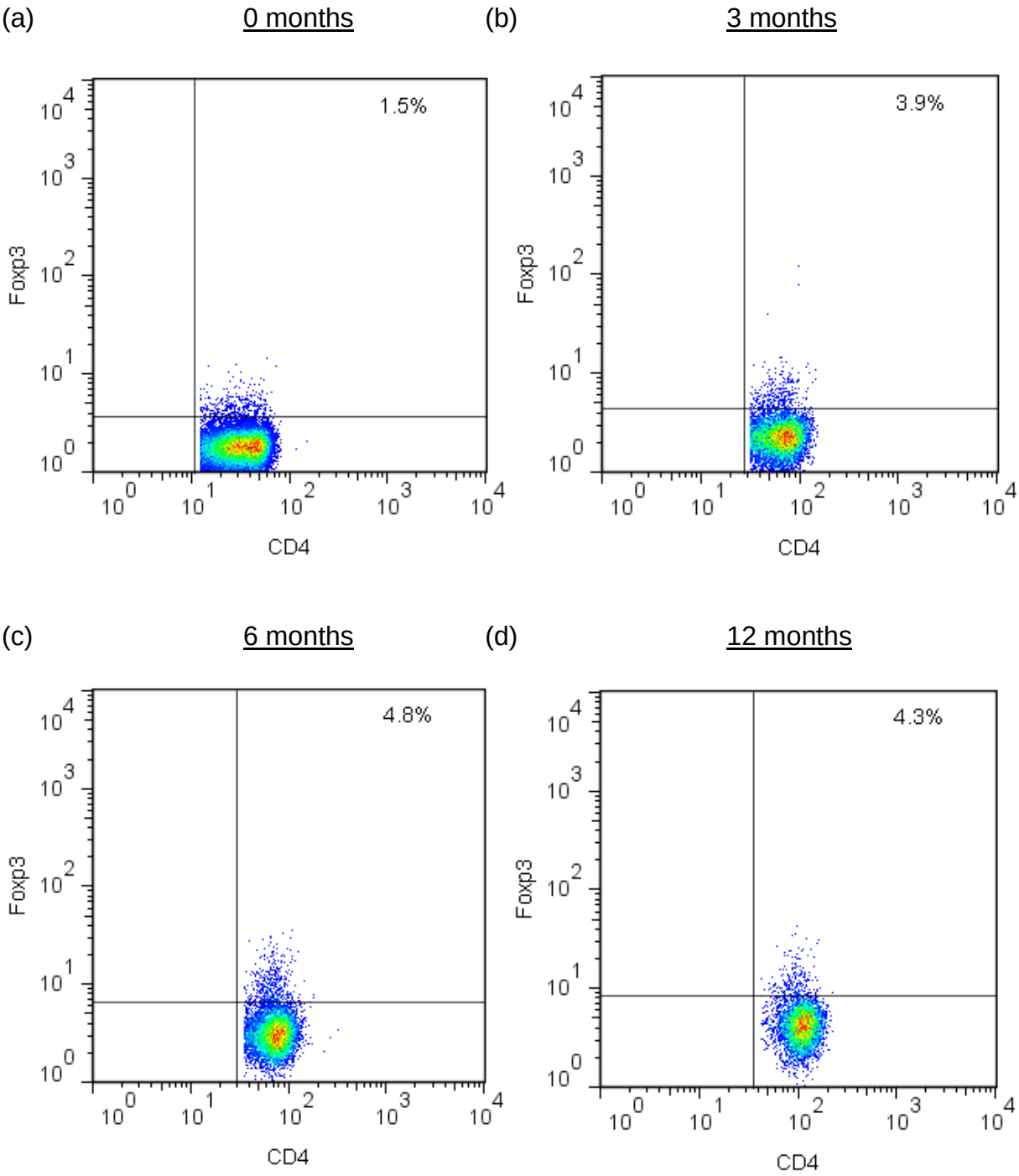


Fig 4.3: CD4$^+$ T cells expressing Foxp3 in a BD patient on IFN-α2b therapy

Whole blood samples were prepared using intracellular cytokine staining method. Lymphocytes were selected according to forward- and side-scatter properties and CD4$^+$ T cells were gated on. The graphs show representative data on the expression of Foxp3 within CD4$^+$ T cells in a patient who was on IFN-α2b therapy. The data are from (a) the baseline visit, (b) 3 month assessment visit, (c) 6 month assessment visit, and (d) 12 month assessment visit. The numbers in the histograms indicate the percentages of cells positive for Foxp3. All samples shown were from unstimulated cultures.

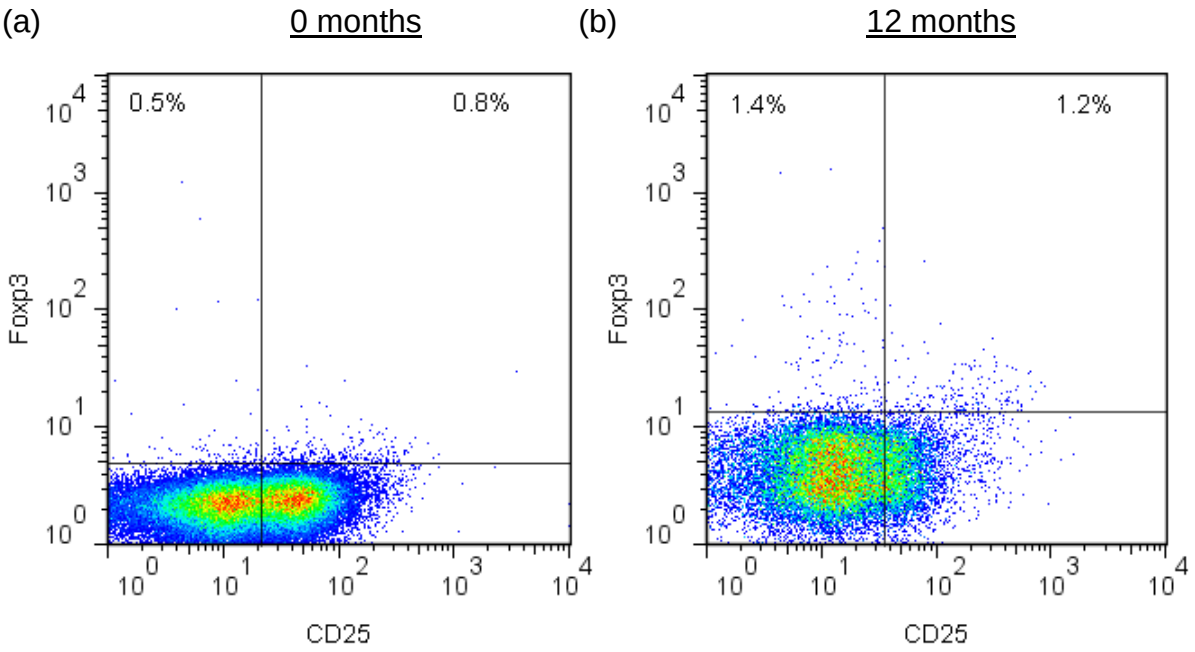
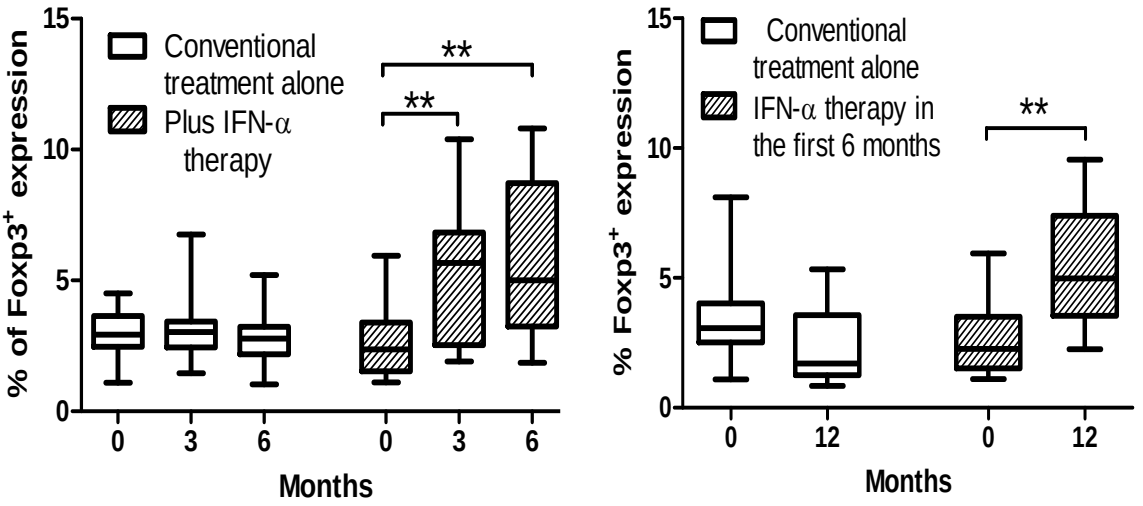


Fig 4.4: The influence of IFN-α2b therapy on the expression of CD25 and Foxp3

Whole blood samples were prepared using intracellular cytokine staining method. Lymphocytes were selected according to forward- and side-scatter properties and $CD4^+$ T cells were gated on. The graphs show representative data on the Foxp3 expression within either the $CD25^+$ or $CD25^-$ T cell populations in a patient who was on IFN-α2b therapy. The data are from (a) the baseline visit, and (b) 12 month assessment visit. The numbers in the histograms indicate the percentages of cells positive for Foxp3. Samples shown were from unstimulated cultures.

(a) Untreated



(b) PMA/ionomycin stimulation

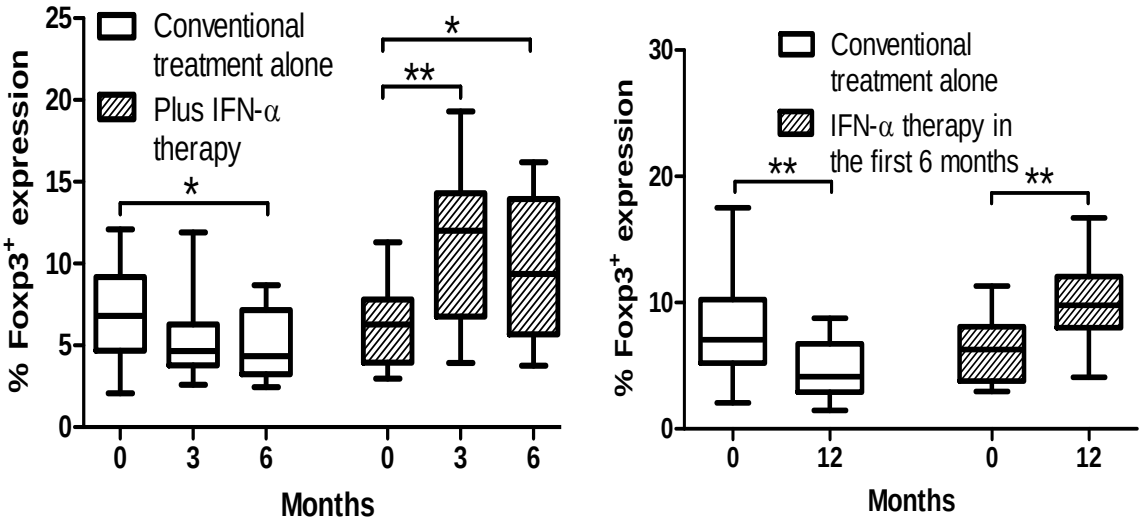
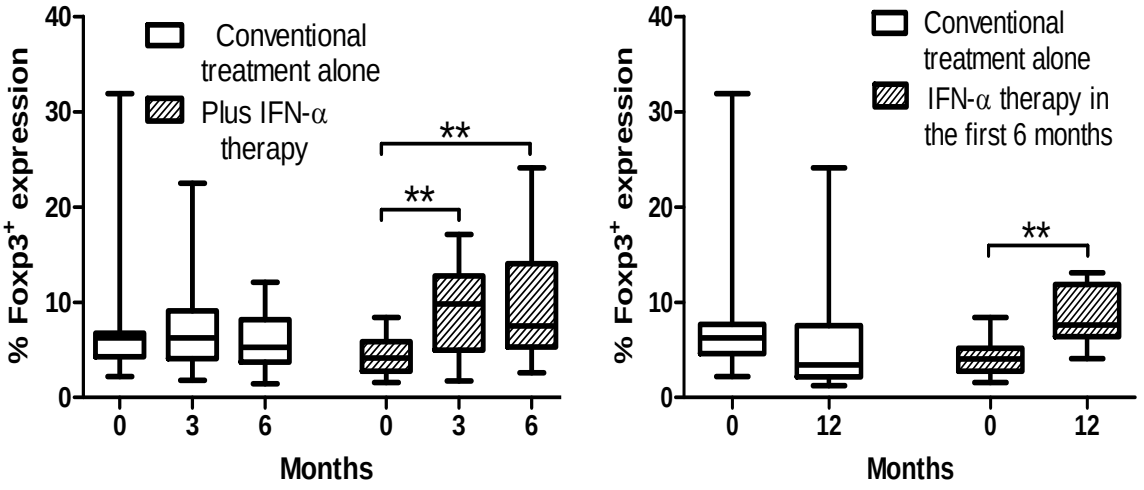


Fig 4.5: Upregulation of Foxp3 expression with IFN-α therapy in CD4$^+$ T cells

Whole blood samples from BD patients were obtained at baseline visits and follow-ups at 3, 6, and 12 months and prepared using intracellular cytokine staining method. Cells were stained with anti-CD4 surface antibodies before intracellularly with anti-Foxp3 antibodies. The data are shown as percentages of Foxp3$^+$ cells among CD4$^+$ lymphocytes. Of the patients presented here, 16 (14) BD patients were on conventional treatment alone and 13 (12) were on IFN-α2b therapy as well. The number in brackets denotes the number of patients represented in the 12 month data. In (a), data were from untreated cell cultures; in (b), data were from cell cultures stimulated with PMA and ionomycin. The results are presented as boxplots.

(a) Untreated



(b) PMA/ionomycin stimulation

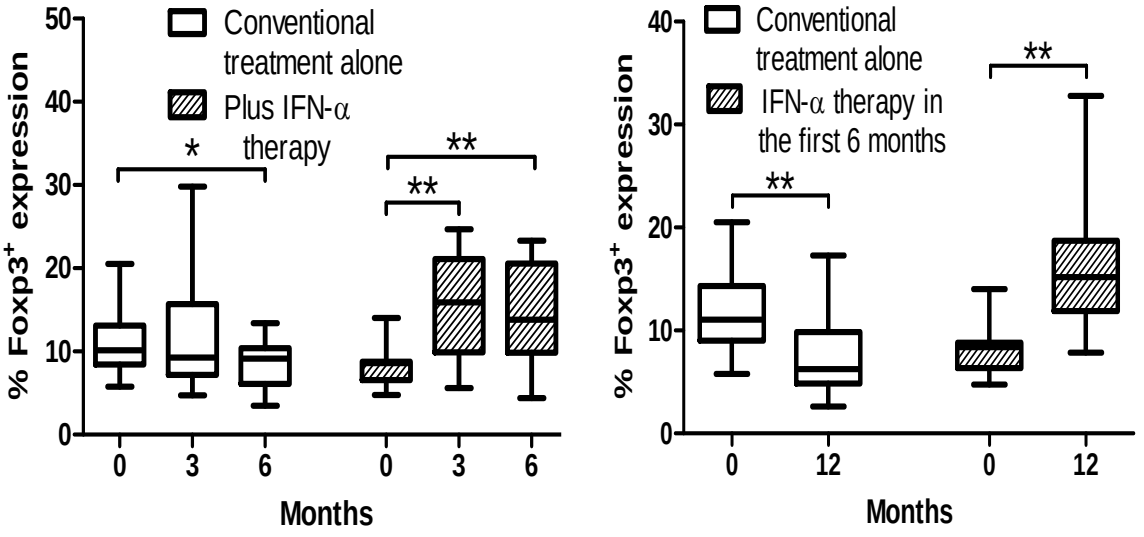
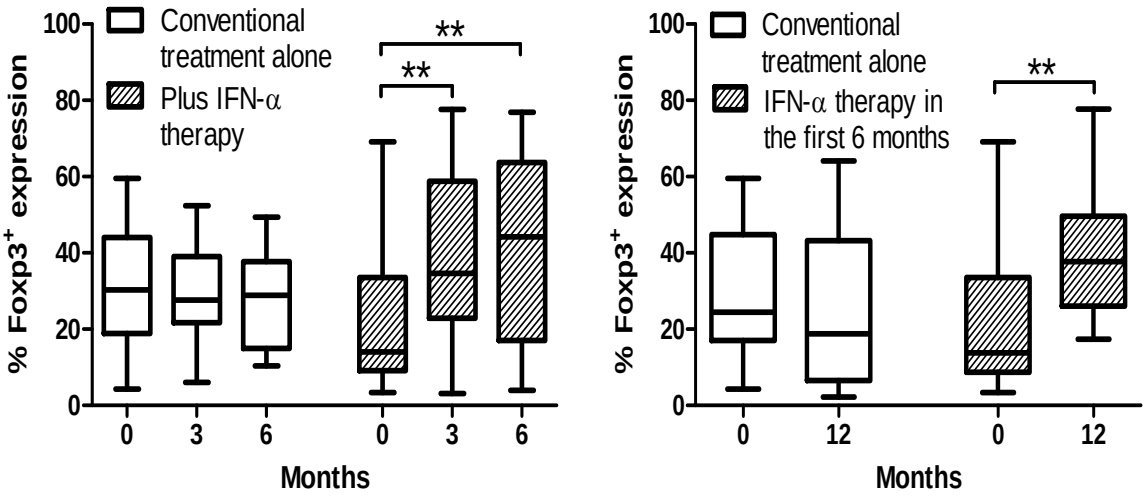


Fig 4.6: Upregulation of Foxp3 expression with IFN-α therapy in $CD4^+CD25^+$ T cells

Whole blood samples from BD patients were obtained at baseline visits and follow-ups at 3, 6, and 12 months and prepared using intracellular cytokine staining method. Cells were stained with anti-CD4 and anti-CD25 surface antibodies before intracellularly with anti-Foxp3 antibodies. The data are shown as percentages of $Foxp3^+$ cells among $CD4^+CD25^+$ lymphocytes. Of the patients presented here, 16 (14) BD patients were on conventional treatment alone and 13 (12) were on IFN-α2b therapy as well. The number in brackets denotes the number of patients represented in the 12 month data. In (a), data were from untreated cell cultures; in (b), data were from cell cultures stimulated with PMA and ionomycin. The results are presented as boxplots.

(a) Untreated



(b) PMA/ionomycin stimulation

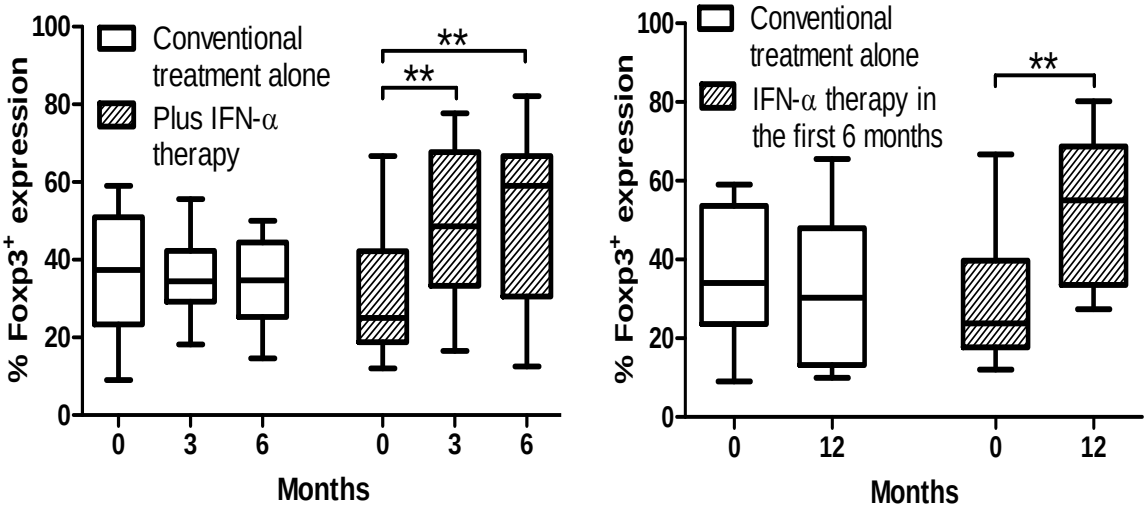
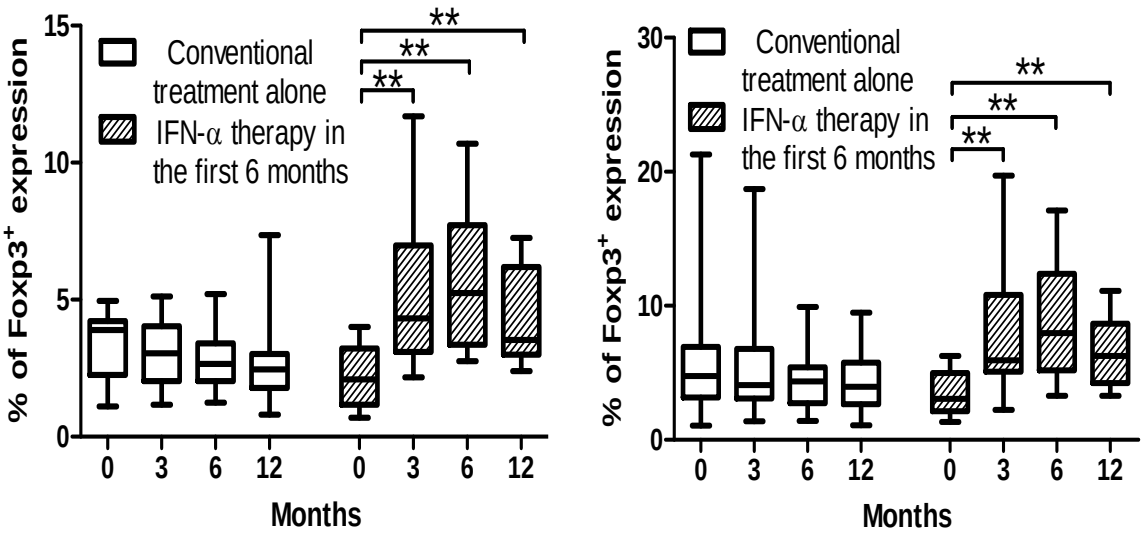


Fig 4.7: Upregulation of Foxp3 expression with IFN-α therapy in $CD4^+CD25^{high}$ T cells

Whole blood samples from BD patients were obtained at baseline visits and follow-ups at 3, 6, and 12 months and prepared using intracellular cytokine staining method. Cells were stained with anti-CD4 and anti-CD25 surface antibodies before intracellularly with anti-Foxp3 antibodies. The data are shown as percentages of $Foxp3^+$ cells among $CD4^+CD25^{high}$ lymphocytes. Of the patients presented here, 16 (14) BD patients were on conventional treatment alone and 13 (12) were on IFN-α2b therapy as well. The number in brackets denotes the number of patients represented in the 12 month data. In (a), data were from untreated cell cultures; in (b), data were from cell cultures stimulated with PMA and ionomycin. The results are presented as boxplots.

(a) $CD4^+$ T cells

(b) $CD4^+CD25^+$ T cells



(c) $CD4^+CD25^{high}$ T cells

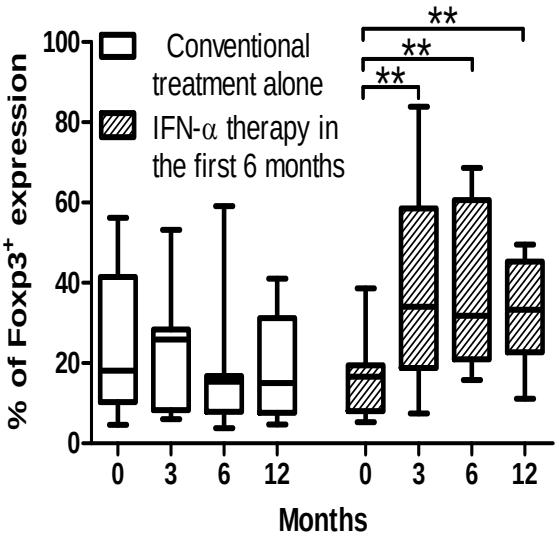


Fig 4.8: Phenotyping experiments showing augmentation of Foxp3 expression with IFN-α therapy in $CD4^+$ T cells

Whole blood samples from BD patients were obtained at baseline visits and follow-ups at 3, 6, and 12 months. Cells were stained with anti-CD4 and anti-CD25 surface antibodies before with anti-Foxp3 antibodies. The data are shown as percentages of $Foxp3^+$ cells among subpopulations of $CD4^+$ lymphocytes. Of the patients presented here, 17 BD patients were on conventional treatment alone and 16 were on IFN-α2b therapy as well. In (a), results were from all $CD4^+$ T cells; in (b), results were from $CD4^+CD25^+$ T cells. In (b), results were from $CD4^+CD25^{high}$ T cells. The results are presented as boxplots. ns=not significant.

4.3.3 IFN-α2b therapy shows a tendency to increase IL-10 expression by T cells in BD patients

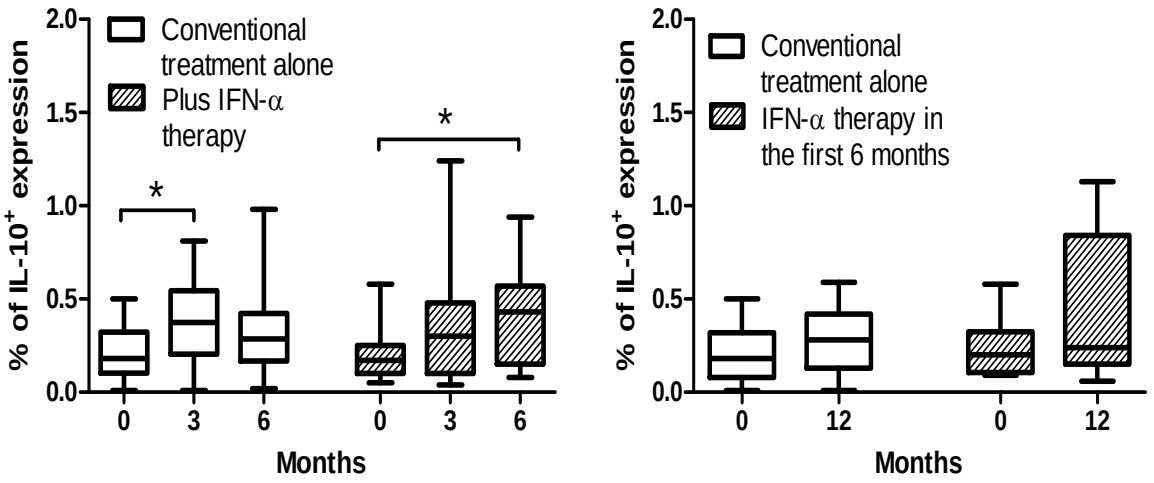
Analysis of the effects of IFN-α on IL-10 expression by T cells was approached using intracellular cytokine staining on whole blood samples as described in Materials and Methods. Cell cultures were either untreated or activated with anti-CD3/CD28 Abs or PMA/ionomycin for 24 hours before staining. Cytokine expression was analysed by gating on $CD3^+$ or $CD4^+$ lymphocytes. Within the $CD4^+IL\text{-}10^+$ cells, Foxp3 positivity was studied as well.

To start with, the $CD3^+$ T cells were examined. In untreated cultures (Fig 4.9a), an upregulation of IL-10 levels was noticed at 6 months (median 0.4% and IQR 0.2-0.6%; $p<0.05$) when compared to initial visits (median 0.2% and IQR 0.1-0.3%) in the IFN-α treated group. In CD3/CD28 stimulated cultures (Fig 4.9b), a significantly heightened proportion of IL-10 expressing cells was shown at follow-up visits at 6 (median 0.3% and IQR 0.2-0.6%; $p<0.05$) and 12 months (median 0.4% and IQR 0.2-0.7%; $p<0.05$) when evaluated against baseline values(median 0.2% and IQR 0.1-0.3%) in the IFN-α treated group. Significant changes were not evident in cultures stimulated with PMA/ionomycin (data not shown). No meaningful alteration in the IL-10 expression level was noticed in the group on conventional treatment alone except an increase noticed at 3 months (median 0.4% and IQR 0.2-0.5%; $p<0.05$) from baseline values (median 0.2% and IQR 0.1-0.3%).

Afterwards, the $CD4^+$ T cell populations were analysed. In untreated cultures (Fig 4.10a), a raised level of IL-10 expression was found at 3 (median 0.3% and IQR 0.1-0.7%; $p<0.05$), 6 (median 0.3% and IQR 0.2-0.7%; $p<0.05$), and 12 month (median 0.3% and IQR 0.1-0.7%; $p<0.01$) follow-ups relative to the results from initial visits (median 0.1% and IQR 0.1-0.2%) in the IFN-α treated patients. These $IL\text{-}10^+$ cells were found to be mostly $Foxp3^-$ and hence an upregulation of this subpopulation was noted as well. The relative proportions of $CD4^+IL\text{-}10^+Foxp3^-$ (Fig 4.10b) were shown to be elevated at 3 (median 0.2% and IQR 0.1-0.5%; $p<0.05$), 6 (median 0.2% and IQR 0.1-0.6%; $p<0.05$), and 12 months (median 0.3% and IQR 0.1-0.6%; $p<0.05$) when compared to results from initial visits (median 0.1% and IQR 0.1-0.2%). In contrast, there was no significant alterations of cells with either $IL\text{-}10^+$ or IL-

$10^{+}Foxp3^{-}$ expression in the group on conventional treatment alone. Also, no change in the $CD4^{+}IL\text{-}10^{+}Foxp3^{+}$ subpopulation was revealed in our patients whether they received IFN-α treatment or not (data not shown).

(a) Untreated



(b) CD3/CD28 stimulation

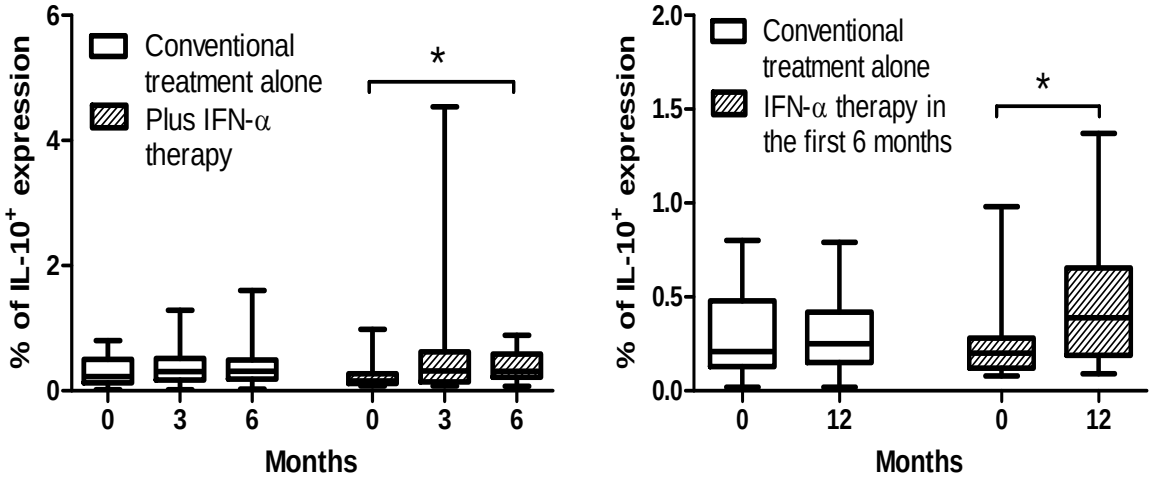
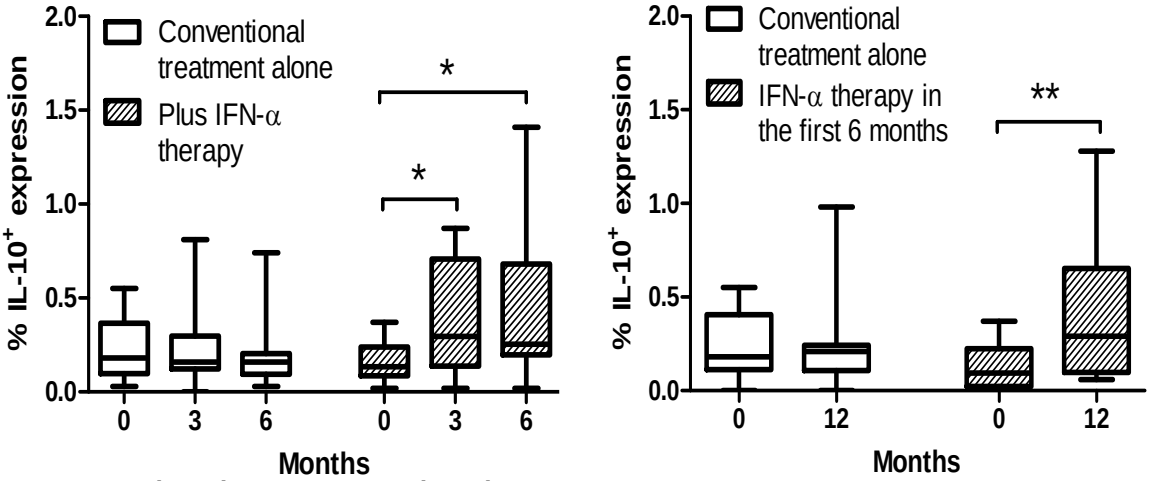


Fig 4.9: Upregulation of IL-10 expression with IFN-α therapy in $CD3^+$ T cells

Whole blood samples from BD patients were obtained at baseline visits and follow-ups at 3, 6, and 12 months and prepared using intracellular cytokine staining method. Cells were stained with anti-CD3 surface antibodies before intracellularly with anti-IL-10 antibodies. The data are shown as percentages of $IL\text{-}10^+$ cells among $CD3^+$ lymphocytes. Of the patients presented here, 18 (15) BD patients were on conventional treatment alone and 15 (13) were on IFN-α2b therapy as well. The number in brackets denotes the number of patients represented in the 12 month data. In (a), data were from untreated cell cultures; in (b), data were from CD3/CD28 stimulated cell cultures. The results are presented as boxplots.

(a) $CD4^+IL\text{-}10^+$



(b) $CD4^+IL\text{-}10^+Foxp3^-$

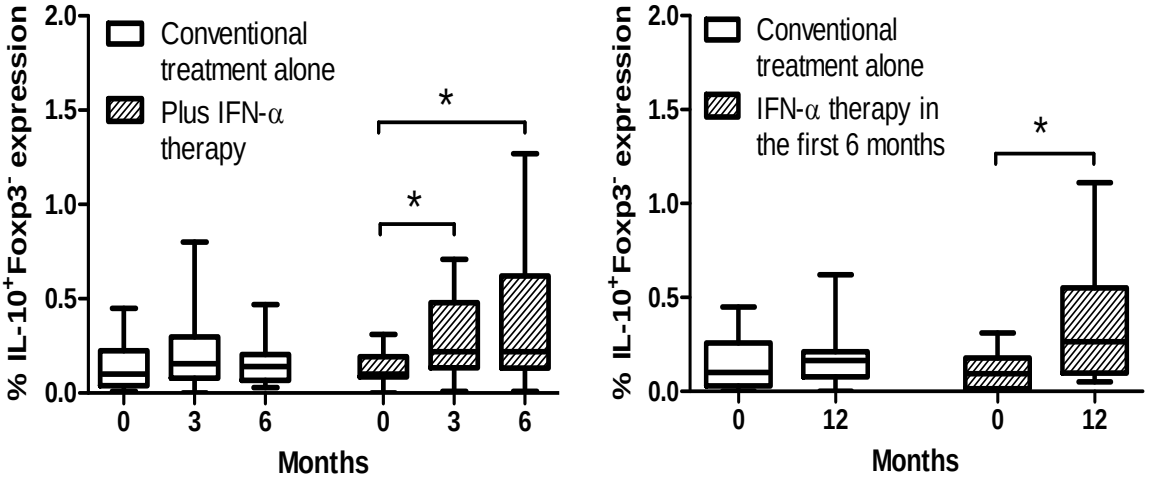


Fig 4.10: Upregulation of $IL\text{-}10^+$ and $IL\text{-}10^+Foxp3^-$ expression with IFN-α therapy in $CD4^+$ T cells

Whole blood samples from BD patients were obtained at baseline visits and follow-ups at 3, 6, and 12 months and prepared using intracellular cytokine staining method. Cells were stained with anti-CD4 surface antibodies before intracellularly with anti-IL-10 and anti-Foxp3 antibodies. The data are shown as percentages of positive cells among $CD4^+$ lymphocytes. Of the patients presented here, 16 (14) BD patients were on conventional treatment alone and 10 (12) were on IFN-α2b therapy as well. The number in brackets denotes the number of patients represented in the 12 month data. In (a), data were from cells with $IL\text{-}10^+$ expression; in (b), data were from cells with $IL\text{-}10^+Foxp3^-$ expression. All cultures were untreated and the results are presented as boxplots.

4.3.4 IFN-α2b therapy shows the tendency to increase TGF-β expression by T cells in BD patients

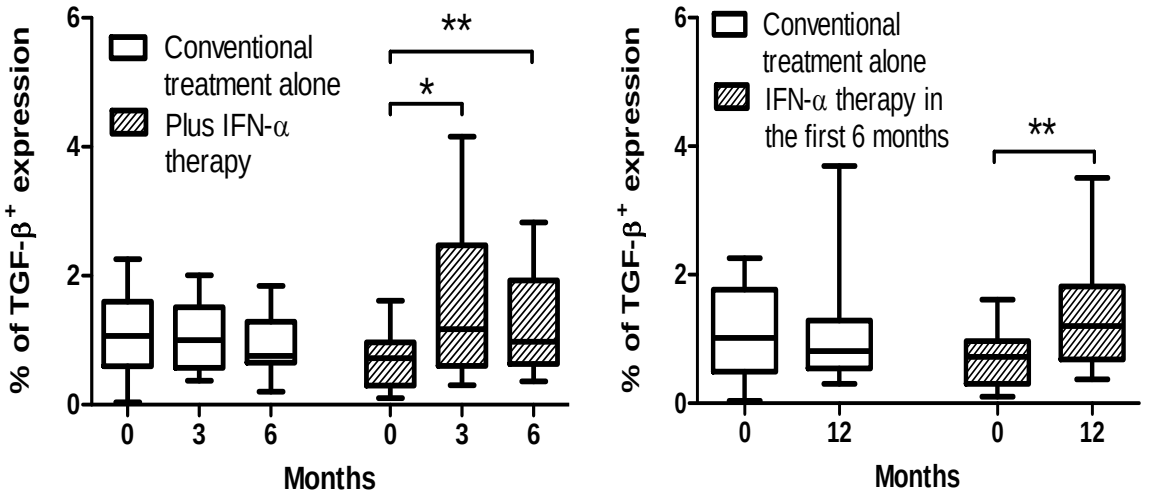
Using intracellular cytokine staining method as described in Materials and Methods, the influence of IFN-α on TGF-β expression by T cells were examined. Cell cultures were either untreated or activated with anti-CD3/CD28 Abs or PMA/ionomycin for 24 hours before staining. Cytokine expression was analysed by gating on $CD3^+$ lymphocytes.

In the IFN-α treatment group, there was no significant alteration of the amounts of TGF-β-expressing $CD3^+$ T cells with PMA/ionomycin stimulation (Fig 4.11b). However, in untreated cultures (Fig 4.11a), the enhancement of TNF-α expression was clearly shown at 3 (median 1.2% and IQR 0.6-2.5%; $p<0.05$), 6 (median 1.0% and IQR 0.6-1.9%; $p<0.01$), and 12 month (median 1.2% and IQR 0.7-1.8%; $p<0.01$) follow-ups when evaluated against the baseline visit data (median 0.7% and IQR 0.3-1.0%). Similarly, in CD3/CD28 stimulated cultures, the median levels of TGF-β expression was raised from 1.1% (IQR 0.6-1.6%) initially to 1.4% (IQR 0.7-2.5%; $p<0.05$) at 3 months (data not shown). Despite being statistically insignificant ($p=0.08$), an tendency to upregulate of the TGF-β expression was seen at 12 months as well (data not shown).

On the other hand, no consistent alteration was noted in patients on conventional treatment alone whether cells were cultured with or without CD3/CD28 stimulation (Fig 4.11a). However, in cultures stimulated with PMA/ionomycin (Fig 4.11b), the proportion of $CD3^+$ T cells expressing TGF-β was downregulated at 3 (median 2.1% and IQR 1.4-4.3%; $p<0.01$) and 6 months (median 1.6% and IQR 1.1-2.9%; $p<0.01$) when compared to the baseline visit data (median 3.8% and IQR 2.5-6.7%). At 12 months (median 1.7% and IQR 1.4-3.3%; $p<0.01$), the trend continued when evaluated against initial assessment results (median 4.7% and IQR 3.0-8.9%).

The proportion of $CD3^+$ T cells expressing both IL-10 and TGF-β was investigated as well, and their levels were found to be very low. No significant change was found in either group of BD patients whether they were on IFN-α or not (data not shown).

(a) Untreated



(b) PMA/ionomycin stimulation

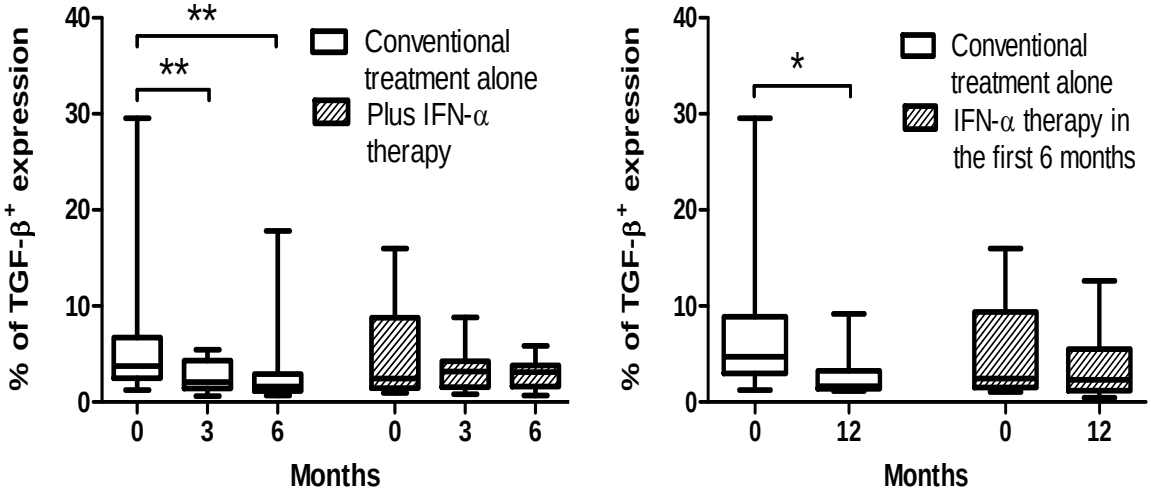


Fig 4.11: The effects of IFN-α therapy on TGF-β expression by CD3$^+$ T cells

Whole blood samples from BD patients were obtained at baseline visits and follow-ups at 3, 6, and 12 months and prepared using intracellular cytokine staining method. Cells were stained with anti-CD3 surface antibodies before intracellularly with anti-TGF-β antibodies. The data are shown as percentages of TGF-β$^+$ cells among CD3$^+$ lymphocytes. Of the patients presented here, 18 (15) BD patients were on conventional treatment alone and 18 (16) were on IFN-α2b therapy as well. The number in brackets denotes the number of patients represented in the 12 month data. In (a), data were from untreated cell cultures; in (b), data were from PMA/ionomycin stimulated cell cultures. The results are presented as boxplots.

4.3.5 IFN-α2b therapy does not increase $CD56^+$ expression by T cells in BD patients

To examine the influence of IFN-α therapy on NKT cells in BD patients, serial whole blood samples from BD patients receiving conventional immunosuppressive therapy with or without recombinant IFN-α2b treatment were phenotyped. The phenotyping results were obtained by analysing $CD56^+$ cells after gating on the $CD3^+$ lymphocytes.

As shown in Figure 4.12, there was no significant change in the levels of CD56 expression within the $CD3^+$ T lymphocyte populations in BD patients whether they were on IFN-α or not. The only exception found was an increased percentage of the $CD3^+CD56^+$ T cells in the group on conventional treatment alone when 3 month levels (median 6.0% and IQR 4.3-10.2%; $p<0.01$) were compared to the baseline ones (median 3.3% and IQR 2.4-6.1%).

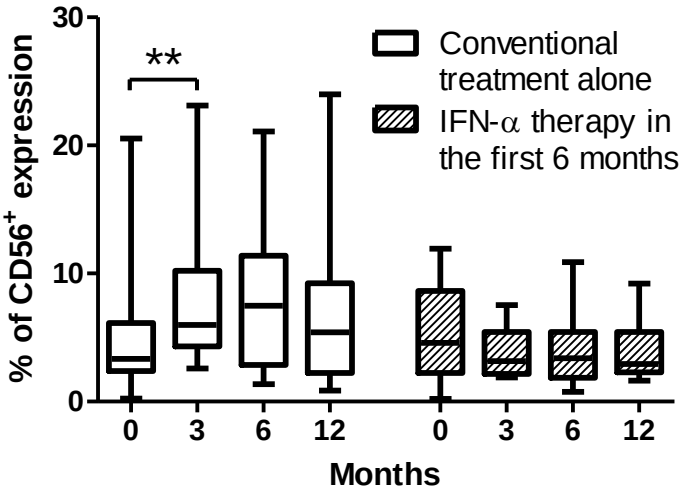


Fig 4.12: Phenotyping experiments showing CD56 expression by $CD3^+$ T cells in BD patients

Whole blood samples from BD patients were obtained at baseline visits and follow-ups at 3, 6, and 12 months. Cells were stained with anti-CD3 and anti-CD56 surface antibodies. The data are shown as percentages of $CD56^+$ cells within $CD3^+$ lymphocytes. Of the patients presented here, 16 BD patients were on conventional treatment alone and 15 were on IFN-α2b therapy as well. The results are presented as boxplots.

4.4 Discussion

Inflammation is characterised by a complex interaction of pro- and anti-inflammatory mediators. A favourable balance leads to a recovery of homeostasis while an unfavourable balance, associated with an exacerbated release of pro-inflammatory cytokines, leads to damaging effects. Individual background seems to govern this equilibrium, and it is known that a great heterogeneity exists between individuals.

4.4.1.1 Biology of IL-17

IL-17 has assorted biological functions, but the most notable role of IL-17 is its involvement in inducing and mediating pro-inflammatory responses. As well as promoting neutrophil recruitment and macrophage activation and survival, IL-17 stimulates the production of many other pro-inflammatory cytokines, chemokines and prostaglandins from a variety of immune and non-immune cells (Crome, Wang, & Levings 2010). Recently it was described that IL-17 itself can also function as a chemokine, acting directly on the IL-17RA and IL-17RC of monocytes to induce their migration (Shahrara et al. 2009). Due to these roles, the IL-17 family has been linked to many immune/autoimmune related diseases.

4.4.1.2 The immunomodulatory effect of IFN-α on IL-17

From our data, a downregulation of IL-17 expression was seen after cells cultures were activated with PMA/ionomycin in the IFN-α2b-treated group compared to patients on conventional treatment alone. This was evident at all follow-up visits up to 12 months compared to the data before initiation of treatment. This is in accordance to a recent study which found that IFN-α significantly decreased IL-17 production by anti-CD3/CD28 stimulated PBMCs in cell culture supernatants from BD patients (Liu et al. 2011). These findings are supported by other studies on inflammatory conditions. In ulcerative colitis patients, treatment with pegylated IFN-α2b therapy was reported to result in a significant decrease of colonic IL-17A mRNA expression after 4 weeks and the downregulation of IL-17A expression in colonic

tissue was associated with clinical remission (Moschen et al. 2008). Similarly, direct IFN-β1a treatment of $CD4^+CD45RA^+$ naive T cells cultured in Th17-polarising conditions demonstrated decrease in RORc, IL-17A, IL-23R and CCR6 levels in both MS patients and healthy controls (Ramgolam et al. 2009).

4.4.1.3 Mechanisms leading to the inhibitory effects of IFN-α on IL-17

Like IFN-γ, IFN-α mediates its signal through phosphorylation of Stat1 resulting in induction of T-bet, which is critical for Th1 cell development, and subsequent inhibition of Th17 responses (Harrington et al. 2005). In addition to the direct inhibitory effect of IFN-α on Th17 development, IFN-α binds to the type 1 IFN receptor on DCs, macrophages and microglia cells within the CNS. In these cells, IFN-α-mediated signalling inhibits the activation of an intracellular isoform of osteopontin, followed by the induction of IL-27 (Guo, Chang, & Cheng 2008). Although IL-27 induces Th1 differentiation, the same cytokine has immunosuppressive properties which depend on IL-2 suppression, induction of IL-10 production as well as inhibition of Th17 differentiation (Yoshida, Nakaya, & Miyazaki 2009).

IFN-γ plays an important role in Th17 cell biology. The development of Th17 cells from naive precursor cells is strongly suppressed by IFN-γ both *in vitro* and *in vivo* (Harrington et al. 2005;Meyers et al. 2006;Park et al. 2005). Anti-IFN-γ added during *in vitro* Th17 differentiation leads to upregulated IL-17 expression, and increased expression of the IL-23R is displayed by the treated cells as well. Therefore, it was proposed that IFN-γ signaling might exert its inhibitory effect on pathogenic Th17 cells by thwarting IL-23R expression (Harrington et al. 2005). It has been observed that both IL-4 and IFN-γ suppress RORC2 by upregulating GATA-3 and T-bet, respectively, thereby inhibiting Th17 differentiation. However, IL-17 does not significantly alter expression of IL-4 or IFN-γ but does induce IL-2 expression in $CD4^+$ T cells, suggesting that this cytokine may promote T cell proliferation and survival (Crome, Wang, & Levings 2010). All these mechanisms, by which autoreactive Th17 cells can be regulated, may explain the molecular basis of IFN-α therapy for Behcet's disease. As described in Chapter 3, we have observed upregulation of

both IFN-γ and IL-4 expression by T cells, and this could help explain why a suppression of Th17 cells was found in patients treated with recombinant IFN-α.

4.4.1.4 T cells co-expressing IL-17 and IFN-γ

Although Th17 lineage development is independent of that of Th1, they can often be found side-by-side, suggesting that the interplay between Th17 and Th1 is likely to be important for our understanding of autoimmune disorders (Annunziato et al. 2007). In our experiments, the so-called Th1/Th17 cells were detected only in very low amount and IFN-α2b therapy did not seem to have any influence on this cell population. In the future it will be important to determine if these Th1/Th17 cells are a distinct and stable lineage of $CD4^+$ T effector cells, or whether they are an intermediate state destined to become true Th1 or Th17 cells.

4.4.2.1 Biology of regulatory T cells

Treg cells are pivotal for the maintenance of peripheral self-tolerance and imbalances in this T cell compartment have been shown to contribute to various autoimmune diseases (Allan et al. 2008b). $Foxp3^+$ Tregs regulate a wide variety of different immune cells including naive and memory $CD4^+$ and $CD8^+$ T effector cells, B cells, NK cells, monocytes, and DCs *in vitro* and *in vivo* (Grossman et al. 2004;Lim et al. 2005). Currently, it is not clear whether $Foxp3^+$ Tregs of direct thymic origin differ in phenotype or function from those of peripheral origin. What is clear is that cells of both types can specifically recognise a wide variety of Ags (Fazilleau et al. 2007b).

In peripheral blood, roughly 50% of lymphocytes are $CD4^+$ and the $CD4^+CD25^{high}$ T cell subset comprises 1-2% of circulating $CD4^+$ T cells in humans. nTreg cells mainly reside within the $CD4^+CD25^{high}$ cell population (Baecher-Allan et al. 2001). In adult human peripheral blood, $CD4^+CD25^{high}$ Treg cell compartment contains not only antigen-experienced, $CD45RA^-$ memory cells, but also a subpopulation of $CD45RA^+$ naive recent thymic emigrants without previous exposure to extrathymic self or foreign antigens (Seddiki et al. 2006). Analysis of naive Treg cell prevalence in

peripheral blood indicates that their proportion among $CD4^+$ T cells declines with age, starting from a range of 4-10% in cord blood and decreasing to 1-4% in young adults and to 0.5-1.5% in healthy elderly donors. However, the prevalence of $CD45RA^-$ Treg cells slightly increases with age starting from a range of 0-0.5% in cord blood and increasing to 1-2.5% in young adults and to 1-4% in elderly health donors (Miyara et al. 2009; Sakaguchi et al. 2010).

4.4.2.2 CD25 expression and its relationship with $Foxp3^+$ Treg cells

Although there is not one particular cell surface marker that defines Treg cells, the CD25 surface molecule is at least expressed on the vast majority of cells that express the Foxp3 transcription factor (Apostolou et al. 2008). Given that the expression levels of CD25 and Foxp3 are usually proportional in human $Foxp3^+$ T cells, one shortcoming of examining T cells expressing high levels of CD25 is the exclusion of $CD25^{int}Foxp3^{low}$ naive Treg cells (Miyara et al. 2009). Therefore, in our experiments, $CD4^+CD25^+$ T cells were examined as well as $CD4^+CD25^{high}$ T cells in order to cover the effects of IFN-α2b on these naive Treg populations. However, one must be aware that $CD4^+$ T cells with intermediate CD25 expression ($CD25^{int}$) are made up mainly of recently activated non-regulatory effector T cells, with only 5 to 15 % $Foxp3^+$ Treg cells (Hoffmann et al. 2006).

In addition, it has been reported that non-regulatory $CD45RO^+Foxp3^{low}$ T cells that produce pro-inflammatory cytokines may constitute 30-50% of the $CD4^+Foxp3^+$ T cells, which contain the $CD4^+CD25^-Foxp3^+$ cell population (Sakaguchi et al. 2010). Committed regulatory T cells with the $CD25^-$ phenotype are present in the periphery as a minor fraction of $CD4^+$ T cells but they may become $CD25^+$ upon activation. Data have shown that $CD4^+CD25^-$ cells exhibit an approximately 60-fold lower level of Foxp3 mRNA expression than $CD4^+CD25^+$ cells as measured by real-time quantitative RT-PCR (Yagi et al. 2004).

In accordance to these findings, our data consistently demonstrated that $Foxp3^+$ expression levels were much lower in either $CD4^+$ or $CD4^+CD25^+$ cell subpopulations compared to $CD4^+CD25^{high}$ cell population. One important point to bear in mind

though is that there is no firm criterion suggested from previous studies as to where the boundary lies between intermediate and high CD25 expression. This vague boundary has complicated the reproducibility of clinical data analysing the number and function of Treg cells, particularly under inflammatory conditions in which activated T cells express CD25.

In order to reduce populations with transient expression of Foxp3 and determine the true fraction of stable $Foxp3^+$ Tregs among $CD25^{high}$ cells, it may be an idea to incorporate steps to rest cells obtained from patients with inflammatory disorders during experiments. Alternatively, analysis of CD69 expression on Treg subsets has been suggested in order to determine whether these cells are contaminated with recently activated $CD4^+$ T cells as these would be mainly $CD69^+$ (Valencia et al. 2006).

