STUDIES ON THE DIFFERENTIATION OF INFLAMMATORY AND REGULATORY T CELLS

By

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ABSTRACT

Low vitamin D is associated with an increased risk of autoimmune diseases, whose pathology might involve T_{Reg} and T_h17 dysregulation. Thus, understanding how vitamin D modifies $CD4^+$ T cell responses holds therapeutic potential. I therefore investigated the effect of $1,25(OH)_2D_3$, the active form of vitamin D, upon human $CD4^+$ T cell differentiation.

$1,25(OH)_2D_3$ acted directly upon human $CD4^+$ T cells, suppressing inflammatory cytokines (IL-17, IL-21, IFNγ and IL-22) whilst enhancing regulatory markers (CTLA-4, CD25, FoxP3 and IL-10). Consistently, $1,25(OH)_2D_3$-treated T cells suppressed division of naive T cells stimulated by dendritic cells (DCs). Strong up-regulation of CTLA-4 by $1,25(OH)_2D_3$ reduced B7 expression by DCs, suggesting that enhanced CTLA-4 could be important mechanistically in $1,25(OH)_2D_3$-modified immunity. Furthermore, pro-regulatory effects of $1,25(OH)_2D_3$ were maintained under inflammatory conditions and modest suppression of established IL-17 by $1,25(OH)_2D_3$ was observed, supporting ability of $1,25(OH)_2D_3$ to control T cell phenotype at inflammatory sites. DCs could also efficiently convert $25(OH)D_3$ to drive $1,25(OH)_2D_3$-modified T cell responses, which might be important *in-vivo*, given the low level of $1,25(OH)_2D_3$ in serum.

Whether dysregulation of the T_{Reg}/T17 balance or response to $1,25(OH)_2D_3$ was associated with disease outcome in early synovitis patients was also studied. Although the T_{Reg}/T17 ratio did not stratify with outcome, T cell responses to $1,25(OH)_2D_3$ were observed in all patients,

suggesting that their VDR signalling is intact and that $1,25(OH)_2D_3$ might be useful in the treatment of synovitis.

DEDICATION

This thesis is dedicated to my mother and late father who gave me the opportunity to receive a higher education and encouraged me all of the way and to my sister Hannah, who has been a continual support and inspiration throughout my studies.

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I, Miss Louisa Elizabeth Jeffery, was responsible for all of the work described in this thesis with the exception of blood and synovial fluid collection from synovitis patients. These were collected by Dr Karim Raza and Dr Andrew Filer.

Firstly, I would like to thank my supervisors, Dr David Sansom and Dr Karim Raza, for their excellent supervision: I really appreciate the time that they took to meet with me on a weekly basis to discuss my project and help me to design experiments. I am also very grateful for the advice that they gave to me when writing abstracts, articles or presentations about my work and especially for their willingness to proof read my thesis. They really helped to make the three years of my project feel an exciting and rewarding time during which my ambition to pursue a career in medical research has been strengthened.

I would also like to thank all of the members of the Sansom lab, both past and present, for their general help with laboratory duties and for making the lab feel a happy and friendly place to work. I would particularly like to thank Dr Omar Qureshi for teaching the principles of flow cytometry to me during the early stages of my project and for his advice on the design of some experiments.

I would also like to thank all of those who donated blood or synovial fluid for my studies.

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ABBREVIATIONS

$1,25(OH)_2D_3$	1,25-dihydroxyvitamin D_3
$25(OH)_2D_3$	25-hydroxyvitamin D_3
APC	Antigen Presenting Cell
ATRA	All Trans Retinoic Acid
BTLA	B and T Lymphocyte Activator
CTLA-4	Cytotoxic T lymphocyte Associated-4
DC	Dendritic Cell
GALT	Gut Associated Lymphocyte Tissue
G-CSF	Granulocyte Colony Stimulating Factor
GM-CSF	Granulocyte -Macrophage Colony Stimulating Factor
IBD	Inflammatory Bowel Disease
ICOS	Inducible Costimulator
IFN□	Interferon Gamma
IL	Interleukin
IRF	Interferon Regulatory Factor Transcription Factor
ITAM	Immunoreceptor Tyrosine Based Activation Motif
JAK	Janus Kinase
MHC	Major Histocompatibility
MMP	Matrix Metalloproteinase
MS	Multiple Sclerosis
NF-AT	Nuclear Factor of Activated T cell
NFkB	Nuclear Factor Kappa B
NKT	Natural Killer T Cells

PAMP	Pathogen Associated Molecular Pattern
PBMC	Peripheral Blood Monocuclear Cells
PD-1	Programmed Cell Death
PRR	Pattern Recognition Receptor
RA	Rheumatoid Arthritis
RAR	Retinoic Acid Receptor
RF	Rheumatoid Factor
ROR	Retinoic Acid Receptor Related Orphan
RORE	ROR Response Element
RXR	Retinoid X Receptor
SFMC	Synovial Fluid Mononuclear Cells
SLE	Systemic Lupus Erythromatosis
SOCS	Suppressor of Cytokine Signalling
STAT	Signal Transducer and Activator of Transcription
T1D	Type 1 Diabetes
TCR	T Cell Receptor
TGF	Transforming Growth Factor
Th	T Helper
TNF	Tumour Necrosis Factor
T_{Reg}	Regulatory T Cell
VDBP	Vitamin D Binding Protein
VDR	Vitamin D Receptor
ZAP-70	Zeta Associated Protein Kinase

1 INTRODUCTION

1.1 Role of the immune system

The prime responsibility of the immune system is to protect the body from infection by pathogenic organisms ranging from viruses, through unicellular bacteria, to multi-cellular parasites (Bettelli et al., 2006). It also serves to rid the body of altered self-cells, the uncontrolled division of which can lead to cancer. To achieve such functions, the immune system is comprised of several organs and many cell types, which communicate through cell surface and soluble molecules such as cytokines and chemokines. Immune defence operates at two levels: the innate and adaptive. The former, which involves both cellular and molecular mechanisms, is constitutively present and deals non-specifically with the invading pathogen. By contrast, the latter, which involves the activation and differentiation of antigen specific $CD4^+$ or $CD8^+$ T lymphocytes and antibody secreting B lymphocytes is a delayed response tailored to the pathogen. $CD8^+$ T cells recognise endogenous antigen displayed as linear peptides by Class I Major Histocompatibility (MHC) antigens on the surface of all body cells. As such, $CD8^+$ T cells deal with the removal of virally infected or altered self-cells. Conversely, $CD4^+$ T cells recognise processed exogenous antigens that have been taken up primarily by professional antigen presenting cells (APCs), including monocytes, dendritic cells (DCs), macrophages and B lymphocytes and presented to them by MHC class II molecules. Therefore, $CD4^+$ T cells are important in responses to bacterial, fungal and parasitic pathogens. The adaptive immune response also displays the feature of memory. This ensures that subsequent infection by the same pathogen is dealt with quickly, usually before symptoms develop (Delves and Roitt, 2000a).

Introduction

The essential role of the immune system in protection from disease is clear, since abnormalities that cause the loss of its activity result in recurrent infections and severe cases of immunodeficiency can lead to death. Equally, inappropriate, excessive activation of immune responses can result in debilitating and life threatening autoimmune diseases in which the immune system attacks normal self-cells. Thus, the immune system might be viewed as a double-edged sword, which whilst serving to protect has the potential to harm. Maintaining a balanced immune state is therefore essential and is the key to the prevention and treatment of immune disease. By deciphering the molecular and cellular basis of immune reactions, and identifying factors that can shift the position of immune homeostasis, potential therapeutic targets for immune-related diseases can be determined.

In recent years, a number of environmental factors including dietary vitamins have been studied in the context of immunity and seen to affect its balance (Benson et al., 2007; Elias et al., 2008; Quintana et al., 2008; Sun et al., 2007; Veldhoen et al., 2009; Veldhoen et al., 2008a; Xiao et al., 2008). Such information could be useful in the design of prophylactic therapies as well as treatments for active disease. Vitamin D is one such factor, which despite its longstanding association with calcium and phosphate homeostasis and the avoidance of childhood rickets and osteoporosis, has been shown to have immunological effects, influencing both innate and adaptive systems (Adams and Hewison, 2008; Holick, 2007). This project is particularly concerned with the effect of 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), the active form of vitamin D, upon $CD4^+$ T cell differentiation to the T helper 17 (T_h17) & and inducible T_{Reg} (iT_{Reg}) effector lineages.

To put the work of this project in context, subsequent paragraphs within this section overview the principles behind T cell activation and differentiation, focussing upon the factors required for iT_{Reg} and T_h17 development and their roles in health and disease. Lastly, an overview of vitamin D metabolism and evidence that suggests its involvement in the regulation of innate and adaptive immune networks is provided.

1.2 T cell activation

1.2.1 Antigen recognition and co-stimulation signals

T cell activation, as illustrated in **figure 1.1**, requires the induction of two signals within the T cell. The first stems from the engagement of the T cell receptor (TCR) variable domains with the linear peptide antigen displayed by an MHC class II molecule at the surface of an APC. All of the TCRs expressed by a single T cell have the same antigen specificity but as a consequence of gene rearrangement within the variable domain during T cell development, approximately 10^{10} unique antigen specificities can be generated, which is sufficient to cater for the diversity of pathogen motifs. The random nature of the process, however, results in the formation of self-reactive T lymphocytes (Delves and Roitt, 2000b). Extensive autoimmune attack is avoided as these cells are largely removed in the thymus at the negative selection stage and those cells that escape are suppressed in the periphery by tolerance mechanisms. (Delves and Roitt, 2000a).

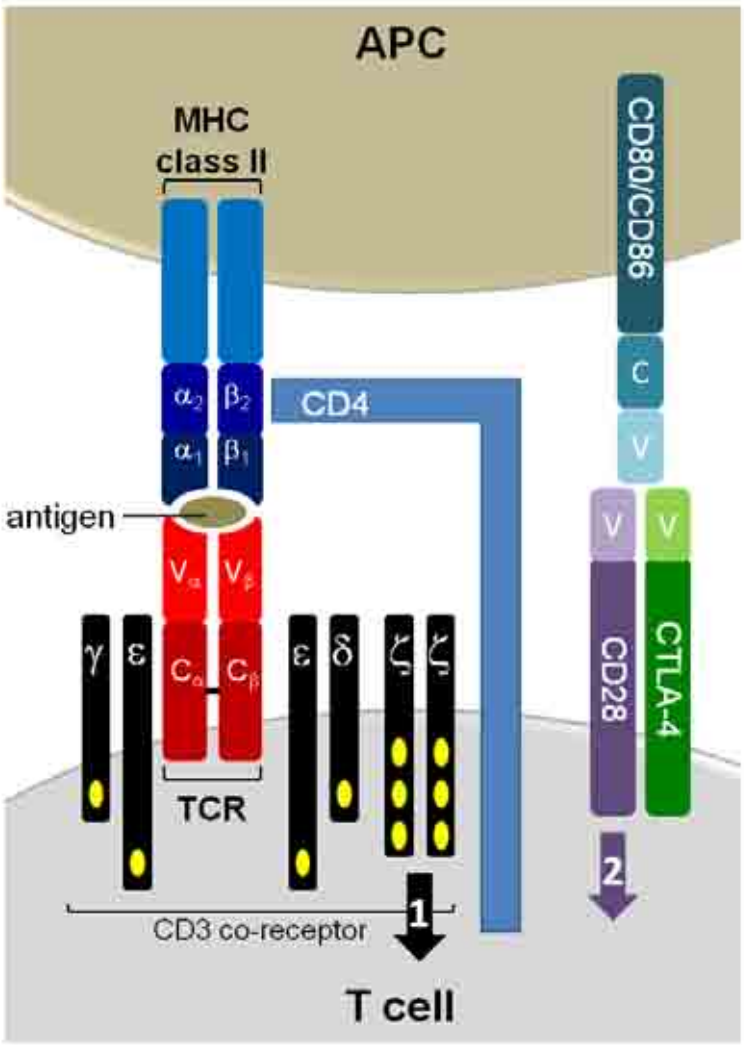

Figure 1.1. The immune synapse. Major Histocompatibility complex (MHC) II molecules on antigen presenting cells (APCs) display processed linear antigen to $CD4^+$ T cells. The variable domains of the T cell receptor (TCR) α and β chains form the antigen binding groove and CD4 co-receptor binds to an invariant site on the MHC II molecule. The CD3 co-receptor, whose component chains (γ,δ,ε and ζ) contain intracellular immunoreceptor tyrosine based activation motifs (ITAMs), mediates intracellular signalling following antigen recognition by the TCR (signal 1). Interaction of co-stimulatory CD28 with its B7 ligands, CD80 or CD86, is also required for T cell activation (signal 2). CD28 family member, CTLA-4, is induced upon T cell activation to inhibit T cell activation. Abbreviations: IgV domain (V), IgC domain (C).

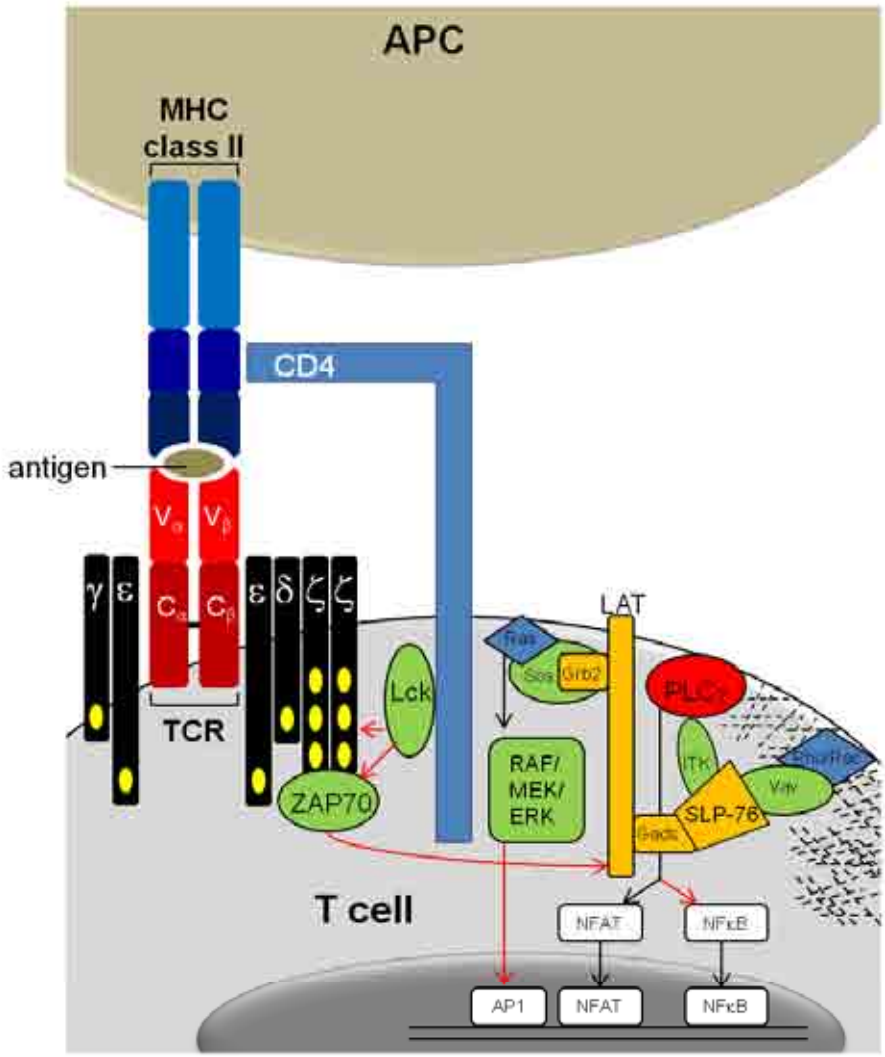

Figure 1.2. Signalling pathways activated upon T cell receptor ligation. Upon TCR-antigen-MHCII engagement the TCR-CD3 complex associates with CD4. $p56^{Lck}$ that is bound to the cytoplasmic tail of CD4 phosphorylates the ITAMs of the CD3 ζ chains, creating a docking site for tyrosine kinase, ZAP-70, that in turn phosphorylates a series of adaptor molecules including LAT, SLP-76 and Vav-1. Mitogen activated protein kinase (MAPK) and Phospholipase Cγ (PLCγ) pathways are induced leading to the activation of transcription factors AP-1, NFAT and NFκB and a change in gene expression (red arrows indicate phosphorylation involvement). The expression of IL-2 is one early response, required to instigate T cell proliferation. Activation of the small G protein, Rho/Rac-GTPase, results in cytoskeletal remodelling that is essential for migration of the T cell and its closer interaction with its target cell.

Signal transduction cannot extend directly from the TCR due to the lack of appropriate motifs within its short cytoplasmic tails. Instead signal transduction is achieved through the six chain CD3 co-receptor with which the TCR associates (Delves and Roitt, 2000b). Transcription factors AP-1, NF-AT and NF-κB are activated downstream of CD3 signalling to induce T cell activation. One early response is the induction of cytokines such as interleukin-2 (IL-2), which is important for the initiation and maintenance of T cell proliferation (**figure 1.2**) (Razzaq et al., 2004).

The second signal, defined as a co-stimulatory signal is apparently required to prevent clonal anergy, in which the T cell is incapable of proliferation and IL-2 secretion (Schwartz, 1996). It stems from the engagement of the transmembrane receptor molecule, CD28 with its cognate B7 ligand, either CD80 (B7-1) or CD86 (B7-2) expressed by the APC. Downstream responses to CD28 stimulation are the induction of glucose metabolism, high IL-2 production, T cell proliferation and resistance to apoptosis (reviewed in (Carreno and Collins, 2002; Lenschow et al., 1996)). The importance of CD28 co-stimulation is seen in the phenotype of the CD28 knock out mouse, which has decreased sensitivity to antigen, reduced IL-2 production and a lack of maintained T cell proliferation (Shahinian et al., 1993). Additionally, immunoglobulin concentrations are much lower in CD28-deficient mice compared to wildtype controls and CD28-deficient mice show defects in isotype class switching and germinal centre formation (Ferguson et al., 1996; Lucas et al., 1995). Likewise, CD80 and CD86 knockout mice show defects in humoral and cellular immune responses (Borriello et al., 1997). The biological significance of the two CD28 ligands, which have strong structural homology (reviewed in (Collins et al., 2005)), is not known but their conservation across species (Collins et al., 2005)

argues for redundancy in the system or that the two ligands have distinct functions. *In-vitro* evidence supports the later, since resting T cells when stimulated with anti-CD3 in the presence of CD80 or CD86 alone show differences in cytokine production and the expression of surface molecules (Odobasic et al., 2008).

1.2.2 CTLA-4 mediated suppression of T cell activation

Induction of the CD28 family member, cytotoxic T-lymphocyte associated-4 (CTLA-4), is one response that lies downstream of T cell activation. CTLA-4 expression is evident as early as six hours and reaches its maximum level by 36 hours (Lindsten et al., 1993). In addition to TCR signalling, CD28 and IL-2 independently and additively enhance CTLA-4 expression (Alegre et al., 1996). Although, CTLA-4 shares 30% homology with CD28, they have opposite roles, as CTLA-4 is required to suppress T cell activation rather than to promote it. This function is evident from the fact that CTLA-4 knockout mice die from hyperlymphoproliferative disorders (Tivol et al., 1995; Waterhouse et al., 1995). Thus, together, the phenotypes of CD28 and CTLA-4 knockout mice highlight the importance of both stimulatory and suppressive immune mechanisms for survival.

In contrast to CD28, CTLA-4 is localised primarily in intracellular vesicles (Egen and Allison, 2002; Linsley et al., 1996) but in response to TCR activation it is trafficked to the cell surface, towards the sites of TCR activation. New protein is not synthesised (Schneider et al., 1999) but the extent of surface expression is dependent upon the strength of the TCR signal (Egen and Allison, 2002). This mechanism therefore allows for balance in the strength of stimulatory and inhibitory signals. CTLA-4 exocytosis might depend upon the activities of

GTPase ADP-ribosylation factor 1 and phospholipase D (Mead et al., 2005). By contrast, endocytosis of CTLA-4 from the plasma membrane involves binding of the μ2 chain of AP-2 clathrin associated adaptor complex to the phosphotyrosine motif YVKM, within the cytosolic tail of CTLA-4 (Shiratori et al., 1997). Interestingly, the same YVKM motif could be the means by which CTLA-4 delivers a cell-intrinsic negative signal, since tyrosine phosphatases such as Syp (Marengere et al., 1996) and SHP-1 (Guntermann and Alexander, 2002) can be recruited to it and might in turn inactivate components of the TCR signalling cascade. Since AP-2 μ2 binds to the YVKM motif in the absence of phosphorylation it appears that the phosphorylation status of a single tyrosine could determine whether CTLA-4 delivers a negative signal or whether it is internalised (Shiratori et al., 1997). The clathrin adaptor protein complex, AP-1, can also associate with the YVKM motif of CTLA-4. However, in contrast to AP-2, AP-1 and CTLA-4 associate predominately within the golgi compartment, although binding to AP-1 may also regulate the distribution of CTLA-4 to lysosomal compartments. Thus, by directing excess CTLA-4 to the lysosomes for degradation, AP-1 might be a homeostatic regulator of intracellular CTLA-4 (Schneider et al., 1999).

Consistent with its high homology to CD28, CTLA-4 interacts with CD80 and CD86 and in agreement with the role of CTLA-4 in suppressing B7-CD28 co-stimulatory signals, mice triply deficient for CD80, CD86 and CTLA-4 did not show the autoimmune phenotype of CTLA-4 knockout mice (Mandelbrot et al., 1999). The affinity of CTLA-4 for both CD80 and CD86 is greater than the affinity of CD28 for both ligands (Carreno et al., 2005; Carreno and Collins, 2002; van der Merwe et al., 1997) (**figure 1.3**). Therefore, absence of CTLA-4 prior

to simulation is important to permit CD28-driven T cell activation but when CTLA-4 is subsequently expressed, its high affinity for CD80 and CD86 enables it to efficiently suppress CD28-mediated co-stimulation. In addition, during the course of stimulation, CD80 expression by the APC is increased relative to CD86. Because the CTLA-4/CD28 relative affintiy is greater for CD80 than CD86, this change in the CD80/CD86 ratio could help to maximise the efficiency of CTLA-4-mediated suppression. These differences in binding affinities and temporal patterns of B7 expression could contribute to differences in T cell responses following stimulation by CD80 or CD86 alone (Lenschow et al., 1996; Odobasic et al., 2008). As well as simply competing with CD28 for binding to B7, *in-vitro* and *in-vivo* data by others in my lab (Qureshi et al., manuscript in preparation) strongly suggest a mechanism of transendocytosis, in which engagement of B7 with CTLA-4 leads to internalisation of the ligand by the T cell and its subsequent lysosomal degradation. Such a mechanism is clearly consistent with the continual cycling of CTLA-4 between the plasma membrane and intracellular compartments upon TCR stimulation. In addition, by this model, one CTLA-4 expressing cell could suppress itself and neighbouring cells rather than itself only, as the model of binding competition suggests.

1.2.3 Other CD28 and B7 family co-stimulatory systems

The CD28/CTLA-4–CD80/CD86 co-stimulatory system represents the first and best understood of several CD28 family-B7 co-stimulatory systems, which over the past decade have been identified and become recognised participants in the regulation of T cell activation. Other CD28 family receptors expressed by leukocytes include: inducible co-stimulator (ICOS), programmed cell death-1 (PD-1) and B and T lymphocyte attenuator (BTLA), and

other B7 ligands include: ICOS-L (B7-H2), PD-L1 (B7-H1), PD-L2 (B7-DC), B7-H3 and B7-H4 (Carreno et al., 2005). Both haematopoietic and non-hematopoietic cells express these ligands. The importance of these molecules in the regulation of T cell activation and their roles as either inducers or inhibitors has been confirmed through knockout mice studies in which immunosuppressive or autoimmune disorders developed respectively in their absence (reviewed in (Carreno et al., 2005; Carreno and Collins, 2002)). **Figure 1.3** summarises the distribution and binding partners of CD28 receptor and B7 ligand family members. Although, the precise functions and relative requirements of these co-stimulatory receptors and ligands is not yet known, their differential expression patterns might allow for finer regulation of T cell activation than is possible through the CD28/CTLA-4-CD80/CD86 system alone. Heterotypic binding, has also been seen within this family of ligands and receptors, for example between CD80 and the PD-1 ligand, PD-L1 (Butte et al., 2007). Conceivably, such alternative interactions could permit even finer tuning of T cell activation.

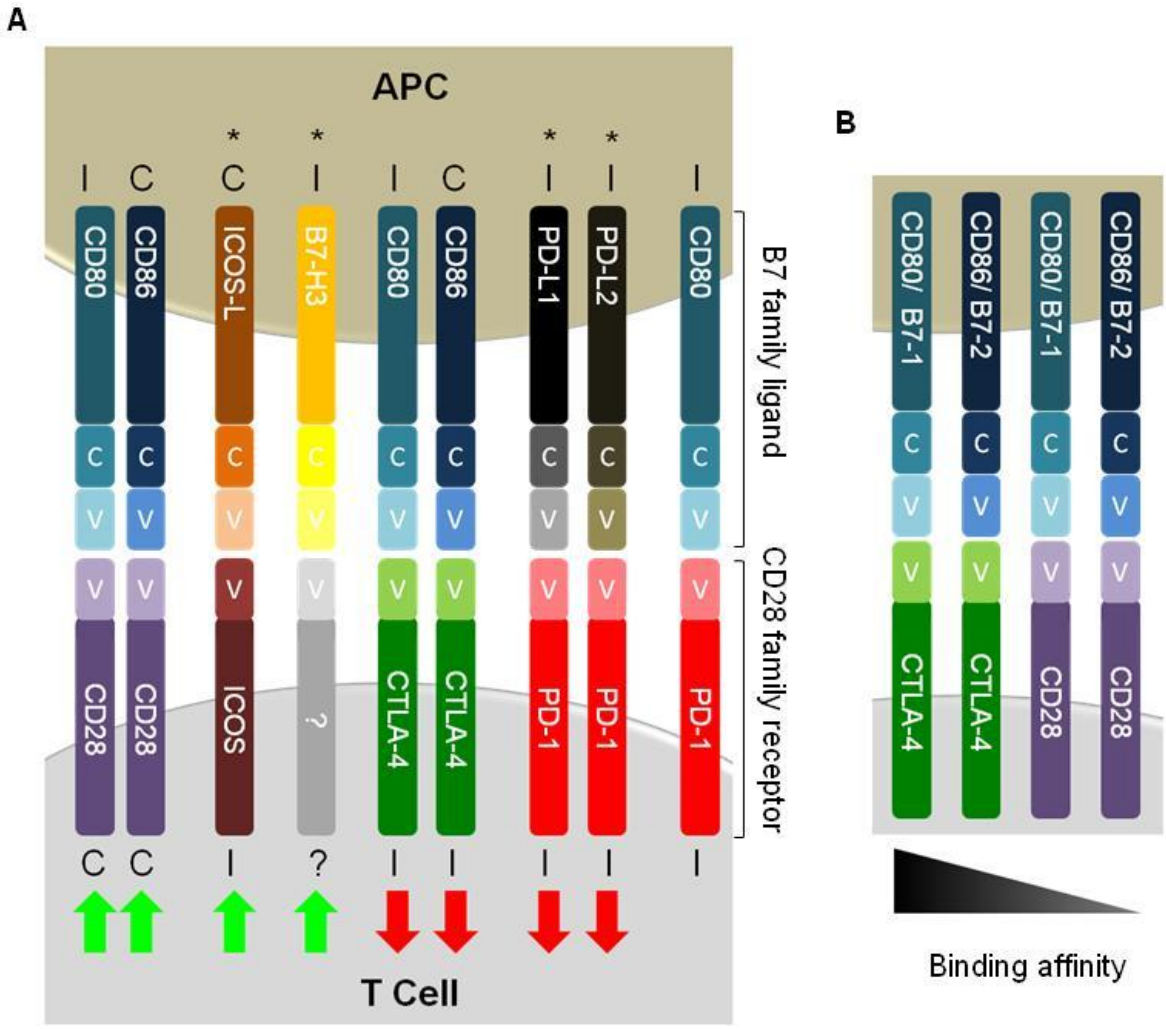

Figure 1.3. CD28 receptor family members and their B7 ligands. A) CD28 family receptors and their B7 family ligands have conserved structures: The receptors are single pass transmembrane proteins with a single IgV motif in their extracellular IgV domain. The B7 ligands have one IgV and one IgC motif in their extracellular domain. Interaction between the ligands and receptors is largely via their IgV motifs. Both co-stimulatory (↑) and co-inhibitory (↓) interactions are seen. With the exception of CD28 and CD86 and ICOS-L expression is not constitutive (**C**) but is induced upon activation (**I**). Receptors are expressed by T cells, although PD-1 is also expressed on monocytes and B cells. B7 ligands are expressed by DCs, B cells and monocytes, although expression by non-haematopoietic cells is also observed for some ligands (*). **B)** CD28 and CTLA-4 both bind to CD80 and CD86 but their binding affinities are different. The order of their binding affinities is shown.

T cell differentiation

The role of $CD4^+$ T cells in the adaptive immune response is to orchestrate the activities of other effector cells whose reactions in turn kill the pathogen. They achieve this in the main by soluble factors including cytokines and chemokines that they release and it is according to their cytokine profile that $CD4^+$ T cells are classified into differentiation lineages (**figure 1.4**). Mossmann and Coffman introduced the concept of T cell differentiation over twenty years ago. They proposed a binary model wherein a naive $CD4^+$ (Th0) cell can differentiate into one of two terminal effector fates: T helper 1 (T_h1) or T helper 2 (T_h2) (Mosmann and Coffman, 1989). T_h1 cells release interferon gamma (IFNγ) and tumour necrosis factor (TNF), through which they instigate delayed-type-hypersensitivity responses effective in the clearance of intracellular pathogens as well as virally infected or cancerous cells. Aberrant activation of these cells is associated with inflammatory disease. By contrast, T_h2 cells release cytokines including IL-4, IL-5, IL-9, IL-10 and IL-13 that induce B cell antibody class switching to IgE, mast cell proliferation and degranulation, and eosinophilia. These reactions facilitate the killing of extracellular parasites such as helminths (Bettelli et al., 2007; Harrington et al., 2005; Park et al., 2005). Aberrant activities of these cells in response to innocuous antigens are associated with allergy and asthma.

Much work has been done to determine the signals that govern the differentiation of $CD4^+$ T cells into each type. These studies have led to the hypothesis that polarisation is primarily controlled at the level of the APC, which matures to a particular phenotype following its direct activation through detection of pathogen products by Pattern Recognition Receptors (PRRs) or the detection of tissue factors that are released from neighbouring cells in response

to their detection of the pathogen. As such, the terms DC1 and DC2 have been assigned to mature DCs that direct the development of T_h1 and T_h2 cells respectively (Kaiko et al., 2008; Rissoan et al., 1999). However, how particular pathogens promote each type of T cell response is still poorly understood.

1.2.4 T_h1 and T_h2 differentiation

An environment enriched in DC and macrophage derived IL-12, type 1 interferons (IFNα and IFNβ) and potentially IL-18 (Kaiko et al., 2008) favours T_h1 development, as IL-12 signals through signal transducer and activator of transcription factor 4 (STAT4) to drive IFNγ expression. This in turn signals through STAT1 to promote T-bet expression, the T_h1 signature transcription factor. T-bet fixes the T_h1 phenotype by itself driving IFNγ and IL-12 receptor expression (**figure 1.5**). By contrast, IL-4, which might be released by natural killer T cells, eosinophils and mast cells, drives T_h2 development. Through STAT6, IL-4 induces GATA-3, the hallmark T_h2 transcription factor. GATA-3 induces T_h2 associated cytokines and suppresses IL-12Rβ2, thereby desensitizing T cells to pro-T_h1 conditions. Similarly, during T_h1 development, T-bet down-regulates IL-4. In addition to GATA-3, the transcription factor c-MAF supports T_h2 differentiation by inducing IL-4 expression (**figure 1.5**) (Dong, 2006).

1.2.5 Other CD4$^+$ T helper cell lineages

In recent years, it has become apparent that the binary model of CD4$^+$ T cell differentiation is not sufficient. Rather, as shown in **figure 1.4**, the CD4$^+$ T cell differentiation map now includes inducible T_{Regs} (iT_{Reg}), T helper 17 (T_h17), T follicular helper (T_{fh}) (Vogelzang et al., 2008), T helper 9 (T_h9) (Veldhoen et al., 2008b) and T helper 22 (T_h22) (Trifari et al., 2009) effector T cells. The increased availability of fluorophores and cytokine staining reagents may well lead to the discovery of additional subsets with specific cytokine and cell surface protein expression patterns and subsequently to the designation of additional CD4$^+$ lineages. Of the newer CD4$^+$ lineages, the immunoregulatory iT_{Reg} and pro-inflammatory T_h17 cells are the best studied.

The cytokines required for iT_{Reg} and T_h17 differentiation in humans and mice are largely resolved and their significance in health and disease is being studied. Their potential to be reciprocally regulated by a number of exogenous factors makes them particularly attractive therapeutic targets for the restoration and maintenance of immune homeostasis. Since this study is concerned with the differentiation of CD4$^+$ T cells to the iT_{Reg} and T_h17 lineages, the following paragraphs overview the history of their discoveries, function and differentiation.

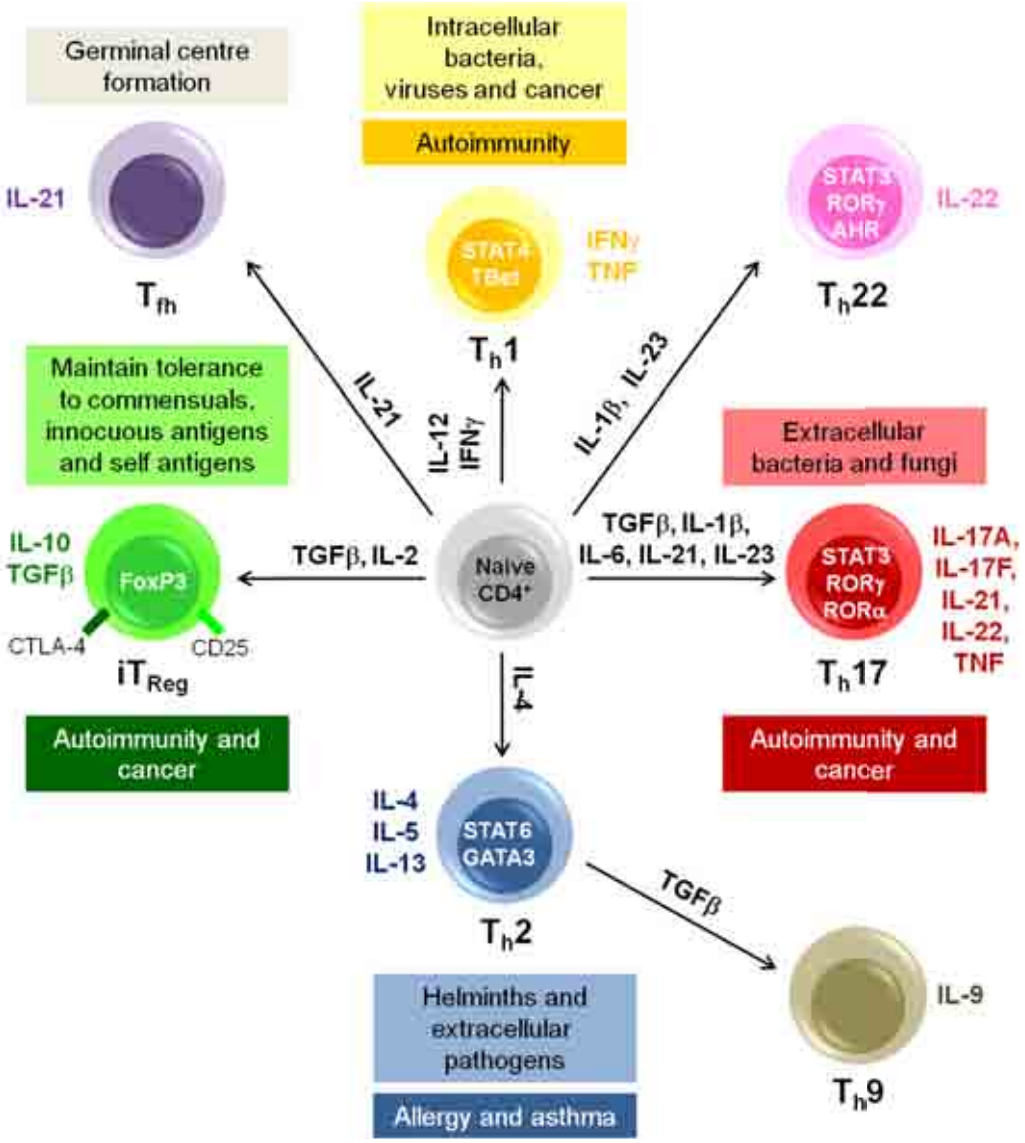

Figure 1.4. $CD4^+$ T cell classes. Naive $CD4^+$ T cells activated in the periphery differentiate into effector $CD4^+$ T cells that by their release of certain cytokines direct an immune response that is tailored to a particular pathogen. Cytokines present at the time of antigen presentation (indicated on arrows) are largely responsible for the lineage chosen as they control the expression and activities of lineage transcription factors (indicated in nuclei). The effector classes presently defined include: T helper 1 (T_h1), T helper 2 (T_h2), T helper 17 (T_h17), T helper 9 (T_h9) and T helper 22 (T_h22). $CD4^+$ T cells also differentiate into regulatory T cells (T_{Reg}). The hallmark cytokines of each lineage are coloured the same as the cell that releases them. Dysregulation of $CD4^+$ T cell activities leads to disease. Functions of the $CD4^+$ T cell lineages in immune homeostasis and disease are detailed in like-colour boxes above or beneath them. Pale boxes indicate pathogens cleared by the cells. Dark boxes list diseases associated with the T cell lineage.

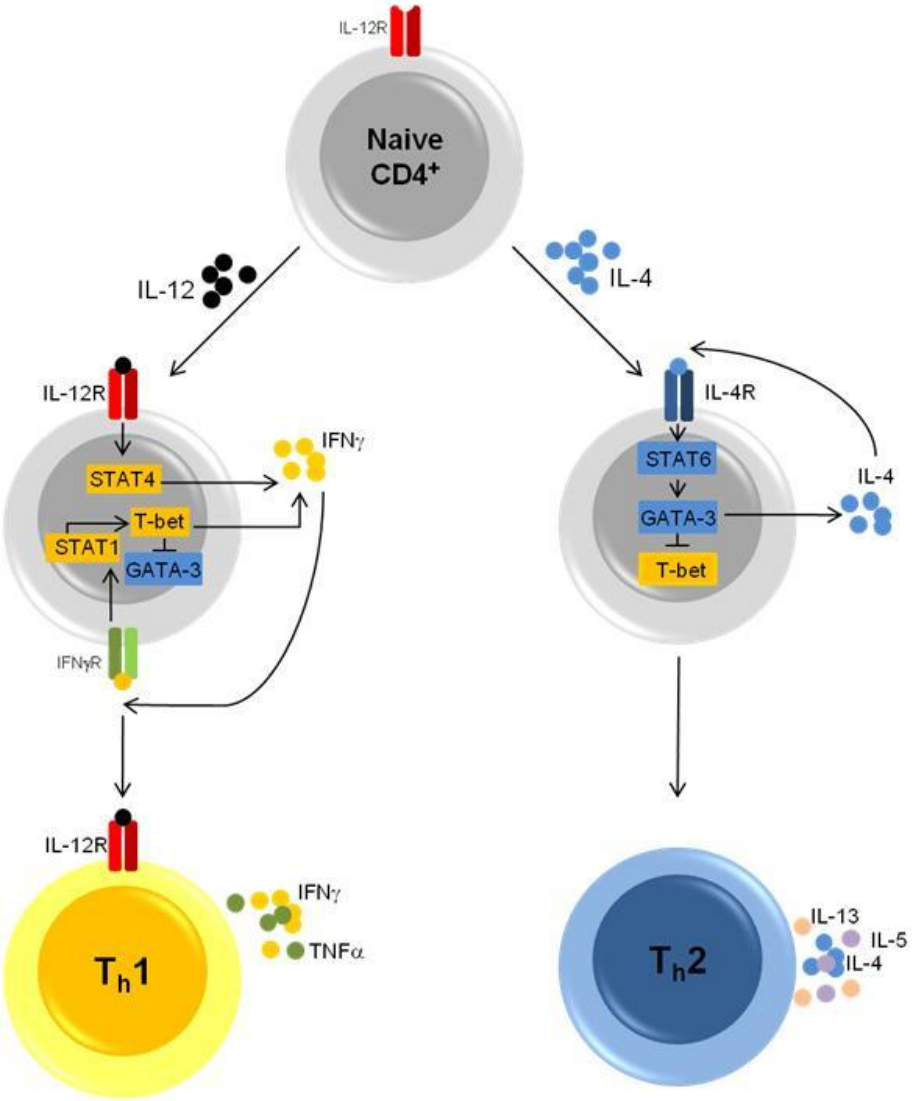

Figure 1.5. Cross regulation in T_h1 and T_h2 differentiation. Cytokines in the environment at the time of naïve T cell activation are largely responsible for the differentiation pathway chosen. Cross regulation ensures the efficient expansion of one lineage. In T_h1 differentiation, IL-12, produced by the APC, induces the activation of signal transducer and activator of transcription 4 (STAT4) that induces IFNγ transcription and expression. IFNγ feeds back positively, activating transcription factor T-bet via STAT1. T bet promotes IFNγ production and inhibits T_h2 cytokine expression via GATA-3. T_h2 differentiation is promoted in an IL-4 rich environment. IL-4 signals via STAT6 to increase expression of the T_h2 lineage transcription factor, GATA-3. GATA-3 drives the expression of T_h2 cytokines but suppresses T-bet and therefore T_h1 development. IL-4 expression is also promoted by transcription factor c-Maf.

1.2.6 Regulatory CD4$^+$ T cells (T_{Regs})

The concept of a suppressor cell was introduced in the 1970s (Gershon and Kondo, 1970), however its potential existence lost presidency as researchers struggled to define the molecular identity of these cells. In 1995 Sakaguchi et al. showed that CD4$^+$ T cells, which constitutively expressed the IL-2 receptor α chain, CD25, had immunosuppressive functions both *in-vivo* and *in-vitro* (Sakaguchi et al., 1995). Since then, the existence of a regulatory T cell subset (T_{Reg}) has become widely accepted and their importance in both health and disease acknowledged. It is now believed that T_{Regs} potently suppress the proliferation or functions of CD4$^+$, CD8$^+$, NK cells, NK-T cells, DCs and B cells (Cederbom et al., 2000; Piccirillo and Shevach, 2001; Seo et al., 2002; Thornton and Shevach, 1998; Trzonkowski et al., 2004). Furthermore, it is recognised that suppression of T cell responses is independent of antigen specificity (Marie et al., 2005), although T_{Regs} require antigen stimulation to function. T cells with regulatory capacity have been identified in both the CD4$^+$ and CD8$^+$ divisions (Jiang and Chess, 2006; Kaiko et al., 2008) but most work has focussed on those from the former class, for which two subsets, the natural (nT_{Regs}) and iT_{Regs}, are defined. Together, these cells account for 5-10% of peripheral CD4$^+$ cells (Marie et al., 2005; Thompson and Powrie, 2004) but to what extent they use similar mechanisms is not established.

nT_{Regs} develop in the thymus during the early stages of foetal and neonatal T cell development. Although the TCR repertoire of nT_{Regs} is as varied as that of naive T cells and there is some overlap, in general, it is skewed towards the recognition of self-antigen. This implies a major role for nT_{Regs} in protection from autoimmunity (Hsieh et al., 2006; Jordan et al., 2001; Sakaguchi et al., 2003). Consistent with this, depletion of nT_{Regs} led to the

development of a variety of autoimmune disorders such as thyroiditis, gastrititis, orchitis, oophoritis, Type 1 diabetes (T1D) and inflammatory bowel disease (IBD) which could be prevented by provision of normal $CD4^+CD25^+$ T cells (reviewed in (Piccirillo, 2008)). However, nT_{Regs} also provide tolerance to commensual bacteria, innocuous environmental antigens and provide foeto-maternal tolerance during pregnancy (reviewed in (Piccirillo, 2008; Sakaguchi, 2004; Sakaguchi et al., 2006)).

The highly conserved forkhead/winged helix transcription factor, FoxP3, has been defined the master regulator of the T_{Reg} lineage. This follows identification that loss of the FoxP3 protein due to a natural X-linked mutation in the *foxP3* gene accounts for the lethal Scurfy phenotype of mice, in which $CD4^+$ T cells are hyperactive and show enhanced cytokine production (Fontenot et al., 2003; Khattri et al., 2003). Likewise, in humans, the rare disease, immune dysfunction, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome, that is characterised by autoimmune diseases, allergies, hyper IgE production and many haematological and infectious diseases, occurs as a result of mutations in the *foxP3* gene (Bennett et al., 2001; Gambineri et al., 2003; Sojka et al., 2008). Furthermore, FoxP3 is expressed at high levels by both murine and human T_{Regs} and over expression of FoxP3 converted naive T cells to a regulatory phenotype (Hori et al., 2003), suppressed diabetes in NOD mice (Jaeckel et al., 2005) and delayed disease in CTLA-$4^{-/-}$ mice (Khattri et al., 2003). Indeed, FoxP3 is presently the most reliable marker for T_{Regs} in both rodents and humans (Ziegler, 2006). However, whilst dual expression of CD25 and FoxP3 clearly delineates T_{Regs} in mice, this may not be true in humans, since CD25 is expressed on pro-inflammatory as well as anti-inflammatory cells (Kleinewietfeld et al., 2009) and human $CD4^+CD25^-$ T cells up-regulate FoxP3 transiently upon activation (Allan et al., 2007). To aid the purification of T_{Reg}

cells for functional assays, additional biomarkers are being investigated. Whilst a number have been proposed, including high expression of glucocorticoid induced tumour necrosis factor receptor (GITR), IL-2Rβ (CD122), CD103 and low levels of CD45RB, CD127 and CD49d (Liu et al., 2006b; Seddiki et al., 2006; Vignali et al., 2008) they too are present on activated T cells. Nonetheless, it is now considered that negative selection for $CD49d^{-}CD127^{-}$ cells, provides an acceptably pure, untouched T_{Reg} population from peripheral human blood that is devoid of $CD25^{+}$ effector cells (Kleinewietfeld et al., 2009).

The importance of FoxP3 in T_{Reg} function, might be that it establishes the molecular programme of these cells. This it achieves through interaction with a range of other transcription factors including NFAT, AML-1 (acute myeloid leukaemia-1)/Runx-1 (runt related transcription factor- 1) the histone-acetyltransferase (HAT)/histone deacetyltransferase (HDAC) complex and NF-κB. Following activation, NFAT complexes with AP-1 and NF-κB to promote the expression of genes such as *Il-2, Il-4* and *ctla*-4 (reviewed in (Piccirillo, 2008)).

In addition, to FoxP3, IL-2 appears to play a significant role in nT_{Reg} development, since mice deficient for IL-2, IL-2Rα (CD25) or IL-2Rβ (CD122) show similar symptoms to Scurfy mice, dying from severe lymphoproliferative and autoimmune disorders and hyper-reactivity to commensual microbes (Malek et al., 1984; Malek et al., 2002; Wolf et al., 2001). Likewise, CD25 deficiency in humans results in a disease similar to IPEX (Caudy et al., 2007). However, it seems that IL-2 is necessary for maintenance of nT_{Regs} in the periphery rather than their induction in the thymus, since T cells from IL-2 deficient mice expressed FoxP3

and suppressed T cell proliferation *in vitro*, even though reduced numbers of these cells were found *in-vivo* (Fontenot et al., 2005). It is possible that other common-gamma chain cytokines substitute for IL-2 in the thymus, since common γ chain-deficient mice showed complete absence of $FoxP3^+$ nT_{Regs}. In particular, a role for IL-15 is suggested as T_{Reg} production is greatly reduced in IL-2 and IL-15 double deficient mice or in mice deficient in IL-2Rβ, which is required for both IL-2 and IL-15 signalling (Burchill et al., 2007; Soper et al., 2007). In addition, a role for IL-7 in nT_{Reg} development has been proposed, although the finding that IL-7 deficient mice have normal numbers of nT_{Regs} suggests that IL-7 might be redundant in nT_{Reg} development (Liston and Rudensky, 2007). Lastly, strong co-stimulation through CD28-B7 is required for nT_{Reg} induction. This might be to induce sufficient IL-2 but a cell intrinsic requirement for CD28 is also suggested (Bennett et al., 2001; Tai et al., 2005).

In contrast to nT_{Regs} that are released from the thymus as active self-antigen specific suppressor cells, iT_{Regs} develop in the periphery from $CD4^+CD25^-$ T cells. Initial evidence for the existence of iT_{Regs} was the finding that $FoxP3^+$ T cells expressing CD25, CTLA4 and GITR that had suppressive activity could be isolated from lymphopenic mice that had received polyclonal $CD4^+CD25^-$ T cells (Curotto de Lafaille, 2004). It is now contended that various other types of $CD4^+$ iT_{Regs} exist, including IL-10 and TGFβ expressing Tr1 that do not stably express FoxP3 (reviewed by (Battaglia et al., 2006)) and TGFβ expressing T_h3 (Chen et al., 1994). In addition, $CD4^+$ T cells that expressed LAG-3 but had low CTLA-4, FoxP3 and GITR expression have been identified and might represent another iT_{Reg} class but distinctions between iT_{Reg} classes are still not clear (reviewed in (Lan et al., 2005)). Since iT_{Regs} derive from conventional T cells, their TCRs should be specific to non-self antigens

(Hsieh et al., 2006; Pacholczyk et al., 2006). Thus, iT_{Regs} might be responsible for establishing tolerance to commensuals, and innocuous antigens, rather than providing tolerance to self alongside their nT_{Reg} counterparts. Therefore, iT_{Regs} may be particularly important at mucosal sites such as the gut. However, it has been shown that T_{Regs} can transfer tolerance (Qin et al., 1993), thus, iT_{Regs} may mediate suppression following nT_{Reg} stimulation to self-antigen and thus provide protection from autoimmunity. Differentiating between the functions of nT_{Regs} and iT_{Regs} has been hampered by the lack of distinguishing markers (Curotto de Lafaille and Lafaille, 2009). In addition, much work on the features of iT_{Regs} has utilised *in-vitro* generated iT_{Regs}, which may not truly reflect the properties of *in-vivo* induced iT_{Regs}. Indeed, it is contended that *in-vitro* induced T_{Regs} might not be as suppressive as nT_{Regs}, with human *in-vitro* generated iT_{Regs} requiring several rounds of stimulation to become suppressive (Curotto de Lafaille and Lafaille, 2009; Horwitz et al., 2008b; Shevach et al., 2008). By contrast, *in-vivo* generated iT_{Regs} are considered effective suppressors (Apostolou and von Boehmer, 2004; Cobbold et al., 2004; Mucida et al., 2005). Consistent with distinct behaviours for nT_{Regs} and *in-vitro* induced iT_{Regs}, gene expression analysis revealed that iT_{Regs} lack a component of the nT_{Reg} signature genes (Hill et al., 2007). In addition, *in-vitro* generated iT_{Regs} lack demethylation of the conserved T_{Reg} cell specific demethylated region (TSDR) region, located upstream of exon 1 of the *foxp3* gene, which is demethylated in nT_{Regs} (Floess et al., 2007). By contrast, efficient demethylation of this locus was observed in *in-vivo* generated iT_{Regs} (Polansky et al., 2008), which further supports the view that *in-vitro* and *in-vivo* generated iT_{Regs} might not be mirror images of each other. However, it is possible that respective analysis at six days versus three weeks post antigen exposure in these studies could explain the discrepancy. Nonetheless, lack of TSDR demethylation might account for the instability of FoxP3 expression in *in-vitro* generated iT_{Regs}. Another recently identified distinction

between natural and induced T_{Regs} is the selective expression of Helios, a member of the Ikaros transcription factor family, in the former group (Thornton et al., 2010).

The conditions under which iT_{Regs} differentiate *in-vivo* are not clearly defined, indeed multiple micro-environments could be employed (Curotto de Lafaille and Lafaille, 2009). However, it is well established that both mouse and human iT_{Regs} can develop *in-vitro* by TCR stimulation in the presence of TGFβ and IL-2 and in contrast to nT_{Reg} development co-stimulation might not be required (Apostolou and von Boehmer, 2004; Kretschmer et al., 2005). This might explain why presentation of antigen by immature DCs promotes T_{Reg} development (Wakkach et al., 2003). TGFβ signalling involves receptor dependent phosphorylation of receptor associated Smads (2 or 3) followed by their heterodimerisation with the co-Smad, Smad4 to form a complex that binds to regulatory elements in its target genes and interacts with other DNA binding factors to control gene expression (O'Shea et al., 2009). Consistent with the requirement of TGFβ for iT_{Reg} development, recent ChIP analysis of the *foxP3* locus identified a Smad3 binding site in an enhancer site upstream of the FoxP3 ATG start site (Tone et al., 2008; Xu et al., 2010). Binding of Smad3 at this site was found to precede its recruitment to the promoter region where it completed the assembly of an enhanceosome complex. Whilst Smads 2, 3 and 4 all appear to have non-redundant function in iT_{Reg} development, deficiency in any single gene does not impair nT_{Reg} function and only mildly affects nT_{Reg} numbers (Malhotra et al., 2010; Martinez et al., 2009; Takimoto et al., 2010; Yang et al., 2008c). This is perhaps consistent with the fact that TGFβ is required for peripheral maintenance but not the induction of nT_{Regs} (Marie et al., 2005). The importance of

TGFβ in T_{Reg} development is further evident, as deficiencies in TGFβ lead to pathologies similar to those associated with *foxp3* deficiency (Marie et al., 2005) and (Chen et al., 2003).

1.2.7 Suppressive mechanisms employed by T_{Regs}

Since their discovery, much work has been done to identify the mechanisms by which T_{Reg} cells suppress the functions of their target cells. Most studies have employed *in-vitro* assays. Direct effects of T_{Regs} upon responder T cells versus indirect effects via APCs have been investigated with APC free and APC present systems respectively. In the former, suppression typically required high T_{Reg} to responder ratios and low concentrations of the stimulation reagent. Direct mechanisms suggested include, IL-2 consumption, release of inhibitory cytokines such as IL-10, TGFβ and the novel cytokine, IL-35, direct granzyme A and perforin or granzyme B mediated cytolysis or the induction of cell cycle arrest and apoptosis by soluble or cell surface galectin-1 (**figure 1.6**) (Shevach, 2009). However, contention surrounds many of the mechanisms proposed. For example, although IL-2 consumption by T_{Regs} is an appealing mechanism, given that IL-2R chains (CD25, CD122 and CD132) are expressed at high levels by $T_{Regs,}$ the suggestion that exogenous IL-2 could not overcome suppression of IL-2 mRNA transcription by $CD4^+CD25^-$ T cells co-cultured with $CD4^+CD25^+$ T cells might imply that IL-2 consumption is not a critical mechanism (Thornton et al., 2004). Furthermore, blocking CD25 did not affect suppression by human T_{Reg} cells when TCR and CD28 signals were sufficiently strong (Tran et al., 2009). The importance and mechanism of TGFβ mediated suppression is likewise not clear: suppression by TGFβ could involve soluble or cell-contact mechanisms, since latent TGFβ has been detected on the surface of resting and activated $CD25^+$ T cells, coupled to the latency-associated peptide

(LAP) (Andersson et al., 2008; Nakamura et al., 2001). However, most *in-vitro* studies have failed to inhibit suppression with anti-TGFβ, thus questioning its role. Conversely, *in-vivo* studies suggest a non-redundant role for TGFβ in suppression of IBD. Whilst it might be that T_{Regs} are not the source of TGFβ (reviewed in (Shevach, 2009)), mice with a T cell specific defect in TGFβ production or processing developed IBD-like symptoms (Li et al., 2007; Pesu et al., 2008), therefore supporting the involvement of T cell produced TGFβ. The story for IL-10 is similar to that of TGFβ, as *in-vitro* studies have generally failed to demonstrate its requirement in T_{Reg} mediated suppression (Shevach, 2009), whilst *in-vivo* studies again demonstrate importance of IL-10 secretion by T_{Regs} (Belkaid, 2007; McGeachy et al., 2005). Ultimately, *in-vitro* suppression needs to be interpreted cautiously.