4.4.2.3 Foxp3 expression levels in $CD4^+CD25^{high}$ T cells

Approximately 60% to 90% of $CD25^{high}$ cells are $Foxp3^+$ (Tang & Bluestone 2006). It was reported that $CD45RA^+CD4^+CD25^{high}$ T cells homogeneously maintained their Treg cell phenotype and function even after long-term culture (up to 3 weeks). In contrast to $CD45RA^+CD4^+CD25^{high}$ Treg cells, $CD4^+$ T cells selected solely for $CD25^{high}$ expression, were highly variable with respect to Foxp3 expression (50.5 to 72.8% $Foxp3^+$ cells) after long-term *in vitro* expansion (Hoffmann et al. 2006). This was despite that more than 90% of the sorted cells expressed Foxp3 after isolation.

In our experiments the percentages of $Foxp3^+$ cells in $CD25^{high}$ cells were observed to be substantially lower. This was evident whether whole blood samples were cultured over 24 hours or not as shown in the phenotyping and intracellular cytokine staining experiments. One possible explanation could be the presence of non-regulatory effector T cells that preferentially expand in culture over time. Another explanation could be the presence of a substantial number of ‘‘induced’’ Tregs that only transiently express Foxp3 after recent *in vivo* stimulation and revert to effector T cells during culture or before phenotyping could be performed (Tran, Ramsey, & Shevach 2007;Walker et al. 2003). The BD patient blood samples used in our experiments

were not usually processed on the day of collection. They were stored at room temperature and processed in the morning the following day. One other possible factor to consider could be that our BD patients had lower basal levels of $CD25^{high}Foxp3^{+}$ cells to begin with.

Additional contributing factor leading to the observed lower $Foxp3^{+}$ expression has been provided by recent findings that Foxp3 mRNA and protein expression in cell lines derived from $CD45RA^{-}CD4^{+}CD25^{high}$ Treg cells is particularly prone to be lost after 2 to 3 weeks in culture upon repetitive polyclonal TCR and CD28 co-receptor-mediated stimulation. After long-term culture, there can be parallel increase in cytokine-secreting cells within this cell population, even in the absence of a pro-inflammatory milieu (Hoffmann et al. 2009). Despite gaining the ability to secrete inflammatory cytokines (IL-2, IFN-γ, or both), these cytokine-secreting cells do not convert completely to effector T cells, as they also produce substantial amounts of IL-10. Thus, such cells may still be regulatory but mediate their effects via cytokines rather than cell-contact-dependent mechanisms. Of note, these data have been obtained using cell cultures with high-dose exogenous IL-2 present. As IL-2 has been demonstrated to have important, non-redundant effects on the maintenance and survival of $Foxp3^{+}$ Treg cells (Burchill et al. 2008), the loss of $Foxp3^{+}$ expression in our experiments could be greater and over shorter period of time as a result of absence of exogenous IL-2 in cultures.

4.4.2.4 Upregulation of Foxp3 expression in BD patients treated with IFN-α

Our data from phenotyping and intracellular cytokine staining experiments showed a significant upregulation of Foxp3 expression in $CD4^{+}CD25^{high}$ T cell subsets as early as after 3 months of subcutaneous IFN-α2b therapy. The enhanced expression of Foxp3 in BD patients was also observed in the $CD4^{+}$ and $CD4^{+}CD25^{+}$ T cell populations. Importantly, these positive findings were observed consistently in both phenotyping experiments and unstimulated cultures where no cell activation was involved. Consequently, expansion of recently activated non-regulatory $CD45RO^{+}Foxp3^{low}$ T cells was unlikely to be a major contributor to these results. Collectively, these results are likely to signify that Treg cell populations were promoted in BD patients

treated with IFN-α2b. In BD patients receiving conventional treatment alone, however, no change or even a decrease in the proportion of Foxp3 expressing cells were detected. This may indicate that the augmentation of Foxp3 expression in IFN-α2b-treated patients was not associated with the influence of systemic prednisolone or other immunosuppressants.

It remains to be determined how stable the enhanced expression of Foxp3 is and whether the status can be sustained even after stopping the IFN-α2b therapy. Encouragingly, Foxp3 expression levels were significantly elevated in our IFN-α2b-treated BD patients at 6 month assessments and these changes were still evident at 12 month visits, 6 months after ending the treatment.

Our findings of Foxp3 upregulation in peripheral blood lymphocytes by IFN-α2b therapy are in contrast to a recent study on melanoma patients receiving 4 weeks of IFN-α2b treatment where no statistically significant change in $Foxp3^+$ Treg level was detected (Ascierto et al. 2010). This could be explained in part by different patient characteristics at recruitment as Behcet's disease patients may have normal or even decreased levels of $CD4^+CD25^{high}$ Treg cells in peripheral blood to start with (Nanke et al. 2008) compared to increased levels in melanoma patients (Ascierto et al. 2010). Also, the treatment duration for our patients on IFN-α2b therapy was for 6 months, which was substantially longer.

In accordance to our results, an upregulation of $Foxp3^+$ Treg cells, associated with increase of Stat5 but downregulation of Stat3, was demonstrated in lymph node biopsies of melanoma patients with nodal metastasis after a course of high-dose IFN-α2b therapy over 48 weeks (Wang et al. 2008). Similarly, in a study on MS patients, treatment with IFN-β increased the expression of Foxp3 mRNA in PBMCs significantly after 6 months but not Foxp3 protein on $CD4^+CD25^+$ T cells (Vandenbark et al. 2009). Although a significant reduction was demonstrated in the capability of patients' plasmacytoid dendritic cells (pDCs), known inducers of Tregs, to produce IFN-α in a group of heterogeneous uveitis patients including BD (Plskova et al. 2006), it was suggested that the deficiency could be overcome with the administration of exogenous IFN-α.

4.4.2.5 Foxp3 and Treg function

Hypoproliferative ('anergic') and immune suppressive activities are two defining properties for Foxp3 expressing Treg cells. Intriguingly, $Foxp3^{low}$ expressing T cells were demonstrated to remain anergic upon TCR stimulation *in vitro*, but their immunosuppressive activities were greatly impaired (Wan & Flavell 2007). Thus, anergy and immune suppression are two separable properties of Treg cells that are affected differentially by the expression levels of Foxp3. While Foxp3 does not seem to control the entire Treg transcriptome, it is clearly essential for Treg differentiation and/or function, as its absence leads to a lack of Tregs and fatal autoimmune disease (Buckner & Ziegler 2008). Notably, previous studies have shown that transfection of human Foxp3 into naive $CD4^+$ T cells can induce Treg functions (Oswald-Richter et al. 2004;Yagi et al. 2004).

Foxp3 is expressed by both activated and regulatory T cells in the peripheral blood as are other known Treg cell biomarkers such as CD25. Virtually all activated $CD4^+$ and $CD8^+$ T cells have been shown to transiently upregulate Foxp3 and acquire suppressive properties (Pillai et al. 2007). However, it has been indicated that only those cells with sustained, high level Foxp3 expression go on to differentiate into Treg cells (Buckner & Ziegler 2008). In one study, it was noted that not all $CD25^+$ T cells in typical activation cultures had regulatory function and the great majority of these cells became $CD25^-$ after 10 days. Only those T cells that remained $CD25^+$ 10 days after activation were $Foxp3^+$ and showed suppressive function (Walker et al. 2005). Data from another group suggested that the human cells, unlike mouse cells, need more time to become fully differentiated $CD4^+CD25^+Foxp3^+$ suppressor cells. It was observed that upon repeated stimulation, the cells became anergic, some then expressed membrane TGF-β, and T cell proliferation was strongly suppressed subsequently (Horwitz et al. 2008).

Activated T cells possibly require additional factors, as well as the functional and phenotypic changes generated by Foxp3 expression, to acquire full suppressive activity. It has been reported that the maintenance of intrinsic Treg stability likely involves the epigenetic modification of the Foxp3 locus, especially the two highly conserved CpG-rich regions located in the Foxp3 first intron and up-stream Foxp3

promoter. The methylation status of these two regions is tightly associated with stable gene expression of Foxp3 (Miyara et al. 2009). In $Foxp3^+$ Tregs with suppressive activity, CpGs in these regions are fully demethylated, whereas the CpG's of $Foxp3^{low}$ non-regulatory T cells are incompletely demethylated. More recently, the expression of Helios, a member of the Ikaros transcription factor family, has been shown to be unique in thymus-derived $CD4^+CD25^+Foxp3^+$ Treg cells and this enables nTreg to be distinguished from iTreg cells and, most importantly, recently activated cells (Thornton et al. 2010).

Interaction of Foxp3 with other transcription factors has also been suggested to be critical to its action. Foxp3 has been demonstrated to directly interact with NFAT, a key regulator of T cell activation and anergy, and the NF-κB subunit p65. Several residues in the forkhead domain of Foxp3 interact with NFAT directly, and mutagenesis of these residues result in a decrease in the ability of Foxp3 to inhibit IL-2 production (Buckner & Ziegler 2008). Therefore, at least one mechanism of Foxp3-mediated transcriptional repression involves direct contact with NFAT and subsequent inhibition.

Requirement of other transcription factors, such as Runx, has been described. There are three putative Runx binding sites in the proximal Foxp3 promoter. It has been suggested that these binding sites have partially redundant functions, but binding to at least two sites seems to be necessary for full promoter activation. In suppression experiments, reduced suppressive activity has been shown to be caused by reduced Foxp3 expression in $CD4^+CD25^+Foxp3^+$ iTreg cells by small interfering RNA (siRNA) inhibition of Runx1 and Runx3. These results demonstrate the important role of Runx1 and Runx3 not only for the induction of Foxp3, but also for the suppressive capacity of Treg cells (Klunker et al. 2009).

It remains to be determined whether the induced $Foxp3^+$ T cells in our experiments are functionally competent suppressive cells. Maintenance of sustained Foxp3 expression as T cell activation wanes may be key for acquisition of suppressor function and development of peripherally induced Tregs. Nevertheless, we can speculate that a small subset of effector T cells with activation induced Foxp3 may not downregulate Foxp3 and therefore ultimately develop into functional Treg cells.

4.4.3.1 Biology of IL-10

IL-10 is a multi-functional regulatory cytokine first described as a molecule secreted by Th2 cells that could inhibit cytokine synthesis by Th1 cells (Zhu & Paul 2008). All the $CD4^+$ T cell subsets including Th1, Th2, Th17, as well as Treg cells are capable of producing IL-10 under certain conditions (Anderson et al. 2007;Jankovic et al. 2007;McGeachy et al. 2007). The principal role of IL-10 seems to be keeping immune responses in check and preventing the detrimental effects of excessive inflammatory reactions.

IL-10 is a potent inhibitor of antigen presentation, and its main biological function appears to be exerted on DCs and macrophages. It inhibits MHC class II expression as well as the upregulation of costimulatory molecules CD80 (B7-1) and CD86 (B7-2), which profoundly affects the ability of APCs to activate T cells. IL-10 inhibits the maturation of DCs and differentiation of DCs from monocyte precursors. Thus, many of the immuno-inhibitory characteristics of IL-10 can be attributed to their effect on APCs to downregulate the production of the Th1-associated cytokines IL-2 and IFN-γ, and also the Th2-associated cytokines IL-4 and IL-5. Thus, the original description of IL-10 as a crossregulator of Th1/Th2 immunity is probably no longer applicable. The other profound effect of IL-10 is to suppress the production of pro-inflammatory cytokines and mediators from macrophages and DCs, including IL-1, IL-6, IL-12, TNF-α. IL-10 can further inhibit inflammation by increasing the release of IL-1 receptor antagonist by macrophages (Akdis & Blaser 2001;Mosser & Zhang 2008).

IL-10 can target naive $CD4^+$ T cells, possibly via an inhibition of CD28 signalling pathway. It has little or no direct effects on activated or memory T cells due to the reduction of IL-10R upon T cell activation (Akdis & Blaser 2001). Inflammatory chemokines of both the CC and CXC type are suppressed by IL-10, as is the production of macrophage matrix metalloproteases. Then again, it is important to note that the biologic functions of IL-10 are not solely restricted to suppression of the immune system. IL-10 can augment B cell proliferation, prolong B cell survival, and contribute to class switching in B cells. It can also costimulate NK cell proliferation and cytokine production. IL-10 can act as a growth factor to stimulate the proliferation of certain subsets of $CD8^+$ T cells (Mosser & Zhang 2008). Furthermore,

T cells are protected from apoptotic death by IL-10 through upregulation of Bcl-2, a protooncogene (Cohen et al. 1997).

4.4.3.2 The immunomodulatory effect of IFN-α on IL-10

Analysing our data, a tendency to upregulate IL-10 production by $CD3^+$ and $CD4^+$ T cells was observed. The finding is in line with a recent study which showed that IFN-α significantly increased IL-10 production by anti-CD3/CD28 stimulated PBMCs in cell culture supernatants from BD patients (Liu et al. 2011). Our results are also in agreement with earlier studies. In patients with uveitis, IL-10 production by $CD3^+/CD4^+$ T cells was reduced but was restored to normal after IFN-α therapy (Ito et al. 2001;Plskova et al. 2006). In patients with corticosteroids-resistant asthma, the observation of increased IL-10 expression in venous blood T cells and monocytes as a consequence of IFN-α therapy has been described (Simon et al. 2003). In addition, IFN-α was found to induce mild upregulation of IL-10 production by $CD4^+CD3^-$ Th2-like clones isolated from peripheral blood of patients with hypereosinophilic syndrome (Schandene et al. 1996). Stimulation of DCs with LPS induced mainly production of IL-10 that was further amplified by IFN-α (Tamir et al. 2005).

4.4.3.3 Biology of Tr1 cells

For the systemic control of autoreactive T effector cells in a multi-system inflammatory disease, apart from $Foxp3^+$ Treg subsets that function mainly via contact-dependent mechanism, distinct induced Tr1 cells that lack Foxp3 expression also regulate T cell functions. Recent evidence suggests that, like in the case of human effector T cells, Tr1 cells can express Foxp3 upon activation, albeit transiently (Roncarolo & Gregori 2008). Human Tr1 cells generated *in vitro* do not constitutively express Foxp3, but upon activation can upregulate this transcription factor to levels similar to those observed in activated effector T cells. Interestingly, functional suppressor Tr1 cells can be differentiated *in vitro* from naive T cells of IPEX patients lacking Foxp3 (Roncarolo & Gregori 2008). It has been indicated that one of the mechanisms of action of $Foxp3^+$ Treg cells may relate to the induction of Tr1

cells *in vivo*, as there is evidence that Foxp3$^+$ Treg cells induce the development of Tr1 cells *in vitro* (Dieckmann et al. 2002).

Through the capacity to secrete IL-10 by Tr1 cells, infectious tolerance can be mediated effectively. Also, Tr1 cells may enhance the function of Foxp3$^+$ Treg cells by inhibiting production of pro-inflammatory cytokines such as TNF-α, as the presence of these cytokines may compromise the suppressive capacity of Foxp3$^+$ Tregs (Thunberg et al. 2007). Currently, there are no known specific cell-surface markers for Tr1 cells, and it is therefore difficult to identify and track these cells. Although many cell types produce IL-10, Tr1 cells characteristically produce high levels of IL-10 as well as lower levels of TGF-β (Roncarolo et al. 2006). In addition to their cytokine production profile, Tr1 cells are functionally characterised by their intrinsic low proliferative capacity (Levings et al. 2001). Tr1 cells are found *in vivo* in the chronic presence of low levels of antigen. Although Tr1 cells must encounter their antigen to exert these regulatory effects, once activated, they seem to suppress in an antigen non-specific manner and bystander inhibition by cytokines such as IL-10 (Groux et al. 1997;Roncarolo, Levings, & Traversari 2001). Furthermore, Tr1 cells can be induced from naive CD4$^+$ T cells activated by tolerogenic APCs in the periphery in the presence of IL-10 (Roncarolo et al. 2006).

4.4.3.4 The immunomodulatory effect of IFN-α on Tr1 cells

From our experiments, a tendency to enhance IL-10 production by CD3$^+$ and CD4$^+$ T cells was detected. This is likely to support induction of a larger population of Tr1 cells. Notably, the increase in the CD4$^+$IL-10$^+$ T cell populations was mostly accounted for by the CD4$^+$IL-10$^+$Foxp3$^-$ cells, some of which were likely to be Tr1 cells. Not surprisingly, an upregulation of CD4$^+$IL-10$^+$Foxp3$^-$ T cells themselves were also observed.

4.4.4.1 Biology of TGF-β

Previous studies revealed that TGF-β plays a key role in inducing tolerance and maintaining T cell homeostasis. It can target proliferation and differentiation programs in T cells independently. TGF-β has been found to inhibit T cell proliferation via multiple pathways, including blocking IL-2 expression (Wan & Flavell 2008). However, inclusion of IL-2 in T cell cultures relieves TGF-β inhibition of T cell proliferation. Of note, TGF-β-mediated inhibition of Th cell differentiation can still take place in the presence of IL-2 despite the fact that T cell proliferation is encouraged (Sad & Mosmann 1994;Wan & Flavell 2008). This indicates that TGF-β can inhibit T cell proliferation through yet to be defined IL-2-independent mechanisms.

TGF-β is a powerful regulator of effector T cell differentiation, and it generally impedes the acquisition of Th cell functions (Gorelik & Flavell 2002). TGF-β has been shown to inhibit both Th1 and Th2 differentiation, possibly through downregulation of Th1 and Th2 master regulators, T-bet, and GATA3 (Gorelik, Constant, & Flavell 2002;Gorelik, Fields, & Flavell 2000;Park, Letterio, & Gorham 2007). However, the activity of TGF-β is influenced by the environmental milieu. Also, TGF-β is intimately associated with the survival and function of Treg cells (Li et al. 2006). Nevertheless, it is now generally accepted that whereas TGF-β alone induces Foxp3 and inhibits Th17 differentiation, if inflammatory cytokines such as IL-6 are present, TGF-β-induced Foxp3 is suppressed and the expression of Th17-defining transcription factors is favoured (Yang et al. 2008b).

4.4.4.2 The immunomodulatory effect of IFN-α on TGF-β

Our *ex vivo* data showed the tendency to upregulate TGF-β by $CD3^+$ T cell populations as early as 3 months after starting IFN-α therapy and the trend was still evident at 12 month visits. This is in contrast to patients on conventional treatment alone as no change or even a downregulation of TGF-β expression by T cells was observed. The positive effects of IFN-α therapy on TGF-β levels have not been reported previously.

In a study on myeloma patients treated with 2 weeks of low dose IFN-α2a, no significant change in TGF-β level was detected with IFN-α administration (Sonmez et al. 2004). Also, there was no significant effect observed on the levels of TGF-β in patients with melanoma treated with 4 weeks of IFN-α2b either (Ascierto et al. 2010). The difference could be related to the dosage of IFN-α administered and the shorter period of IFN-α therapy compared to our study. Importantly, the production and action of IL-10 and TGF-β are interrelated and likely involve a positive feedback loop in which IL-10 enhances expression of TGF-β and vice versa. IL-10 not only enhances production of TGF-β, but it can also modulate the ability of cells to respond to TGF-β. This involves the IL-10-mediated restoration of TGF-βR2 expression on recently activated T cells, which typically downregulate this molecule and become resistant to the inhibitory effects of TGF-β (Cottrez & Groux 2001). Therefore, the production and function of IL-10 and TGF-β may be interdependent, and the enhancement of one cytokine may also lead to the augmentation of the other as observed in our data.

4.4.5 Relationships between Th17 and Treg cells

The developmental relationship between Treg and Th17 subsets is complex and has not been completely elucidated. Previous studies have suggested the existence of not only a functional antagonism between the two subsets, but also of a dichotomy, mutually exclusive, in their generation. During development, both Treg and Th17 cells require TGF-β for differentiation, albeit at different levels (Manel, Unutmaz, & Littman 2008). Also of note is that aTreg cells specifically expressed high amounts of AHR repressor transcripts and the ensuing constraint on Th17 cell development might play an important part in promoting Treg cell differentiation (Miyara et al. 2009). In the presence of IL-1β, IL-2, IL-21 and IL-23, $CD4^{+}CD25^{hi}Foxp3^{+}$ Tregs were found to differentiate into IL-17-producing cells, when stimulated by allogeneic APCs (Koenen, Smeets, Vink, van Rijssen, Boots, & Joosten 2008). In agreement, another study reported that Treg cells converted to Th17 cells when cultured in the presence of monocyte-derived IL-1β and IL-2 and lost their suppressive abilities (Deknuydt et al. 2009).

Interestingly, memory Tregs ($CD25^+CD127^{low}CD45RA^-Foxp3^+$) display more a tendency to be converted to Th17 cells than conventional memory $CD4^+$ T cells. In addition, memory Tregs demonstrate a more pronounced proficiency to give rise to Th17 cells than natural naive Tregs ($CD25^+CD127^{low}CD45RA^+Foxp3^+$) (Deknuydt et al. 2009). In the peripheral blood, up to 3% of $Foxp3^+$ Tregs cells in peripheral blood are capable of producing IL-17 upon activation and these cells co-express transcription factors RORc2 and Foxp3 (Voo et al. 2009). These cells are suggested to be generated in the periphery. Intriguingly, these $IL\text{-}17^+Foxp3^+$ Treg cells are reported to function as both effector and Tregs cells and are capable of suppressing the proliferation of $CD4^+$ responder cells *in vitro*. Compared to $Foxp3^+IL\text{-}17^-$ cells, the levels of Foxp3 expression in the $Foxp3^+IL\text{-}17^+$ cells are significantly lower (Voo et al. 2009). It is likely $CD4^+$ T cells positive for IL-17 in our experiments contain some cells with $Foxp3^+IL\text{-}17^+$ phenotype as a large part of our experiments involved some kinds of polyclonal activation. Further evaluation will need to be carried out in the future in order to clarify the roles of this subset of cells in relation to our findings.

A disputable question is whether Th17 cells are susceptible to the suppressive effects of Tregs. As exacerbation of Behcet's disease may be linked to Th17 cells, it is crucial to understand whether an upregulation of Treg cells would ameliorate this aspect of inflammation. Consistent data from human studies show that Th17 cells are resistant to Treg-mediated suppression at the level of proliferation, as well as cytokine production (Annunziato et al. 2007;Evans et al. 2007;Lohr et al. 2006). Further examination revealed that although Treg cells could limit TNF-α and IL-6 produced by RORc2-transduced T cells, they failed to suppress IL-17 (Crome et al. 2009). IL-17 has been shown to induce effector T cell proliferation and also impede the ability of Treg cells to regulate this proliferation. Also, the fact that IL-17 could stimulate IL-2 production by effector T cells all may help explain the resistance of Th17 cells to suppression by Treg cells (Crome et al. 2009).

4.4.6.1 Biology of NKT cells

NKT cells are a special kind of lymphocyte that bridges the adaptive immune system with the innate immune system. Once activated, these cells can perform functions ascribed to both Th and Tc cells, including cytokine production and release of cytolytic molecules (Godfrey & Kronenberg 2004;Kawano et al. 1999). NKT cells may promote the functions of $CD4^+CD25^{high}$ T cells by secreting cytokines, including IL-2 and TGF-β, and modulate both Th1 and Th2-associated immunity (Godfrey et al. 2000;La Cava, Van Kaer, & Fu 2006). Similar to $CD4^+CD25^+$ Tregs, iNKT cells have been identified to express CD25 and regulate a range of immune responses (Godfrey & Kronenberg 2004). Innate production of IL-17 by this group of cells was discovered recently in human as well (Rachitskaya et al. 2008).

4.4.6.2 NKT cells and Behcet's disease

In BD, a decreased frequency of NKT cells in the peripheral circulation was observed in patients with neurological manifestations, contrasting with a high percentage of these cells within the CSF mononuclear cells. The difference was considered due to migration of unimpaired NKT cells toward the inflammatory sites to exert their regulatory function. These NKT cells were found to be biased towards a Th1 phenotype (Hamzaoui et al. 2006). However, in a separate study, $CD3^+CD56^+$ cells, especially the $CD8^+CD56^+$ subset, were reported to increase significantly in both the aqueous humor and peripheral blood of BD patients with uveitis compared with healthy controls and patients with idiopathic anterior uveitis (Yu et al. 2004). Similar increase of $CD4^+CD56^+$ T cells in the peripheral blood of BD patients were described as well (Eksioglu-Demiralp et al. 1999a). Therefore, these cells may play a role in the pathogenesis of BD, but their precise role remains to be clarified.

Analysing our data, no significant influence on NKT cell populations by IFN-α therapy was detected.

4.4.7 The potential influence of systemic steroids and immunosuppressants

As the majority of our BD patients were still taking various combinations of steroids and other immunosuppressants. Their potential effects on Th17 and Treg cells are discussed in this section.

4.4.7.1 The immunomodulatory effects on IL-17

In one report, methylprednisolone (MP), a synthetic glucocorticoid, was shown to partially block PMA/ionomycin-triggered IL-17 production by lymphocytes from healthy donors *in vitro* (Ziolkowska et al. 2000). Interestingly, IL-17 production was reported to be less sensitive than IFN-γ production to the influence of MP (Momcilovic et al. 2008). Also, it was recently demonstrated that intravenous MP enhanced Treg cells and their expression of functional molecules including IL-10 in patients with MS two days after treatment. However, the levels returned to baseline values by 6 weeks post-treatment (Braitch et al. 2008).

Cyclosporine A was shown to significantly block IL-17 production by polyclonally stimulated PBMCs as well as $CD4^+$ and $CD8^+$ T cells from both BD patients and normal controls. The *ex vivo* production of IL-17 by PBMCs was also significantly inhibited in BD patients with active uveitis after 1 and 3 months of cyclosporine A treatment in ELISA experiments (Chi et al. 2010). Compared to corticosteroids, cyclosporine A was found to have even greater inhibitory action on IL-17. Furthermore, the presence of cyclosporine A in cell cultures was observed to significantly downregulate IL-17 production by memory Th17 cells at both protein and mRNA levels in healthy individuals and RA patients (Zhang et al. 2008).

Mycophenolic acid was shown to inhibit IL-17 production induced by IL-15 in peripheral blood lymphocytes. Methotrexate, however, did not exhibit any effect on the production of IL-17 (Gonzalez-Alvaro et al. 2009).

4.4.7.2 The immunomodulatory effects on Treg cells

It was reported that steroid treatment resulted in a significant increase in Foxp3 and IL-10 mRNA expression by freshly isolated and unstimulated $CD4^+$ T cells in patients with moderate to severe asthma. *In vitro*, 6 and 24 hours of dexamethasone treatment of healthy donor $CD4^+$ T cells also led to an upregulation in Foxp3 mRNA expression (Karagiannidis et al. 2004).

On the other hand, calcineurin inhibitors such as cyclosporine were found to be damaging to $Foxp3^+$ Tregs as high blood levels of these correlated with lower $Foxp3^+$ Treg frequency in liver transplant patients (San Segundo et al. 2007).

Conversion of immunosuppressive treatment from calcineurin inhibitors to mycophenolate mofetil in liver transplant recipients, however, resulted in the enrichment of $CD25^+Foxp3^+$ T cells in peripheral blood after 6 months (Demirkiran et al. 2009).

4.5 Summary of findings

We demonstrated that 6 months of subcutaneous IFN-α2b therapy can result in lower amount of peripheral blood Th17 cells in BD patients. On the other hand, $CD4^+Foxp3^+$ Treg cell populations, especially $CD4^+CD25^{high}$ T cells, were augmented following IFN-α2b treatment in these patients. Notably, both of these effects were enduring as they were observed even after cessation of IFN-α therapy. Also, a tendency to upregulate the production levels of IL-10 and TGF-β by T cells was detected after treatment with IFN-α. This increase in immunoregulatory cytokine levels could assist in the regulation of Th cells and promote the number and function of Treg subsets. Regarding NKT cells, however, no significant result was able to be observed.

Chapter 5

The *in vitro* effects of interferon-alpha 2a & 2b on IFN-γ and IL-17 expression by $CD4^+$ and $CD8^+$ T cells in healthy donors

5.1 Introduction

Before the discovery of Th17 subset as a distinct $CD4^+$ effector population, Th1 cells were long considered to be the major effectors in multiple autoimmune and immune-mediated diseases. Preferential skewing of the immune response toward the Th1 pathway was also demonstrated in BD patients (Li et al. 2003). As IL-17 has profound pro-inflammatory effects and induces tissue damage during the course of various immune-mediated diseases, Th17 cells are increasingly being recognised to have a key role in autoimmune and inflammatory diseases (Kunz & Ibrahim 2009).

IL-17A and IL-17F can form homo- or hetero-dimers. This can give rise to three forms of IL-17: IL-17A/A, IL-17A/F and IL-17F/F, with potentially different biological activities. A study on mouse Th17 cells showed that IL-17A/A appears to have the strongest function in tissue inflammation, followed by IL-17A/F. However, polarised Th17 cells express IL-17F/A in higher amounts than either homodimer (Liang et al. 2007). IL-17 (A and F) induces production of a broad range of pro-inflammatory cytokines and chemokines, including IL-6, colony-stimulating factors, CXC chemokines, human β-defensin-2 and metalloproteinases, by a variety of cells (Weaver et al. 2007). Dysregulated IL-17 has been implicated to cause severe pathogenic consequences. Recently, increased expression of IL-17 and IL-23 has been identified in BD patients with active uveitis compared to normal controls and patients in remission (Chi et al. 2008).

As a result, when investigating the immunomodulatory effects of IFN-α treatment, analysing changes in T cell subsets producing IFN-γ and IL-17 became one of our main focus. In Chapter 3, we have shown that subcutaneous IFN-α2b therapy has the tendency to augment IFN-γ levels in T cells and suppress Th17 cells in BD patients *in vivo*. In this chapter, we concentrate on exploring the *in vitro* effects of exogenous IFN-α on IL-17- and IFN-γ-producing T cells. In order to establish

adequate amount of Th17 cells to test the effects of exogenous IFN-α, various stimuli, including LPS, zymosan, Candida albicans, alpha-tropomyosin, PDS-Ag, and all-trans retinoic acid (ATRA) were used in an attempt to induce healthy donor PBMCs to produce IL-17 in the preliminary tests. These stimuli were chosen based on previously reported links with Th17 or Behcet's disease. The only exception is ATRA, which is expected to have a suppressive rather than augmentation effect on Th17 cells. Polyclonal stimulation using anti-CD3/CD28 Ab and PMA/ionomycin was evaluated as well.

LPS is the major component of the outer membrane of Gram-negative bacteria, and activates TLR4. It promotes the secretion of pro-inflammatory cytokines in many cell types. Low titre of antibodies against LPS have been found in BD patients' sera (Cuchacovich et al. 2005), and LPS-stimulated production of TNF-α, IL-1, IL-6 and IL-8 has been observed to be significantly increased in patients with BD (Mege et al. 1993). LPS-conditioned medium was found to exhibit strong IL-17 inducing activity in naive human $CD4^+$ T cells after 5 days in culture with CD3/CD28 polyclonal activation. Conditioned medium was derived from supernatants of PBMCs cultured for 72 hours with LPS, and IL-17 concentrations were measured by ELISA (Kattah et al. 2008). Similarly, stimulation of healthy donor PBMCs with 1mg/ml LPS has been shown to induce increased concentration of IL-17 in supernatants collected after 24 hours of culture as measured by ELISA (Moschen et al. 2008).

Zymosan, a yeast cell-wall component, binds to TLR2 and human dectin-1 (β-glucan receptor) (Dillon et al. 2006). It was demonstrated that the supernatants of zymosan-stimulated DCs contained IL-1β and IL-23 and induced production of IL-17 and IL-22 in naive human $CD4^+$ T cells stimulated with anti-CD3 and anti-CD28. In the experiments, naive cells were initially stimulated with anti-CD3 and anti-CD28 Ab and were washed and stimulated again with anti-CD3 Ab and 10ng/ml PMA after 5 days. Supernatants were collected another 18 hours later (Gerosa et al. 2008).

C. albicans is a dimorphic organism that can take either a yeast form (spherical morphology) or hyphal form (rod morphology). In the presence of heat-killed C. albicans yeast or hyphae, ELISA measurements of IFN-γ and IL-17 concentrations in 5 day culture supernatants of naive $CD4^+$ T cells stimulated with autologous

monocytes and anti-CD3 were performed. C. albicans in the yeast form induced the differentiation of IFN-γ-producing cells, whereas C. albicans in the hyphal form primed naive T cell differentiation toward Th17 cells. Also, the yeast form of this fungus induced monocyte-derived DCs to produce IL-12 and at high concentrations, IL-23, as revealed in 48 hour culture supernatants. On the other hand, C. albicans hyphae selectively triggered IL-23 production (Acosta-Rodriguez et al. 2007).

Retinal-S antigen (S-Ag) is a candidate autoantigen in BD. The protein is found mainly in the retina, and an immune mediated response to this antigen can only occur after eye damage due to uveitis. T cell responses against S-Ag are present in various types of human uveitis, including BD. Among the immunodominant epitopes of S-Ag, an epitope named PDS-Ag (aa 342–355) is found to share homology with HLA-B51 and HLA-B27, which are linked to uveitis in BD (Direskeneli 2001).

Tropomyosins are a family of proteins expressed in muscle as well as in non-muscle cells including epithelial cells, fibroblasts, and platelets. A significantly increased lymphoproliferative response to alpha-tropomyosin as well as autoantibodies to tropomyosin have been reported in BD patients with posterior uveitis (Mahesh et al. 2005). These abnormalities support a possible role for alpha-tropomyosin as a self-antigen in patients with BD.

A crucial regulatory role for retinoic acid in promoting Foxp3 induction and inhibiting Th17 cell differentiation has been identified (Elias et al. 2008;Mucida et al. 2007). This is in accordance to a recent report that IL-17 secretion by naive human $CD4^+$ T cells was partially inhibited by addition of exogenous retinoic acid after 5 days in culture with polyclonal activation in the presence of Th17 polarising mileu (TGF-β, IL-1β, IL-6 and IL-23) (Kattah et al. 2008).

5.2 Aims

Having established the capacity of subcutaneous IFN-α2b to upregulate T cells producing IFN-γ and suppress Th17 cells *in vivo* in Chapters 3 and 4, the aim of this chapter is to investigate the *in vitro* immunomodulatory effects of IFN-α on $CD4^+$ as

well as $CD8^+$ T cells producing IFN-γ and IL-17. As the effect of IFN-α on Th1-type cytokines is more established, the focus is therefore more on T cells producing IL-17 in this chapter. Assays are set up using healthy donor PBMCs to test the influence of various concentrations of IFN-α2a and IFN-α2b on T cells and the kinetics of the interactions.

5.3 Results

5.3.1 Preliminary experiments to establish Th1/Th17 test models with antigens and polyclonal stimulation

In order to develop *in vitro* models with adequate amount of Th17 cells to test the effects of IFN-α on these cells, healthy donor PBMCs were either activated with various concentrations of antigens or stimulated with polyclonal stimuli for 24 hours before intracellular cytokine staining and preparation for flow cytometry. Untreated cultures were included as part of the experiments as well. Cytokine expression was analysed by gating on $CD4^+$ lymphocyte populations.

It was found that antigens, including LPS, zymosan and candida, promoted very low levels of IFN-γ (Fig 5.1a) and IL-17 (Fig 5.1b) expression in $CD4^+$ T cells regardless of the concentrations of antigens used, and as expected the levels were even lower in the case of ATRA. Subsequently, extra 15 hours of incubation was tested for cell cultures with LPS and candida yeast extract so there would be more time for interactions between cells and antigens. However, the increase in production levels of IFN-γ and IL-17 achieved was far from satisfactory. Concurrent use of LPS and CD3/CD28 stimulation was evaluated as well, but the effect was no more than using anti-CD3/CD28 Ab alone.

Thus, it was decided to test BD patient PBMCs with BD-related antigens, such as alpha-tropomyosin and PDS-Ag as a trial. However, similar results were observed and IFN-γ and IL-17 production levels achieved were no better than using LPS on healthy donor PBMCs. Originally, HSP60 was going to be tested as well if the trials on alpha-tropomyosin and PDS-Ag were successful. Because of the very low

production levels achieved, it was decided not to use up any more of the precious BD patient PBMCs to test the immunomodulatory ability of HSP60 and other BD-related antigens.

Fortunately, substantially higher production levels of both IFN-γ and IL-17 were able to be induced in $CD4^+$ T cells consistently with polyclonal stimulation such as anti-CD3/CD28 Ab and especially PMA/ionomycin (Fig 5.1a and Fig 5.1b). These results were repeatable as shown by the data in nine separate experiments each for Th1 and Th17 cells despite the fact that the experiments were performed in singles.

As a result, healthy donor PBMCs activated with anti-CD3/CD28 Ab and/or PMA/ionomycin were chosen as the basis for test models in subsequent *in vitro* experiments and further preliminary experiments with antigens were not carried out.

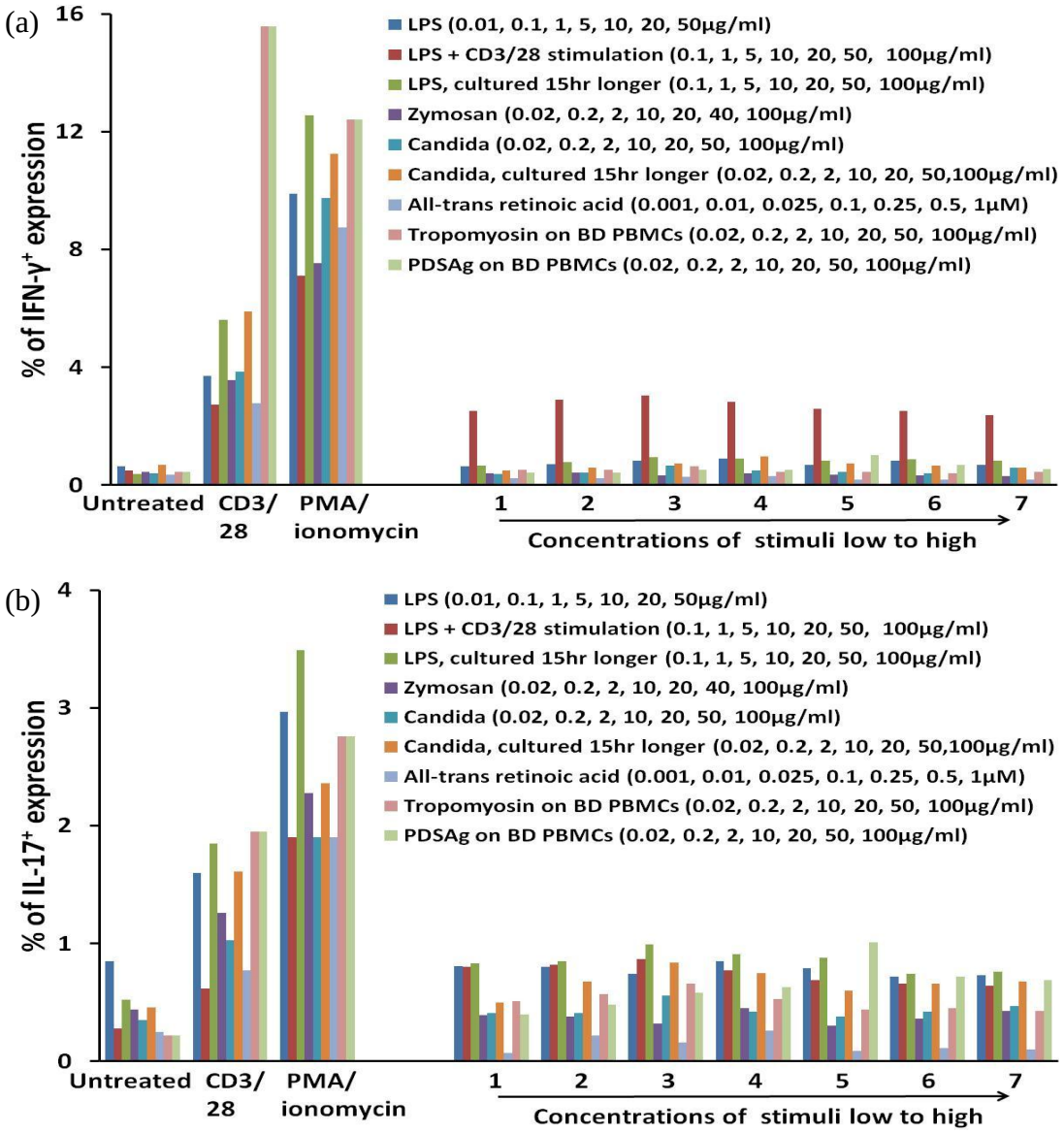


Fig 5.1: The effect of a selection of stimuli on the levels of Th1 and Th17 cells in healthy donors

Healthy donor PBMCs were untreated, activated with anti-CD3 (5ng/ml) & anti-CD28 (1µg/ml), activated with PMA (25ng/ml) & ionomycin (1µg/ml) or stimulated with various stimuli including LPS, zymosan, candida, and all-trans retinoic acid for 24 hours or 39 hours. In one experiment, both LPS and CD3/CD28 stimulation were administered. Also, in 2 of the experiments, PBMCs from BD patients were used instead and stimulated with either polyclonal stimuli or α-tropopmyosin or PDS-Ag for 24 hours. Results are shown as proportion of $CD4^+$ T cells expressing cytokines. In (a), percentage of IFN-γ^+ cells and in (b), percentage of IL-17^+ cells are shown. Each colour represent a separate experiment and all experiments were done only once in singles. 1 is the lowest antigen concentration used and 7 is the highest. The actual concentrations of stimuli are shown inside the brackets in the legend.

5.3.2 Preliminary experiments to establish the needs to rest cells in the medium after thawing and the effects of adding suboptimal IL-2

Next, it was examined whether resting healthy donor PBMCs for one day in culture medium with or without addition of suboptimal amount of IL-2 after thawing could lead to recovery of functional ability by "stunned" cells and thus enhance the proportions of T cells producing cytokines.

In terms of IFN-γ production by $CD4^+$ T cells (Fig 5.2a), a small upregulation of cytokine levels was evident in PHA stimulated cultures with one day resting and presence of IL-2 ($p<0.01$). In PMA/ionomycin stimulated cultures, an increase in the proportion of $CD4^+$ T cells expressing IFN-γ was observed with one day resting whether IL-2 was added or not. However, these results with PMA/ionomycin stimulation were not statistically significant. On the other hand, in CD3/CD28 stimulated cultures, IFN-γ production by $CD4^+$ T cells was downregulated with resting, whether IL-2 was added ($p<0.05$) or not ($p<0.01$). These inconsistent results may be able to be resolved with repeating experiments, but these were not carried out as the main focus was more on setting up reliable Th17 models using the limited resources available.

In terms of IL-17 production by $CD4^+$ T cells (Fig 5.2b), one day resting in culture medium with or without the addition of IL-2 after thawing did not have any significant impact on the proportion of $CD4^+$ T cells expressing IL-17. Anti-CD3/CD28 Ab and PHA were comparable in their capacity to stimulate IL-17 expression and IL-17-enhancing effect achieved was far greater with PMA/ionomycin stimulation. Overall, the findings suggest there is no advantage in resting PBMCs or adding suboptimal concentration of IL-2 for experiments involving Th17 cell populations.

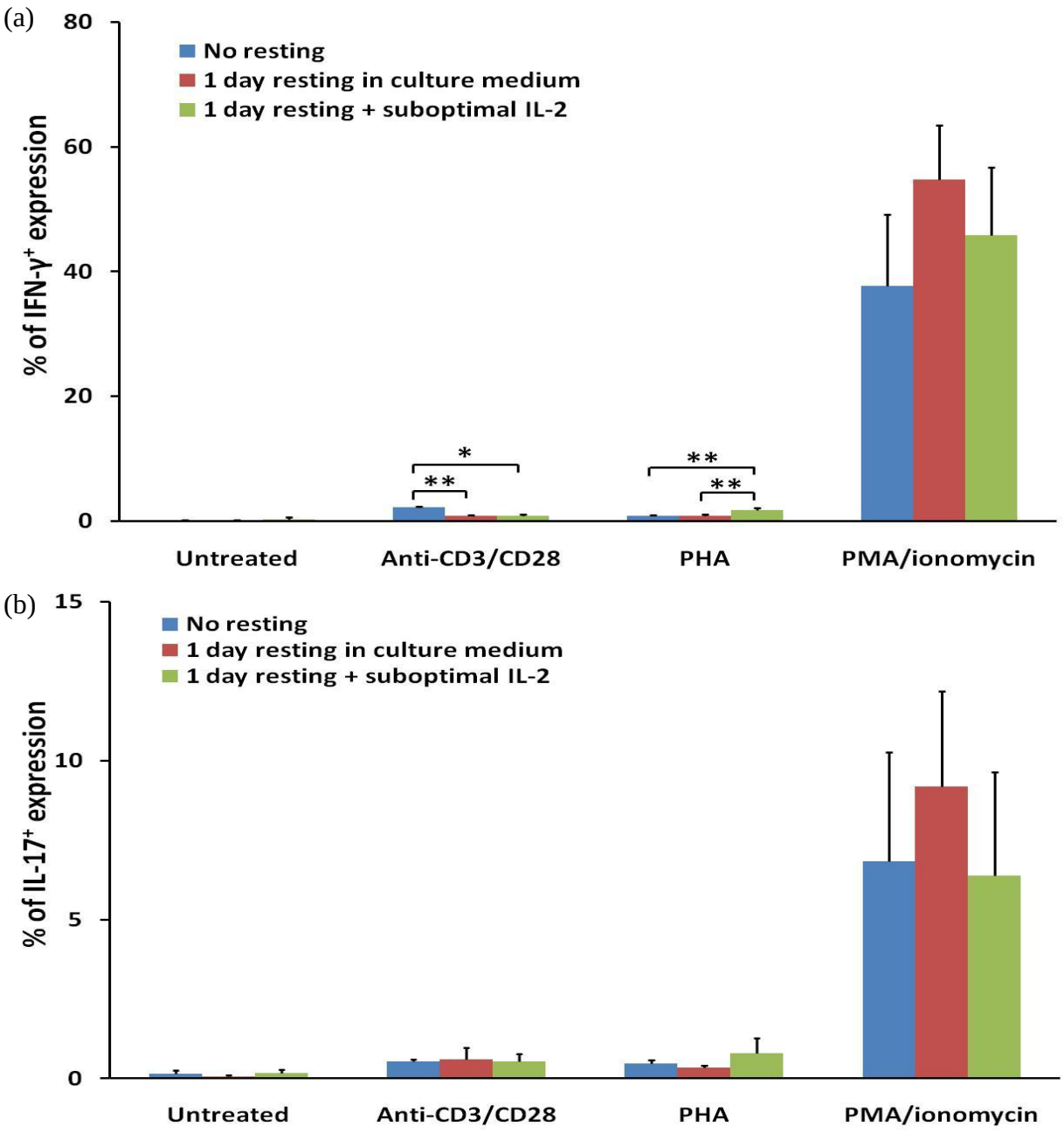


Fig 5.2: The effect of resting PBMCs and suboptimal concentration of IL-2 on the proportion of Th1 and Th17 cells

Healthy donor PBMCs were either stimulated straight away or rested for 1 day in culture medium (RPMI & supplements) ± 0.5 U/ml of IL-2 before stimulation. Under all 3 conditions, PBMCs were untreated, or stimulated with anti-CD3 (5ng/ml) & anti-CD28 (1µg/ml), PMA (25ng/ml) & ionomycin (1µg/ml) or PHA (5µg/ml) for 24 hours. Results are shown as proportion of $CD4^+$ T cells expressing cytokines. In (a), percentage of IFN-γ^+ cells and in (b), percentage of IL-17^+ cells are shown. The experiment was done only once in triplicates and the results are shown as means ± SD.

5.3.3 Preliminary experiments to establish the choice of culture medium

The choice of medium was scrutinised as part of the preliminary experiments since it has been reported that Iscove's modified Dulbecco's medium (IMDM) can result in higher Th17 expansion (Veldhoen et al. 2009). Experiments were set up to test healthy donor PBMCs cultured in RPMI- and IMDM-based T cell media in parallel. In agreement, it was found in PMA/ionomycin stimulated cultures that the proportion of Th17 cells was enhanced from 1.6 ± 0.9% to 5.1 ± 3.5% ($p=0.06$) when the culture medium was switched from RPMI-based medium to IMDM-based medium (Fig 5.3b). However, no significant change was noticed in untreated or CD3/CD28 stimulated cultures.

Notably, IMDM-based medium appeared to have greater influence on the levels of IFN-γ in $CD4^+$ T cells (Fig 5.3a). In comparison to cultures incubated in RPMI-based medium, the proportions of IFN-γ expressing $CD4^+$ T cells were raised from 0.1 ± 0.1% to 0.3 ± 0.2% ($p<0.05$) in untreated cultures and from 3.0 ± 1.6% to 13.3 ± 1.5% ($p<0.01$) in PMA/ionomycin stimulated cultures by IMDM-based culture medium.

Consequently, majority of the experiments involving investigations of Th1/Th17 were performed using IMDM-based culture medium.

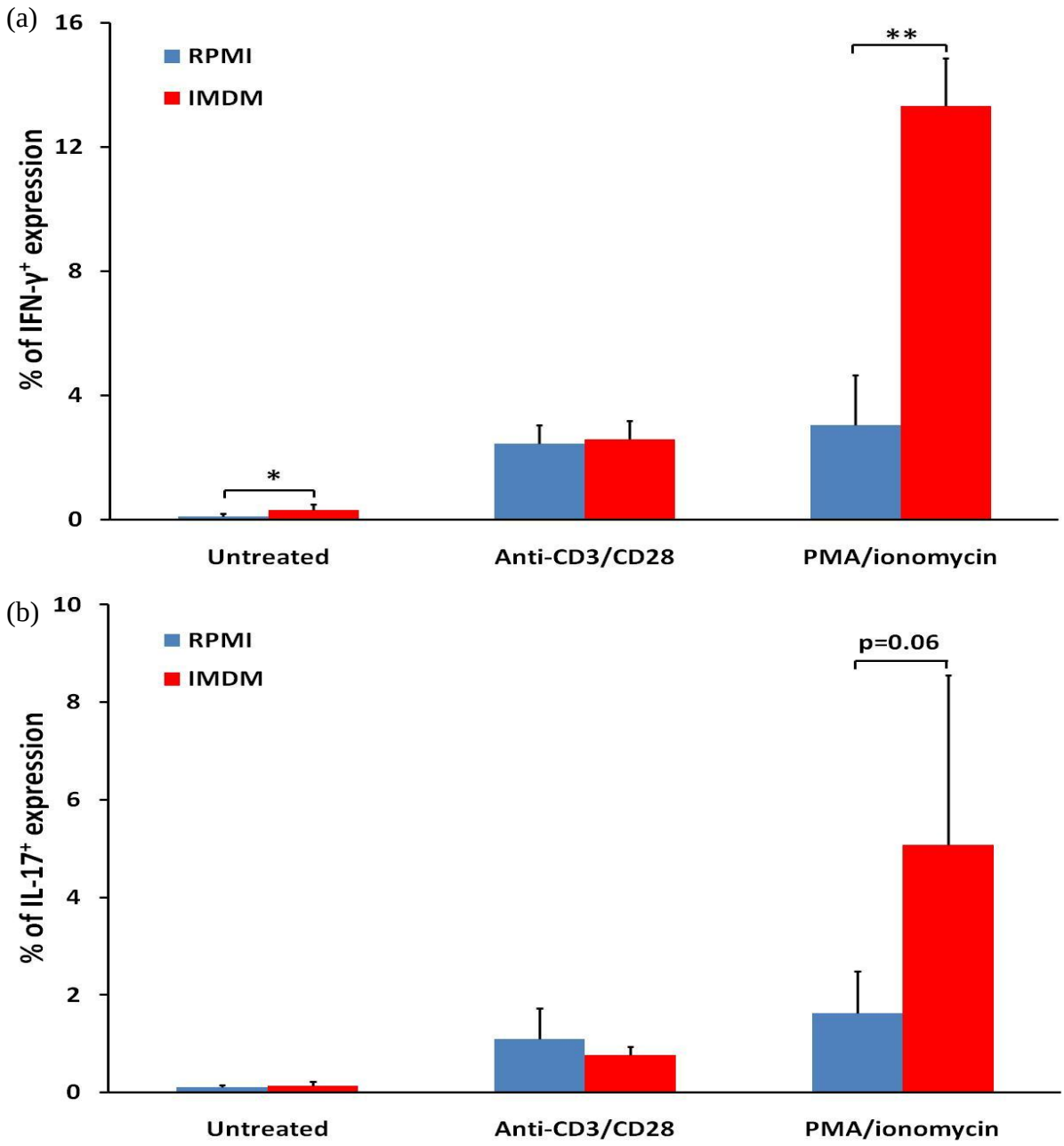


Fig 5.3: The effect of different culture media on the levels of Th1 and Th17 cell populations

Healthy donor PBMCs were either cultured in RPMI- or IMDM-based T cell medium. Cell stimulation was provided by adding anti-CD3 (5ng/ml) & anti-CD28 (1µg/ml) or PMA (25ng/ml) & ionomycin (1µg/ml) for 24 hours. Results are shown as proportion of $CD4^+$ T cells expressing cytokines. In (a), percentage of IFN-γ^+ cells and in (b), percentage of IL-17^+ cells are shown. The histograms show means ± SD from 3 independent experiments. One experiment was done in duplicates rather than triplicates and did not test cells stimulated with PMA/ionomycin.

5.3.4 Preliminary experiments to establish incubation time with polyclonal stimuli

The last of the preliminary experiments were carried out to investigate whether longer incubation with polyclonal stimuli would encourage greater expansion of Th1 and Th17 cell populations. Cultures of healthy donor PBMCs were performed over either 24 or 39 hours in these experiments.

In terms of Th1 cells, longer culture time appeared to result in decreased proportion of IFN-γ expression by $CD4^+$ T cells (Fig 5.4a). This was evident in CD3/CD28 stimulated cultures as the expression level was reduced from 23.5 ± 0.5% to 8.1 ± 0.7% ($p<0.01$) with extra 15 hours of cultures. Likewise, in cultures stimulated with PMA/ionomycin, the percentage of $CD4^+$ T cells expressing IFN-γ decreased from 66.5 ± 1.5% to 41.2 ± 10.5% ($p<0.05$).

Regarding Th17 cell population (Fig 5.4b), longer culture showed the tendency to promote higher levels of IL-17 expression in CD3/CD28 stimulated culture as the percentage of Th17 cells was raised from 1.9 ± 0.3% to 3.6 ± 0.9% (p=ns). However, in cultures stimulated with PMA/ionomycin, such increase was not evident and instead a slight reduction was observed with longer culture time as the levels of Th17 cells was lowered from 8.8 ± 1.7% to 6.6 ± 2.1% (p=ns). As there was no significant advantage detected with longer incubation time, a decision was made to perform Th1 and Th17 experiments over 24 hours.