Several indirect, APC mediated suppression mechanisms have also been proposed that in general involve suppression of antigen presentation or co-stimulatory capacities. Accumulating evidence from both *in-vitro* and *in-vivo* work suggests that the high constitutive expression of CTLA-4 by T_{Regs} contributes greatly to their suppressive action (Friedline et al., 2009; Kolar et al., 2009; Onishi et al., 2008; Read et al., 2006; Schmidt et al., 2009; Tivol et al., 1995; Waterhouse et al., 1995; Wing et al., 2008; Zheng et al., 2008b). Importantly, CTLA-4 blockade or deletion of CTLA-4 in T_{Regs}, prevented the down regulation of CD80 and CD86 seen when T_{Regs} were co-cultured with DCs (Onishi et al., 2008; Wing et al., 2008). Furthermore, selective deletion of CTLA-4 from T_{Regs} induced systemic autoimmunity (Read et al., 2006). Although the biochemical nature of *ex-vivo* suppression awaits clarification, mechanisms by which CTLA-4 regulates T cell activation, including competition with CD28 for B7 engagement and transendocytic depletion of B7, as described previously, are probably

involved. It has also been suggested that the engagement of CTLA-4 with CD80/86 leads to induction of indoleamine 2,3-dioxygenase (IDO) by the DC (Grohmann et al., 2002). IDO catabolises tryptophan to pro-apoptotic metabolites, such as kinurenins, that might in turn suppress the activation of responder T cells. However, there is little clear *in-vivo* or *in-vitro* evidence for the function of IDO in T_{Reg} mediated suppression (Shevach, 2009). Through their expression of LAG-3, a CD4 homologue that binds to MHC class II with high affinity, T_{Reg} cells might suppress DC maturation and stimulatory capacity (Liang et al., 2008). Yet another mechanism of suppression could involve the ability of T_{Regs} to limit ATP induced induction of CD86, since T_{Regs}, express the ectoenzyme, CD39, which hydrolyses ATP to ADP and AMP (Borsellino et al., 2007). ATP is released from damaged cells and might therefore be at high concentration at sites of inflammation. By metabolising ATP, T_{Regs} would help to reduce the ATP-enhanced stimulatory capacity of the APC. Lastly, imaging studies suggest that T_{Regs}, which are more mobile than naive cells outcompete them for clustering around DCs (Onishi et al., 2008). In addition, T_{Regs}, reduced the time of association between DCs and T effector cells *in-vivo* (Tadokoro et al., 2006). Consistent with these findings, T_{Regs} suppressed the clustering of diabetogenic TCR-transgenic T cells in pancreatic lymph nodes, thus reducing their chance of activation (Tang et al., 2006). One molecule that might favour the interaction of DCs with T_{Regs} versus effector T cells is neuropillin (Nrp-1), since it is preferentially expressed on T_{Regs} and enhances their interactions with dendritic cells during antigen recognition (Sarris et al., 2008). A role for leukocyte function-associated antigen-1 (LFA-1) in this mechanism is also proposed (Onishi et al., 2008). Taken together, the *in-vitro* studies imply that T_{Regs} employ a multitude of suppressive mechanisms, however, whether they are all relevant *in-vivo* and whether others exist, remains to be established.

Understanding how, when and where T_{Regs} function could be important therapeutically, given that deregulation of T_{Reg} frequencies, defective T_{Reg} function or effector cell resistance to T_{Reg} suppression is implicated in a number of diseases. These include: chronic infections and cancer as well as autoimmune conditions such as, type 1 diabetes (T1D), multiple sclerosis (MS), rheumatoid arthritis (RA), psoriasis, inflammatory bowel disease (IBD) and systemic lupus erytheromatosus (SLE) (reviewed in (Buckner, 2010; Piccirillo, 2008)). However, it would appear that with the exception of IPEX syndrome, human diseases do not obviously reveal a general deficiency of T_{Reg} numbers. Indeed, for T1D and MS, most studies indicate that the frequency of T_{Regs} in the peripheral blood of patients does not differ from that of healthy controls, whilst in RA and IBD mixed results have been obtained, including reports of increased and decreased frequencies. Most studies, however agree that T_{Reg} frequencies are increased at the disease site. Although, counterintuitive, the demonstration that T_{Reg} suppressive function is less effective in the listed autoimmune conditions, with the exception of IBD, could explain the finding. Given that activated T cells express markers of T_{Regs}, including FoxP3 (Walker et al., 2003), it might be hypothesised that the apparent enrichment of $CD25^{+}FoxP3^{+}$ cells at inflammatory sites represents an accumulation of activated effector T cells. However, the fact that these cells could exert suppressive effects, albeit defective in some cases, might argue against this hypothesis. The accumulation of T_{Reg} cells as well as effector cells at inflammatory sites could be explained instead by the fact that they share the expression of some chemokine receptors that are responsible for homing to inflamed sites (Lim et al., 2006). Examples include CCR4, associated with skin homing (Campbell et al., 1999), CCR6 that could home cells to an inflamed joint (Hirota et al., 2007) and $\alpha4\beta7$ that directs T cells to the gut (Hamann et al., 1994). In addition, IFNγ inducible CXCR3, that is expressed by T_h1 cells as well as $CD8^{+}$, NK and NKT cells to direct their movement to

inflamed sites, has been detected along with T-bet on a subset of $FoxP3^+$ cells at sites of T_h1 inflammation (Koch et al., 2009). Although, it is difficult to establish a causative link between T_{Reg} cells and inflammatory disease, their involvement is supported by the fact that depletion of T_{Regs} in animal models exacerbates disease supports their involvement. Furthermore, some clinically effective treatments such as anti-TNFα (infliximab) in RA (Ehrenstein et al., 2004), IFNβ in MS (Venken et al., 2008) and corticosteroids in SLE (Suarez et al., 2006) were able to increase T_{Reg} cell frequencies. Whether local defects in T_{Reg} function are the cause or consequence of the inflammation in these conditions is presently not understood but it will be important to determine the mechanisms involved. It will also be important to identify if dysregulation of T_{Regs} results from cell intrinsic or extrinsic environmental factors. If environmental factors are to blame, adoptive transfer of *ex-vivo* cultured T_{Regs} in therapy might not be particularly successful. In this regard, it has been shown that $FoxP3^+$ T_{Regs} can be converted to inflammatory effector populations, including T_h1 and T_h17 (Beriou et al., 2009; Koenen et al., 2008; Zhou et al., 2009b), the outcome being determined by the microenvironment (Zhou et al., 2009b). Understanding the involvement of T_{Reg} cells in response to infection could have implication in the design of vaccination strategies, as it is generally the case that T_{Regs} ($CD4^+FoxP3^+$ cells) are present at increased frequencies in response to bacterial, parasitic or viral infection (reviewed by (Curotto de Lafaille and Lafaille, 2009)). Their presence could therefore impede successful vaccination. Likewise, the action of T_{Regs} could prevent the success of cancer immunotherapy. Strategies to limit T_{Reg} responses locally might therefore aid the success of vaccination treatments.

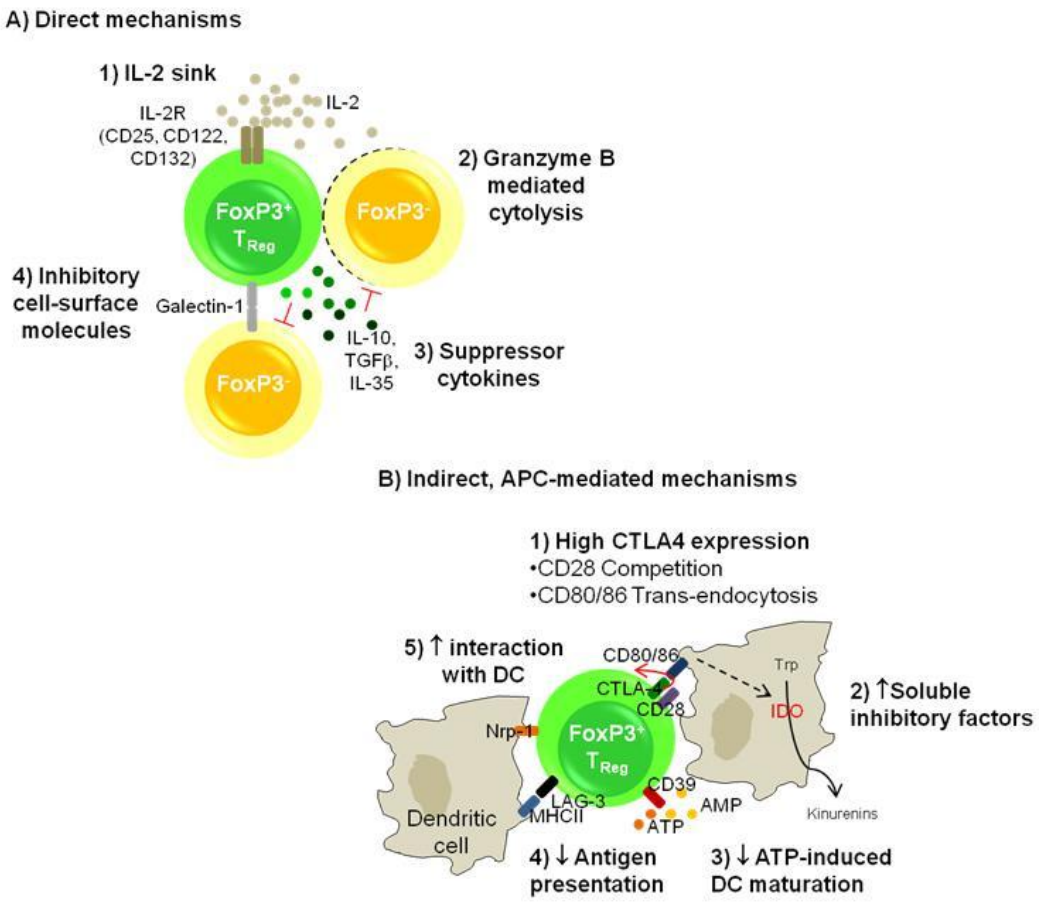

Figure 1.6. Mechanisms of suppression by regulatory T cells (T_{Reg}). T_{Reg} suppression mechanisms can be classified as direct and indirect, APC-mediated. A) Direct mechanisms include 1) dominant use of pro-survival IL-2 as T_{Regs} express high levels of IL-2R. Insufficient IL-2 at the effector T cell could lead to its apoptosis. 2) Production of granzyme B and perforin by the T_{Reg} permits killing of effector T cells by cytolysis. 3) Expression of suppressive cytokines by the T_{Reg} could inhibit activation of the effector T cell and /or promote its differentiation to an inducible T_{Reg}. 4) Molecules such as galectin-1 or TGF expressed on the surface of T_{Regs} could suppress effector T cells by cell-cell contact signalling. B) Indirect mechanisms could involve 1) high CTLA-4 expression by the T_{Reg}. CTLA-4 could reduce co-stimulatory signals by i) competing with CD28 for binding CD80/86, ii) reducing CD80/86 expression, possibly by trans-endocytosis. CTLA-4 engagement with CD80/86 might also lead to indoleamine 2,3-dioxygenase (IDO) activation and the breakdown of tryptophan to kinurenins that could cause apoptosis of the T cells (2). 3) CD39 expressed by T_{Regs} hydrolyses ATP to AMP, thereby reducing ATP- induced DC maturation. 4) LAG-3 binding to MHC-II reduces DC maturation and its antigen presenting capacity. 5) Enhanced duration of T_{Reg} association with DCs, maybe through Nrp-1, reduces the opportunity for T effector cells to associate with DCs and become stimulated.

T_h17 cells were defined a novel $CD4^+$ lineage in 2005 on the basis that they require different transcription factors and polarising cytokines to T_h1 and T_h2 cells for their development. They were named according to their hallmark cytokine, IL-17, which as described below has many targets and can therefore instigate a wide range of inflammatory responses. However, T_h17 cells can also release other pro-inflammatory cytokines including IL-17F, IL-21, IL-22, TNF and IL-6, which either support the development of the T_h17 lineage or feed into the inflammatory response (Kaiko et al., 2008).

1.2.8 The IL-17 cytokine

IL-17 (also known as IL-17A) was originally identified by a subtractive hybridisation screen in a rodent T cell library (Rouvier et al., 1993) and is the founding member of a group of cytokines including: IL-17A, IL-17B, IL-17C, IL-17D, IL-25 (IL-17E) and IL-17F, which were discovered later by sequence alignment. Although pro-inflammatory responses are suggested for most members (with the exception of IL-17E), IL-17A and IL-17F are the best characterised and are both expressed by T_h17 cells (Gaffen, 2009). Both IL-17A and IL-17F exist as covalent homodimers but they can also form heterodimers. Indeed, IL-17A-IL-17F is produced more abundantly *in-vitro* by peripheral blood mononuclear cells than IL-17A homodimer (Wright et al., 2007). IL-17A, IL-7F and the heterodimer signal through the same receptor, which is a heteromeric complex of the single-transmembrane domain proteins IL-17RA and IL-17RC (Kuestner et al., 2007; Wright et al., 2008). Interestingly, although IL-17A and IL-17F share a common receptor, the phenotypes of IL-17A and IL-17F knockout mice suggest that they have distinct functions (Ishigame et al., 2009; Yang et al., 2008b). It would appear that IL-17A contributes more strongly to autoimmunity than IL-17F. This might

reflect the different affinities of the IL-17RA and IL-17RC subunits for IL-17A and IL-17F and/or the greater strength of signalling induced by IL-17A (Ishigame et al., 2009; Yang et al., 2008b).

IL-17RA is expressed ubiquitously (Moseley et al., 2003), with particularly high expression in hematopoietic tissues. The majority of IL-17 induced responses are described for non-hematopoietic cells, including epithelial and endothelial cells and fibroblasts, although macrophages and dendritic cells also respond to IL-17 (Dong, 2008; Gaffen, 2009; Miossec et al., 2009; Park et al., 2005; Shen and Gaffen, 2008). In general a pro-inflammatory response has been seen, involving expression of pro-inflammatory cytokines such as IL-1β, IL-6, TNFα, G-CSF and GM-CSF and the production of chemokines including CCL2, CCL7, CCL20, CXC-chemokine ligand 1 (CXCL1) and CXC8 (IL-8). From *in-vivo* studies, the recruitment and activation of neutrophils following induction of IL-8 and GM-CSF appears to be an early hallmark of the IL-17 response (reviewed in (Dong, 2008; Kolls and Linden, 2004; Park et al., 2005)). IL-17 further enhances innate immune responses by inducing cyclooxygenase (COX) and inducible Nitric Oxide synthase (iNOS) expressions for respective generation of the inflammatory mediators prostaglandin E2 (PGE2) and nitric oxide (NO). In addition, it promotes acute phase protein production and antimicrobial peptides (reviewed by (Shen and Gaffen, 2008)). Matrix metalloproteinases (MMPs) are also released in response to IL-17, leading to extracellular matrix destruction that can facilitate cell movement (Shen and Gaffen, 2008). In bone, matrix destruction by MMPs would contribute to bone re-sorption. Osteoclastogenesis could be further enhanced by IL-17 as it promotes RANKL expression on osteoblasts (Nakashima et al., 2000). RANKL engagement with

osteoclast RANK leads to osteoclast activation and bone re-sorption. T and B cells also express RANKL (Kong et al., 1999) and higher levels of RANKL have been found on T_h17 cells than other subsets (Sato et al., 2006), although whether they engage with osteoclasts has not been confirmed (Miossec et al., 2009). Interestingly, very few IL-17 regulated genes are reported for lymphocytes. Furthermore, they are distinct from those induced by IL-17 in other cell types (Gaffen, 2009).

The pro-inflammatory responses to IL-17 are similar to responses to stimuli such as Toll Like Receptor ligands (TLRLs) and IL-1 receptor signalling, both of which utilise NF-κB pathways. Consistent with a role for NF-κB in the regulation of IL-17 responses, NF-κB binding sites are found in the promoters of IL-17 target genes (Shen et al., 2006) and IL-17 was able to activate the p50 and p65 components of the canonical NF-κB pathway (Shen and Gaffen, 2008). In addition, bioinformatics has revealed a conserved motif in the cytoplasmic domains of the IL-17R family members that is homologous to the Toll/IL-1R (TIR) domain present in TLRs (Novatchkova et al., 2003). This motif could permit the docking of intracellular adaptors involved in the NF-κB signalling pathway, such as MYD88 (myeloid differentiation primary response protein-88) (Gaffen, 2009). Thus, NF-κB signalling appears to contribute to IL-17 responses. Besides NF-κB, IL-17A might signal via mitogen activated protein kinase pathways (MAPK), since ERK (extracellular signal-related kinase) and p38 MAPK were phosphorylated following IL-17 stimulation (Shen and Gaffen, 2008). MAPK pathways are implicated in mRNA stabilisation by inhibiting mRNA destabilising proteins (reviewed by (Shen and Gaffen, 2008)). Several chemokines and cytokines regulated by IL-17 contain 3'destabilising motifs and IL-17 increases their transcript levels. Thus, IL-17 might

regulate cell responses by controlling mRNA stability. Similar to NF-κB binding sites, binding motifs for CCAAT/enhancer binding proteins (C/EBP) are over-represented in the IL-17 target gene promoters (Shen et al., 2006) and a functional role for C/EBP promoter binding has been demonstrated in the regulation of IL-17 target, IL-6 (Ruddy et al., 2004). There are also suggestions that JAK-STAT signalling might mediate some IL-17 responses (Kao et al., 2004; Subramaniam et al., 1999), although secondary effects from STAT signalling cytokines such as IL-6, released upon primary response to IL-17, could explain the data from pharmacological JAK inhibition studies. Lastly, PI3Kinase signalling may contribute to IL-17 signalling, as IL-17 responses in lung epithelial cells were associated with elevated lipid and AKT phosphorylation and could be inhibited by PI3Kinase specific inhibitors (Huang et al., 2007). Taken together, a range of signalling systems might co-operate to regulate IL-17 responses in target cells.

Since the discovery of IL-17, a range of cell types have been found to express it. $CD4^+$ αβ T cells are the best characterised source but it is also expressed by $CD8^+$ T cells, Natural Killer cells (NK), invariant NK T cells (iNKT), γδ T cells, neutrophils, eosinophils, Lymphoid tissue inducer cells (LTi) and mast cells (Dong, 2008; Hueber et al., 2010).

1.2.9 Evidence for T_h17 cells as a novel T helper cell lineage

The initial studies that led to the conclusion that T helper cells expressing IL-17 represent a distinct lineage from T_h1 and T_h2 cells were conducted in mice. Interestingly, the first studies that spurred this consideration did not focus on IL-17 itself but involved analysis of the effects

of IL-12 family cytokines, IL-12 and IL-23, upon the establishment of autoimmune disease (reviewed in (Dong, 2008; Kaiko et al., 2008). The importance of IL-12 and by inference IFNγ secreting T_h1 cells in autoimmune pathology had been concluded previously from the observation that neutralising antibodies to IL-12p40 could alleviate autoimmune disease in mouse models. However, in 2000, IL-12p40 was found to complex with p19, an IL-12p35 and IL-6 related molecule, to form the novel cytokine IL-23. Like IL-12, IL-23 is expressed by activated DCs and monocytes but signals through a unique receptor that is a complex of IL-12Rβ1 with IL-23R (Oppmann et al., 2000). p19 and p35 deficient mice were thus used to determine if IL-12 and/or IL-23 are responsible for the autoimmune phenotype of IL-12p40 depleted mice. p19 deficient mice were less susceptible to autoimmune disease than p35 deficient mice, suggesting that IL-23 rather than IL-12 is responsible for the autoimmune condition (Murphy et al., 2003; Oppmann et al., 2000). Subsequently, IL-23 was shown to induce IL-17 but not IFNγ expression (Murphy et al., 2003) and the co-expression of IL-17 and IFNγ was not observed. Thus, the concept that IL-17 producing T cells are a distinct lineage grew.

To test whether T_h17 cells are distinct from T_h1 or T_h2 cells, the involvement of T_h1 and T_h2 associated cytokines was studied in the context of IL-17 production. Two groups reported that inhibition of IFNγ and IL-4 enhances IL-17 production. These studies made use of neutralising antibodies and mice deficient in IFNγ or IFNγR (Harrington et al., 2005; Park et al., 2005). Analysis of the roles of T_h1 and T_h2 associated transcription factors in the generation of IL-17 producing cells further confirmed that T_h17 cells are a distinct lineage. Firstly, whilst T cells from STAT4 and STAT6 deficient mice did not express IFNγ and IL-4

respectively, the frequency of IL-17 expressing cells was not altered (Harrington et al., 2005; Park et al., 2005). Secondly, STAT1 and T-bet deficiencies increased the frequency of IL-17 expressing cells, consistent with the hypothesis that IFNγ suppresses IL-17 expression (Harrington et al., 2005). That T_h2 and T_h17 cells represent separate lineages was similarly confirmed through observation that c-Maf over-expression increases IL-4 but reduces IL-17 and IFNγ (Park et al., 2005).

1.2.10 Cytokines critical for T_h17 development

The finding that STATs 1 and 4 are not required for T_h17 development came as a surprise given that IL-23 signals through STATs 1, 3, 4 and 5. However, in contrast to IL-12, which mainly signals through STAT4 homodimers, IL-23 makes less use of STAT4 and might signal through STAT3 homodimers to promote T_h17 development (Lankford and Frucht, 2003). Consistent with this hypothesis, following IL-23 stimulation, IL-17 was increased in suppressor of cytokine signalling-3 (SOCS3) deficient cells (Chen et al., 2006) and over-expression of active STAT3 amplified T_h17 frequencies (Lankford and Frucht, 2003; Yang et al., 2007). However, it is now suggested, that IL-23 is not required for commitment to the T_h17 lineage but rather functions in the survival and expansion of T_h17 cells. This is consistent with the activation dependent expression of IL-23R by T cells (Mangan et al., 2006). The significance of STAT3 upon T_h17 differentiation was subsequently explained when it was shown that IL-6, which signals via STAT3 in consort with TGFβ drives T_h17 generation. Pro-inflammatory cytokines including IL-1β and Tumour Necrosis Factor (TNF), were also shown to synergise with IL-6 and TGFβ to increase T_h17 frequencies (reviewed in (Dong, 2008)). The release of IL-6, TNF and IL-1β by IL-17 target cells, therefore provides

positive feedback for IL-17 production by T_h17 cells. Likewise, IL-21, which is a major product of T_h17 cells, can feed-forward on their development in a manner analogous to IFNγ and IL-4 in the positive regulation of T_h1 and T_h2 lineages respectively.

The importance of TGFβ for the generation of a pro-inflammatory helper cell was counterintuitive, given its role in effector T cell suppression. Nevertheless, studies in mice faithfully showed a requirement for TGFβ in T_h17 differentiation. Thus, T_h17 and T_{Reg} lineages were said to be reciprocally related with IL-6 as the switch factor (Bettelli et al., 2006). Human studies initially contradicted the observations in mouse suggesting that TGFβ and IL-6 inhibit T_h17 differentiation (Acosta-Rodriguez et al., 2007a; Evans et al., 2007). However, later studies, conducted under serum free conditions refuted this claim, showing that low TGFβ in consort with pro-inflammatory cytokines such as IL-1β, IL-6, IL-21,TNF and IL-23, is essential (Manel et al., 2008; Veldhoen et al., 2006; Volpe et al., 2008; Yang et al., 2008a). Each pro-inflammatory cytokine might uniquely impact the cytokine profile of the differentiated T cell (Volpe et al., 2008). The discrepant findings of earlier and later studies were suggested to involve the presence of human compatible TGFβ in serum media (Volpe et al., 2008). Indeed, whilst high levels of TGFβ might be inhibitory to T_h17 differentiation, low levels appear to be inductive (Yang et al., 2008a). Another factor might have been kinetics, since TGFβ could increase in culture over time. Lastly, the proportion of naive and central memory T cells could have influenced the cytokine requirements. For example, Yang et al (2008) showed that highly purified central memory $CD4^+$ cells could induce IL-17 expression in serum free medium following IL-1β, IL-6 and IL-23 supplement, whilst naive cells from peripheral and cord blood required TGFβ and IL-21 (Yang et al., 2008a).

The role of TGFβ for naive T cell differentiation to the T_h17 lineage can be explained by its ability to promote expression of the hallmark T_h17 lineage transcription factor, RORγt (RORC2 in humans) (Ivanov et al., 2006). TGFβ also induces T_{Reg} associated FoxP3, which in the absence of pro-inflammatory cytokines interacts with RORγt inhibiting its transcriptional activity. Therefore, a post-translational mechanism might contribute to the reciprocal development of T_h17 and T_{Reg} cells in the presence versus absence of pro-inflammatory cytokines respectively (Zhou et al., 2008; Zhou and Littman, 2009). However, via STAT3, pro-inflammatory cytokines can also increase RORγt expression. Interestingly, the IL-17 promoter itself contains a STAT3 binding site (Chen et al., 2006), thus in addition to increasing IL-17 expression via RORγt, STAT3 could directly enhance IL-17 transcription. A recent study in mice further showed that whilst TGFβ does not alter RORγt expression in differentiating and committed T_h1 and T_h2 wildtype cells, it inhibits STAT4 and GATA-4. Additionally, Stat-$6^{-/-}$Tbet$^{-/-}$ mice, that are incapable of developing T_h1 or T_h2 responses, could generate vigorous T_h17 responses in IL-6 supplemented cultures irrespective of TGFβ presence (Das et al., 2009). Together these data suggest that TGFβ might promote T_h17 differentiation by inhibiting T_h1 and T_h2 differentiation programs rather than being required for T_h17 differentiation. This finding has recently been strengthened by others, who demonstrated that permissive epigenetic modifications of the *il-17a-il-17f* and *rorc* loci could occur in the absence of TGFβ, providing IL-23 was supplied in combination with IL-6 and IL-1β (Ghoreschi et al., 2010). Mechanistically, they demonstrated that IL-6 induces IL-23R expression in naive T cells to permit IL-23 signalling that in turn promotes RORγt and IL-17 expression. In addition, IL-23 itself could enhance IL-23R expression to increase T_h17 frequencies. Thus, the previous view that IL-23 is required for maintenance of T_h17 cells rather than their induction (Evans et al., 2007), might need to be revised. Consistent with the

inhibitory effect of TGFβ upon T-bet and IFNγ expression, fewer T_h17 cells were generated in the absence of TGFβ (Ghoreschi et al., 2010), therefore a role for TGFβ in establishing T_h17 populations appears to be upheld. However, the greatest proportion of T_h17 cells isolated from the CNS of EAE mice had an IL-23 driven phenotype, showing dual expression of RORγt and T-bet. Furthermore, adoptive transfer of IL-23-induced T_h17 cells instigated stronger disease than TGFβ induced T_h17 cells in the EAE model (Ghoreschi et al., 2010). Cytokine profile analysis on T cells from human disease tissues including the gut of Crohn's patients (Annunziato et al., 2007) and joints of arthritis patients (Nistala et al., 2008) similarly revealed enrichment of IL-17^+IFNγ^+ T cells ($T_h17/1$) at diseased sites. Thus, it seems likely that the $T_h17/1$ population is particularly pathogenic, which might explain previously conflicting findings that both T_h17 cells and T-bet are pathogenic in EAE (Bettelli et al., 2004). However, it is possible that T_h1 and T_h17 cells are relevant at different stages of the disease and that inter-conversion between the two occurs. In support of this hypothesis, a number of groups have investigated the ability of T_h17 and T_h1 cells to interconvert or become $T_h17/1$ hybrids that coincidently express IL-17 and IFNγ. Reasonably pure T_h17 starting populations were derived either from T cell clones (Annunziato et al., 2008), by reporter gene expression and FACS sorting (Lee et al., 2009) or through use of cytokine detection and capture assays (Nistala et al., 2010). All studies agreed that TGFβ maintains IL-17 expression. By contrast, IL-12 alone promoted a switch to the T_h1 phenotype and whilst the presence of TGFβ with IL-12 tended to favour the hybrid phenotype, in the absence of TGFβ, IL-23 favoured the $T_h17/1$ profile. Thus, TGFβ in consort with pro-inflammatory cytokines might establish a cohort of IL-17^+ cells, which can then be converted under conditions of IL-12 or IL-23 to $T_h17/1$ or T_h1 cells. The relevance of this conversion has been demonstrated *in-vivo* as following transfer of purified T_h17 populations, IFNγ^+ T cells could

be detected in T cell deplete hosts (Bending et al., 2009; Kurschus et al., 2010; Lee et al., 2009; Martin-Orozco et al., 2009). In addition, spectratyping of the TCR-β variable chain (TRBV) determined that $T_h17/1$ cells from the synovial fluid of arthritis patients might share ancestry with T_h17 cells but not T_h1 cells (Nistala et al., 2010). Furthermore, all of the T_h1 clones that shared a TCR spectratype with T_h17 cells expressed CD161, which is a surface marker that identifies the T_h17 precursor pool in cord blood (Cosmi et al., 2008). This suggests that some T_h1 cells in the joint originated from T_h17 cells. However, one study has recently shown that purified T_h1 cells could convert to T_h17 cells following adoptive transfer (Kurschus et al., 2010).

1.2.11 Negative regulation of T_h17 development

Besides IFNγ and IL-4, IL-27, IL-25 (IL-17E) and the novel cytokine IL-35 appear to suppress the development of T_h17 cells and in mouse models of autoimmune disease their absence has resulted in increased disease susceptibility (Dong, 2008; Niedbala et al., 2007). A function for the proliferative cytokine, IL-2 in T_h17 suppression has also been suggested and is consistent with its pro-regulatory effect when supplied in consort with TGFβ (Acosta-Rodriguez et al., 2007a; Laurence et al., 2007). However, exogenous IL-2 might expand differentiated T_h17 populations (Volpe et al., 2008).

1.2.12 T_h17 lineage specific transcription factors

As mentioned, RORγt is the hallmark transcription factor of the T_h17 lineage in both mice and man. It is a member of the retinoic-acid-receptor-related orphan nuclear hormone receptor

family and is encoded by the RORC locus. Evidence for its importance includes RORγt induction under T_h17 polarizing conditions, the loss of T_h17 cells and attenuation of autoimmune disease in RORγt deficient mice. Nonetheless, the mechanism by which RORγt facilitates IL-17 expression remains unclear, although the presence of a ROR response element (RORE) within a non-coding region of the IL-17-IL-17F gene locus implies that direct binding might be involved (reviewed in (Dong, 2008)). In addition, it appears that RORγt is not solely responsible for T_h17 development, since residual numbers of T_h17 cells are present in RORγt deficient mice (Dong, 2008). Recently, a minor but synergistic role for the ROR family member, RORα, has been proposed (Yang et al., 2008d). Transcription factor, interferon regulatory factor four (IRF-4), might also be involved, lying upstream of RORs in the signalling network (Brustle et al., 2007). However, with the possible exception of T_h1 cells, IRF4 appears to contribute to the development of all currently known T helper cells, thus it might function upstream of the lineage determination factors (reviewed by (Veldhoen, 2010)). Consistent with this, IRF4 deficiency reduced RORγt and RORα. However, IRF4 might also influence the function of RORγt or RORα, since ectopic expression of both factors only partially rescued T_h17 differentiation in the absence of IRF4. Alternatively, this could be explained by the need for IRF4 in IL-21 responsiveness (Veldhoen, 2010). Similarly, promotion of IL-21 by cMaf, could explain the role of ICOS-induced cMaf in T_h17 differentiation (Bauquet et al., 2009). Runx1 (runt related transcription factor -1) is another transcription factor that may control T_h17 development, since it too can complex with RORγt and was shown to co-operate with RORγt to promote T_h17 differentiation (Zhang et al., 2008). Similar to IRF4, however, Runx1 activity might not be unique to T_h17 cells, since in T_{Regs}, Runx1 interacts with FoxP3, up-regulates T_{Reg} associated molecules, suppresses IL-2 and IFNγ and permits their suppressive activity (Hu et al., 2007).

The aryl hydrocarbon receptor (AHR), which similar to ROR proteins is a member of the steroid nuclear receptor family, is also highly expressed in T_h17 cells (Quintana et al., 2008; Stockinger et al., 2009; Veldhoen et al., 2008a). Through interaction with RORγt, AHR might enhance IL-17 expression. Furthermore, AHR is required for induction of the T_h17 associated cytokine, IL-22, which is also the hallmark cytokine of the novel T_h22 lineage (Trifari et al., 2009). Nuclear factor of activated T cell (NFAT) proteins, which are induced soon after T cell receptor activation in response to calcium signalling via calcineurin activation, also play a role in lineage development. Although they are induced in a relatively non-specific manner following TCR stimulation, NFAT proteins and their partners can access specific loci following chromatin remodelling. In T_h17 development it is suggested that NFAT co-operates with the above mentioned transcription factors for RORγt, IL-17, IL-21,IL-22 and IL-23R transcription (Hermann-Kleiter and Baier, 2010).

1.2.13 T_h17 cells in disease

As discussed, the existence of the T_h17 lineage grew from the discovery that the pathogenesis of autoimmune disease in certain mouse models involved IL-17 secreting $CD4^+$ T cells rather than IFNγ expressing cells (Murphy et al., 2003). Since then, the involvement of T_h17 cells in a number of mouse models of autoimmune and allergic disease has been studied and pathogenic effects concluded (reviewed in (Korn et al., 2009)). T_h17 cells have also been associated with inflammatory human diseases including RA, asthma, SLE, MS, Crohn's disease and allograft rejection (Korn et al., 2009; Park et al., 2005; Steinman, 2007; van Beelen et al., 2007). However, these associations are largely circumstantial, based upon elevated IL-17 mRNA or IL-17 protein in the tissues or fluids of patients with the disease.

Additionally, increased levels of other T_h17-associated cytokines, factors associated with T_h17 differentiation and molecules expressed by target cells following IL-17 have been taken as evidence of T_h17 activity in disease. However, a few reports have shown that T cells express IL-17 at sites of inflammation. For example, IL-17 was expressed by T cell clones generated from the skin of individuals with contact dermatitis (Pene et al., 2008) and increased T_h17 frequencies, as measured by flow cytometry, were found *ex-vivo* in synovial fluid of RA patients (Colin et al., 2010; Leipe et al., 2010). and the gut of Crohn's disease patients (Annunziato et al., 2007) relative to controls. For psoriasis and some arthropathies neutralising antibodies to IL-17 or T_h17 inducing cytokines have also been shown to reduce disease severity (Allantaz et al., 2007; Genovese et al., 2010; Miossec, 2009; Tak, 2009; Yokota et al., 2008).

Besides their pathogenic effect in autoimmune and allergic conditions, T_h17 cells, have a protective role in host defence against certain microbial infections including the gram positive bacterium, *Propionibacterium acnes* and gram negative *Klebsiella pneumoniae* (Ye et al., 2001), *Citrobacter rodentium* (Mangan et al., 2006), *Bacteriodes spp.*, *Borrelia spp.*, acid-fast *Mycobacterium tuberculosis* (Khader et al., 2007) as well as fungal like pathogens: *Pneumocystis carinii* (Rudner et al., 2007) and *Candida albicans* (Huang et al., 2004). It might be that T_h17 cells deal with the removal of different pathogens to T_h1 cells. This is supported by the finding that human memory T_h17 cells could react against antigens derived from *Candida albicans* whilst the memory T cells specific for *Mycobacterium tuberculosis* were of the T_h1 phenotype (Acosta-Rodriguez et al., 2007b).

1.3 Reciprocal regulation of the T_h17/iT_{Reg} balance

Besides identifying the cytokines that are conducive to the development of one T effector lineage relative to another, the effective treatment and prevention of disease requires the identification of other physiological and environmental factors that favour the acquisition of one cell lineage over another.

1.3.1 Regulation of the T_h17/iT_{Reg} balance by steroid nuclear receptor ligands

Recently a number of steroid nuclear receptor ligands have been studied for their effects upon $CD4^+$ T cell differentiation, in particular their ability to regulate the T_h17/iT_{Reg} balance. Amongst these are all-trans-retinoic acid (ATRA), the active form of vitamin A and a number of AHR ligands including the environmental toxins 2,3,7,8-tetracholorodibenzi-p-dioxin (TCDD) and β-napthoflavone and the endogenous molecules formylindolo[3,2-b]carbazole (FICZ), and 2-(1'H-indole-3'carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE). Specifically, ATRA enhances the TGFβ-dependent conversion of naïve T cells into T_{Reg} cells but suppresses T_h17 differentiation under conditions of TGFβ and IL-6 (Schambach et al., 2007). Such ATRA induced tolerance might be of particular relevance in the gut, contributing to oral tolerance, since firstly $CD103^+$ DCs in gut associated lymphoid tissue (GALT) express high levels of retinal dehydrogenases for ATRA synthesis; secondly, ATRA signalling induces the expression of gut homing molecules α4β7 and CCR9 on the T cell surface (Coombes et al., 2007).

In contrast to ATRA, analysis of AHR deficient cells in the presence of dioxin or FICZ concluded a role for the AHR in T_h17 induction (Veldhoen et al., 2008a; Volpe et al., 2008; Zhou and Littman, 2009). However, other studies suggest that AHR effects upon the T_h17/iT_{Reg} balance are ligand specific (Quintana et al., 2008): TCDD promoted development of $FoxP3^+$ cells that were functionally suppressive *in-vitro* and inhibited EAE pathogenesis, coincident with decreased $CD4^+IL\text{-}17^+$ and $CD4^+IFN\gamma^+$ cells. Similarly, ITE significantly reduced EAE severity. Conversely, FICZ did not induce FoxP3 expression but rather significantly worsened EAE and enhanced IL-17 and IFNγ secretion *in-vitro*. Since AHR can interact with both FoxP3 and RORγt, such AHR ligand specific effects might relate to their differing abilities to modulate these interactions. However, different AHR ligand toxicities and their influence upon AHR degradation could equally explain the converse effects of TCDD and FICZ (Veldhoen et al., 2008a). Nonetheless, it is clear that steroid nuclear receptor ligands can profoundly influence the position of the T_h17/ iT_{Reg} balance. Thus, they represent environmental factors that could influence susceptibility to immune disease.

1.4 Vitamin D

Vitamin D was identified in the early 1900s for its ability to cure childhood rickets and treat osteoporosis. In this capacity it functions as a hormone in the regulation of blood calcium and phosphorus, promoting increased calcium absorption at the intestine and proximal kidney tubule when calcium levels are low and in consort with parathyroid hormone vitamin D triggers calcium mobilisation from bone (reviewed in (Holick, 2007)). More recently, a range of non-classical activities for vitamin D have been suggested, including regulation of cell proliferation, differentiation, apoptosis and angiogenesis and of particular pertinence to this

study, immunomodulatory effects. Discovery of the vitamin D receptor (VDR) in tissues other than those involved in calcium and phosphate regulation persuaded the acceptance of such non-classical endocrinological functions for vitamin D (Mullin and Dobs, 2007). Amongst VDR-expressing cells are cells of the immune system, including: monocytes, macrophages, dendritic cells and activated T and B cells (Veldman et al., 2000).

1.4.1 Vitamin D metabolism

Few foods naturally contain or are supplemented with vitamin D (where D refers to D_2 (plant derived ergocalciferol) or D_3 (cholecalciferol)). Highest levels are found in oily fish and fish based products that provide 300–500 international units (IU) per serving (Mullin and Dobs, 2007). Low levels are also present in butter, cream and egg yolk and some foods including milk, orange juice, cereals, breads, yogurts, and cheeses can be fortified with vitamin D (Baeke et al., 2008). The majority of vitamin D_3 is attained through solar ultraviolet B mediated conversion of 7-dehydrocholesterol, present at high level in the cell membranes of keratinocytes of the stratum basal and stratum spinosum layers of the skin epidermis. Exposure to one minimal erythemal dose of UVB radiation whilst wearing a bathing suit is equivalent to ingesting 20,000IU vitamin D_3 (Holick, 2007).

Similar to other sterol hormones such as oestrogen, vitamin D requires further enzymatic conversion for activation: Two sequential hydroxylations at C1α and C25 are required (**figure 1.7**). The first, to yield 25(OH)D_3 (Calcidiol) is catalysed primarily in the liver by the 25-hydroxylase activity of cytochrome–P450 enzyme, CYP27A1. The active metabolite,

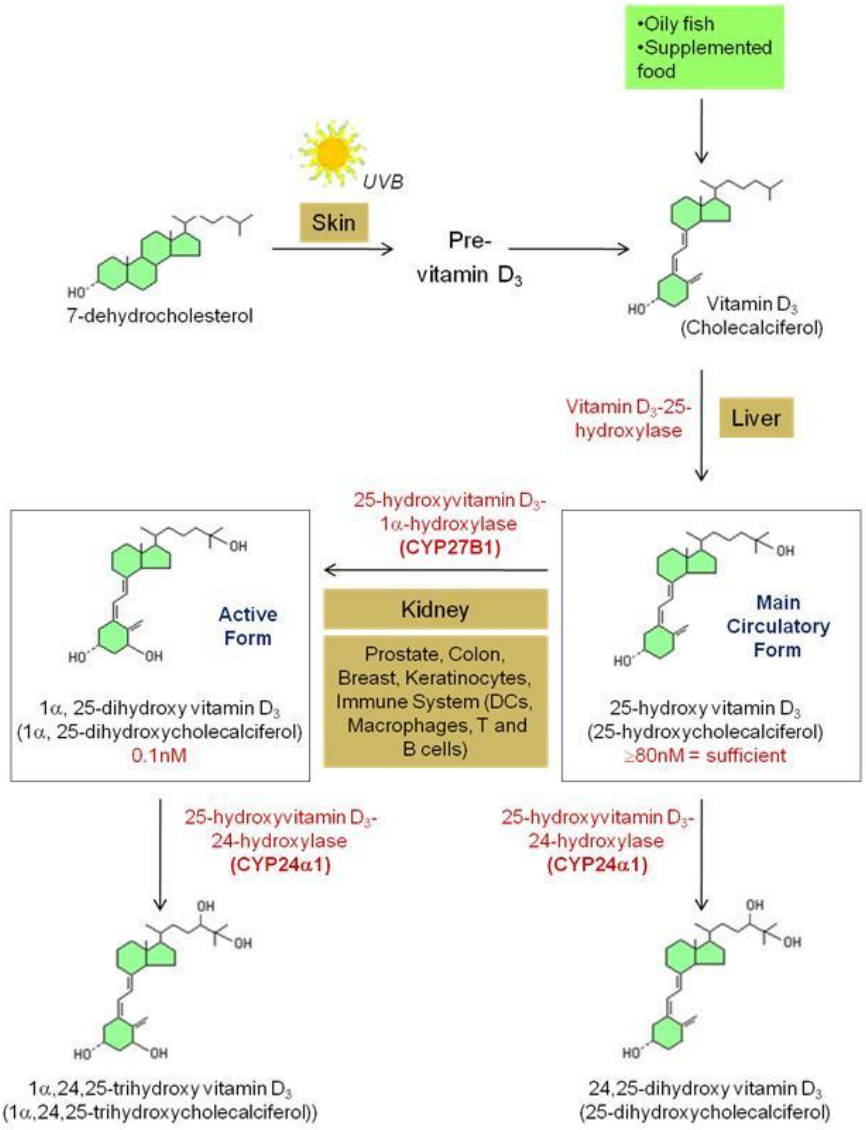

Figure 1.7. Synthesis and metabolism of vitamin D. Ultraviolet B (UVB) radiation from sunlight converts 7 dehydrocholesterol in the skin to pre-vitamin D_3 which is immediately converted to vitamin D_3 in a heat-dependent process. Vitamin D_3 can also be obtained from the diet. Excessive photo exposure degrades vitamin D_3. Vitamin D_3 is carried in blood by vitamin D binding protein (VDBP). In the liver, vitamin D-25-hydroxylase converts vitamin D_3 to 25-hydroxyvitamin D_3, an inactive intermediate that is the major circulating form of vitamin D_3 and used to determine vitamin D status. 25-hydroxy vitamin D_3 is converted to the active form, 1α,25–dihydroxyvitamin D_3, in a second hydroxylation reaction catalysed by 25-hydroxyvitamin D_3 1α-hydroxylase (CYP27B1). That converted in the kidneys regulates calcium and phosphate homeostasis but VDR is also expressed at other body sites. Excess 1α,25–dihydroxyvitamin D_3 and 25-hydroxyvitamin D_3 is hydroxylated and inactivated by 25-hydroxyvitamin D_3 24-hydroxylase (CYP24α1).

$1,25(OH)_2D_3$ (Calcitriol), which has 1000 fold greater activity than $25(OH)D_3$, is finally synthesised via 25-hydroxyvitamin-D_3-1α-hydroxylase (CYP27B1). CYP27B1 expression was first discovered in the kidney but similar to the VDR, its much broader expression in prostate, breast, colon, immune, skin and β cells is now acknowledged. Despite being structurally identical, renal and extra-renal CYP27B1 demonstrate different mechanisms of regulation: Renal-CYP27B1 is suppressed by calcium, phosphate, PTH, phosphaturic factors, phosphatonins and $1\alpha\text{-}25(OH)_2D_3$, consistent with the classical role of vitamin D in calcium homeostasis. By contrast, local agents, including cytokines and growth factors regulate extra renal-CYP27B1 (Leventis and Patel, 2008). As such, it is contended that extra-renal generated $1,25(OH)_2D_3$ regulates the functions of the tissues in which it is produced via autocrine and paracrine mechanisms. The comparatively short half life of $1,25(OH)_2D_3$, which is in the order of hours rather than weeks, as measured for $25(OH)D_3$, further supports the need for local CYP27B1 activity (Mullin and Dobs, 2007). Serum $1,25(OH)_2D_3$ levels are very low (~0.1nM) and remain relatively constant due to tight homeostatic regulation in which 25-hydroxyvitamin D-24-hydroxylase (CYP24A1) hydroxylates both $25(OH)D_3$ and $1,25(OH)_2D_3$ to yield the inactive metabolites $24,25(OH)_2D_3$ and $1\alpha,24,25(OH)_2D_3$. Interestingly, vitamin D deficient persons usually show normal, even elevated $1,25(OH)_2D_3$ serum levels due to secondary hyperparathyroidism (Holick, 2007). By contrast, $25(OH)D_3$ levels vary and are used to define vitamin D status. Most experts define vitamin D deficiency as a $25(OH)D_3$ serum level of less than 50nM (20ng/ml), although up to 80nM is considered relative insufficiency with respect to calcium regulation and bone health (Hollis, 2005). Vitamin D intoxication associated with hypocalcaemia and hyperphosphatemia is not observed until serum $25(OH)D_3$ peaks 374nM (150ng/ml). According to these parameters, one billion persons worldwide are estimated to be vitamin D deficient (Holick, 2007). The

Institute of Medicine recommends a daily dietary vitamin D intake of 200IU/day for children and adults up to 50 years, 400IU for adults between 51 and 70 years and 600 IU for persons of 71 years and older.

The majority (80-85%) of the lipid soluble $25(OH)D_3$ and $1,25(OH)_2D_3$ metabolites are transported around the body in the blood bound with high affinity to vitamin D binding protein (VDBP) (White and Cooke, 2000), which was originally classified as group-specific component or Gc globulin (Hirschfeld, 1959). The remainder travels free or bound to albumin. Three common structural VDBP polymorphisms have been identified that are encoded by co-dominant alleles known as GC^*1F, GC^*1S and GC^*2. In addition to these common variants, over 120 variant VDBP alleles are described worldwide (Cleve and Constans, 1988; White and Cooke, 2000). Interestingly, serum levels of vitamin D metabolites vary according to GC genotype suggesting differences in their abilities to bind vitamin D (Janssens et al., 2010). In addition to its role in vitamin D transport, the monomeric VDBP is a macrophage-activating factor, a scavenger of actin, and it augments the chemotactic effect of C5a for macrophages and neutrophils. In addition, VDBP has been detected on lymphocytes, neutrophils and monocytes and its expression might be regulated by cytokines (reviewed in (White and Cooke, 2000)). Thus, VDBP appears to have immunological functions besides those influenced by vitamin D metabolites themselves. Consistent with a range of immunological effects, VDBP polymorphisms have been associated with inflammatory conditions such as chronic obstructive pulmonary disorder (COPD), tuberculosis, RA and diabetes in some cohorts (Chishimba et al., 2010; Papiha and Pal, 1985). Free vitamin D metabolites can diffuse directly across lipid bilayers into cells whilst VDBP bound molecules are transported via a megalin/cubulin-mediated endocytic

process (Chishimba et al., 2010; Nykjaer et al., 1999; Nykjaer et al., 2001; Papiha and Pal, 1985) (**figure 1.8**). Once inside the cell within clathrin-coated vesicles, VDBP is transported to endosomes where it is released from its receptors. These are recycled back to the plasma membrane and VDBP transported on to lysosomes where it is degraded and its cargo released into the cell (Chishimba et al., 2010; Papiha and Pal, 1985). Although this process was first detected in renal cells, it is reported in other tissues including macrophages (Chun et al., 2008), implying that it has relevance for vitamin D modulated immune responses.

1.4.2 Genomic actions of 1,25(OH)$_2$D$_3$

1,25(OH)$_2$D$_3$ mediates most of its effects by directly regulating the transcription of its target genes (Deeb et al., 2007) (**figure 1.8**). In this mechanism, VDR, which is a member of the class II family of nuclear receptors becomes activated upon ligand binding and typically heterodimerises with the retinoid X receptor (RXR). In this way, ATRA and 1,25(OH)$_2$D$_3$ signalling pathways are related as Retinoic Acid Receptors (RAR) α,β and γ that recognise ATRA also control gene responses through binding to RXR (Hughes et al., 2006). The VDR-RXR complex binds to vitamin D response elements (VDRE) in target genes, most of which are composed of two hexanucleotide repeats interspaced with varying numbers of nucleotides. In the main, for gene activation, VDR occupies the 3' half of the VDRE and RXR the 5' site. In the absence of ligand, co-repressor proteins complex with VDR to inhibit gene expression. They promote this inhibition by virtue of intrinsic chromatin remodelling functions, or by serving as a recruitment platform for chromatin remodelling proteins and basal transcription factors. Upon ligand binding, VDR is phosphorylated and undergoes conformational changes that lead to the release of repressor proteins. In addition, its activation function (AF2) sites

become exposed allowing the binding of stimulatory co-activators as well as the chromatin modifiers, CREB binding protein (CBP-p300) and PBAF (polybromo-and SWI-2-related gene associated factor). Subsequent histone acetylation opens the chromatin allowing the entrance of vitamin D receptor interacting proteins (DRIPs) that interact with the AF2 domains of VDR and with transcription machinery such as TF2B (transcription factor 2B) and RNA polymerase II, to promote transcription. For gene repression, the VDR-RXR heterodimer associates with the VDR-interacting repressor at E-box type negative VDREs (nVDREs) and histone acetyltransferase (HAT) coactivator is exchanged for the histone deacetylase (HDAC) co-repressor.

1.4.3 Non-genomic actions of 1,25(OH)$_2$D$_3$

In addition to direct genomic regulation, 1,25(OH)$_2$D$_3$ can have very rapid, non-genomic effects exemplified by the rapid induction of calcium absorption at the intestine. Although these signalling pathways are poorly understood, it is suggested that they can begin at the plasma membrane with reception of 1,25(OH)$_2$D$_3$ by a non-classical membrane receptor (memVDR) or a G protein coupled receptor. Activation of these receptors could lead to calcium influx via SOCS channels (in muscle cells), PI3Kinase, adenylate cyclase, phospholipase Cγ and PKC activation. Phosphorylation of Ras or Raf could in turn activate the mitogen-activated protein kinase (MAPK)-extracellular signal regulated kinase (ERK) 1 and 2 cascade. For some transcriptional responses, the Raf-MAPK-ERK pathway and the classical VDR pathway could co-ordinately regulate gene transcription and phosphorylation of VDR by PKC could enhance its stability. Thus, 1,25(OH)$_2$D$_3$ regulation appears to involve cooperation between genomic and non-genomic pathways (Deeb et al., 2007) (**figure 1.8**).

1.4.4 Immunomodulatory effects of 1,25(OH)$_2$D$_3$

Immunomodulatory effects of 1,25(OH)$_2$D$_3$ are described for both the innate and adaptive arms of the immune system. In the innate system 1,25(OH)$_2$D$_3$ demonstrates antimicrobial properties inhibiting for example the growth of *Mycobacterium tuberculosis* in macrophage cultures (Rook et al., 1986). *In-vivo* studies on vitamin D deficient mice further indicate a reduced phagocytic response that is remedied by 1,25(OH)$_2$D$_3$ repletion (Bar-Shavit et al., 1981). Recently the molecular basis for the antimicrobial effect of 1,25(OH)$_2$D$_3$ has been studied and is suggested to involve Toll Like Receptor 2 (TLR2) dependent up-regulation of macrophage VDR and CYP27B1 followed by induction of the defensin cathelicidin, whose promoter contains a VDRE (Liu et al., 2006a). In addition to cathelicidin, 1,25(OH)$_2$D$_3$ can induce other factors associated with bacterial killing, such as Nitric Oxide Synthase (Adams and Hewison, 2008). The induction of 1,25(OH)$_2$D$_3$ by human macrophages in response to signalling through other TLRs, such as the lipopolysaccharide (LPS) Pattern Recognition Receptor, TLR4, is also reported (Reichel et al., 1987a) but the broader involvement of TLRs in 1,25(OH)$_2$D$_3$ induction remains to be established. TLR2 mediated induction of CYP27B1 and cathelicidin is also observed in keratinocytes wherein the initial induction of TLR2 might be driven by TGFβ released for example during wound repair (Sadeghi et al., 2006). Thus, in the skin, 1,25(OH)$_2$D$_3$ might link wound repair with enhanced innate immune surveillance. Despite such clear antimicrobial responses in these studies, 1,25(OH)$_2$D$_3$ is also reported to down-regulate monocyte TLR2 and TLR4 expression. Such negative feedback might be important to protect against inflammatory damage that would otherwise ensue (Adams and Hewison, 2008).