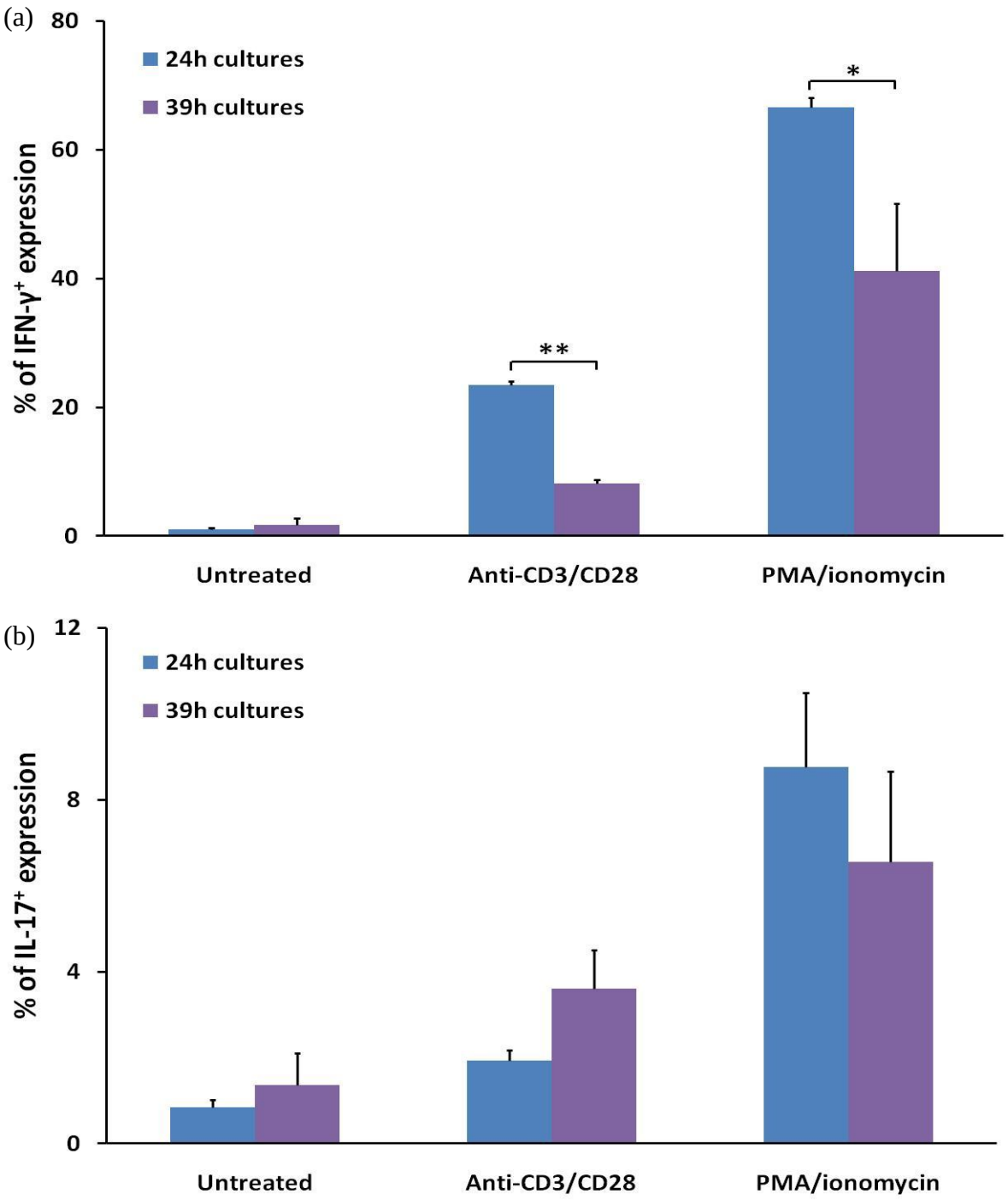


Fig 5.4: The influence of length of culture time on the levels of Th1 and Th17 cell populations

Healthy donor PBMCs were untreated, stimulated with anti-CD3 (5ng/ml) & anti-CD28 (1µg/ml) or PMA (25ng/ml) & ionomycin (1µg/ml) for 24 hours or 39 hours. Results are shown as proportion of $CD4^+$ T cells expressing cytokines. In (a), percentage of IFN-γ^+ cells and in (b), percentage of IL-17^+ cells are shown. The histograms show means ± SD of triplicate wells from one experiment. IMDM-based culture medium was used.

5.3.5 IFN-α has the tendency to promote $CD4^+$ but inhibit $CD8^+$ T cells

To verify the findings in our *ex vivo* experiments and to investigate the effects of IFN-α on T cells further, PBMCs isolated from healthy individuals were either cultured without stimulation or stimulated with anti-CD3 and anti-CD28 Ab or PMA and ionomycin for 24 hours in the presence or absence of IFN-α2a (10^1-10^5 U/ml) and IFN-α2b (10^1-10^4 U/ml) before staining and flow cytometry.

Firstly, the influence of recombinant IFN-α2a and IFN-α2b on the amount of $CD4^+$ and $CD8^+$ T cells were examined. Cytofluorimetric analysis of CD4 surface expression by lymphocytes demonstrated that the addition of IFN-α2a at higher concentrations upregulated the percentage of $CD4^+$ T cells (Fig 5.5a). With PMA/ionomycin stimulation, the proportion of $CD4^+$ T cells increased from 54.9 ± 0.4% to 55.7 ± 0.1% (means ± SEM; $p<0.05$) in the presence of 10^3 U/ml IFN-α2a. This level was further upregulated to 56.9 ± 0.2% ($p<0.05$) with 10^4 U/ml IFN-α2a. Similarly, $CD4^+$ T cell enhancement was observed with the addition of IFN-α2b to cell cultures (Fig 5.5b). With CD3/CD28 stimulation, the percentage of $CD4^+$ T cells was raised from 38.1 ± 0.4% to 39.8 ± 0.2% ($p<0.05$) in the presence of 10^2 U/ml IFN-α2b.

On the contrary, IFN-α was detected to have an inhibitory effect on $CD8^+$ T cells. In terms of IFN-α2a, a concentration of 10^4 U/ml was able to suppress the percentage of $CD8^+$ T cells from 27.9 ± 0.5% to 26.3 ± 0.2% ($p<0.05$) (Fig 5.6a). With CD3/CD28 stimulation, IFN-α2b concentrations of 10^2 to 10^4 U/ml all showed capacity to downregulate CD8 expression (Fig 5.6b). The most prominent effect was seen with 10^3 U/ml IFN-α2b as the levels of $CD8^+$ T cells were reduced from 18.1 ± 0.5% to 17.1 ± 0.2% ($p<0.05$). Likewise, IFN-α2b concentrations of 10^3 and 10^4 U/ml demonstrated capability to inhibit the proportions of $CD8^+$ T cells in cultures stimulated with PMA/ionomycin. The most effective IFN-α2b concentration was observed to be 10^4 U/ml as the levels were downregulated from 26.2 ± 0.1% to 24.8 ± 0.3% ($p<0.05$).

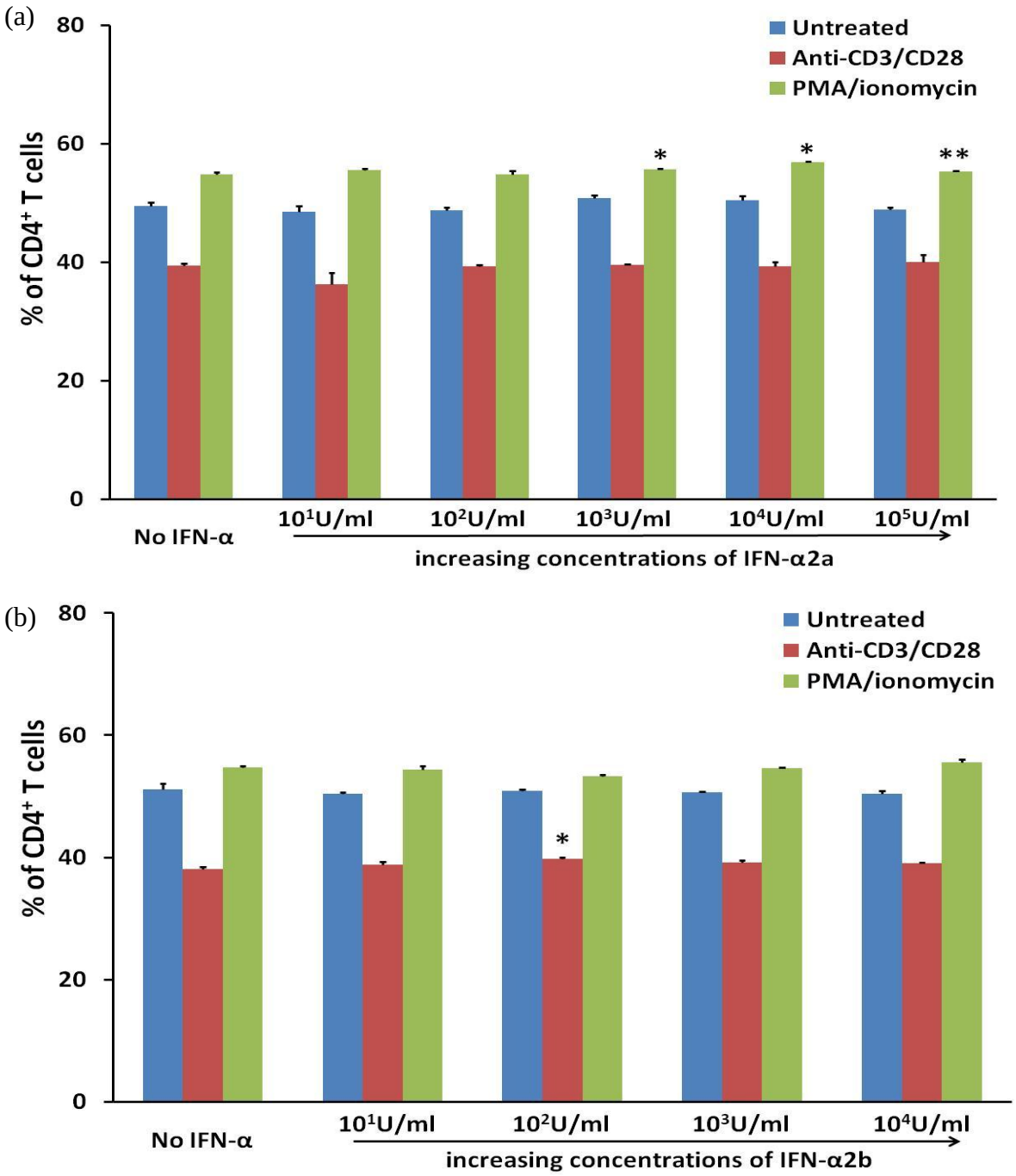


Fig 5.5: The upregulatory effect of recombinant IFN-α on CD4 expression by T cells

PBMCs from one healthy donor were untreated, stimulated with anti-CD3 & anti-CD28 or PMA & ionomycin for 24 hours. Various concentrations of exogenous IFN-α was added at the same time as the polyclonal stimuli to cell cultures. Results are shown as proportion of lymphocytes expressing CD4 surface marker. In (a) and (b), the effects of IFN-α2a and IFN-α2b are shown respectively. The histograms show means ± SEM of triplicate wells from three independent experiments. Statistical analysis was performed using the Student's T-test. *$p<0.05$, **$p<0.01$, compared with controls without IFN-α added.

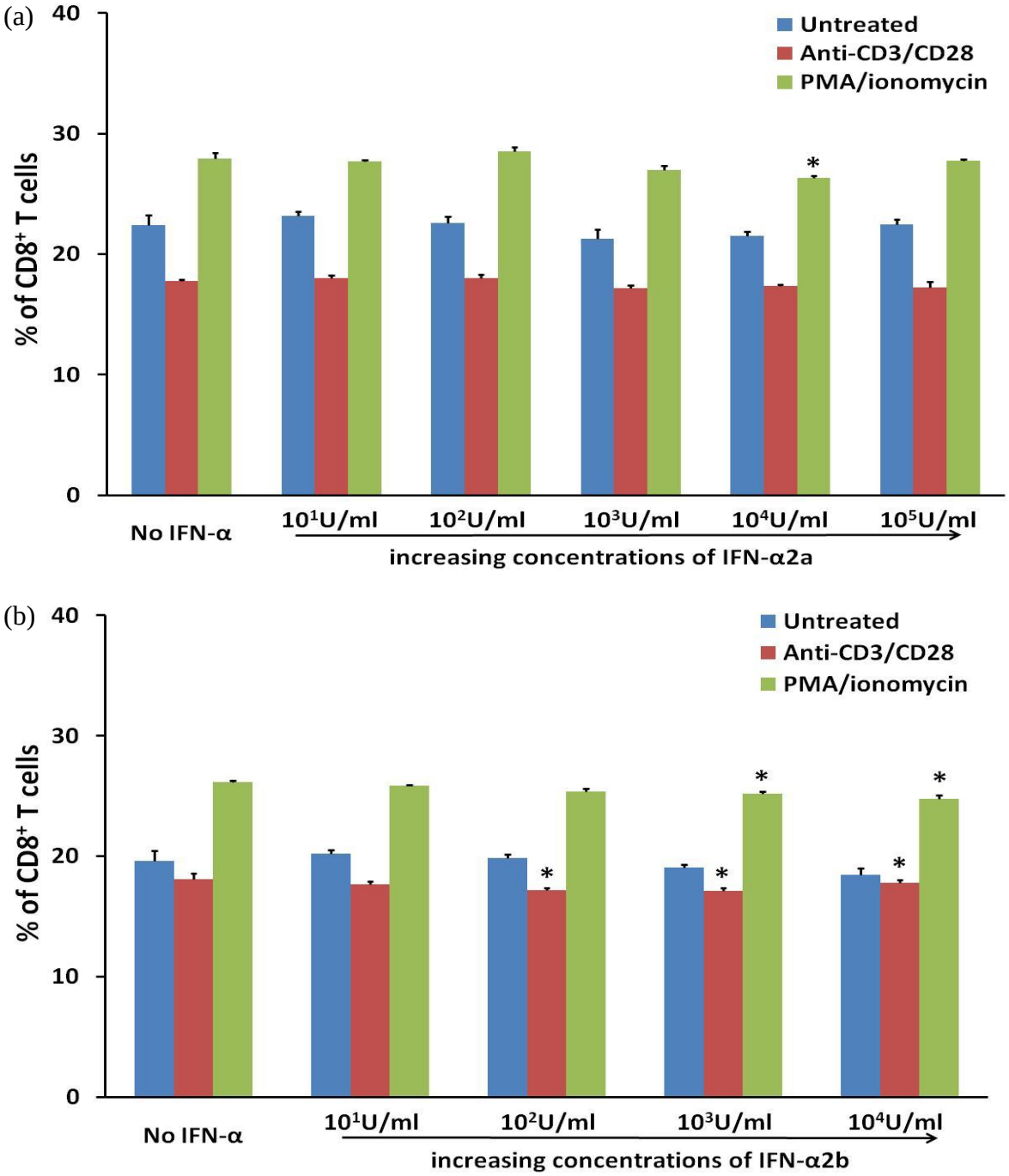


Fig 5.6: The downregulatory effect of recombinant IFN-α on CD8 expression by T cells

PBMCs from one healthy donor were untreated, stimulated with anti-CD3 & anti-CD28 or PMA & ionomycin for 24 hours. Various concentrations of exogenous IFN-α was added at the same time as the polyclonal stimuli to cell cultures. Results are shown as proportion of lymphocytes expressing CD8 surface marker. In (a) and (b), the effects of IFN-α2a and IFN-α2b are shown respectively. The histograms show means ± SEM of triplicate wells from three independent experiments. Statistical analysis was performed using the Student's T-test. *$p<0.05$, compared with controls without IFN-α added.

5.3.6 IFN-α induces IFN-γ production by both $CD4^+$ and $CD8^+$ T cells

Results from Chapter 3 showed that Th1 response can be promoted by 6 months of subcutaneous IFN-α2b therapy. In order to verify this finding, PBMCs isolated from healthy individuals were cultured without stimulation or stimulated with anti-CD3 and anti-CD28 Ab or PMA and ionomycin in the presence or absence of IFN-α2a (10^1-10^5 U/ml) and IFN-α2b (10^1-10^4 U/ml) before intracellular cytokine staining and flow cytometry. Cytokine expression was analysed by gating on either $CD4^+$/$CD3^+CD8^-$ or $CD8^+$ lymphocyte populations to study the ability of IFN-α to induce IFN-γ expression in $CD4^+$ and $CD8^+$ T cells *in vitro*.

The data revealed that Th1 response was consistently promoted by the presence of IFN-α in cultures, especially with higher concentrations. In untreated cultures, the proportion of $CD4^+$ T cells producing IFN-γ was upregulated with 10^5 U/ml of IFN-α2a from 1.4 ± 0.4% to 2.3 ± 0.3% (means ± SD; $p<0.05$) (Fig 5.7a). Similarly, IFN-α2a concentrations of 10^3 and 10^4 U/ml enhanced Th1 cell percentage from 13.5 ± 0.4% to 17.5 ± 0.9% ($p<0.01$) and 18.1 ± 0.7 ($p<0.01$) respectively with CD3/CD28 stimulation. With PMA/ionomycin stimulation, a dose-dependent, upregulating effect of IFN-α2a on Th1 cells was detected. The peak response was seen with a IFN-α2a concentration of 10^5 U/ml, and the proportion of $CD4^+$ T cells producing IFN-γ was raised from a level of 58.8 ± 0.7% to 63.9 ± 0.8% ($p<0.01$). In correspondence, addition of IFN-α2b with concentrations of 10^3 and 10^4 U/ml to cultures increased Th1 cell percentage from 54.5 ± 1.0% to 57.7 ± 0.5% ($p<0.01$) and 60.3 ± 0.6 ($p<0.01$) respectively with PMA/ionomycin stimulation (Fig 5.7b).

The data also showed that $CD8^+$ T cells producing IFN-γ were induced by exogenous IFN-α. In CD3/CD28 stimulated cultures, the proportion of IFN-γ producing $CD8^+$ T cells were elevated in a dose-dependent manner with IFN-α2a (Fig 5.8a). In particular, a IFN-α2a concentration of 10^4 U/ml was demonstrated to increase IFN-γ producing $CD8^+$ T cells from 12.1 ± 0.8% to 20.1 ± 1.1% ($p<0.01$). Similar trend was observed in untreated cultures and cultures activated with PMA/ionomycin as well, but in both cases, the highest IFN-α2a concentration tested of 10^5 U/ml was most the most potent. In agreement, IFN-α2b was found to be equally effective in inducing $CD8^+$ T cells producing IFN-γ, especially with a IFN-α2b

concentration of 10^4 U/ml (Fig 5.8b). With addition of 10^4 U/ml IFN-α2b, the proportion of IFN-γ producing $CD8^+$ T cells was upregulated from 0.9 ± 0.1% to 2.1 ± 0.5% ($p<0.05$) in untreated cultures, from 13.9 ± 1.0% to 20.8 ± 0.5% ($p<0.01$) in CD3/CD28 stimulated cultures, and from 64.7 ± 1.1% to 69.8 ± 1.0% ($p<0.01$) in cultures stimulated with PMA/ionomycin.

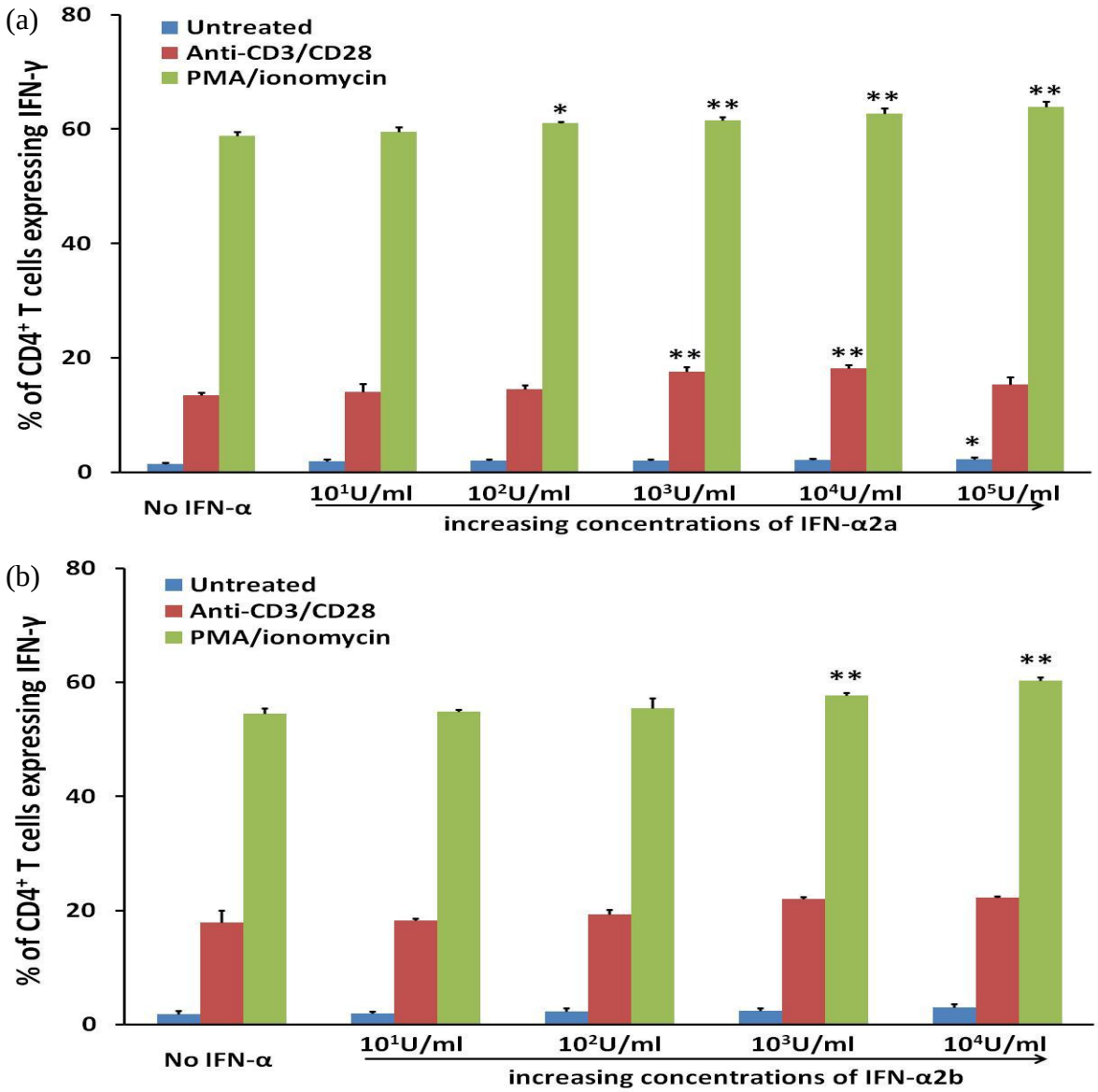


Fig 5.7: The upregulatory effect of recombinant IFN-α on Th1 response

Healthy donor PBMCs were untreated, stimulated with anti-CD3 & anti-CD28 or PMA & ionomycin for 24 hours. Various concentrations of IFN-α was added at the same time as the polyclonal stimuli to cultures. Results are shown as proportion of $CD4^+$ T cells expressing IFN-γ. In (a) and (b), the effects of IFN-α2a and IFN-α2b are shown respectively. Results in (a) and (b) are representative of 3 independent experiments each. PBMC were from three different donors for experiments on both forms of IFN-α. One experiment each did not use CD3/CD28 stimulation and was incubated over 39 hours, and 10^1 U/ml of IFN-α (2a and 2b) and 10^5 U/ml of IFN-α2a were not tested in one experiment. Data are means ± SD values of triplicate wells. Statistical analysis was performed using the Student's T-test. *p<0.05, **p<0.01, compared with controls without IFN-α added.

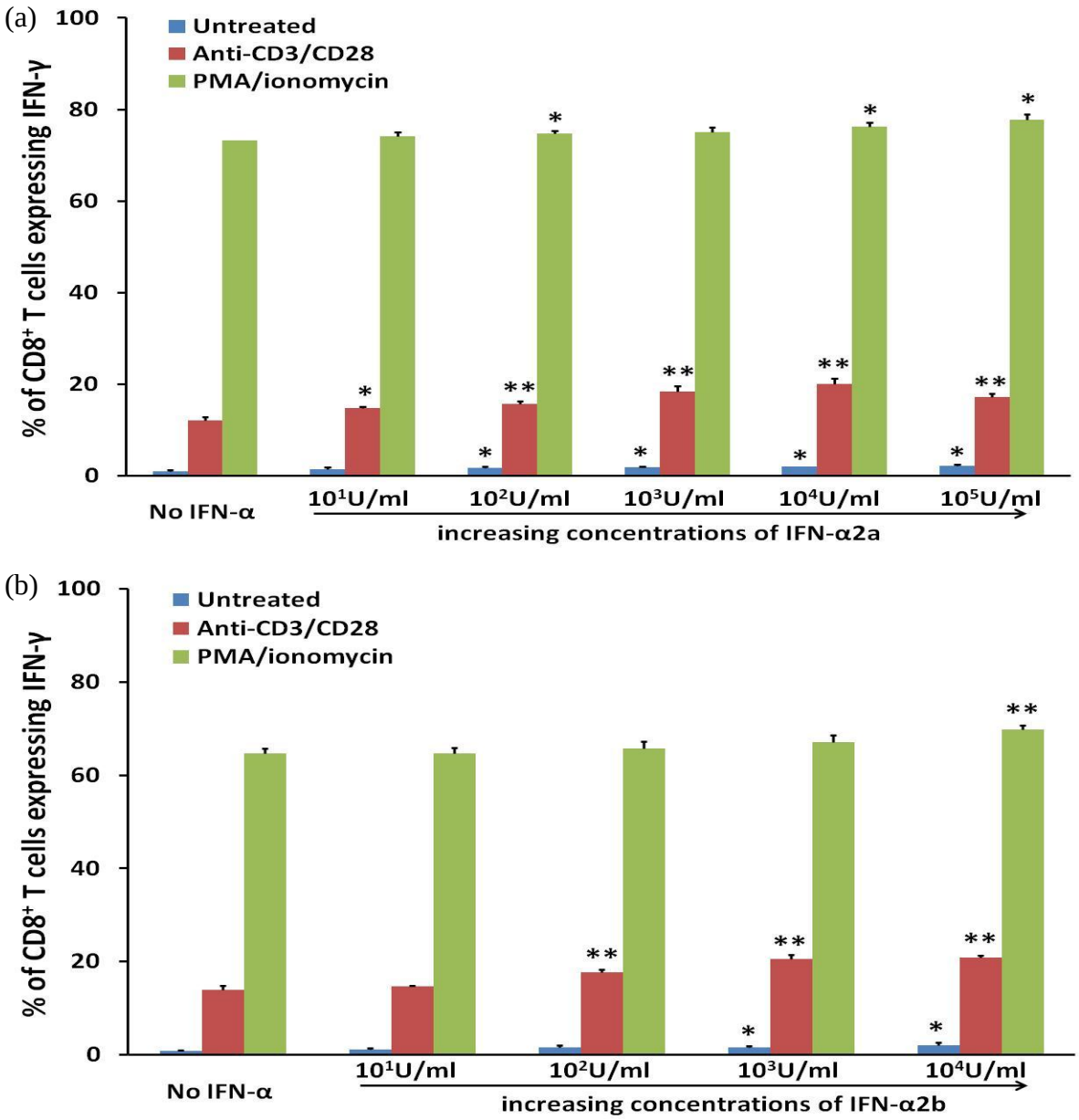


Fig 5.8: The enhancing effect of recombinant IFN-α on $CD8^+$ T cells expressing IFN-γ

Healthy donor PBMCs were untreated, stimulated with anti-CD3 & anti-CD28 or PMA & ionomycin for 24 hours. Various concentrations of IFN-α was added at the same time as the polyclonal stimuli to cultures. Results are shown as proportion of $CD8^+$ T cells expressing IFN-γ. In (a) and (b), the effects of IFN-α2a and IFN-α2b are shown respectively. Results in (a) and (b) are representative of two (a) and three (b) independent experiments respectively, and each experiment for IFN-α2a and IFN-α2b used PBMCs from a different donor. CD3/CD28 stimulation was used in only one experiment for both IFN-α2a and IFN-α2b. 10^1 U/ml of IFN-α (2a and 2b) and 10^5 U/ml of IFN-α2a were tested once in experiments. Data are means ± SD values of triplicate wells. Statistical analysis was performed using the Student's T-test. *$p<0.05$, **$p<0.01$, compared with controls without IFN-α added.

5.3.7 The kinetics of the upregulatory effect of IFN-α on IFN-γ production by T cells

Although IFN-α has been observed to induce an upregulation in IFN-γ production by both $CD4^+$ and $CD8^+$ T cells *in vitro*, it is of interest to investigate whether pre-treatment of PBMCs with IFN-α can exert greater influence when compared to adding IFN-α at the same time as cell activating agents. Also, another question is whether IFN-α can still exert its impact once PBMCs are activated. To aid in answering these questions, PBMCs isolated from healthy individuals were cultured without IFN-α treatment or with 10^4 U/ml IFN-α2a added 24 hours before, 4 hours before, at the same time as, or 4 hours after PMA/ionomycin stimulation. The timing for the introduction of IFN-α to cultures was the same for both unstimulated and stimulated cultures, and PBMCs were incubated for 24 hours after PMA/ionomycin stimulation before intracellular cytokine staining and flow cytometry. IFN-γ expression levels were analysed by gating on either $CD4^+$/$CD3^+CD8^-$ or $CD8^+$ lymphocyte populations.

Surprisingly, it was found that pre-treatment of PBMCs with IFN-α showed the least amount of upregulating potential. In PMA/ionomycin stimulated cultures, IFN-α pre-treatment did not significantly alter the proportion of either $CD4^+$ (Fig 5.9a) or $CD8^+$ (Fig 5.9b) T cells producing IFN-γ when evaluated against controls without IFN-α treatment. In fact, for both $CD4^+$ and $CD8^+$ (Fig 5.9b) T cells, the augmenting effect of IFN-α on IFN-γ levels was strongest when IFN-α was introduced 4 hours after PMA/ionomycin stimulation.

With regards to $CD4^+$ T cells, the proportion of cells producing IFN-γ was appreciably raised from 57.1 ± 2.0% (means ± SEM) without IFN-α treatment to 64.3 ± 0.8% ($p<0.01$) when IFN-α was added 4 hours post PMA/ionomycin activation (Fig 5.9a). This elevation of IFN-γ was statistically significant even when compared to data influenced by 24 hours (56.4 ± 1.6%; $p<0.01$) and 4 hours (59.8 ± 1.8%; $p<0.05$) of IFN-α pre-treatment before polyclonal activation.

Similarly, concerning $CD8^+$ T cells, the percentage of cells producing IFN-γ was considerably elevated from 75.4 ± 2.7% without IFN-α to 82.2 ± 1.2% ($p<0.05$) when

IFN-α was added 4 hours post PMA/ionomycin activation (Fig 5.9b). This increase in IFN-γ was still significant when evaluated against data collected from cultures with 24 hours (74.3 ± 2.7%; $p<0.05$) of IFN-α pre-treatment before polyclonal activation.

These trends were detected in untreated cultures as well, among both $CD4^+$ and $CD8^+$ T cells.

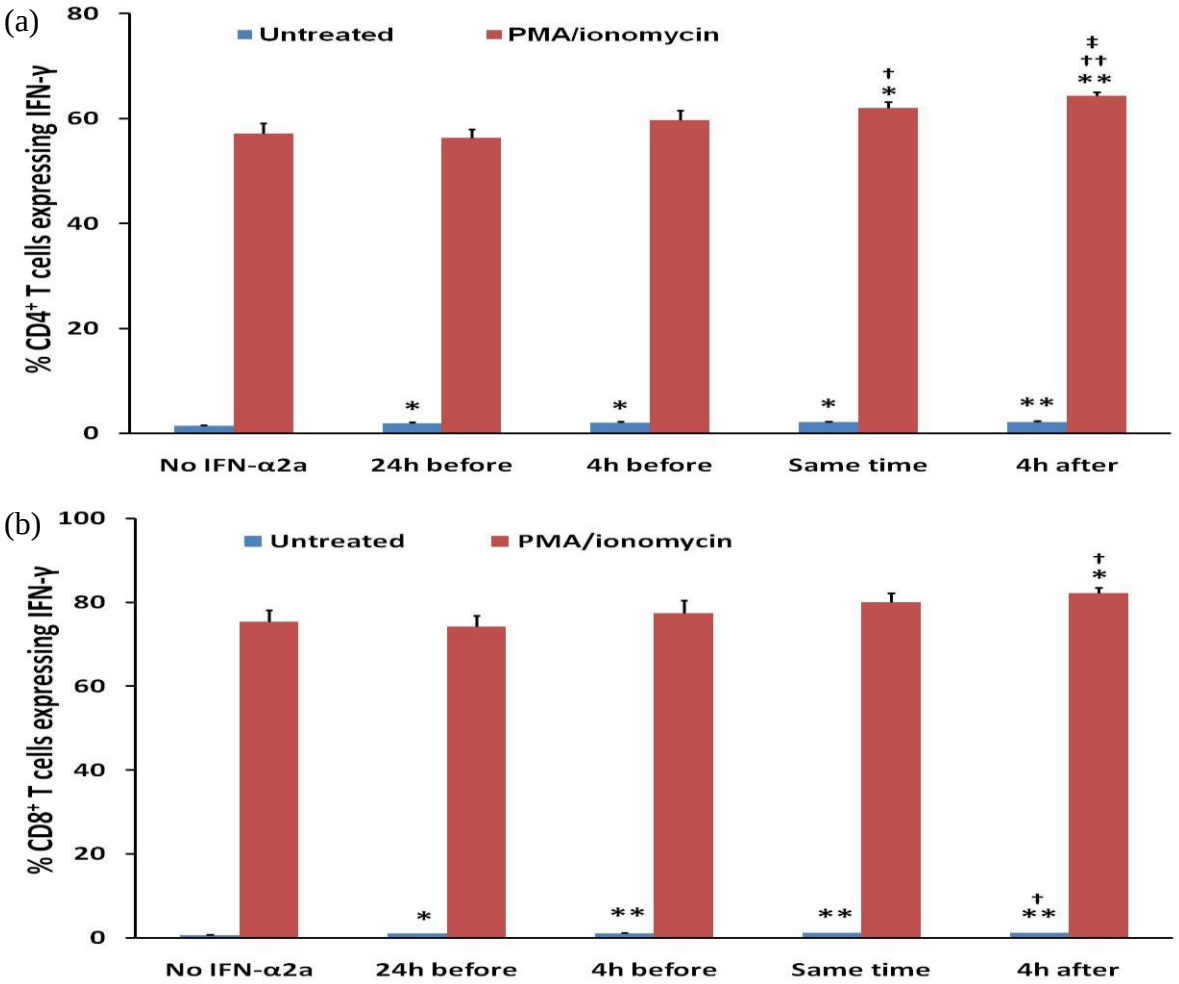


Fig 5.9: The kinetics of upregulatory effect of IFN-α on T cells expressing IFN-γ

Healthy donor PBMCs were either treated without or with 10^4 U/ml IFN-α2a 24 hours before, 4 hours before, at the same time as, or 4 hours after introduction of PMA & ionomycin to cultures. The timing for the addition of IFN-α2a to cultures was the same for both unstimulated and stimulated cultures, and PBMCs were incubated for 24 hours after PMA/ionomycin stimulation before staining. Results are shown as proportion of (a) $CD4^+$ T cells and (b) $CD8^+$ T cells expressing IFN-γ. The histograms show means ± SEM of triplicate wells from three independent experiments. All experiments used PBMCs from the same donor. Statistical analysis was performed using the Student's T-test. *p<0.05, **p<0.01, compared with controls without IFN-α added. †p<0.05, ††p<0.01, compared with data with 24-hour IFN-α pre-treatment. ‡p<0.05, compared with data with 4-hour IFN-α pre-treatment.

5.3.8 IFN-α inhibits IL-17 production by both $CD4^+$ and $CD8^+$ T cells

Results from Chapter 4 revealed that 6 months of subcutaneous IFN-α2b therapy were able to promote a downregulation of Th17 response. Consequently, experiments were set up to test the influence of IFN-α on IL-17 producing $CD4^+$ T cells as well as $CD8^+$ T cells *in vitro*. Again, PBMCs isolated from healthy individuals were either cultured without stimulation or stimulated with anti-CD3 and anti-CD28 Ab or PMA and ionomycin in the presence or absence of IFN-α2a (10^1-10^5 U/ml) and IFN-α2b (10^1-10^4 U/ml) before intracellular cytokine staining and flow cytometry. Cytokine expression was analysed by gating on either $CD4^+$/$CD3^+CD8^-$ or $CD8^+$ lymphocyte populations.

As expected, data showed that Th17 response was inhibited by IFN-α consistently *in vitro* in accordance with the BD patient findings, especially with higher concentrations of IFN-α. With PMA/ionomycin activation, 10^5 U/ml of IFN-α2a and 10^4 U/ml of IFN-α2b induced a downregulation of IL-17 producing $CD4^+$ T cells from 2.1 ± 0.2% (means ± SD) to 1.5 ± 0.2% ($p<0.01$) and 1.2 ± 0.1% ($p<0.01$) respectively (Fig 5.10a and 5.10b). Statistically significant result was evident in CD3/CD28 stimulated cultures as well, but only with IFN-α2b concentration of 10^4 U/ml (Fig 5.10b).

Likewise, IFN-α was observed to exert negative control on $CD8^+$ T cells producing IL-17 *in vitro*. In CD3/CD28 stimulated cultures, the proportion of IL-17 producing $CD8^+$ T cells were suppressed in a dose-dependent manner with IFN-α2b (Fig 5.11b). In particular, a IFN-α2b concentration of 10^4 U/ml was able to reduce IL-17 expressing $CD8^+$ T cells from 0.9 ± 0.1% to 0.5 ± 0.1% ($p<0.05$). In addition, similar trend was observed in cultures stimulated with PMA/ionomycin. The highest IFN-α concentrations used in the *in vitro* experiments were able to promote a downregulation in the percentage of $CD8^+$ T cells producing IL-17 from 0.9 ± 0.1% to 0.6 ± 0.1% ($p<0.05$) in the case of IFN-α2a (Fig 5.11a) and from 0.8 ± 0.1% to 0.4 ± 0.0% ($p<0.05$) in the case of IFN-α2b (Fig 5.11b).

Although $CD4^+$ and $CD8^+$ T cells producing both IFN-γ and IL-17 were detected, they were present in negligible amount and IFN-α2a and IFN-α2b did not have any significant effects on these subpopulations (data not shown).

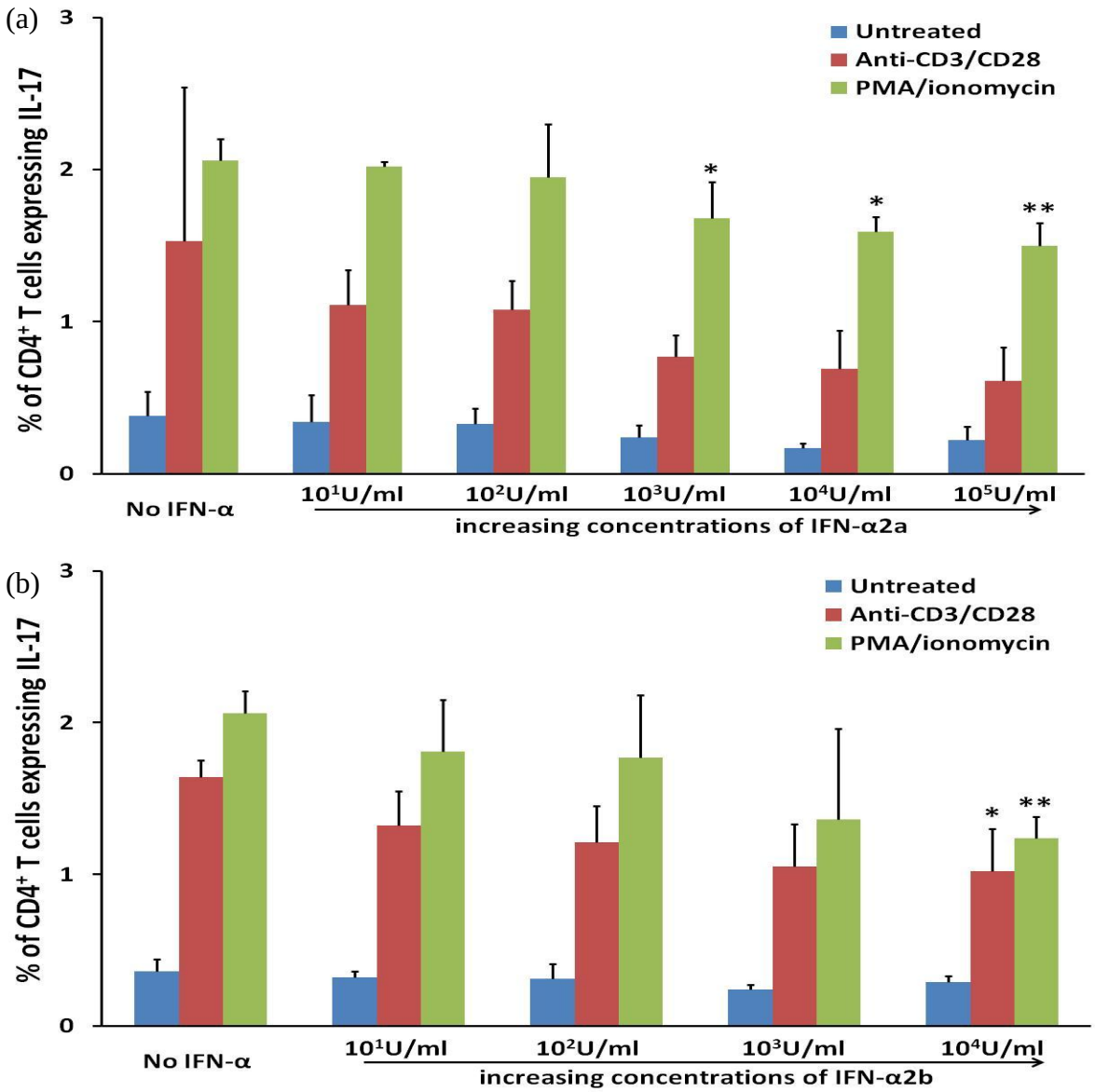


Fig 5.10: The inhibitory effect of recombinant IFN-α on Th17 cell response

Healthy donor PBMCs were untreated, stimulated with anti-CD3 & anti-CD28 or PMA & ionomycin for 24 hours. Various concentrations of IFN-α was added at the same time as the polyclonal stimuli to cultures. Results are shown as proportion of $CD4^+$ T cells expressing IL-17. In (a) and (b), the effects of IFN-α2a and IFN-α2b are shown respectively. Results in (a) and (b) are representative of four independent experiments each. PBMCs were from three and four different donors for IFN-α2a and IFN-α2b respectively. CD3/CD28 stimulation, 10^1 U/ml of IFN-α (2a and 2b) and 10^5 U/ml of IFN-α2a were used in half of the experiments. Also, in one experiment each for both IFN-α2a and IFN-α2b, PBMC cultures were incubated over 39 instead of 24 hours. Data are means ± SD values of triplicate wells. Statistical analysis was performed using the Student's T-test. *p<0.05, **p<0.01, compared with controls without IFN-α added.

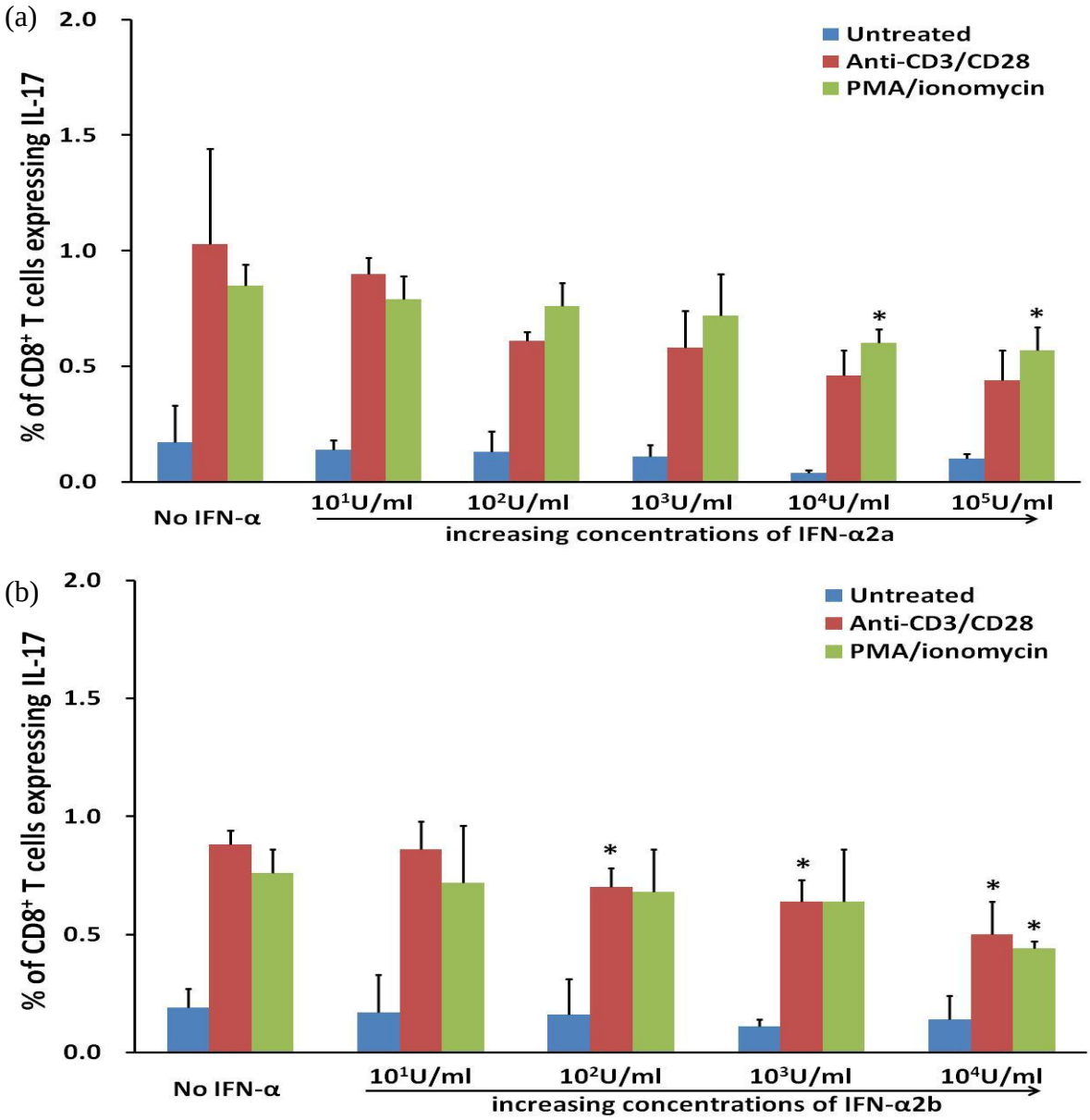


Fig 5.11: The inhibitory effect of recombinant IFN-α on $CD8^+$ T cells expressing IL-17

Healthy donor PBMCs were untreated, stimulated with anti-CD3 & anti-CD28 or PMA & ionomycin for 24 hours. Various concentrations of IFN-α was added at the same time as the polyclonal stimuli to cultures. Results are shown as proportion of $CD8^+$ T cells expressing IL-17. In (a) and (b), the effects of IFN-α2a and IFN-α2b are shown respectively. Results in (a) and (b) are representative of two (a) and three (b) independent experiments respectively, and each experiment for IFN-α2a and IFN-α2b used PBMCs from a different donor. CD3/CD28 stimulation was used in only one experiment for either IFN-α2a or IFN-α2b. 10^1 U/ml of IFN-α (2a and 2b) and 10^5 U/ml of IFN-α2a were tested once in experiments. Data are means ± SD values of triplicate wells. Statistical analysis was performed using the Student's T-test. *$p<0.05$, **$p<0.01$, compared with controls without IFN-α added.

5.3.9 The kinetics of the inhibitory effect of IFN-α on IL-17 production by T cells

Even though IFN-α has been identified to promote an inhibition of IL-17 production by both $CD4^+$ and $CD8^+$ T cells *in vitro*, it is of interest to examine whether pre-treatment of PBMCs with IFN-α can exert stronger effect when compared to adding IFN-α and activating stimuli concurrently. Another question to resolve is whether IFN-α can still exert its influence once PBMCs are activated. To answer these questions, PBMCs isolated from healthy individuals were cultured without IFN-α treatment or with 10^4 U/ml IFN-α2a added 24 hours before, 4 hours before, at the same time as, or 4 hours after PMA/ionomycin stimulation. The timing for IFN-α addition to cultures was the same for both unstimulated and stimulated cultures, and PBMCs was incubated for 24 hours after PMA/ionomycin stimulation before intracellular cytokine staining and flow cytometry. IL-17 expression levels were analysed by gating on either $CD4^+$/$CD3^+CD8^-$ or $CD8^+$ lymphocyte populations.

Although pre-treatment of PBMCs with IFN-α showed a suppression of IL-17 production levels within both $CD4^+$ (Fig 5.12a) and $CD8^+$ (Fig 5.12b) T cell populations, the effect was stronger when IFN-α was added around the same time as the introduction of polyclonal stimulation by PMA/ionomycin. More specifically, the maximum outcome was consistently achieved in both $CD4^+$ and $CD8^+$ T cells with simultaneous introduction of IFN-α and PMA/ionomycin to PBMC cultures.

When 10^4 U/ml IFN-α2a and activating agents were added together, the proportion of $CD4^+$ T cells producing IL-17 was downregulated considerably to 1.0 ± 0.1% (means ± SEM; $p<0.01$) from a level of 1.8 ± 0.2% in the absence of IFN-α (Fig 5.12a). Also, this inhibitory effect was significantly greater than one with 24-hour IFN-α pre-treatment (1.3 ± 0.1%; $p<0.01$).

Similarly, concerning $CD8^+$ T cells, the percentage of cells producing IL-17 was significantly reduced from 0.8 ± 0.2% without IFN-α to 0.3 ± 0.1% ($p<0.05$) with concurrent use of IFN-α and PMA/ionomycin (Fig 5.12b). This suppressive effect was significantly greater than when IFN-α was added 24 hours before polyclonal stimulation (0.5 ± 0.1%; $p<0.05$).

Moreover, in untreated cultures, the addition of IFN-α inhibited IL-17 producing $CD4^+$ and $CD8^+$ T cells, but there was no appreciable difference adding IFN-α early or later during culture.

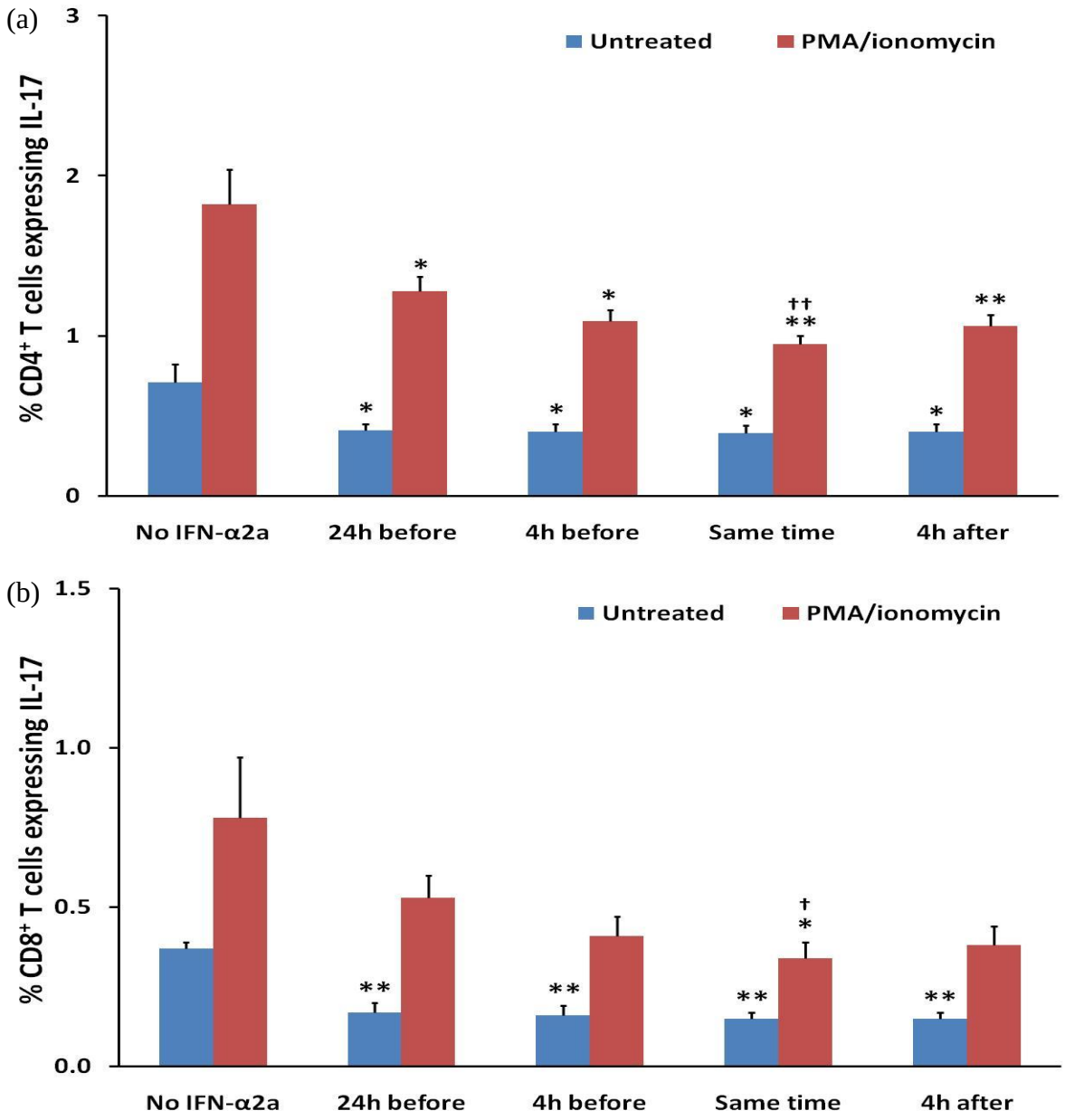


Fig 5.12: The kinetics of inhibitory effect of IFN-α on T cells expressing IL-17

Healthy donor PBMCs were either treated without or with 10^4 U/ml IFN-α2a 24 hours before, 4 hours before, at the same time as, or 4 hours after introduction of PMA & ionomycin to cultures. The timing for the addition of IFN-α2a to cultures was the same for both unstimulated and stimulated cultures, and PBMCs were incubated for 24 hours after PMA/ionomycin stimulation before staining. Results are shown as proportion of (a) $CD4^+$ T cells and (b) $CD8^+$ T cells expressing IL-17. The histograms show means ± SEM of triplicate wells from three independent experiments. All experiments used PBMCs from the same donor. Statistical analysis was performed using the Student's T-test. *p<0.05, **p<0.01, compared with controls without IFN-α added. †p<0.05, ††p<0.01, compared with data with 24-hour IFN-α pre-treatment.

5.4 Discussion

5.4.1.1 Setting up Th1/Th17 *in vitro* test models using antigens and polyclonal stimuli

It has been shown that cytokines present upon T cell activation can shape the differentiation of T cells. However, there are other variables that are known to affect the development of T cell subsets which include the affinity of the TCR for Ag, the duration of the interaction between TCR and Ag, and differential co-stimulation by APCs (Zhu, Yamane, & Paul 2010). In our *in vitro* experiments, selected antigens and polyclonal stimuli were used to induce expansion of Th1 and in particular, Th17 cells in healthy donor PBMCs in order to set up test models for subsequent investigation of the immunomodulatory effects of exogenous IFN-α on these two cell populations. Analysing our data on healthy donor PBMCs, all antigens promoted very low production levels of IL-17 and IFN-γ in $CD4^+$ T cells, even with extra time in culture.

Although LPS, zymosan and candida were previously reported to possess IL-17 inducing activity (Acosta-Rodriguez et al. 2007;Gerosa et al. 2008; Kattah et al. 2008), LPS was the only one which demonstrated some potential. However, even the levels of Th17 cells achieved with LPS were less than one percent of lymphocytes. Also, the commercial candida antigens used in the experiments were mainly made up of components from the yeast form rather the hyphal form, so it is not surprising to induce little Th17 response. ATRA, as expected, resulted in lowest amount of Th17 and this is in accordance to previous report of its ability to inhibit Th17 cell differentiation (Elias et al. 2008;Mucida et al. 2007).

Consequently, two of the BD-related antigens, PDS-Ag and alpha-tropomyosin, were used as a trial but they did not elicit much Th17 response by PBMCs from BD patients either. Thus it was decided to conserve valuable BD patient samples and not to test any other BD-related antigens such as HSP60.

On the other hand, polyclonal stimuli consistently showed good potential to promote Th17 cell populations out of all combinations used, especially in the case of PMA in

combination with ionomycin. Polyclonal stimuli are quite efficient in inducing Th1 cell populations as well. PMA is a mitogen which binds and activates protein kinase C, causing a wide range of effects in cells and tissues (Castagna et al. 1982), and ionomycin is a potent and selective calcium ionophore agent. In human T cells, ionomycin induces hydrolysis of phosphoinositides and synergises with PMA in enhancing the activation of protein kinase C to mediate T cell activation (Chatila et al. 1989).

However, phorbol esters induce CD4 phosphorylation and a consequence of this is enhancement of CD4 internalisation and subsequent degradation of membrane CD4 in lysosomes (Petersen et al. 1992). As a result of this CD4 deficiency, substantial amount of the experiment results which involved PMA/ionomycin stimulation and analysed by gating on CD4 was found to be inconsistent. Therefore, a solution of using CD3 and CD8 instead of CD4 to stain for $CD4^+$ T cells was adopted in later experiments to resolve the problem of PMA-induced CD4 internalisation. $CD3^+CD8^-$ T cells were then considered as equivalent to $CD4^+$ T cells.

5.4.1.2 Testing other aspects of the *in vitro* experimental design

It has been suggested resting PBMCs overnight after thawing before testing effector functions would remove cells destined for death and allow “stunned” cells to recover functional ability (Lamoreaux, Roederer, & Koup 2006). Therefore, instead of stimulating cells straight after thawing, one day resting in culture medium with or without suboptimal level of IL-2 was performed. It was observed that neither resting nor addition of IL-2 augmented the number of Th1 or Th17 cells consistently. This is in agreement with a recent study which showed overnight resting did not improve the $CD4^+$ or $CD8^+$ T cell IFN-γ responses to antigens (Owen et al. 2007). It was thus concluded that it was not necessary to allow time for PBMCs to recover before stimulation.

Concerning the choice of culture medium, it was shown recently that IMDM, a medium richer in aromatic amino acids, consistently resulted in higher Th17 expansion (Veldhoen et al. 2009). In line with the finding, IMDM did display a

tendency to preferentially upregulate Th17 cells as well as enhancing Th1 response when PMA/ionomycin stimulation was applied to cultures of healthy donor PBMC in our experiments, although there was no significant difference between RPMI- and IMDM-based media in untreated and CD3/CD28 stimulated cultures. Consequently, Th17 cell experiments were carried out using IMDM-based culture medium, except some earlier experiments which were performed using RPMI-based culture medium.

Variation of duration of cultures was experimented as well and PBMC cultures were performed over either 24 or 39 hours to examine whether higher proportions of Th1 and Th17 cells could be achieved with longer culture time. In the end, it was decided to perform experiments over 24 hours as there was no advantage detected with extra time in culture with regards to enhancing either IFN-γ or IL-17 production levels by $CD4^+$ T cells.

5.4.2 Choosing the concentrations of IFN-α used for *in vitro* experiments

In our BD patient trial, pegylated IFN-α2b was given according to a dosage table (table 2.1). As the majority of our patients weighed between 50-100kg, 30μg of pegylated IFN-α2b per week was received by most patients. The specific activity of pegylated IFN-α2b is approximately 7.0×10^7 IU/mg protein, compared with 2.6×10^8 IU/mg protein for standard IFN-α2b. As a result, each patient received an equivalent of approximately 2.1 million units every week for 6 months (Schering-Plough 2007). The actual tissue concentrations of IFN-α2b may be difficult to assess following *in vivo* administration, and further studies are required to attain the approximation for pegylated IFN-α2b. However, a dose of $1\text{-}10 \times 10^6$ IU unconjugated IFN-α *in vivo* has been estimated to correlate with a concentration of 100 IU/ml at the tissue level (Krishnaswamy et al. 1996).

It was reported that IFN-α promoted cell-cycle arrest in $CD4^+$ T cells and the inhibitory effect of IFN-α on cell proliferation reached a plateau between concentrations of 10^3 and 10^4 U/ml. Of note, inhibition of proliferation could be associated with a decrease in the production of inflammatory cytokines from cells. Additionally, cell viability was shown to be maintained in PBMCs and T cell clones at

10^3 U/ml of IFN-α (Krishnaswamy et al. 1996). Nevertheless, it is currently unclear whether the anti-proliferative effect of IFN-α is more effective on certain subsets such as Th17 cells than the other ones. Due to these early findings, a concentration of 1000 U/ml of IFN-α was picked as the median concentration for our *in vitro* studies using healthy donor PBMCs.

5.4.3.1 Biology of $CD8^+$ T cells

$CD8^+$ T cells represent an important arm of adaptive immunity and they play a role in the regulation of the activation and differentiation of $CD4^+$ cells (Vukmanovic-Stejic et al. 2000). Notably, dysregulated $CD8^+$ T cells have been implicated in the pathogenesis of various autoimmune diseases (Liblau et al. 2002). $CD8^+$ T cells exert their effector functions through production of inflammatory cytokines such as IFN-γ, and induction of cytotoxic damage of target cells expressing MHC class I molecules and the relevant antigenic peptide via perforin/granzyme and death receptor pathways (Li et al. 2006). As MHC class I molecules are found in every nucleated cells of the body, it is clear that there is potential for considerable amount of tissue damage.

Compares with $CD4^+$ T cells, naive $CD8^+$ T cells acquire effector T cell functions more readily upon antigen stimulation (Seder & Ahmed 2003). Activated $CD8^+$ T cells can produce very high levels of inflammatory cytokines, including TNF-α and IFN-γ (Liblau et al. 2002). All these may contribute greatly to target cell destruction in autoimmune diseases and therefore stringent regulation is essential for $CD8^+$ T cells.

5.4.3.2 Influence of IFN-α on peripheral blood $CD4^+$ and $CD8^+$ T lymphocytes

From our *ex vivo* experiments in Chapter 3, there was no significant alteration of the expression levels of CD4 or CD8 by T cells from BD patients whether they received IFN-α therapy or not. Unlike our *ex vivo* results, there was a tendency for $CD4^+$ T cell upregulation but $CD8^+$ T cell downregulation by addition of either IFN-α2a or

IFN-α2b to healthy donor PBMC cultures in a dose-dependent manner, and IFN-α concentrations of 10^3 or 10^4 U/ml were generally found to be most powerful. The difference could be related to how peripheral blood cells were treated as the *in vitro* cultures were activated with polyclonal stimulation and incubated for 24 hours.

Our results are in part supported by a previous study on patients with severe steroid-resistant asthma. It was described that IFN-α treatment promoted a relative increase in $CD4^+$ T cells but a downregulation of $CD8^+$ T cells was not reported (Simon et al. 2003). In the study, $3x10^6$ IU/day of recombinant IFN-a2a was administered on each patient and systemic corticosteroid treatment was maintained until a clinical improvement was seen and then gradually reduced. An upregulation of $CD4^+$ T cells was observed from 44.2 ± 2.8% within the lymphocyte populations before introduction of IFN-α therapy to 50.2 ± 2.6% two to four weeks after initiation of cytokine therapy.

5.4.4.1 The influence of IFN-α on IFN-γ production by $CD4^+$ T cells

IFN-α was reported to stimulate the production of IFN-γ by resting $CD4^+$ T cells activated by PMA and anti-CD28 in a dose-dependent manner and IFN-α concentration of 10^4 IU/ml was found to be most stimulating (Schandene et al. 1997). Using freshly isolated human cells, it was revealed that IFN-α increased the expression of mRNA for IFN-γ and as a result the frequency of IFN-γ-producing $CD4^+$ T cells within 24 hours of culture (Brinkmann et al. 1993). Also, another study demonstrated that IFN-α augmented IL-12 priming for subsequent IFN-γ production and the generation of Th1 responses (Wenner et al. 1996). These results are in line with our experimental findings that both IFN-α2a and IFN-α2b were proficient in inducing IFN-γ production by $CD4^+$ T cells, especially at higher concentrations.

5.4.4.2 The influence of IFN-α on IFN-γ production by $CD8^+$ T cells

Analysing our data, it was found that $CD8^+$ T cells producing IFN-γ were upregulated by the introduction of IFN-α. This is in contrast to an early study which did not detect

any positive effects on the frequency of IFN-γ-producing $CD8^+$ T cells by IFN-α. The responsiveness of both purified $CD4^+$ or $CD8^+$ T cells to IFN-α was not altered by the addition of monocytes (Brinkmann et al. 1993).

Of note, IFN-γ producing $CD8^+$ T cells may have a significant role in the pathogenesis of BD. It was revealed that the numbers of IFN-γ producing $CD8^+$ T cells in patients with BD were increased, which resulted in a significant increase of the Tc1: Tc2 ratio compared with normal controls (Houman et al. 2004).

5.4.5.1 The influence of IFN-α on IL-17 production by $CD4^+$ T cells

From our *in vitro* experiments, Th17 response was found to be inhibited by IFN-α consistently just like in the case of the BD patient findings. IFN-α suppresses the proportions of Th17 cells in a dose-dependent manner and higher concentrations of both IFN-α2a and IFN-α2b were particularly effective. Notably, in accordance with our BD patient data, the inhibitory effect was seen only in activated cultures stimulated with polyclonal stimuli but not in unstimulated cultures. Our findings are supported by some recent reports. It was shown that pre-treatment with either recombinant IFN-α or IFN-β inhibited PBMC secretion of superantigen-induced IL-17 and CD40 ligand-induced IL-23, which has the capacity to promote IL-17 secretion by memory/effector Th cells. Similar inhibitory effects were observed with pDC TLR9 agonist-induced type I IFN on activation-induced IL-23 and IL-17 expression (Meyers et al. 2006).

Another study demonstrated that IFN-α potently reduced IL-17A mRNA expression levels in healthy donor PBMCs 8 hours after CD3/CD28 stimulation. IFN-α downregulated IL-17 release from PBMCs and Th17 cells in a dose-dependent manner and the highest IFN-α concentration used, 10^3 IU/ml, was found to exert strongest influence. Similar to IL-17A, IL-17F mRNA expression was inhibited by IFN-α as well (Moschen et al. 2008). Also, IFN-α was found to significantly reduce IL-17 production by either anti-CD3/CD28 stimulated PBMCs or $CD4^+$ T cells in culture supernatants from healthy patients (Liu et al. 2011). Moreover, research on mice reported that both IFN-αA/D and IFN-β can inhibit the differentiation of murine

naive Th cells into effector Th17 cells. The αA/D molecule consists of the N-terminal portion of IFN-α2 and the C terminal portion of IFN-α1. However, like IFN-γ, the suppressive effects were limited to the inductive phase and polarised Th17 cells were resistant to IFN-mediated suppression of IL-17 production as a result (Harrington et al. 2005).

5.4.5.2 The influence of IFN-α on IL-17 production by $CD8^+$ T cells

IL-17 production was initially reported within $CD4^+$ αβ T cells and more recently IL-17 producing cells within $CD8^+$ αβ T cells (Tc17) was described in healthy human donor blood. Tc17 cells are able to produce IL-17 alone or in combination with IFN-γ. They share many common differentiation patterns with Th17 cells, including key transcription factors, chemokine receptors, and cytokine receptors (Kondo et al. 2009). However, little is known about the role of IL-17 producing $CD8^+$ lymphocytes in a normal immune response or in disease conditions.

From our *in vitro* experiments, the levels of $CD8^+$ T cells producing IL-17 was observed to be suppressed by IFN-α consistently as well, and like in $CD4^+$ T cells, in a dose-dependent manner. Again, higher concentrations of both IFN-α2a and IFN-α2b had the strongest influence, and the suppressive effect was seen only in activated cultures stimulated with polyclonal stimuli but not in unstimulated cultures.

5.4.6 Cytokine levels in comparison to other studies

From our data, the proportions of $CD4^+$ T cells producing IL-17 in healthy donor PBMCs were around 2% even after stimulation with PMA/ionomycin for 24 hours. The proportions of $CD8^+$ T cells producing IL-17 were even lower. The levels achieved are similar to a recent study where purified human $CD4^+$ T cells were stimulated with PMA/ionomycin and stained 6 hours later with anti-IL-17A. The percentage of $CD4^+$ T cells expressing IL-17A was shown to be around 1% in healthy donors (Brucklacher-Waldert et al. 2009).

In a study where naive $CD4^+$ T cells were stimulated with anti-CD3/CD28 Abs and cultured with medium, IL-12 or IL-23 for 5 days before restimulation with PMA/ionomycin. Again, the level of Th17 cells is comparable to our data and small changes in cell populations were described to be significant. The mean percentage of IL-17 producing $CD4^+$ cells was increased from 0.9% in cultures without IL-23 to 1.8% in the presence of IL-23. However, the level of Th17 cells was decreased in the presence of IL-12 to 0.7%. On the other hand, measurement of the number of IFN-γ producing $CD4^+$ cells showed reciprocal data, and a decrease of Th1 cells in the presence of IL-23 from 16.3% to 13.6% was detected when compared to cultures without added IL-23 (Hoeve et al. 2006).

Also, in a recent study where peripheral blood samples from normal and SLE patients were incubated with PMA and ionomycin for 4 hours before immunofluorescence staining. The levels of Th17 within $CD4^+$ T cells were ranging between 1.6 ± 0.7% for normal controls and 2.0±1.2% for SLE patients with inactive disease. On the other hands, the proportions of Tc17 within $CD8^+$ T cells were ranging between 0.3 ± 0.1% for normal controls and 0.6 ± 0.4% for SLE patients with active disease. All the levels reported are comparable to our study and the very small increase in active SLE patients was reported to be significant compared to healthy controls (Henriques et al. 2010).

5.4.7 The effect of IFN-α on the kinetics of IFN-γ and IL-17 production by T cells

The kinetics of PBMC response under the influence of IFN-α was measured with regards to both IFN-γ and IL-17 producing $CD4^+$ and $CD8^+$ T cells. This was carried out to obtain some indications in the potential roles of IFN-α not only as a treatment in chronic inflammatory diseases but possibly as a prophylaxis option in susceptible patients to chronic inflammatory diseases like BD.

Unexpectedly, IFN-α pre-treatment of healthy donor PBMCs did not demonstrate as much upregulatory effect on either $CD4^+$ or $CD8^+$ T cells producing IFN-γ as when IFN-α was added later on in the cultures. The highest level of T cells positive for

IFN-γ was achieved with introduction of IFN-α at the last time-point tested. Observations from PMA/ionomycin stimulated cultures raised the question whether exogenous IFN-α exerted greater control of IFN-γ production on activated PBMCs than unstimulated cells. However, this wouldn't explain the results analysed using untreated cultures. Another possible explanation is that IFN-γ was produced in greater quantity earlier during incubation in response to IFN-α treatment and the results were simply a reflection of the experimental design picking up data at various stages of cytokine production.

Similar stories were found with regards to IL-17 producing $CD4^+$ and $CD8^+$ T cells. No significant difference in terms of IL-17 production levels was observed regardless of when exogenous IFN-α was added to unstimulated cell cultures. With PMA/ionomycin stimulation, however, IFN-α pre-treatment of healthy donor PBMCs was not able to produce as much inhibitory effect on either $CD4^+$ or $CD8^+$ T cells producing IL-17 as when IFN-α was added later on in the cultures. Regarding IL-17 production by T cells, concurrent use of IFN-α and PMA/ionomycin was detected to result in strongest downregulatory effect. Again, this could be related to PBMC incubation time chosen before cell preparation and staining for flow cytometry. It is likely that the cytokine IL-17 was produced later by T cells during cultures in comparison to IFN-γ.

5.5 Summary of findings

Under the influence of higher doses of either recombinant IFN-α2a or IFN-α2b, there was a small tendency for $CD4^+$ T cell upregulation but $CD8^+$ T cell downregulation in healthy donor PBMCs. Regarding impact on inflammatory cytokine production by T cells *in vitro*, IFN-α2a and IFN-α2b were demonstrated to be equally effective. Both forms of IFN-α showed the tendency to upregulate IFN-γ producing $CD4^+$ and $CD8^+$ T cells and suppress $CD4^+$ and $CD8^+$ T cells producing IL-17. IFN-α appeared to exert the strongest influence on IFN-γ producing and IL-17 producing T cells when it was added 20 hours before and 24 hours before intracellular cytokine staining and flow cytometry respectively. Notably, IFN-α was found to be able to exert its effects on Th17 cells both before and after cell activation.

Chapter 6

The *in vitro* effects of interferon-alpha 2a & 2b on $CD4^+$ and $CD8^+$ regulatory T cells and associated regulatory cytokines in healthy donors

6.1 Introduction

The existence of self-reactive T cells in healthy individuals suggests that many self-reactive T cells that escape clonal deletion in the thymus are subsequently released into the periphery, where they are capable of differentiating into potentially pathogenic effector cells (Bouneaud, Kourilsky, & Bousso 2000). Thus, peripheral mechanisms exist in order to control immune responses and to dampen potential autoimmunity.

It has been recognised Treg cells play prominent roles in the maintenance of lymphoid homeostasis, and these cells maintain tolerance to self and control autoimmune deviation by restraining overzealous immune responses (Allan et al. 2008b). Treg cells, therefore, represent an attractive target for therapeutic immune strategies in various clinical situations, and the interest in this field has exploded the past 5-10 years. However, the increasing number and the heterogeneity of T cell subsets with immunosuppressive properties make it difficult to fully characterise and determine their importance in various clinical conditions. The lack of knowledge of the mechanism of suppression by many of these Treg cell subsets further contributes to the complexity and hampers the development of therapeutic strategies that could have clinical applications.

$CD4^+$ Treg cells can be subdivided into naturally occurring Treg cells that originate from the thymus and adaptive Treg cells that are induced in the periphery. Whereas $CD4^+$ nTreg cells functionally require constitutive expression of the transcription factor Foxp3, adaptive $CD4^+$ Treg cells are frequently $Foxp3^-$ (Allan et al. 2008a). Apart from $CD4^+CD25^{high}FOXP3^+$ Treg cells, there are other T cell subsets with regulatory function. Most of these cells, including IL-10-secreting Tr1 cells and TGF-β-secreting Th3 cells, acquire regulatory functions following specific antigenic stimulation in particular cytokine milieus (Sakaguchi et al. 2010).

Nevertheless, regulatory T cells are not strictly confined to the $CD4^+$ T cell compartment. Naturally occurring $CD8^+$ Treg cells originate from the thymus and are often considered to represent a separate T cell lineage. Postnatal human thymus contains a defined population of $CD8^+$ Treg cells. They express the phenotype $CD8^+CD25^+CTLA\text{-}4^+GITR^+Foxp3^+$ and suppress in a contact-dependent manner (Cosmi et al. 2003). These cells should have a broad repertoire of antigenic specificities. Conversely, adaptive $CD8^+$ Treg cells originate from the postthymic T cell pool and are induced through a variety of *in vivo* or *in vitro* stimuli. Adaptive $CD8^+$ Treg cells can be further subclassified into those that have antigen specificity and those that display their immunoregulatory function in an antigen-non-specific fashion (Scotto et al. 2004).

According to a recent study, it is relatively easy to generate non-antigen-specific adaptive $CD8^+CD28^-Foxp3^-CD56^-$ Treg cells (Filaci et al. 2004). Culturing $CD8^+CD28^-$ T cells in a cytokine cocktail containing IL-2, IL-10 and GM-CSF is sufficient to expand $CD8^+$ Treg cells; antigen recognition is not required. Induction of this cell, however, is monocyte dependent, possibly reflecting the requirement for GM-CSF. Antigen non-specific CD8 Treg cells are able to survive in culture for more than 1 month, broadening their potential therapeutic application. Such non-antigen-specific $CD8^+$ Treg cells suppress APC-mediated antigen presentation, T-cell proliferation, and CTL-mediated cytotoxicity via IL-10 secretion, a cytokine that has been linked to anergy induction in activated T cells (Filaci et al. 2004; Xystraks et al. 2004). Likewise, adaptive alloantigen-specific $CD8^+$ Treg cells can also be generated *in vitro* from naive $CD8^+$ T cells and are present in human transplant patients (Manavalan et al. 2004).

The concept that T cells are capable of downregulating immunity is now firmly established. Both within the CD4 and the CD8 T cell subsets, specialised populations apply sophisticated mechanisms to inhibit early and late steps of immune responses.

6.2 Aims

In this chapter, we set out to explore the *in vitro* effects of IFN-α on regulatory T cell populations, expression of Foxp3, and production of immunoregulatory cytokines, that serve as critical gatekeepers in immune homeostasis. The *ex vivo* data from chapter 4 demonstrated the tendency to upregulate IL-10 and TGF-β in T cells after 6 months of IFN-α2b therapy which was preserved even at 12 month visits. Assays are set up using healthy donor PBMCs to test the influence of various concentrations of IFN-α2a and IFN-α2b on $CD4^+$ and $CD8^+$ Treg subsets, production of IL-10 and TGF-β and the kinetics of the cytokine production.

6.3 Results

6.3.1 Preliminary experiments on Treg cells with antigens and polyclonal stimuli

Having tested the influence of various antigens and polyclonal stimuli on Th1 and Th17 cell populations as described in chapter 5, the same panel of stimuli was applied to healthy donor PBMC cultures for 24 hours before intracellular cytokine staining to test their effects on Treg cells and IL-10 production as a comparison. Foxp3 expression was analysed by gating on $CD4^+$ or $CD4^+CD25^{high}$ T lymphocyte populations, whereas IL-10 expression was analysed by gating on $CD4^+$ T lymphocytes only.

It was found that anti-CD3/CD28 Ab and especially, PMA/ionomycin consistently promoted higher levels of Foxp3 (Fig 6.1) and IL-10 (Fig 6.2) expression by $CD4^+$ T cells. Also, as expected, ATRA showed the potential to induce Foxp3 expression in both $CD4^+$ or $CD4^+CD25^{high}$ T lymphocytes, and the optimal concentration of ATRA appeared to be 0.01μM. However, ATRA had little effect on IL-10 expression. At 5-20μg/ml, LPS demonstrated the tendency to augment Foxp3 expression by $CD4^+CD25^{high}$ T cells (Fig 6.1b) with longer incubation time. Concurrent use of LPS and CD3/CD28 stimulation was evaluated as well, but the effect was no more than using anti-CD3/CD28 Ab alone. Candida antigens were another candidate showing

the capacity in inducing Foxp3 expression by both $CD4^+$ (Fig 6.1a) and $CD4^+CD25^{high}$ (Fig 6.1b) T lymphocytes. Unlike most other antigens tested, candida antigens, especially at lower concentrations, also showed the tendency to induce IL-10 production by $CD4^+$ T cells (Fig 6.2). Furthermore, LPS, when used at a concentration of 5µg/ml, displayed some tendency to increase IL-10 producing $CD4^+$ T cells with longer incubation time.

In addition, PBMCs from BD patients were cultured with BD-related antigens, such as alpha-tropomyosin and PDS-Ag as a trial. However, no significant effect on Foxp3 and IL-10 expression levels was noticed with either antigen on BD patient PBMCs, regardless of the concentrations of antigens used. Therefore, it was decided not to use up any more of the precious BD patient PBMCs to test the immunomodulatory ability of other BD-related antigens, including HSP60. Notably, Foxp3 (Fig 6.1b) and IL-10 (Fig 6.2) expression levels in PBMCs from BD patients were particularly low without stimulation when compared to healthy donor PBMCs, but the levels did increase considerably with polyclonal stimuli, especially in the case of PMA/ionomycin stimulation.

Based on the findings in preliminary tests, PBMCs activated with anti-CD3/CD28 Ab and/or PMA/ionomycin were selected as the basis for our subsequent *in vitro* experiments and further preliminary experiments with antigens were not carried out. The positives results with polyclonal stimulation were repeatable as shown by the data in nine separate experiments despite the fact that the experiments were performed in singles.

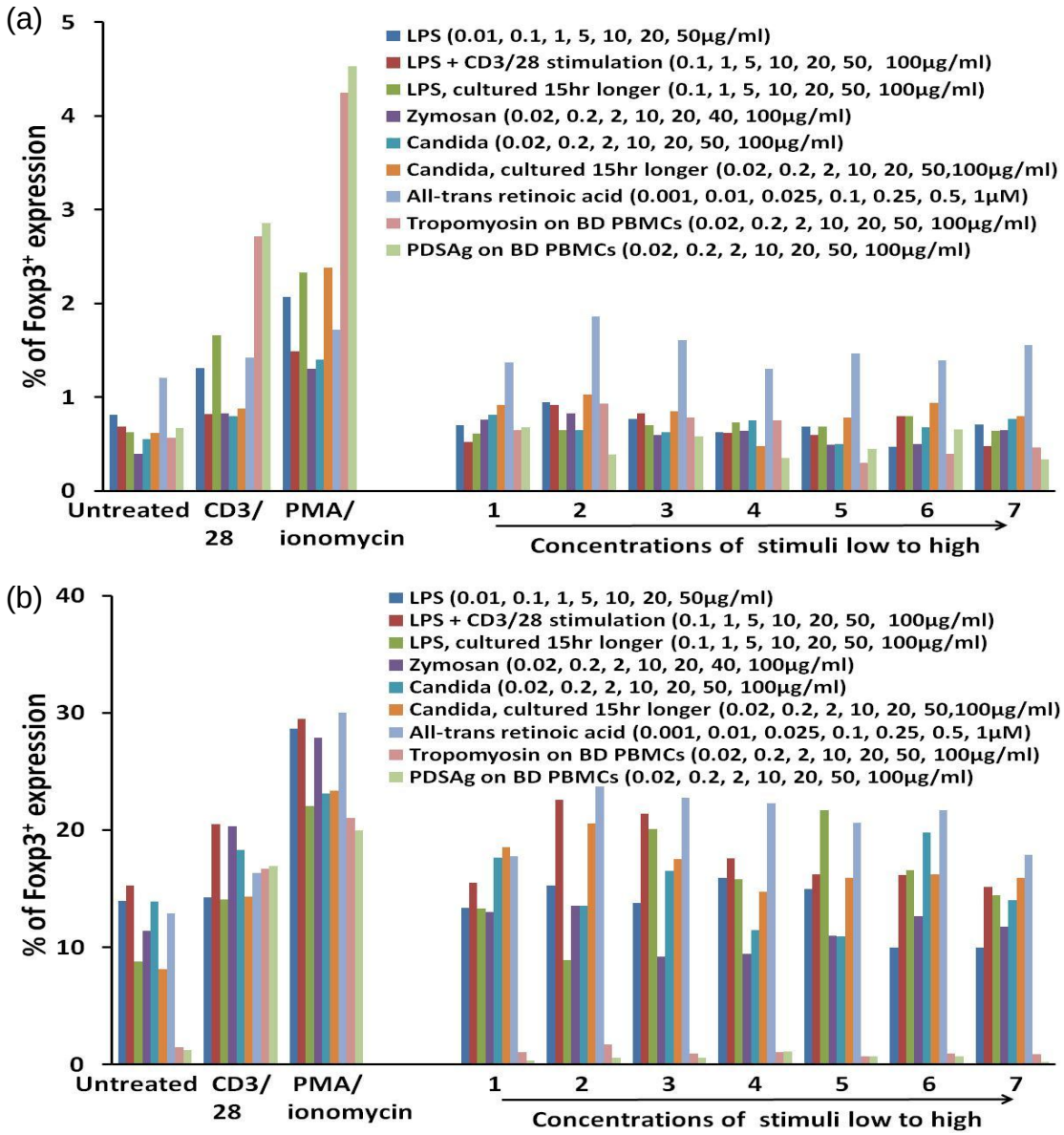


Fig 6.1: The effect of a selection of stimuli on Foxp3$^+$ expression levels in healthy donor PBMCs

Healthy donor PBMCs were untreated, activated with anti-CD3 (5ng/ml) & anti-CD28 (1µg/ml), activated with PMA (25ng/ml) & ionomycin (1µg/ml) or stimulated with various stimuli including LPS, zymosan, candida, and all-trans retinoic acid for 24 hours or 39 hours. In one experiment, both LPS and CD3/CD28 stimulation were administered. Also, in 2 of the experiments, PBMCs from BD patients were used instead and stimulated with either polyclonal stimuli or α-tropopmyosin or PDS-Ag for 24 hours. In (a), percentage of CD4$^+$ T cells expressing Foxp3 and in (b), percentage of CD4$^+$CD25^{high} T cells expressing Foxp3.are shown. Each colour represent a separate experiment and all experiments were done only once in singles. 1 is the lowest antigen concentration used and 7 is the highest. The actual concentrations of stimuli are shown inside the brackets in the legend.

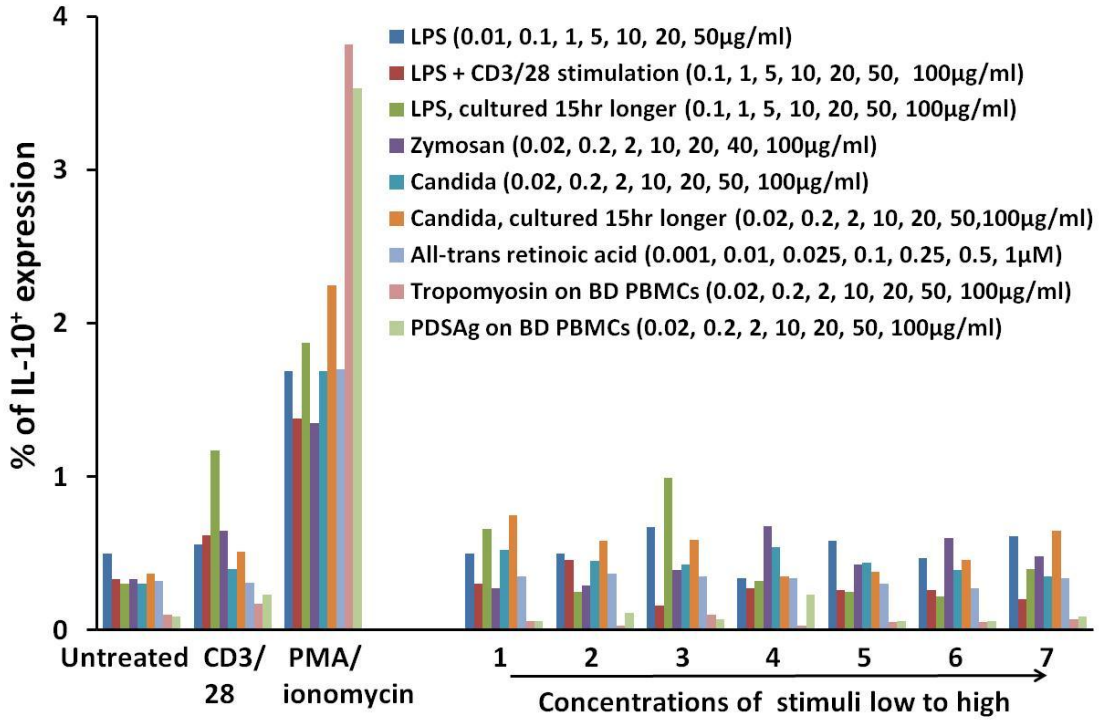


Fig 6.2: The effect of a selection of stimuli on IL-10$^+$ expression by CD4$^+$ T cells

Healthy donor PBMCs were untreated, activated with anti-CD3 (5ng/ml) & anti-CD28 (1µg/ml), activated with PMA (25ng/ml) & ionomycin (1µg/ml) or stimulated with various stimuli including LPS, zymosan, candida, and all-trans retinoic acid for 24 hours or 39 hours. In one experiment, both LPS and CD3/CD28 stimulation were administered. Also, in 2 of the experiments, PBMCs from BD patients were used instead and stimulated with either polyclonal stimuli or α-tropopmyosin or PDS-Ag for 24 hours. Percentage of CD4$^+$ T cells producing IL-10 is shown. Each colour represent a separate experiment and all experiments were done only once in singles. 1 is the lowest antigen concentration used and 7 is the highest. The actual concentrations of stimuli are shown inside the brackets in the legend.

6.3.2 IFN-α induces Foxp3 expression by $CD4^+$ T cells

The results from chapter 4 demonstrated that Foxp3 expression by $CD4^+$ Tcells in patients with BD was upregulated under IFN-α2b therapy. In order to verify these findings, experiments were set up with PBMCs isolated from healthy individuals to study the influence of IFN-α on Foxp3 expression *in vitro*. PBMCs were cultured without stimulation or stimulated with anti-CD3 and anti-CD28 Ab or PMA and ionomycin in the presence or absence of IFN-α2a (10^1-10^5 U/ml) and IFN-α2b (10^1-10^4 U/ml) before intracellular cytokine staining and flow cytometry. Foxp3 expression was analysed by gating on $CD4^+$, $CD4^+CD25^+$ and $CD4^+CD25^{high}$ lymphocyte populations.

In agreement with data from chapter 4, consistent augmentation of Foxp3 expression by $CD4^+$ T cells in a dose-dependent manner was detected. With regards to IFN-α2a, concentrations between 10^3 to 10^5 U/ml were demonstrated to enhance Foxp3 expression significantly (Fig 6.3a). In the presence of 10^3 U/ml IFN-α2a, the proportion of $CD4^+$ T cells expressing Foxp3 was elevated from 0.8 ± 0.3% to 2.4 ± 0.5% (means ± SD; $p<0.05$) with PMA/ionomycin stimulation. Similarly, the Foxp3 expression levels by $CD4^+$ T cells were raised from 1.0 ± 0.3% to 2.7 ± 0.3% ($p<0.01$) in the presence of 10^3 U/ml IFN-α2b and PMA/ionomycin stimulation (Fig 6.3b). Within $CD4^+CD25^+$ T cell population, Foxp3 expression was increased by addtion of IFN-α as well and this was especially evident in cultures activated with PMA/ionmycin in the presence of 10^4 U/ml IFN-α. The percentage of $CD4^+CD25^+$ T cells expressing Foxp3 was upregulated from 1.2 ± 0.4% to 3.0 ± 0.5% ($p<0.01$) with 10^4 U/ml IFN-α2a (Fig 6.4a) and from 1.5 ± 0.3% to 3.0 ± 0.3% ($p<0.01$) with 10^4 U/ml IFN-α2b (Fig 6.4b).

In addition, within $CD4^+CD25^{high}$ T cells, a population enriched in Tregs, a dose-dependent increase in Foxp3 expression was induced by IFN-α consistently. Results from cultures activated with PMA/ionomycin showed that the proportion of $CD4^+CD25^{high}$ T cells expressing Foxp3 was elevated from 4.7 ± 1.2% up to 8.0 ± 0.7% ($p<0.05$) with 10^4 U/ml IFN-α2a (Fig 6.5a) and from 4.4 ± 1.2% up to 9.3 ± 0.5% ($p<0.01$) with 10^3 U/ml IFN-α2b (Fig 6.5b). Similar trend could be observed in unstimulated cultures as well.

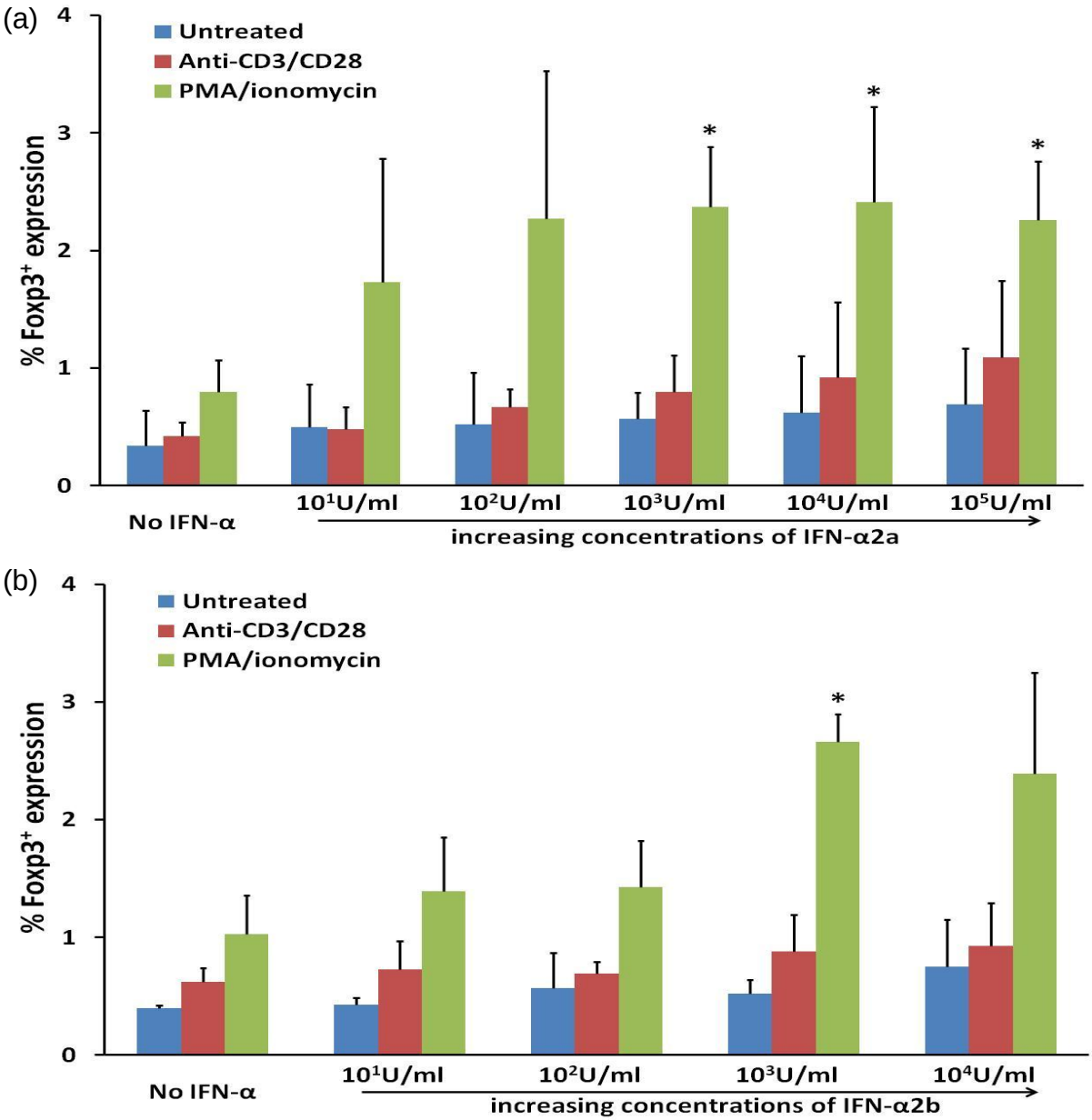


Fig 6.3: IFN-α induces Foxp3 expression by CD4$^+$ T cells

Healthy donor PBMCs were untreated, stimulated with anti-CD3 & anti-CD28 or PMA & ionomycin for 24 hours. Various concentrations of IFN-α was added at the same time as the polyclonal stimuli to cultures. Results are shown as proportion of CD4$^+$ T cells expressing Foxp3. In (a) and (b), the effects of IFN-α2a and IFN-α2b are shown respectively. Results in (a) and (b) are representative of four (a) and five (b) independent experiments. PBMCs were from three different donors for experiments on both forms of IFN-α. Two of the IFN-α2a and IFN-α2b experiments used both CD3/CD28 and PMA/ionomycin stimulation. Also, PBMCs were cultured over 39 instead of 24 hours in two experiments, and 10^1 U/ml of IFN-α (2a and 2b) and 10^5 U/ml of IFN-α2a were only tested twice in these experiments. Data are means ± SD values of triplicate wells. Statistical analysis was performed using the Student's T-test. *p<0.05, **p<0.01, compared with controls without addition of IFN-α.

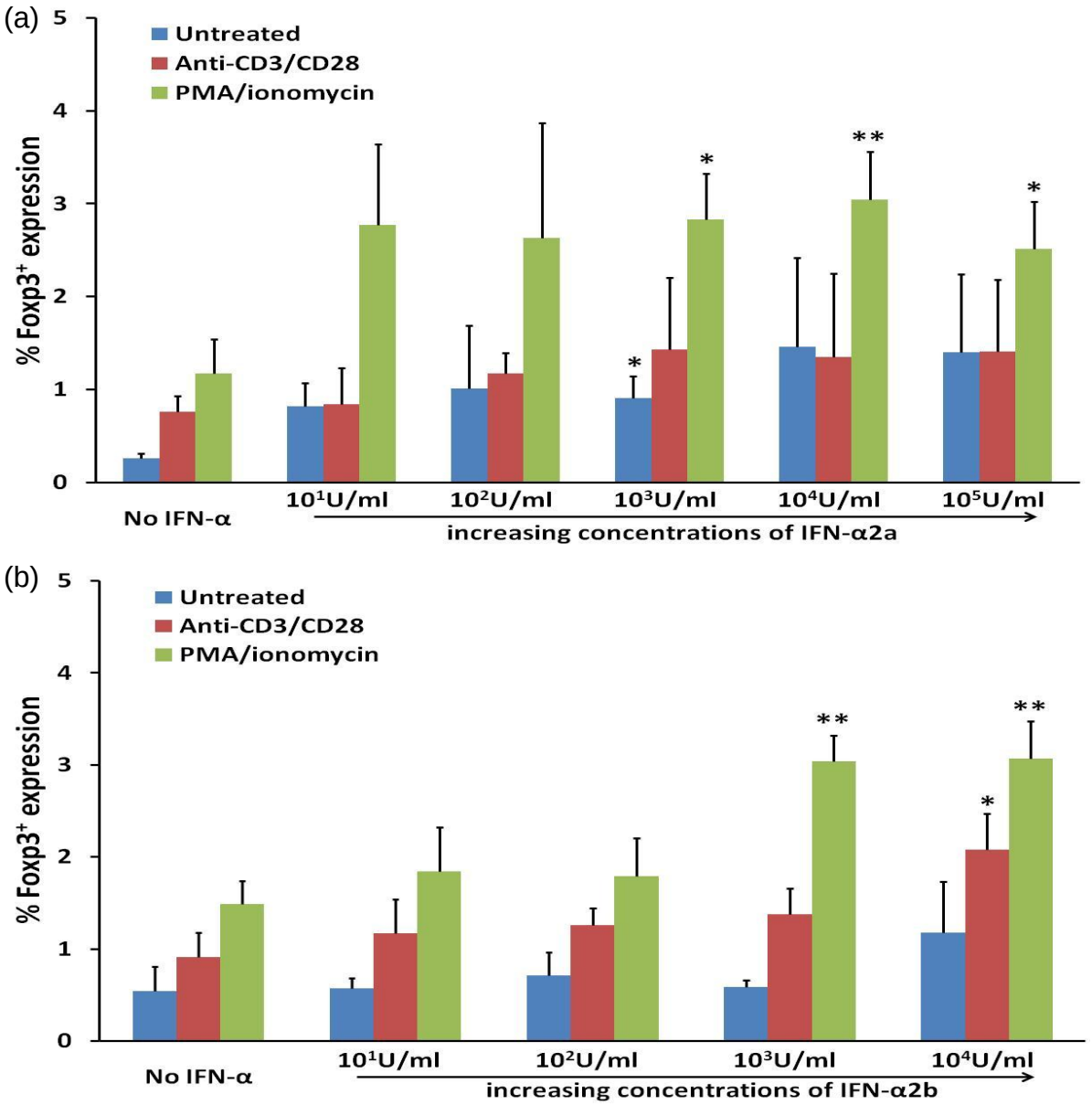


Fig 6.4: IFN-α induces an upregulation of Foxp3 expression by $CD4^+CD25^+$ T cells

Healthy donor PBMCs were untreated, stimulated with anti-CD3 & anti-CD28 or PMA & ionomycin for 24 hours. Various concentrations of IFN-α was added at the same time as the polyclonal stimuli to cultures. Results are shown as proportion of $CD4^+CD25^+$ T cells expressing Foxp3. In (a) and (b), the effects of IFN-α2a and IFN-α2b are shown respectively. Results in (a) and (b) are representative of four (a) and five (b) independent experiments. PBMCs were from three different donors for experiments on both forms of IFN-α. Two of the IFN-α2a and IFN-α2b experiments used both CD3/CD28 and PMA/ionomycin stimulation. Also, PBMCs were cultured over 39 instead of 24 hours in two experiments, and 10^1 U/ml of IFN-α (2a and 2b) and 10^5 U/ml of IFN-α2a were only tested twice in these experiments. Data are means ± SD values of triplicate wells. Statistical analysis was performed using the Student's T-test. *p<0.05, **p<0.01, compared with controls without addition of IFN-α.

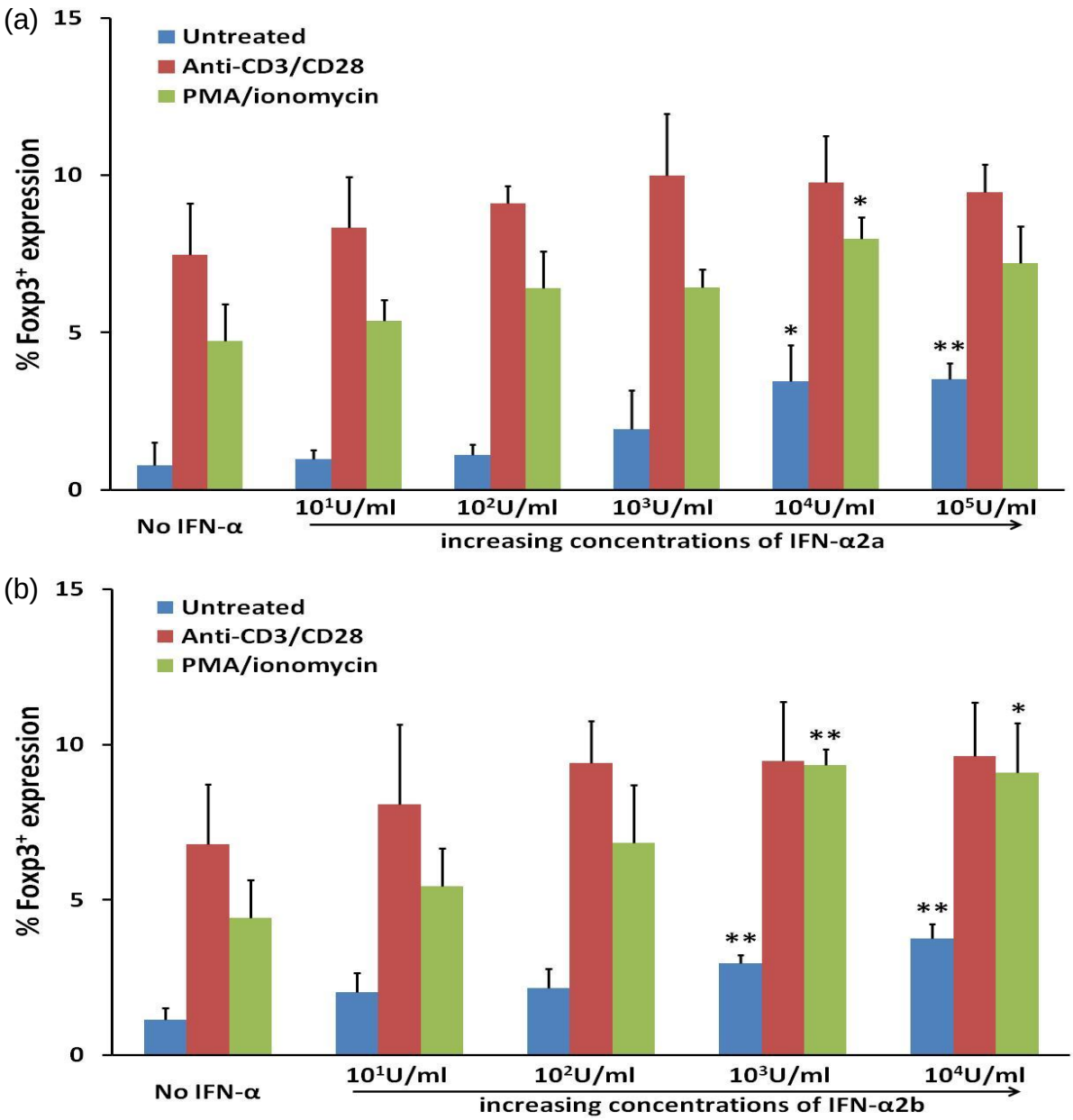


Fig 6.5: IFN-α induces an upregulation of Foxp3 expression by $CD4^+CD25^{high}$ T cells

Healthy donor PBMCs were untreated, stimulated with anti-CD3 & anti-CD28 or PMA & ionomycin for 24 hours. Various concentrations of IFN-α was added at the same time as the polyclonal stimuli to cultures. Results are shown as proportion of $CD4^+CD25^{high}$ T cells expressing Foxp3. In (a) and (b), the effects of IFN-α2a and IFN-α2b are shown respectively. Results in (a) and (b) are representative of four (a) and five (b) independent experiments. PBMCs were from three different donors for experiments on both forms of IFN-α. Two of the IFN-α2a and IFN-α2b experiments used both CD3/CD28 and PMA/ionomycin stimulation. Also, PBMCs were cultured over 39 instead of 24 hours in two experiments, and 10^1 U/ml of IFN-α (2a and 2b) and 10^5 U/ml of IFN-α2a were only tested twice in these experiments. Data are means ± SD values of triplicate wells. Statistical analysis was performed using the Student's T-test. *p<0.05, **p<0.01, compared with controls without addition of IFN-α.

6.3.3 IFN-α induces Foxp3 expression by $CD8^+$ T cells

Having established the effect of IFN-α on inducing Foxp3 expression within $CD4^+$ T cells, the next goal was to explore whether similar effect was present with $CD8^+$ T cells. Again, experiments were set up with PBMCs isolated from healthy individuals, and cells were cultured without stimulation or stimulated with anti-CD3 and anti-CD28 Ab or PMA and ionomycin for 24 hours in the presence or absence of IFN-α2a (10^1-10^5 U/ml) and IFN-α2b (10^1-10^4 U/ml) before intracellular cytokine staining and flow cytometry. Foxp3 expression was analysed by gating on $CD8^+$, $CD8^+CD25^+$ and $CD8^+CD25^{high}$ lymphocyte population.

From our experiments, it was revealed that although the Foxp3 expression levels by $CD8^+$ T cells were very low to begin with, an upregulation was still able to be detected. Following activation with PMA/ionomycin, the proportion of Foxp3 expressing $CD8^+$ T cells was elevated from 0.4 ± 0.1% up to 1.3 ± 0.4% (means ± SD; $p<0.05$) in the presence of 10^4 U/ml IFN-α2a (Fig 6.6a) and from 0.4 ± 0.2% up to 1.0 ± 0.3% ($p<0.05$) in the presence of 10^4 U/ml IFN-α2b (Fig 6.6b). Similarly, within $CD8^+CD25^+$ cell population, Foxp3 expression levels were raised enhanced from 0.5 ± 0.1% up to 1.1% ($p<0.01$) in the presence of 10^5 U/ml IFN-α2a (Fig 6.7a) and from 0.6 ± 0.1% up to 1.5 ± 0.2% ($p<0.05$) in the presence of 10^4 U/ml IFN-α2b (Fig 6.7b) with PMA/ionomycin stimulation. Similar trend of Foxp3 augmentation could be detected in cultures stimulated with anti-CD3/CD28 Ab as well. Notably, within $CD8^+CD25^{high}$ lymphocytes, although there appeared to be a tendency for inreased Foxp3 expression levels induced by IFN-α, none of the changes was statistically significant (Fig 6.8).

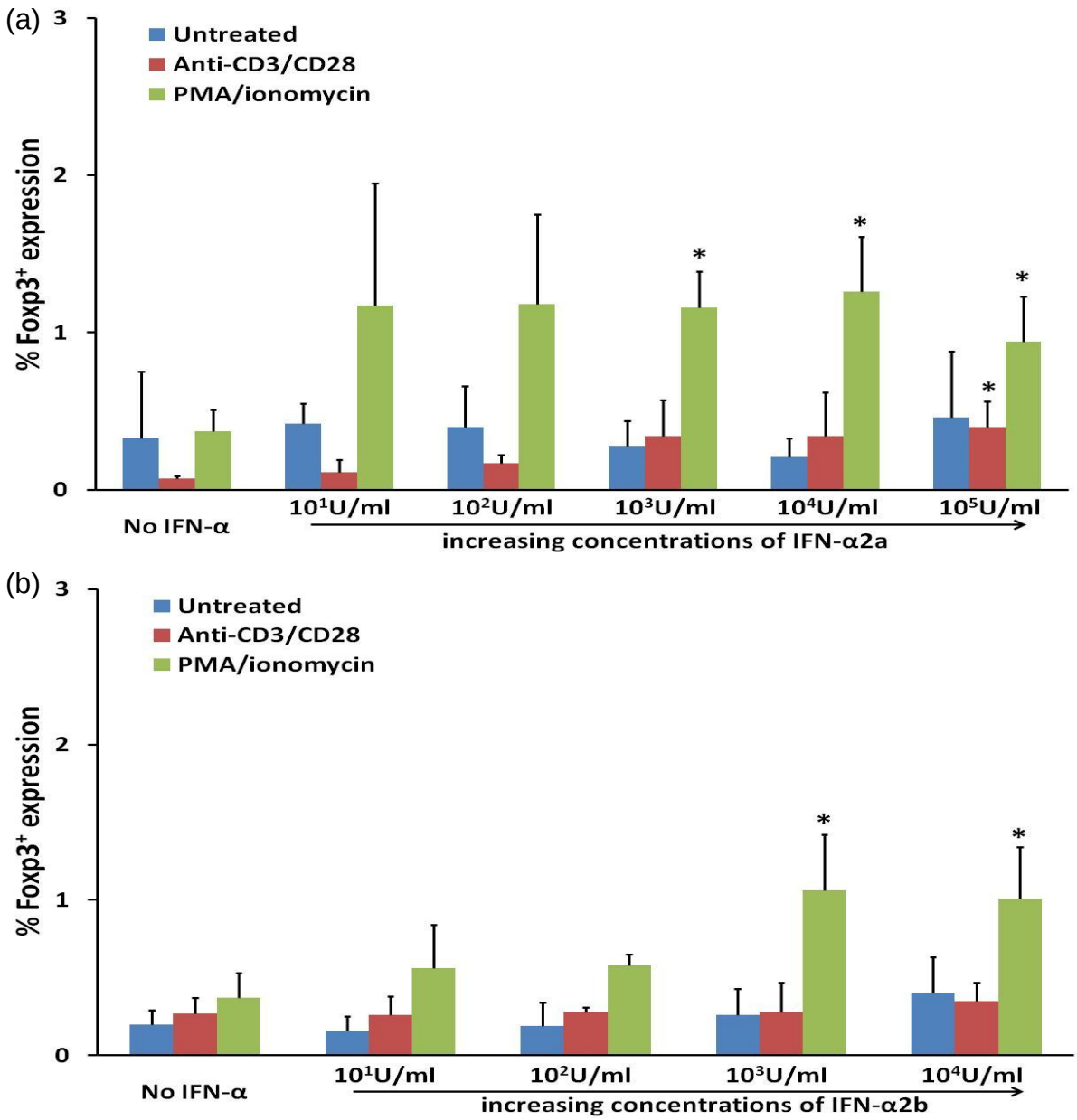


Fig 6.6: IFN-α induces Foxp3 expression by $CD8^+$ T cells

Healthy donor PBMCs were untreated, stimulated with anti-CD3 & anti-CD28 or PMA & ionomycin for 24 hours. Various concentrations of IFN-α was added at the same time as the polyclonal stimuli to cultures. Results are shown as proportion of $CD8^+$ T cells expressing Foxp3. In (a) and (b), the effects of IFN-α2a and IFN-α2b are shown respectively. Results in (a) and (b) are representative of two (a) and three (b) independent experiments. PBMCs were from two different donors for experiments on both forms of IFN-α. For both forms of IFN-α, only one experiment used both CD3/CD28 and PMA/ionomycin stimulation. 10^1 U/ml of IFN-α (2a and 2b) and 10^5 U/ml of IFN-α2a were tested once in these experiments. Data are means ± SD values of triplicate wells. Statistical analysis was performed using the Student's T-test. *p<0.05, **p<0.01, compared with controls without addition of IFN-α.