In the adaptive immune system, similar to vitamin A, $1,25(OH)_2D_3$ demonstrates modulatory properties. Specifically a number of *in-vitro* studies have shown that $1,25(OH)_2D_3$ suppresses monocyte to DC differentiation resulting in $CD1a^-CD14^+$ cells. It also inhibits their maturation, down-regulating MHC II and co-stimulatory molecules, CD40 and CD80 (Berer et al., 2000; Canning et al., 2001; Penna and Adorini, 2000), although contrasting effects upon CD86 have been shown (Canning et al., 2001; Penna and Adorini, 2000). Nevertheless, $1,25(OH)_2D_3$ conditioned DCs demonstrate reduced ability to stimulate T cells and take up soluble antigen. In addition, $1,25(OH)_2D_3$ suppresses DC production of IL-12 but enhances their production of IL-10 (Penna and Adorini, 2000). A switch of T cell phenotype from T_h1 to T_h2 and T_{Reg} in response to $1,25(OH)_2D_3$ has also been suggested, since attenuation of IL-2 (Bhalla et al., 1984) and IFNγ (Lemire, 1992; Penna and Adorini, 2000) upon $1,25(OH)_2D_3$ supplementation has been observed, whilst IL-4 (Boonstra et al., 2001), TGFβ1 (Cantorna et al., 1998) and IL-10 (Barrat et al., 2002; Urry et al., 2009) were increased. However, the effect of $1,25(OH)_2D_3$ upon the T_h2 lineage remains controversial, since inhibition of IL-4 by $1,25(OH)_2D_3$ is also reported (Staeva-Vieira and Freedman, 2002). Most studies on lymphocyte responses to $1,25(OH)_2D_3$ have been conducted in the presence of APC and could be consistent with the effect of $1,25(OH)_2D_3$ upon APC cytokine profiles. However, in a few more recent studies effects of $1,25(OH)_2D_3$ on T cells in the absence of APC were described (Barrat et al., 2002; Boonstra et al., 2001). Such findings are consistent with induction of the VDR in T cells upon stimulation (Bhalla et al., 1983). In addition, the presence of a VDRE in the IFNγ gene promoter (Cippitelli and Santoni, 1998) and VDR binding at the IL-2 gene promoter (Alroy et al., 1995) imply the involvement of direct effects of $1,25(OH)_2D_3$ in T cell differentiation.

Consistent with the immunosuppressive properties of $1,25(OH)_2D_3$ manifest *in-vitro*, population studies suggest that low dietary vitamin D intake, high latitude and VDR polymorphisms associate with increased risk of autoimmune diseases including: RA, Crohn's disease, MS and T1D (reviewed by (Arnson et al., 2007; Cantorna and Mahon, 2004)). Such associations highlight the importance of understanding mechanisms by which $1,25(OH)_2D_3$ influences the adaptive immune system, including the development of T_h17 and T_{Reg} cells that appear critical players in the pathology of autoimmune diseases.

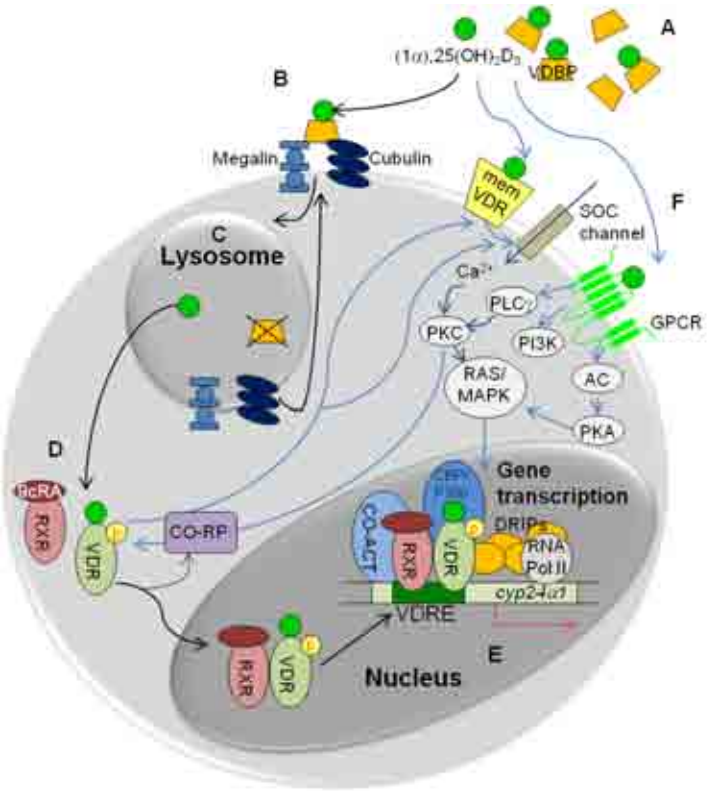

Figure 1.8. Vitamin D signalling. A) The majority of vitamin D metabolites are transported in the blood bound to vitamin D binding protein (VDBP). The remainder travel free or bound to albumin. B) Uptake of VDBP-bound vitamin D by endocytosis is mediated by megalin and cubulin. C) VDBP is released from megalin and cubulin in the lysosome and degraded. Vitamin D is metabolised by the cell. D) 1,25 dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), the active form of vitamin D, binds to the vitamin D receptor (VDR) which becomes phosphorylated and heterodimerises with Retinoid X Receptor (RXR). Co-repressor proteins (CO-RP) are released, permitting VDR transcription factor activity. E) VDR-RXR heterodimer interacts with vitamin D response elements (VDRE) and recruits co-activator proteins (CO-ACT) and chromatin modifiers such as CBP-P300. Histone acetylation opens the chromatin. Vitamin D receptor interacting proteins (DRIPs) are recruited to the open chromatin and help to assemble the transcription factor machinery, including RNA-Polymerase II (RNA Pol II). Target genes, such as cyp24α1, are transcribed. Although most responses to vitamin D involve direct gene regulation by VDR, vitamin D also has non-genomic effects (blue lines). These can be mediated by VDR but might also involve signalling from a membrane VDR or G protein-coupled receptor (GPCR) (F). Adenylate cyclase (AC), PI-3-kinase (PI3K), phospholipase C γ (PLCγ) and mitogen activated protein Kinase (MAPK) signalling pathways can be activated. In muscle cells, store-operated calcium channel opening can also contribute. The expression of some genes might involve genomic and non-genomic VDR signalling due to activation of MAPK signalling.

Aims of the thesis

In recent years, the model of $CD4^+$ T cell differentiation has developed considerably from the binary T_h1/T_h2 model proposed by Mosmann and Coffman, with an increasing number of studies focussing on anti-inflammatory iT_{Reg} and pro-inflammatory T_h17 subsets. Whilst these cells have clear roles in host protection, it is considered that dysregulation of their activities underlies many inflammatory diseases. Therefore, identifying factors that regulate their development holds therapeutic value.

It is becoming apparent that as well as cytokines, environmental and dietary factors can regulate $CD4^+$ T cell differentiation. Epidemiological studies suggest that low vitamin D status is a risk factor for autoimmunity and anti-inflammatory effects of $1,25(OH)_2D_3$, have been demonstrated for APCs. Effects of $1,25(OH)_2D_3$ upon T_h1, T_h2 and T_{Reg} cells in the presence of APCs are also described but the ability of $1,25(OH)_2D_3$ to influence $CD4^+$ T cell differentiation directly has not been clearly shown, neither is the effect of $1,25(OH)_2D_3$ upon T_h17 cells reported. The first two aims of this thesis were therefore to:

1) **Establish APC containing and APC-free systems for generating T_h17 and iT_{Reg} cells *in-vitro* from $CD4^+CD25^-$ T cells purified from healthy human blood.**

2) **Investigate the effect of $1,25(OH)_2D_3$ upon T_{Reg} and T_h17 lineages in the presence and absence of APCs.**

Strong homeostatic mechanisms maintain $1,25(OH)_2D_3$ at a very low level in serum which does not vary markedly between individuals. Thus, local conversion of $25(OH)D_3$ by immune cells might be important for immune-modulation *in-vivo*. A third aim of this thesis was therefore to:

3) **Investigate whether immune cells can convert $25(OH)D_3$ sufficiently for immune-modulatory effects of $1,25(OH)_2D_3$ to be measured.**

Rheumatoid arthritis (RA) is one autoimmune condition in which IL-17 might be pathogenic. Defective T_{Reg} activity is also described in this condition. Interestingly, the concentration of IL-17 in synovial fluid stratified with disease outcome in a cohort of early synovitis patients, thus dysregulation of the T_h17/T_{Reg} balance might predict disease outcome in these patients. VDR polymorphisms are also reported in RA, suggesting that lack of response to $1,25(OH)_2D_3$ could be involved in RA pathogenesis or influence the use of vitamin D in therapy. Therefore, this thesis also aimed to:

4) **Investigate the relationship between T_{Reg} and T_h17 cells in early synovitis patients that develop RA versus those who develop non-RA forms of synovitis or show self-limiting disease.**

5) **Study the ability of lymphocytes from synovitis patients to respond to $1,25(OH)_2D_3$ *in-vitro*.**

2 METHODS

2.1 Cell Culture

All cells were cultured at 37°C, 95% humidity and 5% CO_2, in a MCO-17A1C Sanyo CO_2 incubator.

2.1.1 Primary cells

Primary human T cells, monocytes, DCs and macrophages were cultured in RPM1 1640 medium (Invitrogen) supplemented with 2mM L-glutamine (Sigma), 10% foetal bovine serum (FBS) (Biosera or Invitrogen) and 1% penicillin and streptomycin (Invitrogen) (referred to as **RPMI-FBS**). For serum free conditions, CellGro®SCGM media supplemented with 1% penicillin and streptomycin (Invitrogen) was used (referred to as **serum free medium**).

2.1.2 CD86-GFP CHO transfectants

Human CD86 with mutated stop codon was generated by PCR and cloned into the EGFP-N1 expression vector (Clontech). Chinese Ovarian Hamster (CHO) cells were transfected with the plasmid vector by electroporation. Cells were cultured as below. Those stably expressing the plasmid were selected using G418 (500μg/ml) and fluorescence activated cell sorting (FACS).

Chinese Hamster Ovarian (CHO) cells were cultured in a T75 culture flask in DMEM medium supplemented with 2mM L-glutamine, 10% foetal FBS and 1% penicillin and streptomycin (**DMEM-FBS**). Cells were passaged every 2-3 days by trypsinisation. For this,

medium containing non-adherent cells was aspirated and adherent cells washed once with 5ml phosphate buffered saline (PBS). Following PBS removal, 2ml Trypsin-EDTA (ethylenediamine tetra-acetic acid) (Invitrogen) were added and cells incubated 2-3mins at 37°C. Trypsin was quenched by addition of 8ml DMEM-FBS. To continue the culture, 1ml cell suspension was retained in the flask or transferred to a new flask and 19ml DMEM-FBS added.

2.2 Cell Purification

Unless otherwise stated, all washes involved centrifugation at 490g for 5 minutes.

2.2.1 Isolation of PBMCs by Ficoll-Paque Plus centrifugation

Primary cells were isolated from peripheral blood mononuclear cells (PBMC) obtained from buffy coats or leukocyte reduction system cones (LC) provided by the Birmingham National Blood Service. PBMC isolation was performed by the Ficoll-Paque PLUS method of density gradient centrifugation (GE Healthcare).

Buffy coats or LCs were diluted 1:4 with phosphate buffered saline (PBS) and 25ml layered onto 15ml Ficoll-Paque PLUS in a 50ml Falcon tube. Following 25 minutes centrifugation at 1060g, without brake, the upper plasma layer was removed by aspiration and PBMCs at the Ficoll-plasma interface transferred to a second 50ml Falcon tube using a Pasteur pipette. Cells

were re-suspended in 50ml PBS and centrifuged for 10 minutes at 1060g with brake. Cells were washed a second time with 50ml PBS and centrifuged for 5 minutes at 260g. A further 50ml PBS wash was performed with centrifugation at 490g for 5 minutes followed by two washes with MACS® buffer (2mM EDTA, 0.5% Bovine Serum Albumin (BSA) in PBS). Cells were counted after each wash and the number of washes adjusted according to the extent of debris/platelet contamination.

2.2.2 Isolation of monocytes from PBMCs using StemCell Technologies enrichment methods

2.2.2.1 StemSep® CD14+ monocyte enrichment method (used to purify monocytes for studies reported in chapters three and four)

$CD14^+$ monocytes were isolated from PBMCs by magnetic colloid antibody based negative selection. The hapten antibody human monocyte enrichment cocktail included monoclonal antibodies against CD2, CD3, CD19, CD56, CD66b and glycophorin A. From PBMCs, a monocyte yield of 15% was typically obtained. The protocol was as follows. Note that all steps were performed in MACS® buffer.

PBMCs were suspended at 100 x 10^6/ml and incubated with the monocyte enrichment cocktail (100µl/ml) at 4°C for 30 minutes in a 25ml universal. Following a single 25ml wash, they were re-suspended at 100 x 10^6/ml and incubated for a further 30 minutes at 4°C with magnetic colloid (60µl/ml). After washing, PBMCs were re-suspended at 150 x 10^6/ml and 1ml applied to a primed LS column (Miltenyi Biotec) attached to a magnetic stand. The

column had been primed by rinsing with 3ml MACS® buffer immediately prior to the application of the PBMC suspension. Monocytes were collected from the column in 3 x 3ml washes. The purified monocytes were finally re-suspended in RPMI-FBS at 2 x 10^6/ml and incubated at 37°C. Purity was checked by flow cytometry for CD4, CD14 and CD11c. Monocytes were $CD4^{mid}$, $CD14^{high}$ $CD11c^{+}$.

2.2.2.2 EasySep® $CD14^{+}CD16^{-}$ monocyte enrichment method (used to purify monocytes for studies reported in chapters five and six)

The EasySep® hapten antibody human monocyte enrichment cocktail used for the negative selection of $CD14^{+}CD16^{-}$ monocytes included monoclonal antibodies against CD2, CD3, CD16, CD19, CD20, CD56, CD66b, CD123, glycophorin A and dextran. The protocol was as follows. Note that all steps were performed in MACS® buffer.

PBMCs were suspended at 100 x 10^6/ml and incubated with the monocyte enrichment cocktail (100µl/ml) at 4°C for 10 minutes in a 15ml Falcon tube. EasySep® magnetic particles were vortexed thoroughly and added to the cells at 100µl/ml. Cells and particles were mixed well and incubated at 4°C for 5 minutes. MACS® buffer was added to a total volume of 9ml and the tube placed into the EasySep® magnet with cap removed. After 5 minutes, the magnet holding the tube was picked up and cells poured into a fresh 15ml Falcon tube. The tube was left inverted for 2-3 seconds. Magnetically labelled, unwanted cells remained in the tube. The magnetic removal step was repeated and negatively selected cells poured into a 25ml universal. Cells were then washed in 25ml MACS® buffer at 260g for 5 minutes, re-

suspended in culture medium at a density of 2 x 10^6/ml and incubated at 37°C. Purity was checked by flow cytometry for CD4, CD14 and CD11c. Monocytes were $CD4^{mid}$, $CD14^{high}$ $CD11c^+$.

2.2.3 Isolation of $CD4^+$ cells from PBMCs using StemCell Technologies enrichment methods

2.2.3.1 StemSep® $CD4^+$ enrichment method (used to purify $CD4^+$ T cells for studies reported in chapters three and four)

A similar approach to that used to select $CD14^+$ monocytes from PBMCs was used for the selection of $CD4^+$ T cells. The hapten antibody human $CD4^+$ T cell enrichment cocktail included monoclonal antibodies against CD8, CD14, CD16, CD19, CD56 and glycophorin A. Typically, a 20% $CD4^+$ T cell yield was achieved from PBMC. The method used was as described in **2.2.2.1** with the exception that incubations with the enrichment cocktail and magnetic colloid were performed at room temperature for 15 minutes. Cells were finally re-suspended in MACS® buffer at 100x10^6/ml for CD25 labelling (**2.2.4**).

2.2.3.2 EasySep® $CD4^+$ enrichment method (used to purify $CD4^+$ T cells for studies reported in chapters five and six)

The hapten antibody human $CD4^+$ T cell enrichment cocktail used in this procedure included monoclonal antibodies against CD8, CD14, CD16, CD19, CD20, CD36, CD56, CD123,

TCRγδ, glycophorin A and dextran. The protocol was as follows. Note that all steps were performed in MACS® buffer.

PBMCs were suspended at a density of 100 x 10^6/ml and incubated with the $CD4^+$ T cell enrichment cocktail (50µl/ml) at room temperature for 10 minutes in a 15ml Falcon tube. EasySep® magnetic particles were vortexed thoroughly and added to the cells at 100µl/ml. Cells and particles were mixed well and incubated at room temperature for 5 minutes. MACS® buffer was added to a total volume of 9ml and the tube placed into the EasySep® magnet with cap removed. After 5 minutes, the magnet containing the tube was picked up and the cells poured into a fresh 15ml Falcon tube by inverting the magnet and tube for 2-3 seconds. Magnetically labelled, unwanted cells remained in the tube. The magnetic removal step was repeated and negatively selected cells poured into a 30ml universal. Cells were counted and pelletted by centrifugation at 490g for 5 minutes and re-suspended in MACS® buffer at 100x10^6/ml for CD25 labelling.

2.2.4 Selection of $CD4^+CD25^-$ $CD45RA^+$ or $CD45RO^+$ T cells

$CD4^+CD25^-$ T cells were sorted from $CD4^+$ T cells by negative selection using magnetic microbeads coated with monoclonal antibody to human CD25 (Miltenyi Biotec). Similarly, magnetic microbeads coated with monoclonal antibody to CD45RA or CD45RO (Miltenyi Biotec) were used to negatively sort $CD4^+CD25^-$ T cells into memory and naive populations respectively. The general protocol involved incubation of the starter population in MACS® buffer at a density of 100 x 10^6cells/ml with the microbeads (100µl/ml) for 25 minutes at 4°C.

Following one wash in MACS® buffer, the cells were re-suspended in 1ml MACS® buffer and applied to a primed LS column attached to a magnetic stand (Miltenyi Biotec). Three x 3ml MACS® buffer washes were used to flush the non-labelled cells from the column. The cells were pelletted by centrifugation and re-suspended at 2 x 10^6/ml in the appropriate culture medium.

2.3 Cell Preparation Methods

Unless otherwise stated, all washes involved centrifugation at 490g for 5 minutes.

2.3.1 Generation of monocyte derived dendritic cells (DCs)

$CD14^+$ monocytes were cultured with granulocyte macrophage-colony stimulating factor (GM-CSF) (800U/ml, Peprotech) and IL-4 (400U/ml, Peprotech) for seven days in 12 well plates. At two days, cultures were supplemented with fresh medium containing GM-CSF and IL-4. When required, DCs were matured by overnight treatment with lipopolysaccharide (LPS) (1μg/ml, Sigma).

DCs used for stimulations in the presence of $25(OH)D_3$ (**chapter 6**) were cultured in RPMI supplemented with human plasma (20%). Human plasma was obtained from the top layer of the Ficoll-Paque Plus gradient separation. The plasma was filtered using a 2μm gauge filter and heat inactivated by heating to 65°C for 20 minutes. Debris was pelletted by centrifuging at 1060g for 25 minutes and the supernatant used to supplement the culture medium.

When collecting DCs for use in stimulations, the culture plate was placed on ice for 20 minutes to reduce DC adherence. Wells were washed with ice-cold PBS and cells suspended by gentle pipetting. The DCs were transferred to a 25ml universal, washed twice with PBS and re-suspended in culture medium at 0.5 x 10^6/ml. The DC phenotype was confirmed by flow cytometry for surface markers CD80, CD86, CD14, HLA-DR and CD11c. Immature DCs (iDCs) were $CD80^{low}$, $CD86^{low}$, $CD14^-$ and $HLA\text{-}DR^+$. Mature DCs (mDCs) were $CD80^+$, $CD86^+$, $CD14^-$ and $HLA\text{-}DR^+$.

2.3.2 Carboxyfluorescein succinimidyl ester (CFSE) cell labelling

Cells were washed twice with PBS and re-suspended in PBS at a density of 5-10 x 10^6/ml in a 30ml universal. They were then incubated with 1μM CFSE (Molecular Probes) at room temperature for 10 minutes in the dark. The universal was filled with RPMI-FBS to quench the labelling reaction. Cells were then washed a further two times with RPMI-FBS and re-suspended in the appropriate culture medium.

2.3.3 CellTrace Far Red DDAO-SE labelling

Cells were washed twice with PBS and re-suspended in PBS at a density of 5-10 x 10^6/ml in a 30ml universal. They were then incubated with 5μM CellTrace Far Red DDAO-SE (Molecular Probes) at room temperature for 25 minutes. The universal was filled with RPMI-FBS to quench the labelling reaction. Cells were then washed a further two times with RPMI-FBS and re-suspended in RPMI-FBS.

2.4 $CD4^+CD25^-$ T cell stimulation assays

All T cell stimulation assays were carried out in flat bottom 96 well plates unless otherwise specified. 90,000 T cells were used per well in a total volume of 200μl.

Bead stimulations

CD3/CD28 T Cell Expander Dynabeads® (Invitrogen) were used. Unless otherwise stated a 1:4 bead: cell ratio was prepared by adding 0.5μl beads to each well of 90,000 cells.

Prior to re-stimulating bead cultures, old beads were removed by transferring cells to a 15ml Falcon tube and placing them in the EasySep® magnet for 5 minutes. Cells were then decanted into a universal and re-suspended in the appropriate culture medium.

Monocyte stimulations

Monocytes and T cells were cultured together at a ratio of 1:4 in the presence of anti-CD3 (clone OKT) at 0.5μg/ml.

2.4.1 Supplements

Where indicated, the stimulations were supplemented with

IL-1β (10ng/ml, Peprotech)

IL-2 (200U/ml or as specified, Peprotech)

IL-6 (20ng/ml, Immunotools)

IL-23 (10ng/ml, R and D Systems)

TGFβ (1ng/ml, R and D Systems)

$1,25(OH)_2D_3$ (Sigma) was used at 100nM in studies carried out in RPMI-FBS but a change to 10nM was implemented for later studies in serum free medium since this concentration gave similar outcomes and is more physiologically relevant than 100nM.

$25(OH)D_3$ (Sigma) was used at 50nM unless otherwise stated.

For stimulations carried out in the presence of vitamin D, the plate was covered with tin foil to protect from light.

Ketoconazole (Sigma) was used at the indicated concentrations.

2.4.1.1 Preparation of supplement stocks

IL-1β (Peprotech). Lyophilized protein was dissolved in sterile H_2O to 100μg/ml then diluted to 10μg/ml with 0.1% BSA-PBS. Working aliquots were stored at -20°C.

IL-6 (Immunotools): Lyophilized protein was dissolved in sterile H_2O to 100μg/ml then diluted to 10μg/ml with 0.1% BSA-PBS. Working aliquots were stored at -20°C.

IL-23 (R&D): Lyophilized protein was dissolved in 0.1% BSA-PBS to 20μg/ml. Working aliquots were stored at -20°C.

IL-2 (Peprotech): Lyophilized protein was dissolved in 100mM acetic acid to $1x10^6$Units/ml . Working aliquots were stored at -20°C.

TGFβ (R&D): Lyophilized protein was dissolved in 4mM HCL containing carrier protein BSA at 1mg/ml to a concentration of 10μg/ml.

1,25(OH)$_2$D$_3$ (Sigma): 4mM stock was prepared in isopropanol and diluted to 0.1mM in absolute ethanol. Stock solutions were stored at -20°C protected from light.

25(OH)D$_3$ (Sigma): 4mM stock was prepared in isopropanol and diluted to 0.1mM in absolute ethanol. Stock solutions were stored at -20°C protected from light.

Ketoconazole (Sigma): 50mM stock was prepared in methanol and stored at -20°C protected from light.

2.5 Flow Cytometry

At least 80,000 cells were used for each stain, although for stimulations carried out in a 96 well plate, all of the cells in a single well at the time point monitored were typically stained. Staining was done in 5ml round bottom polystyrene tubes for FACScalibur™ analysis or 5ml polypropylene tubes for DakoCyan analysis. 1ml PBS was used for each wash unless otherwise stated and centrifugation performed at 710g for 5 minutes. Wash solutions were removed by brief inversion of the tube and cells re-suspended in the remaining buffer by gentle vortex. The volume of each antibody used for staining is given in **Table 2.1**. All staining steps were carried out in the absence of light and after staining cells were stored at 4°C in the dark until analysis.

Flow cytometry was performed on a FACScalibur™ (Becton Dickinson UK) or Dako Cyan fluorescence activated cell scanner. Data was analysed using FlowJo™ software (Tree Star, U.S.A). At least 10,000 events were collected for each sample.

Table 2-1. Antibodies used in flow cytometry

Antibody	Company	Catalogue No.	Isotype	Clone	Volume/ labelling (µl)
CD11c-APC	BD	559877	Mouse $IgG_{1,\kappa}$	Bly6	3
CD11c-PE	BD	555392	Mouse $IgG_{1,\kappa}$	Bly6	3
CD14-PE	Serotec	MCA596PE	Mouse IgG_{2a}	UCHM1	2
CTLA4-PE	BD	555853	Mouse $IgG_{2a,\kappa}$	BN13	1
CTLA4-APC	BD	555855	Mouse $IgG_{2a,\kappa}$	BN13	2
CD25-APC	BD	555434	Mouse $IgG_{1,\kappa}$	M-A251	3
CD25-Fitc	BD	345796	Mouse IgG1,k	2A3	2
CD25-PE	BD	555482	Mouse $IgG_{1,\kappa}$	M-A251	3
CD3-FITC	BD	345763	Mouse $IgG_{1,\kappa}$	SK7	2
CD3-PerCP	BD	345766	Mouse $IgG_{1,\kappa}$	SK7	4
CD3-PE	BD	555340	Mouse $IgG_{2a,\kappa}$	HIT3a	3
CD45RA-FITC	BD	31264X	-	-	4
CD45RO-FITC	BD	555492	Mouse $IgG_{2a,\kappa}$	UCHL1	4
CD4-APC	BD	555349	Mouse $IgG_{1,\kappa}$	RPA-T4	3
CD4-FITC	BD	555346	Mouse $IgG_{1,\kappa}$	RPA-T4	3
CD4-PE	BD	555347	Mouse $IgG_{1,\kappa}$	RPA-T4	3
CD69-FITC	BD	555530	Mouse $IgG_{1,\kappa}$	FN50	3
Foxp3-APC	ebioscience	17-4776-73	Rat $IgG_{2a,\kappa}$	PCH101	2.5
IFNγ-APC	BD	554702	Mouse $IgG_{1,\kappa}$	B27	0.5
$IgG_{\gamma 1}$-PE	BD	345816	Mouse $IgG_{\gamma 1}$	X40	2
$IgG_{2a\kappa}$-PE	BD	555574	Mouse $IgG_{2a,\kappa}$	G155-178	3
$IgG_{1,k}$-Fitc	BD	555748	Mouse $IgG_{1,k}$	MOPC-21	-
$IgG_{1,k}$-APC	ebioscience	51-4714-80	Mouse $IgG_{1,k}$		-
$IgG_{2a,k}$-APC	BD	555576	Mouse $IgG_{2a,k}$	G155/178	-
IL-17-PE	ebioscience	12 7178-71	Mouse $IgG_{1\,\kappa}$	eBio64CAP17	2
IL-2-PE	BD	554566	Rat $IgG_{2a,\kappa}$	MQ1-17H12	0.5
IL-10-PE	BD	559337	Rat IgG_1	JES3-9D7	5
IL-21-647	ebioscience	51-7219	Mouse $IgG_{1,k}$	eBio3A3-N2	3
IL-21-PE	ebioscience	12-7219	Mouse $IgG_{1,k}$	eBio3A3-N2	3
IFNγ-Pacific Blue	ebioscience	57-7319	Mouse IgG1,k	4S.B3	3

2.5.1 Staining for surface antigens

Surface antigens were stained on live cells. After transferring cells to FACS tubes they were washed once and buffer aspirated to leave a damp pellet. Cells were re-suspended in 75µl 2% goat serum (Sigma-Aldrich) in PBS and incubated at room temperature for 30 minutes to block Fc binding sites. Directly conjugated antibodies were then added in a 25µl total volume adjusted with PBS and cells incubated on ice for 30 minutes. Following staining, cells were washed twice with PBS and re-suspended in 200µl PBS. Cells were either analysed immediately or fixed with 3% paraformaldehyde-PBS (PFA-PBS) as described in **2.5.2**.

2.5.2 Paraformaldehyde-PBS (PFA-PBS) fixation

Cells were re-suspended in 1ml 3% PFA-PBS by gentle vortexing and incubated at room temperature for 12 minutes. Cells were then pelletted, washed once with 1ml PBS and re-suspended in 200µl PBS. Samples were covered with foil and stored at 4°C until FACS analysis.

2.5.3 Cycling CTLA-4 staining

Cells were transferred to FACS tubes, washed once and re-suspended in APC or PE conjugated anti-CTLA-4 (BD), prepared in 100µl culture medium. Cells were incubated for 30 minutes at 37°C before being washed twice with PBS. Cells were either FACSed immediately or fixed with PFA-PBS for later analysis.

2.5.4 Intracellular cytokine staining

Cells were re-stimulated for 6-7 hours by the addition of phorbol myristate acetate (PMA) (50ng/ml, Sigma-Aldrich) and ionomycin (1μM, Sigma-Aldrich) to the stimulation well. Brefeldin A (10μg/ml, Sigma-Aldrich) was added during the last 4 – 5 hours to suspend protein trafficking. Cells were re-suspended and transferred to FACS tubes. They were fixed with 3% PFA-PBS. Cells were either stored overnight at 4°C or permeabilised and stained as described below.

Cells were permeabilised by washing with 1ml 0.1% saponin in PBS. Supernatant was aspirated and cells blocked at room temperature for 30 minutes in 75μl 2% goat serum prepared in 0.1% saponin-PBS. Antibodies were added in a 25μl volume, adjusted with 0.1% saponin-PBS, and cells stained in the dark for 40 minutes at room temperature. Excess antibody was removed by one wash with 0.1% saponin-PBS followed by two PBS washes. Finally, cells were re-suspended in 200μl PBS and collected by flow cytometry.

2.5.5 Intracellular staining by the ebioscience FoxP3 staining protocol

All buffers for this protocol were freshly prepared from the stock solutions provided in the ebioscience FoxP3 staining kit:

Buffer 1 (fixation/permeabilisation buffer): Prepared by mixing 1 part fixation/permeabilisation concentrate with 3 parts fixation/permeabilisation diluent.

Buffer 2 (1 X permeabilisation buffer): Prepared by diluting 10X permeabilisation buffer with PBS.

Cells were washed once with PBS and re-suspended by vortexing. Note that cells were not vortexed again during the staining process until final analysis. The re-suspended cells were fixed by incubation in 500µl buffer 1 at 4°C for at least one hour or overnight. Following one PBS wash, cells were permeabilised with 1ml buffer 2. After pelletting, buffer 2 was removed by tipping, rather than aspiration, to avoid cell drying. Antibodies were diluted in buffer 2 and added as a 10µl volume to the cells. Goat serum (Sigma Aldrich) was also added to a final concentration of 2% to reduce non-specific antibody binding and the extent of auto-fluorescence. Cells were stained in the fridge for 45 minutes, after which they were washed once with buffer 2 then twice with PBS. Finally, the cells were re-suspended in 200µl PBS for collection by flow cytometry.

When cells were stained for surface proteins and FoxP3, the surface stain was performed first. After two PBS washes to remove excess antibody, cells were fixed and stained according to the ebioscience FoxP3 staining protocol. When FoxP3 and total CTLA-4 were stained together, antibodies for FoxP3 and CTLA-4 were added together.

2.6 RNA Extraction

Cells were gently re-suspended in culture medium by pipetting and transferred to 1.5ml eppendorfs. They were pelletted by centrifugation for 5 minutes at 490g in a microcentrifuge. The supernatant was removed completely by aspiration and cells homogenised in TRIZOL® Reagent (Invitrogen) ($<1x10^6$ cells = 800µl TRIZOL, $\geq 1x10^6$ cells = 1000µl TRIZOL). Cells were homogenised by pipetting up and down more than 40 times with a P200 pipette. To further aid lysis, cells were frozen at -80°C for at least two hours prior to RNA isolation.

Typical cell numbers used

Un-stimulated T cells: 500,000 – $1x10^6$

Stimulated T cells: 250,000-$1x10^6$

Antigen presenting cells 500,000-$1x10^6$

Homogenised samples were thawed and held at room temperature (15-25°C) for 5 minutes. 200µl chloroform (Sigma-Aldrich) were added and tubes shaken vigorously for 15 seconds by hand. After leaving to stand for 2-3 minutes at room temperature, samples were centrifuged at 12,000g for 15 minutes at 2-8°C. The upper aqueous phase was removed and transferred to an RNase free eppendorf. 1µl glycoblue (Ambion) was added to allow later detection of the RNA. RNA was precipitated by mixing with 500µl isopropyl alcohol (Sigma-Aldrich). Either samples were left to stand at room temperature for 10 minutes or when few cells were available, they were incubated overnight at -80°C to increase yield. Precipitated RNA was pelletted by centrifugation at 12,000g for 10 minutes at 2-8°C. Supernatants were removed and the gel like RNA pellets, washed with 1ml 75% ethanol (Sigma-Aldrich) prepared from

absolute ethanol and nuclease free water (Fisher). After vortexing, samples were centrifuged at 7,500g for 5 minutes at 2-8°C and the majority of supernatant removed. Remaining supernatant was collected by a further 1 minute spin at 7,500g supernatant. Samples were left to air-dry for a few minutes before re-suspending in nuclease free water. Finally, the RNA concentration was measured with a Nano-Drop 3300 (Thermo Scientific).

2.7 cDNA synthesis

2.7.1 SuperScript III Reverse Transcriptase system (Invitrogen)

This system was used to prepare samples analysed in **chapter four**.

RNase free water was added to 1μg RNA to a total volume of 11μl in a RNase free 0.2ml eppendorf. 1μl random hexamers (Fermentas Life Sciences 0.2μg/ml) and 1μl 10mM dNTPS (Invitrogen) were added and the samples heated to 65°C for 5 minutes. The samples were immediately placed on ice for at least 1 minute and the following four components added to each tube:

4μl 5x First strand buffer
1μl 0.1M DTT,
1μl RNase OUT
1μl Superscript III Reverse Transcriptase

When preparing multiple samples a master mix of the above reagents was prepared and 7μl added to each reaction. Minus Reverse Transcriptase controls were also prepared to check for genomic DNA contamination. For these, Superscript III Reverse Transcriptase was replaced

with RNase free water. Samples were finally incubated at 25°C for 5 minutes followed by 60 minutes at 50°C in a PTC-225 Peltier thermal cycler. Finally, to inactivate reverse transcriptase, samples were heated to 70°C for 15 minutes.

cDNA was aliquotted and stored at -20°C.

2.7.2 Multiscribe TaqMan® Reverse Transcription system (Applied Biosystems)

This system was used to prepare samples analysed in **chapters five** and **six**.

For samples within a single experiment, equivalent amounts of RNA were reverse transcribed but between experiments, amounts converted ranged between 200ng and 700ng total RNA.

Reactions were prepared in nuclease free 200µl PCR strips. RNA and nuclease free water were mixed to a total volume of 6.95µl. The following kit components were then prepared as a master mix and added to each tube. Volumes stated are for a single reaction:

2µl 10X RT buffer
4.4µl 25mM $MgCl_2$
4µl dNTPs
1µl random hexamers
0.4µl RNase inhibitor
1.25µl Multiscribe reverse transcriptase

Minus reverse transcriptase reactions were also prepared by replacing Multiscribe Reverse Transcriptase with nuclease free water. The reactions were mixed thoroughly and collected by

brief centrifugation. They were then passed through the following incubation steps in a PTC-225 Peltier thermal cycler.

25°C for 10 minutes
37°C for 60 minutes
48°C for 30 minutes
95°C for 5 minutes
4°C hold

cDNA was aliquoted and stored at -20°C

2.8 Quantitative real-time PCR analysis

Quantitative real-time PCR assays were performed using 20X assay on demand kits from Applied Biosystems including: IL-17A (Hs99999082_m1), IFNγ (Hs00174143_m1), CTLA-4 (Hs00175480_m1), FoxP3 (Hs00203958_m1), IL-10 (Hs00174086_m1), CYP27B1 (Hs00168017_m1), VDR (Hs00172113_m1). VIC-labelled Ribosomal RNA control reagents (Applied Biosystems, 4308320) or a 20X VIC labelled 18S gene assay (Applied Biosystems 4319413E) was used to measure 18S as an endogenous control. Reactions were performed in duplex or singleplex depending upon primer compatibility as determined below. Relative gene expressions were analysed by the Pfaffl method (Pfaffl, 2001) for which the equation is given below.

E = efficiency, target = target gene, ref = endogenous control reference, Ct = number of cycles required to reach threshold fluorescence.

$$Ratio = \frac{E_{target}^{(\Delta Ct\,target(control-treated))}}{E_{ref}^{(\Delta Ct\,ref(control-treated))}}$$

The independence of the target gene with 18S in a duplex reaction was assessed by comparing slopes of C_t value against concentration for singleplex and duplex reactions performed over a cDNA dilution series. Independence was concluded if the slopes differed by less than 0.1. Independence to 18S Ribosomal RNA control reagents was satisfied for IL-17A, IFNγ, CTLA-4, IL-10 and FoxP3. VDR and CYP27B1 did not satisfy independence with the 18S gene assay. Therefore, these reactions were run in singleplex. 10µl reactions were prepared as below in a 96 well MicroAMP™ reaction plate with barcode (Applied Biosystems). The volume of cDNA to use was determined by titration for the particular gene assay.

Singleplex qPCR reaction composition

5µl TaqMan® 2 x PCR master Mix (Applied Biosystems)
0.5µl FAM labelled gene assay
cDNA
nuclease free water to a total volume of 10µl

Duplex qPCR reaction composition

5µl TaqMan® 2 x PCR master Mix (Applied Biosystems)
0.5µl FAM labelled gene assay
0.2µl 18S Forward Primer (10µM)
0.2µl 18S Reverse Primer (10µM)
0.0625µl VIC-labelled 18S Probe (40µM)
cDNA
nuclease free water to a total volume of 10µl

cDNA and master mix were collected by centrifugation for 1 minute at 1970g and reactions passed through the following incubation steps in an Applied Biosystems 7500 machine.

50°C for 2minutes (1 cycle)
95°C for 10 minutes (1 cycle)
95°C for 15 seconds, 60°C for 1 minute (40 cycles)

2.9 Chapter specific methods

2.9.1 Responder T cell suppression assay (chapter four)

$1,25(OH)_2D_3$ plus IL-2 suppressors and untreated control suppressors, were generated by stimulating $CD4^+CD25^-$ T cells with monocytes and anti-CD3 (OKT3) (0.1μg/ml) in the presence or absence of $1,25(OH)_2D_3$ (100nM) and IL-2 (200U/ml). Stimulations were prepared in a 24 well plate. 1 million T cells and 250,000 monocytes (4:1 ratio) were added per well to a total volume of 2ml. For IL-2 treated cultures, IL-2 was supplemented at four days. At seven days, the stimulated cells were re-suspended and transferred to 30ml universals. Their CTLA-4/FoxP3 phenotypes were assessed by flow cytometry as described in **2.5.5** and the remaining cells labelled with cell tracking dye DDAO-SE as described in **2.3.3**.

Monocyte derived DCs, autologous to the stimulated suppressor cells were cultured for six days. They were then matured overnight with 1μg/ml LPS (Sigma-Aldrich). At day seven, the DCs were removed from the culture plate, washed and re-suspended in RPMI-FBS as described in **2.3.1**.

At seven days, allogenic responder $CD4^+CD25^-$ T cells were freshly isolated from a buffy coat and labelled with CFSE as described in **2.3.2**. Responder T cells, suppressor T cells and mature dendritic cells were then combined in 96 well flat bottom plates with anti-CD3 (0.1μg/ml). During assay development, a range of responder to suppressor ratios and DC to responder ratios were tested. 10 responders (70,000 cells/well) to 1 suppressor (7,000 cells/well) to 2 DCs (14,000 cells/well) was determined optimum. After five days of culture,

cells were re-suspended, and FACSed live in PBS. $CFSE^{+}DDAO\text{-}SE^{-}$ responder cells were selected by gating (**figure 4.18B**) and their median CFSE fluorescence intensity measured as an indication of division.

2.9.2 T_h17 selection and enrichment (Chapter five)

2.9.2.1 Preparation of T_h17 enriched cultures

An $IL\text{-}17^{+}$ enriched $CD4^{+}$ T cell culture was prepared by stimulating >13million $CD4^{+}CD25^{-}$ T cells with CD3/CD28 T Cell Expander Dynabeads® (Invitrogen) at a ratio of 1 bead to 32 T cells. Serum free medium supplemented with recombinant T_h17 polarising cytokines (TGFβ 1ng/ml, IL-1β 10ng/ml, IL-6 20ng/ml, IL-23 10ng/ml) was used. Stimulations were prepared in 24 well plates with 900,000 T cells and 0.625μl beads per well. At six days, cells were re-suspended and beads removed using the EasySep® magnet. Cells were counted, pelletted and re-suspended in the six day culture medium at 1×10^{6}/ml. 2ml of cells were plated per well of a 24 well plate. 4 million cells were left un-stimulated. The remainder were re-stimulated with phorbol 12, 13 dibutyrate (PdBU) (Sigma-Aldrich) and ionomycin for 2 hours. After this period, 100,000 cells from each culture were treated with brefeldin A for estimation of cytokine expression by intracellular staining. The protocol used included dead cell exclusion, as described in **2.9.2.3**.

2.9.2.2 IL-17 secretion analysis and capture

All centrifugation steps in this protocol were carried out for 7 minutes at 4°C, 490g in an accuSpin^{TM}3R centrifuge (Fischer Scientific) unless otherwise stated.

Re-stimulated and non-re-stimulated cells, prepared as above, were transferred to 15ml Falcon tubes for cytokine secretion staining. Cells were washed with cold MACS® buffer and re-suspended in ice cold medium (RPMI-FBS) at 1x10^{7} cells/80µL. 20µl of IL-17 catch reagent/80µl cells were then added and the cells incubated for 5 minutes on ice.

For the secretion phase of the assay, cells were transferred to pre-warmed RPMI-FBS at 1x10^{5}cells/ml. The cell suspension was distributed as 25ml aliquots into 50ml Falcon tubes. Cells were incubated for 40 minutes at 37°C, throughout which the tubes were turned several times every 5 minutes to maintain cell separation. The tubes were then topped up with ice-cold MACS® buffer and surrounded with ice for 10 minutes to slow cell secretion, thus reducing the risk of cross labelling during the following centrifugation steps. Cells were pelletted, the majority of supernatant removed and like treated cells combined in a 15ml Falcon. Following a second wash in MACS® buffer cells were re-suspended in MACS® buffer at 1x10^{7}cells/80µl and 20µl of IL-17-PE antibody added. Cells were incubated for 10 minutes on ice before being washed with 10ml MACS® buffer. Cells were re-suspended in 1ml MACS® buffer, counted and IL-17^{+} cells captured by MoFlo technology. FSC-W and SSC-W parameters were used to exclude non-singlet cells and the IL-17^{+} acceptance gate set upon cells that had not been re-stimulated with PdBU and ionomycin.

25,000 sorted cells were stimulated for three days in 96 well flat bottom plates under T_h17 polarising conditions (1:32 bead: cell ratio, serum free medium, TGFβ (1ng/ml), IL-1β (10ng/ml), IL-6 (20ng/ml), IL-23 (10ng/ml), IL-2 (50U/ml)) in the presence or absence of $1,25(OH)_2D_3$ (10nM).

2.9.2.3 Intracellular cytokine staining with live/dead discrimination

At three days, cells were re-stimulated with PMA and ionomycin and blocked with brefeldin A as described in **2.5.4**. Prior to fixation, dead cells were labelled with the LIVE/DEAD® Fixable Near-IR dead cell staining kit (Invitrogen). For this, cells were washed once with PBS. Buffer was removed by aspiration and cells re-suspended in 250μl LIVE/DEAD amine reactive fluorescent dye freshly prepared by diluting 1μl of the reconstituted dye in 1ml PBS. Cells were protected from light and incubated 30 minutes on ice. They were then washed twice with PBS before being fixed with 3% PFA-PBS. Intracellular staining with IL-17-FITC, IFNγ-Pacific Blue and IL-21-APC was performed as described in **2.5.4** with the exception that cells were not blocked with goat serum prior to staining.

2.9.3 CD86-GFP acquisition assay (Chapter five)

Preparation of stimulated T cells for CD86-GFP acquisition assay

$CD4^+CD25^-$ T cells were stimulated with CD3/CD28 T Cell Expander Dynabeads® for four days in serum free medium. A ratio of one bead to four T cells was used. For this, 900,000 T

cells were plated per well of a 24 well plate and 5μl beads added per well. To obtain cells with different levels of CTLA-4 they were stimulated under the following conditions:

- T_h17 polarising: 1ng/ml TGFβ (R&D), 10ng/ml IL-1β (Peprotech), 20ng/ml IL-6 (Immunotools), 10ng/ml IL-23 (R&D)
- Control: no supplement
- $1,25(OH)_2D_3$ (10nM)

At four days, cells were re-suspended and transferred to a 15ml falcon. Beads were removed using the EasySep® magnet and cells tipped into 30ml universals. Cells were washed once with PBS and re-suspended in serum free medium at $1x10^6$/ml.

100,000 T cells from each population were stained for total CTLA-4 using the ebioscience FoxP3 staining protocol (**2.5.5**). 100,000 T cells were also stained for cycling CTLA-4 following stimulation with CD3/CD28 T Cell Expander Dynabeads® (**2.5.3**). These stimulations were carried out for 3 hours in 96 well round bottom plates at a ratio of 1 bead:1 T cell.

500,000 CTLA-4-blocked T cells were prepared from each culture by incubating with blocking antibody to CTLA-4 (40μg/ml) at 37°C for at least 40 minutes immediately prior to their use in the CD86-GFP acquisition assay.

Preparation of CHO-CD86-GFP cells

CHO-CD86-GFP cells were prepared and cultured as described in **2.1.2**. Following removal from the culture flask $5x10^6$ cells were labelled with DDAO-SE as described in **2.3.3**. Labelled cells were re-suspended in serum free medium at a density of $3x10^6$/ml.

150,000 DDAO-SE^+ CHO-CD86-GFP cells were mixed with 50,000 T cells (+/- antiCTLA-4 treatment) in a 96 well round bottom plate and anti-CD3 (0.5μg/ml) added to promote CTLA-4 cycling. Control wells were prepared in which CHO cells were absent. These were used to set the baseline GFP fluorescence for each population. Cells were cultured for 3 hours at 37°C after which the plate was placed on ice and cells FACSed live. Cells were analysed on the DakoCyan to permit selection of singlet populations based upon pulse width as shown in **figure 2.1**.

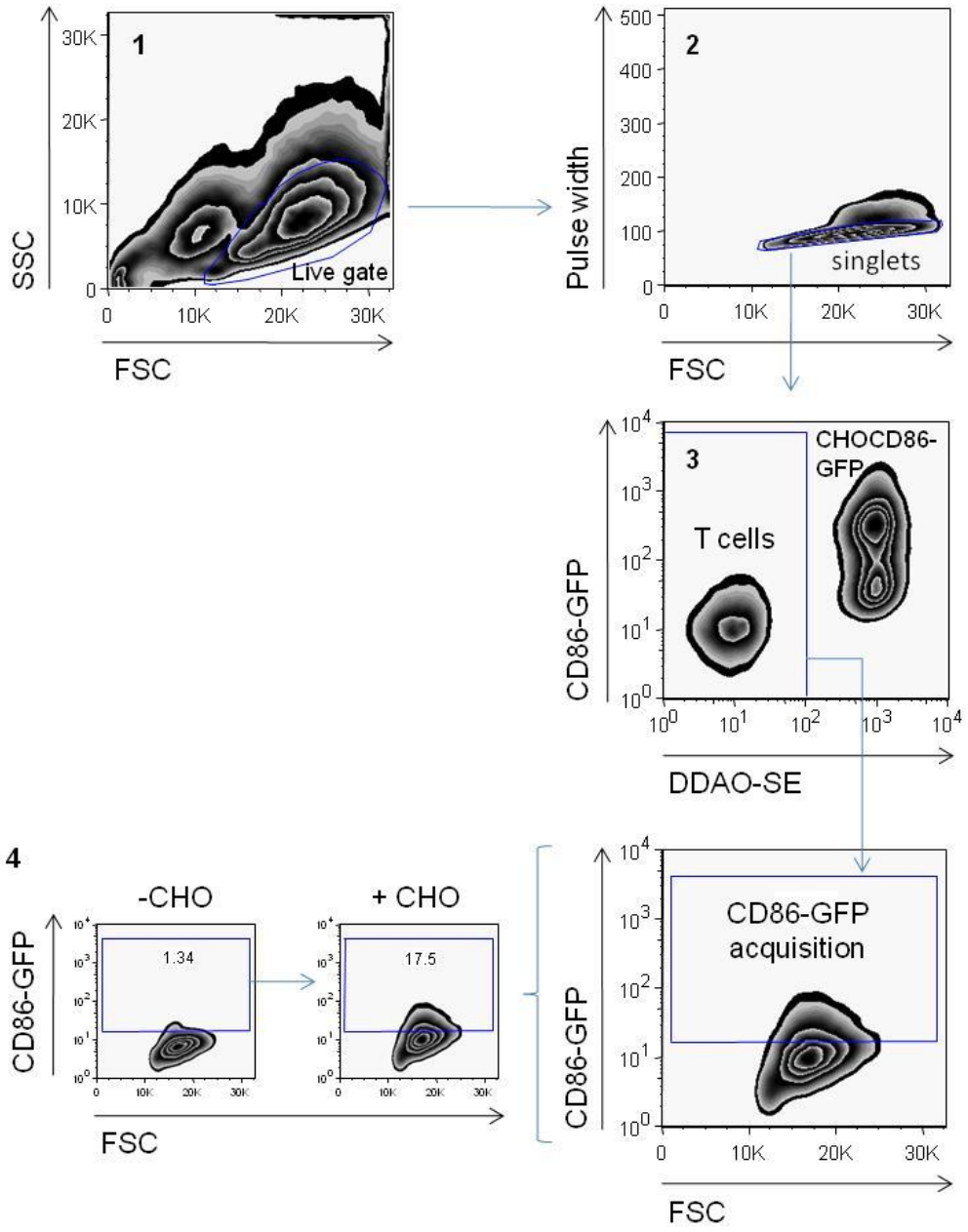

Figure 2.1. Gating strategy for analysis of CD86-GFP acquisition by T cells. 1) Live cells were gated by FSC vs SSC. **2)** Single cells were selected by low pulse width. **3)** DDAO-SE labelled CHO-CD86-GFP cells were excluded. **4)** CD86 acquisition was determined by % GFP^{+ve} cells. The negative gate was set according to the autofluorescence of T cells that had not been co-cultured with CHO cells.

2.9.4 B7 depletion assay (Chapter five)

Preparation of SEB reactive $CD4^+$ T cells

$20x10^6$ PBMCs were suspended in RPMI-FBS at $2x10^6$/ml. $2x10^6$cells were plated per well of a 24 well plate. Medium was supplemented with IL-2 (200U/ml) and cells stimulated with *Staphylococcus aureus enterotoxin B* (SEB) superantigen (100ng/ml). $10x10^6$ cells were treated with $1,25(OH)_2D_3$ (10nM). At 4 days, cultures were divided and 500μl of fresh medium added to each well. For $1,25(OH)_2D_3$-treated cultures, IL-2 and $1,25(OH)_2D_3$ were also re-supplemented. At six days, $1,25(OH)_2D_3$ cultures were supplemented a third time with $1,25(OH)_2D_3$ (10nM final). On day seven, PBMCs were re-suspended and dead cells depleted by gradient centrifugation. For this, 10ml Ficoll-Paque PLUS was aliquot into 30ml universals and cells layered on top. Cells were centrifuged for 25 minutes at 1060g without brake. The PBMC layer at the Ficoll-plasma interface was then transferred to a second 30ml universal. Cells were re-suspended in MACS® buffer and centrifuged for 10 minutes at 1060g, with brake. Cells were re-suspended in 10ml MACS®buffer, counted and re-suspended at a density of $100x10^6$/ml. $CD4^+$ T cells were isolated using the EasySep® $CD4^+$ enrichment method as described in **2.2.3.2**. Because cell numbers were low, after incubation with magnetic particles cells were re-suspended in 4ml MACS® buffer and placed in the EasySep® magnet. Purified cells were washed with PBS and labelled with CFSE as described in **2.3.2**. After labelling, cells were re-suspended in RPMI-FBS at 750,000/ml. The phenotype of the cells was checked by surface staining for CD4, CD3 and CD8 and intracellular staining for total CTLA-4 and FoxP3 (**figure 2.2**).

Half of the control and 1,25$(OH)_2D_3$-treated SEB reactive $CD4^+$ T cells were blocked with anti-CTLA-4 (40μg/ml) for >40 minutes at 37°C immediately prior to co-culture with the SEB pulsed DCs as described below.

Preparation of SEB-pulsed DCs

Immature DCs were removed from the culture plate and transferred to a 25ml universal. DCs were washed with RPMI-FBS and re-suspended in 1ml RPMI-FBS. SEB was added at 1μg/ml and incubated at 37°C for 2 hours. DCs were then washed once with medium and re-suspended at 150,000/ml, 75,000/ml and 37,500/ml.

SEB pulsed DCs and stimulated T cells were mixed at ratios of 1:5, 1:10 and 1:20 in a 96 well round bottom plate. To enable a sufficient number of DCs to be collected for FACS, one, two and four wells were prepared for each ratio respectively with 150,000 T cells being added per well. DCs and T cells were cultured for 24 hours. The plate was then placed on ice to reduce DC adherence. Wells were also rinsed twice with ice cold PBS and rinsings combined to maximise adherent cell collection. Cells were surface stained for CD80-PE and CD86-APC and FACSed live on the DakoCyan. Cells were gated on pulse width and CFSE negative cells excluded. CD80 and CD86 mean fluorescence intensities were recorded.

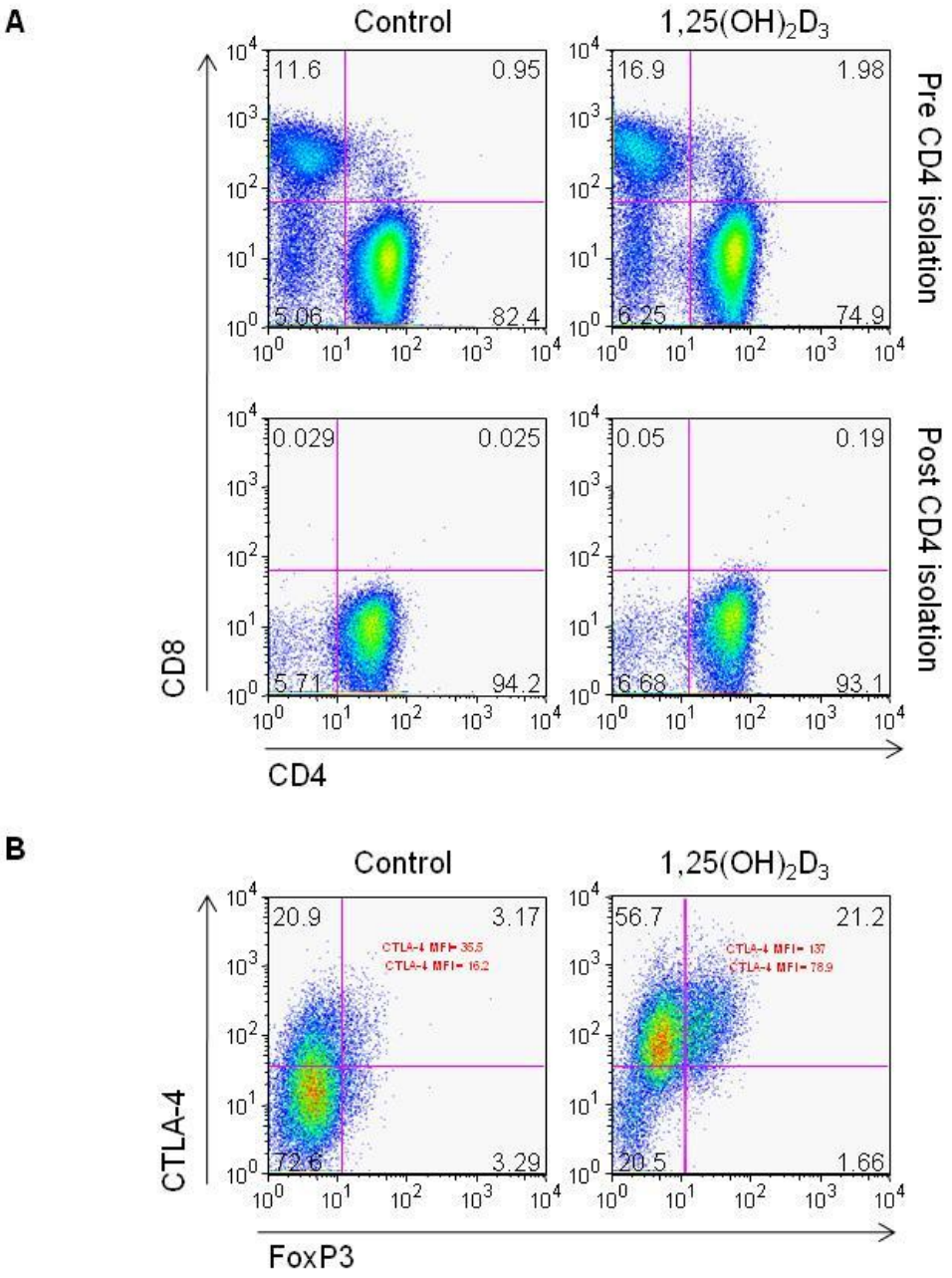

Figure 2.2. CTLA-4 and FoxP3 expression by SEB reactive CD4$^+$ T cells. PBMCs were stimulated with staphylococcus aureus enterotoxin B with IL-2 in the presence of 1,25$(OH)_2D_3$ or vehicle control. At seven days CD4$^+$ T cells were isolated by the EasySep® CD4$^+$ T cell enrichment method. **A)** CD8 and CD4 expression by CD3$^+$ T cells pre and post CD4$^+$ T cell isolation. **B)** CTLA-4 and FoxP3 expression by purified CD4$^+$ T cells.

2.10 Methods for analysis of samples from synovitis patients (chapter seven)

The Solihull Local Research Ethics Committee (REC reference number 07/Q2706/2) granted ethical approval for the study. All of the patients enrolled gave written informed consent before samples were taken. Patients were seen by consultant rheumatologists, Dr Karim Raza or Dr Andrew Filer and were recruited as early synovitis patients if they had at least one swollen joint and had experienced symptoms for ≤3 months. A number was assigned to each sample so that it could be dealt with anonymously. After 18 months, the patient's condition was diagnosed as i) rheumatoid arthritis (RA), ii) non-RA persistent synovitis, iii) self-limiting. For some patients, adequate follow-up data were not available and these patients were classified as "outcome unknown". Patients were diagnosed with RA if their condition satisfied at least four of the American College of Rheumatology 1987 criteria (Arnett et al., 1988) as listed in **table 2.2**. Non-RA-persistent synovitis was diagnosed if patients continued to show clinical evidence of joint inflammation but did not satisfy the 1987 ACR criteria for RA. Self-limiting disease was diagnosed if synovitis was no longer clinically apparent at the 18-month assessment and the patient had not received glucocorticoid or disease modifying anti-rheumatic drug treatment within the previous 3 months. For comparison, patients presenting at first assessment with established RA were also included in the study and age-matched colleagues without a history of illness or joint inflammation and who were not taking medication were recruited as healthy controls.

Table 2-2. American College of Rheumatology 1987 criteria for the classification of rheumatoid arthritis (RA). At least four criteria must be fulfilled for classification as RA. Abbreviations: Proximal Interphalangeal (PIP), Metacarpophalangeal (MCP), Metatarsophalangeal (MTP).

Criteria	Definition
Morning stiffness	Morning stiffness in and around the joints lasts for at least 1 hour.
Arthritis of 3 joints	Soft-tissue swelling or fluid in at least three of the following areas: the left or right PIP, MCP, wrist, elbow, knee, ankle or MTP joints.
Arthritis of the hand joints	Swelling of the wrist, MCP or PIP joints.
Symmetric arthritis	Simultaneous involvement of the same joint areas (as above) on both sides of the body.
Rheumatoid nodules	Subcutaneous nodules present
Rheumatoid factor	Detected by a method that yields positive findings in <5% or normal cohorts.
Radiographic changes	Erosions or unequivocal bony decalcification localised to the joints of the hands and wrists.

At initial assessment, the disease activity score 28 (DAS28), which has been validated for use in the assessment of disease activity in RA, was calculated (Prevoo et al., 1995). Serum C Reactive Protein (CRP) was measured and included in the DAS28 calculation (Paulus et al., 1999). Blood samples were also analysed for Rheumatoid Factor (RF), anti-cyclic citrullinated peptide (CCP), erythrocyte sedimentation rate (ESR) and 25(OH)D_3 level. EDTA anti-coagulated peripheral blood (PB) samples (10ml) and, where possible, synovial fluid (SF) samples, were taken for the isolation of mononuclear cells as described in **2.10.1**. SF was extracted by ultrasound guidance as described in (Raza, 2004) or by palpation guidance. In

general, SF was obtained from the joint, which contained the largest amount of SF according to ultrasonographic assessment.

2.10.1 Isolation of PBMCs and SFMCs from early synovitis patient blood and synovial fluid samples

Prior to SFMC isolation, SF was treated with hyaluronidase (10U/ml) for 30 minutes at 37°C.

PBMCs and SFMCs were purified from PB and SF samples respectively, by the Ficoll-Paque Plus method (GE Healthcare) as described in **2.3.1**, with the exception that the top layer of the gradient (plasma or synovial fluid) was filtered with a 2μm gauge filter and used to supplement the culture medium. A 1ml sample of the fluid was also stored at -20°C. All washes were carried out with PBS except the last, for which RPMI 1640 supplemented with 10% self-plasma or SF, 1% penicillin and streptomycin (Invitrogen), 2mM L-glutamine (Sigma), was used. After washing, the cells were re-suspended to a density of $1x10^6$/ml and allocated as follows (**2.10.2**-**2.10.5**)

2.10.2 Surface staining for CD3, CD4 and CD45-RO

80,000-100,000 cells were surface stained with CD45-RO-FITC, CD3-PE and CD4-APC directly *ex-vivo* and analysed live by flow cytometry.

2.10.3 Ex-vivo T17 analysis

100,000-150,000 cells were plated per well of a 96 well plate for intracellular cytokine staining following PMA and ionomycin stimulation in the presence of brefeldin A as described in **2.5.4**. During assay development, it was confirmed that the *ex-vivo* T17 frequency was not influenced by resting the cells overnight prior to stimulation. Since samples were generally received late in the day, overnight resting was adopted for routine analysis. After stimulation, cells were intracellular stained with CD4-FITC, IL-17-PE, CD3-PerCP and IFNγ-APC. Because PMA and ionomycin treatment often reduced CD4 expression to very low levels, it was decided to exclude CD4 distinction from the analysis and quote IL-17 frequencies for $CD3^+$ cells, defined as T17, rather than for $CD3^+CD4^+$ cells.

2.10.4 Ex-vivo T_{Reg} analysis and CTLA-4 expression

80,000-100,000 cells were surface stained with (i) CD4-FITC, CD25-PE and CD3-PerCP or (ii) CD4-FITC, and CD3-PerCP or (iii) appropriate isotype controls and fixed overnight in FoxP3 staining buffer A. The following day, samples were permeabilised with buffer B and processed as described in **2.5.5**. Samples (i) and (ii) were stained with FoxP3-APC. Sample (ii) was also stained with CTLA-4-PE and the appropriate isotype control antibody was added to sample (iii). To enable CTLA-4 expression levels to be compared between individuals over time, the PE signal was normalised prior to collecting the samples, by passing PE fluorescent beads (Caltag Laboratories) through the cytometer and finely adjusting the laser voltage to set their MFI within 90 and 100 units. T_{Reg} frequencies were calculated as the frequency of $CD3^+$ cells that stained positive for CD4, CD25 and FoxP3.

2.10.5 Post-culture T17 analysis and 1,25(OH)$_2$D$_3$ sensitivity

250,000 cells were plated per well of a 24 well plate and stimulated for seven days with anti-CD3 (0.5μg/ml, clone OKT) in the presence or absence of 1,25(OH)$_2$D$_3$ (100nM) in a total 1ml volume. At seven days, cultures were assessed for stimulation by (i) presence of cell clusters by light microscopy, (ii) CD25 expression by CD3$^+$ cells as measured by flow cytometry. For stimulated cultures, IL-17 and IFNγ expression by CD3$^+$ cells was measured by intracellular staining and flow cytometry. 1,25(OH)$_2$D$_3$ sensitivity of IL-17$^+$IFNγ$^-$ and IL-17$^+$IFNγ$^+$ cells was calculated by the equation below. Thus, the larger the value, the greater the 1,25(OH)$_2$D$_3$ sensitivity of the subset.

$$1{,}25(OH)_2D_3\ sensitivity = \frac{subset\ frequency_{(control)}}{Subset\ frequency_{1.25(OH)2D3}}$$

2.11 Statistical Analysis

Graphpad Prism version 3.02 was used to assess normality of samples by the Kolmogorov-Smirnov (KS) test and to check equivalence of variance between two samples by the F statistic. If both conditions were satisfied, two-tailed paired, or unpaired T tests, as appropriate, were conducted to check for the difference between the means of two populations. If these conditions were not satisfied, two-tailed, non-parametric Wilcoxon or Mann-Whitney U tests for paired and unpaired data sets respectively, were used to compare the medians of the two populations in question.

When comparing more than three data sets in **chapter 7**, one-way Analysis of Variance (ANOVA) was performed using SPSS 14.0 after checking for normality by the Kolmogorov-Smirnov (KS) test. If equivalence of variance was not satisfied, as determined by the Levene's statistic, data was transformed by calculating $Log_{10}X$ and equivalence of variance re-tested. For all cases where equivalence of variance was met and a post-test was required, the Hochberg test was used to account for differences in sample size. If equivalence of variance was not satisfied, the Games Howell post-test was used. To specifically test for differences between early RA and established RA patients in the post-test analysis, the Bonferroni post-test in Graphpad Prism version 3.02 was used.

For correlation analysis, Pearson's correlation coefficient (r) was calculated using Graphpad Prism version 3.02 after checking for normality by the Kolmogorov-Smirnov (KS) test.

2.12 Reagent and equipment suppliers

Table 2-3. Addresses of reagent and equipment suppliers

Company	Address
AbD Serotec	Endeavour House, Langford Lane, Kidlington, OX5 1GE. United Kingdom.
BD	The Danby Building, Edmund Halley Road, Oxford Science Park Oxford, OX4 4DQ. United Kingdom.
eBioscience Ltd	2nd Floor, Titan Court, 3 Bishop Square, Hatfield, AL10 9NA. United Kingdom.
Fisher Scientific UK Ltd	Fisher Scientific UK Ltd, Bishop Meadow Road, Loughborough, Leicestershire, LE11 5RG. United Kingdom.
Fermentas Life Sciences	Fermentas UK, Sheriff House, Sheriff Hutton Industrial Park, York, Y060 6RZ. United Kingdom
ImmunoTools	Altenoyther Strasse 10, 26169 Friesoythe. Germany.
Life Technologies Corporation (Includes Applied Biosystems and Invitrogen)	5791 Van Allen Way, PO Box 6482, Carlsbad, 92008. California.
Miltenyi Biotec	Miltenyi Biotec Ltd. Almac House, Church Lane Bisley, Suffey. GU24 9DR. United Kingdom
Peprotech EC	PeproTech EC Ltd, PeproTech House, 29 Margravine Road, London W6 8LL. United Kingdom
R & D Systems	R&D Systems Europe Ltd. 19 Barton Lane, Abingdon, Science Park, Abingdon, OX14 3NB. United Kingdom
Sarstedt	SelectScience Ltd, Church Farm Business Park, Corston, Bath, BA2 9AP. United Kingdom.
Sigma-Aldrich Company Ltd	Dorset, United Kingdom

3 ESTABLISHING SYSTEMS TO GENERATE T_H17 AND T_{REG} CELLS IN-VITRO

3.1 Generating T_h17 cells in-vitro

Initial studies compared the abilities of monocytes and monocyte-derived dendritic cells (DCs) to promote IL-17 expression in $CD4^+$ T cells. Intracellular cytokine staining with flow cytometry was chosen for cytokine expression analysis because it would allow the co-expression of cytokines with other markers to be studied. **Figure 3.1** shows that monocytes and DCs permitted the development of IL-17^+ (T_h17) cells, which included IFNγ^+ and IFNγ^- subsets. However, monocytes repeatedly supported a greater T_h17 population than did DCs. Therefore, monocytes were chosen to study the effect of $1,25(OH)_2D_3$ upon APC-driven T_h17 differentiation in later studies. The efficiency of monocytes for promoting T_h17 differentiation had been demonstrated by others (Acosta-Rodriguez et al., 2007a; Evans et al., 2007), which supports the relevance of the monocyte system for *in-vitro* T_h17 studies. Others had also demonstrated that T_h17 cells derive primarily from the memory population. To further compare my system with theirs, I negatively selected CD45-$RA^+$and CD45-RO^+ cells from $CD4^+CD25^-$ cells and measured their IL-17 expression following monocyte stimulation. As shown in **figure 3.2**, greater T_h17 frequencies arose from the CD45-RO^+ fraction than the CD45-RA^+ fraction, whilst mixed cultures gave an intermediate T_h17 frequency.

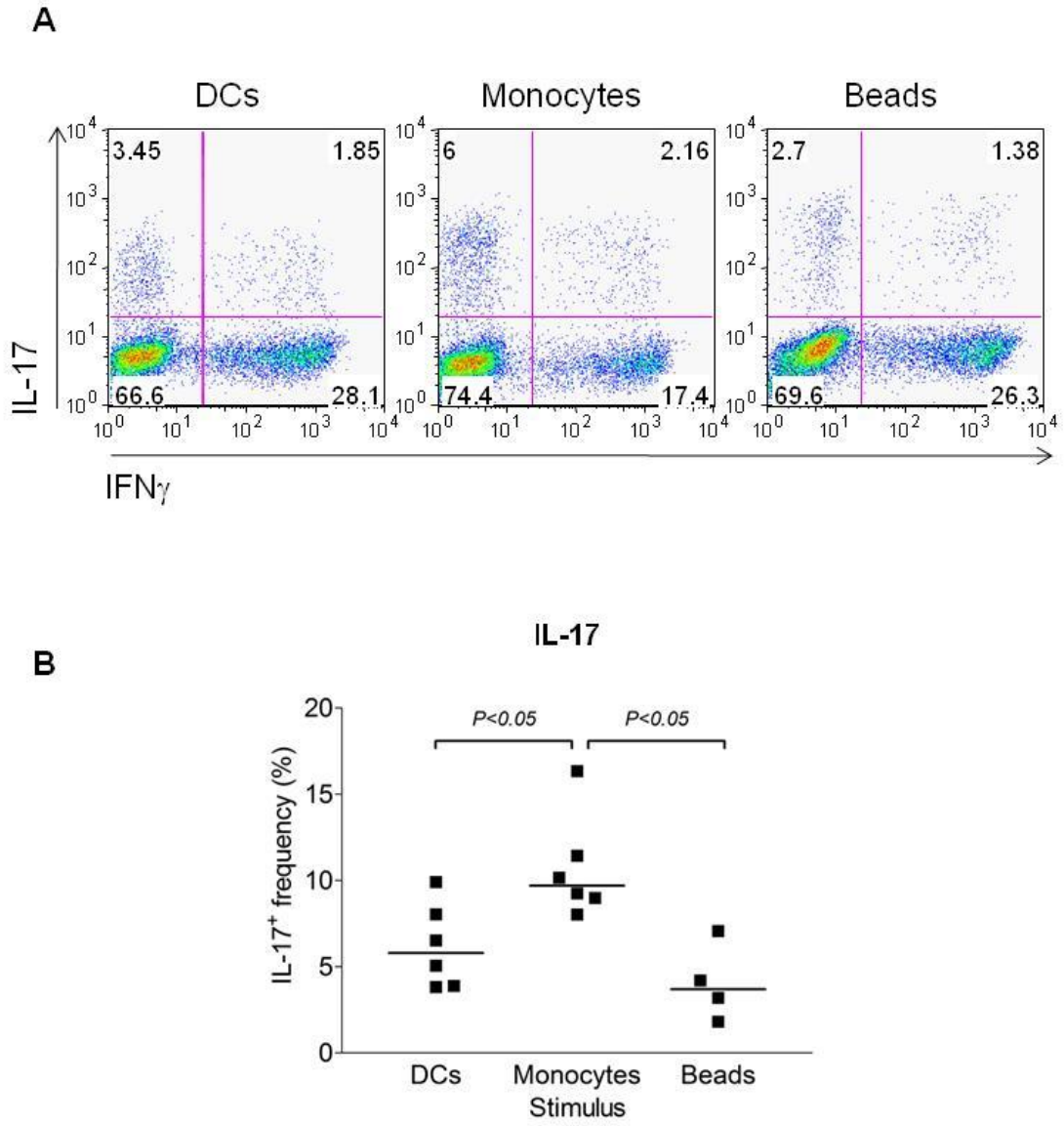

Figure 3.1. Monocytes induce greater T_h17 frequencies than beads or dendritic cells. $CD4^+CD25^-$ T cells were stimulated with **(i)** dendritic cells (DC) + anti-CD3 **(ii)** monocytes + anti-CD3 or **(iii)** anti-CD3/CD28 beads. At five days cells were intracellular stained for CD3, IL-17 and IFNγ and analysed by flow cytometry. **A)** Representative FACS plots to illustrate IL-17 and IFNγ expression by $CD3^+$ cells under each stimulation. **B)** Percentage of $IL\text{-}17^+$ T cells generated by DC, monocyte or bead stimulation for multiple donors. Horizontal bars represent the median percentage of IL-17 producing cells. Significance was tested by two-tailed Mann Whitney U tests.

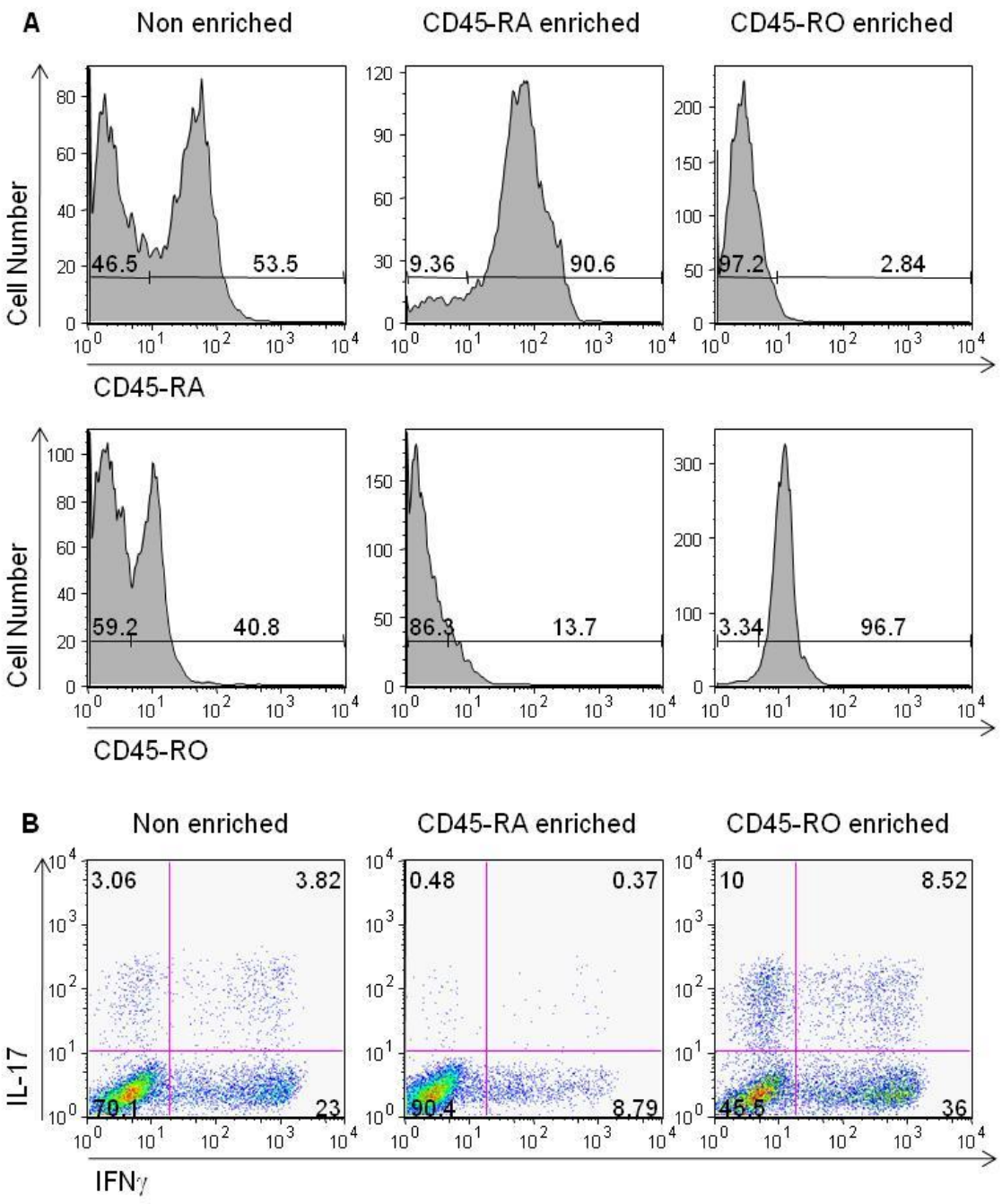

Figure 3.2. IL-17$^+$ cells derive mainly from the CD45-RO$^+$ fraction. CD45-RA and CD45-RO enriched fractions were obtained from CD4$^+$CD25$^-$ T cells. Population purities were assessed by flow cytometry. Gates were positioned by the isotype control **(A).** At five days, cells were analysed for IL-17 and IFNγ expression by flow cytometry **(B).** FACS plots are from one experiment representative of two performed.

The effect of $1,25(OH)_2D_3$ upon T cells stimulated in the absence of APCs was also to be investigated in this thesis. Therefore IL-17 and IFNγ expression by $CD4^+$ T cells was measured following stimulation with dynabeads coated with agonistic antibodies to CD3 and CD28 (referred to as beads). Stimulation with beads induced a small T_h17 population, which similar to T_h17 populations generated by monocytes or DCs included both $IFN\gamma^+$ and $IFN\gamma^-$ cells (**figure 3.1**). These data are consistent with those by Evans et al. as they also observed low frequencies of $IL\text{-}17^+$ cells when stimulating $CD4^+$ T cells with anti-CD3/CD28 coated beads (Evans et al., 2007).

3.2 Time course of IL-17 expression

To determine the optimum time at which to measure T_h17 frequencies, IL-17 expression was monitored by flow cytometry over a period of twelve days. **Figure 3.3** shows that $IL\text{-}17^+$ cells occurred at very low frequencies (0.5-1%) during the first two days of stimulation and generally lacked IFNγ expression. At three days, $IL\text{-}17^+$ frequencies began to increase co-incident with the onset of cell division, as monitored by CFSE dilution. Analysis further showed that $IL\text{-}17^+$ cells accumulated in the dividing populations (**figure 3.4**). $IL\text{-}17^+$ frequencies continued to rise reaching 10% by seven days. At four days, $IL\text{-}17^+IFN\gamma^+$ cells became evident and the proportion of $IL\text{-}17^+$ cells expressing IFNγ increased further over time (**figure 3.3**). Based upon this analysis and the finding that cell survival was greatest at earlier time points, it was decided to measure T_h17 frequencies at five days.

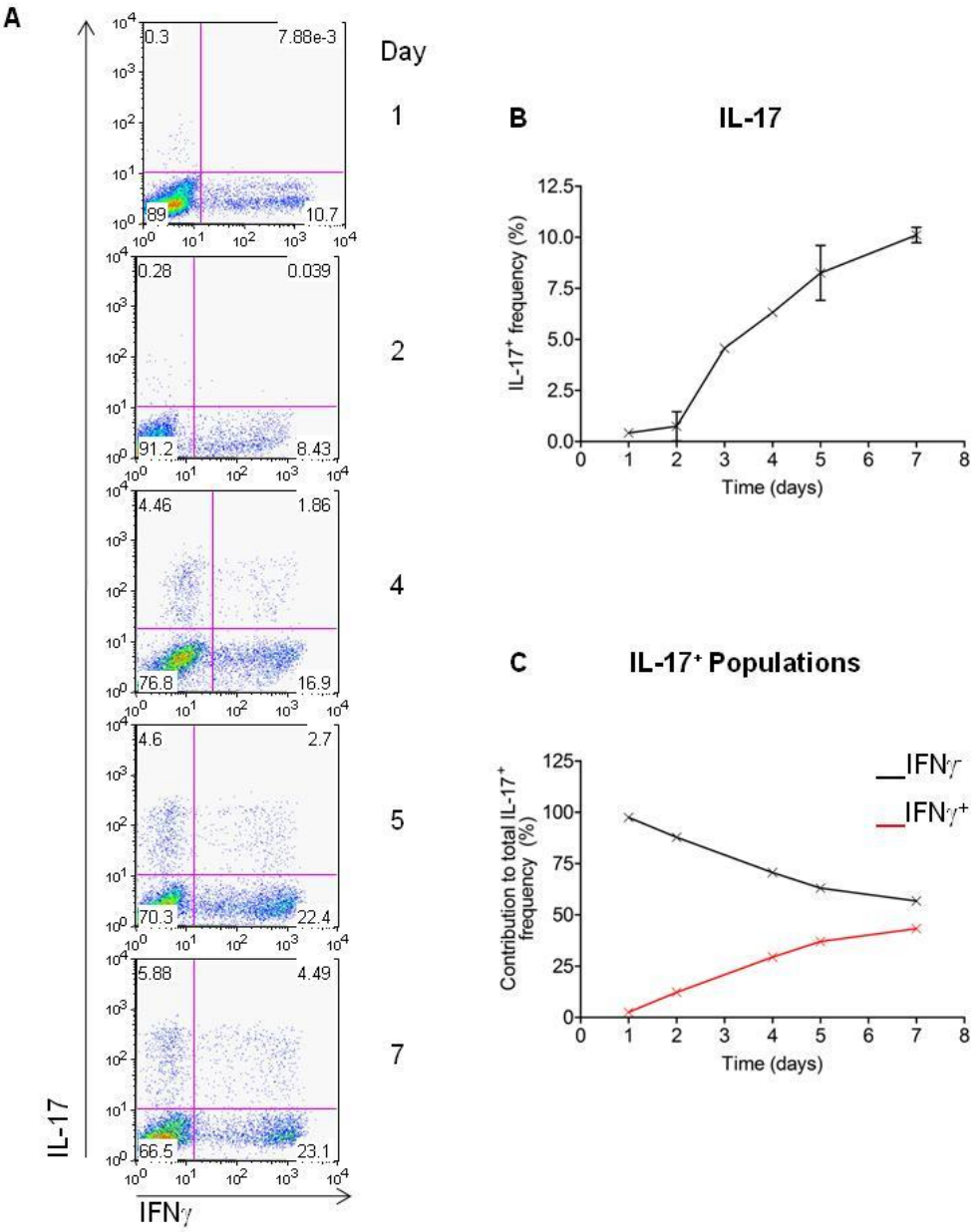

Figure 3.3. Time course of IL-17 expression. $CD4^+CD25^-$ T cells were stimulated with monocytes and anti-CD3. At the times stated, cells were stained for CD3, IL-17 and IFNγ and analysed by flow cytometry. **A)** FACS plots from one experiment representative of two performed. **B)** A summary of the frequencies of $IL\text{-}17^+$ cells from both experiments (n=1 for days 3 and 4). **C)** The contributions of $IFN\gamma^-$ and $IFN\gamma^+$ subsets to the total $IL\text{-}17^+$ cell frequency (data are from the FACS plots shown).

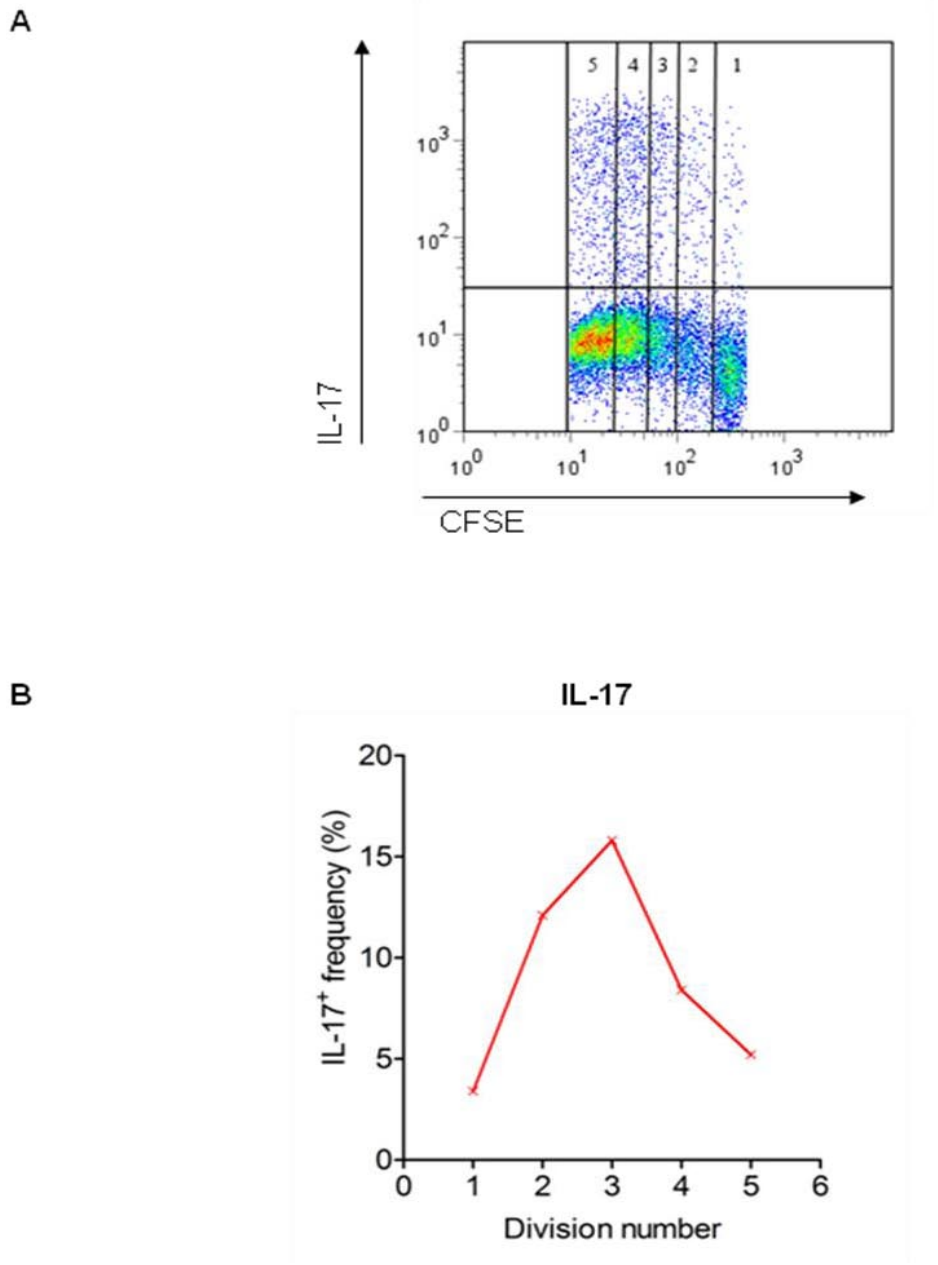

Figure 3.4. Dividing cells express IL-17. $CD4^+CD25^-$ T cells were CFSE labelled and stimulated with anti-CD3/CD28 beads or monocytes plus anti-CD3. At four days, cells were intracellular stained for IL-17 and analysed by flow cytometry. **A)** FACS plot of IL-17 against CFSE. Vertical lines mark division stages where 1 is undivided cells. **B)** The percentage of $IL\text{-}17^+$ cells in each division. Data are from one experiment representative of n>3.

To examine the possibility that soluble factors accounte for the different abilities of monocyte and bead stimulations to generate T_h17 cells, supernatants were periodically collected from monocyte and bead stimulations during one round of stimulation and used to supplement primary stimulations of the opposite type. The transfer of bead supernatants onto monoycte stimulations reduced T_h17 frequencies, whilst early stage monocyte supernatants permitted a small increase in bead driven T_h17 frequencies. However, late stage monocyte supernatants were inhibitory to bead driven T_h17 differentiation (**figure 3.5**). These data suggested that soluble factors inhibitory to T_h17 differentiation were present at the early stage of bead stimulations and accumulated during the course of monocyte stimulations.

3.3 IL-2 is inhibitory to T_h17 differentiation

One soluble factor that might be inhibitory to T_h17 differentiation is IL-2 (Acosta-Rodriguez et al., 2007a; Laurence et al., 2007). Analysis of IL-2 production by T cells following bead or monocyte stimulation revealed a higher frequency of IL-2^+ cells at an early stage of bead compared to monocyte stimulations. In addition, the level of IL-2 expressed by IL-2^+ cells was greater for bead-stimulated cells (**figure 3.6**). Consistent with higher IL-2 levels, the IL-2 receptor alpha chain, CD25, which is enhanced by IL-2 signalling, was expressed more strongly by bead versus monocyte-stimulated cells (**figure 3.7**). Thus, to investigate whether high IL-2 might contribute to low T_h17 differentiation, monocyte stimulations were supplemented with increasing doses of IL-2 and the frequencies of IL-17^+ and IFNγ^+ T cells generated by five days measured. **Figure 3.8** shows that IL-2 reduced IL-17^+ and IFNγ^+ frequencies by monocyte stimulated T cells. Interestingly, whilst IL-2 expression was low in monocyte stimulated T cells at early stages of stimulation, the frequency of IL-2^+ cells

increased in these cultures over time (**figure 3.6**). Given the inhibitory effect of IL-2 upon T_h17 differentiation, the accumulation of IL-2 in monocyte cultures supported the inhibitory effect of late stage monocyte supernatants upon T_h17 differentiation (**figure 3.5**). Since increased IL-2 contributed to T_h17 suppression in the bead system, it was hypothesised that reducing bead stimulation might increase T_h17 frequencies. **Figure 3.9** shows that by reducing bead to T cell frequencies over the range 1:1 to 1:32 the proportion of cells that committed to division was decreased but the T_h17 frequency was increased. Taken together, these data suggest that strong stimulation in the bead system might yield high IL-2, which contributes to low T_h17 frequencies.

3.4 Generating T_{Reg} cells in-vitro

FoxP3 and CTLA-4 are two molecules that are expressed at high levels on T_{Reg} cells, thus to monitor the development of T_{Regs} from $CD4^+CD25^-$ T cells were stimulated and total stained for CTLA-4 and FoxP3. Since FoxP3 is known to be transiently expressed in activated T cells (Horwitz et al., 2008b) its expression and that of CTLA-4 was monitored over time (**figure 3.10**). For both bead and monocyte stimulations, CTLA-4 induction was observed within 36 hours. Its expression gradually increased over four days but then decreased. Consistent with expectation, FoxP3 expression was transient. Although the level of FoxP3 was quite variable, those cells that acquired highest FoxP3 had greatest CTLA-4. In keeping with the finding that bead stimulated cells had higher CD25 than monocyte stimulated cells, the maximal CTLA-4^+FoxP3^+ population was three fold greater in bead versus monocyte stimulated T cells. Since the CTLA-4^+FoxP3^+ population peaked at four days, this time was chosen for routine T_{Reg} analysis.

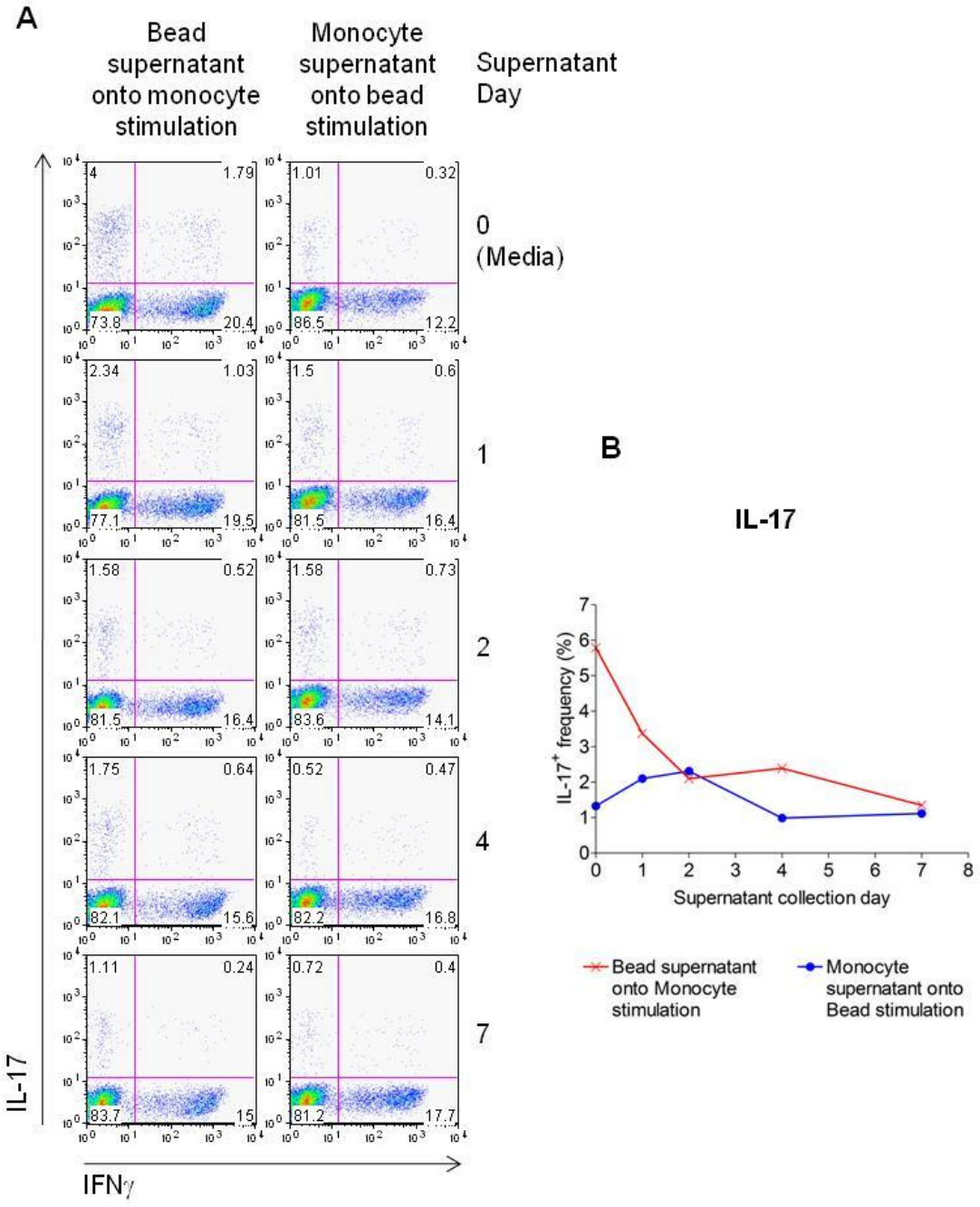

Figure 3.5. Bead supernatants inhibit monocyte induced T_h17 differentiation. A) FACS plots of IL-17 and IFNγ expression by $CD4^+$ T cells stimulated with monocytes or anti-CD3/CD28 beads in the presence of supernatant collected from the alternative stimulation type after the time stated. Numbers in quadrants refer to percentage of $CD3^+$ cells. **B)** Line graph summary of the IL-17^+ frequencies determined from FACS plots in A. (n=1).

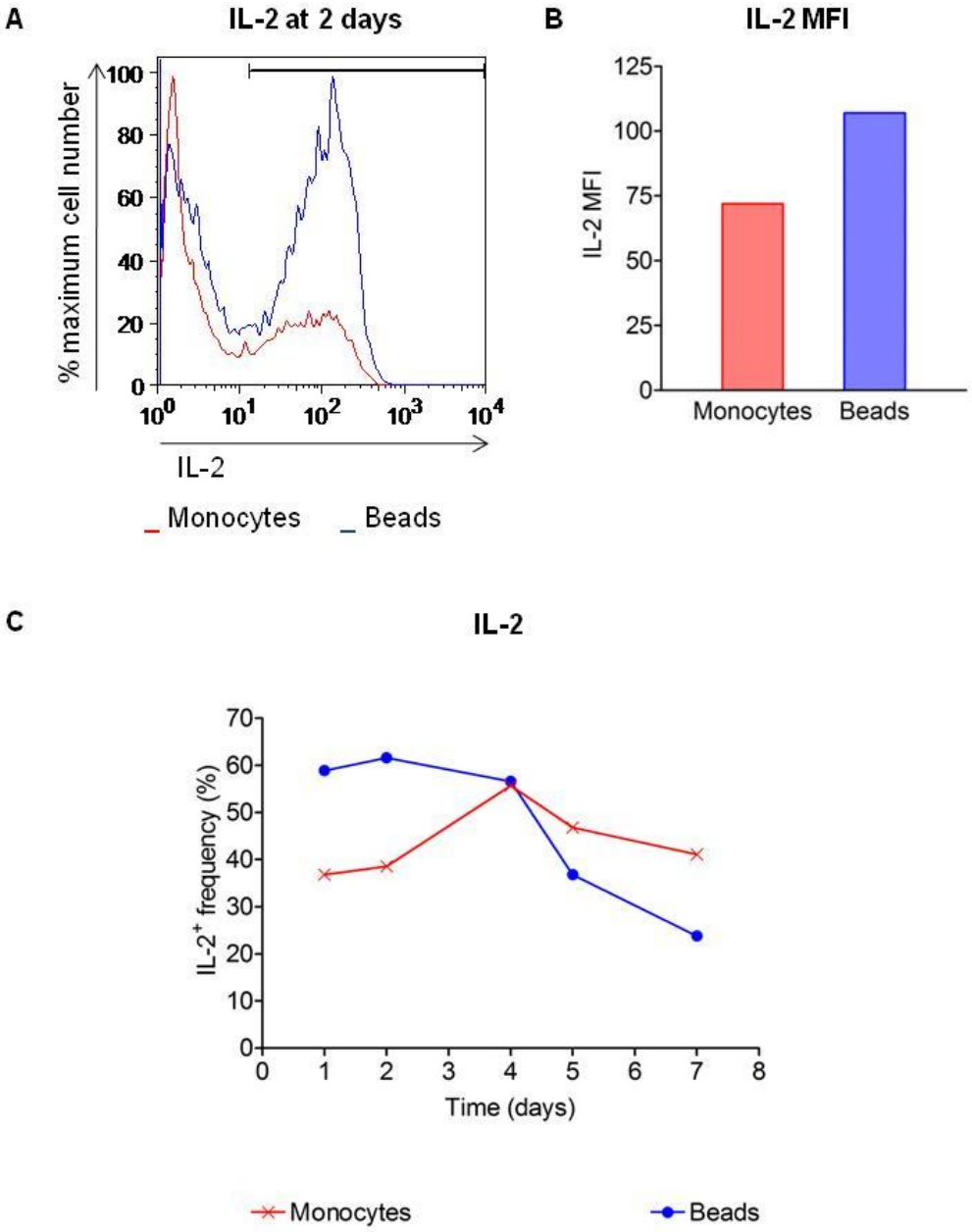

Figure 3.6. Bead stimulated T cells produce more IL-2 than monocyte stimulated cells at early time points. $CD4^+CD25^-$ T cells were stimulated with monocytes plus anti-CD3 (red) or anti-CD3/CD28 coated beads (blue) for two days. Cells were stained for CD3, IL-2 and IFNγ and analysed by flow cytometry. **A)** Histogram of IL-2 expression by $CD3^+$ cells. Horizontal bar indicates $IL\text{-}2^+$ cells whose median IL-2 fluorescence intensity is graphed in **B**. **C)** Time course of IL-2 expression by bead and monocyte stimulated T cells. (n=1).

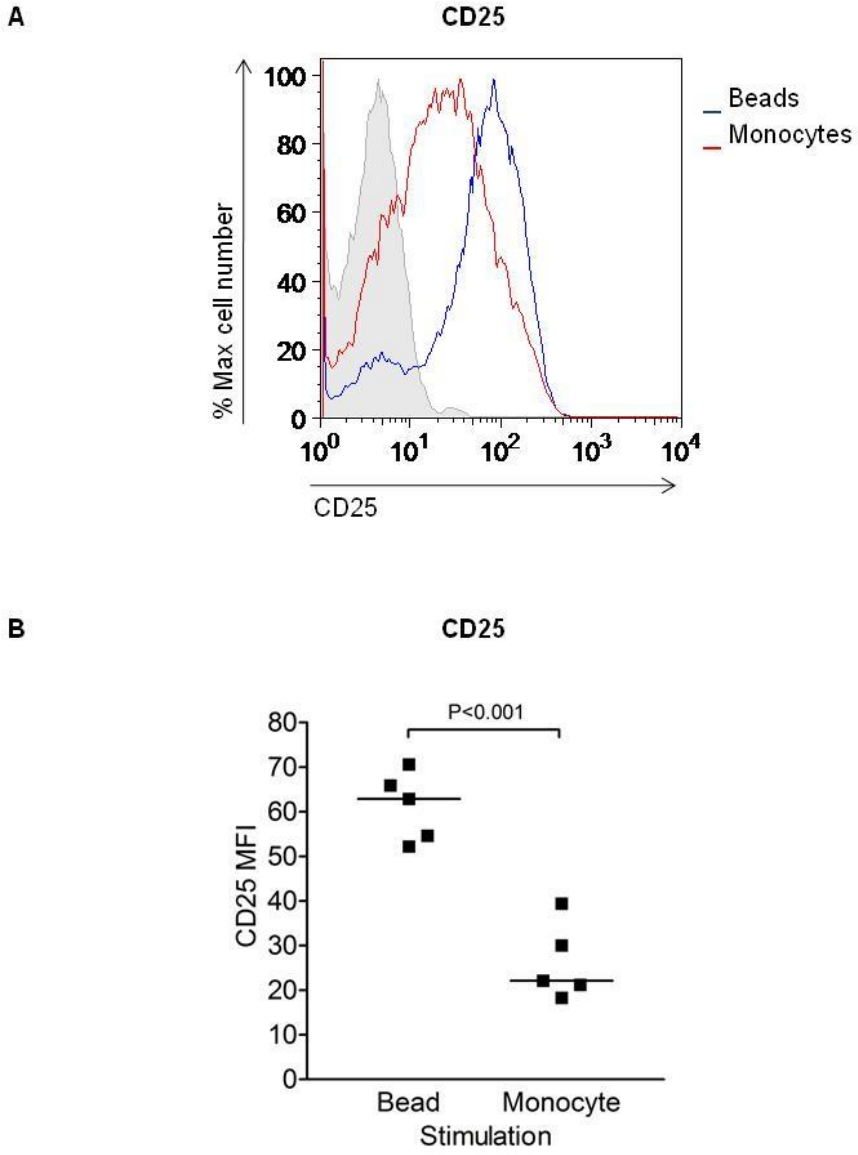

Figure 3.7 Bead stimulation induces greater CD25 expression than monocyte stimulation. $CD4^+CD25^-$ T cells were stimulated with beads or monocytes plus anti-CD3 for four days, stained for CD25 and analysed by flow cytometry. **A)** Representative histograms of CD25 expression. Shaded histogram indicates isotype control. **B)** CD25 median fluorescence intensity for multiple donors. Horizontal bars indicate median expression. Significance was tested by a two-tailed Wilcoxon matched pairs test.

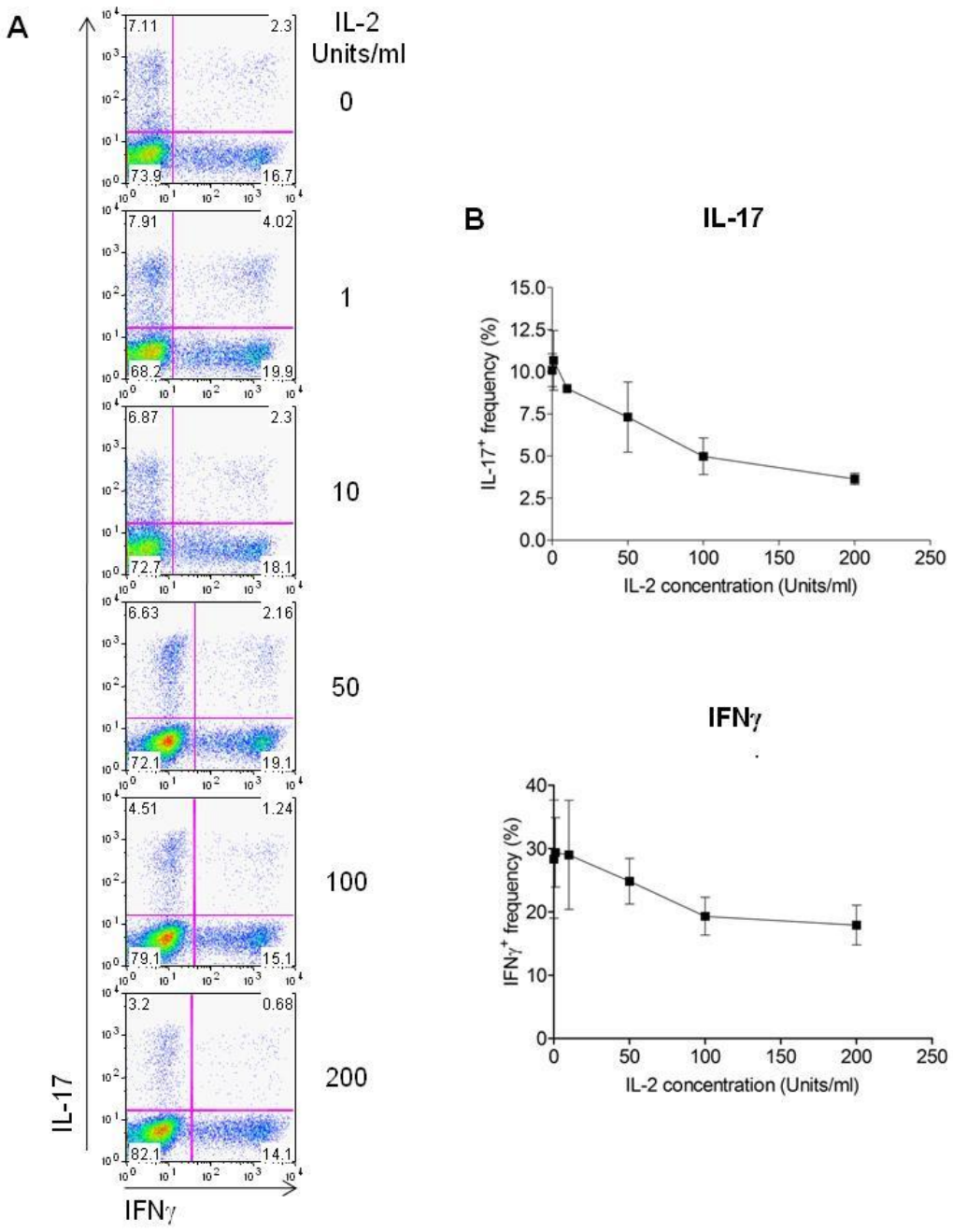

Figure 3.8. IL-2 suppresses IL-17 expression. $CD4^+CD25^-$ T cells were stimulated with monocytes plus anti-CD3 in the presence of IL-2 over the range 0-200U/ml. At five days, cells were stained for CD3, IL-17 and IFNγ and analysed by flow cytometry. **A)** Representative FACS plots from one experiment. Numbers in quadrants refer to percentage of $CD3^+$ cells. **B)** IL-2 concentration effect upon $IL\text{-}17^+$ and $IFN\gamma^+$ frequencies (n=3)**.** Error bars show standard deviation.

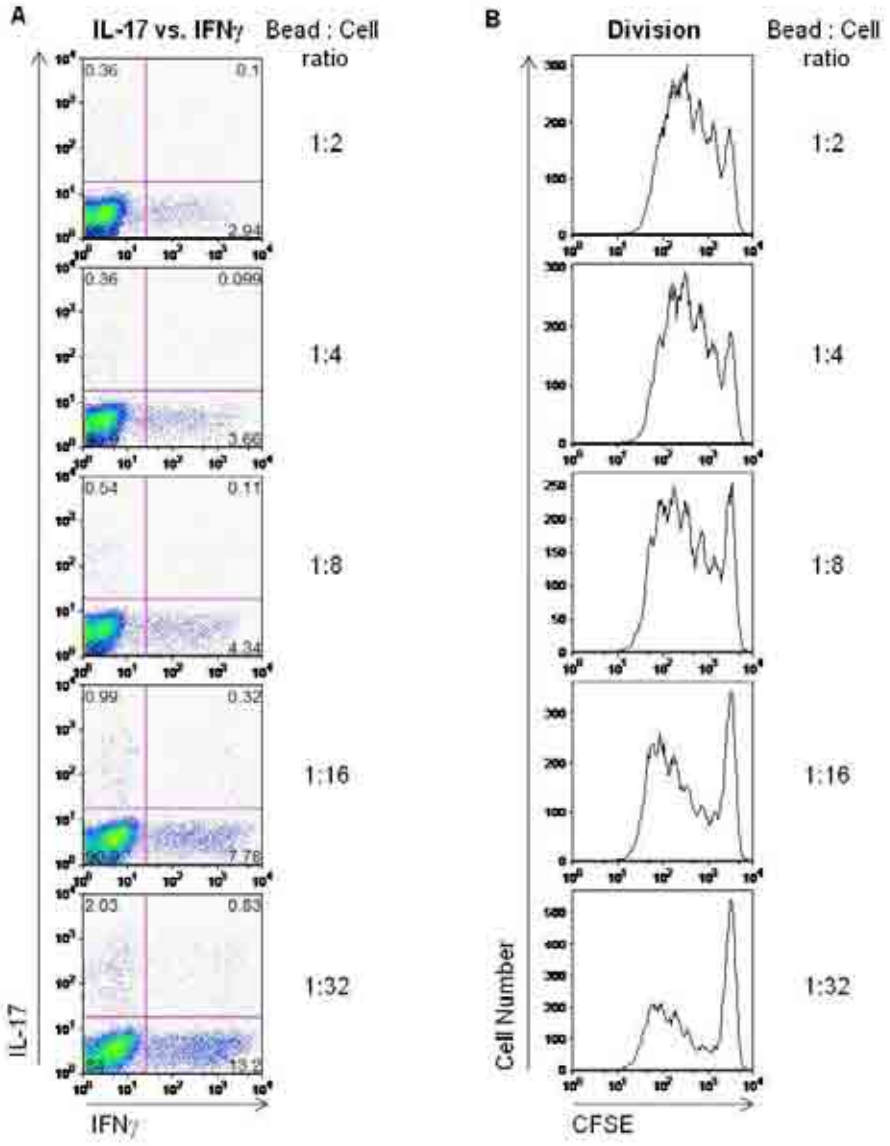

Figure 3.9. T_h17 frequencies are inversely related to bead: cell ratio. A) $CD4^+CD25^-$ T cells were stimulated with beads at the given ratio. At five days cells were stained for intracellular IL-17 and IFNγ and analysed by flow cytometry. **B)** CFSE labelled $CD4^+CD25^-$ T cells were stimulated with beads at the given ratio. CFSE dilution was monitored by flow cytometry after 5 days. Data are from a single experiment representative of n>3.