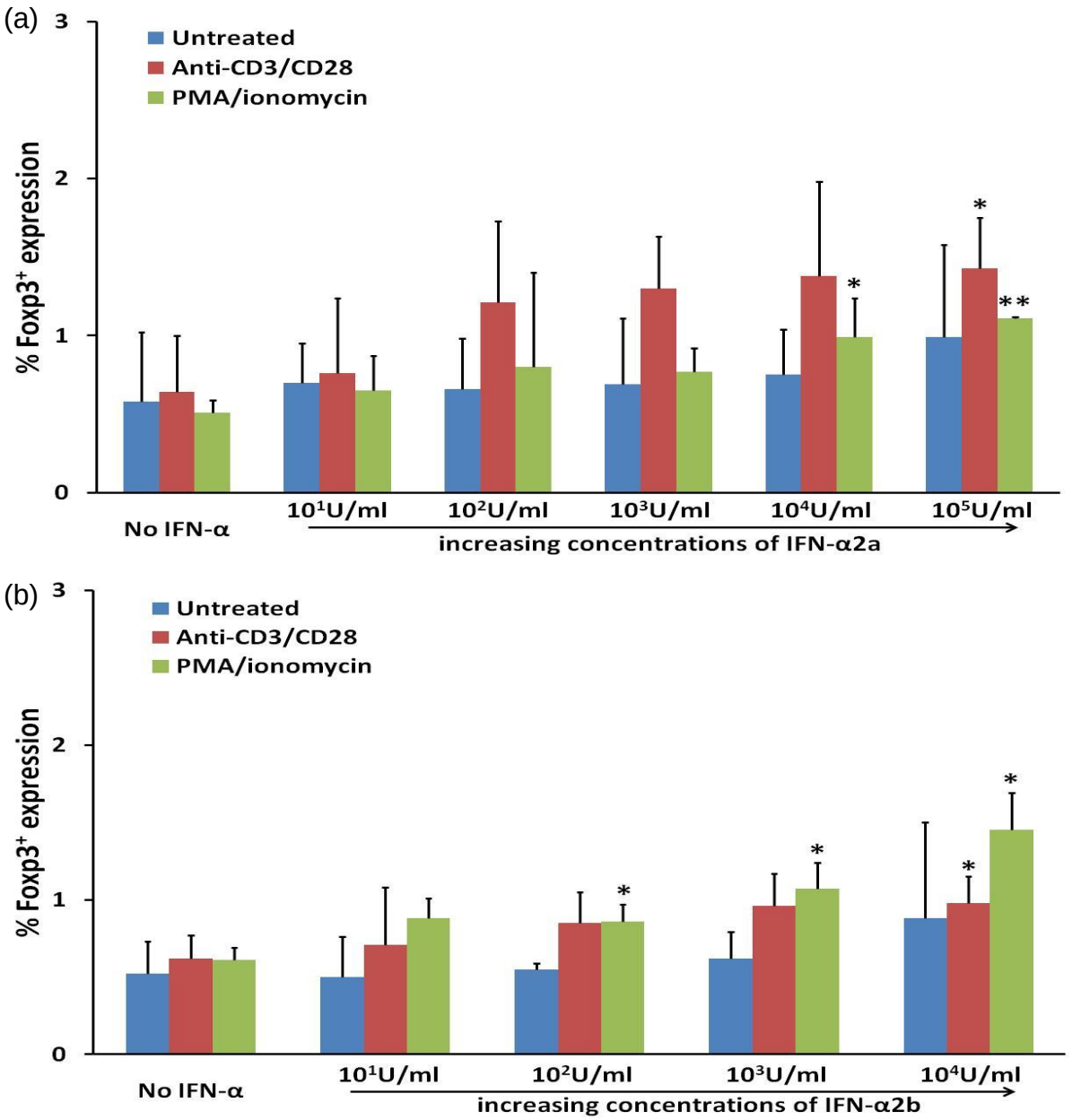


Fig 6.7: IFN-α induces an upregulation of Foxp3 expression by $CD8^+CD25^+$ T cells

Healthy donor PBMCs were untreated, stimulated with anti-CD3 & anti-CD28 or PMA & ionomycin for 24 hours. Various concentrations of IFN-α was added at the same time as the polyclonal stimuli to cultures. Results are shown as proportion of $CD8^+CD25^+$ T cells expressing Foxp3. In (a) and (b), the effects of IFN-α2a and IFN-α2b are shown respectively. Results in (a) and (b) are representative of two (a) and three (b) independent experiments. PBMCs were from two different donors for experiments on both forms of IFN-α. For both forms of IFN-α, only one experiment used both CD3/CD28 and PMA/ionomycin stimulation. 10^1 U/ml of IFN-α (2a and 2b) and 10^5 U/ml of IFN-α2a were tested once in these experiments. Data are means ± SD values of triplicate wells. Statistical analysis was performed using the Student's T-test. *$p<0.05$, **$p<0.01$, compared with controls without addition of IFN-α.

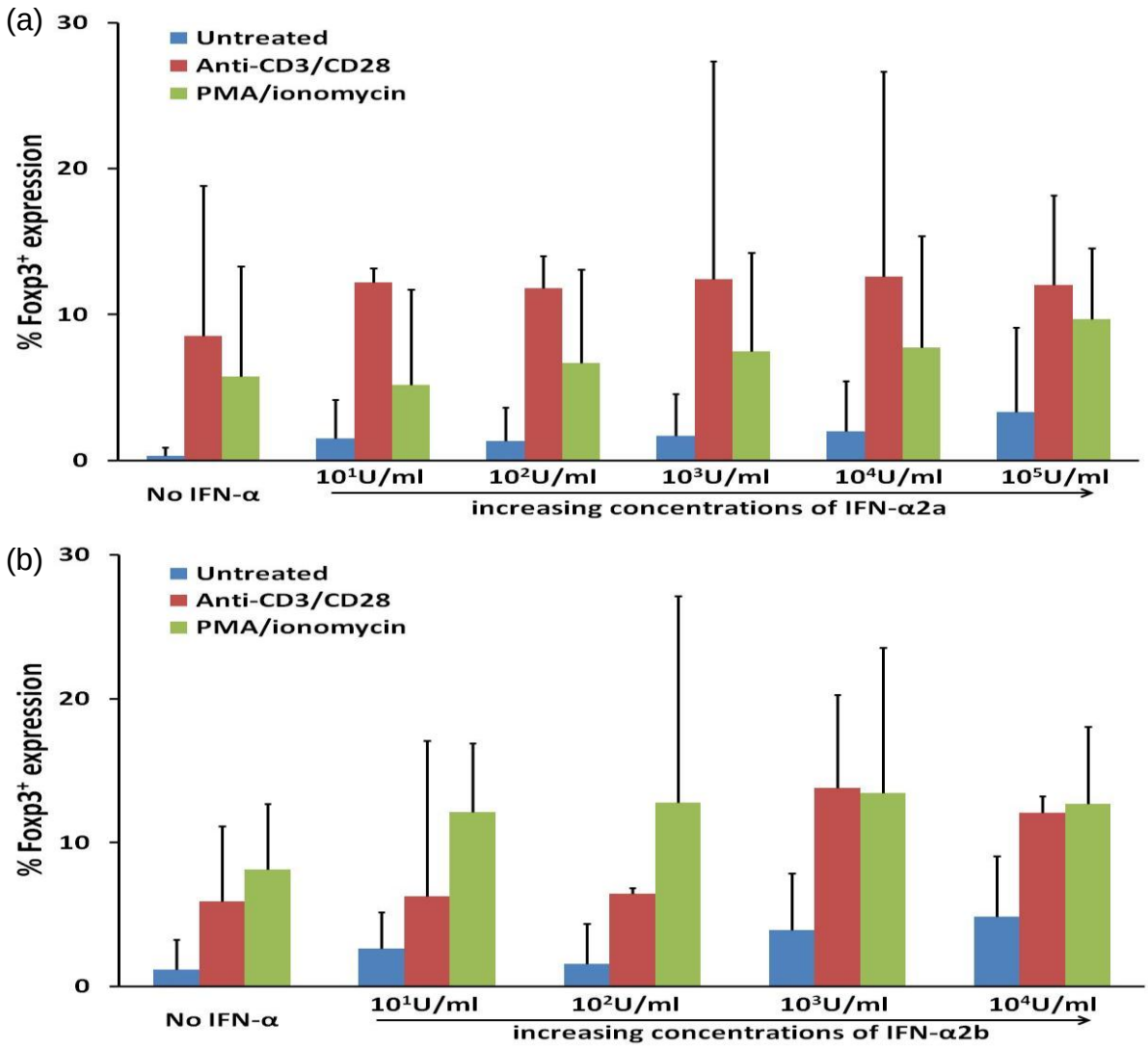


Fig 6.8: IFN-α induces an upregulation of Foxp3 expression by $CD8^{+}CD25^{high}$ T cells

Healthy donor PBMCs were untreated, stimulated with anti-CD3 & anti-CD28 or PMA & ionomycin for 24 hours. Various concentrations of IFN-α was added at the same time as the polyclonal stimuli to cultures. Results are shown as proportion of $CD8^{+}CD25^{high}$ T cells expressing Foxp3. In (a) and (b), the effects of IFN-α2a and IFN-α2b are shown respectively. Results in (a) and (b) are representative of two (a) and three (b) independent experiments. PBMCs were from two different donors for experiments on both forms of IFN-α. For both forms of IFN-α, only one experiment used both CD3/CD28 and PMA/ionomycin stimulation. 10^{1} U/ml of IFN-α (2a and 2b) and 10^{5} U/ml of IFN-α2a were tested once in these experiments. Data are means ± SD values of triplicate wells. Statistical analysis was performed using the Student's T-test. *p<0.05, **p<0.01, compared with controls without addition of IFN-α.

6.3.4 The kinetics of the upregulatory effect of IFN-α on Foxp3 expression by T cells

Although IFN-α has been observed to induce an upregulation in Foxp3 expression by both $CD4^+$ and $CD8^+$ T cells *in vitro*, the kinetics of this particular effect by IFN-α remained to be explored. PBMCs isolated from healthy individuals were cultured without IFN-α treatment or with 10^4 U/ml IFN-α2a added 24 hours before, 4 hours before, at the same time as, or 4 hours after PMA/ionomycin stimulation. The timing for the introduction of IFN-α to cultures was the same for both unstimulated and stimulated cultures, and PBMCs were incubated for 24 hours after addition of PMA/ionomycin before intracellular cytokine staining and flow cytometry. Foxp3 expression levels were analysed by gating on $CD4^+$, $CD4^+CD25^+$, $CD4^+CD25^{high}$, $CD8^+$, $CD8^+CD25^+$ and $CD8^+CD25^{high}$ lymphocyte populations in turn.

Clearly, adding IFN-α to PBMC cultures at all time-points tested was effective in inducing an upregulation of Foxp3 expression by $CD4^+$, $CD4^+CD25^+$, and $CD4^+CD25^{high}$ T cells (Fig 6.9). Despite not being statistically significant, pre-treatment of PBMCs with 10^4 U/ml IFN-α2a showed more efficacy in Foxp3 induction within all three cell populations than adding IFN-α2a after polyclonal activation by PMA/ionomycin or at the last time-point in the case of untreated cultures. Following activation with PMA/ionomycin, the proportion of $CD4^+$ T cells expressing Foxp3 was upregulated from 1.0 ± 0.1% up to 2.4 ± 0.2% (means ± SEM; $p<0.01$) with 24 hours of IFN-α pre-treatment and only up to 1.8 ± 0.4% ($p<0.05$) with 4 hours of IFN-α post-treatment (Fig 6.9a). Similar results were observed within $CD4^+CD25^+$ T cells as well as the Foxp3 expression levels were increased from 1.6 ± 0.1% up to 3.0 ± 0.3% ($p<0.01$) following 24 hours of IFN-α pre-treatment and PMA/ionomycin stimulation (Fig 6.9b). In accordance, within $CD4^+CD25^{high}$ T cells, the Foxp3 expression levels were elevated from 4.6 ± 0.4% up to 7.6 ± 0.7% ($p<0.01$) with 24 hours of IFN-α pre-treatment and activation by PMA/ionomycin (Fig 6.9c).

With regards to $CD8^+$ T cells, Foxp3 expression levels were quite low relative to $CD4^+$ T cells, but they were able to be brought up with the introduction of 10^4 U/ml IFN-α2a. Adding IFN-α at any time-points to PBMC cultures was demonstrated to be equally efficacious as the proportion of $CD8^+$ T cells expressing Foxp3 was raised

from 0.5% to 0.8 ± 0.1% ($p<0.05$) following IFN-α treatment and PMA/ionomycin stimulation (Fig 6.10a). However, within $CD8^+CD25^+$ T cells, pre-treatment with IFN-α again showed the tendency to be more potent in upregulating Foxp3 expression than adding IFN-α later during incubation (Fig 6.10b). With PMA/ionomycin stimulation, the proportion of $CD8^+CD25^+$ T cells expressing Foxp3 was enhanced from 0.8 ± 0.1% to 1.3 ± 0.1% ($p<0.05$) after 24 hours of IFN-α pre-treatment but only up to 1.1 ± 0.1% (p=ns) when IFN-α was added 4 hours after polyclonal stimulation with PMA/ionomycin. In contrast, there was no appreciable augmentation of Foxp3 expression within $CD8^+CD25^{high}$ with the introduction of IFN-α to cell cultures, although adding IFN-α earlier in cultures showed greater potential than doing so later on (Fig 6.10c).

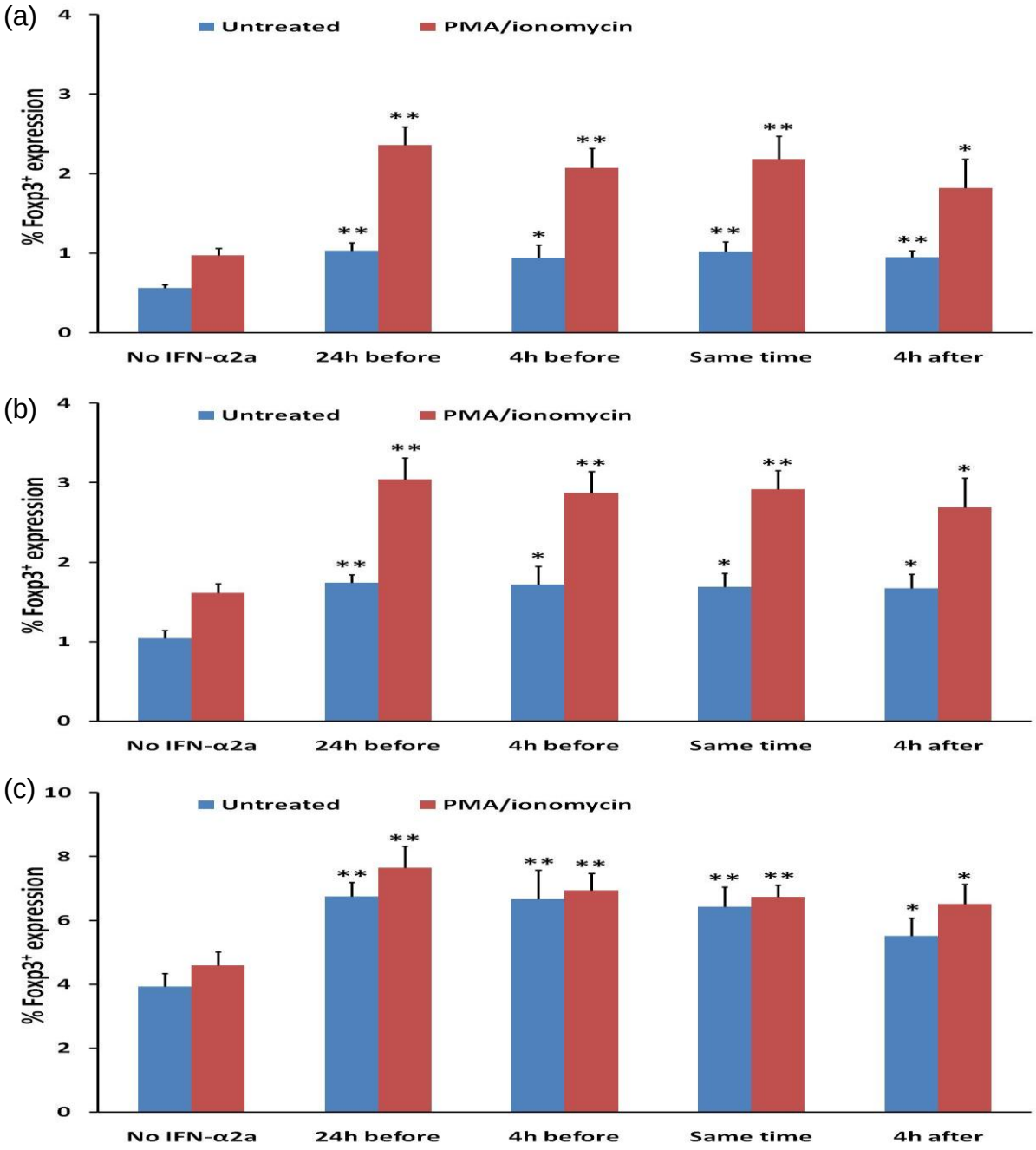


Fig 6.9: The kinetics of upregulatory effect of IFN-α on $CD4^+$ T cells expressing Foxp3

Healthy donor PBMCs were either treated without or with 10^4 U/ml IFN-α2a 24 hours before, 4 hours before, at the same time as, or 4 hours after introduction of PMA & ionomycin to cultures. The timing for the addition of IFN-α2a to cultures was the same for both unstimulated and stimulated cultures, and PBMCs were incubated for 24 hours after PMA/ionomycin stimulation before staining. Results are shown as proportion of (a) $CD4^+$, (b) $CD4^+CD25^+$, (c) $CD4^+CD25^{high}$ T cells expressing Foxp3. The histograms show means ± SEM of triplicate wells from three independent experiments. All experiments used PBMCs from the same donor. Statistical analysis was performed using the Student's T-test. *p<0.05, **p<0.01, compared with controls without IFN-α added.

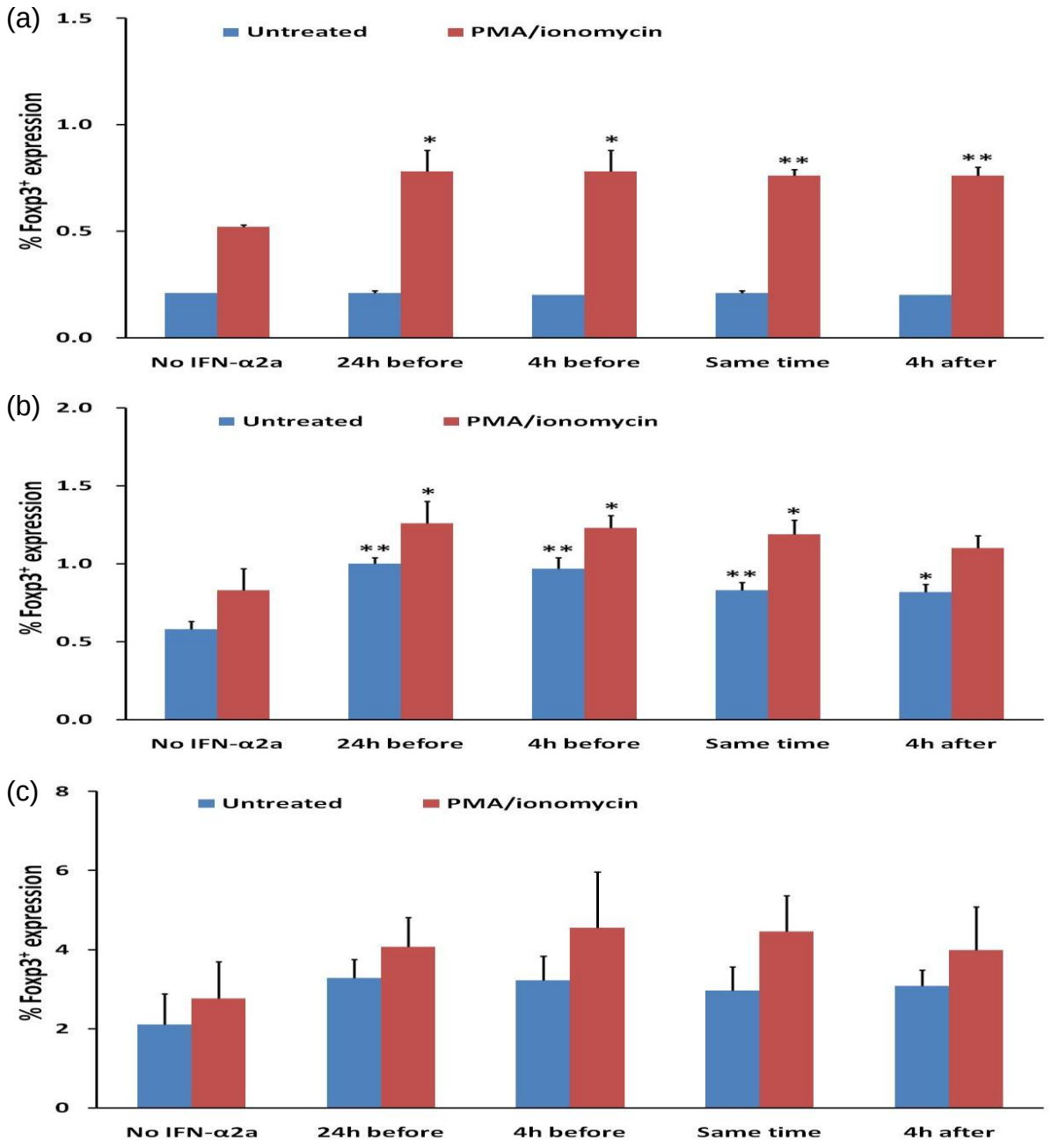


Fig 6.10: The kinetics of upregulatory effect of IFN-α on $CD8^+$ T cells expressing Foxp3

Healthy donor PBMCs were either treated without or with 10^4 U/ml IFN-α2a 24 hours before, 4 hours before, at the same time as, or 4 hours after introduction of PMA & ionomycin to cultures. The timing for the addition of IFN-α2a to cultures was the same for both unstimulated and stimulated cultures, and PBMCs were incubated for 24 hours after PMA/ionomycin stimulation before staining. Results are shown as proportion of (a) $CD8^+$, (b) $CD8^+CD25^+$, (c) $CD8^+CD25^{high}$ T cells expressing Foxp3. The histograms show means ± SEM of triplicate wells from three independent experiments. All experiments used PBMCs from the same donor. Statistical analysis was performed using the Student's T-test. *p<0.05, **p<0.01, compared with controls without IFN-α added.

6.3.5 IFN-α upregulates IL-10 production by both $CD4^+$ and $CD8^+$ T cells

Results from chapter 4 demonstrated a tendency to upregulate the levels of IL-10 producing T cells in BD patients who received subcutaneous IFN-α2b therapy. In order to verify these findings, experiments were set up to test the influence of recombinant IFN-α on $CD4^+$ T cells as well as $CD8^+$ T cells producing IL-10 *in vitro*. Healthy donor PBMCs were cultured without stimulation or stimulated with anti-CD3 and anti-CD28 Ab or PMA and ionomycin in the presence or absence of IFN-α2a (10^1-10^5 U/ml) and IFN-α2b (10^1-10^4 U/ml) before intracellular cytokine staining and flow cytometry. Cytokine expression was analysed by gating on either $CD4^+$/$CD3^+CD8^-$ or $CD8^+$ lymphocyte populations.

Within $CD4^+$ T cells, although proportions of IL-10 producing cells were quite low even after CD3/CD28 or PMA/ionomycin stimulation, the addition of IFN-α was able to induce an upregulation of IL-10 production in a dose-dependent manner under all three culture conditions. When 10^5 U/ml of IFN-α2a was added to untreated PBMC cultures, the proportion of $CD4^+$ T cells expressing IL-10 was increased from 0.6 ± 0.1% to 0.9 ± 0.1% (means ± SD; $p<0.05$) (Fig 6.11a). In correspondence, the percentage of IL-10 producing $CD4^+$ T cells was elevated from 0.9 ± 0.1% to 2.1 ± 0.6% ($p<0.05$) with CD3/CD28 stimulation and from 0.7 ± 0.1% to 1.3 ± 0.1% ($p<0.01$) following PMA/ionomycin stimulation. Similarly, when 10^4 U/ml of IFN-α2b was added to untreated PBMC cultures, the IL-10 production levels by $CD4^+$ T cells was raised from 0.6% to 1.1 ± 0.2% ($p<0.01$) (Fig 6.11b). Correspondingly, the proportion of IL-10 producing $CD4^+$ T cells was upregulated from 1.0 ± 0.2% to 1.7 ± 0.3% ($p<0.05$) with CD3/CD28 stimulation and from 0.8 ± 0.1% to 1.5 ± 0.1% ($p<0.01$) following PMA/ionomycin stimulation.

Having established enhancement of IL-10 levels by $CD4^+$ T cells, data were further analysed to investigate Foxp3 expression in these IL-10 producing cells. It was revealed that the increase in IL-10 production levels was mainly due to $Foxp3^-$ cells. Following CD3/CD28 stimulation, the percentage of $CD4^+$ T cells with IL-10^+Foxp3^- expression was augmented from 0.5 ± 0.3% up to 1.7 ± 0.6% ($p<0.05$) in the presence of 10^5 U/ml of IFN-α2a (Fig 6.12a) and from 0.4 ± 0.2% up to 1.3 ± 0.3% ($p<0.01$) in the presence of 10^4 U/ml of IFN-α2b (Fig 6.12b). On the other hand, very low levels of

$CD4^+$ T cells displayed IL-10^+Foxp3^+ expression and IFN-α was not observed to have any effects on these cells, regardless of the concentrations used (data not shown).

Within $CD8^+$ T cells, an upregulation of IL-10 production by IFN-α in a dose-dependent manner could be detected consistenly as well under all three culture conditions. In the presence of 10^5 U/ml of IFN-α2a, the proportions of $CD8^+$ T cells producing IL-10 were raised from 1.1 ± 0.2% up to 1.9 ± 0.1% ($p<0.01$) in untreated cultures, from 1.9 ± 0.3% up to 4.6 ± 0.4% ($p<0.01$) in CD3/CD28 stimulated cultures, and from 1.6 ± 0.2% up to 2.1 ± 0.1% ($p<0.05$) in cultures activated with PMA/ionomycin (Fig 6.13a). Likewise, in the presence of 10^4 U/ml of IFN-α2b, the percentages of IL-10 producing $CD8^+$ T cells were elevated from 1.1 ± 0.2% up to 1.5 ± 0.1% ($p<0.05$) in untreated cultures, from 1.0 ± 0.2% up to 2.9 ± 0.8% ($p<0.05$) in CD3/CD28 stimulated cultures, and from 1.0 ± 0.1% up to 1.8 ± 0.3% ($p<0.05$) with PMA/ionomycin stimulation (Fig 6.13b).

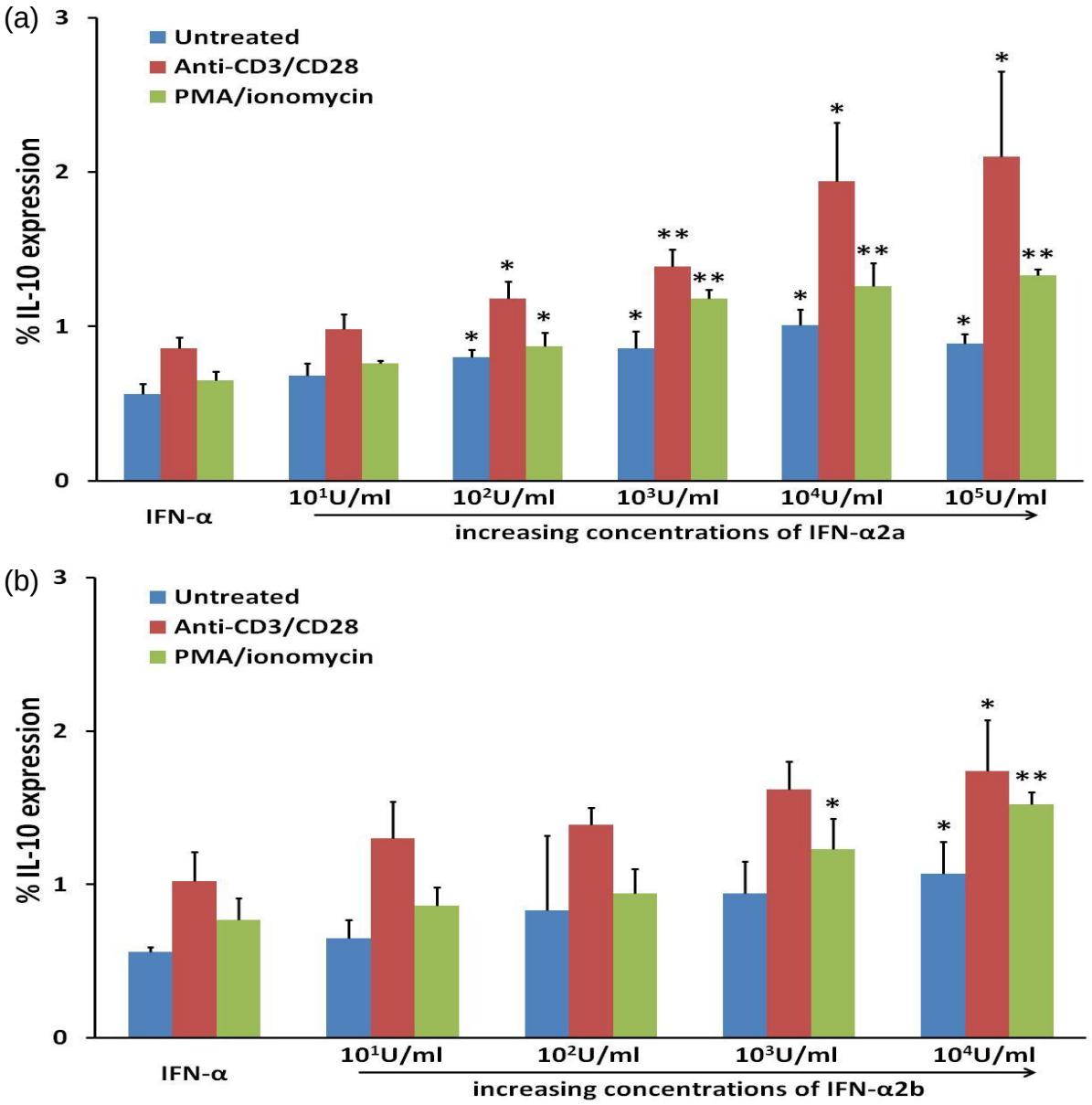


Fig 6.11: IFN-α upregulates IL-10 expression by $CD4^+$ T cells

Healthy donor PBMCs were untreated, stimulated with anti-CD3 & anti-CD28 or PMA & ionomycin for 24 or 39 hours. Various concentrations of IFN-α was added at the same time as the polyclonal stimuli to cultures. Results are shown as proportion of $CD4^+$ T cells producing IL-10. In (a) and (b), the effects of IFN-α2a and IFN-α2b are shown respectively. Results in (a) and (b) are representative of six independent experiments each. PBMCs were from four different donors for both forms of IFN-α. Only one experiment for both IFN-α2a and IFN-α2b used both CD3/CD28 and PMA/ionomycin stimulation. 10^1 U/ml of IFN-α (2a and 2b) and 10^5 U/ml of IFN-α2a were tested only in half of these experiments. Data are means ± SD values of triplicate wells. Statistical analysis was performed using the Student's T-test. *p<0.05, **p<0.01, compared with controls without addition of IFN-α.

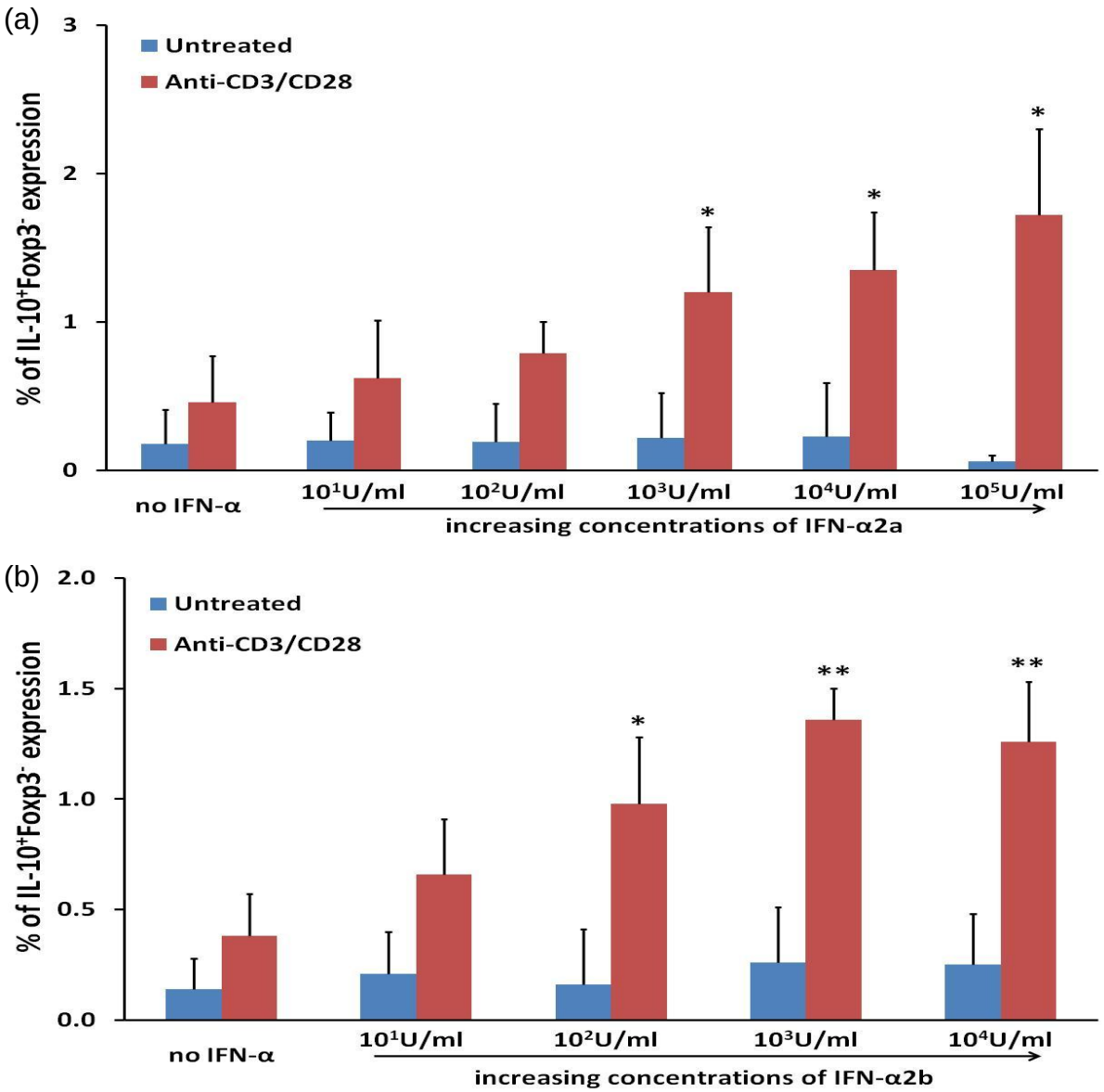


Fig 6.12: Upregulation of IL-10 expression by IFN-α is mainly in the Foxp3$^-$ subset in CD4$^+$ T cells

Healthy donor PBMCs were either untreated or stimulated with anti-CD3 and anti-CD28 Ab for 39 hours. Various concentrations of IFN-α was added at the same time as the polyclonal stimuli to cultures. Results are shown as proportion of CD4$^+$ T cells expressing IL-10$^+$Foxp3$^-$ phenotype. In (a) and (b), the effects of IFN-α2a and IFN-α2b are shown respectively. Results in (a) and (b) are representative of three independent experiments each. PBMCs were from two different donors for both forms of IFN-α. Data are means ± SD values of triplicate wells. Statistical analysis was performed using the Student's T-test. *p<0.05, **p<0.01, compared with controls without addition of IFN-α.

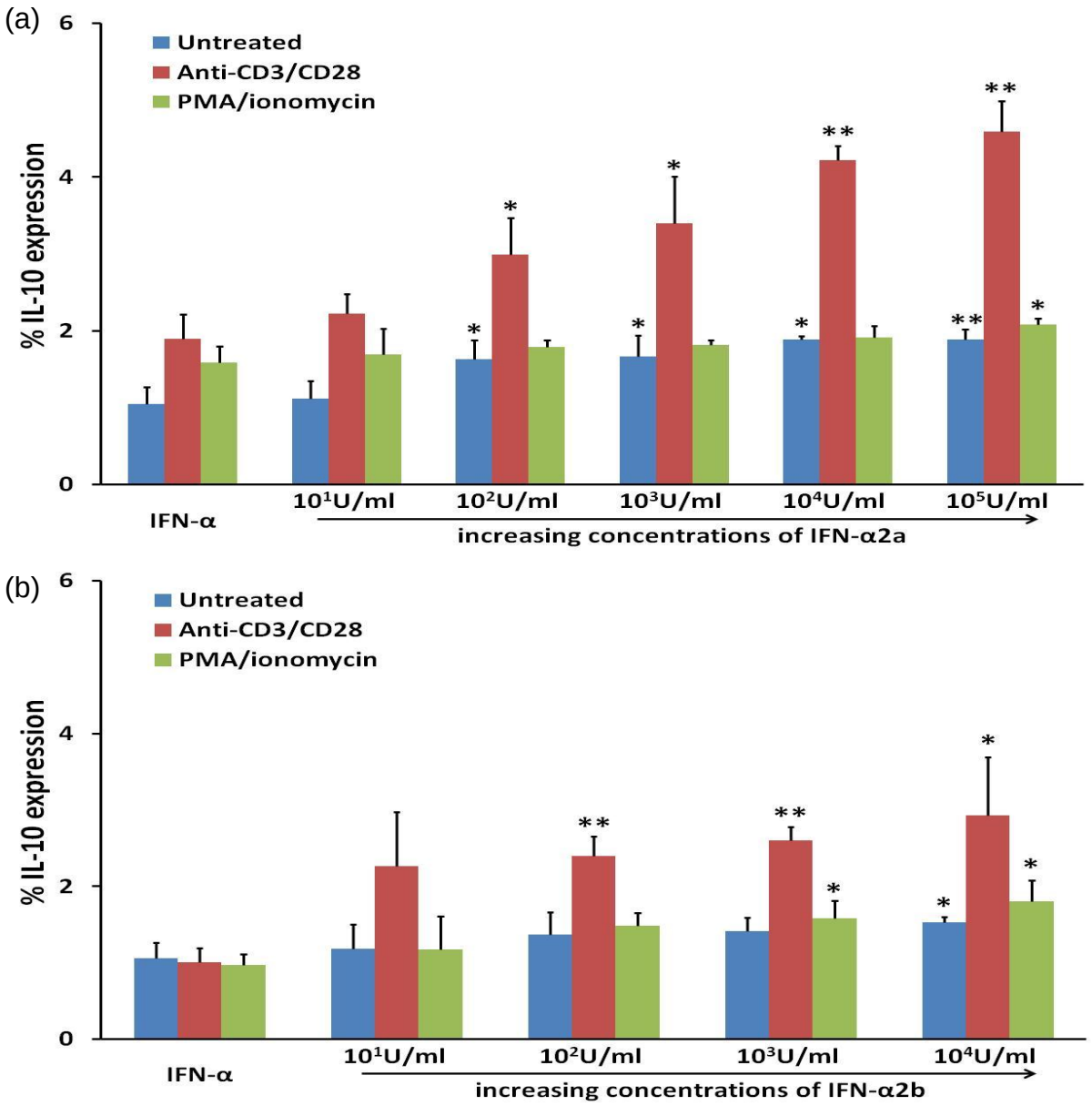


Fig 6.13: IFN-α upregulates IL-10 expression by $CD8^+$ T cells

Healthy donor PBMCs were untreated, stimulated with anti-CD3 & anti-CD28 or PMA & ionomycin for 24 hours. Various concentrations of IFN-α was added at the same time as the polyclonal stimuli to cultures. Results are shown as proportion of $CD8^+$ T cells producing IL-10. In (a) and (b), the effects of IFN-α2a and IFN-α2b are shown respectively. Results in (a) and (b) are representative of three independent experiments each. PBMCs were from two different donors for both forms of IFN-α. Only one experiment for both IFN-α2a and IFN-α2b used both CD3/CD28 and PMA/ionomycin stimulation. 10^1 U/ml of IFN-α (2a and 2b) and 10^5 U/ml of IFN-α2a were tested only once in these experiments. Data are means ± SD values of triplicate wells. Statistical analysis was performed using the Student's T-test. *p<0.05, **p<0.01, compared with controls without addition of IFN-α.

6.3.6 The kinetics of the upregulatory effect of IFN-α on IL-10 production by T cells

Having established the potential of IFN-α to induce IL-10 production by both $CD4^+$ and $CD8^+$ T cells *in vitro*, the next objective was to investigate the influence of IFN-α on kinetics of IL-10 production. PBMCs isolated from healthy individuals were cultured without IFN-α treatment or with 10^4 U/ml IFN-α2a added 24 hours before, 4 hours before, at the same time as, or 4 hours after PMA/ionomycin stimulation. The timing for the introduction of IFN-α to cultures was the same for both unstimulated and stimulated cultures, and PBMCs were incubated for 24 hours after addition of PMA/ionomycin before intracellular cytokine staining and flow cytometry. IL-10 expression levels were analysed by gating on $CD4^+$/$CD3^+CD8^-$ or $CD8^+$ lymphocyte populations.

Evidently, the introduction of 10^4 U/ml IFN-α2a to PBMC cultures at all time-points tested showed a positive effect on IL-10 production by $CD4^+$ T cells. Despite not being statistically significant, pre-treatment of PBMCs with IFN-α demonstrated better capacity in IL-10 induction within $CD4^+$ T cells than adding IFN-α after polyclonal activation with PMA/ionomycin or at the last time-point in the case of untreated cultures. Following activation with PMA/ionomycin, the proportion of $CD4^+$ T cells producing IL-10 was elevated from 1.0 ± 0.1% to 1.7 ± 0.1% (means ± SEM; $p<0.01$) with 24 hours of IFN-α pre-treatment (Fig 6.14a), and only up to 1.3 ± 0.1% ($p<0.05$) when IFN-α was added after PBMC activation. In accordance, the IL-10 expression levels were upregulated from 0.7 ± 0.1% up to 1.3 ± 0.1% ($p<0.01$) with 24 hours of IFN-α pre-treatment in untreated cultures and only to 1.1 ± 0.1% when IFN-α was added at the last time-point.

Similarly, IL-10 expression levels by $CD8^+$ T cells were augmented significantly with the introduction of 10^4 U/ml IFN-α2a at any time points following PMA/ionomycin stimulation. However, 24 hour of IFN-α pre-treatment was the most effective as proportion of $CD8^+$ T cells producing IL-10 was raised from 1.2 ± 0.1% up to 1.9 ± 0.2% ($p<0.01$) when compared to just 1.5 ± 0.1% ($p<0.05$) with IFN-α added at the last time-point (Fig 6.14b). In untreated cultures, although an upregulation of IL-10 levels within $CD8^+$ T cell population was observed with IFN-α treatment at all time-

points, the increase was only statistically significant when comparing 24 hours pre-treatment data (1.9 ± 0.2%; $p<0.05$) to ones without IFN-α treatment (0.9 ± 0.2%). In fact, the levels of IL-10 producing $CD8^+$ T cells were significantly lower when IFN-α were added at later time-points (1.2 ± 0.2% and 1.1 ± 0.2%; $p<0.05$) when evaluated against data with 24 hours of IFN-α pre-treatment (Fig 6.14b).

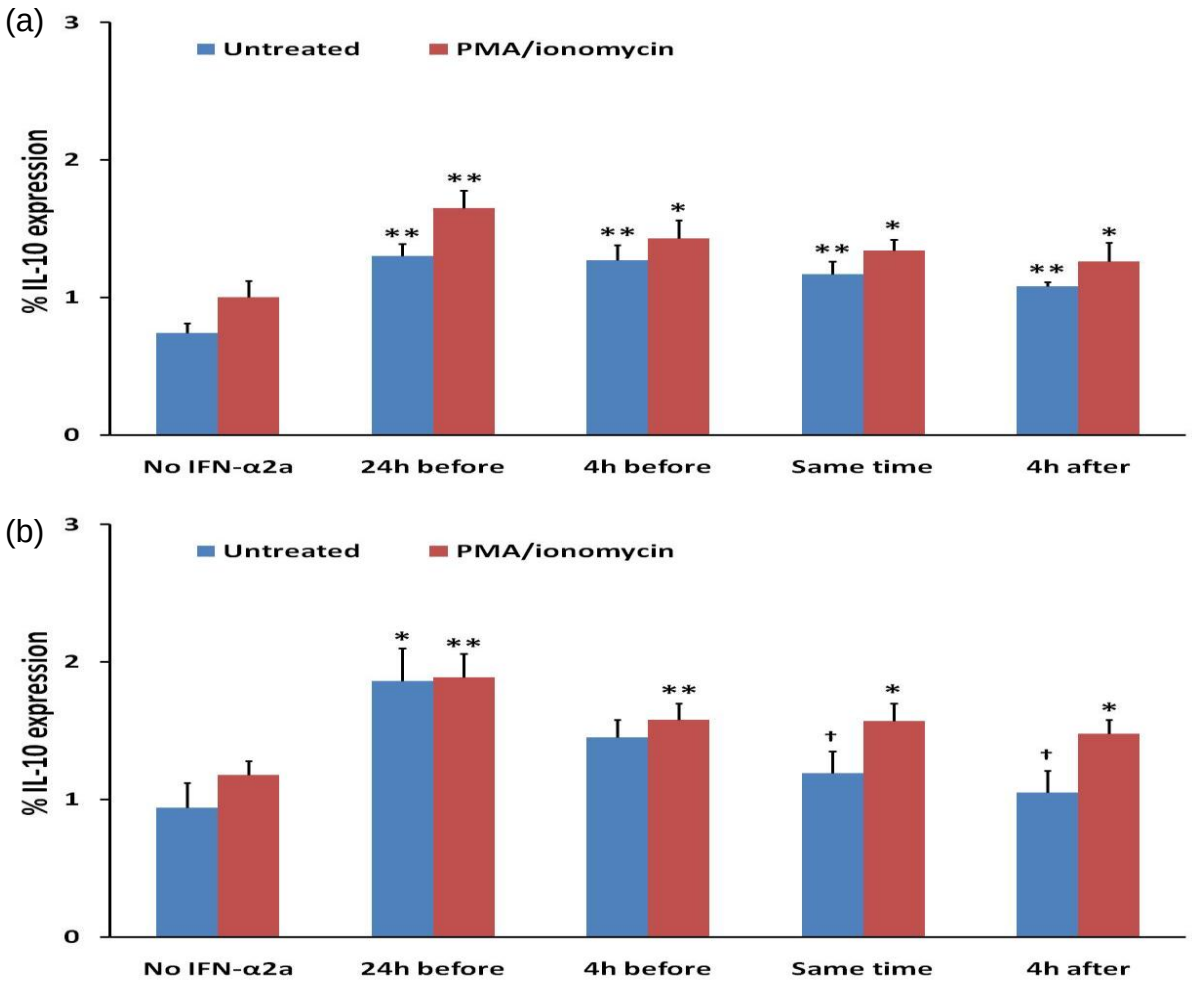


Fig 6.14: The kinetics of upregulatory effect of IFN-α on IL-10 expression by T cells

Healthy donor PBMCs were either treated without or with 10^4 U/ml IFN-α2a 24 hours before, 4 hours before, at the same time as, or 4 hours after introduction of PMA & ionomycin to cultures. The timing for the addition of IFN-α2a to cultures was the same for both unstimulated and stimulated cultures, and PBMCs were incubated for 24 hours after PMA/ionomycin stimulation before staining. Results are shown as proportion of (a) $CD4^+$ and (b) $CD8^+$ T cells producing IL-10. The histograms show means ± SEM of triplicate wells from three independent experiments. All experiments used PBMCs from the same donor. Statistical analysis was performed using the Student's T-test. *p<0.05, **p<0.01, compared with controls without IFN-α added.

6.3.7 IFN-α upregulates TGF-β production by both $CD4^+$ and $CD8^+$ T cells

A tendency of T cells to display a heightened TGF-β level in BD patients under subcutaneous IFN-α2b therapy was detected in chapter 4. In order to verify this effect, *in vitro* experiments were performed to examine the influence of recombinant IFN-α on $CD4^+$ and $CD8^+$ T cells producing TGF-β. Healthy donor PBMCs were cultured without stimulation or stimulated with anti-CD3 and anti-CD28 Ab or PMA and ionomycin for 24 hours in the presence or absence of IFN-α2a (10^1-10^5 U/ml) and IFN-α2b (10^1-10^4 U/ml) before intracellular cytokine staining and flow cytometry. Cytokine expression was analysed by gating on either $CD4^+$/$CD3^+CD8^-$ or $CD8^+$ lymphocyte populations.

Regarding $CD4^+$ T cells, higher concentrations of IFN-α were able to induce an enhancement of TGF-β levels, especially after polyclonal activation. When 10^5 U/ml of IFN-α2a was added to CD3/CD28 stimulated cultures, the proportion of $CD4^+$ T cells producing TGF-β was upregulated from 1.4 ± 0.4% to 2.6 ± 0.5% (means ± SD; $p<0.05$) (Fig 6.15a). In correspondence, the percentage of TGF-β expressing $CD4^+$ T cells was elevated from 1.7 ± 0.5% to 2.9 ± 0.4% ($p<0.05$) with PMA/ionomycin stimulation. Similarly, when IFN-α2b was added to PBMC cultures stimulated with PMA/ionomycin, a dose-dependent increase in TGF-β expression levels by $CD4^+$ T cells was consistently observed. The most potent dose of IFN-α2b was at a concentration of 10^4 U/ml and the TGF-β expression levels by $CD4^+$ T cells was raised from 0.7 ± 0.1% without the influence of IFN-α to 2.0 ± 0.3% ($p<0.01$) (Fig 6.15b).

With regards to $CD8^+$ T cells, an upregulation of TGF-β expression by higher concentrations of IFN-α could be observed consistenly as well. In the presence of 10^5 U/ml of IFN-α2a, the proportion of $CD8^+$ T cells producing TGF-β was increased from 1.0 ± 0.6% to 2.8 ± 0.4% ($p<0.01$) in untreated cultures and from 3.2 ± 0.8% to 5.1 ± 0.3% ($p<0.05$) in cultures activated with anti-CD3/CD28 Ab (Fig 6.16a). Similar trend was detected in cultures stimulated with PMA/ionomycin as well in the presence of higher concentrations of IFN-α2a. Likewise, in the presence of 10^4 U/ml of IFN-α2b, the proportion of TGF-β expressing $CD8^+$ T cells was upregulated to 3.6 ± 0.6% ($p<0.05$) with CD3/CD28 stimulation and 2.3 ± 0.2% ($p<0.05$) without cell activation from 1.9 ± 0.7% and 1.0 ± 0.5% respectively (Fig 6.16b).

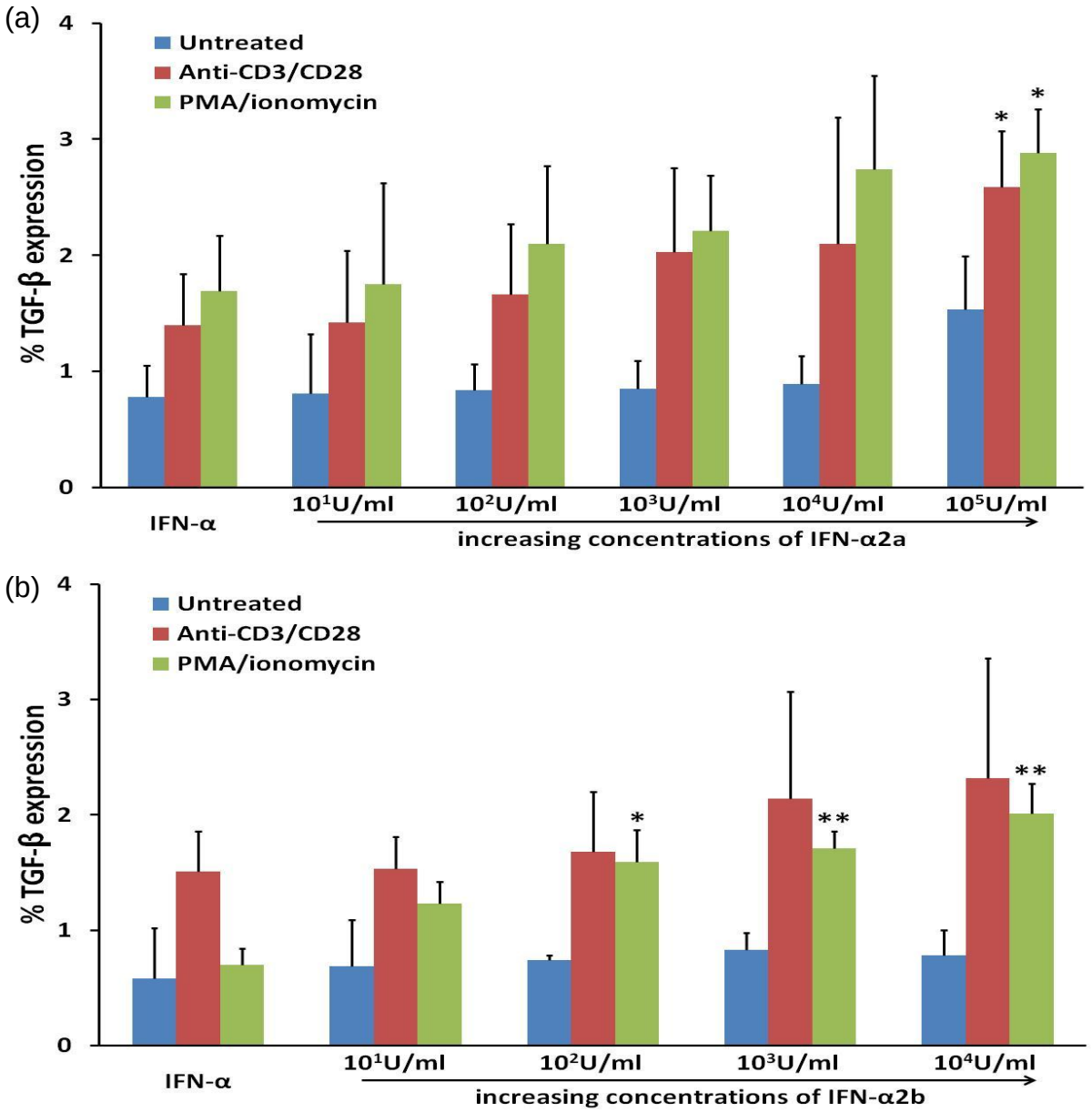


Fig 6.15: IFN-α upregulates TGF-β expression by CD4$^+$ T cells

Healthy donor PBMCs were untreated, stimulated with anti-CD3 & anti-CD28 or PMA & ionomycin for 24 hours. Various concentrations of IFN-α was added at the same time as the polyclonal stimuli to cultures. Results are shown as proportion of CD4$^+$ T cells producing TGF-β. In (a) and (b), the effects of IFN-α2a and IFN-α2b are shown respectively. Results in (a) and (b) are representative of three independent experiments each. PBMCs were from two different donors for both forms of IFN-α. Only one experiment for both IFN-α2a and IFN-α2b used both CD3/CD28 and PMA/ionomycin stimulation. 10^1 U/ml of IFN-α (2a and 2b) and 10^5 U/ml of IFN-α2a were tested only once in these experiments. Data are means ± SD values of triplicate wells. Statistical analysis was performed using the Student's T-test. *p<0.05, **p<0.01, compared with controls without addition of IFN-α.