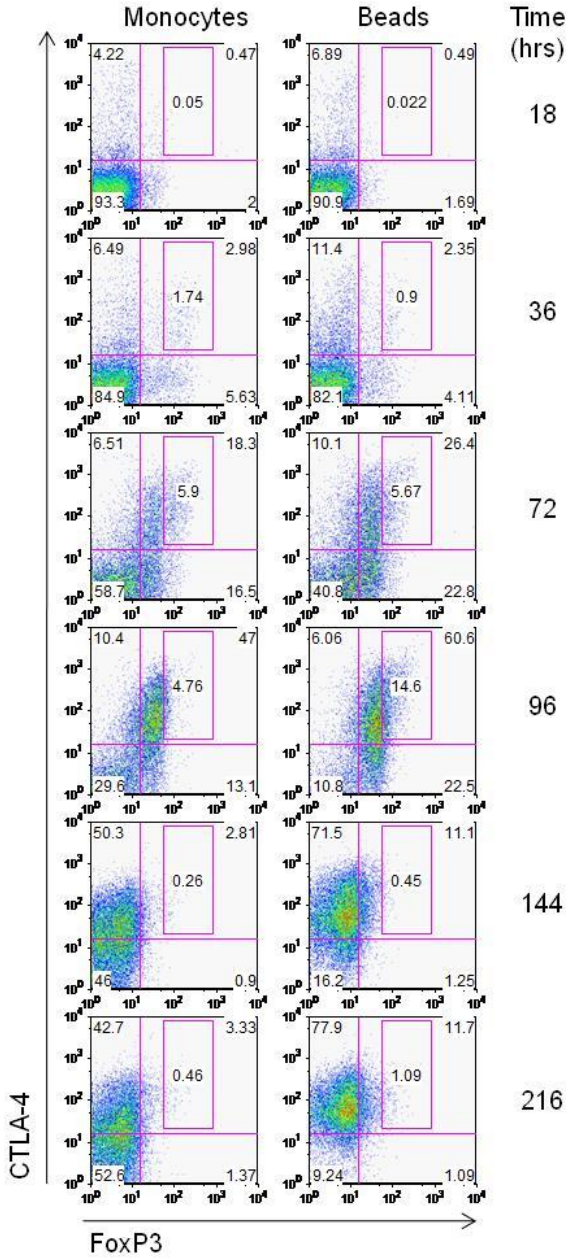

Figure 3.10. Time course of FoxP3 and CTLA-4 expression by monocyte and bead stimulated T cells. $CD4^+CD25^-$ T cells were stimulated with monocytes plus anti-CD3 or anti-CD3/CD28 coated beads. At the indicated times, cells were stained for CD3, CTLA-4 and FoxP3 and analysed by flow cytometry. FACS plots from one experiment representative of two performed are shown. Numbers in quadrants refer to percentage of $CD3^+$ cells and boxes in the top right quadrants indicate CTLA-4^+FoxP3^+ cells with highest FoxP3 expression.

Discussion

The aim of the studies reported in this chapter was to develop systems for the *in-vitro* generation of T_h17 and T_{Reg} cells from human $CD4^+CD25^-$ T cells. Systems were compared in which APCs were present or absent, since this would permit later analysis of the intermediary role of APCs in the response of T cells to different factors, including $1,25(OH)_2D_3$. This revealed that monocytes support higher IL-17^+ frequencies than DCs or beads. Such is consistent with data published by Evans et al (2007). Conversely, beads induced greater CTLA-4^+FoxP3^+ T_{Reg} populations than monocytes.

The pro-T_h17 effect of monocytes appears to relate largely to the cytokines that they release upon stimulation. Consistent with this hypothesis, using antibody blockade and caspase-1 inhibition, Acosta-Rodriguez et al. demonstrated the importance of monocyte derived, STAT3 signalling cytokines, IL-1β and IL-6, for T_h17 generation (Acosta-Rodriguez et al., 2007a). They also showed that GM-CSF and IL-4 derived DCs produce comparatively little IL-1β and IL-6 in response to maturation stimuli but release IL-12 that promotes IFNγ expression. In addition, several groups have demonstrated an inhibitory effect of IFNγ upon IL-17 expression (Harrington et al., 2005; Park et al., 2005), thus the lower frequencies of IL-17^+ cells in DC stimulations compared to monocyte stimulations might relate to greater IFNγ expression as well as the comparative absence of STAT3 signalling cytokines. Potentially, competitive binding of STAT3 and STAT1 at the *IL-17a* promoter might underlie the process, as both have been shown to bind there (Laurence et al., 2007). However, it is likely that indirect mechanisms are also involved. For example, in the absence of STAT1 signalling, up

regulation of the IL-23R compared to the IL-12Rβ2 was favoured and might promote a T_h17 outcome (Harrington et al., 2005).

As mentioned, a clear distinction in the abilities of monocytes and beads to promote IL-17 expression by T cells was observed in this study. When investigating possible reasons for this I found that the absence of monocyte derived cytokines in bead stimulations was not solely responsible for their low T_h17 frequencies. Further investigation led to the identification that IL-2, which is expressed at high levels during the early stage of bead stimulations, was inhibitory to IL-17 expression. Consistent with this finding, an inhibitory effect of high IL-2 concentration upon T_h17 differentiation has been suggested by others (Acosta-Rodriguez et al., 2007a; Laurence et al., 2007). Laurence et al. also demonstrated that IL-17 inhibition by IL-2 depends upon STAT5a/b and they identified multiple STAT5 binding sites at the *IL-17a* locus. Interestingly, in support of the hypothesis that IL-2 produced endogenously by bead stimulated cells is sufficient to inhibit T_h17, Laurence et al. showed that when cells were stimulated with antiCD3/CD28 beads, STAT5 bound appreciably to the *IL-17a* gene in the absence of exogenous IL-2 (Laurence et al., 2007).

Analysis of IL-2 expression at later stages of stimulation revealed that IL-2 expression increases over time in monocyte-stimulated T cells. Whilst it is possible that accumulating IL-2 feeds back to avoid persistent T_h17 development, it might be that differentiation fate is governed primarily by the cytokine profile at the start of stimulation (Constant and Bottomly, 1997; Nakamura et al., 1997) with inhibitory cytokines at later stages having little effect. To confirm this for IL-2 upon T_h17 differentiation, it would be necessary to inhibit IL-2 at

increasingly delayed times post stimulation. If enhanced IL-17 frequencies were measured, a role for IL-2 in the avoidance of persistent T_h17 inflammation might be implied.

In this chapter, as well as investigating systems to generate T_h17 cells a protocol to generate inducible T_{Reg} development from $CD4^+CD25^-$ T cells was also studied. Transient FoxP3 induction was observed under both monocyte and bead stimulation. However, it was found that monocyte stimulated cells had lower FoxP3 expression compared to bead stimulated cells, only 5% acquiring a high CTLA-4 and FoxP3 phenotype by four days compared to 14% by beads. This distinction might again be reconciled by the greater level of IL-2 expression during the early stage of bead stimulations, since IL-2 is required for the development and expansion of $FoxP3^+$ iT_{Regs} *in-vitro* (Horwitz et al., 2008b). In addition, the release of STAT3 dependent cytokines by monocytes might have contributed to FoxP3 inhibition in monocyte stimulated cells (Zhou and Littman, 2009; Zhou et al., 2008). Overall, it would seem that the pro-T_{Reg} anti-T_h17 outcome of bead stimulations versus the anti-T_{Reg} pro-T_h17 outcome of monocyte stimulations supports the view that T_{Reg} and T_h17 lineages are reciprocal fates (Bettelli et al., 2006).

In addition to soluble factors, varying surface molecule interactions may contribute to the differing abilities of beads and monocytes to induce IL-17 expression *in-vitro*. Indeed, for T_h1 and T_h2 differentiation, there is evidence that whilst cytokines play a dominant role in lineage decision, factors including the strength and frequency of TCR engagement and the nature of co-stimulatory signals can contribute (reviewed by (Constant and Bottomly, 1997)). The general opinion is that for soluble antigens, low doses favour T_h2 responses whilst high doses

promote a T_h1 outcome. For co-stimulatory signals, CD28 signalling may favour T_h2 but not T_h1 responses and differential effects for CD86 and CD80 upon T_h1 and T_h2 subsets have been observed. However, neither B7 ligand clearly associates with either lineage (Constant and Bottomly, 1997). In this chapter, I observed that reducing bead to T cell ratios from 1:4 to 1:32 increased T_h17 frequencies. Very recently, others supported this finding as they demonstrated that decreased stimulation strength, administered by low antiCD3/anti-CD28 bead or DC ratios dramatically increased human T_h17 frequencies when pro-T_h17 cytokines were present, although, they suggested a need for CD28 stimulation (Purvis et al., 2010). Conversely, *in-vitro* studies with mice imply that strong TCR signals are required for T_h17 differentiation (Bouguermouh et al., 2009; Gomez-Rodriguez et al., 2009). However, whilst Bouguermouh et al. demonstrated an inhibitory effect of CD28 signalling, showing increased IL-17^+ frequencies when T cells were stimulated by DCs in the presence of CTLA-4-Ig, Gomez-Rodriguez et al. positively correlated CD28 signal strength with T_h17 frequencies. Conflicting conclusions are likewise reached from *in-vivo* mouse studies as a range of outcomes for T_h17 associated diseases have been observed following CD28 or B7 deletion (reviewed in (Bouguermouh et al., 2009)). Possibly, such discrepancies relate to differences in genetic background or reflect the importance of a fine balance between TCR and co-stimulatory signal strength for T_h17 differentiation as observed for T_h2 (Tao et al., 1997). Nonetheless, the varying conclusions from these studies make it difficult to define the relative requirements of TCR and co-stimulatory signals for T_h17 development.

It is possible that factors besides stimulation strength could explain the effect of reduced bead to T cell ratios upon T_h17 frequencies that I observed. Indeed, cells that committed to division in the low bead to T cell cultures might have received TCR and co-stimulatory signals of

equal strength to cells stimulated at higher bead to T cell ratio, since they passed through the same number of divisions. Because fewer cells committed to division as the bead to cell ratio decreased, it is likely that cytokines inhibitory to T_h17, such as IL-2 and IFNγ, were at lower concentration in these cultures and governed the outcome. In support of this, Bouguermouh et al. demonstrated that anti-IFNγ or anti-IL-2 abrogated the suppressive effect of CD28 upon T_h17 induction. However, the same was not seen by Purvis et al. rather they and Gomez-Rodriguez et al. related signal strength to the activation of calcium dependent NFATc, that binds at the *IL-17a* promoter (Gomez-Rodriguez et al., 2009; Purvis et al., 2010). Thus, the strength of TCR and co-stimulatory signals as well as cytokines might contribute independently to T_h17 differentiation.

In support of a model in which strong TCR and CD28 signals are inhibitory to T_h17 differentiation, it is likely that both signals were stronger in the bead compared to monocyte system. Indeed, by clustering with the cells, beads probably provided a greater local anti-CD3 concentration than achieved with soluble anti-CD3. Secondly, the affinity of anti-CD28 for CD28 is likely to be greater than that of CD86, via which monocytes would initially stimulate CD28. Thirdly, during the course of stimulation, bead stimulated cells could have continually experienced strong CD28 signals, whilst by CTLA-4 up-regulation and its engagement with B7, CD28 signals in monocyte-stimulated T cells could have subsided. An important role for weak CD86-CD28 interactions in T_h17 development as proposed here, is supported by the presence of high frequencies of $CD86^+$ cells relative to $CD80^+$ cells in rheumatoid tissue and fluid in which IL-17 levels are also elevated (Balsa et al., 1996; Thomas and Quinn, 1996). Besides an importance for CD28/B7 signalling in the regulation of T_h17 development, a study

in mice suggests that signalling through ICOS, which has been associated with T_h2 differentiation, might also enhance T_h17 differentiation (Nurieva et al., 2003). The pro-T_h17 effect of ICOS may relate to reduced CD28 signal strength and IL-2 release as ICOS signals take over (Odobasic et al., 2008; Riley et al., 2001). This system could further favour T_h17 induction in the monocyte system but would be absent from beads.

In summary, the studies in this chapter have shown that CTLA-4^+FoxP3^+ T_{Reg} and IL-17^+ T_h17 populations can be induced by bead and monocyte plus anti-CD3 stimulations, although the former favours T_{Reg} induction whilst the latter promotes a T_h17 outcome. It is likely that these distinctions reflect the release of pro-T_h17, STAT3 signalling cytokines by monocytes compared to the early high expression of anti-T_h17 cytokines such as IL-2 in bead stimulations. Differences between the strengths of TCR and co-stimulatory signals in the two systems might also contribute to their opposing T_h17 and T_{Reg} outcomes.

4 DOES 1,25(OH)$_2$D$_3$ ACT DIRECTLY UPON CD4$^+$ T CELLS TO INFLUENCE THEIR PHENOTYPE?

This chapter considers the effect of the biologically active form of vitamin D, 1,25-dihydroxyvitamin D$_3$ (1,25(OH)$_2$D$_3$) upon CD4$^+$ T cell responses in the presence and absence of antigen presenting cells (APCs). In particular, effects upon T$_h$17 cells were monitored by studying expression of their associated cytokines: IL-17, IL-21 and IFNγ whilst effects upon regulatory T cells were observed by measuring CTLA-4, FoxP3 and IL-10.

4.1 1,25(OH)$_2$D$_3$ directly attenuates CD4$^+$ T cell expression of pro-inflammatory cytokines IL-17, IFNγ and IL-21

The effect of 1,25(OH)$_2$D$_3$ upon pro-inflammatory CD4$^+$ T cell cytokines, including IL-17, IFNγ and IL-21 was investigated by adding 100nM 1,25(OH)$_2$D$_3$ at the start of monocyte and bead driven T cell stimulations. Across multiple donors and under both stimulation methods, all three cytokines were profoundly suppressed, as determined by flow cytometry (**figure 4.1 and figure 4.2**). Notably, for cells expressing IL-17 or IL-21, those co-expressing IFNγ were the most sensitive to 1,25(OH)$_2$D$_3$-mediated suppression. To confirm these findings by a complementary method, quantitative real-time PCR for IL-17 and IFNγ transcripts was used (**figure 4.3**). These data supported the flow cytometry results, demonstrating strong suppression of IL-17 and IFNγ at the mRNA level. Because the anti-inflammatory effects of 1,25(OH)$_2$D$_3$ could occur when T cells were stimulated in the absence of APC, the ability of 1,25(OH)$_2$D$_3$ to modify T cells through direct signalling was concluded.

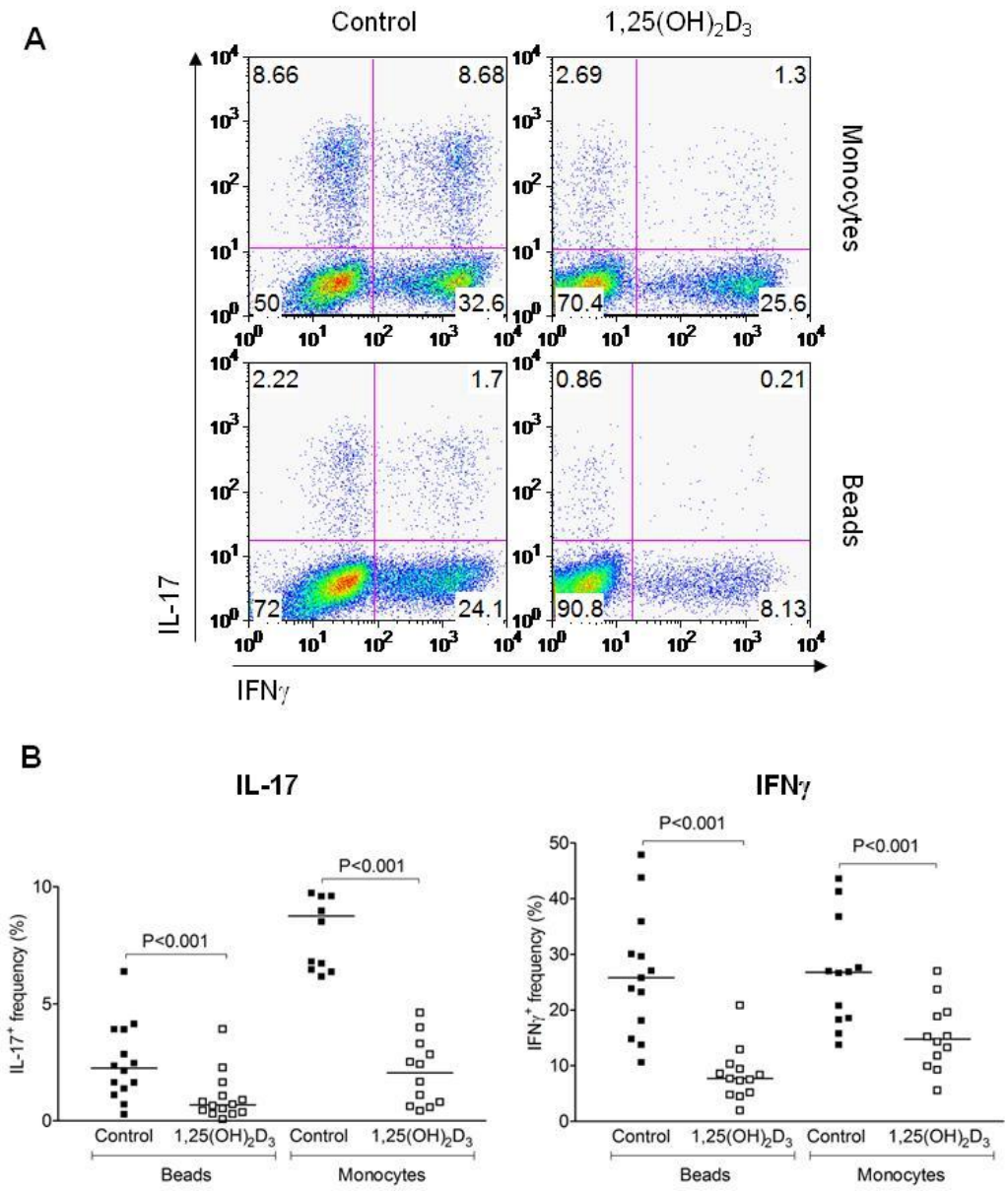

Figure 4.1. 1,25(OH)$_2$D$_3$ directly inhibits IL-17 and IFNγ expression. CD4$^+$CD25$^-$ T cells were stimulated with anti-CD3/CD28 beads or monocytes plus anti-CD3 in the presence of 1,25(OH)$_2$D$_3$ or vehicle control. At five days, cells were stained for CD3, IL-17 and IFNγ and analysed by flow cytometry. **A)** Representative FACS plots from one experiment. Numbers in quadrants refer to percentage of CD3$^+$ cells expressing IL-17 or IFNγ. **B)** IL-17$^+$ frequencies for multiple donors. Horizontal lines indicate median frequency. Significance was tested by a two-tailed Wilcoxon matched pairs test.

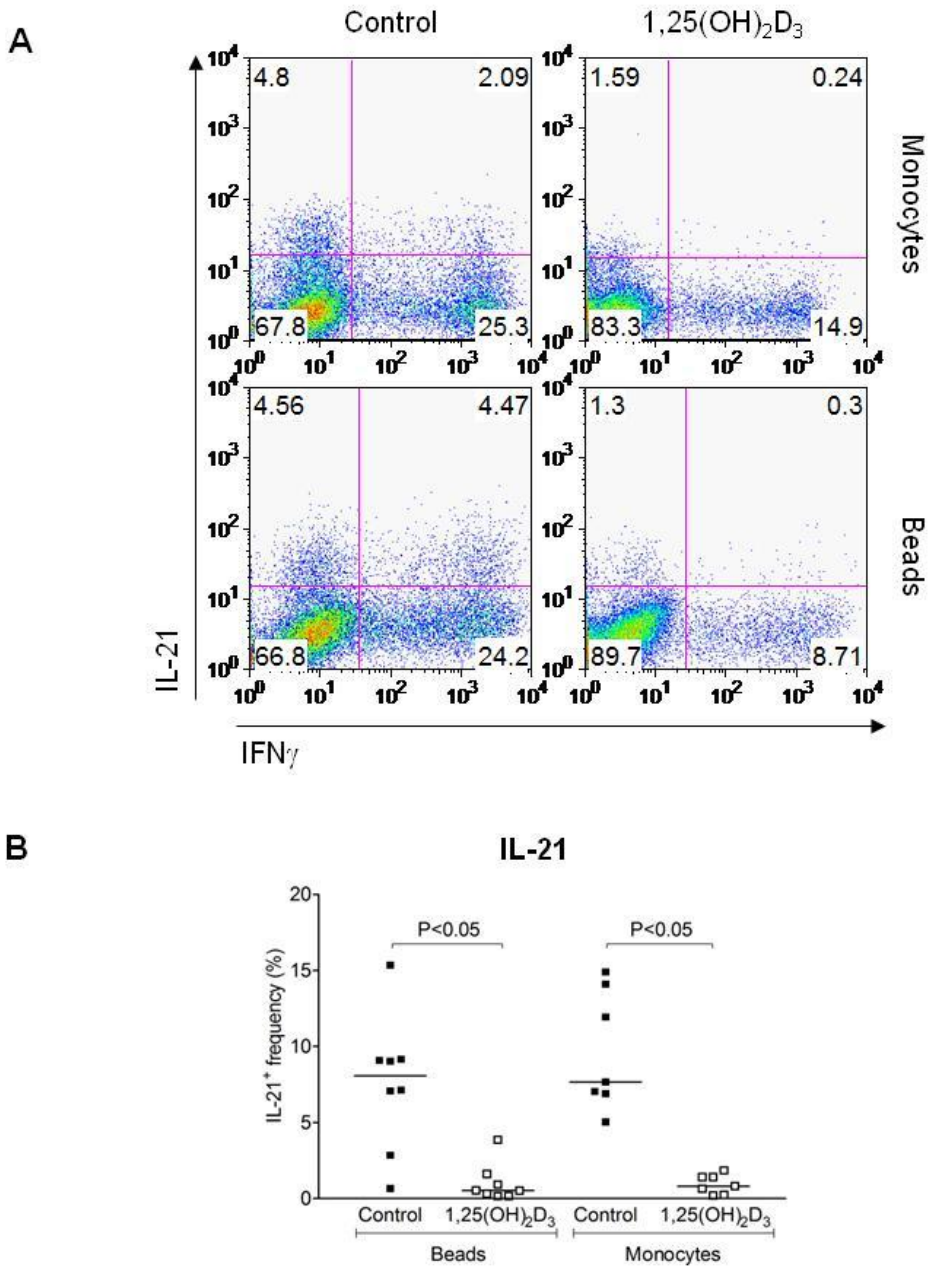

Figure 4.2. IL-21 and IFNγ are directly suppressed by 1,25(OH)$_2$D$_3$. CD4$^+$CD25$^-$ T cells were stimulated with anti-CD3/CD28 beads or monocytes plus anti-CD3 in the presence of 1,25(OH)$_2$D$_3$ or vehicle control. At five days, cells were stained for CD3, IL-21 and IFNγ and analysed by flow cytometry. **A)** Representative FACS plots from one experiment. Numbers in quadrants refer to percentage of CD3$^+$ cells expressing IL-21 or IFNγ. **B)** IL-21$^+$ frequencies for multiple donors. Horizontal lines indicate median frequency. Significance was tested by a two-tailed Wilcoxon matched pairs test.

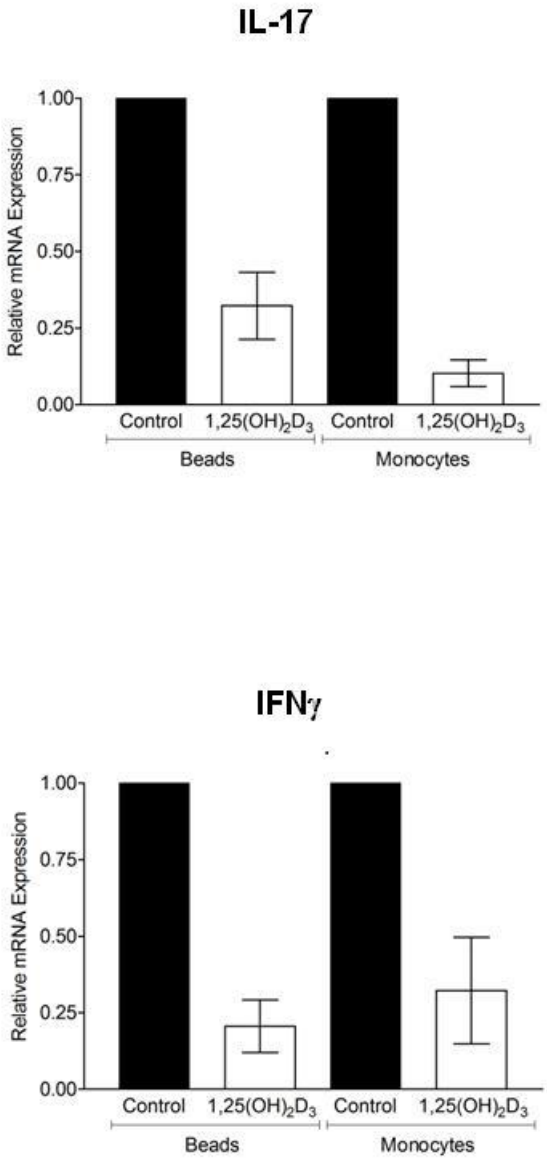

Figure 4.3. 1,25(OH)$_2$D$_3$ directly reduces IL-17 and IFNγ mRNA levels. CD4$^+$CD25$^-$ T cells were stimulated with anti-CD3/CD28 beads or monocytes plus anti-CD3 in the presence of 1,25(OH)$_2$D$_3$ (clear) or vehicle control (black). At four days IL-17 and IFNγ expression was analysed by quantitative real-time PCR. Mean expression is plotted relative to control. Error bars show standard deviation (n≥3).

4.2 Suppression of pro-inflammatory cytokine expression is not a consequence of reduced commitment to activate, divide and differentiate

Since IL-17 expression increased at three days post stimulation and was expressed primarily by dividing cells (**chapter 3**, **figures 3.3 and 3.4**), it was reasoned that an inhibitory effect of 1,25(OH)$_2$D$_3$ upon T cell proliferation (Lemire et al., 1985; Penna and Adorini, 2000) might explain the low frequencies of pro-inflammatory cytokines. To examine this, CD4$^+$CD25$^-$ T cells were labelled with CFSE and cell division measured. Whilst for monocyte stimulations proliferation was inhibited by 1,25(OH)$_2$D$_3$, it was not reduced under bead stimulation (**figure 4.4**). Furthermore, total cell counts confirmed equivalent cell numbers in bead stimulated cultures in the presence and absence of 1,25(OH)$_2$D$_3$. These results indicated that direct suppression of IL-17, IL-21 and IFNγ by 1,25(OH)$_2$D$_3$ was not due to reduced commitment to divide. They also suggest that the suppressive effects of 1,25(OH)$_2$D$_3$ upon T cell proliferation as reported by others (Lemire et al., 1985; Penna and Adorini, 2000) could be an indirect consequence of the reduced ability of the APC to stimulate. The ability of 1,25(OH)$_2$D$_3$ to affect DC phenotype was therefore also assessed. Reduced expression of HLA-DR and CD80 was observed, whilst the monocyte marker, CD14, was maintained at a higher level (**figure 4.5**). These findings were consistent with those of others (Berer et al., 2000; Canning et al., 2001; Penna and Adorini, 2000) and support the hypothesis that 1,25(OH)$_2$D$_3$ reduces the ability of APCs to stimulate T cells. Thus, taken together my data indicate that 1,25(OH)$_2$D$_3$ can act through APCs to influence T cell proliferation and differentiation but it can also act directly upon T cells to affect their differentiation but not their proliferation.

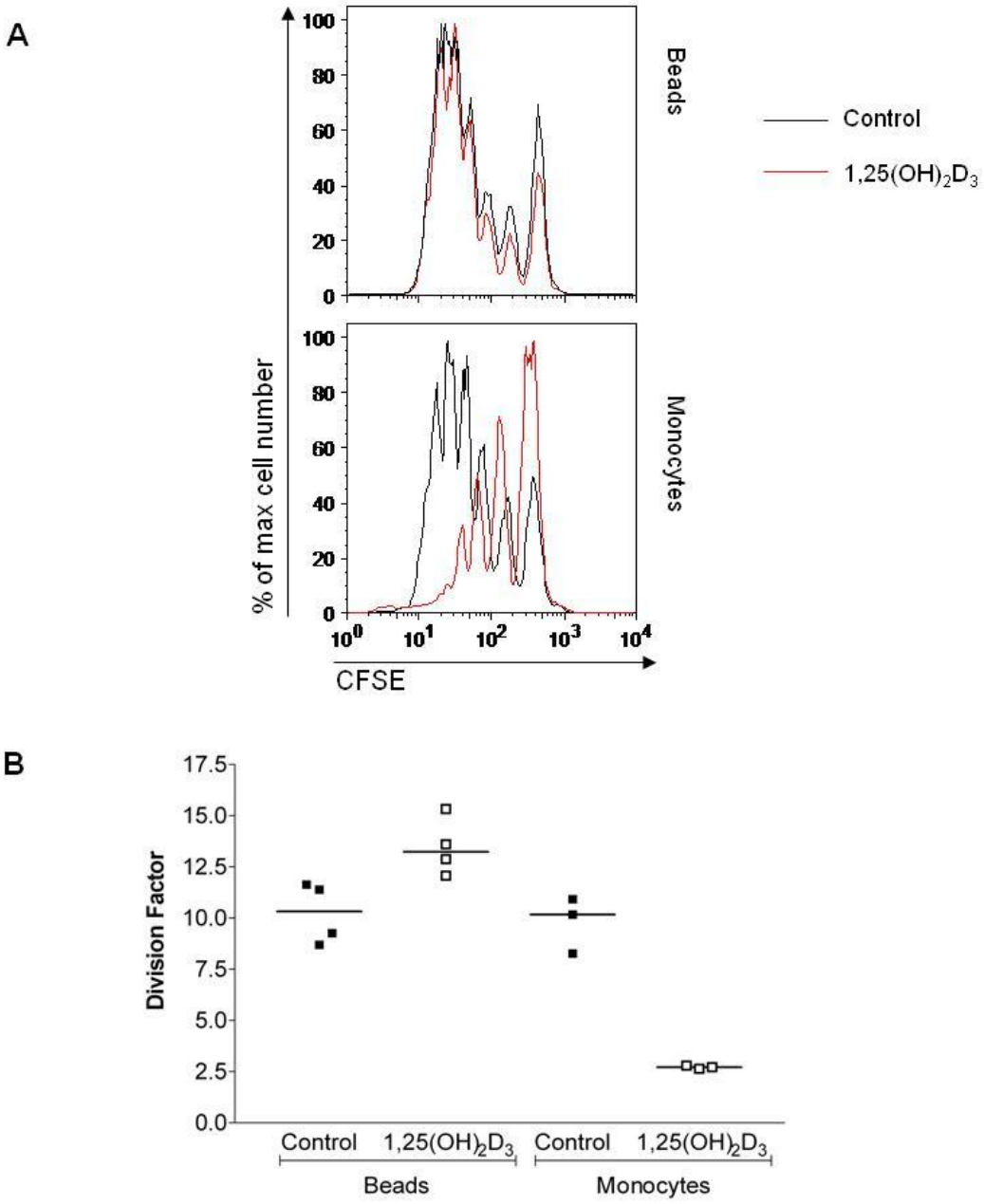

Figure 4.4. 1,25(OH)$_2$D$_3$ indirectly attenuates T cell proliferation through effects upon the APC. CFSE labelled CD4$^+$CD25$^-$ T cells were stimulated with anti-CD3/CD28 beads or monocytes plus anti-CD3 in the presence of 1,25(OH)$_2$D$_3$ or vehicle control for five days and analysed for cell division by flow cytometry. Representative CFSE histograms from one experiment are shown in **(A)** and division data from repeat experiments in **(B)**. Division factor was calculated by CFSE MFI$_{un-stimulated}$ / CFSE MFI$_{stimulated}$. Horizontal lines indicate median value.

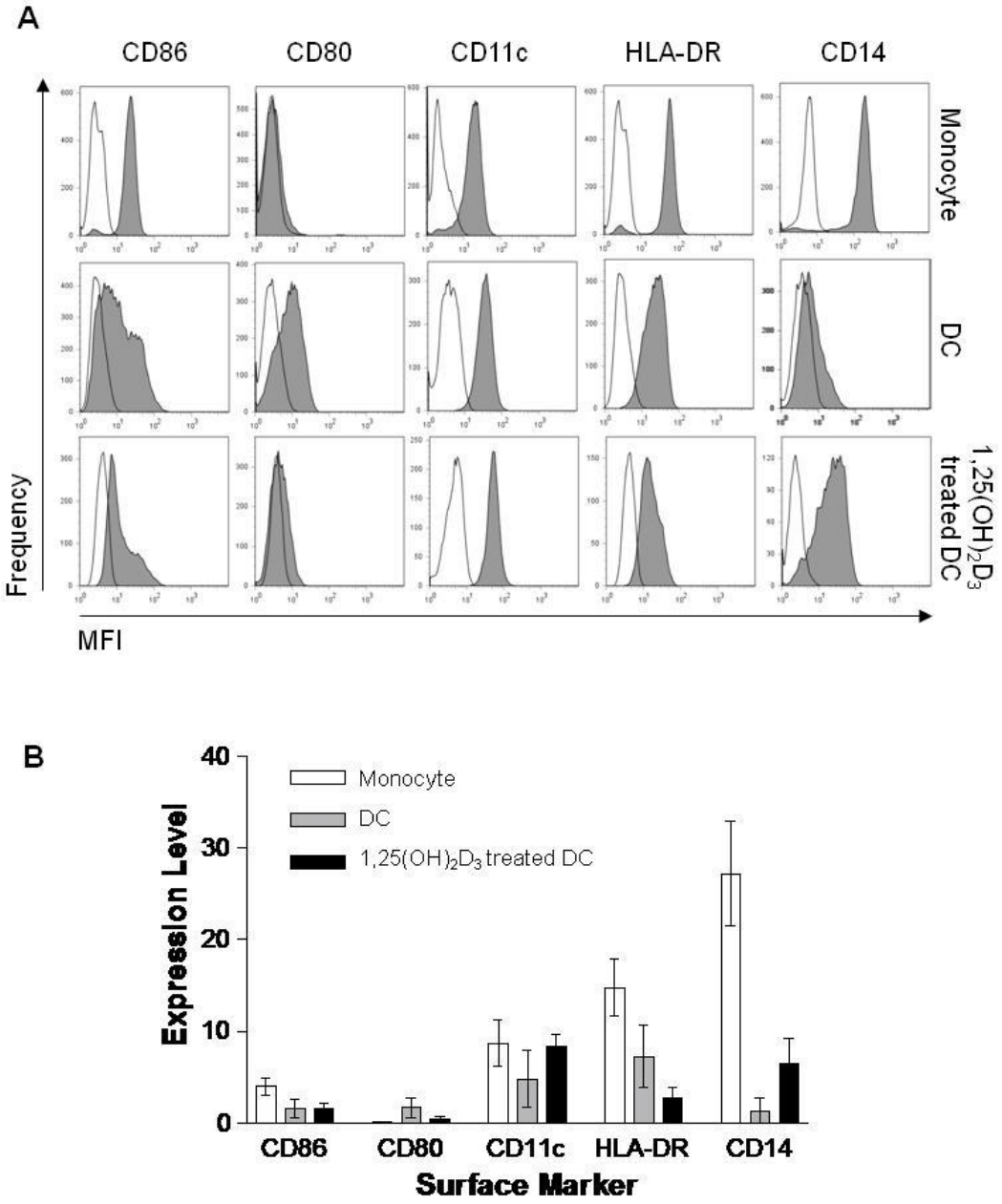

Figure 4.5. 1,25(OH)$_2$D$_3$ affects APC phenotype. A) Representative histograms for the expression of surface markers on monocytes, DCs and DCs cultured in the presence of 1,25(OH)$_2$D$_3$, as assessed by flow cytometry (shaded). The clear histogram represents isotype control staining. **B)** Bar chart summary of APC surface marker expression (median fluorescence intensity). Error bars show standard deviation (n≥3).

4.3 1,25(OH)$_2$D$_3$ promotes a T$_{Reg}$ phenotype, enhancing CTLA-4 expression, CTLA-4$^+$FoxP3$^+$ frequency and promoting IL-10

To test the hypothesis that 1,25(OH)$_2$D$_3$ might also influence T$_{Reg}$ development, the effect of 1,25(OH)$_2$D$_3$ upon the expression of CTLA-4 and FoxP3, was monitored (**figure 4.6 and 4.7**). This revealed that 1,25(OH)$_2$D$_3$ dramatically up-regulated CTLA-4 protein and mRNA transcripts under both bead and monocyte conditions and was highly significant when analysed for multiple donors. Induction was most dramatic under bead stimulation, fold increases in CTLA-4 MFI being 4.6 and 1.8 for bead and monocyte stimulations respectively (**figure 4.6**). However, only bead stimulations supported a 1,25(OH)$_2$D$_3$-driven increase in FoxP3 mRNA and the frequency of FoxP3$^+$ cells. Furthermore, the CTLA-4$^+$FoxP3$^+$ population doubled for bead stimulated cells in the presence of 1,25(OH)$_2$D$_3$ but 1,25(OH)$_2$D$_3$ did not affect the CTLA-4$^+$FoxP3$^+$ population under monocyte conditions. Analysis of CTLA-4 against CFSE further showed that 1,25(OH)$_2$D$_3$ could up-regulate CTLA-4 in undivided cells (**figure 4.8**), supporting the hypothesis that 1,25(OH)$_2$D$_3$ affects T cell differentiation rather than permitting the selective outgrowth of a population of CTLA-4^{high} cells.

I also investigated the anti-inflammatory activity of 1,25(OH)$_2$D$_3$ upon the regulatory associated cytokine, IL-10. This revealed that 1,25(OH)$_2$D$_3$ enhanced the frequency of IL-10$^+$ cells under bead but not monocyte stimulation (**figure 4.9**). However, IL-10 transcript was increased by 1,25(OH)$_2$D$_3$ in both systems (**figure 4.10**). Overall, these data, together with

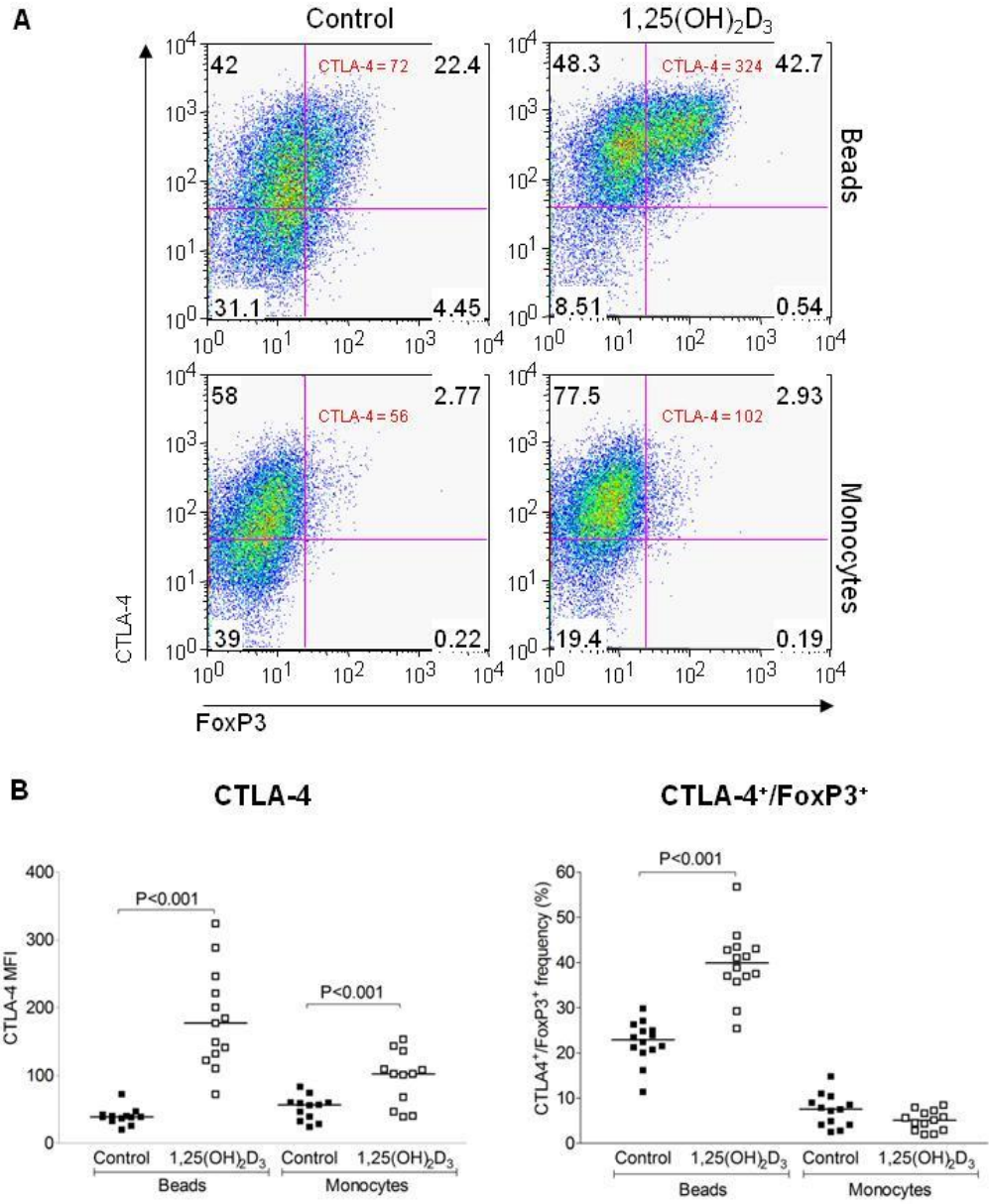

Figure 4.6. CTLA-4 and FoxP3 are directly enhanced by 1,25(OH)$_2$D$_3$. CD4$^+$CD25$^-$ T cells were stimulated with anti-CD3/CD28 beads or monocytes plus anti-CD3 in the presence of 1,25(OH)$_2$D$_3$ or vehicle control. At four days, cells were stained for CD3, total CTLA-4 and FoxP3 and analysed by flow cytometry. **A)** Representative FACS plots from one experiment. Numbers in quadrants refer to percentage of CD3$^+$ cells expressing CTLA-4 or FoxP3. CTLA-4 median fluorescence intensity (MFI) is shown. **B)** CTLA-4 MFI and CTLA4$^+$FoxP3$^+$ frequency data for multiple donors. Horizontal lines indicate median values. Significance was tested by a two-tailed Wilcoxon matched pairs test.

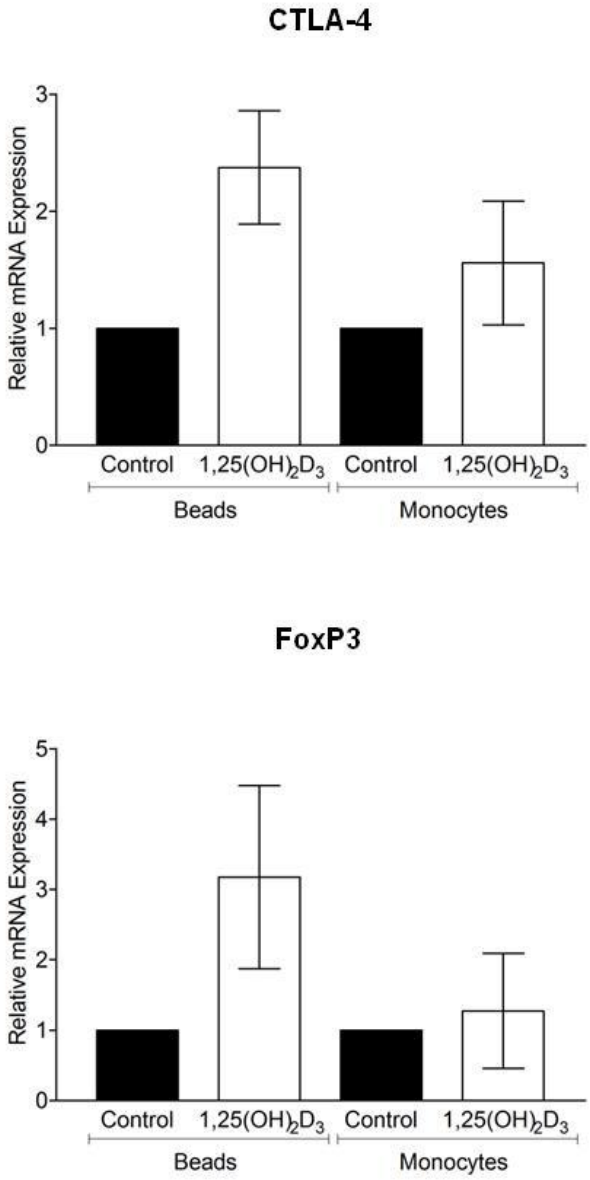

Figure 4.7. 1,25(OH)$_2$D$_3$ directly enhances CTLA-4 and FoxP3 mRNA levels. CD4$^+$CD25$^-$ T cells were stimulated with anti-CD3/CD28 beads or monocytes plus anti-CD3 in the presence of 1,25(OH)$_2$D$_3$ (clear) or vehicle control (black). At four days, cells were re-stimulated and CTLA-4 and FoxP3 expression analysed by quantitative real-time PCR. Mean expression is plotted relative to control. Error bars show standard deviation (n≥3).

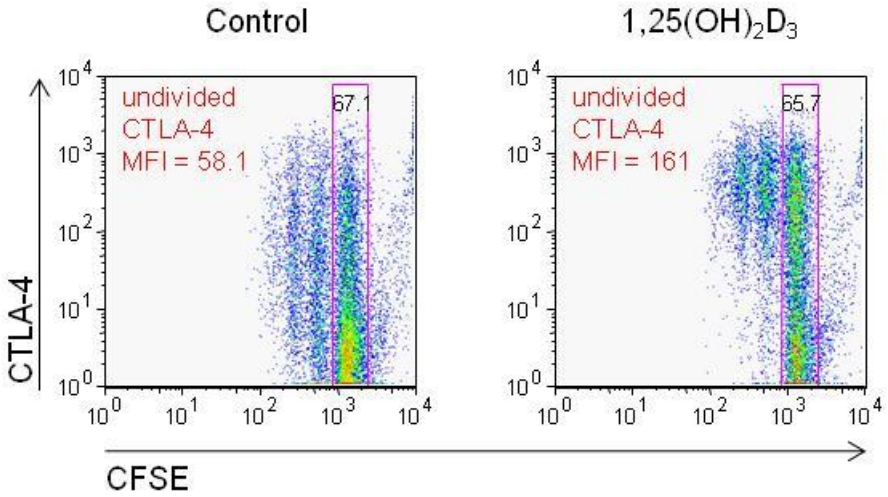

Figure 4.8. 1,25(OH)$_2$D$_3$ increases CTLA-4 expression in undivided cells. CD4$^+$CD25$^-$ T cells were labelled with CFSE and stimulated with anti-CD3/CD28 beads in the presence of 1,25(OH)$_2$D$_3$ or vehicle control. Total CTLA-4 expression against division was analysed at three days by flow cytometry. Undivided cells are gated and their frequency shown. Their median fluorescence intensity (MFI) for CTLA-4 is also given. Data are representative of n>3.

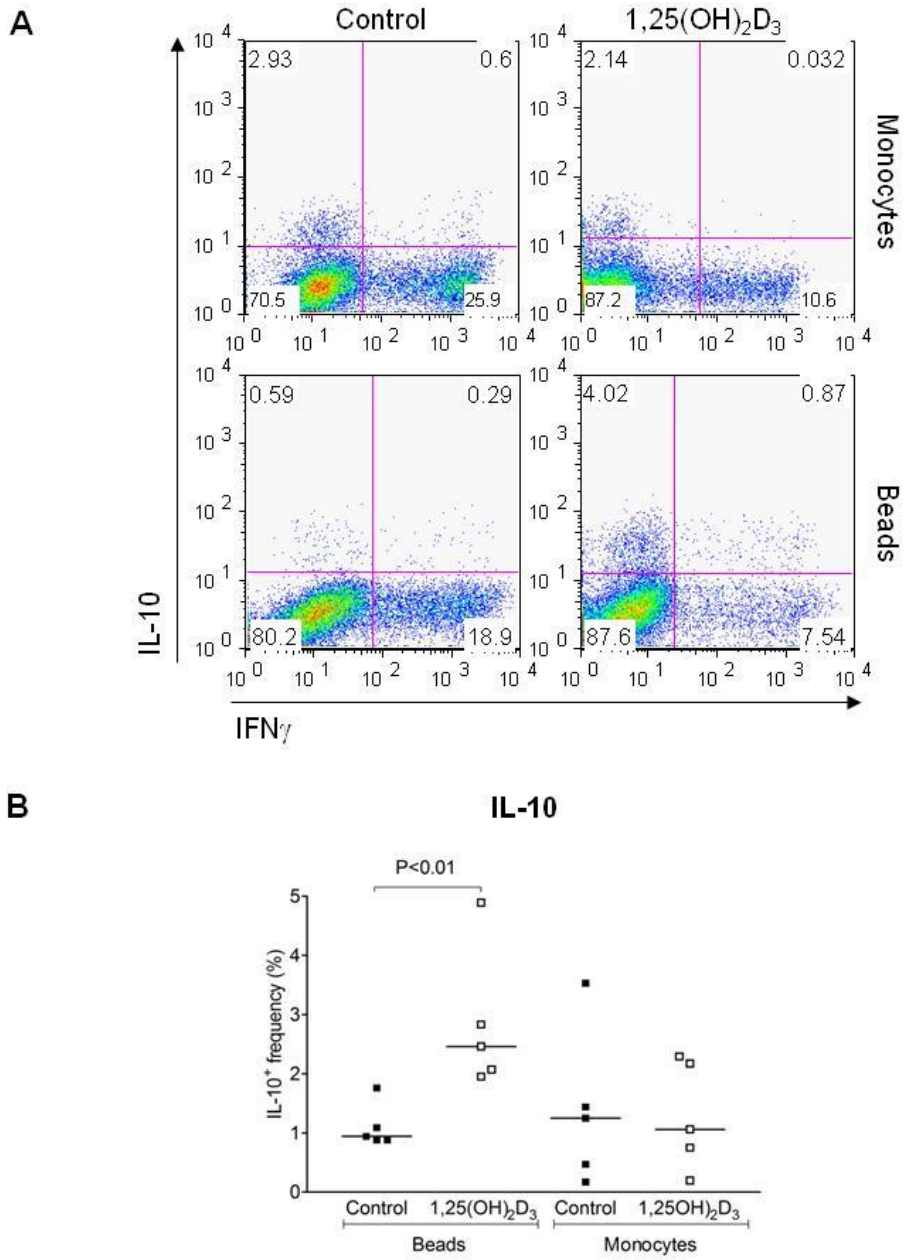

Figure 4.9. 1,25(OH)$_2$D$_3$ increases IL-10 expression by CD4$^+$ T cells. CD4$^+$CD25$^-$ T cells were stimulated with anti-CD3/CD28 beads or monocytes plus anti-CD3 in the presence of 1,25(OH)$_2$D$_3$ or vehicle control. At five days, cells were stained for CD3, IL-10 and IFNγ and analysed by flow cytometry. **A)** Representative FACS plots from one experiment. Numbers in quadrants refer to percentage of CD3$^+$ cells expressing IL-10 or IFNγ. **B)** IL-10$^+$ frequencies for multiple donors. Horizontal lines indicate median frequency. Significance was tested by a two-tailed Wilcoxon matched pairs test.

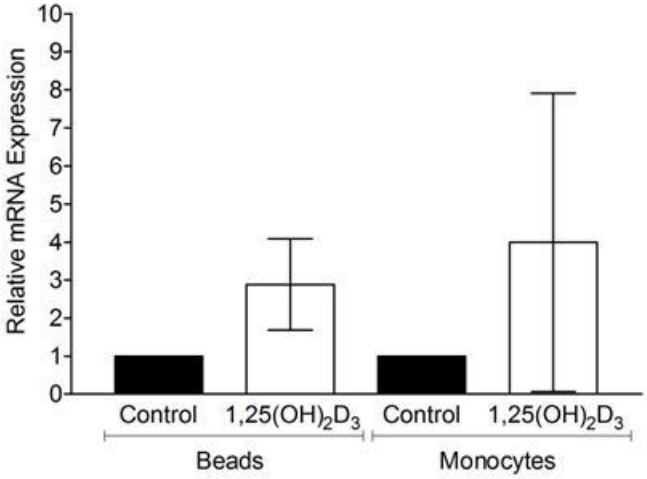

Figure 4.10. 1,25(OH)$_2$D$_3$ directly enhances IL-10 mRNA level. CD4$^+$CD25$^-$ T cells were stimulated with anti-CD3/CD28 beads or monocytes plus anti-CD3 in the presence of 1,25(OH)$_2$D$_3$ (clear) or vehicle control (black). At four days, cells were re-stimulated, and IL-10 expression analysed by quantitative real-time PCR. Mean expression is plotted relative to control. Error bars show standard deviation (n≥3).