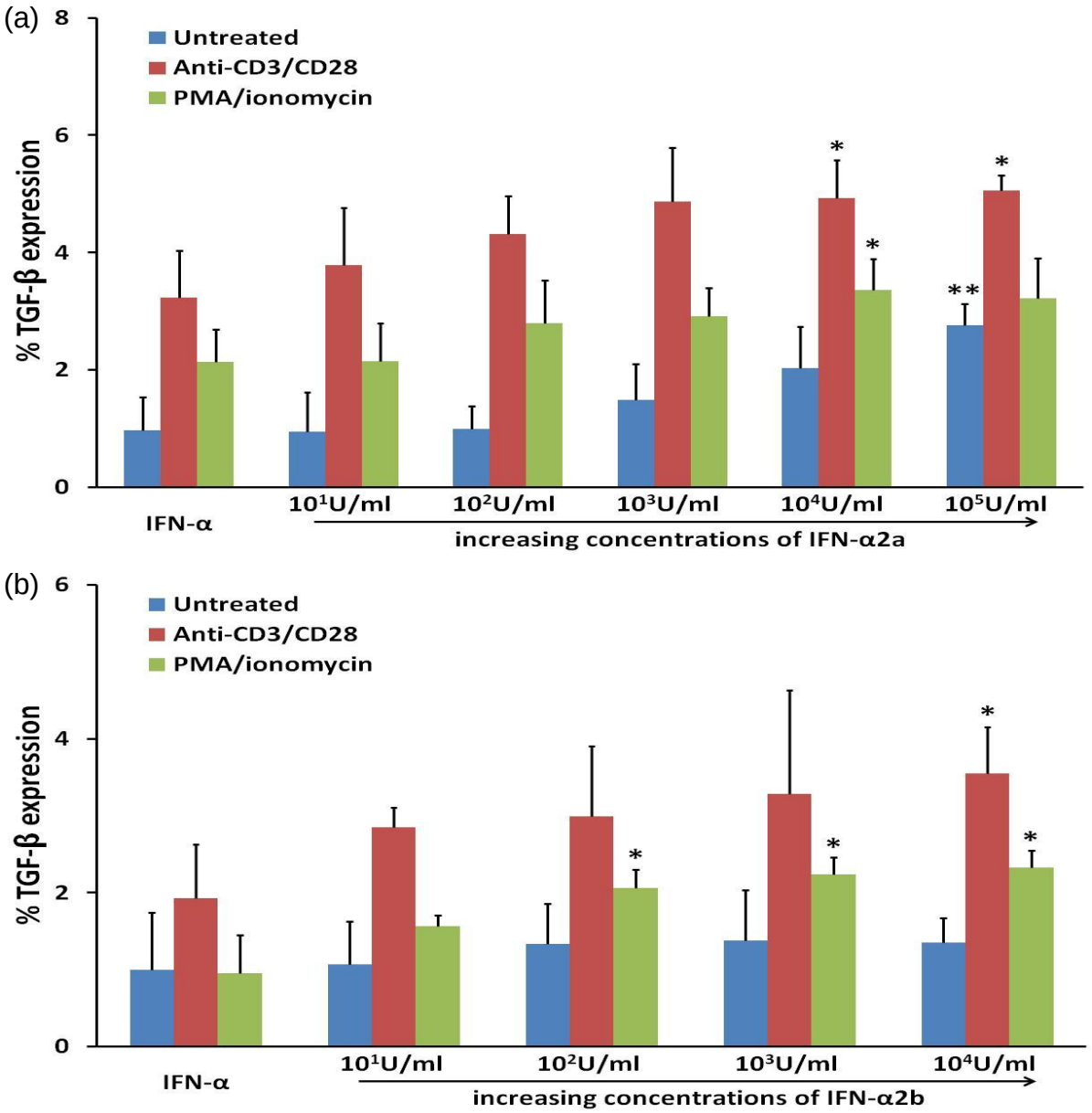


Fig 6.16: IFN-α upregulates TGF-β expression by $CD8^+$ T cells

Healthy donor PBMCs were untreated, stimulated with anti-CD3 & anti-CD28 or PMA & ionomycin for 24 hours. Various concentrations of IFN-α was added at the same time as the polyclonal stimuli to cultures. Results are shown as proportion of $CD8^+$ T cells producing TGF-β. In (a) and (b), the effects of IFN-α2a and IFN-α2b are shown respectively. Results in (a) and (b) are representative of three independent experiments each. PBMCs were from two different donors for both forms of IFN-α. Only one experiment for both IFN-α2a and IFN-α2b used both CD3/CD28 and PMA/ionomycin stimulation. 10^1 U/ml of IFN-α (2a and 2b) and 10^5 U/ml of IFN-α2a were tested only once in these experiments. Data are means ± SD values of triplicate wells. Statistical analysis was performed using the Student's T-test. *p<0.05, **p<0.01, compared with controls without addition of IFN-α.

6.3.8 The kinetics of the upregulatory effect of IFN-α on TGF-β production by T cells

After confirming the ability of IFN-α to promote higher TGF-β levels in both $CD4^+$ and $CD8^+$ T cells *in vitro*, the next goal was to explore the kinetics of the effect induced by IFN-α. PBMCs isolated from healthy individuals were cultured without IFN-α treatment or with 10^4 U/ml IFN-α2a added 24 hours before, 4 hours before, at the same time as, or 4 hours after PMA/ionomycin stimulation. The timing for the introduction of IFN-α to cultures was the same for both unstimulated and stimulated cultures, and PBMCs were incubated for 24 hours after addition of PMA/ionomycin before intracellular cytokine staining and flow cytometry. TGF-β expression levels were analysed by gating on $CD4^+$/$CD3^+CD8^-$ or $CD8^+$ lymphocyte populations.

In accordance to IL-10 kinetics data, pre-treatment of PBMCs with 10^4 U/ml of IFN-α2a appeared to have greater positive effect on TGF-β production by $CD4^+$ T cells than treating cells with IFN-α post activation or at later time-point (Fig 6.17a). In untreated cultures, the proportion of $CD4^+$ T cells producing TGF-β was upregulated from 0.5% up to 1.1% ($p<0.01$) with IFN-α added at the earliest time-point, compared to only 0.9% ($p<0.01$) with IFN-α added at the last time-point. With PMA/ionomycin stimulation, TGF-β expression levels by $CD4^+$ T cells were raised from 1.1 ± 0.1% up to 1.6 ± 0.2% (means ± SEM; $p<0.01$) with 24 hours of IFN-α pre-treatment and only 1.4 ± 0.2% (p=ns) with IFN-α post-treatment.

With regards to $CD8^+$ T cells, comparable amount of TGF-β expression enhancement was observed in untreated cultures when IFN-α was added at any time-points tested (Fig 6.17b). However, with PMA/ionomycin stimulation, pre-treatment of PBMCs with IFN-α again demonstrated better capacity in TGF-β induction within $CD8^+$ T cells than adding IFN-α after polyclonal activation. In the presence of PMA/ionomycin activation, the proportion of $CD8^+$ T cells producing TGF-β was elevated from 0.9 ± 0.1% without IFN-α treatment up to 1.3 ± 0.1% ($p<0.01$) with 24 hours of IFN-α pre-treatment and only 1.1 ± 0.1% (p=ns) with IFN-α added at the last time-point.

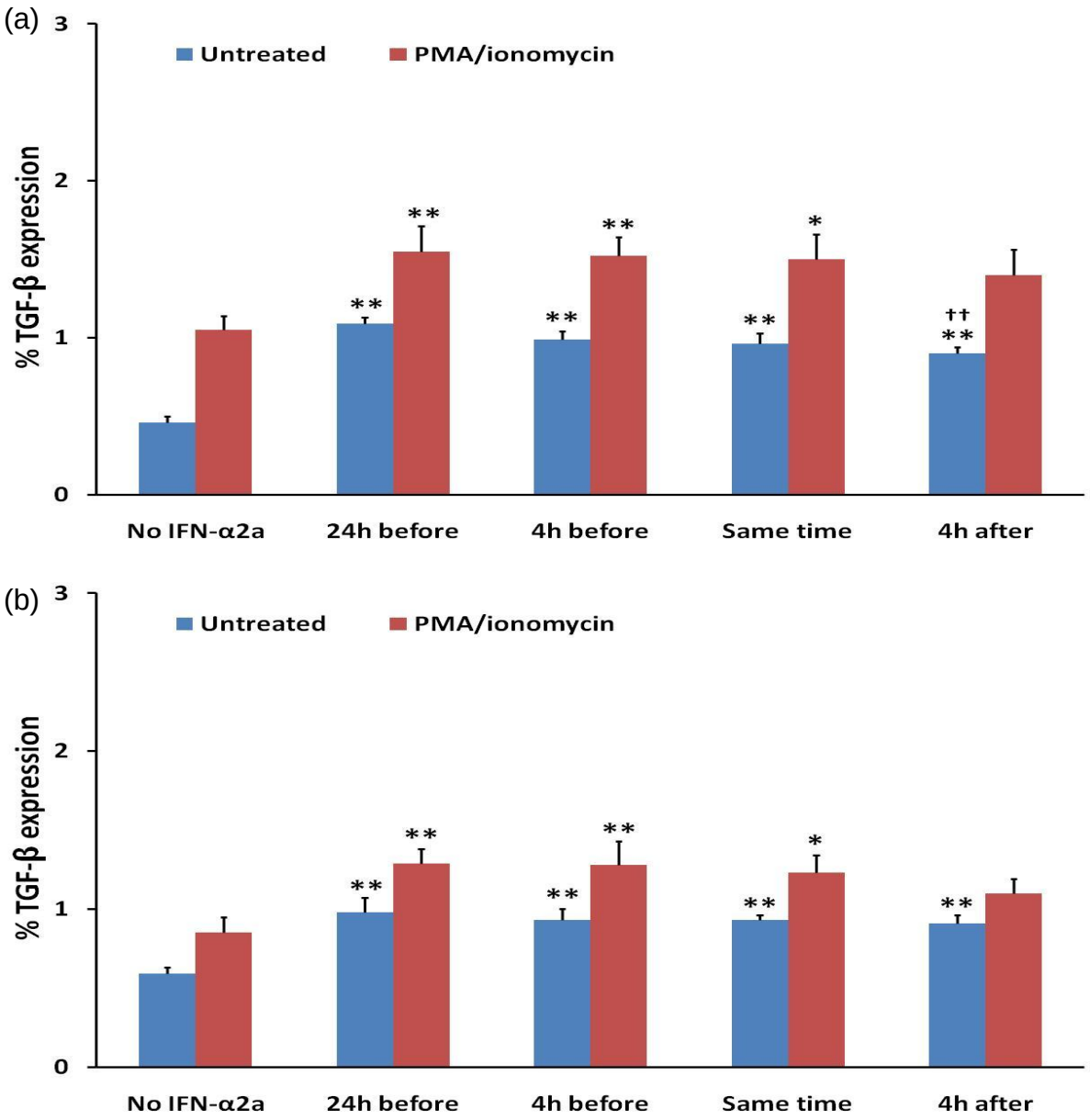


Fig 6.17: The kinetics of upregulatory effect of IFN-α on TGF-β expression by T cells

Healthy donor PBMCs were either treated without or with 10^4 U/ml IFN-α2a 24 hours before, 4 hours before, at the same time as, or 4 hours after introduction of PMA & ionomycin to cultures. The timing for the addition of IFN-α2a to cultures was the same for both unstimulated and stimulated cultures, and PBMCs were incubated for 24 hours after PMA/ionomycin stimulation before staining. Results are shown as proportion of (a) $CD4^+$ and (b) $CD8^+$ T cells producing TGF-β. The histograms show means ± SEM of triplicate wells from three independent experiments. All experiments used PBMCs from the same donor. Statistical analysis was performed using the Student's T-test. *p<0.05, **p<0.01, compared with controls without IFN-α added. ††p<0.01, compared with data with 24-hour IFN-α pre-treatment.

6.4 Discussion

A number of regulatory T cell subsets have been identified, and there is growing evidence that these cells are essential for controlling immune responses. Although we have much to learn about the biology of Treg cells, it has become clear that via expression of inhibitory cell-surface molecules and production of cytokines such as IL-10 and TGF-β, Treg cells maintain peripheral tolerance to self and foreign Ags, and that in their absence the host may succumb to a variety of autoimmune and chronic inflammatory diseases (Levings et al. 2002). Remarkably, Treg cells are able to suppress activated T cells in vitro at a ratio of one Treg to 20 effector cells (Dieckmann et al. 2001). Therefore, a large clone size for Treg cells is less important than for conventional T cells, and induction of naive precursor T cells into Foxp3-expressing cells with regulatory capacity has become an attractive mechanism to dampen an overactive acute inflammation or to curtail the response to a chronic unresolved challenge in theory.

6.4.1 Setting up Treg *in vitro* test models using antigens and polyclonal stimuli

Although immunosuppressive activity of Treg cells is exerted in a non-specific manner, the identification of few Ag recognised by Tregs in a murine study suggests that *in vivo* expansion of these cells could result from Ag-specific stimuli (Thornton & Shevach 2000). Also, it has been described that Tregs express a range of TLRs, stimulation of which can have significant effects on the expansion and suppressive function of Treg cells (Allan et al. 2008b). Tregs treated with HSP60 were reported to be more effective than untreated ones in suppressing $CD4^+CD25^-$ or $CD8^+$ T cells and the enhancing effects of HSP60 on Treg cells involved TLR2 signalling. Besides, it was indicated that the HSP60-treated Tregs induced their target $CD4^+CD25^-$ T cells to secrete IL-10 (Zanin-Zhorov et al. 2006). Zymosan, a stimulus for TLR2 and dectin-1 (Dillon et al. 2006), was demonstrated to promote IL-10 production by DCs at a concentration of 200μg/ml after 18 hours of culture as tested by ELISA of culture supernatants (Gerosa et al. 2008).

In our preliminary experiments, two TLR ligands were used. Zymosan was not found to induce an upregulation of either Foxp3 or IL-10 expression, even at 100μg/ml. LPS, on the other hand, showed the tendency to induce higher expression levels of Foxp3 by $CD4^+CD25^{hi}$ T cells at 5-20μg/ml and IL-10 by $CD4^+$ T cells at 5μg/ml with longer incubation time. Another antigen made from candida extracts also displayed some potential in promoting $Foxp3^+$ T cells and IL-10 cytokine. However, of all the antigens used, there was none better than ATRA, at a range of concentrations, in enhancing Foxp3 expression. This is consistent with reports of a crucial regulatory role for retinoic acid in promoting Foxp3 induction (Elias et al. 2008;Mucida et al. 2007). However, the presence of ATRA in PBMC cultures was not observed to increase IL-10 production.

Due to consistent observation that polyclonal activation by anti-CD3/CD28 Ab and PMA/ionomycin enhanced greater amount of Foxp3 and IL-10 expression by $CD4^+$ T cells, healthy donor PBMCs with or without polyclonal activation were chosen for the subsequent tests on the *in vitro* immunoregulatory effects of IFN-α.

6.4.2.1 $CD8^+$ Treg cells

Like in $CD4^+$ T cells, polyclonal activation of PBMCs can lead to induction of Foxp3 expression by human $CD8^+$ T cells *in vitro* (Gavin et al. 2006;Morgan et al. 2005). Notably, the ability to express Foxp3 has been demonstrated to be inherent to $CD8^+$ T cells, and it is independent of the mode of TCR activation yet critically dependent on IL-2 or IL-15 signalling (Ahmadzadeh, Antony, & Rosenberg 2007).

Recently, it has been indicated that highly suppressive adaptive $CD8^+CD25^+Foxp3^+$ T cells can be induced from $CD8^+CD25^-$ T cells by continuous antigen stimulation in the presence of monocytes as well. These adaptive $CD8^+$ Treg cells express markers associated with a regulatory phenotype, and inhibit $CD4^+$ and $CD8^+$ T cell proliferation and cytokine production. A contact-dependent mechanism appears to be the key for their suppressive activity, even though these regulatory cells express prostaglandin E2, IL-10, and TGF-β (Mahic et al. 2008). An alternative way to induce $CD8^+$ Treg cells is to stimulate PBMCs from primed donors with the

mycobacterial vaccine Bacillus Calmette Guerin (BCG) yielding $CD8^+CD25^+$lymphocyte-activation gene 3 $(LAG\text{-}3)^+Foxp3^+CCL4^+$ cells. The suppressive function of such $CD8^+$ Treg cells was assigned to secretion of the chemokine CCL4 (Joosten et al. 2007).

6.4.2.2 The influence of IFN-α on Foxp3 expressing $CD4^+$ and $CD8^+$ T cells

A dose-dependent upregulation of Foxp3 expression by either exogenous IFN-α2a or IFN-α2b was clearly evident in $CD4^+$, $CD4^+CD25^+$, and $CD4^+CD25^{high}$ T cells in our experiments. Also, a dose-dependent enhancement of Foxp3 expression by either exogenous IFN-α2a or IFN-α2b was observed in $CD8^+$, $CD8^+CD25^+$ T cells, but not $CD8^+CD25^{high}$ T cells. The difference in results with $CD8^+CD25^{high}$ T cell population could be related to the much smaller number of cells detected with flow cytometry and hence larger variation in data as a result. Moreover, the upregulation in the proportion of $CD8^+$ T cells expressing Foxp3 observed in our data was likely contributed not only by the promotion of $CD8^+CD25^+Foxp3^+$ T cells but also other Treg subsets such as the antigen-specific adaptive $CD8^+CD28^-$ Tregs, which have been described to express both CTLA-4 and Foxp3 (Scotto et al. 2004).

In support of our results, it was reported that *in vitro* treatment of PBMCs from MS patients with IFN-α could induce an increased frequency of $CD4^+CD25^{high}$ T cells and a linear correlation between the CD25 mRNA and Foxp3 mRNA expression was also observed (Penton-Rol et al. 2008). No study was found to describe the effect of *in vitro* IFN-α treatment on Foxp3 expressing $CD8^+$ T cells.

Of note, there are two isoforms of human Foxp3, a full-length form and a smaller form lacking exon 2, and they were reported to be induced equally in freshly activated $CD4^+CD25^-$ T cells (Allan et al. 2005). It is likely that the two isoforms are expressed in different Treg cell subsets. However, further analysis of our data could not be performed as reagents that distinguish between the isoforms by flow cytometry are yet to be developed.

6.4.2.3 Foxp3 levels in comparison to other studies

In our experiments on healthy donor PBMCs, polyclonal stimulation with anti-CD3/CD28 and/or PMA/ionomycin was applied for 24 hours but sometimes 39 hours to PBMC cultures. Notably, a recent study demonstrated that activation with anti-CD3/CD28-coated beads led to induction of newly expressing $Foxp3^+$ cells as early as 24 hours, which peaked at 72 hours and then decreased over a period of 7-10 days (Allan et al. 2007). From our data, the mean proportions of T cells expressing Foxp3 were generally less than 3% for $CD4^+$ T cells and less than 1.5% for $CD8^+$ T cells with or without activation. The Foxp3 expression levels achieved are comparable to a recent study. It was reported that the percentages of $CD4^+$ T cells expressing Foxp3 were between 3-6% depending on the types of antibodies used and the proportions of $CD8^+$ T cells expressing Foxp3 were around 0.5% in normal subjects (Gavin et al. 2006).

6.4.3.1 Biology of IL-10 and Tr1 cells

Suppression of specific immune response and induction of anergy in T cells are essential processes in regulation of immune defense. T cell anergy can be induced when $CD4^+$ or $CD8^+$ T cells are activated in the presence of IL-10 or with APCs which have been pre-treated with IL-10 (Groux et al. 1996;Steinbrink et al. 2002). IL-10, a suppressor cytokine of T cell proliferative and cytokine responses, thus plays an important role in peripheral T cell tolerance and contributes to the homeostasis of the immune system. This effect of IL-10 is not only due to downregulation of co-stimulatory molecules on APCs, but also dependent on direct effects on the T cells themselves (Moore et al. 2001).

IL-10-anergised T cells become non-responsive and fail to produce cytokines. In addition, these cells acquire regulatory activity and attenuate T cell responses. This function is not dependent on production of immunosuppressive cytokines, but rather is mediated by cell-cell contact-dependent mechanisms via suppressive molecules expressed on the cell surface (Groux et al. 1996;Steinbrink et al. 2002) Interestingly, when IL-10-anergised T cells are put through forced proliferation, and likely following

repetitive exposure to antigen, the cells regain some ability to proliferate and IL-10-producing Tr1 cells are induced as a result (Allan et al. 2008b).

Fully differentiated Tr1 cells may be more potent suppressor cells than anergic T cells *in vivo*, since they have been shown to inhibit both APCs and T cells, and may even induce the development of Tr1 cells from naive $CD4^+$ T cells via their ability to produce IL-10 (Roncarolo et al. 2001). Cultures containing Tr1 cells have been demonstrated to display reduced responses to alloantigens via a mechanism that was partially mediated by IL-10 and TGF-β. Compared to IL-10, which has been considered essential for both the differentiation and effector function of Tr1 cells, TGF-β appeared to play little part in the enhancement of Tr1 cell development (Levings et al. 2001). Of note, $CD4^+CD25^+$ Treg cells, which were observed to be upregulated in our experiments, may actively promote the differentiation of Tr1 cells as well (Levings et al. 2002).

6.4.3.2 The influence of IFN-α on IL-10 producing $CD4^+$ and $CD8^+$ T cells

From our experimental data, a dose-dependent upregulation of IL-10 level was consistently detected in both $CD4^+$ and $CD8^+$ T cells with IFN-α treatment *in vitro*. These results are in line with an early study evaluating the effects of IFN-α on resting $CD4^+$ cells purified from normal PBMCs and stimulated with PMA in conjunction with anti-CD28 Ab, which found IFN-α enhanced IL-10 production in a dose-dependent manner with a four-fold increase in IL-10 levels in the presence of 10^4 IU/ml IFN-α (Schandene et al. 1996). Increased IL-10 mRNA accumulation was found in the system as well. Also, another early study reported that IFN-α enhanced IL-10 expression by unseparated PBMCs. Upregulation of IL-10 production by either activated $CD4^+$ T cells or activated monocytes were demonstrated as well, but these purified cells failed to express IL-10 with IFN-α treatment unless they were activated with appropriate stimuli. Activation with PHA and PMA was required for purified $CD4^+$ T cells and LPS was required for purified monocytes (Aman et al. 1996). In contrast to IFN-α, IL-4 and IFN-γ were not observed to regulate IL-10 synthesis by CD3/CD28 stimulated $CD4^+$ T cells. Recently, IFN-α was found to significantly

increase IL-10 production by either anti-CD3/CD28 stimulated PBMCs or $CD4^+$ T cells in culture supernatants from healthy patients (Liu et al. 2011).

In accordance to our *ex vivo* data, the upregulation in IL-10 expression level in $CD4^+$ T cells was mostly attributed to $Foxp3^-$ cells. It has been reported that $CD4^+$ T cells from cord blood or peripheral blood in the presence of IFN-α and IL-10 resulted in a population of cells that display a Tr1 phenotype of cytokine production: $IL\text{-}10^+IFN\text{-}\gamma^+IL\text{-}2^{-/low}IL\text{-}4^-$. The data demonstrated that the additive effects of IL-10 and IFN-α acted directly on T cells to induce differentiation of Tr1 cells (Levings et al. 2001). In keeping with the observation, it was reported that IL-10-producing regulatory T cells can be induced after priming of mature $CD11c^+$ DCs by IFN-α (Ito et al. 2001). Therefore, it is likely the enhanced IL-10 production synergised with exogenous IFN-α to promote a larger and more stable population of Tr1 cells in our experiments.

Furthermore, a population of $CD8^+CXCR3^+$ cells have recently been identified and suggested as the counterpart of murine $CD8^+CD122^+$ Tregs. These cells are $CD28^+$ and able to produce IL-10 (Shi et al. 2009). It is quite possible that the increased number of $CD8^+IL\text{-}10^+$ T cells observed in our experiments contain some cells from such population.

6.4.3.3 IL-10 levels in comparison to other studies

The percentages of $CD4^+$ or $CD8^+$ T cells producing IL-10 were low and mostly less than 3% in our experiments. These levels were comparable to a recent study using PBMCs stimulated with increasing concentrations of bacterial superantigens of Streptococcus pyogenes over 5 days. Of note, it was indicated that IL-10 production appeared to occur later with a much greater proportion of $CD4^+$ T cells producing IL-10 at day 5 (up to 15%) than at day 1 (<2%) (Taylor & Llewelyn 2010). Thus, it is likely that longer incubation time may be required to generate higher percentage of IL-10 producing $CD4^+$ or $CD8^+$ T cells.

In accordance to our results, PMA/ionomycin was not shown to be very effective in the stimulation of significant levels of intracellular IL-10 production in an earlier study.

PBMCs were cultured for 48 hours in the presence of PMA/ionomycin and were labelled for CD3. The proportions of IL-10 producing $CD3^+$ T cells were upregulated slightly from 0.4% in culture medium alone to 1.0% in representative results from a healthy donor (Baran et al. 2001).

In another recent study, PBMCs were cultured with anti-CD3 for 5 days and were labelled for CD8. The percentages of $CD8^+$ T cells producing IL-10 in healthy subjects were found to be around 2.5% and the levels increased to around 6.5% with 6 hours of PMA/ionomycin restimulation (Ablamunits, Bisikirska, & Herold 2010).

6.4.4.1 Biology of TGF-β

Studies of animal models and human patients have revealed the importance of TGF-β in regulating leukocyte functions in autoimmune diseases. Although TGF-β can modulate the activity of multiple cell types, it exerts the greatest impact on T lymphocytes. It inhibits the production of most effector cytokines in T cells (IFN-γ, IL-2, IL-4 and IL-10), and ectopic expression of TGF-β1 in T cells induces enhanced production of the anti-inflammatory cytokine IL-10. This effect is associated with TGF-β activation of the IL-10 promoter via Smad4 (Kitani et al. 2003). However, studies suggest that endogenous TGF-β has little contribution in the induction of anergy by IL-10 in human cells (Levings et al. 2002).

Like in Th1 cells, the presence of TGF-β greatly attenuates IFN-γ expression in $CD8^+$ T cells. Also, TGF-β regulates cytotoxic T lymphocyte functions of $CD8^+$ T cells by inhibition of perforin expression in activated $CD8^+$ T cells (Li et al. 2006). Of note, TGF-β regulation of $CD8^+$ T cell differentiation is context dependent and is modulated by co-stimulatory receptor and cytokine signaling pathways (Ahmadzadeh & Rosenberg 2005). Furthermore, TGF-β is a critical regulator of B lymphocyte activity. TGF-β inhibits B cell proliferation, provokes apoptosis of immature or resting B cells, and opposes B cell activation and class switching to most isotypes except for IgA (Li et al. 2006).

6.4.4.2 The influence of IFN-α on TGF-β producing $CD4^+$ and $CD8^+$ T cells

In line with findings in our *ex vivo* experiments, our *in vitro* data showed that TGF-β expression was upregulated in both $CD4^+$ and $CD8^+$ T cells in the presence of IFN-α, especially at higher concentrations.

Increased TGF-β expression by $CD4^+$ T cells could be an indicator of expansion of Th3-like cells. Th3 are a subset of antigen-induced regulatory T cells reported to be predominantly found at the tissue site of inflammation. The degree to which Th3 and Tr1 cells come from similar precursors or are different types of regulatory T cells is not known (Weiner 2001). TGF-β itself can promote the differentiation of Th3 cells, a process that can be enhanced by IL-4 and IL-10 (Seder et al. 1998).

6.4.4.3 TGF-β levels in comparison to other studies

The percentages of $CD4^+$ or $CD8^+$ T cells producing TGF-β were low and mostly less than 5% in our experiments. The levels are in keeping with a recent study where PBMCs were cultured with anti-CD3 for 5 days and labelled for CD8. The proportions of $CD8^+$ T cells producing TGF-β were observed to be less than 1% with or without PMA/ionomycin restimulation (Ablamunits, Bisikirska, & Herold 2010).

6.4.5 The effect of IFN-α on the kinetics of Foxp3, IL-10 and TGF-β expression by T cells

The kinetics of PBMC response under the influence of IFN-α was measured as well for Foxp3, IL-10 and TGF-β levels within $CD4^+$ and $CD8^+$ T cell populations. The idea was to obtain some indications in the potential roles of IFN-α therapy could play as either prophylaxis or treatment options in chronic inflammatory diseases.

In terms of Foxp3, the potential for greater enhancement of its expression within $CD4^+$, $CD4^+CD25^+$, $CD4^+CD25^{high}$ and $CD8^+CD25^+$ T cell populations were observed with longer exposure of PBMCs to IFN-α. With PMA/ionomycin stimulation, IFN-α

showed greater activity in upregulating Foxp3 in T cells when it was added before rather than after cell activation.

Similar trends could also be observed in terms of IL-10 and TGF-β expression by both $CD4^+$ and $CD8^+$ T cells as higher levels were associated with adding IFN-α at the earlier time-points or introduction of IFN-α before cell activation by PMA/ionomycin. This could imply that expression of Foxp3 and regulatory cytokines, including IL-10 and TGF-β, by T cells were induced to a greater degree in response to longer IFN-α exposure or cells required more time to respond to IFN-α treatment and the results were simply a reflection of the experimental design picking up data at different stages of cytokine production.

6.5 Summary of findings

We were able to verify findings from BD patient data and recombinant IFN-α2a and IFN-α2b were found to be equally potent in their augmenting effects on immunoregulatory subsets and cytokines *in vitro*. Both forms of IFN-α were efficacious in inducing Foxp3 expression by $CD4^+$, $CD4^+CD25^+$, $CD4^+CD25^{high}$, $CD8^+$, and $CD8^+CD25^+$ T cell populations. IFN-α2a and IFN-α2b were also observed to consistently upregulate IL-10 and TGF-β levels in $CD4^+$ and $CD8^+$ T cells. Moreover, IFN-α was able to exert its effects both before and after cell activation.

Chapter 7
The immunomodulatory effects of interferon-alpha on γδ T cells

7.1 Introduction

Although the vast majority of mature T lymphocytes express αβ TCR, a small subset (1-5%) of peripheral blood T cells carries the alternative γδ TCR, which is composed of a γ and δ chain heterodimer (Konigshofer & Chien 2006). However, they constitute a major population in other anatomical localisations such as the small intestine and some epithelia (Hayday 2000). The physiological roles fulfilled by γδ T cells are diverse and include protective immunity against extracellular and intracellular pathogens, tumour surveillance, modulation of innate and adaptive immune responses, tissue healing and epithelial cell maintenance, and regulation of physiological organ function (Bendelac et al. 2001;Hayday 2009). γδ T cells are capable of responding to various environmental insults despite having only a limited amount of expressed TCR repertoire, and regulate αβ T cell activation through cytokine production (Kuhl et al. 2009). The functional specialisation of γδ T cell subsets bestows them with a unique ability to carry out a restricted set of tasks with spatial and temporal features that are unmatched by other immune effectors.

In Behcet's disease, the basic aetiopathogenic mechanism consists of a hyperactivation of neutrophils but the primary trigger is unknown. At present, the most accepted hypothesis is that a multifactorial process induces an immune dysregulation that results in exaggerated neutrophils activation (Mendes et al. 2009). γδ T lymphocytes have been suggested as one of the causes of cytokine dysregulation that results in neutrophils hyperactivation in BD patients. This is because that an enhanced level of γδ T cells, especially $CD8^+$ γδ T cells, in active lesions and peripheral blood, has consistently been demonstrated in BD patients (Nishida et al. 1998;Treusch et al. 2004). Compared with healthy donors, there is not only neutrophil hyperactivity, but also a significant elevation of monocytes and γδ T cells in patients with active BD before treatment (Treusch et al. 2004). These γδ T cells have been shown to express activation markers, such as CD25, CD69, and

CD29 and produce inflammatory cytokines including IFN-γ and TNF-α (Freysdottir, Lau, & Fortune 1999).

There are two main γδ T cell subpopulations in humans as $Vδ2^+$ and $Vδ1^+$ account for more than 90% of the total γδ T cells (Carding & Egan 2002). It was reported recently that there is a preferential activation of peripheral blood Vδ1 as compared to Vδ2 γδ T cells in patients with active BD (Yasuoka et al. 2008). In supporting these findings and a key role of these cells in the pathogenesis of BD, increased frequency and number of γδ T cells, predominantly Vγ9Vδ2 T cells have been found in peripheral blood of BD patients (Yamashita et al. 1997) and in the intraocular fluid of BD patients with uveitis (Verjans et al. 2002). Significantly, antigens that activate γδ T cells could play an important role in the pathogenesis of the disease. Indeed, peripheral blood γδ T cells from BD patients can respond specifically to peptides derived from the mycobacterial HSP65 and the homologous human HSP60 (Hasan et al. 1996). In addition, peripheral blood Vγ9Vδ2 T cells from patients with active BD were found to secrete elevated amounts of the serine protease granzyme A when stimulated with phosphoantigens (Accardo-Palumbo et al. 2004).

7.2 Aims

The aim of this chapter is to assess the immunomodulatory effects of 6 months of subcutaneous IFN-α therapy on γδ T cells in BD patients *ex vivo*. *In vitro* assays are also performed to test the influence of various concentrations of IFN-α2a and IFN-α2b on γδ T cells and their IFN-γ and IL-17 cytokine production using healthy donor PBMCs. The kinetics of interactions between IFN-α and IFN-γ and IL-17 cytokine production by γδ T cells are to be explored as well.

7.3 Results

7.3.1 IFN-α therapy helps maintain a stable population of γδ T cells in BD patients

To examine the influence of IFN-α therapy on γδ T cells among peripheral blood T lymphocytes in BD patients, serial whole blood samples from BD patients receiving conventional immunosuppressive therapy with or without recombinant IFN-α2b treatment were obtained for immunofluorescence staining and flow cytometry. PBMCs were not incubated or activated with polyclonal stimuli. Apart from analysing TCRγδ expression by gating on lymphocytes, results were analysed further using CD8 surface protein to acquire data on $CD4^+$ and $CD8^+$ T lymphocyte populations.

Conspicuously, there was no significant alteration of the proportions of γδ T cells in BD patients under IFN-α therapy. This is in contrast to patients on conventional treatment alone, who demonstrated a tendency to have elevated levels of γδ T cells. Among lymphocytes, the percentages of γδ T cells were raised at 6 (median 5.5% and IQR 3.8-8.6%; $p<0.05$) and 12 months (median 5.0% and IQR 2.9-7.8%; $p<0.05$) when compared to values at recruitments (median 4.9 and IQR 3.1-6.5%) (Figure 7.1a).

Within $CD4^+$ T lymphocytes, the levels of $TCR\gamma\delta^+$ cells were increased when comparing values at 6 month visits (median 3.8% and IQR 2.4-7.3%; $p<0.05$) with ones at 3 months (median 2.8% and IQR 2.0-4.9%) (Fig 7.1b). Also, within $CD8^+$ T lymphocytes, the proportions of $TCR\gamma\delta^+$ cells were elevated at 12 month assessment visits (median 1.2% and IQR 0.7-2.5%; $p<0.01$) when evaluated against baseline visit values (median 0.7% and IQR 0.3-1.1%) (Fig 7.1c).

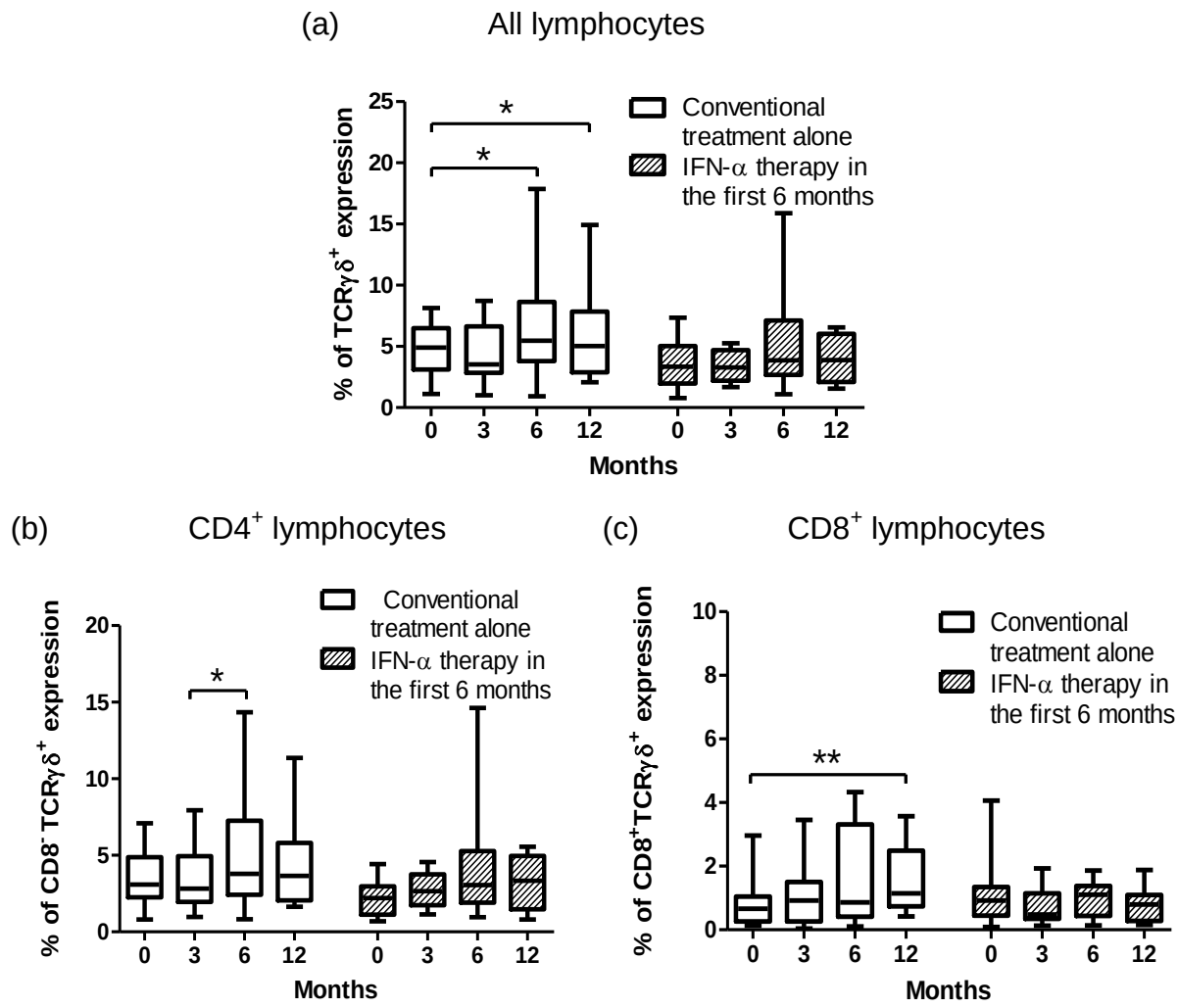


Fig 7.1: IFN-α therapy promotes a stable population of γδ T cells

Whole blood from BD patients receiving either the conventional treatment alone (n=13) or with IFN-α2b therapy (n=12) were obtained at 0, 3, 6 and 12 month assessment visits. These samples were stained with anti-CD8, and anti-TCRγδ antibodies. The graphs show percentages of the TCRγδ expression in (a) all lymphocytes, (b) $CD4^+$ T cells, and (c) $CD8^+$ T cells. The results are presented as boxplots.

7.3.2 Preliminary experiments to establish *in vitro* γδ T test models with antigens and polyclonal stimulation

In order to set up an *in vitro* model to verify findings from BD patients, the same panel of antigens and polyclonal stimuli used in preliminary experiments in Chapter 5 and 6 were applied to healthy donor PBMC cultures for 24 hours before intracellular cytokine staining and flow cytometry. IFN-γ and IL-17 levels were analysed by gating on TCRγδ$^+$ lymphocytes.

Notably, none of the antigens chosen was particularly effective in inducing IFN-γ production by γδ T cells (Fig 7.2a). LPS and longer incubation with candida antigens showed some promise in promoting IL-17 production by γδ T cells, although the levels achieved were less than stimulation with anti-CD3/CD28 Ab (Fig 7.2b). Concurrent use of LPS and CD3/CD28 stimulation was evaluated as well, but the effect was no more than using anti-CD3/CD28 Ab alone.

In addition, PBMCs from BD patients were incubated with BD-related antigens, including alpha-tropomyosin and PDS-Ag, as a trial. PDSAg, when used at 10-50μg/ml, demonstrated some potential in augmenting IL-17 levels in γδ T cells but not to the same degree as stimulated by PMA/ionomycin. All the other antigens tested did not encourage IL-17 production by γδ T cells. In particular, the levels attained with ATRA were similar or lower than ones obtained from untimulated PBMC cultures.

Fortunately, substantially higher production levels of both IFN-γ and IL-17 were able to be induced in γδ T cells consistently with polyclonal stimulation such as anti-CD3/CD28 Ab and especially PMA/ionomycin. These results were repeatable as shown by the data in nine separate experiments each for IFN-γ and IL-17 producing cells despite the fact that the experiments were performed in singles.

As a result, healthy donor PBMCs activated with anti-CD3/CD28 Ab and/or PMA/ionomycin were chosen as the basis for test models in subsequent *in vitro* experiments and further preliminary experiments with antigens were not carried out.

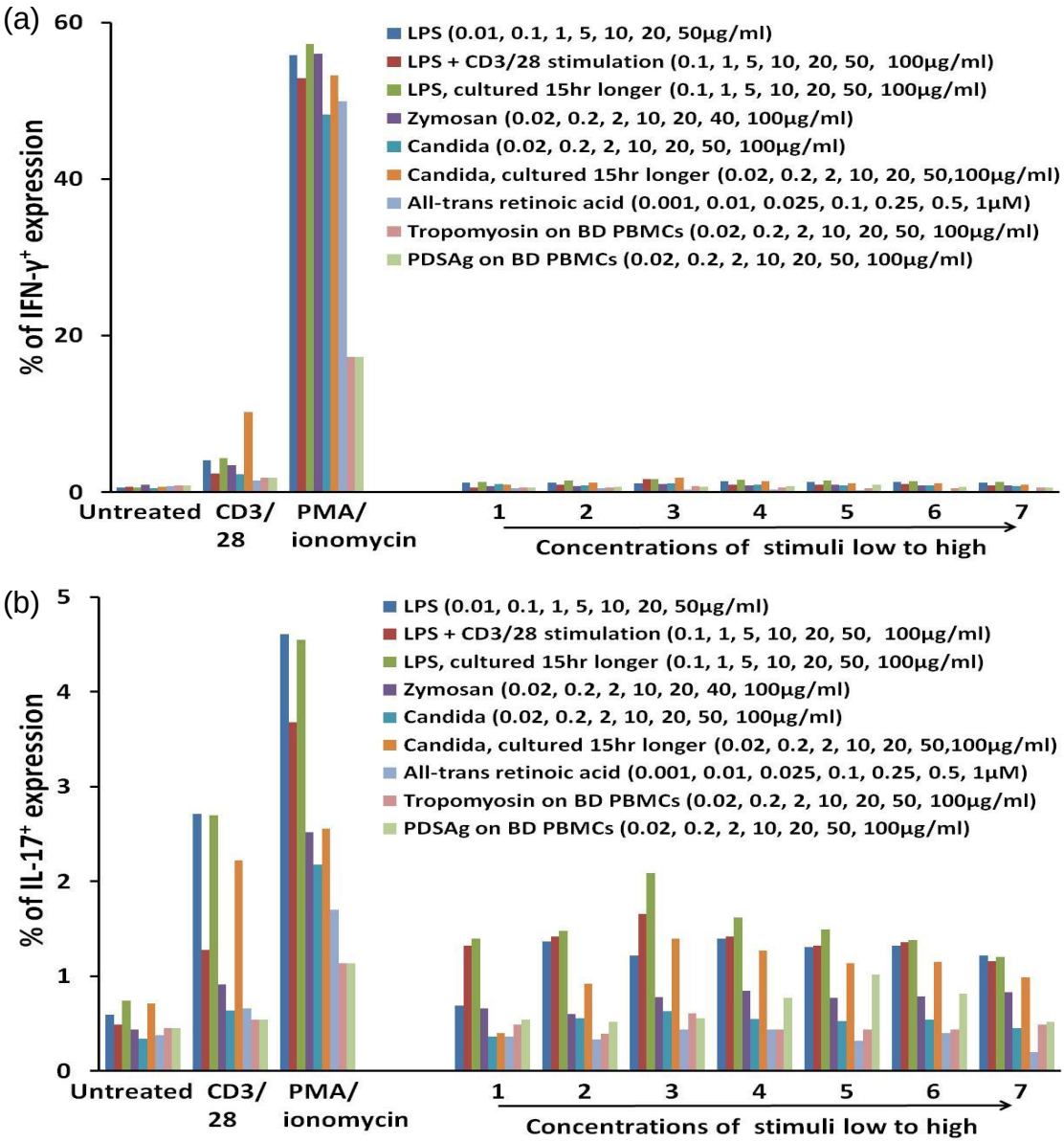


Fig 7.2: The effect of a selection of stimuli on the levels of γδ cells producing IFN-γ and IL-17 in healthy donors

Healthy donor PBMCs were untreated, activated with anti-CD3 (5ng/ml) & anti-CD28 (1µg/ml), activated with PMA (25ng/ml) & ionomycin (1µg/ml) or stimulated with various stimuli including LPS, zymosan, candida, and all-trans retinoic acid for 24 hours or 39 hours. In one experiment, both LPS and CD3/CD28 stimulation were administered. Also, in 2 of the experiments, PBMCs from BD patients were used instead and stimulated with either polyclonal stimuli or α-tropopmyosin or PDS-Ag for 24 hours. Results are shown as proportion of γδ T cells expressing cytokines. In (a), percentage of IFN-γ^+ cells and in (b), percentage of IL-17^+ cells are shown. Each colour represent a separate experiment and all experiments were done only once in singles. 1 is the lowest antigen concentration used and 7 is the highest. The actual concentrations of stimuli are shown inside the brackets in the legend.

7.3.3 Preliminary experiments to establish the needs to rest cells in the medium after thawing and the effects of adding suboptimal IL-2

Next, it was examined whether resting healthy donor PBMCs for one day in culture medium with or without addition of suboptimal amount of IL-2 after thawing could enhance cytokine production.

With regards to IFN-γ producing γδ T cells (Fig 7.3a), there was a tendency for more elevated levels with resting PBMCs for one day without additional IL-2 under all 4 culture conditions, but none of these results were statistically significant.

With regards to IL-17 producing γδ T cells (Fig 7.3b), there was no advantage in resting the cells in three out of four culture conditions and again none of the variations were statistically significant.

Also, PHA and anti-CD3/CD28 Ab were comparable in their ability to induce either IFN-γ or IL-17 expression and the effects of both were not as strong as one exerted by PMA/ionomycin stimulation.

Overall, the findings suggest there is no significant advantage in resting PBMCs or adding suboptimal concentration of IL-2 for experiments involving IFN-γ and IL-17 producing γδ T cells.

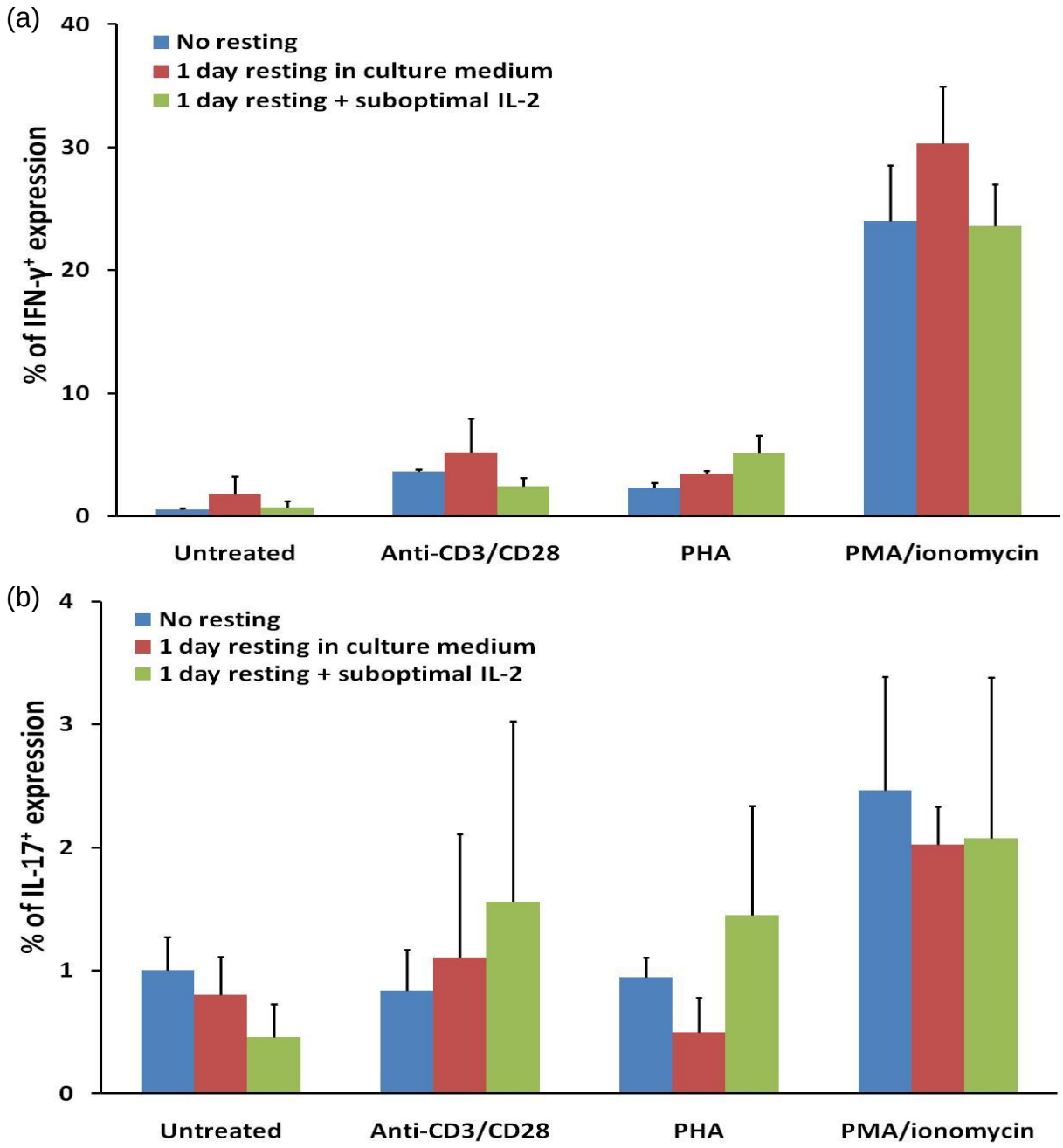


Fig 7.3: The effect of resting PBMCs and suboptimal concentration of IL-2 on the proportion of IFN-γ and IL-17 producing γδ T cells

Healthy donor PBMCs were either stimulated straight away or rested for 1 day in medium (RPMI & supplements) ± 0.5 U/ml of IL-2 before stimulation. Under all 3 conditions, PBMCs were untreated, or stimulated with anti-CD3 (5ng/ml) & anti-CD28 (1µg/ml), PMA (25ng/ml) & ionomycin (1µg/ml) or PHA (5µg/ml) for 24 hours. Results are shown as proportion of γδ T cells producing cytokines. In (a), percentage of IFN-γ^+ cells and in (b), percentage of IL-17^+ cells are shown. The experiment was done only once in triplicates and the results are shown as means ± SD.

7.3.4 Preliminary experiments to establish the choice of culture medium

The choice of medium was scrutinised as it has been reported that Iscove's modified Dulbecco's medium (IMDM) can result in higher Th17 expansion (Veldhoen et al. 2009). Experiments were set up to test healthy donor PBMCs cultured in RPMI- and IMDM-based T cell media in parallel.

Results from these experiments demonstrated that there was no significant advantage with using either types of culture medium to expand IFN-γ and IL-17 producing γδ T cell populations whether polyclonal activation was applied or not.

Therefore, it was decided to perform γδ T cell experiments using IMDM-based culture medium in line with Th1 and Th17 experiments in Chapter 5.

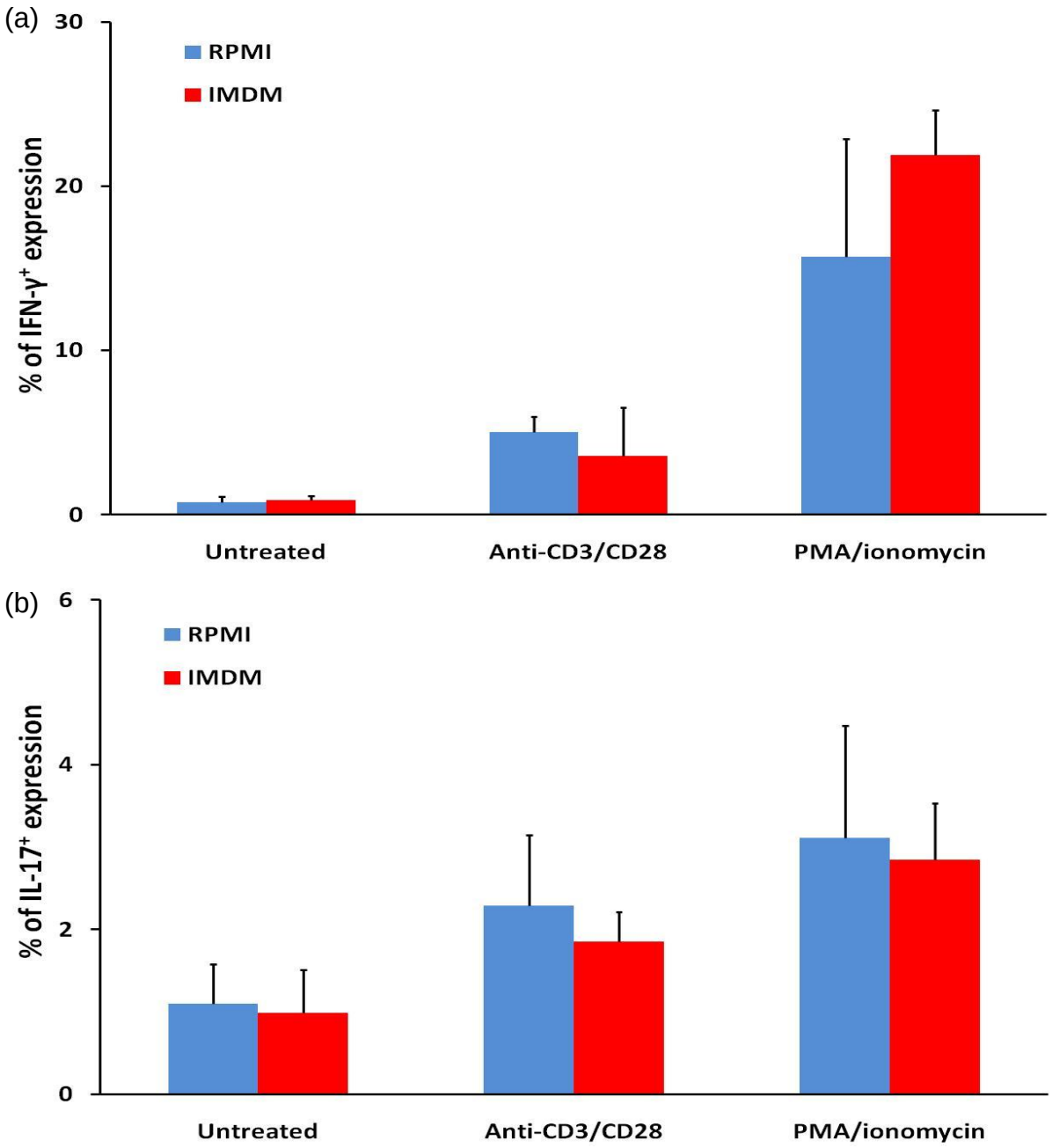


Fig 7.4: The effect of different culture media on the levels of IFN-γ and IL-17 producing γδ T cell populations

Healthy donor PBMCs were either cultured in RPMI- or IMDM-based T cell medium. Cell stimulation was provided by adding anti-CD3 (5ng/ml) & anti-CD28 (1µg/ml) or PMA (25ng/ml) & ionomycin (1µg/ml) for 24 hours. Results are shown as proportion of γδ T cells producing cytokines. In (a), percentage of IFN-γ^+ cells and in (b), percentage of IL-17^+ cells are shown. The histograms show means ± SD from 3 independent experiments. One experiment was done in duplicates rather than triplicates and did not test cells stimulated with PMA/ionomycin.

7.3.5 Preliminary experiments to establish incubation time with polyclonal stimuli

PBMC cultures were performed over 24 and 39 hours to investigate whether longer exposure to polyclonal stimuli would promote greater expansion of γδ T cell population producing either IFN-γ or IL-17.

With regards to γδ T cells producing IFN-γ, longer incubation time actually resulted in decreased levels under all three culture conditions (Fig 7.5a). The levels were lowered from 3.6 ± 0.3% to 2.4 ± 0.6% ($p<0.05$), 7.2 ± 1.0% to 3.2 ± 0.4% ($p<0.05$), and 47.1 ± 1.1% to 25.6 ± 3.4% ($p<0.01$) in untreated cultures, CD3/CD28 stimulated cultures, and PMA/ionomycin stimulated cultures respectively.

Similarly, the proportion of γδ T cells producing IL-17 was reduced with extra 15 hours of incubation time as well (Fig 7.5b). The levels were decreased from 2.4 ± 0.4% to 1.4 ± 0.4% ($p<0.05$) and 4.1 ± 0.5% to 2.0 ± 0.8% ($p<0.05$) in untreated cultures and CD3/CD28 stimulated cultures respectively. Such trend, however, was not evident in PBMC cultures activated by PMA/ionomycin.

Based on the findings, a decision was made to perform γδ T cell experiments with 24 hours of culture time.

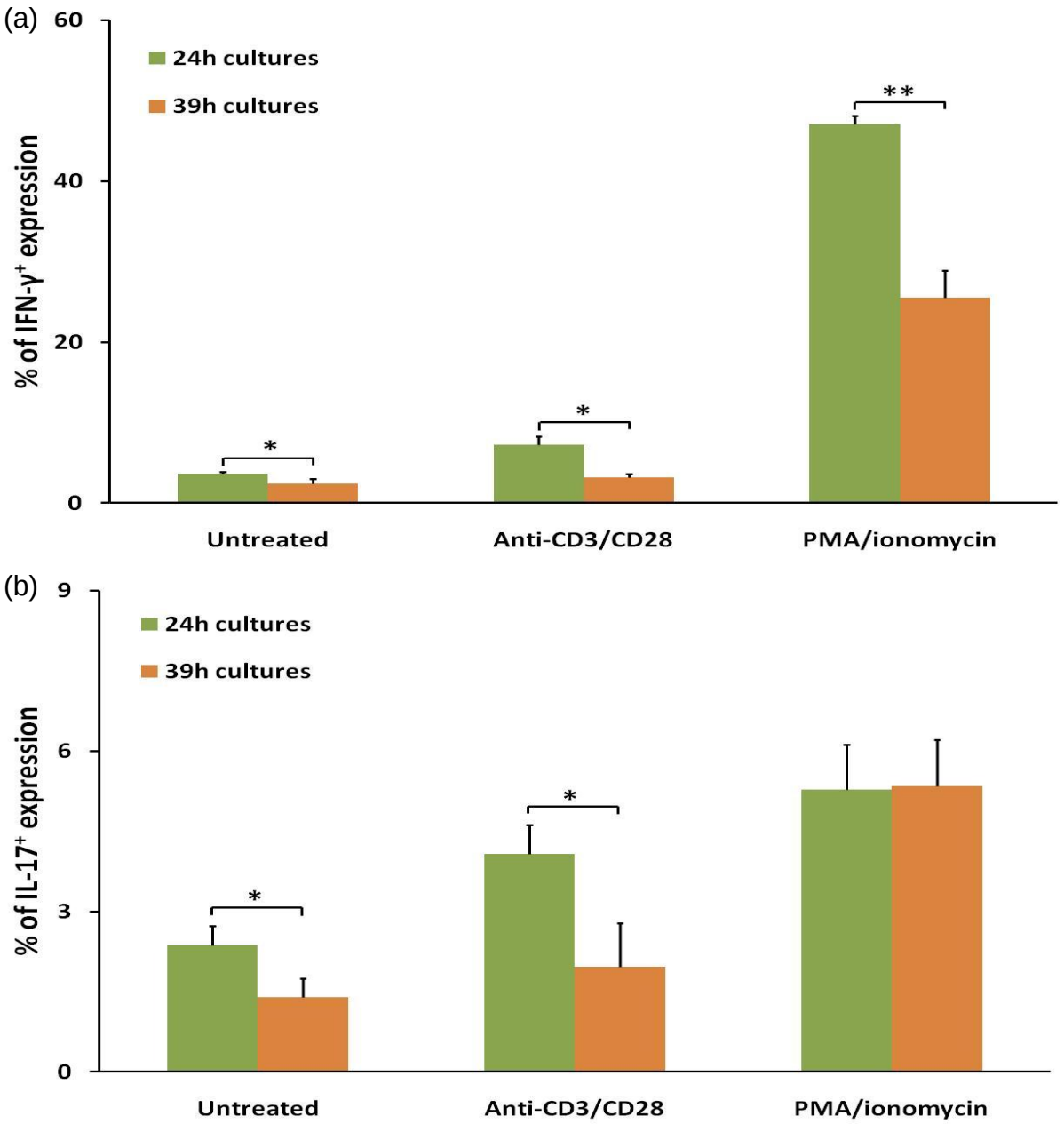


Fig 7.5: The influence of length of culture time on the levels of IFN-γ and IL-17 producing γδ T cell populations

Healthy donor PBMCs were untreated, stimulated with anti-CD3 (5ng/ml) & anti-CD28 (1µg/ml) or PMA (25ng/ml) & ionomycin (1µg/ml) for 24 hours or 39 hours. Results are shown as proportion of γδ T cells producing cytokines. In (a), percentage of IFN-γ^+ cells and in (b), percentage of IL-17^+ cells are shown. The histograms show means ± SD of triplicate wells from one experiment. IMDM-based culture medium was used.

7.3.6 IFN-α induces a downregulation of γδ T cells

To verify the findings in our *ex vivo* experiments on γδ T cells, PBMCs isolated from healthy individuals were cultured without stimulation or stimulated with anti-CD3 and anti-CD28 Ab or PMA and ionomycin in the presence or absence of IFN-α2a (10^1-10^5 U/ml) and IFN-α2b (10^1-10^4 U/ml) before staining and flow cytometry. Data were obtained by examining the population of lymphocytes expressing both CD3 and TCRγδ surface proteins.

Analysis of results revealed that recombinant IFN-α2a and IFN-α2b were equally effective in suppressing the amount of γδ T cells, especially at higher concentrations. In the presence of 10^5 U/ml of IFN-α2a, the proportion of $CD3^+TCR\gamma\delta^+$ T cells within lymphocyte population was downregulated from 1.1 ± 0.1% to 0.8 ± 0.1% (means ± SD; $p<0.05$) in CD3/CD28 stimulated cultures and from 1.2% to 1.1% ($p<0.05$) in PMA/ionomycin stimulated cultures (Fig 7.6a).

Likewise, in the presence of 10^4 U/ml of IFN-α2b, the percentage of $CD3^+TCR\gamma\delta^+$ T cells within lymphocyte population was downregulated from 1.0% to 0.9% ($p<0.05$) in CD3/CD28 stimulated cultures and from 1.2% ± 0.1% to 1.0 ± 0.1% ($p<0.05$) in PMA/ionomycin stimulated cultures (Fig 7.6b).

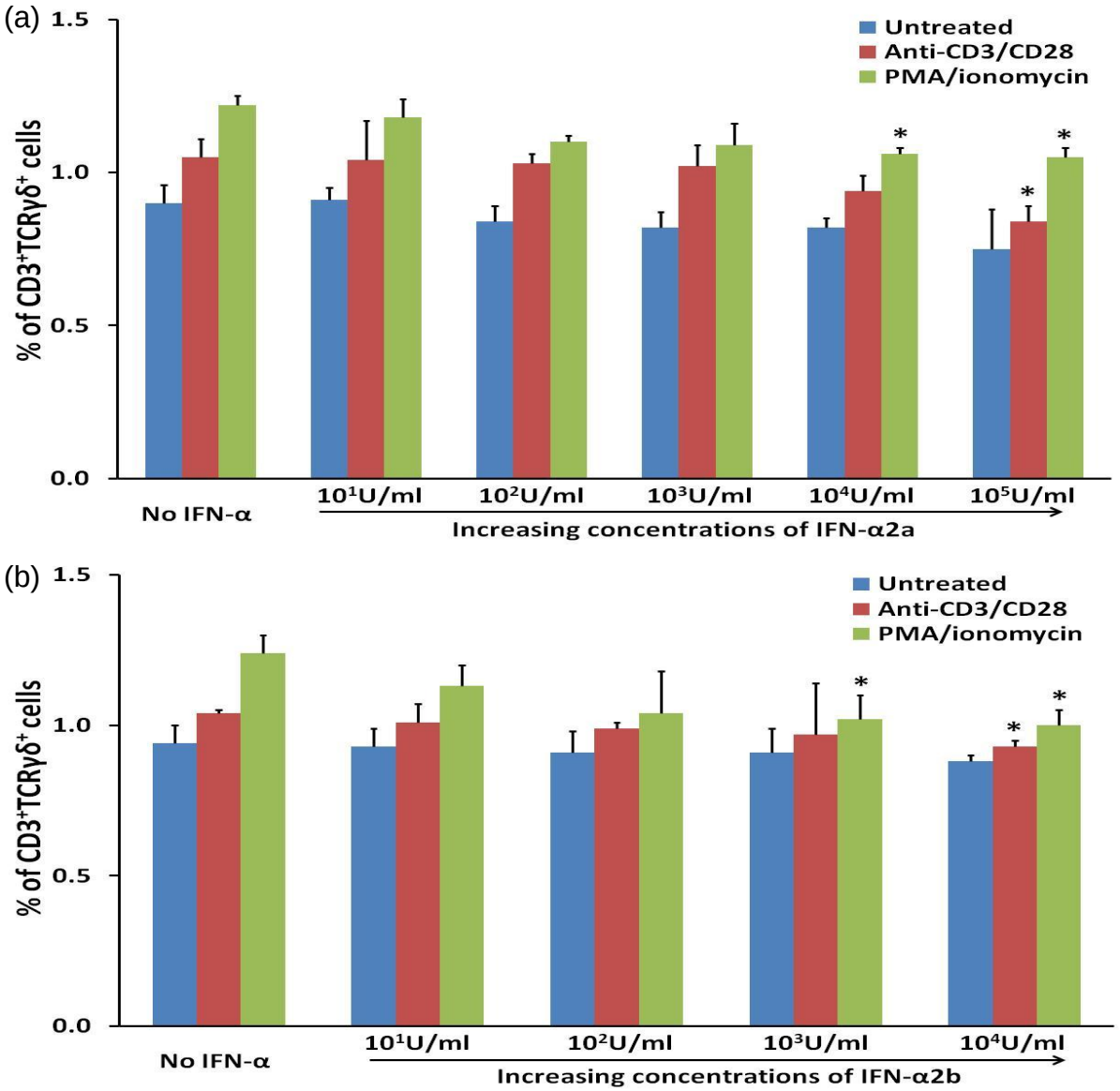


Fig 7.6: The inhibitory effect of recombinant IFN-α on TCRγδ expression by $CD3^+$ T cells

PBMCs from healthy donor were untreated, stimulated with anti-CD3 & anti-CD28 or PMA & ionomycin for 24 hours. Various concentrations of exogenous IFN-α was added at the same time as the polyclonal stimuli to cell cultures. In (a) and (b), the effects of IFN-α2a and IFN-α2b are shown respectively. Results in (a) and (b) are representative of 5 independent experiments each, and PBMCs were from four different donors for experiments on both forms of IFN-α. Two of the IFN-α2a and IFN-α2b experiments used both CD3/CD28 and PMA/ionomycin stimulation. Also, PBMCs were cultured over 39 instead of 24 hours in two experiments, and 10^1 U/ml of IFN-α (2a and 2b) and 10^5 U/ml of IFN-α2a were only tested twice in these experiments. Data are means ± SD values of triplicate wells. Statistical analysis was performed using the Student's T-test. *$p<0.05$, compared with controls without IFN-α added.

7.3.7 IFN-α promotes IFN-γ production by γδ T cells

To study the ability of IFN-α to induce IFN-γ expression by γδ T cells *in vitro*, PBMCs isolated from healthy individuals were cultured without stimulation or stimulated with anti-CD3 and anti-CD28 Ab or PMA and ionomycin in the presence or absence of IFN-α2a (10^1-10^5 U/ml) and IFN-α2b (10^1-10^4 U/ml) before intracellular cytokine staining and flow cytometry. IFN-γ expression was analysed by gating on $CD3^+TCR\gamma\delta^+$ lymphocyte populations.

Evidently, both IFN-α2a and IFN-α2b showed that they could enhance IFN-γ production by γδ T cells at higher concentrations. Following PMA/ionomycin activation, the IFN-γ producing γδ T cells were raised from 69.6 ± 4.9% in the absence of IFN-α up to 78.1 ± 0.9% (means ± SD; $P<0.05$) in the presence of 10^5 U/ml IFN-α2a (Fig 7.7a). Similarly, in the presence of 10^4 U/ml IFN-α2b, the proportions of γδ T cells producing IFN-γ were upregulated from 4.1 ± 0.9% up to 6.4 ± 0.2% ($p<0.05$) in untreated cultures and 70.1 ± 4.8% up to 78.0 ± 2.9% ($p<0.05$) in cultures stimulated with PMA/ionomycin (Fig 7.7b).

A tendency for both IFN-α2a and IFN-α2b to augment IFN-γ could be detected in CD3/CD28 stimulated cultures as well, but the results were not statistically significant.

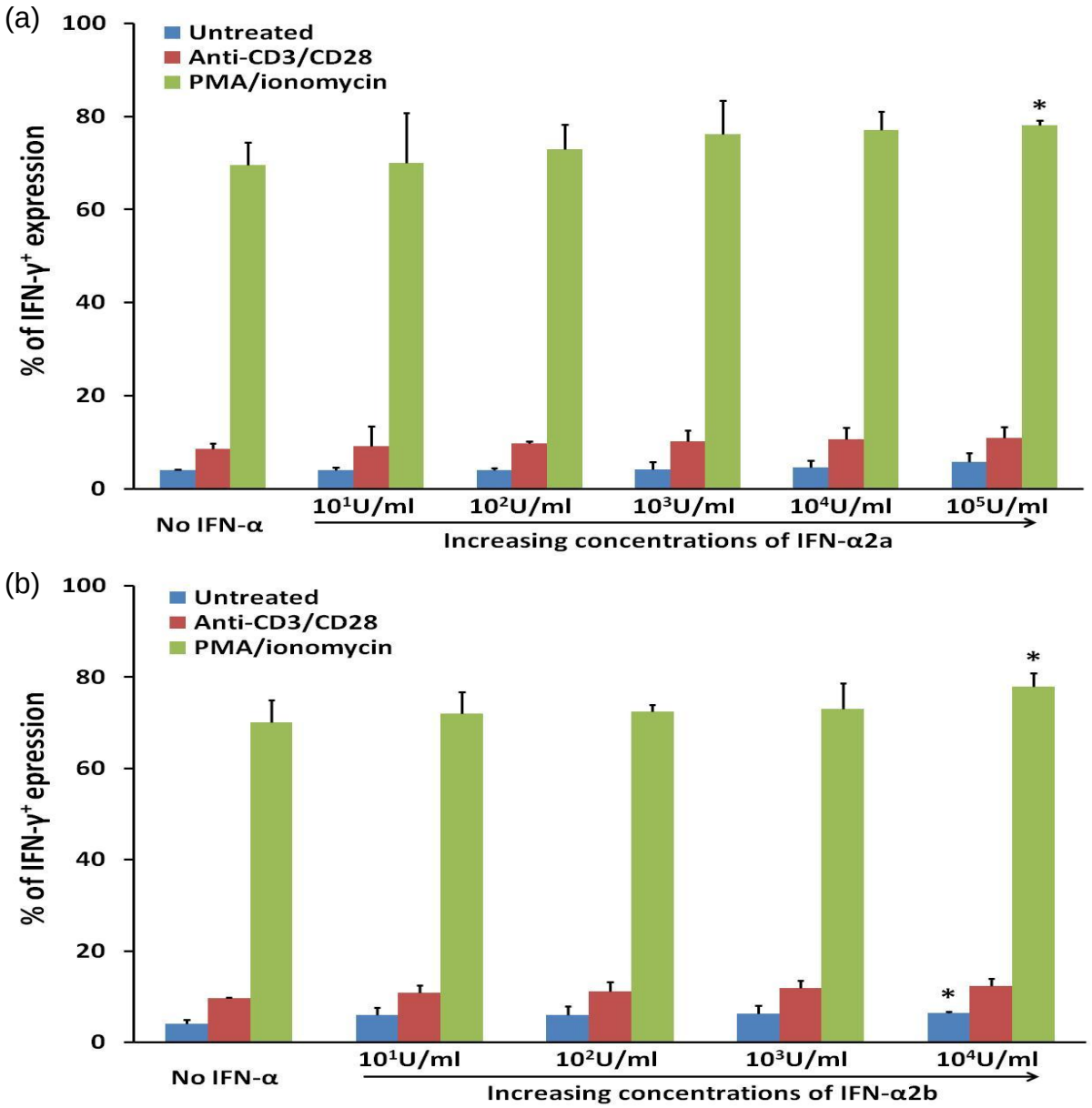


Fig 7.7: The augmenting effect of recombinant IFN-α on γδ T cells producing IFN-γ

Healthy donor PBMCs were untreated, stimulated with anti-CD3 & anti-CD28 or PMA & ionomycin for 24 hours. Various concentrations of IFN-α was added at the same time as the polyclonal stimuli to cultures. Results are shown as proportions of γδ T cells producing IFN-γ. In (a) and (b), the effects of IFN-α2a and IFN-α2b are shown respectively. Results in (a) and (b) are representative of 5 independent experiments each, and PBMCs were from four different donors for experiments on both forms of IFN-α. Two of the IFN-α2a and IFN-α2b experiments used both CD3/CD28 and PMA/ionomycin stimulation. Also, PBMCs were cultured over 39 instead of 24 hours in two experiments, and 10^1 U/ml of IFN-α (2a and 2b) and 10^5 U/ml of IFN-α2a were only tested twice in these experiments. Data are means ± SD values of triplicate wells. Statistical analysis was performed using the Student's T-test. *p<0.05, **p<0.01, compared with controls without IFN-α added.

7.3.8 IFN-α suppresses IL-17 production by γδ T cells

In order to examine the capacity of IFN-α to inhibit IL-17 production by γδ T cells *in vitro*, PBMCs isolated from healthy individuals were cultured without stimulation or stimulated with anti-CD3 and anti-CD28 Ab or PMA and ionomycin in the presence or absence of IFN-α2a (10^1-10^5 U/ml) and IFN-α2b (10^1-10^4 U/ml) before intracellular cytokine staining and flow cytometry. IL-17 expression was analysed by gating on $CD3^+TCR\gamma\delta^+$ lymphocyte populations.

The data demonstrated that both IFN-α2a and IFN-α2b could suppress IL-17 production by γδ T cells in a dose-dependent manner. The trend was particularly evident with PMA/ionomycin activation as the proportions of γδ T cells producing IL-17 were downregulated from 16.1 ± 4.5% in the absence of IFN-α to 7.8 ± 2.5% (means ± SD; $p<0.05$) when 10^5 U/ml of IFN-α2a was added to PBMC cultures (Fig 7.8a). Likewise, the percentages of γδ T cells producing IL-17 was reduced from 17.2 ± 2.8% in the absence of IFN-α to 11.6 ± 2.0% in the presence of 10^4 U/ml of IFN-α2b (Fig 7.8b).

The tendency for IFN-α2a and IFN-α2b to inhibit IL-17 could be observed in untreated and CD3/CD28 stimulated cultures as well, but the results were not statistically significant.

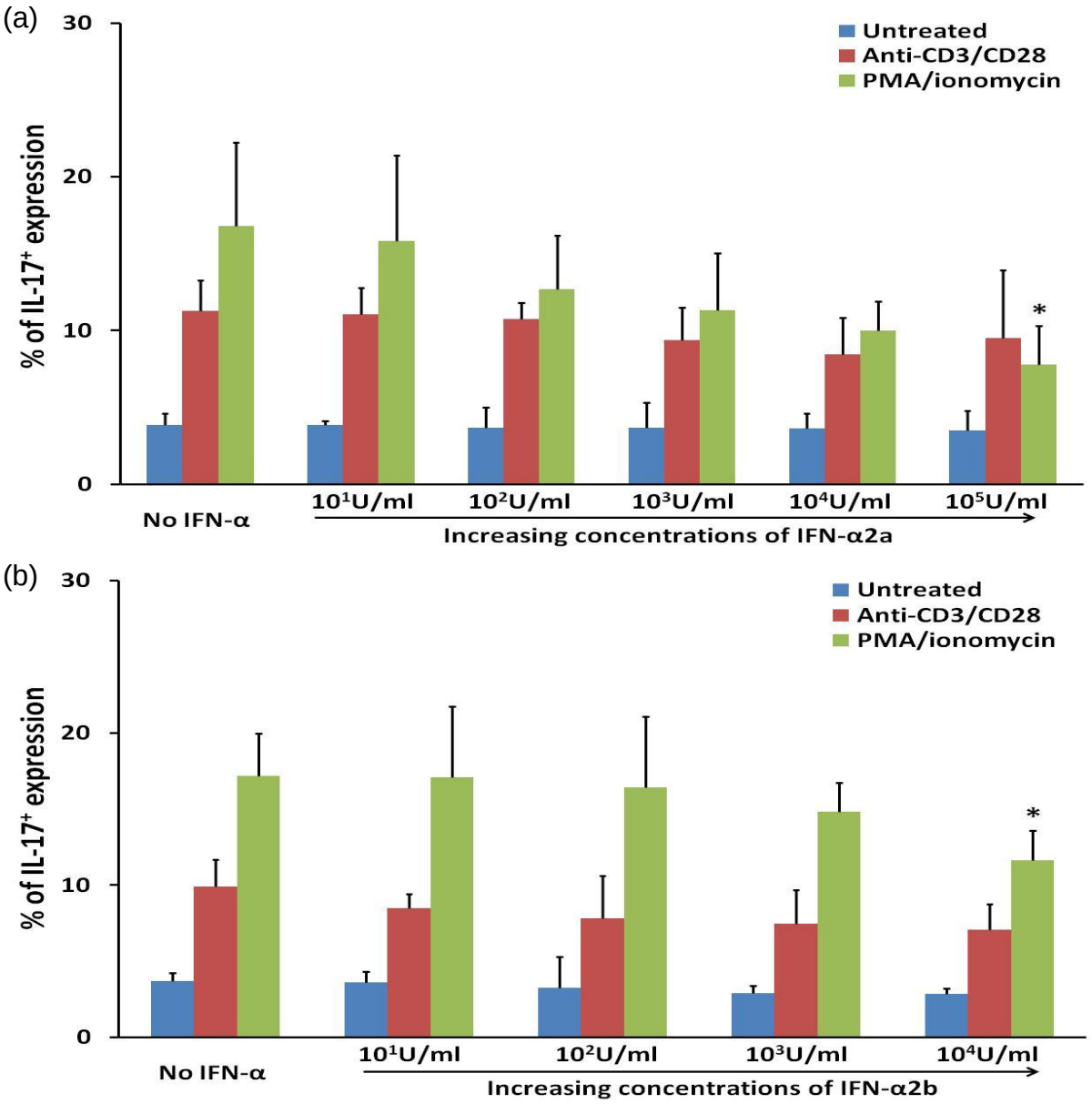


Fig 7.8: The inhibitory effect of recombinant IFN-α on IL-17 producing γδ T cells

Healthy donor PBMCs were untreated, stimulated with anti-CD3 & anti-CD28 or PMA & ionomycin for 24 hours. Various concentrations of IFN-α was added at the same time as the polyclonal stimuli to cultures. Results are shown as proportions of γδ T cells producing IL-17. In (a) and (b), the effects of IFN-α2a and IFN-α2b are shown respectively. Results in (a) and (b) are representative of 5 independent experiments each, and PBMCs were from four different donors for experiments on both forms of IFN-α. Two of the IFN-α2a and IFN-α2b experiments used both CD3/CD28 and PMA/ionomycin stimulation. Also, PBMCs were cultured over 39 instead of 24 hours in two experiments, and 10^1 U/ml of IFN-α (2a and 2b) and 10^5 U/ml of IFN-α2a were only tested twice in these experiments. Data are means ± SD values of triplicate wells. Statistical analysis was performed using the Student's T-test. *p<0.05, **p<0.01, compared with controls without IFN-α added.

7.3.9 The kinetics of immunomodulatory effects of IFN-α on γδ T cells expressing IFN-γ and IL-17

Although IFN-α has been observed to enhance IFN-γ while inhibiting IL-17 production by γδ T cells *in vitro*, it was not clear whether pre-treatment of PBMCs with IFN-α could exert greater influence when compared to adding IFN-α at the same time as activating agents. Also, it was doubtful whether IFN-α could produce its effect once PBMCs were activated. To aid in answering these questions, PBMCs isolated from healthy individuals were cultured without IFN-α treatment or with 10^4 U/ml IFN-α2a added 24 hours before, 4 hours before, at the same time as, or 4 hours after PMA/ionomycin stimulation. The timing for the introduction of IFN-α to cultures was the same for both unstimulated and stimulated cultures, and PBMCs were incubated for 24 hours after PMA/ionomycin stimulation before intracellular cytokine staining and flow cytometry. IFN-γ and IL-17 expression levels were analysed by gating on $CD3^+TCR\gamma\delta^+$ lymphocyte populations.

In terms of IFN-γ producing γδ T cells, although upregulation was evident at all four time-points relative to the levels achieved without the presence of IFN-α, statistical significance was reached only when IFN-α was added at the second last time-point. In the case of untreated cultures, the proportions of IFN-γ producing γδ T cells were raised from 4.4 ± 0.5% in the absence of IFN-α up to 5.9 ± 0.4% (means ± SEM; $p<0.05$) at this time-point (Fig 7.9a). Also, the concurrent introduction of IFN-α and PMA/ionomycin at this time-point was able to enhance the levels of IFN-γ producing γδ T cells from 65.4 ± 3.2% to 75.2 ± 2.1% ($p<0.05$).

With regards to γδ T cells producing IL-17, an inhibitory effect was seen at all time-points. However, the strongest effect was seen when IFN-α was added at the second last time-point (Fig 7.9b). In the case of untreated cultures, the proportions of IL-17 producing γδ T cells were downregulated from 2.8 ± 0.4% without the presence of IFN-α to 1.3 ± 0.1% ($p<0.01$) at this time-point. The decrease was even significant relative to results obtained from the first (2.6 ± 0.5%; $P<0.05$) and second (2.1 ± 0.3%; $p<0.05$) time-points. With PMA/ionomycin stimulation, the concurrent use of IFN-α reduced the levels of IL-17 producing γδ T cells from 8.3 ± 1.1% in the

absence of IFN-α to 5.4 ± 0.7% ($p<0.05$). This result was significant even when evaluated against data with 24 hours of IFN-α pre-treatment (8.7 ± 0.8; $p<0.01$).

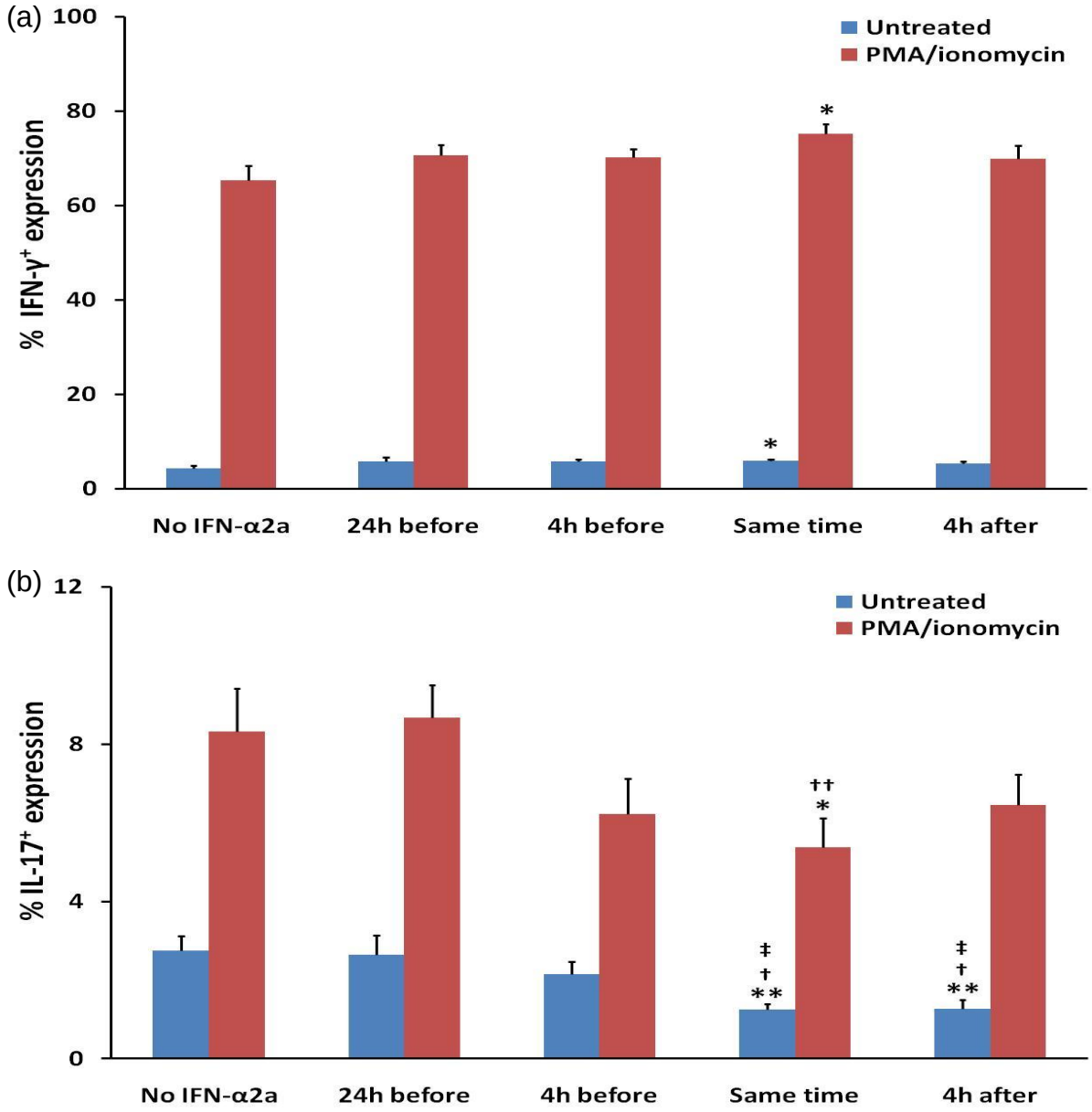


Fig 7.9: The kinetics of immunomodulatory effects of IFN-α on γδ T cells producing IFN-γ and IL-17

Healthy donor PBMCs were treated without or with 10^4 U/ml IFN-α2a 24 hours before, 4 hours before, at the same time as, or 4 hours after introduction of PMA & ionomycin to cultures. The timing for the addition of IFN-α2a to cultures was the same for both unstimulated and stimulated cultures, and PBMCs were incubated for 24 hours after PMA/ionomycin stimulation before staining. Results are shown as proportions of γδ T cells producing (a) IFN-γ and (b) IL-17. The histograms show means ± SEM of triplicate wells from three independent experiments. All experiments used PBMCs from the same donor. Statistical analysis was performed using the Student's T-test. *p<0.05, **p<0.01, compared with controls without IFN-α added. †p<0.05, ††p<0.01, compared with data with 24-hour IFN-α pre-treatment. ‡p<0.05, compared with data with 4-hour IFN-α pre-treatment.

7.4 Discussion

7.4.1.1 γδ T cells and Behcet's disease

γδ T cells make up 1-10% of the peripheral blood T cells (Kaufmann 1996), and may help bridge the gap between early innate and later adaptive immune responses. They represent a unique subset of unconventional lymphocytes, because they display features of both conventional αβ T cells and NK cells (Hayday 2000). γδ T cells localise in specialised areas such as the mucosal tissues and can produce additional cytokines with relevance for the local immune response (Bonneville, O'Brien, & Born 2010). γδ T cells freshly isolated from human peripheral blood tend to be primed toward a Th1 phenotype (Wesch, Glatzel, & Kabelitz 2001).

γδ T cells are mainly $CD4^{-}CD8^{-}$ T lymphocytes characterised by their expression of an antigen receptor encoded by the γ and δ TCR genes. As they do not express CD4 or CD8 co-receptors in most cases, activation of γδ T cells is possible without requirement of Ag processing and restriction by MHC molecules. These characteristics might allow γδ T cells to be highly autoreactive. However, the TCRγδ restriction to small compounds over-expressed after infection or cell stress, the restricted tissue distribution of TCR agonists as well as the short half-life of the effector cells all contribute to the limitation of the inherent self-reactivity of γδ T cells (Nedellec, Bonneville, & Scotet 2010).

In Behcet's disease, γδ T cell numbers has been observed to be significantly higher during active disease than in remission (Bank, Duvdevani, & Livneh 2003), suggesting that the increase in γδ T cells in BD is a result of disease activity, rather than an innately augmented level of γδ T cells in these individuals.

7.4.1.2 Influence of IFN-α on peripheral blood γδ T cells

From our data on BD patients, escalations of both $CD8^{-}$ and $CD8^{+}$ γδ T cell numbers were detected sporadically in individuals on conventional treatment alone, which were likely to coincide with their spontaneous relapses. However, this tendency to

increase appeared to be thwarted by the addition of IFN-α therapy in the treatment group.

Consistently, our *in vitro* results displayed that higher concentrations of both IFN-α2a and IFN-α2b could significantly downregulate the proportions of $CD3^+$ γδ T cells in healthy donor PBMCs following polyclonal activation by anti-CD3/CD28 or PMA/ionomycin.

In agreement with our results, IFN-α2a was reported to decrease the number of circulating $CD3^+$ and $CD8^+$ γδ T cells considerably after 24 weeks of treatment in BD patients with active ocular disease (Treusch et al. 2004). Also, the proportions of $CD8^+$ γδ T cells were observed to be reduced by IFN-α therapy in patients with hepatitis C in an early study (Wejstal et al. 1992).

7.4.2.1 Setting up IFN-γ and IL-17 producing γδ T cell *in vitro* test models using antigens and polyclonal stimuli

IL-17-producing γδ T cells were reported to express TLR1 and TLR2, as well as dectin-1 in mice (Martin et al. 2009). In agreement, a role for TLR2 in the enhancement of IL-17 production by γδ T cells was revealed in another recent murine study. TLR2 activation alone was not sufficient in inducing IL-17 production from γδ T cells, but in combination with IL-23 induced an upregulated IL-17 responses compared to cells treated with IL-23 alone (Reynolds et al. 2010). As a result of these reports, zymosan, a stimulus for TLR2 and dectin-1 (Dillon et al. 2006), was considered likely to have positive influence on IL-17 producing γδ T cells. However, such result was not observed.

Analysing our data, LPS, candida antigens, and PDS-Ag were the ones which demonstrated some activity in the enhancement of IL-17 producing γδ T cells. The tendency of LPS to promote IL-17 in $CD4^+$ T cells was noticed in Chapter 5, and LPS were previously reported to possess IL-17 inducing activity (Kattah et al. 2008;Moschen et al. 2008). Longer cultures with candida antigens also revealed their potential to augment IL-17 production in γδ T cells, which was unexpected as

the commercial candida antigens used in the experiments were mainly made up of components from the yeast form rather the hyphal form. C. albicans in the yeast form were reported to induce differentiation of IFN-γ producing cells rather than IL-17 producing cells as in the case of C. albicans in hyphal form (Acosta-Rodriguez et al. 2007). PDS-Ag, a BD-related antigen, was another surprise as it did not elicit much Th17 response as depicted in Chapter 5. ATRA, as expected, resulted in lowest IL-17 levels in γδ T cells and this is in accordance to previous report of its ability to inhibit Th17 cell differentiation (Elias et al. 2008;Mucida et al. 2007).

On the other hand, all antigens used in our preliminary experiments promoted very low levels of IFN-γ producing γδ T cells, even with extra time in culture. Of all the combinations used to promote cytokine production by γδ T cells, polyclonal stimuli consistently showed greater potential than using antigens alone, especially in the case of PMA in combination with ionomycin.

7.4.2.2 Testing other aspects of the *in vitro* experimental design

It has been suggested resting PBMCs overnight after thawing before testing effector functions would remove cells destined for death and allow “stunned” cells to recover functional ability (Lamoreaux, Roederer, & Koup 2006). Therefore, instead of stimulating cells straight after thawing, one day resting in culture medium with or without suboptimal level of IL-2 was performed. It was noted that neither resting nor addition of IL-2 augmented the levels of IFN-γ producing and IL-17 producing γδ T cells appreciably.

Unlike the Th1 and Th17 experiments in Chapter 5, a significant difference between RPMI- and IMDM-based culture media was not found in terms of ability to induce IFN-γ producing and IL-17 producing γδ T cells, whether PBMC cultures were activated or not. Consequently, majority of the γδ T cell experiments were carried out using IMDM-based culture medium in line with Th1 and Th17 experiments performed previously.

The benefit of extra time in culture was tested as well but 24 hours of culture was shown to be superior to 39 hours of culture with regards to achieving higher levels of IFN-γ and IL-17 cytokine production by γδ T cells in general. The only exception was IL-17 production by γδ T cells following PMA/ionomycin stimulation where the levels achieved were comparable between 24 and 39 hours of culture. Therefore, a decision was made to perform experiments over 24 hours without resting healthy donor PBMCs.

7.4.3 The influence of IFN-α on IFN-γ production by γδ T cells

Classically, IFN-γ has been regarded as a pro-inflammatory cytokine. In recent years, however, IFN-γ has been implicated not only as a mediator of disease but possibly as a protective factor during autoimmunity depending on factors such as the timing, location, and intensity of IFN-γ action (Kelchtermans, Billiau, & Matthys 2008;O'shea, Ma, & Lipsky 2002).

In Chapter 5, our data have consistently shown that both IFN-α2a and IFN-α2b could increase IFN-γ production by $CD4^+$ and $CD8^+$ T cells *in vitro*, especially at higher concentrations. Similarly, the levels of γδ T cells producing IFN-γ were found to be upregulated in our experiments in the presence of increasing dose of IFN-α. The possible mechanism of action has been suggested to be that type I IFNs, including IFN-α, promote IFN-γ signalling via association of type I and type II IFN receptor subunits (Takaoka et al. 2000).

Of note, IFN-γ producing γδ T cells were found to lack expression of TLR2, dectin-1, CCR6 and AHR in comparison to $IL\text{-}17^+$ γδ T cells in a recent murine study (Martin et al. 2009).

7.4.4.1 Biology of IL-17 producing γδ T cells

It now seems that IL-17 is one of the more important villains in the pathogenesis of autoimmune diseases. It is a potent inducer of autoimmunity through the promotion

of tissue inflammation and the mobilisation of the innate immune system. The challenge is to keep IL-17 in check, while maintaining host defence at the same time. Importantly, IL-17 production is not an exclusive characteristic of $CD4^+$ αβ T cells, and γδ T cells are recognised as a powerful cellular source of IL-17 as well and contribute to the initial defence against pathogens (Michel et al. 2007;Roark et al. 2008).

Low but significant numbers of adult human blood Vγ2Vδ2 T cells produce IL-17 or IL-22 *ex vivo*, although few produce both. Induction of IL-17-producing cells from neonatal naive Vγ2Vδ2 T cells requires cytokines including IL-1β, IL-6, TGF-β, but not IL-23. On the other hand, IL-23, not IL-6, is essential for maximal expansion of IL-17-producing memory Vγ2Vδ2 T cells in adults. For both neonatal and adult Vγ2Vδ2 T cells, cells producing IL-17 have higher levels of RORγt compared with non-IL-17 producing cells, demonstrating the importance of RORγt in IL-17 production (Ness-Schwickerath, Jin, & Morita 2010). It has been observed that many γδ T cells already exist as effector memory cells, ready to mount strong IL-17 responses quickly.

Moreover, it was shown that some phenotypic characteristics are shared between Th17 and IL-17-producing γδ T cells including CCR6, RORγt, AHR, and IL-23 receptor in a recent murine study (Martin et al. 2009).

7.4.4.2 The influence of IFN-α on IL-17 production by γδ T cells

Our results from Chapter 4 and 5 demonstrated the capacity of recombinant IFN-α in the suppression of IL-17 production by $CD4^+$ and $CD8^+$ T cells. It was also shown in a study that pre-treatment with recombinant IFN-α could inhibit human PBMC secretion of IL-17 and activation-induced IL-23 as measured by ELISA (Meyers et al. 2006). However, the subgroups of cells capable of producing IL-17, including γδ T cells, were not discriminated.

In accordance with these findings, both IFN-α2a and IFN-α2b were observed in our experiments using healthy donor PBMCs to inhibit IL-17 production by γδ T cells in a

dose-dependent manner. The results are particularly relevant in the understanding of IFN-α therapy being used as a disease-modifying treatment in immune-mediated diseases like BD as elevated levels of γδ T cells and IL-17 have been described to be associated with disease activity and implicated in the pathogenesis of BD (Bank, Duvdevani, & Livneh 2003;Chi et al. 2008).

7.4.5 The effect of IFN-α on the kinetics of IFN-γ and IL-17 production by γδ T cells

The kinetics of PBMC response under the influence of IFN-α was measured with regards to IFN-γ and IL-17 production levels within γδ T cell populations. The idea was to obtain some indications in the ways IFN-α therapy could contribute as either prophylaxis or treatment options in chronic inflammatory diseases.

Unexpectedly, adding IFN-α and PMA/ionomycin simultaneously induced the largest increase in IFN-γ levels in γδ T cells. On the other hand, concurrent use of IFN-α and PMA/ionomycin resulted in the greatest amount of IL-17 inhibition in γδ T cells. However, these immunomodulatory effects were not significant when IFN-α was added as a pre-treatment at earlier time-points. Similar trends were also observed in untreated samples.

A possible explanation is that IFN-γ was produced by γδ T cells in greater quantity relatively early during incubation in response to IFN-α treatment. On the other hand, IL-17 production by γδ T cells occurred later during cultures after longer exposure of PBMCs to IFN-α. The results could be simply a reflection of the experimental design picking up data at various stages of cytokine production.

7.5 Summary of findings

Our data demonstrated that both IFN-α2a and IFN-α2b have the potential to suppress the number of γδ T cells and their production of cytokine IL-17. On the other hand, IFN-γ producing γδ T cells were shown to be induced following treatment

with IFN-α. Of note, IFN-α appeared to exert the strongest influence on IFN-γ producing and IL-17 producing γδ T cells when it was added 24 hours before intracellular cytokine staining and flow cytometry.

Chapter 8
General discussion and future work

8.1 General discussion

IFN-α is a leukocyte-derived cytokine with profound immunomodulatory capabilities. Despite its discovery more than 50 years ago (Isaacs & Lindenmann 1957), the *in vivo* operative mechanisms of actions are still not sufficiently understood. Interferons have diverse effects, influencing both innate and adaptive immune responses and form a network of complex interactions with other cytokines (Baccala, Kono, & Theofilopoulos 2005). Current advances in our understanding of the development of autoimmune responses, including the important roles played by both recently discovered Treg and Th17 cell lineages, have enabled us to investigate the properties of this effective therapy further.

8.1.1 Approaches used in this study

The work presented in this thesis has been directed at exploring the immunomodulatory effects of IFN-α on peripheral blood T cells, with a view to a potential disease-modifying therapy for the treatment of Behcet's disease. In order to achieve this goal, a number of approaches have been utilised. Firstly, serial whole blood samples from BD patients receiving conventional immunosuppressive therapy with or without recombinant IFN-α2b treatment were phenotyped without activation or culture. Secondly, serial whole blood samples from the same group of BD patients were studied using intracellular cytokine staining, elucidating the influence of IFN-α on both conventional T cells and more recently discovered T cell subsets. Thirdly, the phenotype of interferon treated healthy donor PBMCs was analysed *in vitro* using intracellular cytokine staining and flow cytometry.

Human PBMCs usually contain only very low percentages of effector cells which produce relatively high levels of cytokines required for this kind of analysis (Vyas & Noble 2008). It is therefore necessary to activate the T cells to induce rapid

accumulation of cytoplasmic cytokines before analysis. In our study, cell cultures were either treated with anti-CD3/CD28 Abs or PMA/ionomycin for 24 or 39 hours in order to study cytokine production. However, this makes it difficult to analyse the antigen specificity of responding T cells but will indicate the type 1/type 2/type 17 bias of the population, reflecting previous exposures to antigen.

Intracellular cytokine staining directly analyses T cells producing a given cytokine and is a sensitive and convenient way of monitoring the effect of a medication longitudinally. Also, a unique strength of this technique is the ability to assess multiple cytokines simultaneously. The human whole blood assay employed in our *ex vivo* experiments for determining IFN-α effects may resemble the *in vivo* situation most closely, as other essential factors for T lymphocyte activation are present at physiologic concentrations. However, our *in vitro* experiments gave us an indication of the influence of IFN-α in the absence of other immunosuppressants.

8.1.2 Findings from *ex vivo* experiments

Analysing our data, six months of subcutaneous pegylated IFN-α2b therapy was not observed to have any impact on the proportions of $CD4^+$, $CD8^+$ and αβ T cells in BD patients. However, the tendency of γδ T cell number to increase in BD patients was suppressed in those who received IFN-α therapy. In terms of cytokine production, IFN-α therapy demonstrated the tendency to enhance T cell production of IFN-γ but not TNF-α. Under the influence of IFN-α2b therapy, upregulation of IL-2 and IL-4 expression by $CD3^+$ T cells was consistently observed. All these findings have been described in some earlier studies, even though the subjects of most previous studies were not BD patients. Notably, all the positive effects of IFN-α detected in our work were enduring as they were evident even after the termination of IFN-α2b treatment. To our knowledge, this is the first time 12-month longitudinal data concerning BD patients who received IFN-α treatment was presented.

$CD4^+$ T lymphocytes play an essential role in steering immune responses and can contribute to autoimmunity. These cells include effector T cells and regulatory T cells, which protect against effector responses to autoantigens and also against

responses to exogenous antigens. Discoveries made over the past two decades have markedly changed the way we study immunological processes. Not the least of these was the identification and cloning of several new cytokines, including IL-17. These discoveries provided compelling reasons to explore outside the Th1/Th2 cell paradigm in search of answers to explain the effector T cell responses that occur independently of known Th1 and Th2 cell signalling pathways. Understanding the roles played by alternative T cell subsets is critical in avoiding excessive chronic inflammation and tissue damage.

With regards to Th17 cells, it was demonstrated in our experiments that 6 months of subcutaneous IFN-α2b therapy could downregulate these cells in peripheral blood of BD patients. This is in accordance to a very recent study which found that IFN-α significantly suppressed IL-17 production in PBMC culture supernatants from BD patients *in vitro* (Liu et al. 2011). Whereas, $CD4^{+}Foxp3^{+}$ Treg cell populations, especially $CD4^{+}CD25^{high}$ T cells, were upregulated following IFN-α2b treatment in BD patients. To our knowledge, this is the first time that IFN-α treatment has been observed to increase the proportions of $CD4^{+}Foxp3^{+}$ T cells in PBMCs. Importantly, the effects on Th17 and Treg cells were enduring as they were observed even after cessation of IFN-α therapy. These findings could be pivotal in order to understand IFN-α as a disease-modifying treatment for BD.

Furthermore, a tendency to upregulate the production levels of IL-10 and TGF-β by T cells was detected after treatment with IFN-α. This increase in immunoregulatory cytokine levels could assist in the regulation of Th cells and promote the number and function of Treg subsets.

8.1.3 Findings from *in vitro* experiments

In our study, *in vitro* experiments were carried out using healthy donor PBMCs to test the effects of IFN-α in the absence of the influence by corticosteroids and other immunosuppressants. The other aim was to examine whether similar effects by IFN-α on BD patients could be observed in healthy donor cells. Additionally, the influence of IFN-α on different subsets of $CD8^{+}$ and γδ T cells was explored further.

Both recombinant IFN-α2a and IFN-α2b were used and they were demonstrated to be equally effective in their control on the relative proportions of T cell subsets and inflammatory cytokine production by T cells.

Higher doses of IFN-α demonstrated the tendency to upregulate $CD4^+$ but inhibit $CD8^+$ T cells. IFN-α also showed the potential to increase IFN-γ producing $CD4^+$ and $CD8^+$ T cells and suppress $CD4^+$ and $CD8^+$ T cells producing IL-17. On the other hand, IFN-α2a and IFN-α2b were efficacious in inducing Foxp3 expression by $CD4^+$, $CD4^+CD25^+$, $CD4^+CD25^{high}$, $CD8^+$, and $CD8^+CD25^+$ T cell populations. Both forms of IFN-α were observed to consistently upregulate IL-10 and TGF-β levels in $CD4^+$ and $CD8^+$ T cells as well. Notably, IFN-α was found to be able to exert its effects on Th17 and Treg cells both before and after cell activation. This may imply that IFN-α therapy may be effective even when the disease is active. Moreover, our data showed that both IFN-α2a and IFN-α2b have the potential to suppress the number of γδ T cells and their production of cytokine IL-17. On the other hand, IFN-γ producing γδ T cells were observed to be induced following treatment with IFN-α.

To our knowledge, it is the first time that induction of $CD4^+Foxp3^+$ and $CD8^+Foxp3^+$ T cells by IFN-α has been demonstrated *in vitro*. The effects of IFN-α on various $CD8^+$ and γδ T cells subsets described here has not been reported in previous studies either.

8.1.4 The implications of the findings in this study

In chronic inflammatory and autoimmune conditions, the entire cytokine network is in a long-term persistent state of activation. In such situations, either the stimulatory or the counter-regulatory circuits might in the end predominate in determining the net global effect of cytokines (Kelchtermans, Billiau & Matthys 2008). It is possible that acute flares of autoimmune diseases may be explained by a preferential differentiation towards pro-inflammatory lineages or enhanced survival of these phenotypes in the periphery. T cell heterogeneity and plasticity open opportunities for targeting or redirecting specific subsets in immune-mediated diseases. In our study, cytokine production by $CD4^+$ T cells, $CD8^+$ T cells and γδ T cells was

investigated. It is likely that cooperation between different cytokine-producing T cell subsets needs to be regulated to achieve desired immune responses and immune system homeostasis.

It has been suggested that the cytokines secreted by $CD4^+$ T cells play a complementary function with cytokines produced by $CD8^+$ and γδ T cells as they release cytokines at different phase during an inflammatory response. Importantly, even though other cell types could trigger auto-aggressive responses independently of $CD4^+$ T cells, a lack of cooperation from these cells would limit the damage to the host due to the reduced duration of such responses as a result. Indeed, a sustained memory $CD8^+$ T cell response and high-affinity immunoglobulin production by B cells are restricted without $CD4^+$ T cell help (Castellino & Germain 2006).

In comparison to $CD4^+$ T cells, naive $CD8^+$ T cells acquire effector T cell functions more readily upon antigen stimulation (Seder & Ahmed 2003). Activated $CD8^+$ T cells can produce very high levels of inflammatory cytokines, including IFN-γ (Liblau et al. 2002). These may contribute greatly to target cell destruction in autoimmune diseases and therefore regulation is essential for $CD8^+$ T cells. Notably, it has been demonstrated that $CD8^+$ T cells can direct the development of $CD4^+$ T cells into Th1 and Th2 effectors. Tc1 clones have been shown to favour the development of $CD4^+$ effectors that are Th1-biased. Whereas Tc2 clones not only can promote Th2 effectors but also can efficiently suppress the development of Th1 cells (Vukmanovic-Stejic et al. 2000).

γδ T cells are able to prime downstream immune events, including the production of pathogenic TNF-α and T helper type 1 cytokines, it is plausible that the disruption of γδ T cell-mediated immunostimulation could be an important mechanism of action for IFN-α. Antigen-naive γδ T cells are known to produce large amount of effector cytokines within hours upon infection. Also, cytokines produced by γδ T cells can contribute to the regulation of αβ T cell activation (Kuhl et al. 2009;Roark et al. 2008).