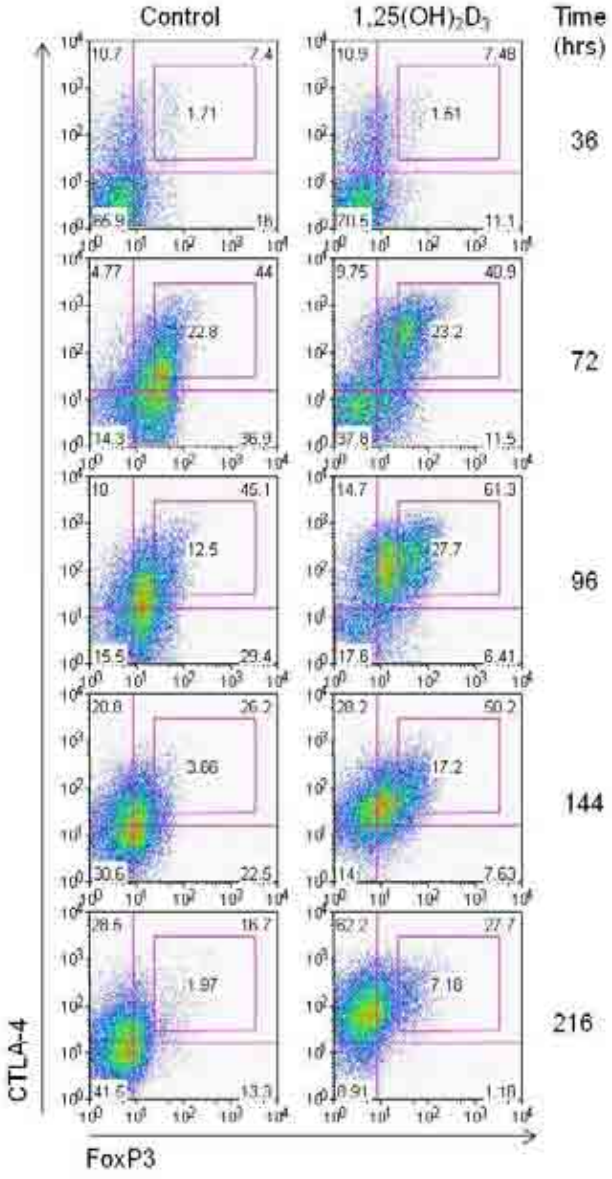

Figure 4.11. 1,25(OH)$_2$D$_3$ does not increase CTLA-4$^+$FoxP3$^+$ phenotype stability during one round of stimulation. CD4$^+$CD25$^-$ T cells were stimulated with anti-CD3/CD28 beads in the presence of 1,25(OH)$_2$D$_3$ or vehicle control. At the indicated times, cells were stained for CD3, total CTLA-4 and FoxP3 and analysed by flow cytometry. Numbers in quadrants refer to percentage of cells expressing CTLA-4 or FoxP3. FACS plots from one experiment representative of three performed are shown. CTLA-4$^+$FoxP3$^+$ cells are indicated by the box in the top right quadrant.

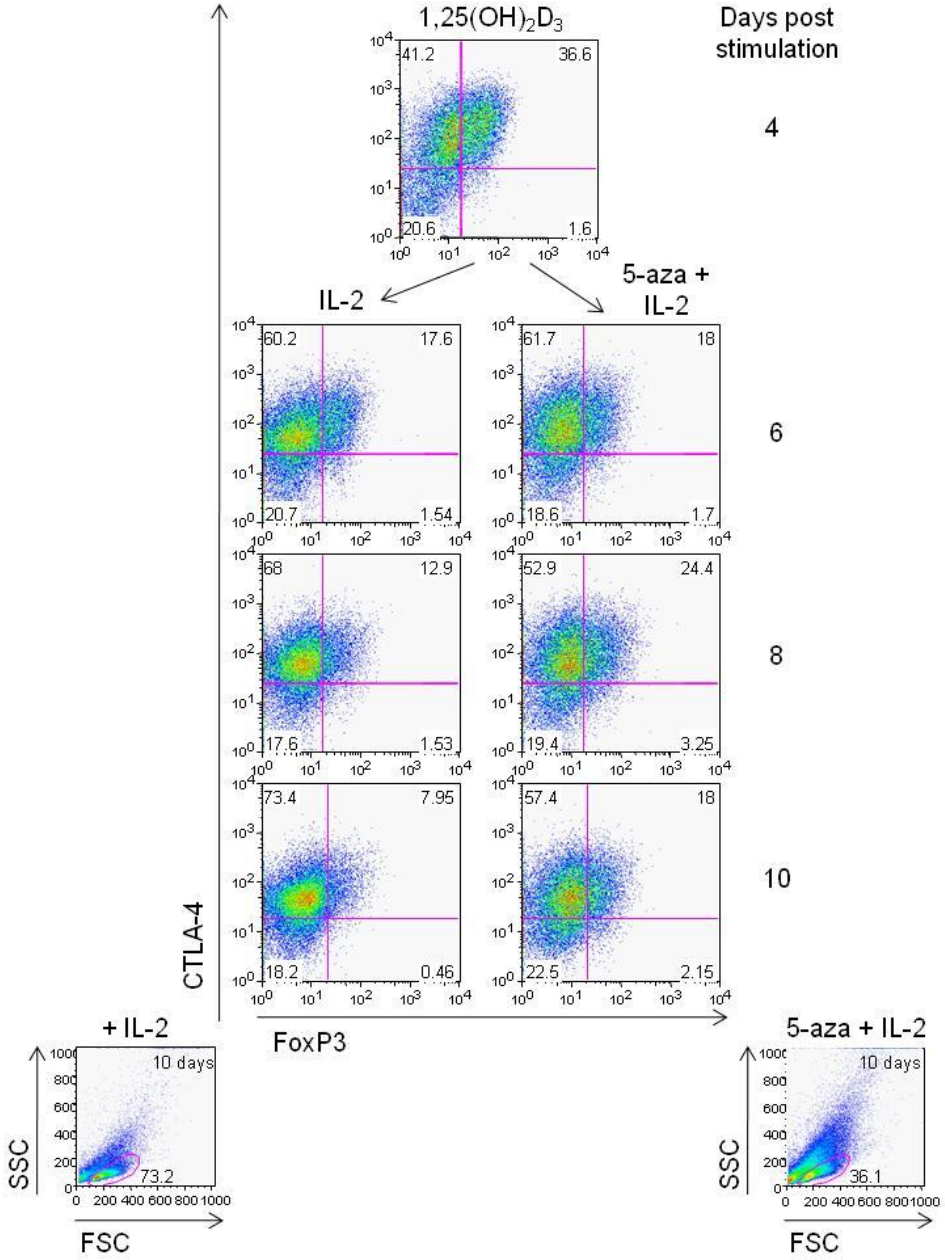

Figure 4.12. 5-aza-2-deoxycytidine reduces the rate of CTLA-4$^+$FoxP3$^+$ decline. CD4$^+$CD25$^-$ T cells were stimulated with anti-CD3/CD28 beads in the presence of 1,25(OH)$_2$D$_3$. At four days cells were treated with IL-2 with or without 5-aza-2-deoxycytidine (5-aza). At the indicated times, cells were stained for total CTLA-4 and FoxP3 and analysed by flow cytometry. FACS plots are from one experiment representative of three performed. FSC vs. SSC plots are shown for ten day cultures. Percentages of live cells based upon scatter are given.

those for CTLA-4$^+$FoxP3$^+$ frequencies, indicate that 1,25(OH)$_2$D$_3$ promotes a T_{Reg} phenotype under bead but not monocyte stimulation.

4.4 1,25(OH)$_2$D$_3$ does not stabilise CTLA-4 or FoxP3 expression.

Although FoxP3 is constitutively expressed at high level by natural T_{Regs} (nT_{Regs}), it appears to be transient in induced T_{Regs} (iT_{Regs}) (Horwitz et al., 2008a). Thus, it was of interest to ascertain whether 1,25(OH)$_2$D$_3$ influenced FoxP3 and CTLA-4 stability. **Figure 4.11** confirms that 1,25(OH)$_2$D$_3$ was not able to prevent the down-regulation of either marker but it did prolong ther expressions.

In nT_{Regs}, demethylation of a region within the FoxP3 locus, the T_{Reg} specific demethylated region (TSDR), is believed to account for the stability of their FoxP3 expression (Floess et al., 2007). Thus, whether the DNA methyltransferase inhibitor, 5-aza-2-deoxycytidine (5-aza), could fix FoxP3 expression by 1,25(OH)$_2$D$_3$-treated cells was investigated. Whilst 5-aza reduced the rate of CTLA-4$^+$FoxP3$^+$ decline, stable CTLA-4$^+$FoxP3$^+$ expression was not achieved (**figure 4.12**). In addition, 5-aza reduced cell survival as evident from FSC versus SSC data.

4.5 1,25(OH)$_2$D$_3$ and IL-2 additively promote a T$_{Reg}$ phenotype

IL-2 is required for the induction and maintenance of FoxP3 expression in the periphery (Davidson et al., 2007). Thus, it was hypothesised that the lack of FoxP3 expression by monocyte-stimulated cells may be a consequence of lower IL-2 at the early stage of these stimulations compared to bead stimulations (**chapter 3, figure 3.6**). Therefore, the effect of IL-2 supplementation upon FoxP3 and CTLA-4 expression was investigated. At IL-2 concentrations of 100U/ml and above, the frequency of CTLA-4$^+$FoxP3$^+$ T cells dramatically increased to 30-50% in 1,25(OH)$_2$D$_3$ treated monocyte cultures (**figure 4.13 and 4.14**). In addition, IL-2 and 1,25(OH)$_2$D$_3$ additively increased CTLA-4 expression. By contrast, even at 200U/ml, IL-2 supplement had limited effect upon the features of bead stimulation (**figure 4.14**). This finding further supported the hypothesis that bead stimulations generate greater IL-2 levels than monocyte stimulations. Corresponding with the T$_{Reg}$ bias under 1,25(OH)$_2$D$_3$ plus IL-2 treatment, IL-17$^+$ and IFNγ^+ frequencies were maintained at a low level under these conditions (**figure 4.15**). Analysis across multiple donors confirmed the significance and reproducibility of these observations. Hence, IL-2 and 1,25(OH)$_2$D$_3$ appear to be a potent cocktail for the induction of CD4$^+$ T cells with regulatory features. It was further observed that periodic IL-2 supplementation throughout one round of stimulation could increase the duration of FoxP3 expression and raise FoxP3 expression frequencies in populations where the level had previously waned. However, combined supplement of 1,25(OH)$_2$D$_3$ and IL-2 could not support a stable CTLA-4$^+$FoxP3$^+$ population.

4.6 Re-stimulation in the presence of 1,25(OH)$_2$D$_3$ and IL-2 can switch the phenotype of a previously T$_h$17 polarised culture to one of regulatory nature

Polarisation of T cell phenotype might be attained by subjecting cells to several rounds of stimulation under differentiation conditions (Grogan et al., 2001). Thus, CD4$^+$CD25$^-$ T cells were passed through two rounds of stimulation with monocytes and anti-CD3 in the presence of 1,25(OH)$_2$D$_3$ and IL-2 to see if this would increase the T$_{Reg}$ polarity of the culture. Although enhanced suppression of IL-17 and IFNγ (**figure 4.16**) and induction of CTLA-4 and FoxP3 occurred (**figure 4.17**), CTLA-4 levels and CTLA-4$^+$FoxP3$^+$ frequencies were lower four days post second stimulation than at four days post primary stimulation. By contrast, repeat stimulation of cells under control conditions dramatically increased the frequency of IL-17$^+$ cells to 30%, and rendered very low levels of CTLA-4 and FoxP3 expression. Interestingly, re-stimulation of cells initially cultured under control conditions in the presence of 1,25(OH)$_2$D$_3$ and IL-2 markedly suppressed IL-17 and IFNγ but increased CTLA-4 and FoxP3. Conversely, primary stimulation in the presence of 1,25(OH)$_2$D$_3$ and IL-2 followed by secondary stimulation under control conditions, permitted increased IL-17$^+$ and IFNγ$^+$ frequencies and suppression of CTLA-4 and FoxP3. These data support an anti-inflammatory, pro-regulatory effect for 1,25(OH)$_2$D$_3$ and IL-2 and confirm that it can switch the phenotype of a previously T$_h$17 polarised culture to one of regulatory nature.

4.7 CD4$^+$ T cells treated with 1,25(OH)$_2$D$_3$ and IL-2 are functionally suppressive

Following observation that 1,25(OH)$_2$D$_3$ and IL-2 enhance T cell expression of regulatory

markers, it was important to determine if cultures enriched with cells of this phenotype were functionally suppressive. Since CTLA-4 was expressed at very high level on 1,25(OH)$_2$D$_3$ treated cells, it was hypothesised that CTLA-4-mediated cell extrinsic mechanisms might contribute greatly to any suppression. Therefore, a suppression assay in which responder T cell division depended upon CD80 or CD86 co-stimulation was designed. Specifically, CD4$^+$CD25$^-$ allogenic responder T cells were labelled with CFSE and stimulated with DCs and anti-CD3. Use of immature DCs (iDCs) versus DCs matured over night with 1μg/ml lipopolysaccharide (LPS) (mDCs) was tested. Suppression effects were greatest when mDCs were used in place of iDCs for stimulation, thus the former were chosen for repeat analyses. DC to responder ratios of 1:5 and 1:10 were also compared because it was hypothesised that suppressor effects might be more apparent when the pool of co-stimulatory ligand was more limited. However, at a 1:10 ratio, responder proliferation was low, thus reducing the power of the assay. Therefore, repeat assays were conducted using mDCs at a 1:5 DC to responder ratio.

During assay development, suppressor to responder ratios of 1:2, 1:4 and 1:10 were also tested (**figure 4.18**). A 1:10 ratio was chosen because although responder division increased as the suppressor: responder ratio decreased, at the highest ratios competitive proliferation by 1,25(OH)$_2$D$_3$ plus IL-2 and untreated suppressor T cells seemed to contribute greatly to the suppression. Whilst this effect was of interest, suggesting that activated T cells can behave as suppressor cells, the lowest ratio of 1:10 enabled the involvement of 1,25(OH)$_2$D$_3$ plus IL-2 induced regulatory features to be observed. **Figure 4.19** confirms that in repeat experiments 1,25(OH)$_2$D$_3$ plus IL-2 conditioned T cell cultures suppressed responder T cell proliferation to

a greater extent than untreated controls. However, untreated control T cells reduced responder division when compared to responders alone.

4.8 1,25(OH)$_2$D$_3$ modifies T cell phenotypes at physiologically relevant concentrations

The concentration of 1,25(OH)$_2$D$_3$ used in the studies described so far was 100nM. However, serum 1,25(OH)$_2$D$_3$ approximates 0.1nM (Mullin and Dobs, 2007) and it is estimated that mDCs synthesise 1,25(OH)$_2$D$_3$ from 25(OH)D$_3$ *in-vitro* to a maximal concentration of 5nM (Fritsche et al., 2003). Thus, within secondary lymphoid tissues or at inflamed sites 1,25(OH)$_2$D$_3$ concentrations might lie within the range 1-10nM. 1,25(OH)$_2$D$_3$ was therefore titrated over the range 1-100nM and its effects upon IL-17 and IFNγ monitored. **Figure 4.20** demonstrates that even at 1nM, 1,25(OH)$_2$D$_3$ suppressed IL-17$^+$ and IFNγ$^+$ frequencies. Thus the effects of 1,25(OH)$_2$D$_3$ reported in this chapter are likely to be relevant *in-vivo*. Nonetheless, since T cell responses to 1,25(OH)$_2$D$_3$ were evident at concentrations lower than 10nM, it was felt appropriate to reduce the 1,25(OH)$_2$D$_3$ concentration in future studies from 100nM to 10nM.

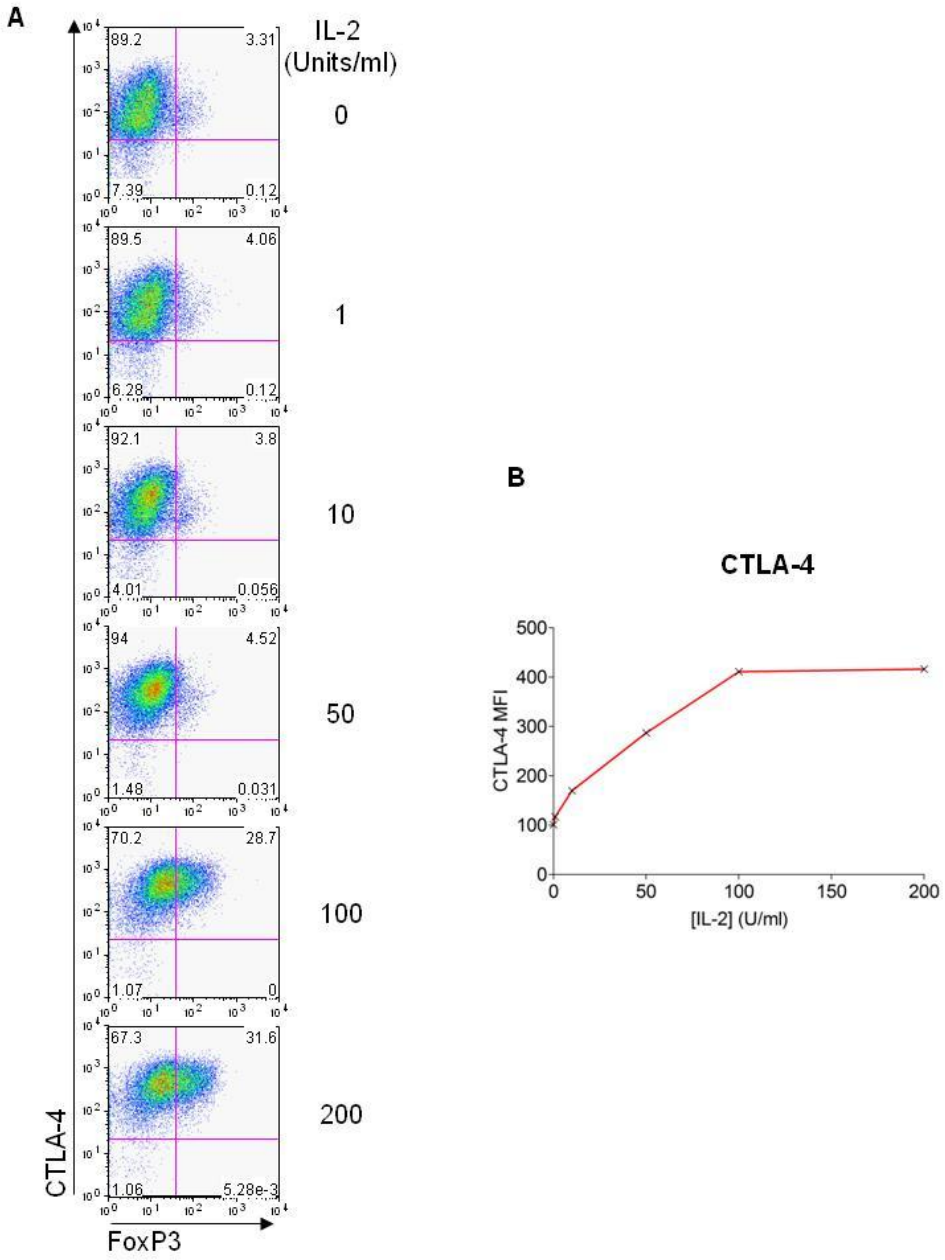

Figure 4.13. The effect of IL-2 upon CTLA-4 and FoxP3. CD4$^+$CD25$^-$ T cells were stimulated with monocytes plus anti-CD3 in the presence of 1,25(OH)$_2$D$_3$ and IL-2 concentrations over the range 0-200U/ml. At four days, cells were stained for CD3, CTLA-4 and FoxP3 and analysed by flow cytometry. **A)** Data are from one experiment representative of two performed. Numbers in quadrants refer to percentage of CD3$^+$ cells expressing CTLA-4 or FoxP3. **B)** CTLA-4 median fluorescence intensity (MFI) from data in **A**.

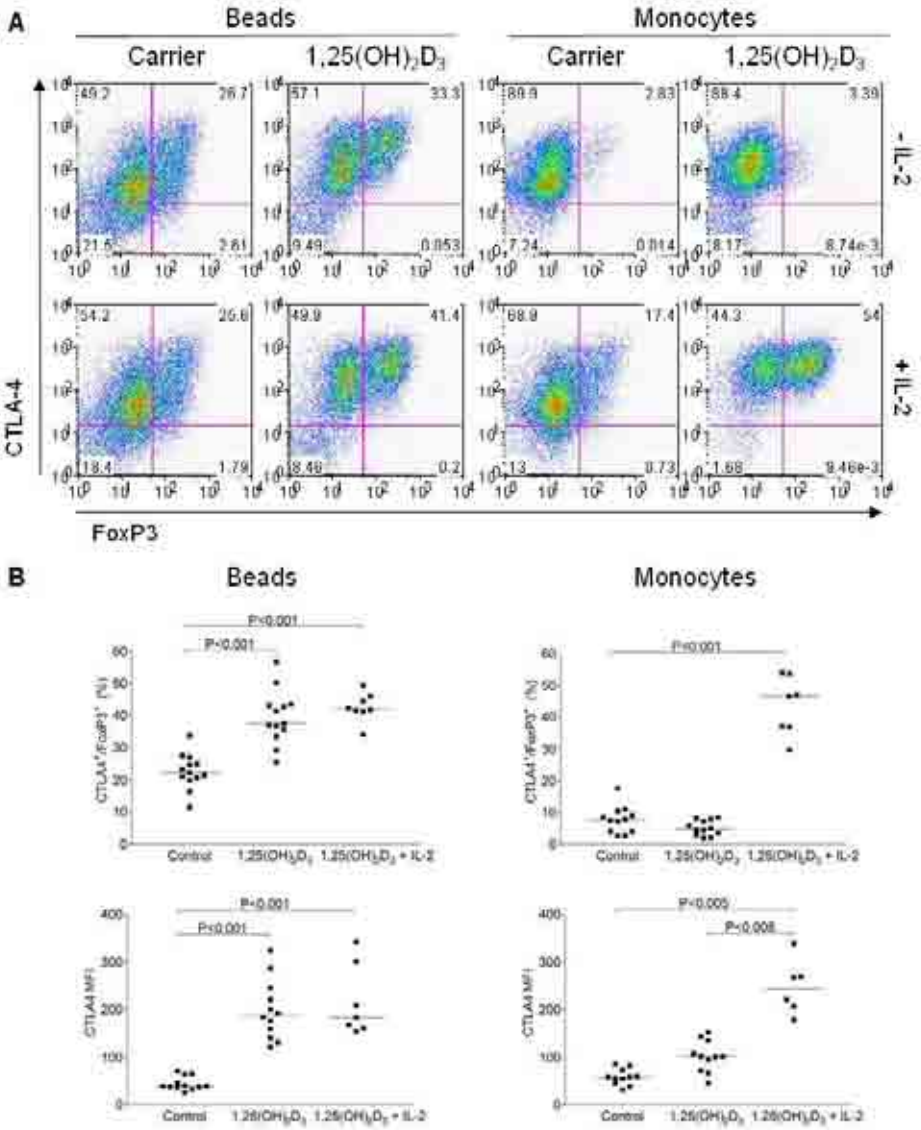

Figure 4.14. 1,25(OH)$_2$D$_3$ and IL-2 additively promote a regulatory phenotype. CD4$^+$CD25$^-$ T cells were stimulated with anti-CD3/CD28 beads or monocytes plus anti-CD3 in the presence of 1,25(OH)$_2$D$_3$ or vehicle control with or without IL-2 (200Units/ml). At four days cells were stained for CD3, total CTLA-4 and FoxP3 and analysed by flow cytometry. **A)** Representative FACS plots from one experiment. Numbers in quadrants refer to percentage of CD3$^+$ cells expressing CTLA-4 or FoxP3. **B)** CTLA4$^+$FoxP3$^+$ frequency and CTLA-4 median fluorescence intensity data for multiple donors. Horizontal lines indicate median values. Significance was tested by a two-tailed Wilcoxon matched pairs test.

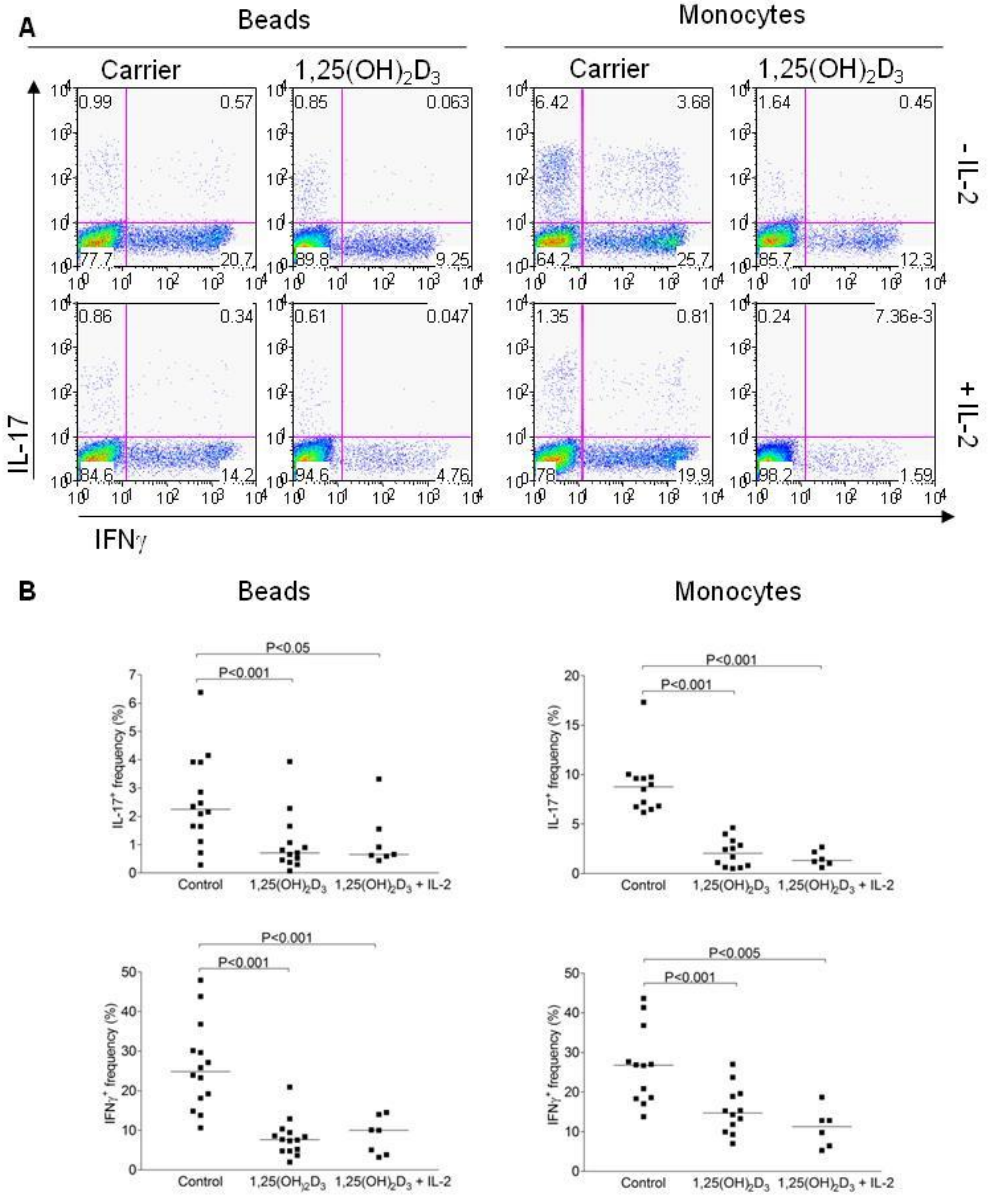

Figure 4.15. 1,25(OH)$_2$D$_3$ and IL-2 inhibit IL-17 and IFNγ expression. CD4$^+$CD25$^-$ T cells were stimulated with anti-CD3/CD28 beads or monocytes plus anti-CD3 in the presence of 1,25(OH)$_2$D$_3$ or vehicle control with or without IL-2 (200Units/ml). At five days, cells were stained for CD3, IFNγ and IL-17 and analysed by flow cytometry. **A)** Representative FACS plots from one experiment. Numbers in quadrants refer to percentage of CD3$^+$ cells expressing IL-17 or IFNγ. **B)** IL-17$^+$ and IFNγ$^+$ frequencies for multiple donors. Horizontal lines indicate median frequencies. Significance was tested by a two-tailed Wilcoxon matched pairs test.

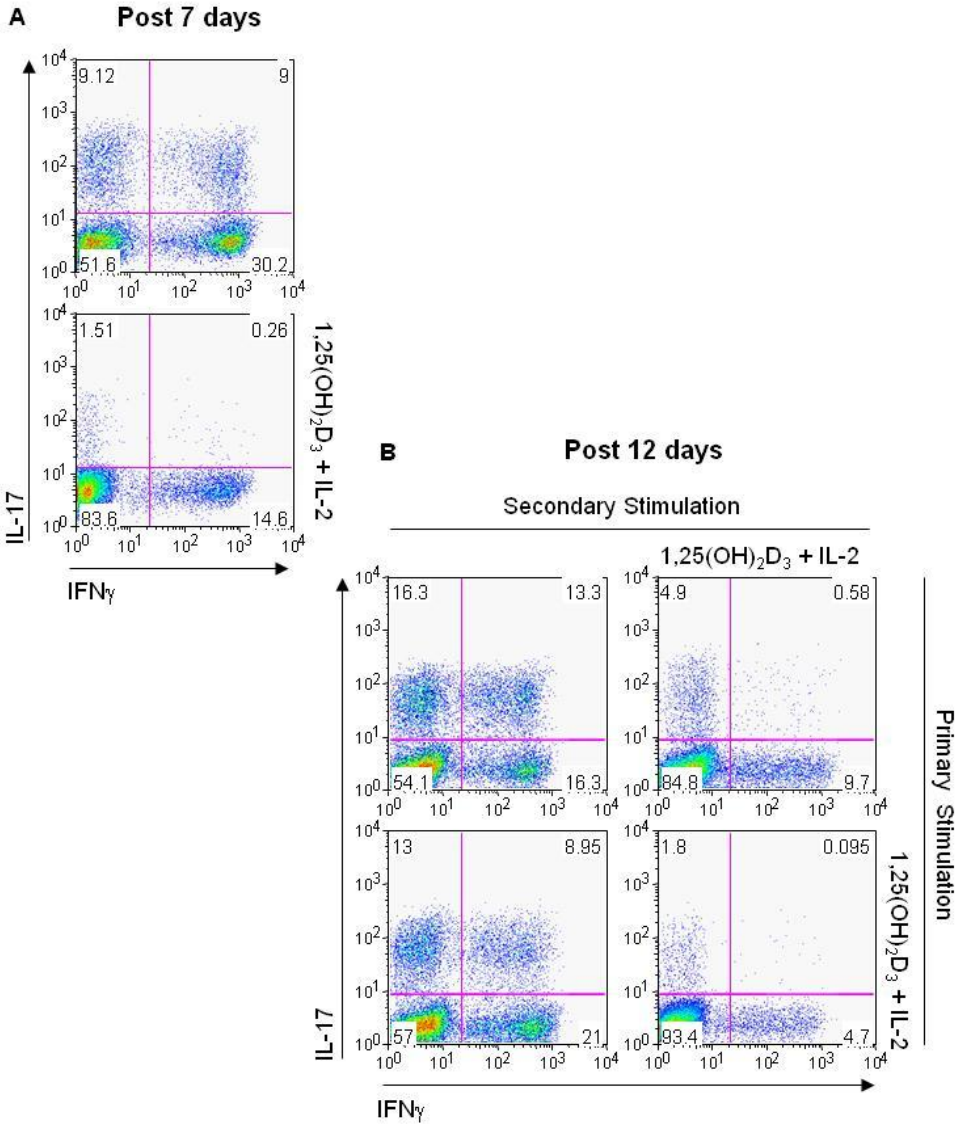

Figure 4.16. IL-17 and IFNγ are suppressed by 1,25(OH)$_2$D$_3$ and IL-2 during a second round of stimulation. CD4$^+$CD25$^-$ T cells were stimulated with monocytes plus anti-CD3 in the presence or absence of 1,25(OH)$_2$D$_3$ plus IL-2 for seven days. IL-2 treated cells were supplemented with IL-2 at day four. At seven days, cells were stimulated a second time with monocytes plus anti-CD3 under the conditions shown. Cells were stained for CD3, IL-17 and IFNγ at the end of the first stimulation period **(A)** and five days after second stimulation **(B)**. Numbers in quadrants refer to percentage of CD3$^+$ cells expressing IL-17 or IFNγ. Data are from a single experiment, representative of three performed.

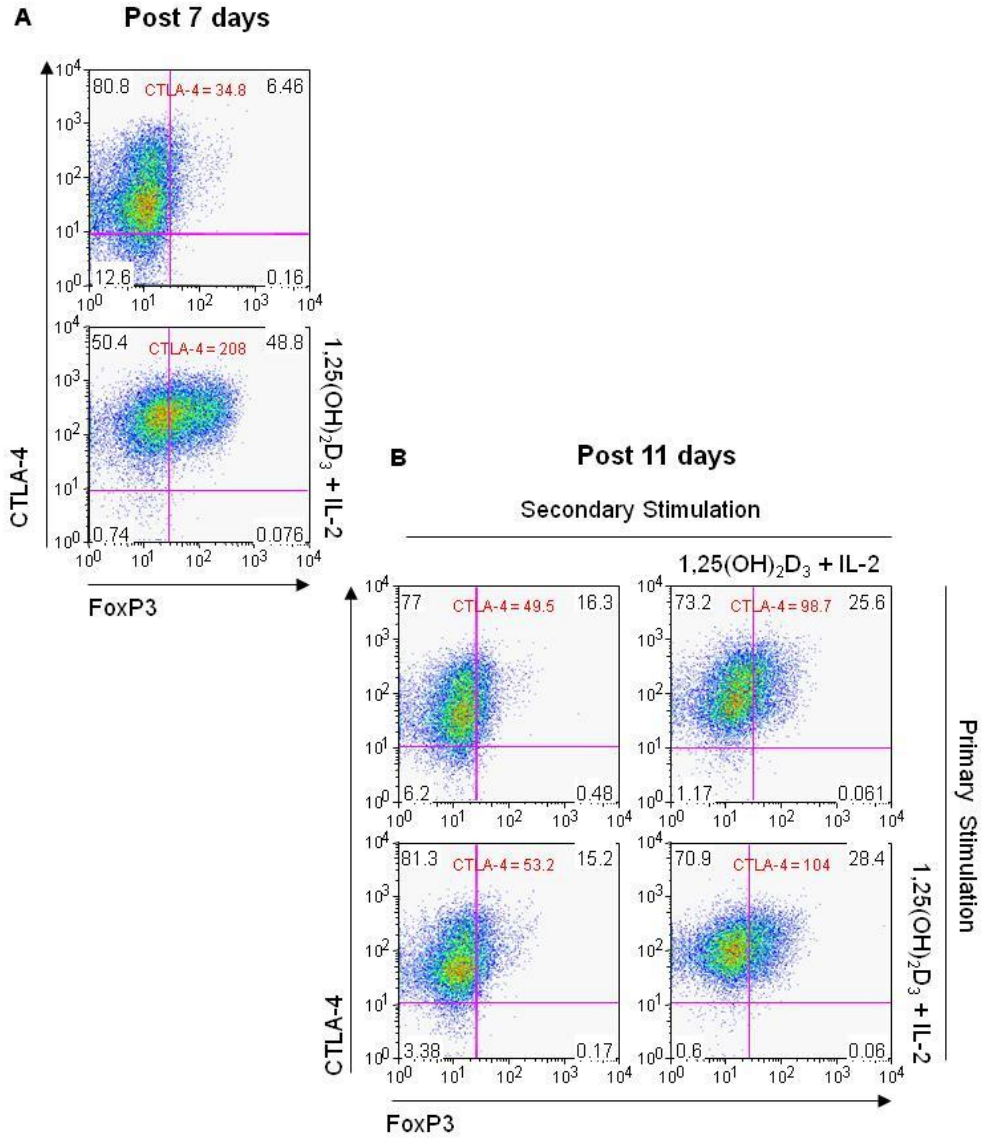

Figure 4.17. 1,25(OH)$_2$D$_3$ and IL-2 promote CTLA-4 and FoxP3 though a second round of stimulation. CD4$^+$CD25$^-$ T cells were stimulated with monocytes plus anti-CD3 in the presence or absence of 1,25(OH)$_2$D$_3$ plus IL-2 for seven days. IL-2 treated cells were supplemented with IL-2 at day four. At seven days, cells were stimulated a second time with monocytes plus anti-CD3 under the conditions shown. Cells were stained for CD3, total CTLA-4 and FoxP3 at the end of the first stimulation period **(A)** and four days after second stimulation **(B)**. Numbers in quadrants refer to percentage of CD3$^+$ cells expressing CTLA-4 or FoxP3 and CTLA-4 median fluorescence intensity (MFI) is shown. Data are from a single experiment, representative of three performed.

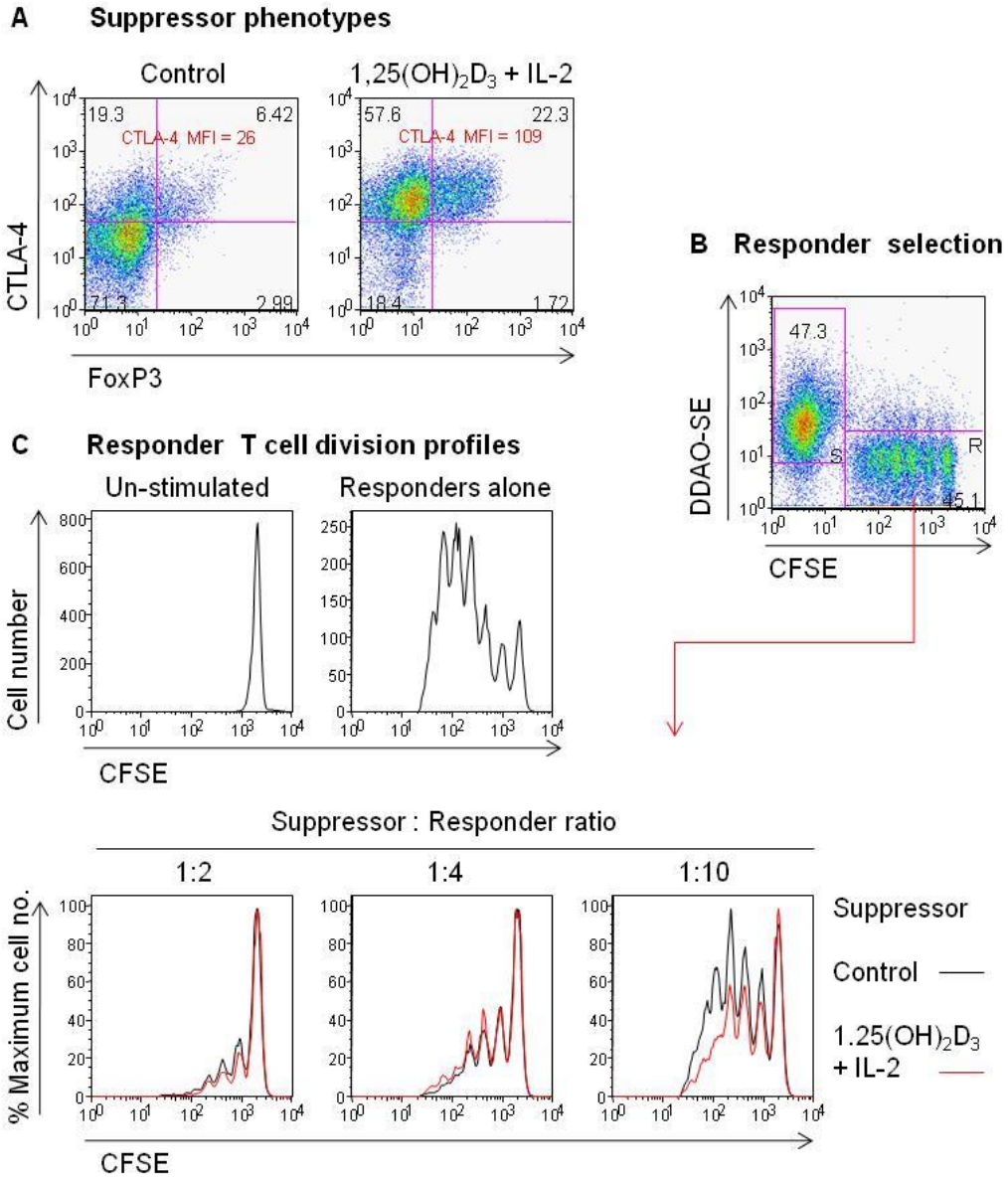

Figure 4.18. 1,25(OH)$_2$D$_3$ and IL-2 conditioned T cells are suppressive. Suppressor T cells (S) were generated by stimulation with monocytes in the presence or absence of 1,25(OH)$_2$D$_3$ and IL-2. Their CTLA-4/FoxP3 phenotype is shown in **(A)**. Suppressors were labelled with DDAO-SE and added to dendritic cell + anti-CD3 driven stimulations of CFSE labelled CD4$^+$CD25$^-$ responder T cells (R), prepared as 1 DC : 5 responders. At five days post stimulation, responder division was analysed by CFSE dilution in flow cytometry. Suppressors were excluded from the analysis **(B)**. Division by responders at different suppressor to responder ratios is shown in **(C)**. Data are from a single experiment, representative of four performed.

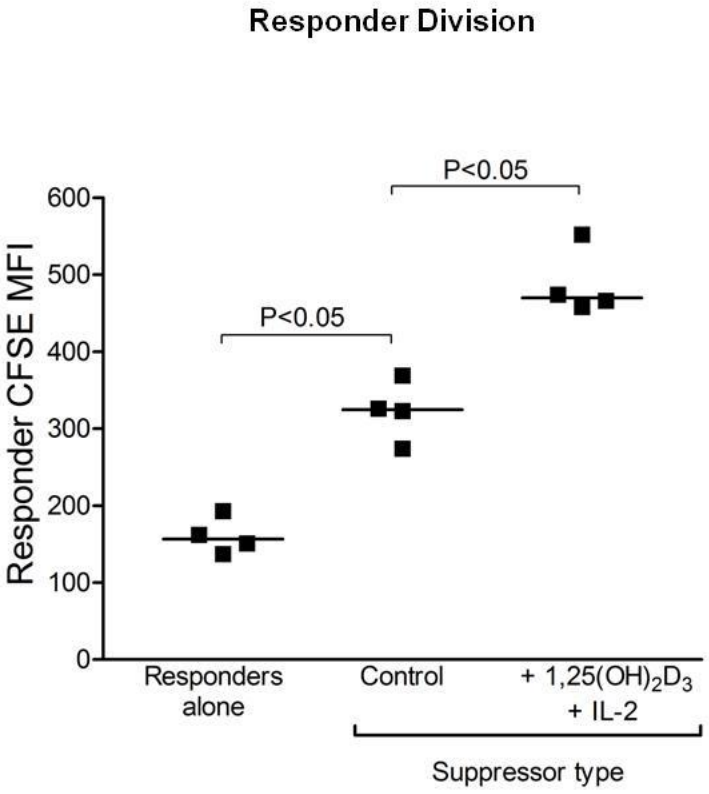

Figure 4.19. T cells cultured in the presence of 1,25(OH)$_2$D$_3$ and IL-2 suppress responder T cell division. Suppressor T cells cultured in the presence or absence of 1,25(OH)$_2$D$_3$ and IL-2 were added to dendritic cell + anti-CD3 driven stimulations of CFSE labelled CD4$^+$CD25$^-$ responder T cells at a 1:10 suppressor : responder ratio. Five days post stimulation, responder division was analysed by CFSE dilution in flow cytometry. Data from several experiments reported as CFSE median fluorescence intensity (MFI) are shown. Horizontal lines show median values. Significance was tested by a two-tailed Mann Whitney U test.

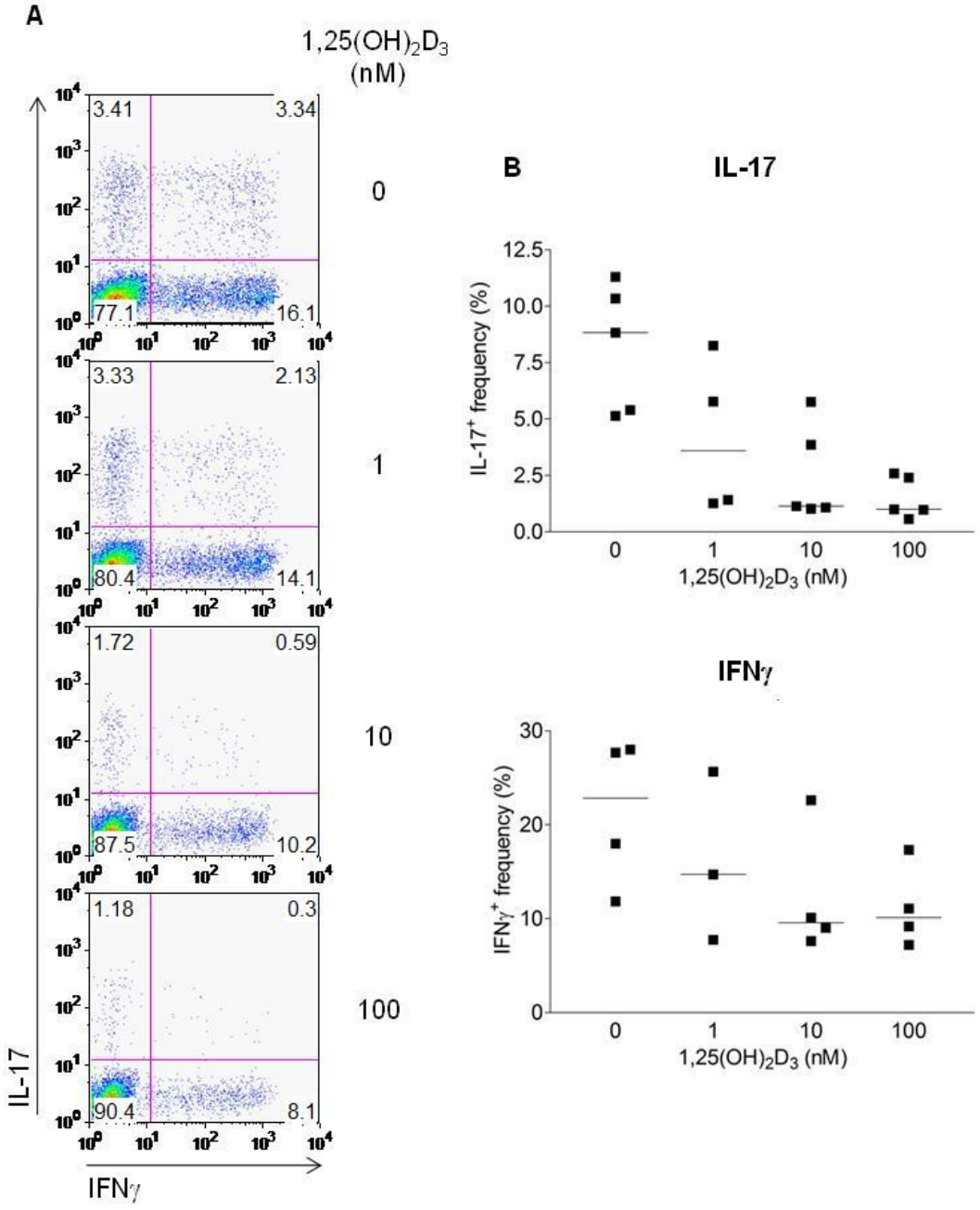

Figure 4.20. 1,25(OH)$_2$D$_3$ concentration effects upon IL-17 and IFNγ. CD4$^+$CD25$^-$ T cells were stimulated with monocytes plus anti-CD3 in the presence of 1,25(OH)$_2$D$_3$ over the concentration range 0-100nM. At five days, cells were stained for CD3, IL-17 and IFNγ and analysed by flow cytometry. **A)** Representative FACS plots from one experiment. Numbers in quadrants refer to percentage of CD3$^+$ cells. **(B)** The effect of 1,25(OH)$_2$D$_3$ concentration upon IL-17 and IFNγ frequencies for multiple donors. Horizontal lines indicate median frequencies.

4.9 Discussion

Studies in this chapter examined the effect of 1,25(OH)$_2$D$_3$ upon CD4$^+$ T cell proliferation and differentiation towards inflammatory versus regulatory lineages. In addition by using monocyte and bead stimulation systems in parallel, direct effects of 1,25(OH)$_2$D$_3$ on T cell responses and indirect effects through the APC were investigated.

Marked 1,25(OH)$_2$D$_3$-mediated suppression of CD4$^+$ T cell proliferation was observed in the presence of monocytes. However, under stimulation with beads no difference in proliferation between control and 1,25(OH)$_2$D$_3$ treatment was found. Recently, Ikeda et al. similarly suggested that 1,25(OH)$_2$D$_3$ does not directly affect T cell proliferation (Ikeda et al., 2010). Thus, 1,25(OH)$_2$D$_3$ does not appear to suppress T cell proliferation directly but indirectly through the APC. Potentially the ability of 1,25(OH)$_2$D$_3$ to down-regulate HLA and co-stimulatory molecule expression by the APC as was shown here and by others (Berer et al., 2000; Canning et al., 2001; Penna and Adorini, 2000), might be mechanistically relevant in this context.

In contrast to proliferation, the impact of 1,25(OH)$_2$D$_3$ upon T cell differentiation was seen for both monocyte and bead stimulations, with strong suppression of the pro-inflammatory cytokines IL-17, IL-21 and IFNγ being seen. All three cytokines are associated with the T$_h$17 lineage, although IFNγ and IL-21 are also markers of T$_h$1 and T$_{fh}$ lineages respectively (Mosmann and Coffman, 1989; Vogelzang et al., 2008). Thus 1,25(OH)$_2$D$_3$ could affect differentiation to each of these lineages. Inhibition of T$_h$17 development by 1,25(OH)$_2$D$_3$ is

supported by recent studies in which injection of 1,25(OH)$_2$D$_3$ reduced expression of IL-17, IL-23p19 and IL-6 in the colons of mice with colitis. This corresponded with reduced disease severity (Daniel et al., 2008). In another study, Tang et al. (2009) observed direct 1,25(OH)$_2$D$_3$-mediated suppression of IL-17 by CD4$^+$ mouse T cells *in-vitro* and the ability of oral 1,25(OH)$_2$D$_3$ to reduce IL-17 secretion by CD4$^+$ T cells in the experimental autoimmune uveitis (EAU) model was shown. In addition, T_h17 suppression in the EAU model was associated with prevention and partial reversion of disease (Tang et al., 2009). These data therefore support the relevance of 1,25(OH)$_2$D$_3$ mediated T_h17 suppression *in-vivo*. Subsequent to the publication of data from this chapter (Jeffery et al., 2009), the direct inhibitory effect of 1,25(OH)$_2$D$_3$ upon human T_h17 cells was demonstrated by others (Ikeda et al., 2010).

Rather than simply globally inhibiting T cell differentiation, I observed that 1,25(OH)$_2$D$_3$ dramatically up-regulates CTLA-4 expression and functions with IL-2 to yield a culture enriched for CTLA4$^+$FoxP3$^+$ cells. Such cultures were able to suppress the division of bystander CD4$^+$CD25$^-$ T cells. In addition, 1,25(OH)$_2$D$_3$ increased regulatory associated IL-10 by bead stimulated cells which is consistent with others' observations (Barrat et al., 2002; Urry et al., 2009). Therefore, 1,25(OH)$_2$D$_3$ appears to counter-regulate inflammatory and regulatory T cell states.

The regulatory effect of 1,25(OH)$_2$D$_3$ that I observed is consistent with results from several *in-vivo* studies. For example Mathieu et al. demonstrated that co-transfer of splenocytes from 1,25(OH)$_2$D$_3$-treated non-obese diabetic (NOD) mice prevented diabetes caused by the

transfer of splenocytes from diabetic NOD mice (Mathieu et al., 1994). More recently the 1,25(OH)$_2$D$_3$ analogue, Ro-26-2198, was shown to arrest diabetes in NOD mice. This response corresponded with an increase in the frequency of CD4$^+$CD25$^+$ T cells in the pancreatic lymph node (Gregori et al., 2002). In addition, oral 1,25(OH)$_2$D$_3$ induced tolerance to islet allografts associated with down-regulation of co-stimulatory molecules on DCs and macrophages surrounding the graft. In addition an increase in the frequency of CD4$^+$CD25$^+$ cells that were able to prevent islet graft rejection was reported (Gregori et al., 2001). Although the FoxP3 status of these cells was not assessed, increased CTLA-4 expression was found. More recently it was reported that immunization through skin treated with the 1,25(OH)$_2$D$_3$ analogue, calcipotriol, induced expansion of antigen specific CD4$^+$CD25$^+$FoxP3$^+$ T cells, that had greater CTLA-4 expression than controls (Ghoreishi et al., 2009). Lastly, the relevance of enhanced CTLA-4 expression by 1,25(OH)$_2$D$_3$ is consistent with the finding that T$_{Reg}$ cells from sarcoidosis patients in whom 1,25(OH)$_2$D$_3$ production is excessive, have high CTLA-4 expression and enhanced activity (Miyara et al., 2006).

In the studies cited above, the induction of CD4$^+$ T cells with regulatory phenotype was largely attributed to the well-established tolerogenic effect of 1,25(OH)$_2$D$_3$ upon DCs. The relevance of indirect regulation is supported by the finding that alloreactive T cells co-cultured with 1,25(OH)$_2$D$_3$ conditioned DCs became hypo-responsive, marked by suppressed IFNγ production upon re-stimulation with untreated mature DCs. They also reported up-regulation of CTLA-4 by T cells stimulated by 1,25(OH)$_2$D$_3$-conditioned DCs (Penna and Adorini, 2000). However, the extent of this up-regulation was modest compared to the fold changes I observed. Furthermore, they did not address the ability of 1,25(OH)$_2$D$_3$ to induce

CTLA-4 by direct action upon the T cell. Overall, my data together with earlier findings, indicate that 1,25(OH)$_2$D$_3$ acts upon CD4$^+$ T cells via both direct and indirect mechanisms to create a suppressive immunological environment.

One of the most striking observations in this chapter was the extent to which 1,25(OH)$_2$D$_3$ could up-regulate CTLA-4. This ability might be important in the mechanism by which 1,25(OH)$_2$D$_3$ promotes iT$_{Reg}$ development because it is suggested that CTLA-4 up regulation early after T cell activation is required for iT$_{Reg}$ formation (Zheng et al., 2006). However, nT$_{Reg}$ development does not appear to require CTLA-4, as CTLA-4 knockout mice have nT$_{Regs}$, albeit of impaired function (Kolar et al., 2009; Schmidt et al., 2009). My observation that 1,25(OH)$_2$D$_3$ enhanced CTLA-4 expression by monocyte stimulated T cells but did not increase FoxP3 expression in the absence of IL-2 could suggest that CTLA-4 alone is not central to FoxP3 induction. In addition, if CTLA-4 promotes FoxP3 expression it might be expected that 1,25(OH)$_2$D$_3$ would stabilise FoxP3 expression longer-term but I did not observe this. Rather, my data support the finding that CTLA-4 over-expression in human T cells could not enhance FoxP3 expression (Zheng et al., 2008b) implying that CTLA-4 is not required for FoxP3 expression. An alternative model for iT$_{Reg}$ development suggests that FoxP3 is required for CTLA-4 expression (Yagi et al., 2004). Thus, if a way to fix FoxP3 could be determined, CTLA-4 retention might be possible. Several studies have demonstrated the importance of CTLA-4 for T$_{Reg}$ function (Friedline et al., 2009; Kolar et al., 2009; Onishi et al., 2008; Read et al., 2006; Schmidt et al., 2009; Tivol et al., 1995; Waterhouse et al., 1995; Wing et al., 2008; Zheng et al., 2008b). Thus, by coupling the ability of 1,25(OH)$_2$D$_3$ to promote high CTLA-4 expression with a mechanism to fix FoxP3, an efficient and stable T$_{Reg}$ population might be generated.

FoxP3 maintenance by nT_{Regs} has been attributed to selective demethylation of a CpG rich intronic region within the FoxP3 locus (Floess et al., 2007) and a highly conserved non-intronic upstream enhancer region (Lal et al., 2009). Other epigenetic modifications unique to nT_{Regs} have also been found at this enhancer site (Lal et al., 2009). Treatment with the DNA methyltransferase inhibitor 5-axa-2-deoxycytidine (5-aza) resulted in demethylation of these regions in mice CD4$^+$CD25$^-$ T cells and led to strong FoxP3 expression in the presence and absence of TGFβ. Enhanced FoxP3 expression in stimulated human CD4$^+$CD25$^-$ T cells was also reported at four days following 5-aza treatment and these cells were functionally more suppressive than TGFβ treated control T cells (Lal et al., 2009). I therefore investigated whether 5-aza could stabilise the 1,25(OH)$_2$D$_3$-induced CTLA4$^+$FoxP3$^+$ phenotype. Although 5-aza did decrease the rate of CTLA4$^+$FoxP3$^+$ decline, long-term stabilisation was not achieved. Possibly, the absence of TGFβ supplement in the culture might have limited the duration of FoxP3 expression, although TGFβ effects are to some extent provided by serum (Manel et al., 2008). Furthermore, others suggest that TGFβ is not required for FoxP3 induction by 5-aza (Lal et al., 2009; Polansky et al., 2008). Further work is therefore required to confirm if 5-aza does induce stable demethylation at the FoxP3 locus in human T cells and to determine if there are conditions that can permit long-term maintenance of CTLA-4$^+$FoxP3$^+$ cells.

In this chapter re-stimulation in the presence of 1,25(OH)$_2$D$_3$ and IL-2 was also investigated as a means to 'fix' the CTLA-4$^+$FoxP3$^+$ phenotype. Whilst by this method a second rise in CTLA-4 expression and the induction of FoxP3 occurred upon secondary stimulation, CTLA-4$^+$FoxP3$^+$ frequencies and the magnitude of the CTLA-4 increase were lower than by primary

stimulation. 1,25(OH)$_2$D$_3$ could also suppress IL-17 and IFNγ in T_h17 polarised cultures. These findings are encouraging in the context of disease treatment and are consistent with the ability of 1,25(OH)$_2$D$_3$ supplement to reduce disease symptoms in models of autoimmune disease (reviewed in (Adorini, 2002; Arnson et al., 2007; Mullin and Dobs, 2007; Smolders et al., 2008). However, my re-stimulation experiments do not definitively demonstrate the action of 1,25(OH)$_2$D$_3$ upon previously committed cells but might represent the selective outgrowth of CTLA-4$^+$FoxP3$^+$ cells balanced by a decline of inflammatory effector cells. Since I observed that 1,25(OH)$_2$D$_3$ could induce CTLA-4 in undivided cells, selective outgrowth alone cannot account for the increase in CTLA-4 in response to 1,25(OH)$_2$D$_3$.

The rapid effect of 1,25(OH)$_2$D$_3$ upon gene expressions is consistent with a mechanism of direct gene regulation. This involves the heterodimeric binding of the vitamin D receptor (VDR) with the retinoid-X-receptor (RXR) to vitamin D response elements (VDREs) within the regulatory portions of target genes (Deeb et al., 2007; Haussler et al., 2010). Very recently, the results of a ChIP-seq-defined genome wide map of VDR binding sites in lymphoblastoid cell lines (LCLs) were published (Ramagopalan et al., 2010). This study identified 2776 VDR binding sites in 1,25(OH)$_2$D$_3$-treated LCLs compared to 623 in untreated LCLs. The binding sites were particularly enriched in intergenic or intronic regions. Consistent with the inhibitory effect of vitamin D upon autoimmune disease, VDR binding sites were enriched in a number of regions previously associated with disease. The *ctla-4* locus at chromosome 2q33 was one such region (Kristiansen et al., 2000; Ramagopalan et al., 2010; Scalapino and Daikh, 2008), thus CTLA4 transcription might be directly regulated by VDR binding. Genes encoding other CD28 family members, CD28 and ICOS, lie either side of the *ctla-4* gene locus at this locus. Interestingly, I have also observed that 1,25(OH)$_2$D$_3$ can

up-regulate CD28 as well as CTLA-4, thus, VDR might co-regulate genes at this locus. However, I have not detected enhanced expression of ICOS by 1,25(OH)$_2$D$_3$. Therefore, regulation by VDR might not extend 3' of *ctla-4* at the 2q33 locus. VDR binding sites have already been characterised in the promoters of genes encoding IFNγ (Cippitelli and Santoni, 1998) and IL-10 (Matilainen et al., 2010). Thus, both pro-inflammatory and regulatory associated genes might be primary VDR targets. However, secondary responses could contribute to the T helper to T_{Reg} switch afforded by 1,25(OH)$_2$D$_3$. Consistent with this, 1,25(OH)$_2$D$_3$ suppressed transcript expressions of receptors for IL-1, IL-6, IL-21 and IL-23 by CD4$^+$ T cells (Ikeda et al., 2010). Thus, 1,25(OH)$_2$D$_3$ could inhibit T_h17 induction and promote T_{Reg} development by suppressing the T cell's response to pro-T_h17 cytokines .

In this chapter, the function of 1,25(OH)$_2$D$_3$ plus IL-2 treated T cells was also assessed. Whilst 1,25(OH)$_2$D$_3$ plus IL-2 treated T cells reduced responder proliferation compared to control suppressor T cells, control T cells also reduced responder proliferation. The greater suppressive effect of 1,25(OH)$_2$D$_3$ plus IL-2 treated cells was most apparent at lower suppressor to responder ratios. As mentioned, CTLA-4 is thought to play an important role in suppression by T_{Regs} (Friedline et al., 2009; Kolar et al., 2009; Onishi et al., 2008; Read et al., 2006; Schmidt et al., 2009; Tivol et al., 1995; Waterhouse et al., 1995; Wing et al., 2008; Zheng et al., 2008b). Hence, its elevated expression by 1,25(OH)$_2$D$_3$ plus IL-2 treated cells could have contributed to their greater suppression efficiency. Another plausible explanation could be enhanced IL-10 and reduced IFNγ secretion by 1,25(OH)$_2$D$_3$ plus IL-2 treated cells, since both cytokine changes could lead to decreased B7 expression by DCs (Fujihara et al., 1996; Moore et al., 2001). General suppression of responder T cells by stimulated T cells

might be explained in a number of ways. Firstly, due to their larger size, stimulated T cells might dominate APC space. Secondly, by their high CD25 expression stimulated cells could act as an IL-2 sink, depriving responder cells of the IL-2 they require for proliferation (Shevach, 2009). Furthermore, both of these mechanisms could be enhanced, as memory cells respond more rapidly than naive T cells to co-stimulation signals (reviewed by (Boesteanu and Katsikis, 2009). Nonetheless, the finding that untreated, stimulated cells could suppress responder cell division is also consistent with the transient expression of T_{Reg} associated markers by activated T cells (Pillai and Karandikar, 2007). Indeed it is now contended that activated T cells that express FoxP3 might have similar functions to T_{Regs} (Pillai and Karandikar, 2007). Physiologically, acquisition of regulatory capacity upon activation could permit negative feedback of the immune response and avoidance of hyperlymphoproliferation.

Taken together, the data presented here indicate that 1,25(OH)$_2$D$_3$ functions in the reciprocal regulation of T_h17 versus T_{Reg} differentiation, acting directly upon CD4$^+$ T cells to inhibit the development of T helper cells expressing IL-17, IL-21 and IFNγ, whilst promoting in consort with IL-2 a population of CD4$^+$CTLA-4$^+$FoxP3$^+$ cells with suppressive activity. Therefore, similar to other steroid nuclear receptors, including RARα (Schambach et al., 2007) and AHR (Quintana et al., 2008), signalling through VDR appears to influence the position of the T_h17/T_{Reg} balance, deregulation of which is associated with immune disease. Data in this chapter therefore provide further insight into the molecular and cellular basis of the epidemiological link between low vitamin D status and autoimmune conditions (reviewed by (Arnson et al., 2007; Cantorna and Mahon, 2004; Mullin and Dobs, 2007)).

5 ARE PRO-REGULATORY EFFECTS OF 1,25(OH)$_2$D$_3$ MAINTAINED IN THE ABSENCE OF SERUM?

The data in chapter four of this thesis demonstrated the ability of 1,25(OH)$_2$D$_3$ to shift the T_h17/T_{Reg} balance in favour of T_{Reg} development. Thus, 1,25(OH)$_2$D$_3$ appears to share immunological properties with the other steroidal vitamin, All Trans Retinoic Acid (ATRA). However, FoxP3 induction by ATRA required TGFβ (Benson et al., 2007; Elias et al., 2008; Mucida et al., 2007; Sun et al., 2007; Xiao et al., 2008). Since TGFβ responses have been observed in serum containing media in the absence of TGFβ supplement (Manel et al., 2008), I considered whether CD4$^+$ T cell responses to 1,25(OH)$_2$D$_3$ likewise involved the interaction of 1,25(OH)$_2$D$_3$ with TGFβ. In this chapter, I therefore investigated the effect of 1,25(OH)$_2$D$_3$ upon CD4$^+$ T cell responses in chemically defined, serum free medium.

5.1 1,25(OH)$_2$D$_3$ influences CD4$^+$ T cell phenotype alone and in consort with TGFβ

To investigate whether CD4$^+$ T cell responses to 1,25(OH)$_2$D$_3$ required TGFβ, bead stimulations were carried out in serum free medium with and without TGFβ in the presence of 1,25(OH)$_2$D$_3$. **Figures 5.1 and 5.2** show that, in the absence of serum, 1,25(OH)$_2$D$_3$ increased CTLA-4 and CD25 expression and suppressed IFNγ. However, IL-17 expression required TGFβ, thus in its absence 1,25(OH)$_2$D$_3$ did not affect IL-17 levels. By contrast, IL-21 was expressed at very high level in the absence of serum and was dramatically suppressed by 1,25(OH)$_2$D$_3$ (**figure 5.3**). Similarly, 1,25(OH)$_2$D$_3$ mediated IL-22 suppression in the absence of serum components or TGFβ (**figure 5.3**). Thus, it was clear that 1,25(OH)$_2$D$_3$ did not

require serum or TGFβ to influence CD4$^+$ T cell phenotype. However, in the absence of serum, FoxP3 expression was very low and 1,25(OH)$_2$D$_3$ had only a modest effect upon its level (**figure 5.4**). By supplying TGFβ, though, FoxP3 was strongly increased. Accordingly, 1,25(OH)$_2$D$_3$ and TGFβ synergistically increased the CTLA-4$^+$FoxP3$^+$ population. Equivalent FoxP3 induction was observed at TGFβ concentrations ≥1ng/ml (**figure 5.5**), hence 1ng/ml TGFβ was used throughout subsequent experiments. Induction of IL-10 by 1,25(OH)$_2$D$_3$ similarly depended upon TGFβ (**figure 5.6**). Interestingly, when supplied individually, TGFβ and 1,25(OH)$_2$D$_3$ suppressed IL-10 relative to non-supplemented cultures but when provided together IL-10$^+$ frequencies were increased.

Although TGFβ was not required for 1,25(OH)$_2$D$_3$-mediated increase in CTLA-4, it tended to enhance it (**figure 5.1**). TGFβ is known to regulate the expression of a number of transcription factors including the hallmark T_{Reg} and T_h17 transcription factors FoxP3 and RORC respectively (Ivanov et al., 2006; Zhou et al., 2008). Thus, whether the additive effects of TGFβ and 1,25(OH)$_2$D$_3$ upon CTLA-4 might involve TGFβ mediated VDR induction was considered. VDR mRNA level was measured for CD4$^+$CD25$^-$ T cells by quantitative real-time PCR after 12 hours stimulation in the presence or absence of TGFβ, with or without 1,25(OH)$_2$D$_3$. As shown in **figure 5.7**, stimulation marginally increased VDR but 1,25(OH)$_2$D$_3$ did not influence this induction. However, TGFβ enhanced VDR in stimulated cells 4.3 fold and together 1,25(OH)$_2$D$_3$ and TGFβ supported highest VDR expression.

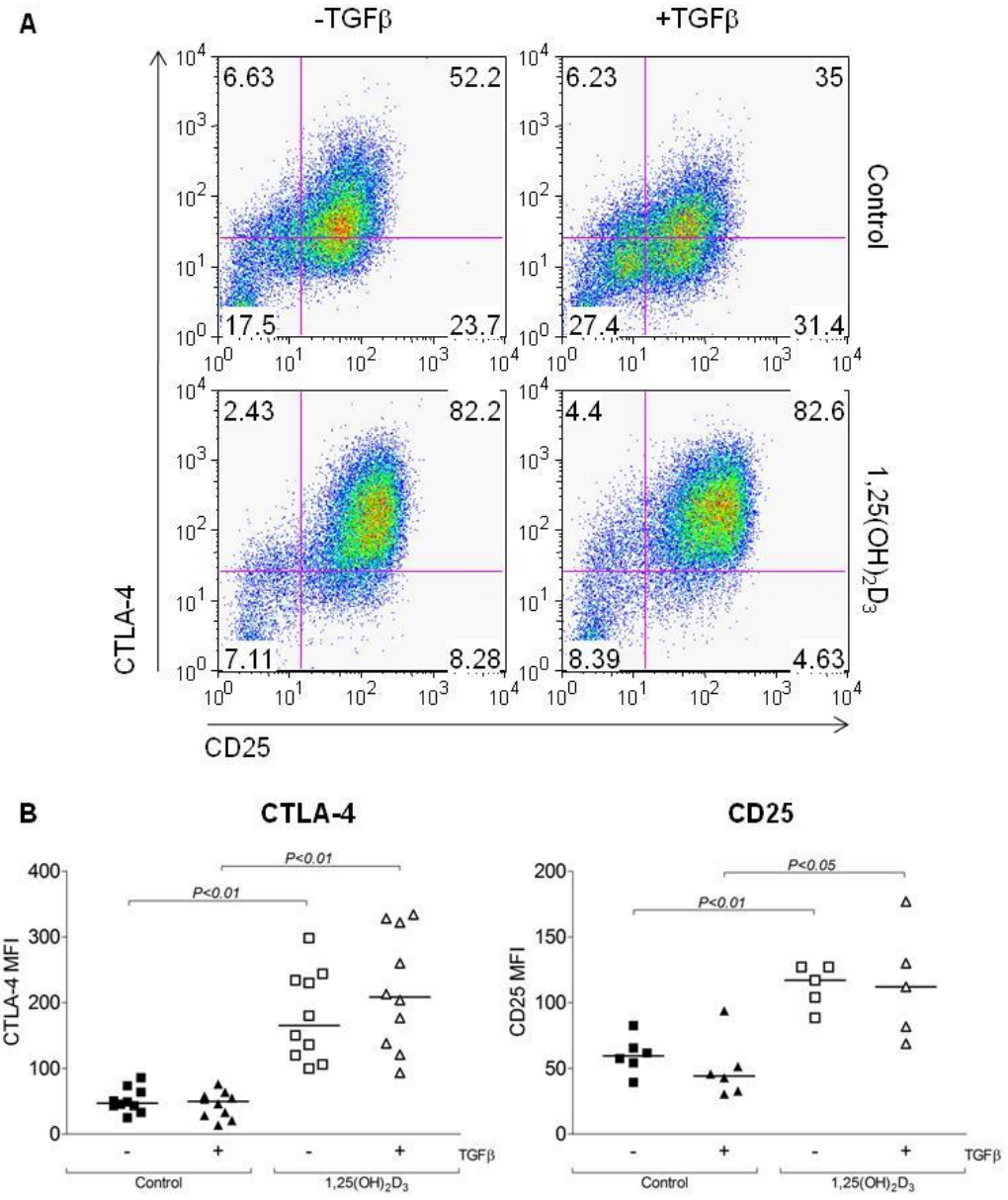

Figure 5.1. 1,25(OH)$_2$D$_3$ increases CTLA-4 and CD25 expression in the absence of TGFβ. CD4$^+$CD25$^-$ T cells were stimulated with anti-CD3/CD28 beads under serum free conditions in the presence of 1,25(OH)$_2$D$_3$ or vehicle control, with or without TGFβ. At four days, cells were stained for total CTLA-4 and CD25 and analysed by flow cytometry. **A)** Representative FACS plots from one experiment. Numbers in quadrants refer to percentage of cells. **B)** CTLA-4 and CD25 median fluorescence intensities (MFI) for multiple donors. Horizontal lines indicate median values. Significance was tested by a two-tailed Wilcoxon matched pairs test.

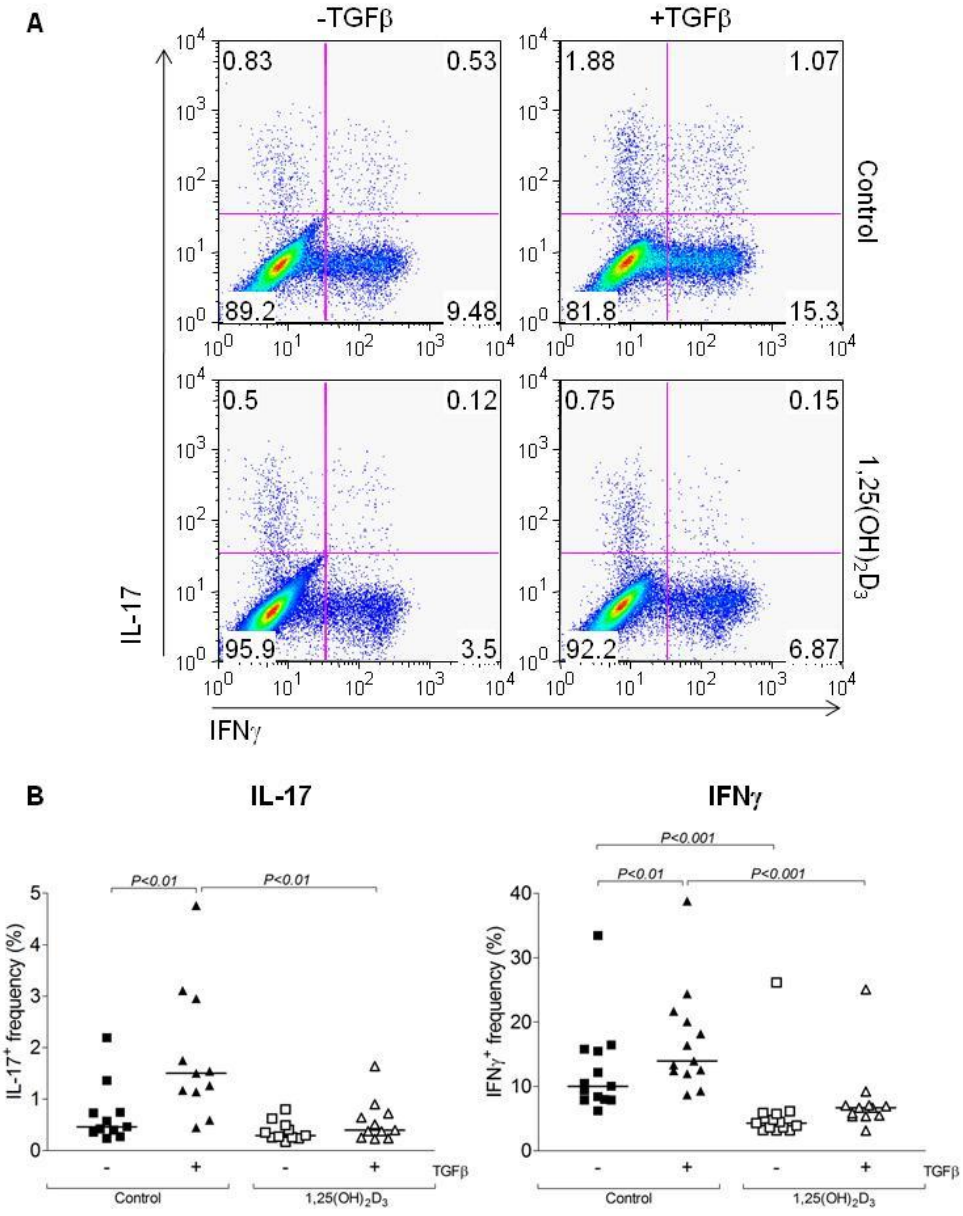

Figure 5.2. 1,25(OH)$_2$D$_3$ suppresses IFNγ in the absence of TGFβ. CD4$^+$CD25$^-$ T cells were stimulated with anti-CD3/CD28 beads under serum free conditions in the presence of 1,25(OH)$_2$D$_3$ or vehicle control with or without TGFβ. At five days, cells were stained for intracellular IL-17 and IFNγ and analysed by flow cytometry. **A)** Representative FACS plots from one experiment. Numbers in quadrants refer to percentage of cells. **B)** IL-17$^+$ and IFNγ$^+$ frequencies for multiple donors. Horizontal lines indicate median frequencies. Significance was tested by a two-tailed Wilcoxon matched pairs test.

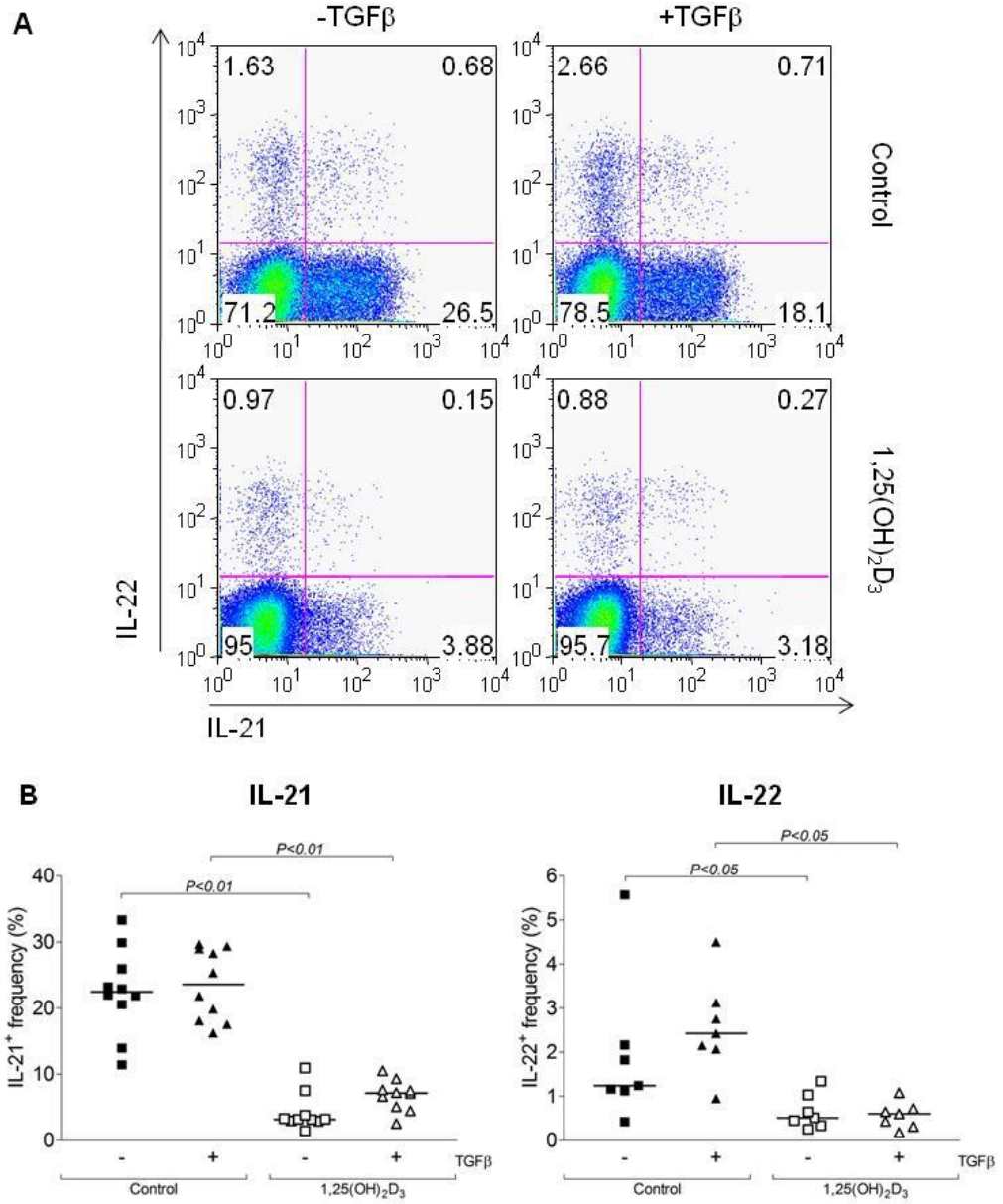

Figure 5.3. IL-21 and IL-22 are suppressed by 1,25(OH)$_2$D$_3$ in the absence of serum. CD4$^+$CD25$^-$ T cells were stimulated with anti-CD3/CD28 beads under serum free conditions in the presence of 1,25(OH)$_2$D$_3$ or vehicle control with or without TGFβ. At five days, cells were stained for IL-21 and IL-22 and analysed by flow cytometry. **A)** Representative FACS plots from one experiment. Numbers in quadrants refer to percentage of cells. **B)** IL-21$^+$ and IL-22$^+$ frequencies for multiple donors. Horizontal lines indicate median values. Significance was tested by a two-tailed Wilcoxon matched pairs test.

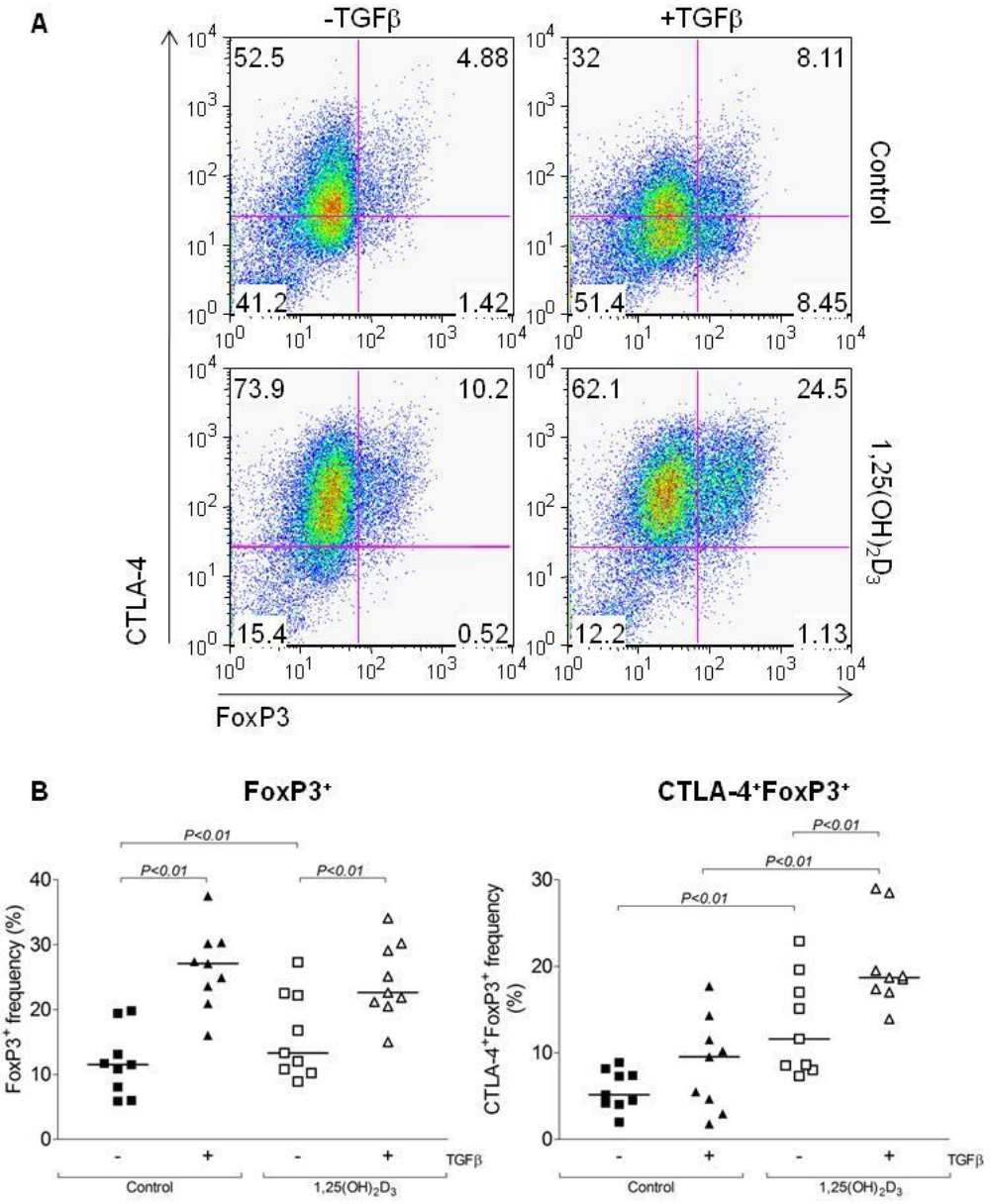

Figure 5.4. TGFβ induces FoxP3 and with 1,25(OH)$_2$D$_3$ enhances CTLA-4$^+$FoxP3$^+$ frequencies. CD4$^+$CD25$^-$ T cells were stimulated with anti-CD3/CD28 beads under serum free conditions in the presence of 1,25(OH)$_2$D$_3$ or vehicle control, with or without TGFβ. At four days, cells were stained for total CTLA-4 and FoxP3 and analysed by flow cytometry. **A)** Representative FACS plots from one experiment. Numbers in quadrants refer to percentage of cells. **B)** FoxP3$^+$ and CTLA-4$^+$FoxP3$^+$ frequencies for multiple donors. Horizontal lines indicate median values. Significance was tested by a two-tailed Wilcoxon matched pairs test.

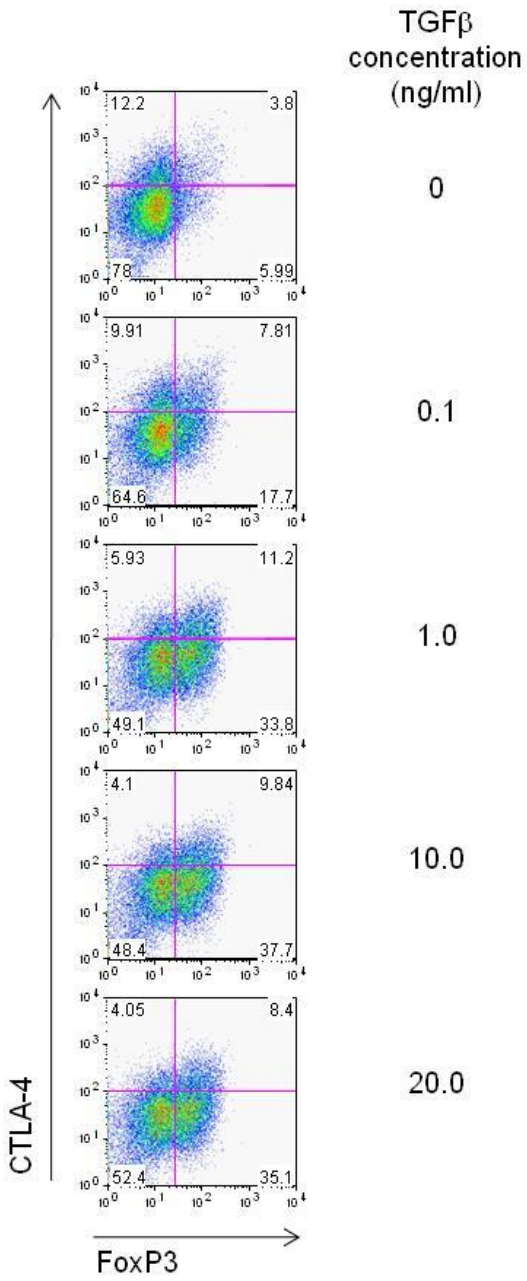

Figure 5.5. Effect of TGFβ upon CTLA-4 and FoxP3 expression. CD4$^+$CD25$^-$ T cells were stimulated with anti-CD3/CD28 beads under serum free conditions at increasing concentrations of TGFβ. At four days, cells were stained for total CTLA-4 and FoxP3 and analysed by flow cytometry. Representative FACS plots from one experiment are shown. Numbers in quadrants refer to percentage of cells.

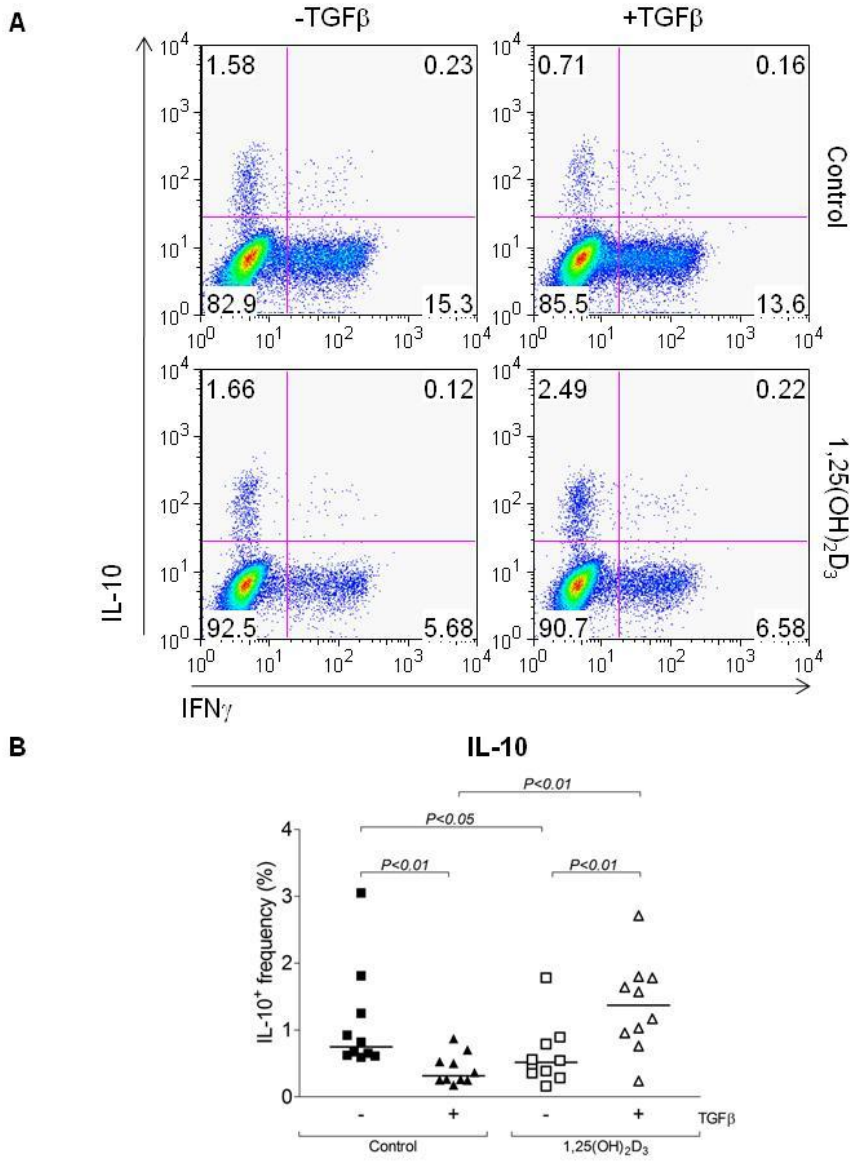

Figure 5.6. TGFβ is required for IL-10 induction by 1,25(OH)$_2$D$_3$. CD4$^+$CD25$^-$ T cells were stimulated with anti-CD3/CD28 beads under serum free conditions in the presence of 1,25(OH)$_2$D$_3$ or vehicle control, with or without TGFβ. At five days, cells were stained for intracellular IL-10 and IFNγ and analysed by flow cytometry. **A)** Representative FACS plots from one experiment. Numbers in quadrants refer to percentage of cells. **B)** IL-10$^+$ frequencies for multiple donors. Horizontal lines indicate median frequencies. Significance was tested by a two-tailed Wilcoxon matched pairs test.

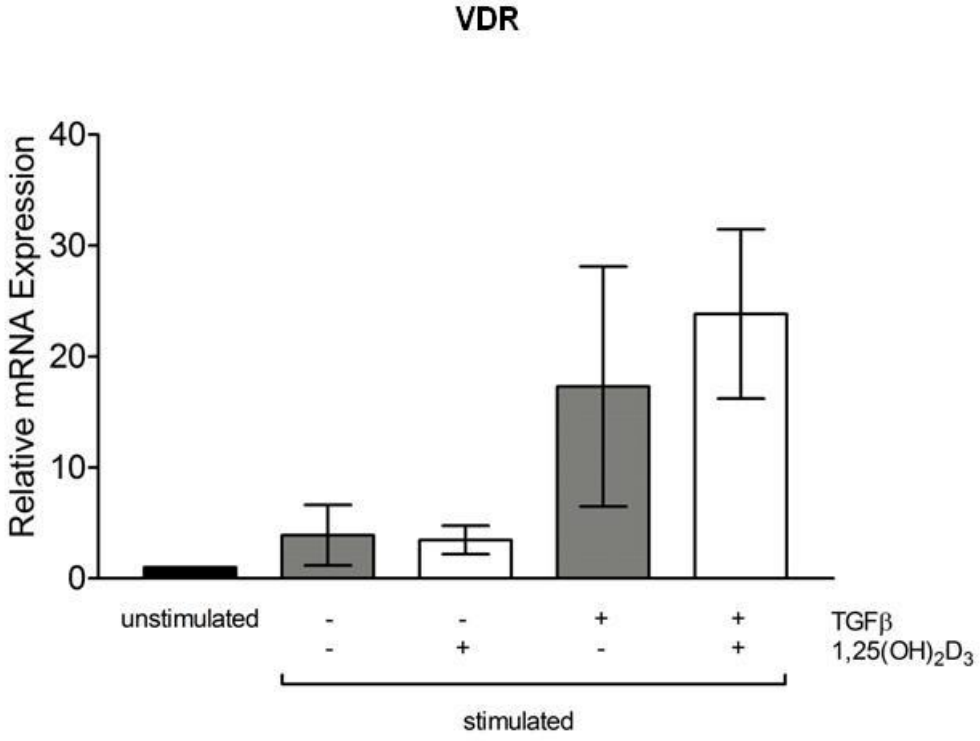

Figure 5.7. TGFβ enhances vitamin D receptor mRNA expression by stimulated T cells. $CD4^+CD25^-$ T cells were stimulated for 12 hours with anti-CD3/CD28 beads under serum free conditions in the presence of $1,25(OH)_2D_3$ or vehicle control, with or without TGFβ. Vitamin D receptor (VDR) expression was measured by quantitative real-time PCR. Expression was normalised to 18S rRNA and plotted relative to the level in autologous unstimulated $CD4^+CD25^-$ T cells. Error bars indicate standard deviation, n=2.

5.2 1,25(OH)$_2$D$_3$ effects are maintained under pro-inflammatory conditions

If 1,25(OH)$_2$D$_3$ is to be of benefit in the control of active disease it might be necessary that it can suppress effector T cell development and promote T_{Reg} populations under pre-established inflammatory conditions. To examine this, bead stimulations were repeated in the presence of T_h17 polarizing cytokines. **Figure 5.8** shows that TGFβ combined with IL-1β, IL-6 and IL-23, enhanced T_h17 frequencies. Therefore, in this study a pro-T_h17 environment was created by supplementing serum free medium with recombinant TGFβ, IL-1β, IL-6 and IL-23. **Figure 5.9** confirms that even under pro-T_h17 conditions, 1,25(OH)$_2$D$_3$ could suppress the expression of inflammatory cytokines IL-17, IFNγ, IL-21 and IL-22. At the same time, its ability to enhance CTLA-4, CD25 and IL-10 and increase CTLA-4$^+$FoxP3$^+$ frequencies was seen (**figure 5.10**). Thus the regulatory effect of 1,25(OH)$_2$D$_3$ could be maintained under pro-inflammatory conditions.

5.3 Pro-inflammatory cytokines suppress CTLA-4 but enhance its 1,25(OH)$_2$D$_3$-dependent induction

When studying the effects of 1,25(OH)$_2$D$_3$ under pro-T_h17 conditions it was seen that pro-inflammatory cytokines significantly suppressed CTLA-4 expression (**figure 5.11 and 5.12**). This observation did not merely reflect faster activation and proliferation kinetics in the pro-T_h17 cultures, since CFSE dilution experiments showed that CTLA-4 expression was reduced at all division stages (**figure 5.11**). Surprisingly, when 1,25(OH)$_2$D$_3$ was added, highest CTLA-4 levels were repeatedly found under the pro-T_h17 conditions (**figure 5.12**). Thus, pro-regulatory effects of 1,25(OH)$_2$D$_3$ might be strengthened under inflammatory conditions.

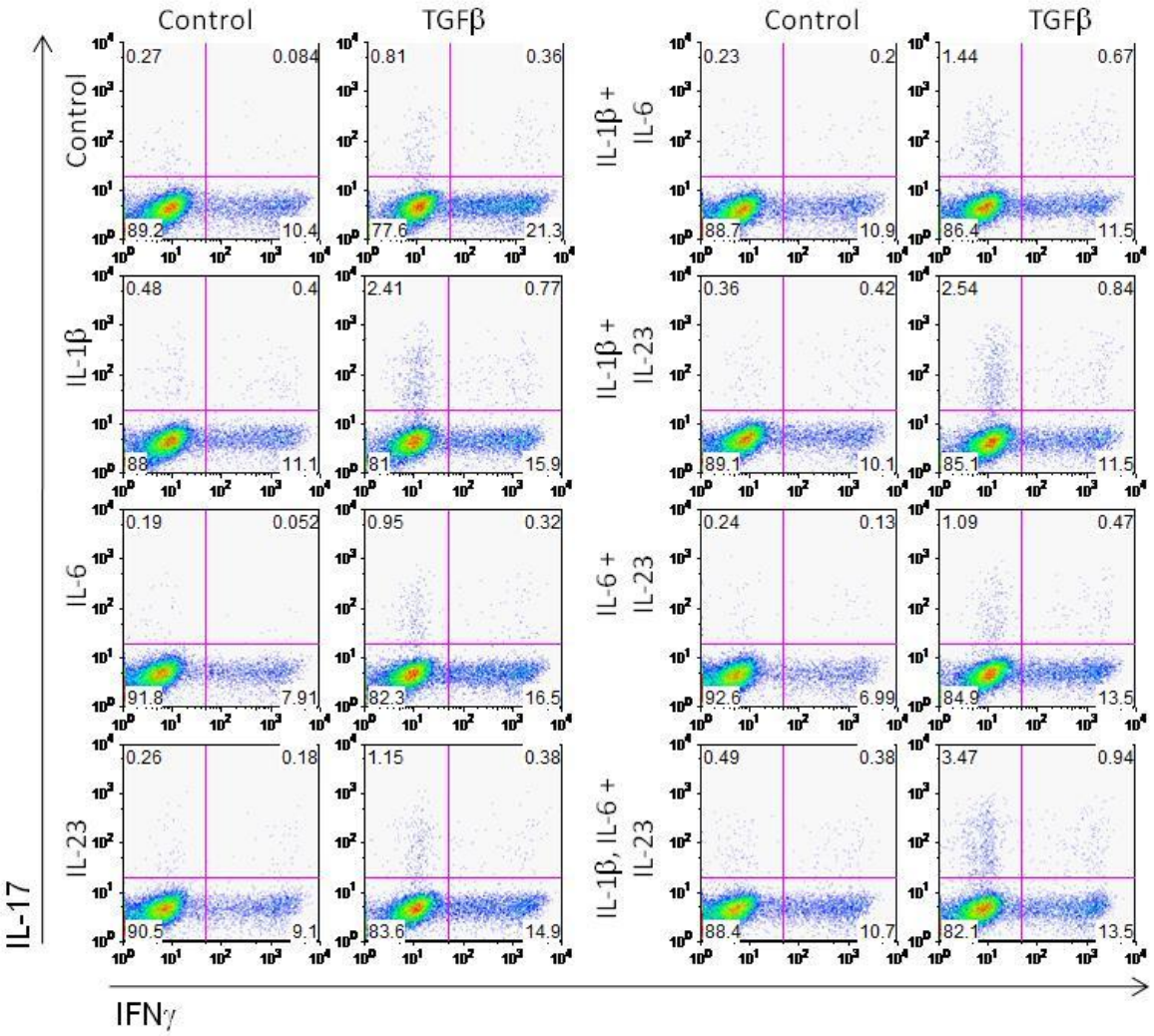

Figure 5.8. TGFβ plus pro-inflammatory cytokines, IL-1β, IL-6 and IL-23, yields the highest IL-17$^+$ frequencies. CD4$^+$CD25$^-$ T cells were stimulated for five days with anti-CD3/CD28 beads in serum free medium supplemented with combinations of the cytokines TGFβ, IL-1β, IL-6 and IL-23 as indicated. Cells were stained for IL-17 and IFNγ and analysed by flow cytometry. Data shown are for a single experiment representative of two performed.

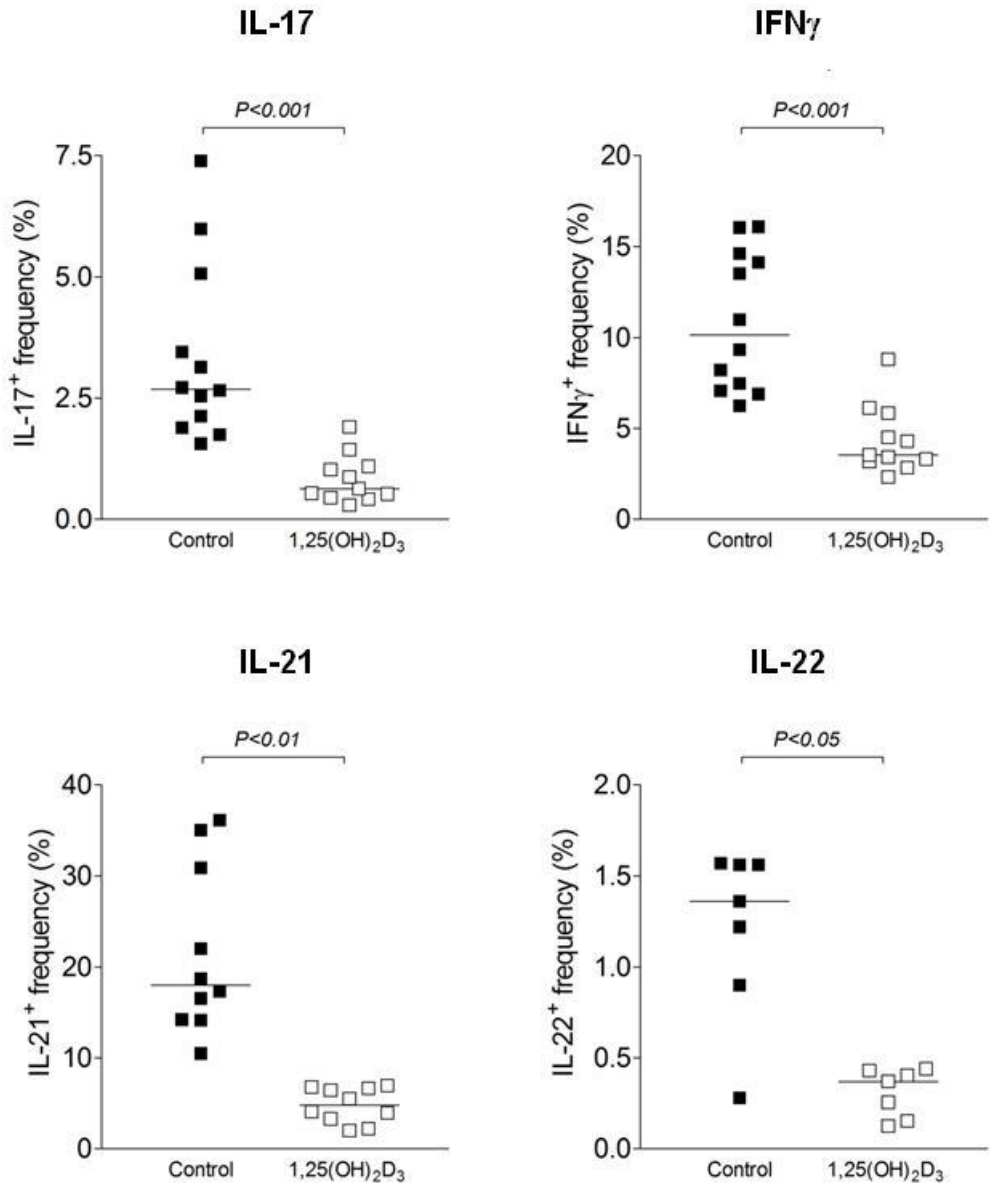

Figure 5.9. Inflammatory cytokines are suppressed by 1,25(OH)$_2$D$_3$ under pro-T$_h$17 conditions. CD4$^+$CD25$^-$ T cells were stimulated with anti-CD3/CD28 beads in serum free medium supplemented with TGFβ, IL-1β, IL-6 and IL-23 in the presence of 1,25(OH)$_2$D$_3$ or vehicle control. At five days, cells were stained for IL-17, IFNγ, IL-21 and IL-22 and analysed by flow cytometry. A summary of the cytokine frequencies for multiple donors is shown. Horizontal lines indicate median frequencies. Significance was tested by a two-tailed Wilcoxon matched pairs test.

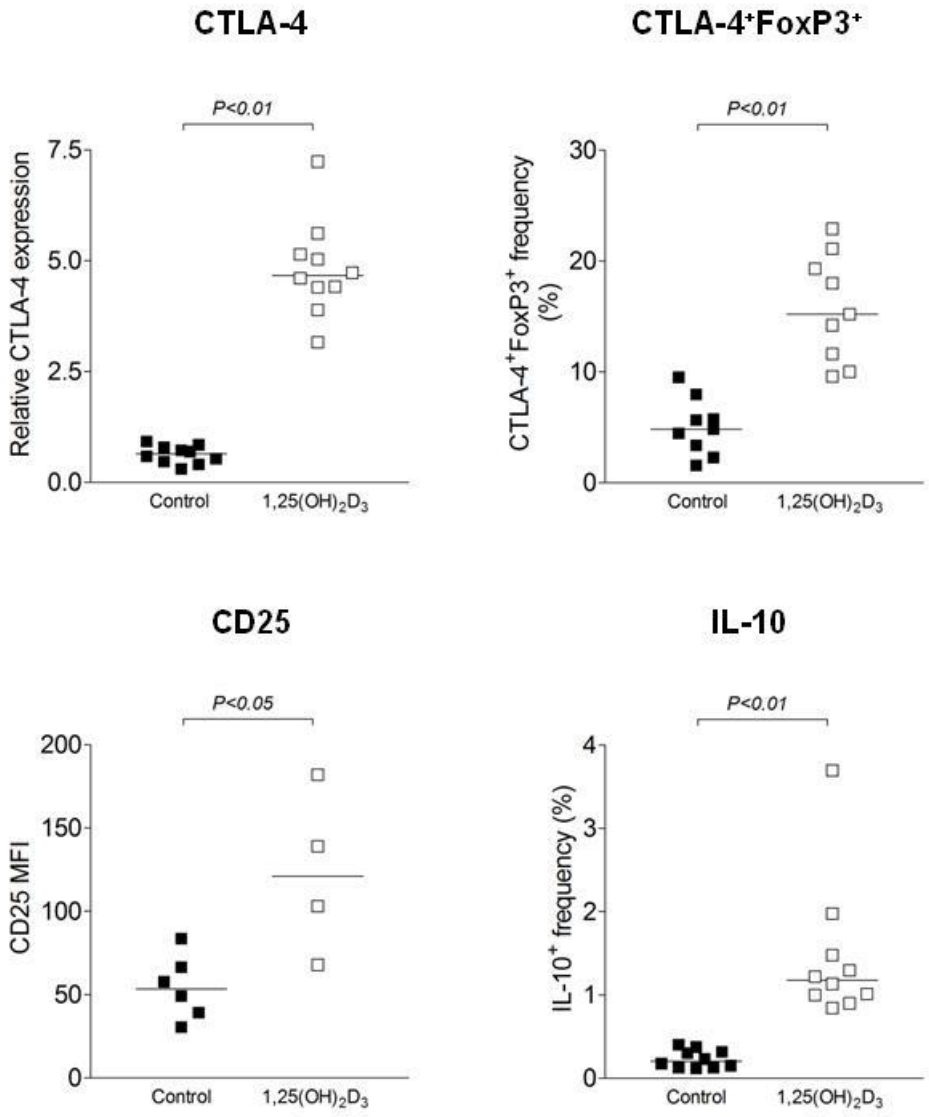

Figure 5.10. 1,25(OH)$_2$D$_3$ enhances regulatory markers under pro-T$_h$17 conditions. CD4$^+$CD25$^-$ T cells were stimulated with anti-CD3/CD28 beads in serum free medium supplemented with TGFβ, IL-1β, IL-6 and IL-23 in the presence of 1,25(OH)$_2$D$_3$ or vehicle control. At four days, cells were stained for total CTLA-4, FoxP3 and CD25. At five days cells were stained for IL-10. All samples were analysed by flow cytometry. A summary of the data for multiple donors is shown. Horizontal bars indicate median values. Significance was tested by a two-tailed Wilcoxon matched pairs test.

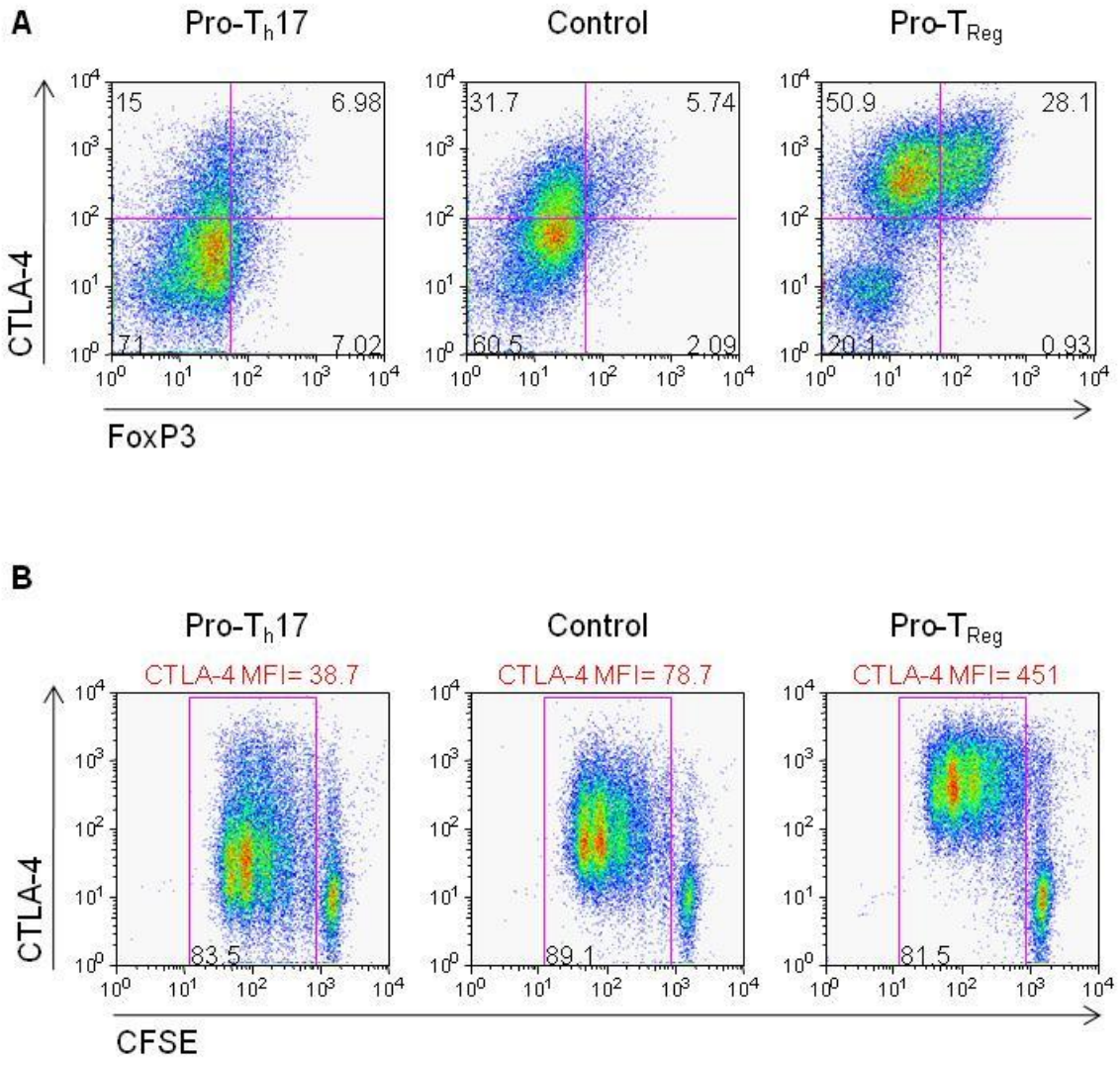

Figure 5.11. Across division stages, pro-T$_h$17 conditions suppress CTLA-4 expression whilst 1,25(OH)$_2$D$_3$ enhances it. CFSE labelled CD4$^+$CD25$^-$ T cells were stimulated with anti-CD3/CD28 beads in serum free medium without supplement (control) or in the presence of Pro-T$_h$17 recombinant cytokines (TGFβ, IL-1β, IL-6 and IL-23) or Pro-T$_{Reg}$ conditions (1,25(OH)$_2$D$_3$ + TGFβ). At four days, cells were stained for CTLA-4 expression and analysed by flow cytometry. **A)** CTLA-4 vs. FoxP3. **B)** CTLA-4 vs. CFSE. Numbers in gates indicate percentage of dividing cells and CTLA-4 median fluorescence intensities (MFI) are given in red. FACS plots are from a single experiment representative of n>3.