From our data, IFN-α was shown to have the tendency to promote Th1 signature cytokine IFN-γ. The proportions of IFN-γ secreting T cells, including $CD4^+$, $CD8^+$ and γδ T cells were all upregulated by IFN-α *in vitro*. This could be due to the

augmentation of IFN-γ signalling by IFN-α via association of type I and type II IFN receptor subunits (Takaoka et al. 2000). It may also be that the effects of IFN-α on one cell subset to secrete IFN-γ induced stimulation of other immune cells to produce IFN-γ in a series of positive feedback loops. IFN-γ lowers host damage during active disease by lowering the recruitment of neutrophils to inflammatory sites and suppressing the production of highly pro-inflammatory molecules, including IL-17, and the development of the Th17 lineage (Kelchtermans, Billiau, & Matthys 2008). Therefore, it was not unexpected that IFN-α was observed to have the capacity to downregulate IL-17 secreting T cells including $CD4^+$, $CD8^+$ and γδ T cells in our experiments. All IL-17 producing cells may have pivotal roles in licensing T cell effector functions that result in inflammatory disease. However, the relative contributions of Th17 cells versus other IL-17 producing T cells to inflammatory responses in Behcet's disease are not clear.

The ratio of IFN-γ : IL-4 secreting cells has been reported to be higher in BD patient with active disease (Misumi et al. 2003). Thus, increased disease activity in BD patients could be associated with higher IFN-γ : IL-4 ratio rather than higher levels of IFN-γ alone. It was observed in our *ex vivo* experiments that IL-4 production by T cells was appreciably augmented by IFN-α therapy, even though IFN-γ production by T cells showed a tendency to be upregulated by IFN-α as well. Thus, our patients who had less active disease after IFN-α therapy might even have lower ratio of IFN-γ : IL-4 secreting cells when compared to before initiation of treatment.

As IL-2 has been shown to be necessary for T cells to acquire IL-4 producing capacity (Paul & Zhu 2010), the enhanced amounts of IL-2 expression detected in our experiments could contribute to IL-4 augmentation. Early in an immune response, IL-2 maintains T cell survival and supports the growth and expansion of T cells. If the response is enduring, as in the case of self-antigens, the prolonged IL-2 exposure makes T cells sensitive to Fas-mediated apoptosis and terminates the response (Abbas 2003) IL-2 is indispensable for the induction of Tregs as IL-2 signalling is required for thymic development, peripheral expansion and suppressive activity of Treg cells (Shevach et al. 2006). The other common γ-chain cytokines including IL-4 were also reported to maintain maximal regulatory function, suggesting a degree of redundancy of the cytokines required to maintain Treg function. However, the concentrations required for IL-4 to achieve the same effect were up 50-fold higher

than for IL-2 *in vitro*, thought to reflect the high density of expression of the IL-2 αβγ receptor by $CD4^+CD25^+$ Tregs (Yates et al. 2007).

Our results are, to our knowledge, the first demonstration of an upregulatory effect on T cell Foxp3 expression in PBMCs by IFN-α treatment. It is possible that proliferation of $Foxp3^{low}$ naive natural regulatory T cells as well as induced Treg cells may have contributed to our findings. Increases in Foxp3 expression can also be a result of activation in effector T cells, and in this case it is likely that the induced Foxp3 expression may provide a way to fine-tune proliferation and effector function of these cells. This is based on the fact that Foxp3-transduced human naive $CD4^+CD25^-CD45RA^+$ T cells have been demonstrated to be hyporesponsive and have a significantly reduced capacity to produce cytokines, including IL-2 and IFN-γ, upon TCR-mediated activation in comparison with controls (Allan et al. 2005). Moreover, Foxp3 may form transcription factor complexes with T-bet, GATA3, or RORγt so that the capacity of these factors to induce effector cytokines is inhibited (Zhu & Paul 2010). Importantly, our findings indicate a previously unrecognised potential mechanism by which IFN-α therapy may provide benefit to BD patients.

Tregs have the capacity to actively block immune responses, inflammation, and tissue destruction and have a major role in suppressing proliferation and effector functions of other immune cells of both the adaptive ($CD4^+$ and $CD8^+$ αβ T cells, and B cells), and the innate (NK cells, NKT cells, monocytes and dendritic cells) immune system (Grossman et al. 2004;Tang & Bluestone 2008). Recently, it was also demonstrated that $CD4^+CD25^{high}Foxp3^+$ T cells isolated from human blood could inhibit both anti-CD3 and antigen-driven activation and IFN-γ production by $Vδ2^+$ T cells (Mahan et al. 2009). Tregs were shown to suppress γδ T cell proliferation via a cell-cell contact-independent mechanism (Kunzmann et al. 2009). However, it was reported that human Th17 cells were less sensitive to Tregs than Th1 cells. Likewise, $CD8^+IL\text{-}17^+$ cells showed little susceptibility to Tregs from peripheral blood (Annunziato et al. 2007;Ortega et al. 2009).

Of note, human Treg cells probably cannot suppress pro-inflammatory cytokine production and proliferation in conditions in which effector T cells are strongly activated (Sakaguchi et al. 2010). It is likely that the loss of suppression observed in

the presence of strongly activating factors is due to both an increase in effector T cells resistance and a decrease in Treg cell function (Koenen et al. 2008). In addition, there is propensity of Tregs to convert to Th17 cells in the context of pro-inflammatory stimuli, such as IL-1β and IL-6, in humans (Abdulahad, Boots, & Kallenberg 2010). Increased Foxp3 expression induced by IFN-α in our study, however, could help stabilise the phenotype and function of $Foxp3^+$ Treg cells and reduce their conversion to pathogenic IL-17 secreting effector T cells during active phase of immune-mediated disease like BD.

The diversity of effector mechanisms affords versatility to a Treg-mediated suppression program capable of restraining diverse types of inflammatory responses in different tissues (Feuerer et al. 2009). In some systems, direct cell-to-cell contact seems to be required; in other systems, suppression seems to involve TGF-β and IL-10 cytokines. Our data have suggested that IFN-α therapy has the tendency to upregulate IL-10 and TGF-β production by T cells. Therefore, besides promoting Treg cell subsets, IFN-α may enhance negative feedback mechanisms ascribed to cytokines such as IL-10 and TGF-β, which limits the inflammatory reactions.

Major T cell sources of IL-10 include subsets of regulatory T cells designated Tr1 as well as Th2, Th1, and Th17 cells. $CD8^+$ T cells also produce IL-10 (Mosser & Zhang 2008). Our results indicated that it was likely some Tr1 cells were induced by IFN-α treatment. Notably, IL-10 production can be promoted by IL-10 itself, IL-27 and TGF-β (Zhu & Paul 2008). As our results showed the tendency for increased TGF-β secretion by T cells of BD and healthy patients *ex vivo* and *in vitro* respectively following IFN-α treatment, it is possible that besides Tr1 cells, Th3 regulatory cells might also be induced by IFN-α therapy. Different types of Treg cells have complementary and overlapping functions in suppression of autoimmunity and associated inflammation (Littman & Rudensky 2010).

TGF-β suppresses immune responses through at least two ways: inhibiting the function of inflammatory cells and promoting the function of Treg cells (Wan & Flavell 2008). Although T cell specific target genes of TGF-β are largely unknown, the expression of genes important for T cell differentiation and function, such as GATA3, T-bet, STAT4, IFN-γ, and granzyme-B, are suppressed by TGF-β (Wan & Flavell

2008). Although nTreg cell commitment is independent of TGF-β signalling, TGF-β has been suggested to promote nTreg cell survival by antagonising T cell negative selection and protecting nTreg cells from apoptosis (Ouyang et al. 2010).

Of note, recent findings have revealed that TGF-β can also have a pro-inflammatory role dependent on the immunological environment. Thus, in the presence of pro-inflammatory cytokines, TGF-β contributes to the generation of immunopathogenic IL-17 secreting T cells (Yang et al. 2008b). Consequently, the prerequisite for the generation of Treg cells, rather than IL-17 secreting T cells, by TGF-β is the removal of pro-inflammatory cytokines, specifically IL-6, but also TNF-α and IL-1. IFN-α can serve this function well as it has been indicated in an early study to suppress the ability of IL-6, the critical partner for TGF-β in the production of IL-17 producing T cells, to bind to the IL-6 receptor by downregulating IL-6 receptor expression (Anthes et al. 1995). Also, it has been shown in some very early studies that IFN-α inhibits IL-1 synthesis by PBMCs (Schindler, Ghezzi, & Dinarello 1990), and induces IL-1 receptor antagonist (Tilg et al. 1993).

Therapy targeting Treg cell populations and immunoregulatory cytokines could be a powerful approach for modulating responses in immune-mediated disease like Behcet's disease. Activated Treg cells can suppress a wide range of immune responses through bystander suppression (Tang & Bluestone 2008), and Treg cells have been known to induce the development of long-lasting immunologic memory via modulation of APCs and *de novo* development of Tregs (Jonuleit et al. 2002;Roncarolo et al. 2006). Thus, even though individual Treg cells have a finite lifespan, their ability to impart a tolerant phenotype to other cells via infectious tolerance means that their transient immunosuppressive signal could be maintained for a long time.

In this study, we have explored the potentiation of Tregs with IFN-α treatment as evident by increases in Foxp3 expression and regulatory cytokine production *ex vivo* and *in vitro*. The resulting $Foxp3^+$ Tregs likely serve to curb escalating effector T cell responses and control tissue damage. Restoring immune homeostasis with upregulation of Tregs would, it is hoped, be effective as at least a short-term therapy, because it would then allow a tolerogenic milieu to develop and ultimately promote the recovery of natural regulatory responses. It is tempting to speculate that increases in Treg cell

subsets after 6 months of IFN-α therapy could be responsible for the long-lived efficacy in patients with BD.

IFN-α therapy holds the promise to replace and/or supplement indiscriminatory immunosuppression by drugs with potentially deadly side effects. This is not to say that immunosuppressants may be completely useless. Their transient application may help to set the immune system to a stage where Treg cells can be more effective in dealing specifically with unwanted immunity. The data presented here could serve as a basis for understanding the mechanism of action of IFN-α and identifying strategies for effective treatment. Our findings may provide new inroads into elucidating the mechanisms involved in the disease-modifying ability of IFN-α therapy. Which of the above mechanisms plays the most important role in the observed beneficial effects of IFN-α in the treatment of BD remains to be elucidated.

In conclusion, IFN-α has unique immunomodulatory properties and is a treatment option for BD. This study provides molecular evidences for possible mechanisms of action of IFN-α in the BD treatment.

8.2 Limitations of this study

The diverse immunosuppressive drugs received by BD patients in our study, especially those with active disease, may have an influence on peripheral blood T cell subsets and contribute to interindividual heterogeneity. Attempts were made in both groups of BD patients to reduce systemic steroid requirement to ≤10mg daily gradually over the initial 3-6 months and to stop the second line immunosuppressive agents. However, a possible implication of the concurrent use of corticosteroids and other immunosuppressants in our BD patients cannot be definitely ruled out. Since similar effects on cytokine production were not consistently detected in patients on immunosuppressants alone and response of our IFN-α treated patients was impressive and enduring, our results suggested the observed effects were predominantly due to IFN-α therapy in BD patients.

Regrettably, larger sample size would have been more ideal as this would enable subgroup analysis of patients on similar immunosuppressants. However, the amount of time required to recruit a much larger number of BD patients meeting the inclusion criteria was not practical. Therefore, the impact of concurrent use of steroids and second line agents was minimised as much as we could under the circumstances through randomisation. Undoubtedly, further studies on the influences of IFN-α monotherapy on the cytokine network in newly diagnosed yet untreated patients with different manifestations of BD would be useful to overcome the limitations of the present study. Moreover, no age- and sex-matched healthy volunteer was enrolled in our study and therefore data on cytokine producing T cells obtained from our trial patient samples could not be evaluated against healthy subjects to determine whether the levels were normal to start with.

It is unfortunate that an investigation of the inflammatory parameters in the active inflammatory sites could not be carried out as specimens other than peripheral bloods were not available. As BD is a multisystem inflammatory disorder, studying peripheral blood samples of patients alone may not help us understand fully the extent of damages caused by the inflammatory response in these patients. Also, due to omission from the original trial design, the *in vivo* effects of 6 months of IFN-α therapy on some potentially interesting T cell subsets such as IL-17 producing γδ and $CD8^+$ T lymphocytes were not included in the investigation of our BD patients.

Following the discovery that Foxp3 expression has a central role in the differentiation and maintenance of Treg cells, the use of flow cytometry-based analysis of Foxp3 expression in T cells became the gold standard for defining Treg cells (Buckner 2010). However, it was discovered that Foxp3 can also be expressed by effector T cells following activation, raising the possibility that any assessment of Treg cell number or function may include recently activated effector T cells in the Treg cell populations (Pillai et al. 2007). The lack of a cell marker that is unique to Treg cells and the multiplicity of Treg cell subsets complicated the task of enumerating Treg cells. In our study, a phenotypical evaluation of Treg cells was the approach used to get an indication of their levels, but no functional assays or Treg suppression assays were performed to assist in the assessment of Treg levels. Of note, as Foxp3 is a nuclear protein, assessment of its expression in T cells requires fixation and

permeabilisation of the cells, resulting in an inability to obtain viable cells for further functional analysis (Buckner 2010).

8.3 Future work

Due to the fact that human effector T cells could transiently express Foxp3 following TCR-mediated activation (Pillai et al. 2007), performing functional assays would be useful to get an indication whether an upregulation of $Foxp3^+$ expression by T cells following IFN-α treatment would also lead to an increase in Treg function.

Studies of Treg cell function in human autoimmune diseases have examined the proliferation of responder cells in response to polyclonal activation in co-cultures with Treg cells isolated from PBMCs. Evaluation is based on the incorporation of either tritiated thymidine (^{3}H-thymidine) or on the dilution of the fluorescent label 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE). Measurements of cytokine production by the responder cells can also be performed in conjunction with these assays. The classical suppression assay involves the co-culture of $CD25^+$ Tregs and $CD25^-$ responder T cells over a range of suppressor : responder ratios in order to measure the functional activity of Tregs (Thornton & Shevach 1998).

CFSE dilution is considered a superior approach for examining Treg cell-mediated suppression, as it shows the number of cell divisions throughout the culture period. On the other hand, ^{3}H-thymidine incorporation assays only demonstrate the level of proliferation that occurs during the period in which the ^{3}H-thymidine is present in the culture, which typically lasts for 12-16 hours. The use of CFSE also has the advantage of allowing simultaneous analysis of cell surface markers and cytokines to better define the character of the proliferating responder cells. However, the quantity of cells that is needed for CFSE assays is much greater than that required for ^{3}H-thymidine incorporation assays, making CFSE assays less useful when only small amounts of blood are available (Buckner 2010).

Of note, the results of these types of studies are influenced by the way by which the Treg cells are isolated. This can affect the composition of the Treg cell pool and

hence the degree of inhibition. The type of selection method (bead based or flow cytometry based) and the stringency of selection (based on the level of CD25 expression and the use of additional Treg cell markers) can lead to large differences in the percentage of isolated cells that express Foxp3 and in the level of suppression. The type and strength of the stimulus used should be taken into account as well when interpreting any of these studies (Buckner 2010).

The specificity and activation status of regulatory and effector T cell populations as well as the cytokines present in the microenvironment and the activation status of antigen-presenting cells (APCs) will influence the capacity of Tregs to suppress *in vivo*. These conditions are often not well modelled *in vitro* and this caveat represents the greatest limitation of this type of assay. Suppressing the proliferation or cytokine production of highly activated polarised T cells is much more challenging. Tregs from the site of inflammation are more suppressive than their peripheral blood counterparts but, simultaneously, the activated responder T cells from the inflammatory foci are more resistant to suppression (de Kleer et al. 2004;van Amelsfort et al. 2004).

To examine whether IFN-α modifies the functional features of peripheral $CD4^{+}CD25^{-}$ T cells and $CD4^{+}CD25^{high}$ T cells (which should be enriched for nTreg) , their proliferative capacity, cytokine production profile, and suppressive function can be tested *in vitro*. One idea is to perform functional assays on the two T cell subsets purified from Behcet's disease patients before and during IFN-α monotherapy. Another idea is to treat either purified Treg cells or responder cell populations from healthy donor PBMCs with IFN-α before performing *in vitro* functional assay using the two T cell subsets and the results can be compared to data collected from untreated assays. These functional assays are likely to give some indication of the potential of IFN-α to enhance Treg cell function and decrease resistance of responder cells to suppression.

As the BD patients in our study are intended to be followed up for 3 years, longitudinal data at two and three year assessment visits will be available for analysis in the near future. This valuable information should help confirm whether the disease-modifying ability of IFN-α therapy persists after termination of treatment.

Moreover, all the clinical details and disease activity of BD patients throughout the trial will become available once the clinical trial has come to an end, correlation between clinical findings and experimental data will then be able to be examined. It would also be of interest to analyse both BD patients with active and inactive disease and investigate whether there is a difference between the two groups in terms of peripheral blood T cell populations.

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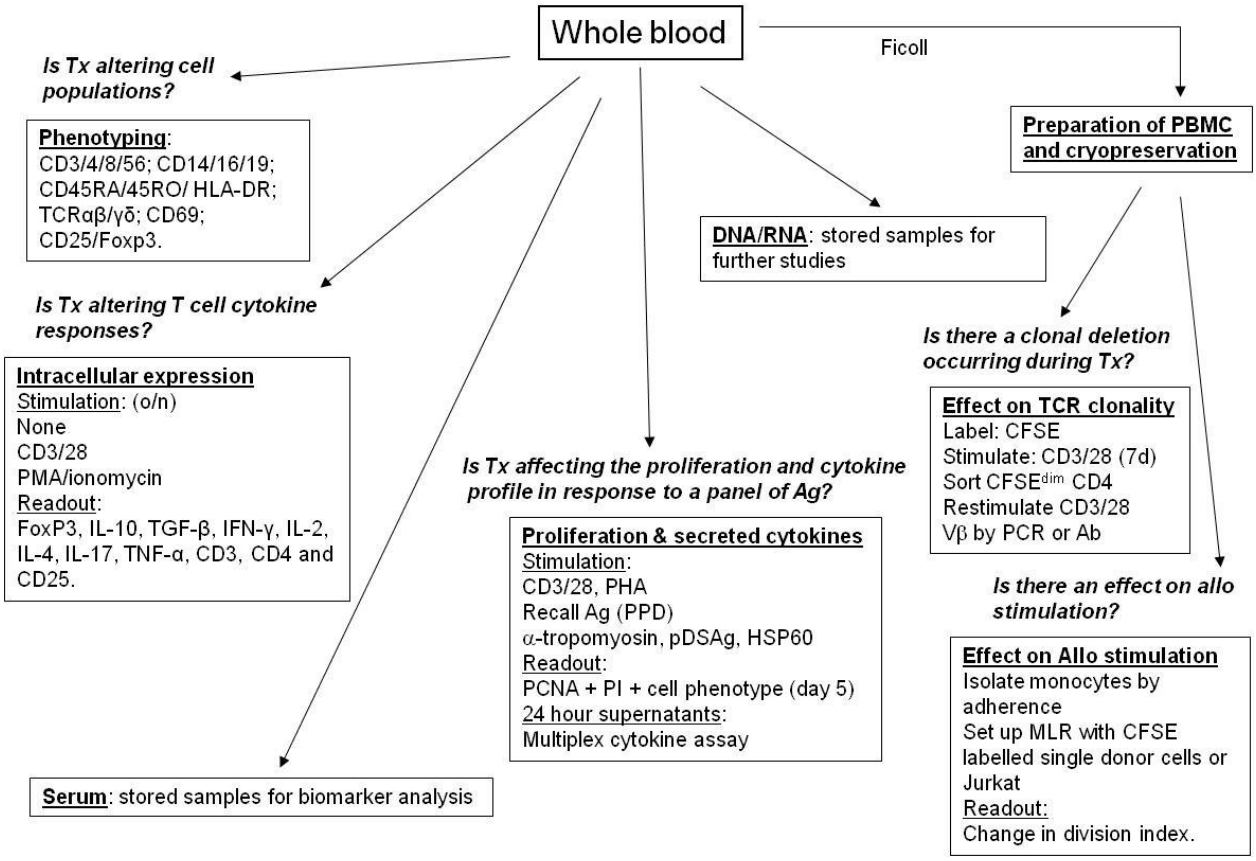
Appendix 1: Overview of the IFN-α Trial

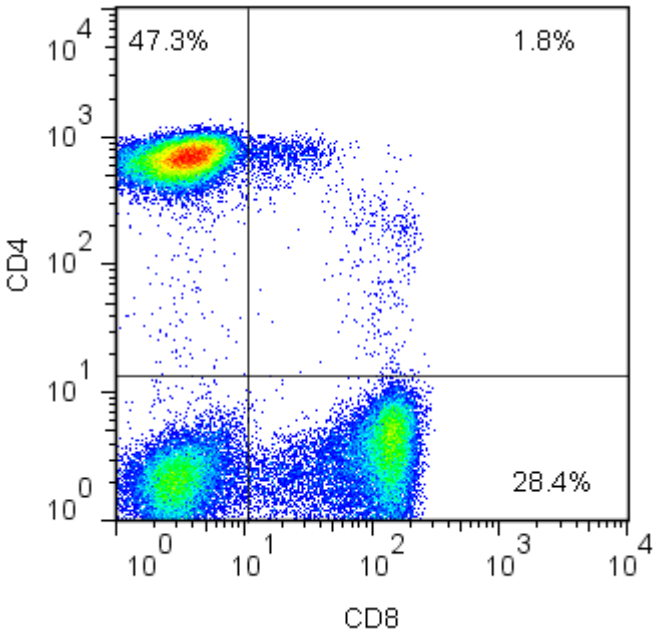


Appendix 2: Fluorescent antibodies

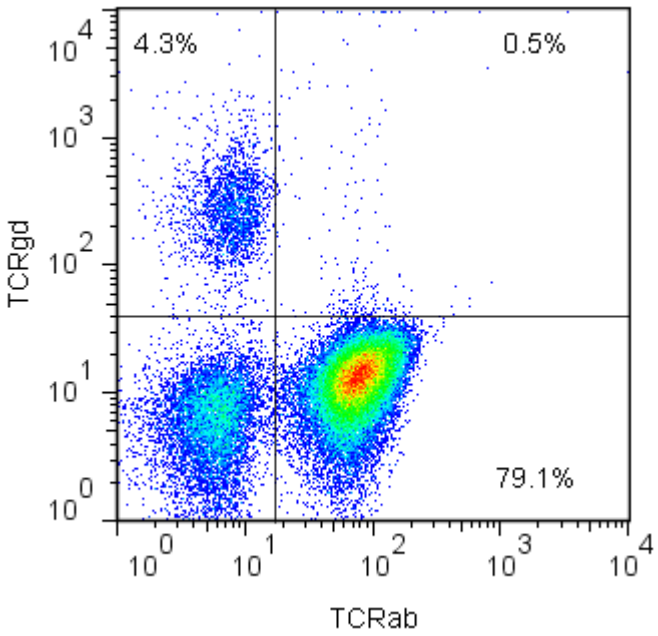
Fluorochrome	Laser excitation line (nm)	Maximal absorbance (nm)	Maximal emission (nm)
Alexa Fluor® 488	488	495	519
FITC	488	490	525
PE	488	490, 565	578
PerCP	488	490	675
APC	633, 635, 647	650	661

Appendix 3: Representative *ex vivo* FACS plots on Behcet's disease patients

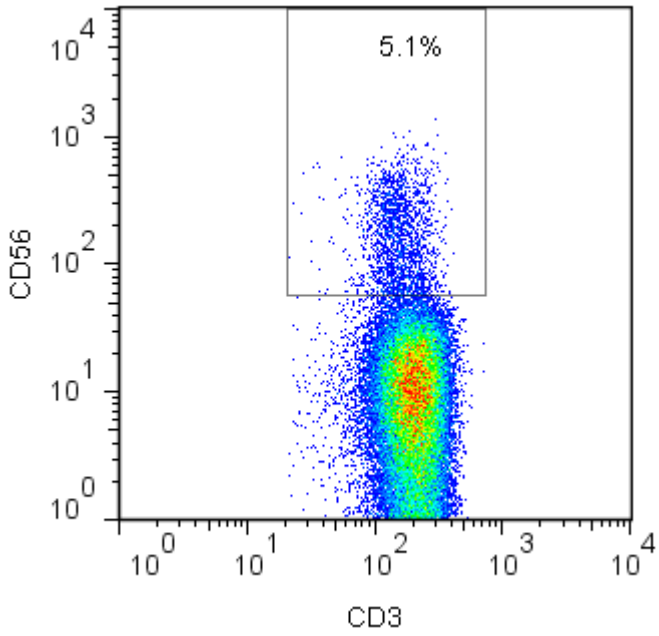
(a) Representative dot plot showing the amount of $CD4^+$ and $CD8^+$ T cells in peripheral blood lymphocytes of patients.



(b) Representative dot plot showing the amount of peripheral blood lymphocytes bearing the αβ and γδ T cell receptors.

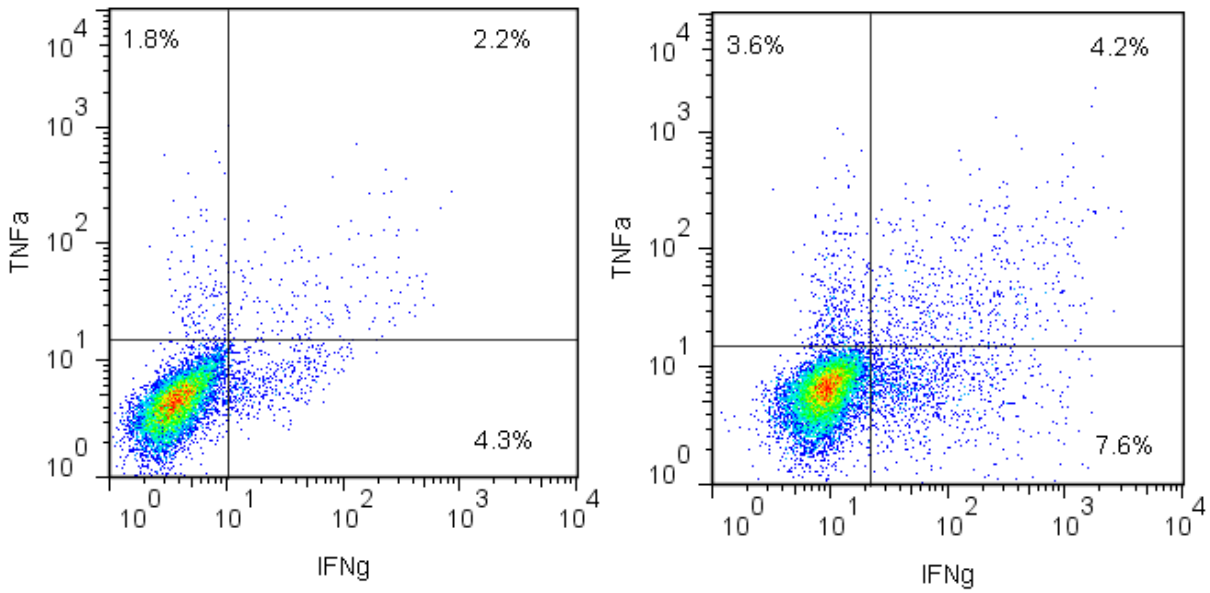


(c) Representative dot plot showing the amount of $CD56^+$ cells in peripheral blood $CD3^+$ T cells.

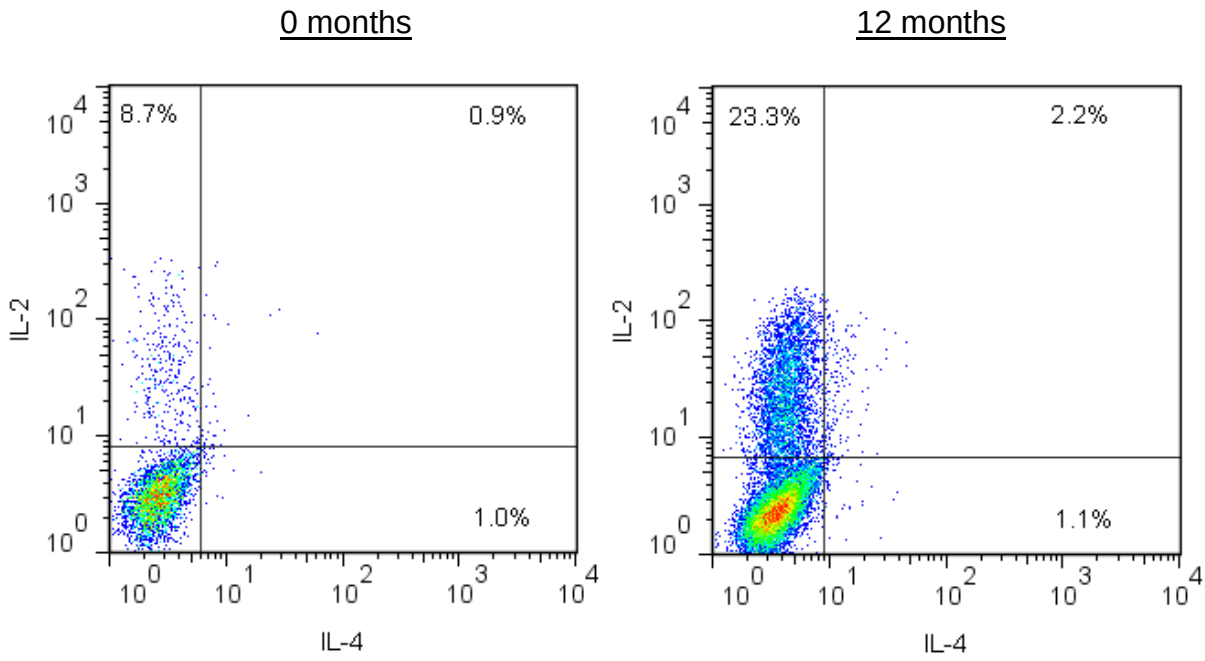


(d) Representative dot plots showing the influence of a 6-month course of IFN-α on the proportions of peripheral blood $CD3^+$ lymphocytes producing IFN-γ and TNF-α in one patient.

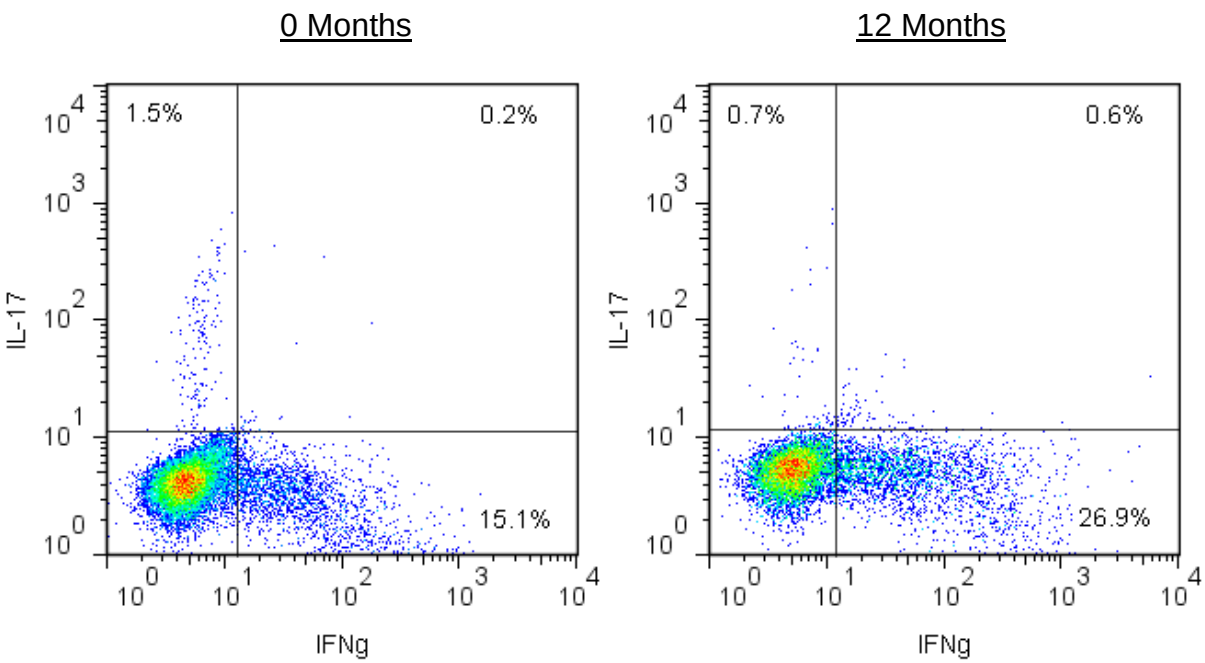
<u>0 months</u> <u>12 months</u>



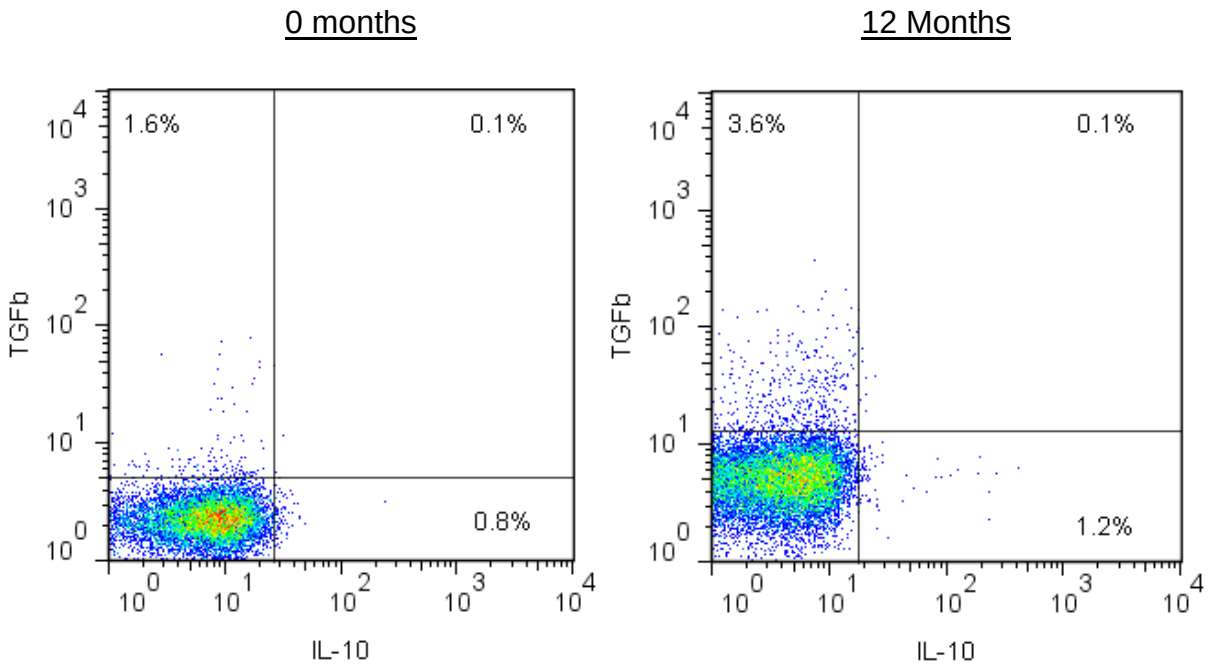
(e) Representative dot plots showing the influence of a 6-month course of IFN-α on the proportions of peripheral blood $CD3^+$ lymphocytes producing IL-2 and IL-4 in one patient.



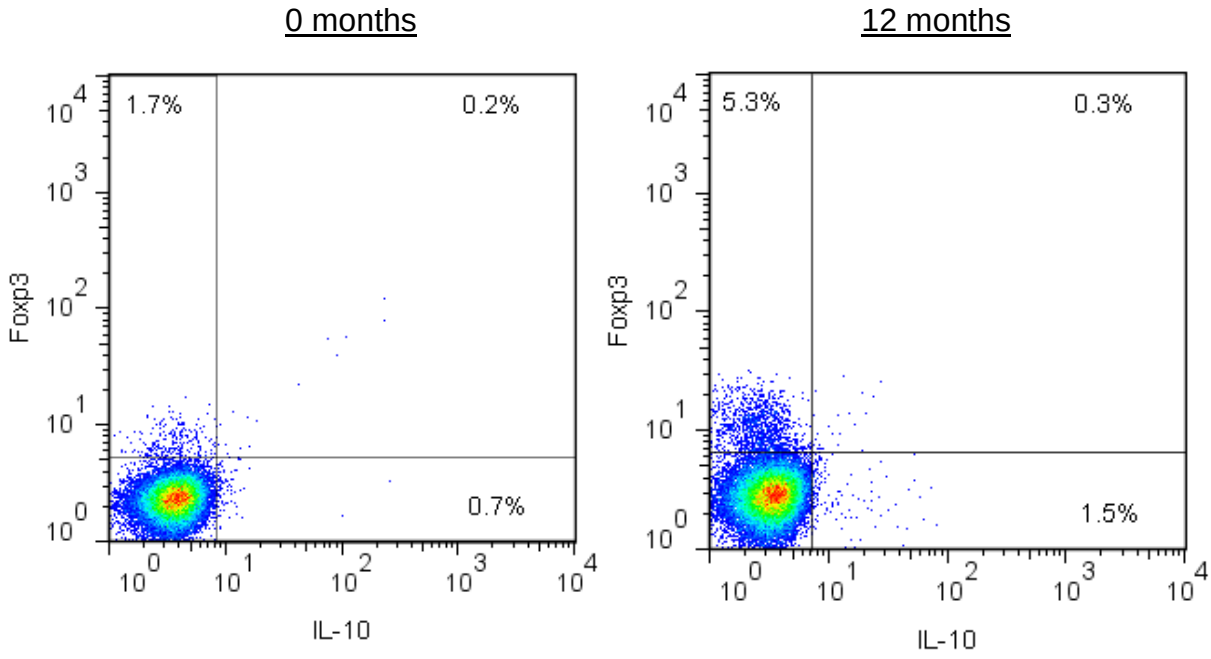
(f) Representative dot plots showing the influence of a 6-month course of IFN-α on the proportions of peripheral blood $CD4^+$ lymphocytes producing IFN-γ and IL-17 in one patient.



(g) Representative dot plots showing the influence of a 6-month course of IFN-α on the proportions of peripheral blood $CD3^+$ lymphocytes producing IL-10 and TGF-β in one patient.

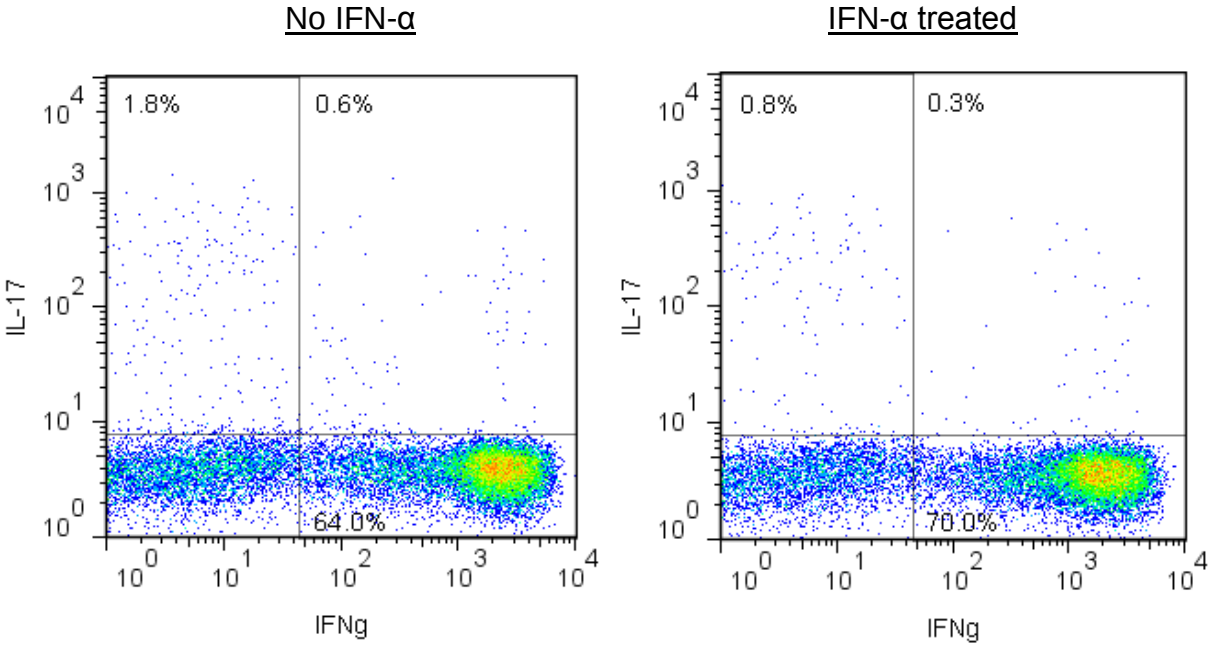


(h) Representative dot plots showing the influence of a 6-month course of IFN-α on the proportions of peripheral blood $CD4^+$ lymphocytes expressing IL-10 and Foxp3 in one patient.

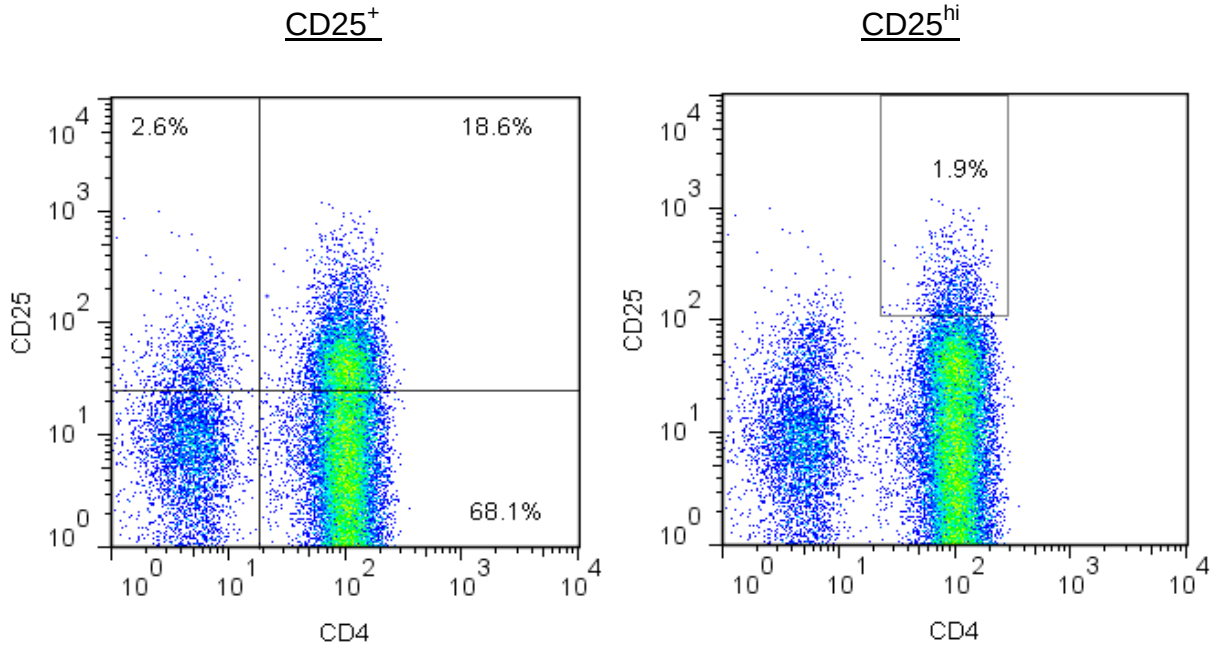


Appendix 4: Representative *in vitro* FACS plots

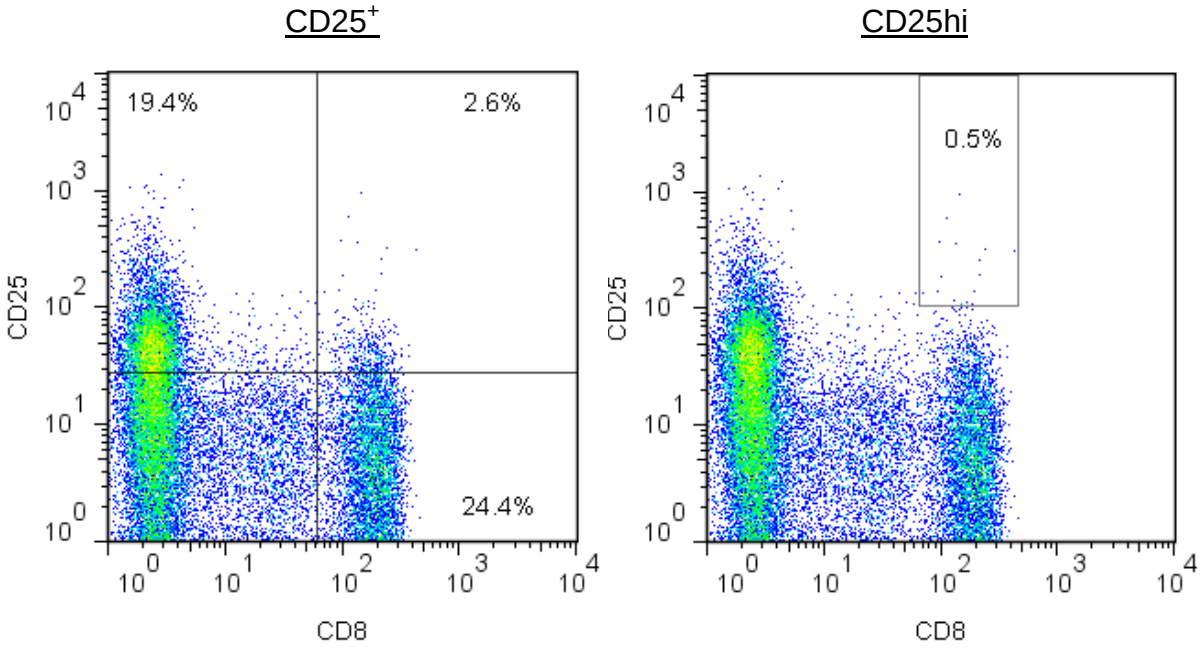
(a) Representative dot plots showing the effect of IFN-α (10^3 U/ml IFN-α2b) on the proportions of peripheral blood $CD4^+$ lymphocytes producing IFN-γ and IL-17. Healthy donor PBMCs were stimulated with PMA and ionomycin for 24 hours. Similar gating strategies were applied to $CD8^+$ and γδ T cells as well to examine the influence of IFN-α on these populations.



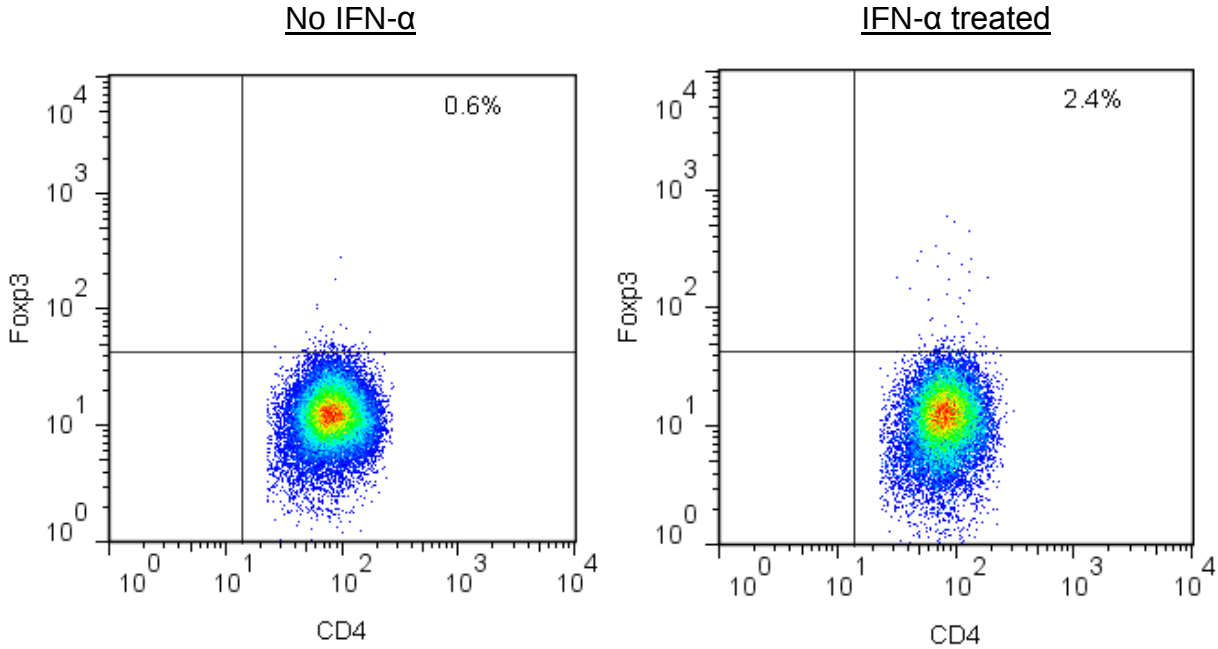
(b) Representative dot plots showing how the $CD4^+CD25^+$ and $CD4^+CD25^{hi}$ T cells are gated within the T lymphocyte populations.



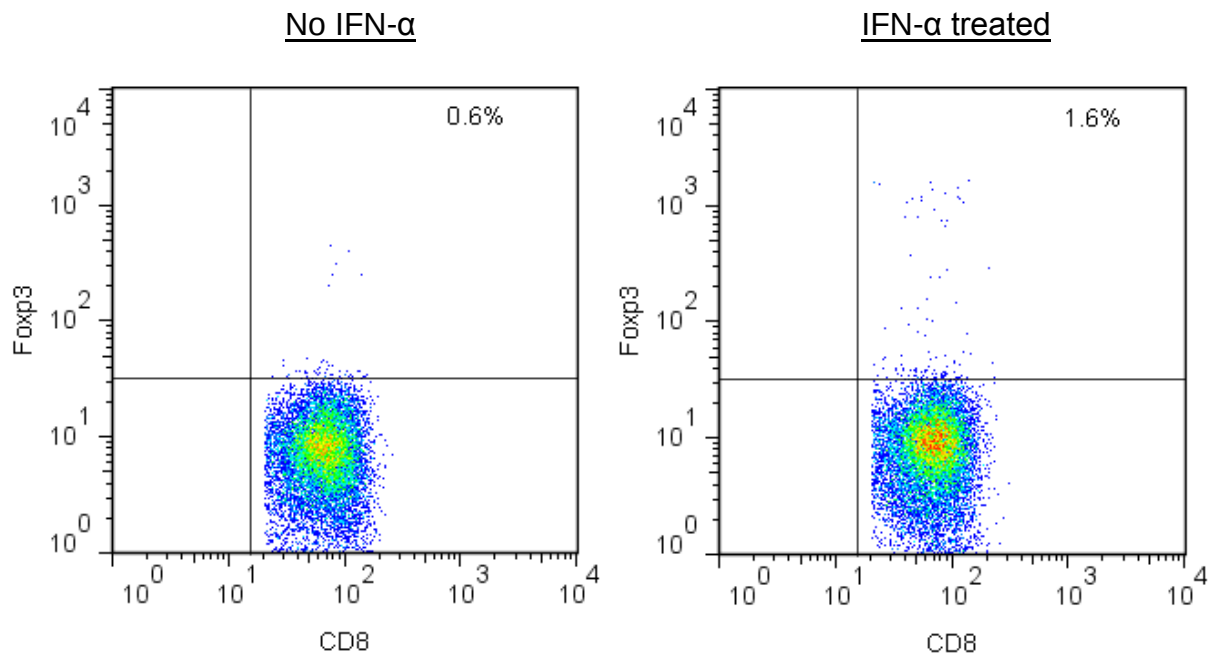
(c) Representative dot plots showing how the $CD8^+CD25^+$ and $CD8^+CD25^{hi}$ T cells are gated within the T lymphocyte populations.



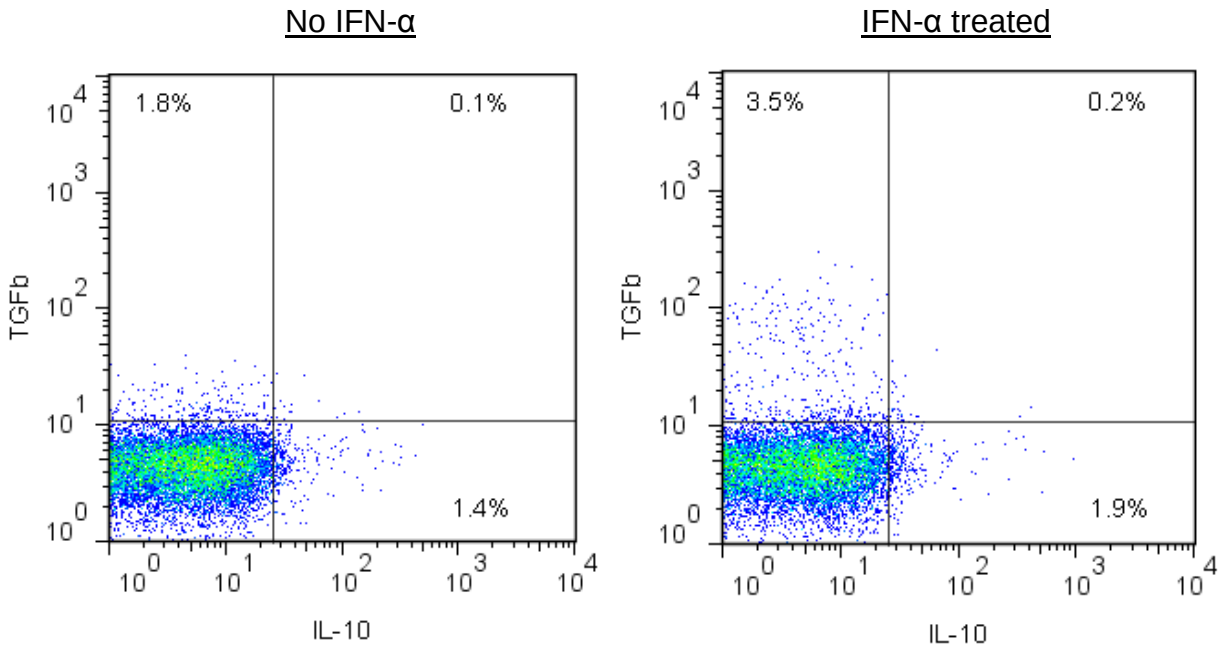
(d) Representative dot plots showing the effect of IFN-α (10^3 U/ml IFN-α2b) on the proportions of peripheral blood $CD4^+$ lymphocytes expressing Foxp3. Healthy donor PBMCs were stimulated with PMA and ionomycin for 24 hours.



(e) Representative dot plots showing the effect of IFN-α (10^3 U/ml IFN-α2b) on the proportions of peripheral blood $CD8^+$ lymphocytes expressing Foxp3. Healthy donor PBMCs were stimulated with PMA and ionomycin for 24 hours.



(f) Representative dot plots showing the effect of IFN-α (10^3 U/ml IFN-α2b) on the proportions of peripheral blood $CD4^+$ lymphocytes producing IL-10 and TGF-β. Healthy donor PBMCs were stimulated with PMA and ionomycin for 24 hours.



(g) Representative dot plots showing the effect of IFN-α (10^3 U/ml IFN-α2b) on the proportions of peripheral blood $CD8^+$ lymphocytes producing IL-10 and TGF-β. Healthy donor PBMCs were stimulated with PMA and ionomycin for 24 hours.