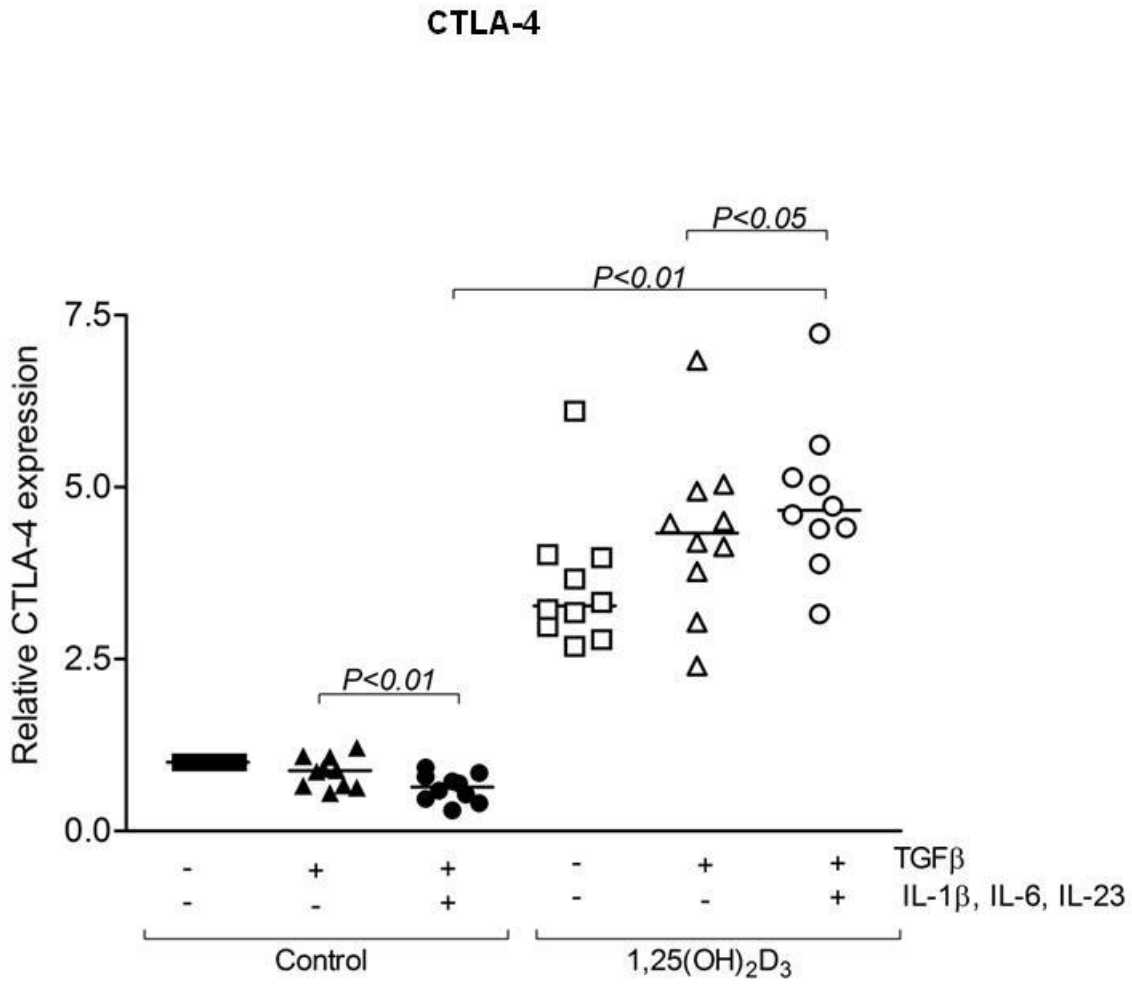

Figure 5.12. Pro-Th17 conditions suppress CTLA-4 expression but synergise with 1,25(OH)$_2$D$_3$ to enhance CTLA-4 up-regulation. CD4$^+$CD25$^-$ T cells were stimulated with anti-CD3/CD28 beads in serum free medium in the absence of recombinant cytokines, with TGFβ alone or with the pro-Th17 cocktail (TGFβ, IL-1β, IL-6 and IL-23). Stimulations were also treated with 1,25(OH)$_2$D$_3$ or vehicle control. At four days, cells were stained for total CTLA-4 and analysed by flow cytometry. Median CTLA-4 fluorescence intensities were calculated relative to the level in medium alone. A summary of the data for multiple donors is shown. Horizontal bars indicate median values. Significance was tested by a two-tailed Wilcoxon matched pairs test. For clarity not all significant comparisons are indicated.

5.4 CTLA-4 expression level affects ligand removal

The involvement of CTLA-4 in suppression is established but its mechanism is controversial. My lab has recently identified that via CTLA-4, T cells can internalise B7 ligands and destroy them by lysosomal degradation (Qureshi et al. manuscript in preparation). This process of internalisation is termed transendocytosis. The varying levels of CTLA-4 displayed by T cells cultured under control, T_h17 and 1,25(OH)$_2$D$_3$ conditions could thus affect their CTLA-4 dependent regulatory capacity. To investigate this, CHO cells stably expressing CD86-GFP were co-cultured with T cells previously stimulated under T_h17, control and 1,25(OH)$_2$D$_3$ conditions. After three hours, T cell acquisition of CD86-GFP was measured by flow cytometry. **Figure 5.13** shows that CD86-GFP acquisition was greatest by T cells expressing the highest levels of CTLA-4 level. To confirm that this trend related to CTLA-4 level, T cells were incubated with anti-CTLA-4 blocking antibody prior to culture with CD86-GFP CHO cells. Anti-CTLA-4 reduced acquisition by 60-70% across conditions, indicating the involvement of CTLA-4 engagement in CD86 acquisition.

5.5 1,25(OH)$_2$D$_3$-conditioned T cells reduce CD80 and CD86 levels on co-cultured DCs in a CTLA-4 dependent manner

Through an artificial system, it was clear that 1,25(OH)$_2$D$_3$-treated T cells have greater ability than control T cells to acquire CD86 from a target cell (**figure 5.13**). However, if co-stimulatory molecule depletion is a mechanism by which 1,25(OH)$_2$D$_3$-conditioned T cells suppress the division of responder T cells in DC stimulations, they should be able to deplete CD86 and CD80 from DCs during co-culture. It was considered that antigen targeting might be important for directing efficient DC and T cell engagement. Therefore, SEB reactive CD4$^+$

T cell populations were generated in the presence of IL-2 and 1,25(OH)$_2$D$_3$ or vehicle control and cultured overnight with SEB pulsed DCs. CD80 and CD86 expression by the DCs was then measured by flow cytometry. Data shown in **Figures 5.14 and 5.15** support the hypothesis that 1,25(OH)$_2$D$_3$ conditioned T cells reduce CD80 and CD86 expression by DCs. Furthermore, pre-treating T cells with anti-CTLA-4, confirmed that depletion of CD80 and CD86 was largely CTLA-4 dependent. However, recovery for CD86 appeared to be less efficient than that for CD80 (**figure 5.15**). It was also of interest that anti-CTLA-4 enhanced CD80 and CD86 expression by DCs cultured with control T cells. This finding corresponds with the previous observation that stimulated control T cells with low CTLA-4 suppressed responder T cell proliferation compared to responders alone (**figures 4.18 and 4.19**).

5.6 1,25(OH)$_2$D$_3$-induced CTLA-4 up-regulation contributes to 1,25(OH)$_2$D$_3$ mediated suppression of dendritic cell driven CD4$^+$ T cell stimulations

1,25(OH)$_2$D$_3$ was previously shown to reduce the extent of T cell proliferation when T cells were stimulated by APCs (**figure 4.4**). Whilst direct signalling of 1,25(OH)$_2$D$_3$ at the APC could limit MHC and co-stimulatory molecule levels, increased CTLA-4 expression by T cells leading to elevated B7 removal from the APC might contribute to reduced responder T cell proliferation. To investigate the role of CTLA-4 in suppressing T cell proliferation under control and 1,25(OH)$_2$D$_3$ conditions, CFSE labelled T cells were stimulated with DCs for five days in the presence and absence of 1,25(OH)$_2$D$_3$ with and without CTLA-4 blocking antibody. **Figure 5.16** shows that across a range of T cell: DC ratios, 1,25(OH)$_2$D$_3$ reduced proliferation relative to controls and anti-CTLA-4 partially overcame this suppression.

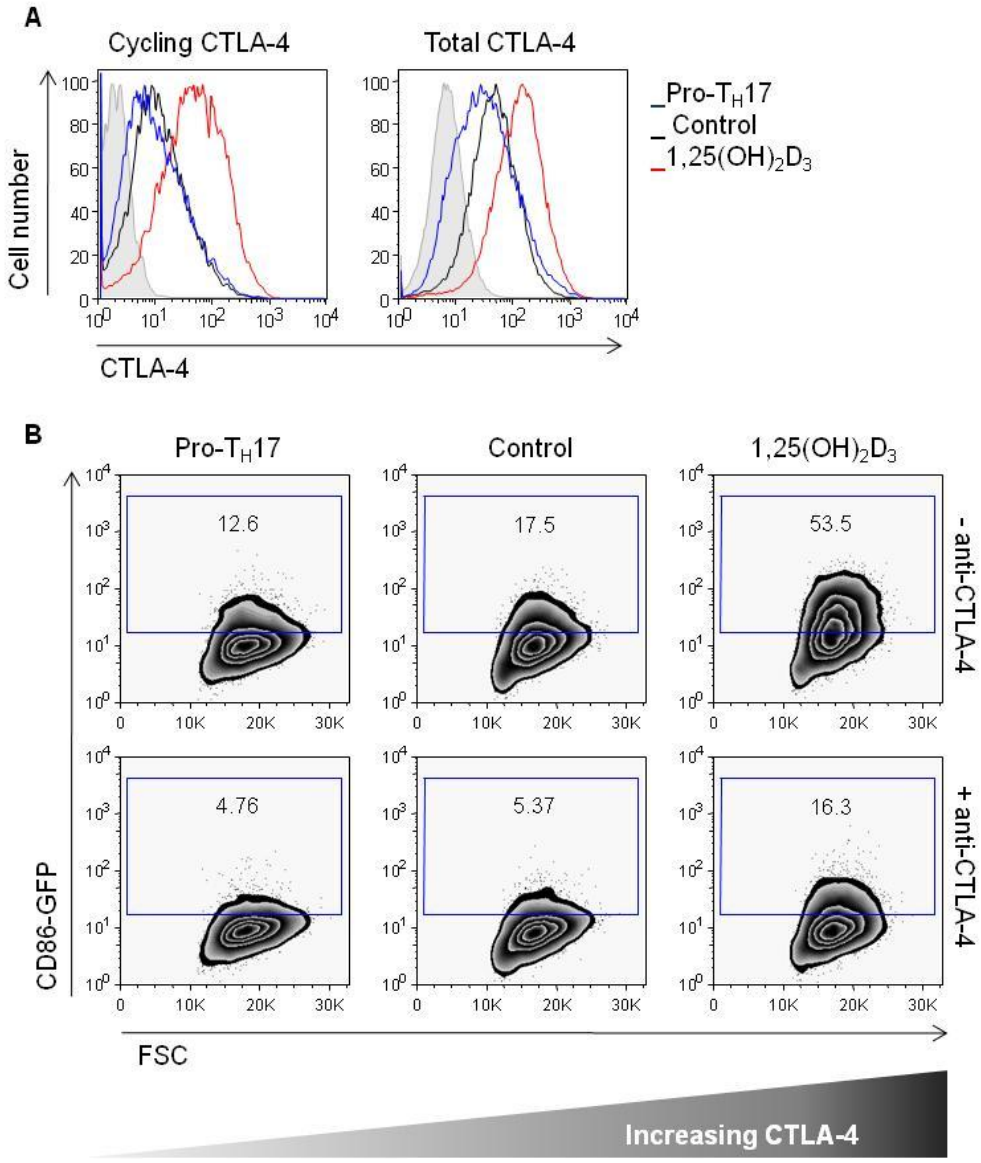

Figure 5.13. CD86 acquisition from a target cell is dependent upon CTLA-4 and relates to the level of CTLA-4 expressed. $CD4^+CD25^-$ T cells stimulated in serum free medium under pro-T_h17, control or 1,25(OH)$_2$D$_3$ conditions were re-stimulated with anti-CD3 and CHO cells expressing CD86-GFP in the presence or absence of anti-CTLA-4 blocking antibody. **A)** Cycling and total CTLA-4 expression for each T cell population as determined by flow cytometry. **B)** CD86 acquisition by the T cells was measured by flow cytometry and is shown as the percentage of GFP^+ T cells . Data are from a single experiment representative of four performed.

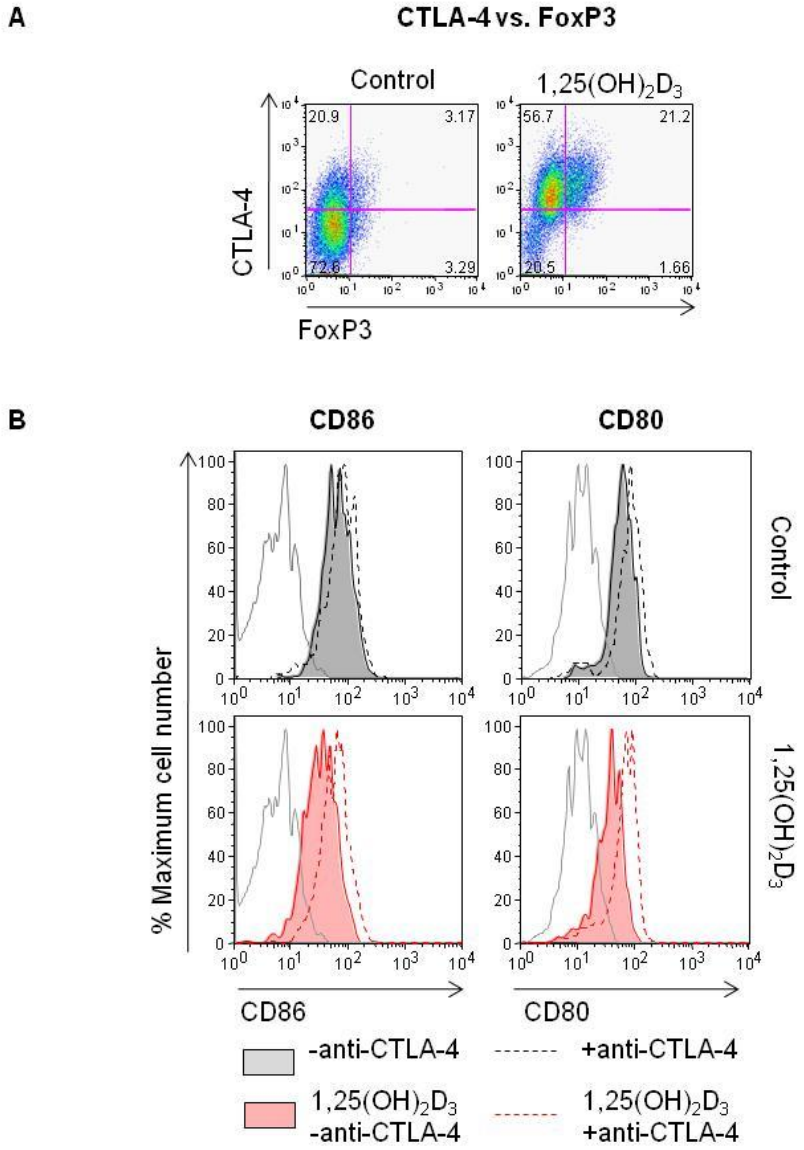

Figure 5.14. 1,25(OH)$_2$D$_3$-treated T cells reduce CD86 and CD80 expression by DCs in a CTLA-4 dependent manner. A) FoxP3 and CTLA-4 expression by SEB reactive CD4$^+$ T cells at the start of co-culture. **B)** CD86 and CD80 expression by SEB-pulsed dendritic cells was monitored by flow cytometry following their overnight culture with SEB reactive control (black) and 1,25(OH)$_2$D$_3$-treated (red) CD4$^+$ T cells in the presence (dashed line) and absence (shaded histogram) of anti-CTLA-4 blocking antibody. Clear histogram shows isotype control. Data are from a single experiment representative of two performed.

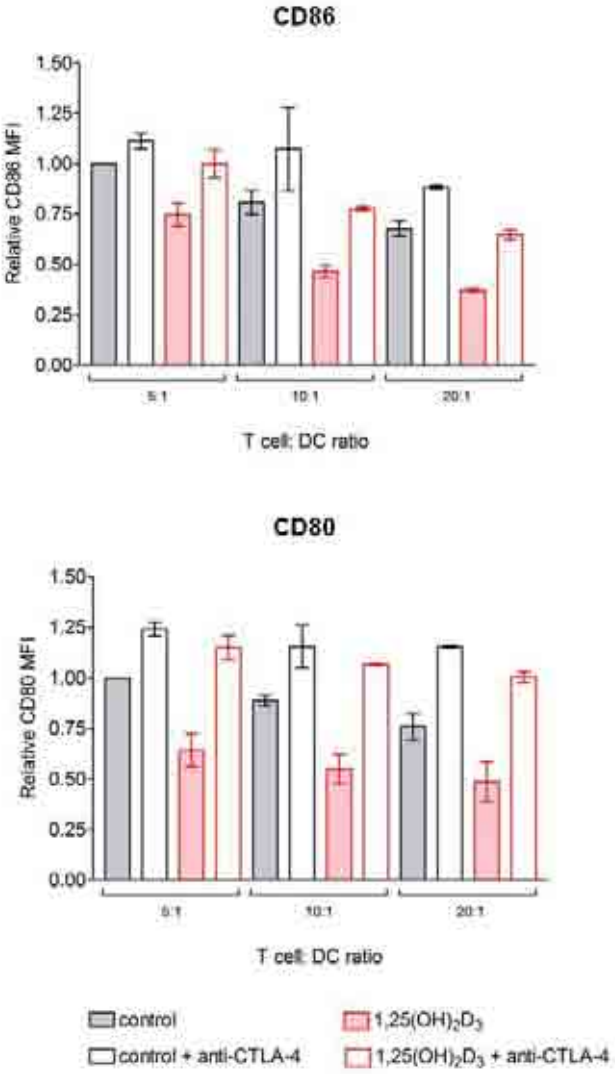

Figure 5.15. 1,25(OH)$_2$D$_3$-treated T cells reduce CD86 and CD80 expression by DCs in a CTLA-4 dependent manner. CD86 and CD80 expression on SEB-pulsed dendritic T cells was monitored by flow cytometry following their overnight culture with increasing ratios of SEB reactive control (black) and 1,25(OH)$_2$D$_3$-treated (red) CD4$^+$ T cells in the presence (clear bar) and absence (shaded bar) of anti-CTLA-4 blocking antibody. CD80 and CD86 expression was calculated relative to the level following co-culture with control T cells at a 5:1 ratio. Mean values for two experiments are graphed. Error bars indicate standard deviation.

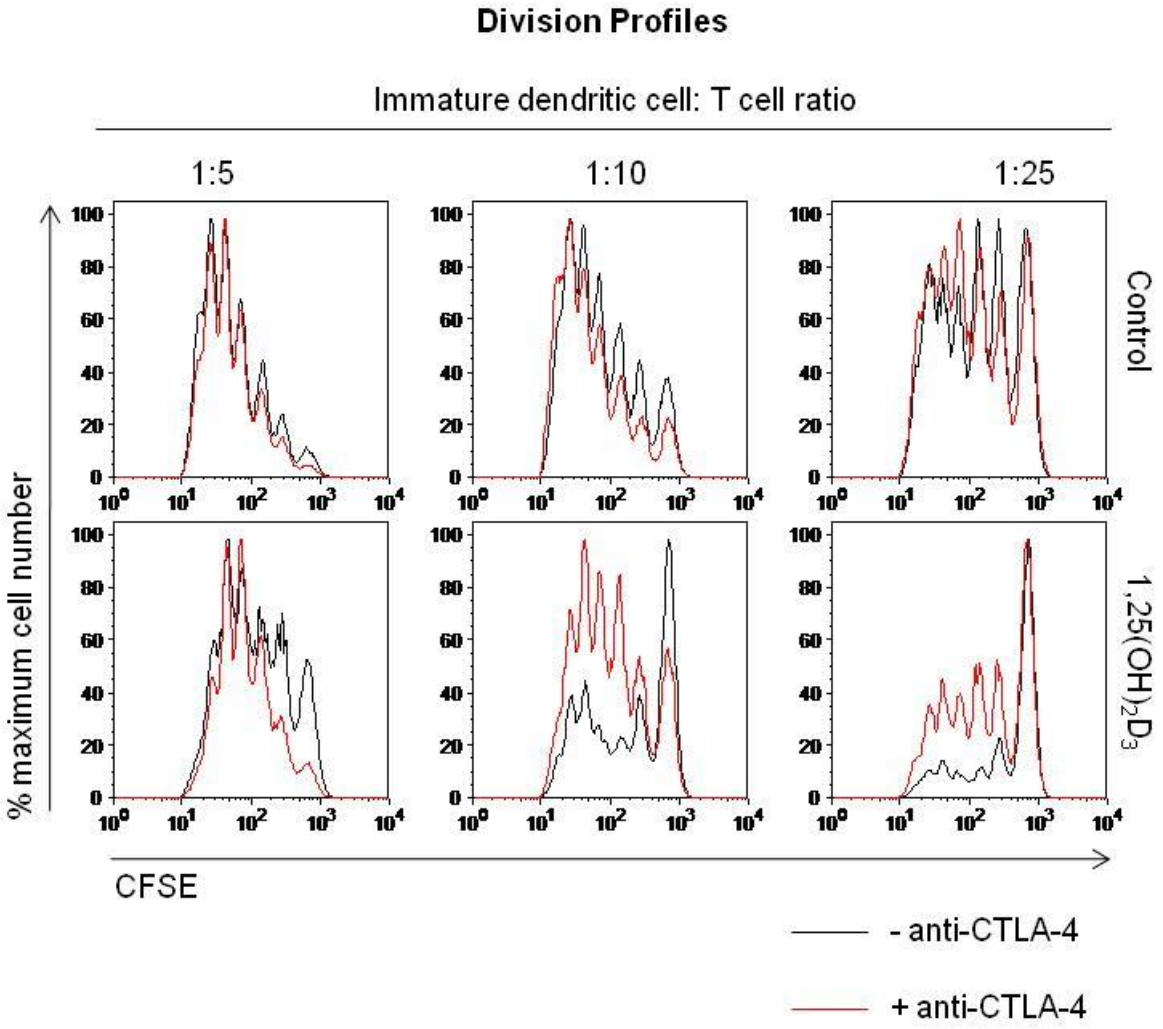

Figure 5.16. 1,25(OH)$_2$D$_3$-induced CTLA-4 up-regulation contributes to 1,25(OH)$_2$D$_3$-mediated suppression of dendritic cell driven CD4$^+$ T cell stimulations. CFSE labelled CD4$^+$CD25$^-$ T cells were stimulated with immature dendritic cells (iDC) and anti-CD3 in the presence of 1,25(OH)$_2$D$_3$ or vehicle control. Parallel stimulations were conducted in the presence (red) versus the absence (black) of blocking antibody to CTLA-4. At five days, proliferation was monitored by CFSE dilution in flow cytometry. Division profiles are from a single experiment representative of three performed.

Therefore, 1,25(OH)$_2$D$_3$-mediated CTLA-4 up-regulation appears to contribute to 1,25(OH)$_2$D$_3$-mediated suppression of T cell proliferation during primary stimulation.

5.7 Can 1,25(OH)$_2$D$_3$ suppress IL-17 expression by committed T$_h$17 cells?

If 1,25(OH)$_2$D$_3$ is to be useful in the control of active inflammatory disease, it might be necessary that it can suppress IL-17 expression by committed T$_h$17 cells. To study this, conditions were optimised for generating T$_h$17 enriched cultures. In earlier studies, I had shown that reducing the bead to cell ratio increased T$_h$17 frequencies (**figure 3.10**). Thus, a 1:32 bead to cell ratio coupled with serum free, T$_h$17 polarising conditions was tested for its ability to generate T$_h$17 cells. Repeatedly ~15% IL-17$^+$ cells were obtained by six days. Since these conditions permitted strong IL-17 enrichment, they were used to generate IL-17$^+$ cells. The ability of 1,25(OH)$_2$D$_3$ to regulate these cells was then studied.

It was considered that if 1,25(OH)$_2$D$_3$ could rapidly modify the phenotype of previously committed cells, for example by direct gene regulation, then 1,25(OH)$_2$D$_3$ might be able to alter cell phenotype before selective outgrowth in a mixed population was possible. 1,25(OH)$_2$D$_3$ responses by an IL-17$^+$ cell-enriched culture were therefore monitored over time. Effects upon CTLA-4 were clear within six hours of treatment and by 24 hours the maximal effect of 1,25(OH)$_2$D$_3$ was reached (**figure 5.17 and 5.20**). Suppression of IL-17, IFNγ and IL-21 by 1,25(OH)$_2$D$_3$ took longer: maximal responses being observed at 48 hours (**figure 5.18, 5.19 and 5.20**). In addition, IFNγ appeared less sensitive to 1,25(OH)$_2$D$_3$ than IL-17 or IL-21 throughout the time course studied (**figures 5.18 and 5.20**). Analysis of

1,25(OH)$_2$D$_3$ effects in the absence of serum

1,25(OH)$_2$D$_3$ effects upon CTLA-4 and IL-17 transcripts likewise suggested that 1,25(OH)$_2$D$_3$ modulates CTLA-4 expression more rapidly than IL-17 (**figure 5.21**). Although further studies are required to confirm this, these data could be indicative of direct versus indirect control of gene transcription for CTLA-4 and IL-17 respectively.

The finding that CTLA-4 induction occurred rapidly suggested that 1,25(OH)$_2$D$_3$ can affect previously activated cells. However, the less rapid responses of cytokines to 1,25(OH)$_2$D$_3$ meant that suppression by selective outgrowth or de-novo commitment could not be discounted. To address these possibilities, an attempt was made to purify live IL-17$^+$ cells from the T$_h$17 polarised population. For this, an IL-17 secretion assay enrichment and detection kit (Miltenyi Biotec) was used followed by MoFlo sorting. 99% IL-17$^+$ purity was achieved (**figure 5.22**). Following culture of the IL-17$^+$ cells for three days under pro-T$_h$17 conditions in the presence of 1,25(OH)$_2$D$_3$ or vehicle control their expression of IL-17 was measured by intracellular staining. In repeat experiments, IL-17 expression was reduced even in the absence of 1,25(OH)$_2$D$_3$ but 1,25(OH)$_2$D$_3$ reduced the frequency of IL-17$^+$ cells or their level of IL-17 expression further. However, IL-17 expression by IL-17$^+$ cells appeared to be less sensitive to 1,25(OH)$_2$D$_3$ than the development of IL-17$^+$ cells in primary stimulations (**figure 5.9**). Therefore, these data suggest that 1,25(OH)$_2$D$_3$ can influence previously stimulated cells but its effect might be weaker than on primary stimulated cultures.

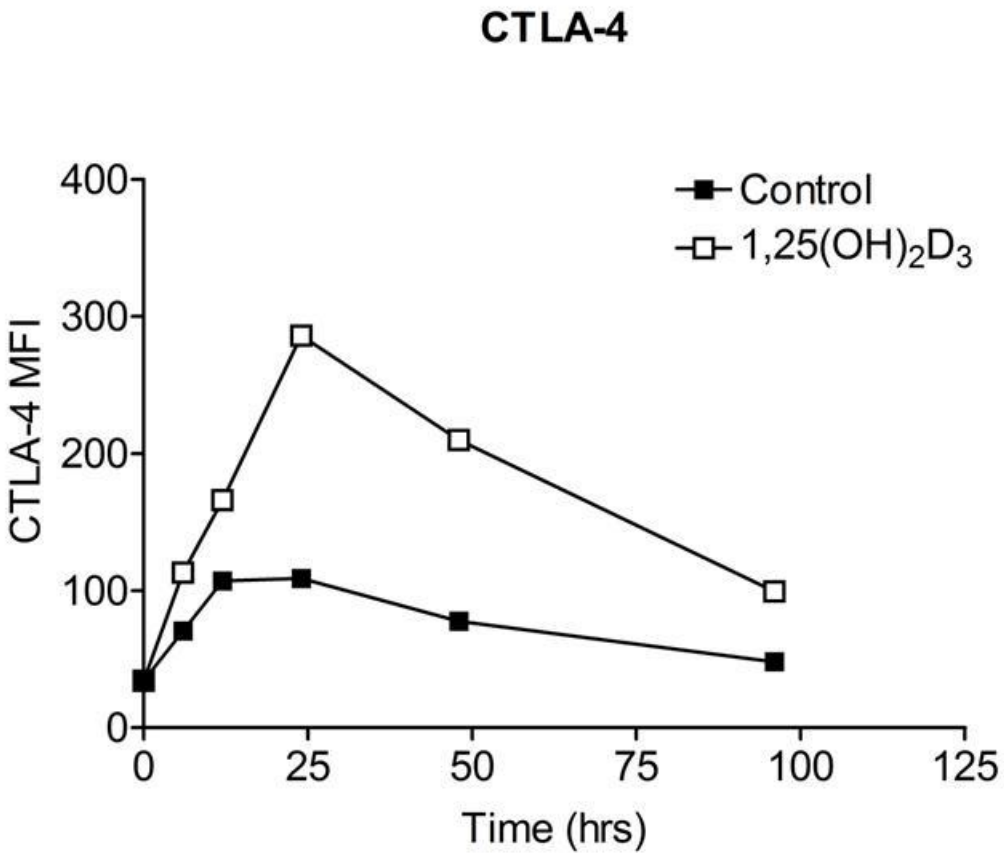

Figure 5.17. 1,25(OH)$_2$D$_3$ enhances CTLA-4 in re-stimulated cells. T$_h$17 polarised CD4$^+$ T cells were re-stimulated with anti-CD3/CD28 beads in the presence of 1,25(OH)$_2$D$_3$ or vehicle control. At the indicated times post second stimulation, cells were stained for total CTLA-4 and analysed by flow cytometry. Data are from a single experiment representative of three performed.

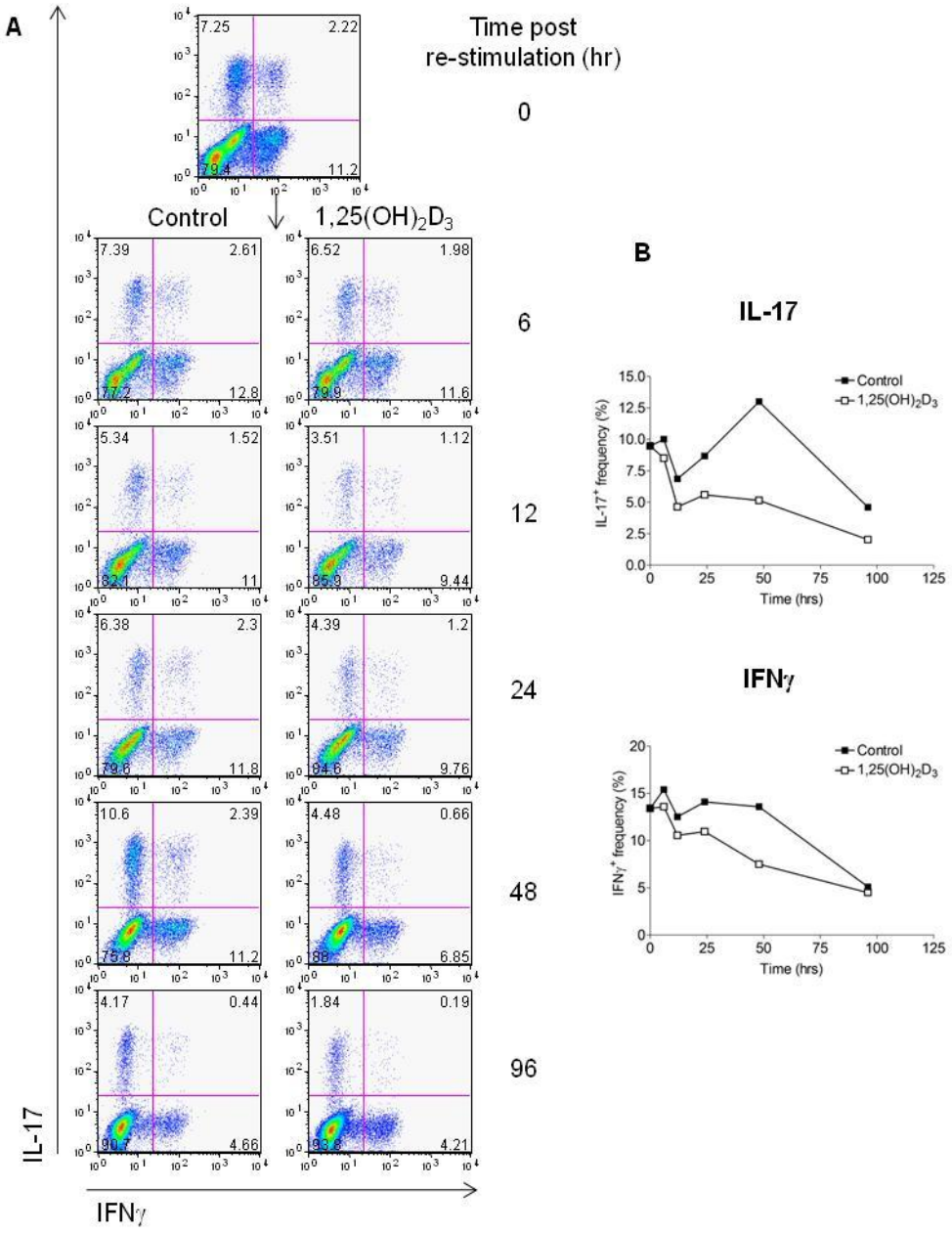

Figure 5.18. Time course of 1,25(OH)$_2$D$_3$ effect upon IL-17$^+$ and IFNγ$^+$ expression. T$_h$17 polarised cultures were re-stimulated with anti-CD3/CD28 beads in the presence of 1,25(OH)$_2$D$_3$ or vehicle control. At the indicated times, cells were stained for IL-17 and IFNγ and analysed by flow cytometry. IL-17$^+$ and IFNγ$^+$ frequencies from the FACS plots in **A** are graphed in **B**. Data are from a single experiment representative of three performed.

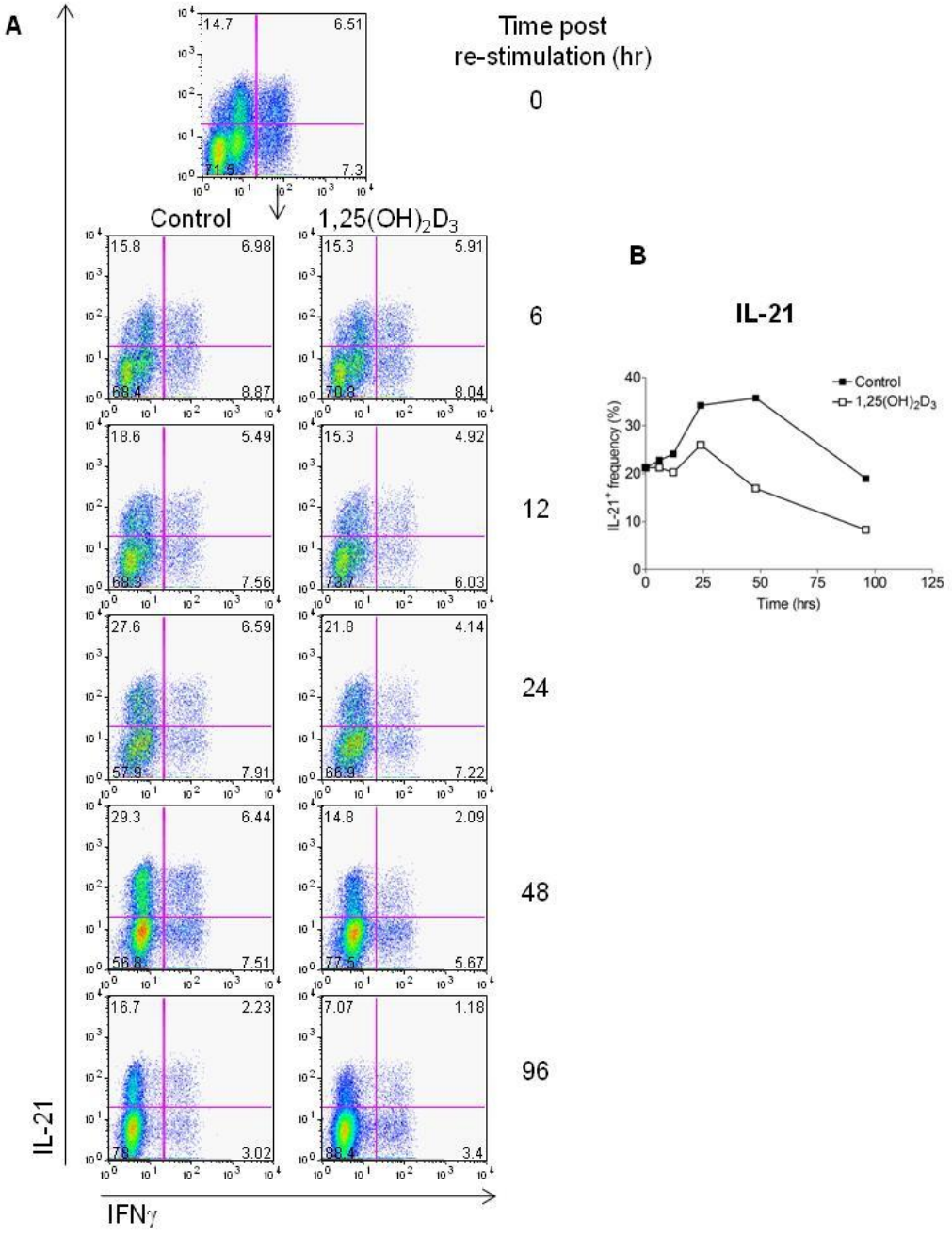

Figure 5.19. Time course of IL-21 depletion by 1,25(OH)$_2$D$_3$. T_H17 polarised cultures were re-stimulated with anti-CD3CD28 beads in the presence of 1,25(OH)$_2$D$_3$ or vehicle control. At the indicated times, cells were stained for IL-21 and IFNγ and analysed by flow cytometry. IL-21$^+$ frequencies from the FACS plots in **A** are graphed in **B.** Data are from a single experiment representative of three performed.

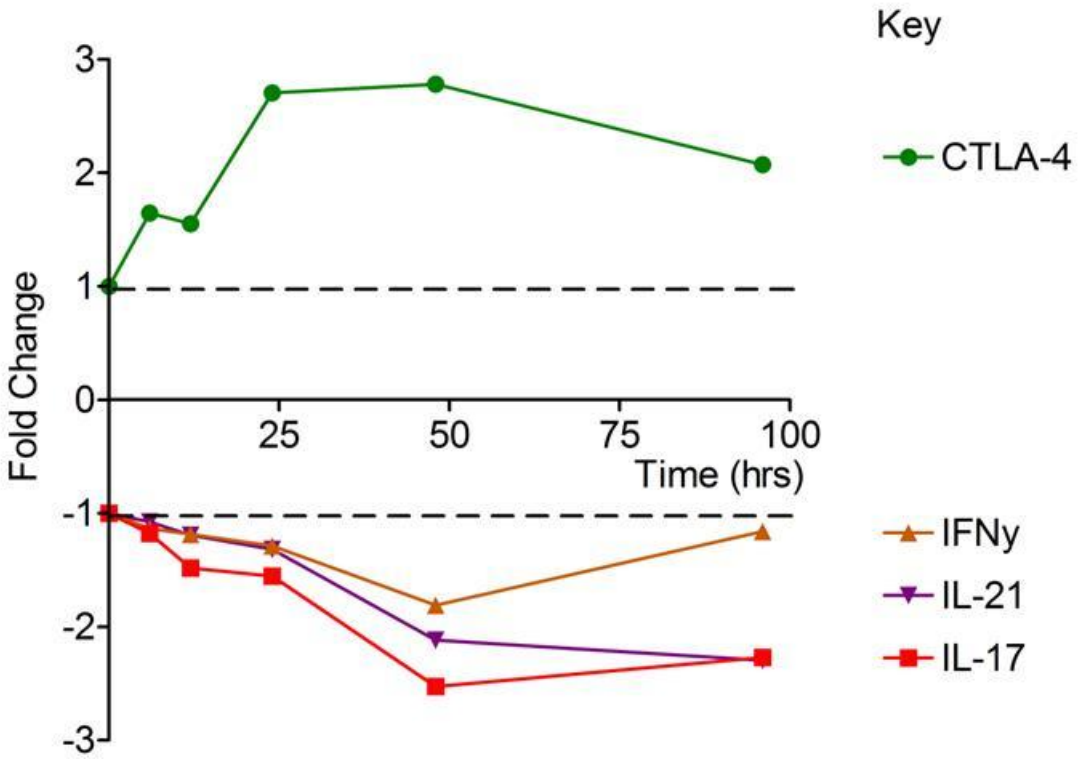

Figure 5.20. Vitamin D sensitivities of CTLA-4 and inflammatory cytokines in T_h17 polarised cultures. T_h17 polarised cultures were re-stimulated with beads in the presence of 1,25(OH)$_2$D$_3$ or vehicle control. Fold differences in level of expression (CTLA-4) or percentage of positive cells (IL-17, IFNγ and IL-21) between control and 1,25(OH)$_2$D$_3$-treated re-stimulation cultures were determined by flow cytometry and are plotted over a period of 96 hours post re-stimulation. Dotted lines indicate the positions of no change. Data are from results shown in figures 5.17 – 5.19.

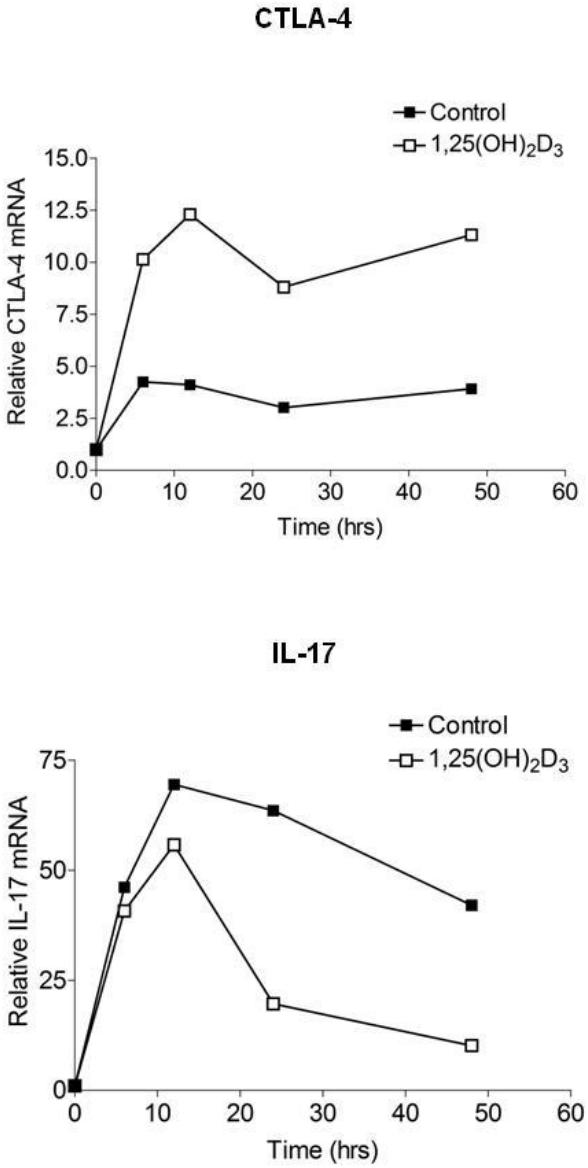

Figure 5.21. Time course of 1,25(OH)$_2$D$_3$ effect upon CTLA-4 and IL-17 mRNA transcripts. T_H17 polarised cultures were re-stimulated with anti-CD3/CD28 beads in the presence of 1,25(OH)$_2$D$_3$ or vehicle control. At the indicated times, CTLA-4 and IL-17 mRNA expressions were determined by quantitative realtime PCR. Expression was normalised to 18S rRNA and plotted relative to the level at the start of re-stimulation (n=1).

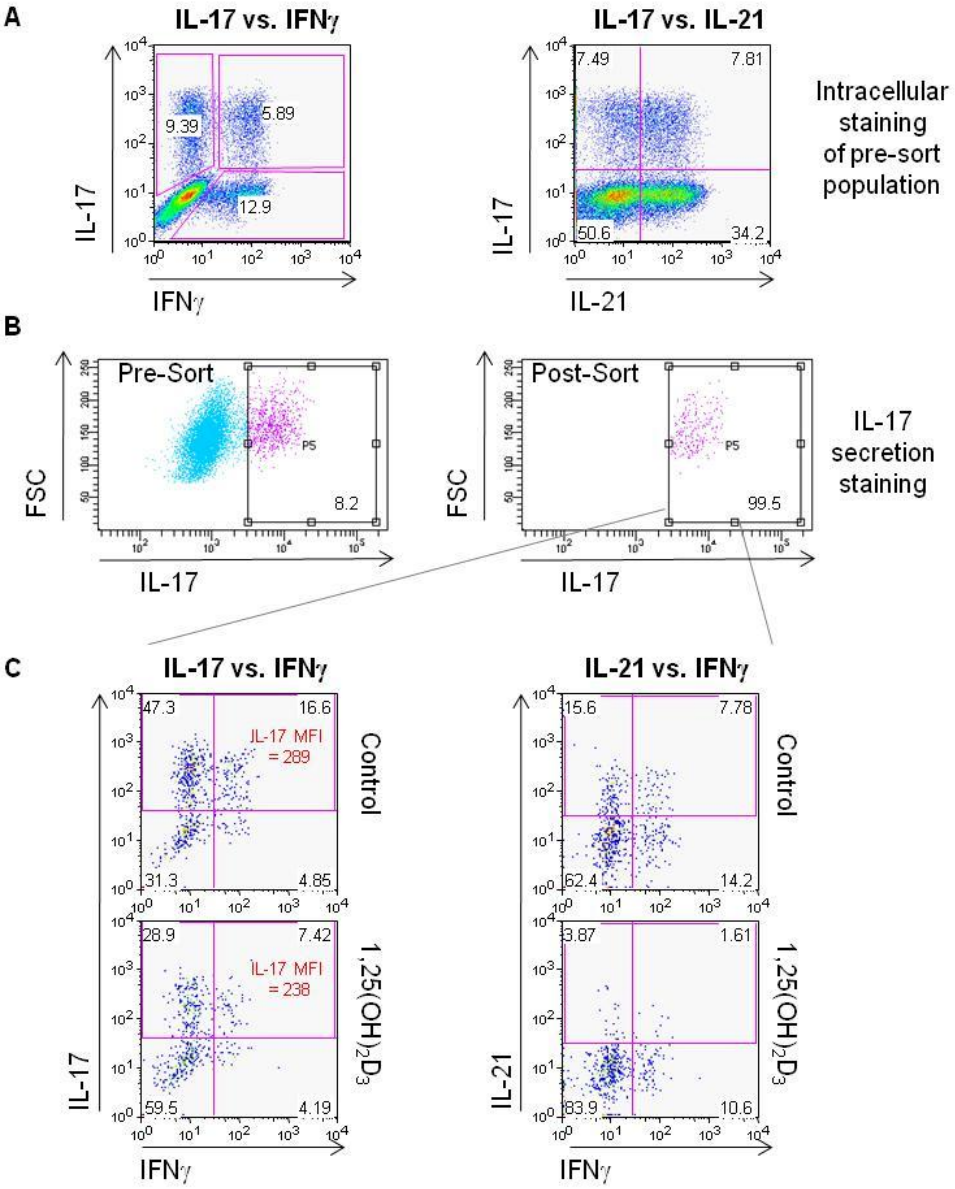

Figure 5.22. 1,25(OH)$_2$D$_3$ reduces IL-17 expression by sorted IL-17$^+$ cells. CD4$^+$CD25$^-$ T cells were stimulated for six days with antiCD3/CD28 beads at a ratio of 1 bead to 32 cells in serum free medium supplemented with T$_h$17 polarising cytokines. **A)** IL-17, IFNγ and IL-21 expression by the culture as measured by intracellular staining and flow cytometry. **B)** Live IL-17$^+$ cells (P5) were sorted from the T$_h$17 culture to 99% purity using an IL-17 secretion, detection and capture kit (Miltenyi Biotec) and MoFlo sorting. Following culture for three days under T$_h$17 polarising conditions in the presence of 1,25(OH)$_2$D$_3$ or vehicle control, cells were stained for intracellular IL-17, IFNγ and IL-21 and analysed by flow cytometry **(C)**.

5.8 Discussion

In this chapter the effect of 1,25(OH)$_2$D$_3$ upon T cell differentiation in the absence of serum was addressed and the role of TGFβ investigated. I observed that when T cells were stimulated with beads under serum free conditions, the effect of 1,25(OH)$_2$D$_3$ upon CTLA-4, CD25, IFNγ, IL-21 and IL-22 was similar to when serum containing medium was used (**chapter four**). This implies that 1,25(OH)$_2$D$_3$ can promote a regulatory T cell phenotype in the presence or absence of factors in serum. However, TGFβ was required for IL-17 expression, as shown by others (Manel et al., 2008; Volpe et al., 2008; Yang et al., 2008a) and TGFβ was needed to stimulate FoxP3 induction. Therefore, TGFβ promoted the 1,25(OH)$_2$D$_3$-dependent formation of a CTLA-4$^+$FoxP3$^+$ population. Interestingly, IL-10 up-regulation by 1,25(OH)$_2$D$_3$ also required TGFβ.

The requirement of TGFβ for FoxP3 expression by iT$_{Regs}$ is now established (Horwitz et al., 2008a; Shevach et al., 2008; Tran et al., 2007) as is the role of TGFβ in maintaining FoxP3 expression by nT$_{Regs}$ in the periphery (Marie et al., 2005). TGFβ signalling involves phosphorylation of receptor associated Smads (2 or 3) that in turn heterodimerise with Smad4. This complex binds to regulatory elements in target genes and interacts with other DNA binding factors to control gene expression (O'Shea et al., 2009). Whilst Smad deficiencies do not impair nT$_{Reg}$ suppression and only mildly affect nT$_{Reg}$ numbers, Smads 2, 3 and 4 are non-redundant in iT$_{Reg}$ differentiation (Malhotra et al., 2010; Martinez et al., 2009; Takimoto et al., 2010; Yang et al., 2008c). The dependency of FoxP3 expression upon TGFβ is supported by the presence of a Smad3 binding site in the FoxP3 promoter and an enhancer element

upstream of the FoxP3 ATG start site (Ruan et al., 2009; Tone et al., 2008). Interestingly, the affinity of the enhancer site for Smad3 is increased by AP-1 or all trans retinoic acid (ATRA) signalling (Xu et al., 2010). The latter is consistent with the synergistic effect of TGFβ and ATRA in FoxP3 induction. In contrast to ATRA, I did not observe synergy between 1,25(OH)$_2$D$_3$ and TGFβ for FoxP3 expression even though both RAR and VDR partner RXR in their signalling. It might be that although two potential RAR-RXR binding sites are present at the FoxP3 locus (Xu et al., 2010), VDR binding cannot substitute RAR. Indeed it has been shown that RARα is important for ATRA enhanced FoxP3 (Schambach et al., 2007). Recently, whether ATRA regulates FoxP3 expression mainly through direct or indirect mechanisms has been a matter of debate (Hill et al., 2008; Mucida et al., 2009). My observations, together with those of others might support the former, since unlike ATRA, 1,25(OH)$_2$D$_3$, did not enhance FoxP3 even though it could influence the same indirect mechanisms by which ATRA might increase FoxP3. For example, like ATRA, 1,25(OH)$_2$D$_3$ could increase Smad3 (Cao et al., 2003b; Xiao et al., 2008) and suppress inflammatory cytokines and receptors for pro-T$_h$17 signals (Cao et al., 2003b; Hill et al., 2008; Ikeda et al., 2010; Xiao et al., 2008). Thus, whilst 1,25(OH)$_2$D$_3$ and ATRA both appear to regulate the T$_h$17/T$_{Reg}$ balance, there are clear differences in their actions.

In this chapter inter-dependency of TGFβ and 1,25(OH)$_2$D$_3$ for IL-10 expression was also observed. One explanation could be that TGFβ-induced Smads and VDR both regulate the *IL-10* gene. Consistent with this, a Smad4 binding site (Kitani et al., 2003) and VDRE (Matilainen et al., 2010) are present at the *IL-10* locus. The importance of TGFβ for IL-10 induction is supported by the observation that induced TGFβ promoted IL-10$^+$ cells in the

spleen and lamina propia of mice (Kitani et al., 2003) and TGFβ was necessary for the development of IL-10$^+$ T$_{Regs}$ from FoxP3$^+$ and FoxP3$^-$ precursor cells (Maynard et al., 2007). Furthermore, the combined importance of TGFβ and IL-10 in regulation was seen as TGFβ induction could not protect against colitis in *il-10* deficient mice (Kitani et al., 2003). Although none of these *in-vivo* studies investigated the combined requirement of 1,25(OH)$_2$D$_3$ with TGFβ for IL-10 expression, as I observed, they do not oppose it, since it is likely that 1,25(OH)$_2$D$_3$ or a replacement factor would be present *in-vivo*.

A number of studies have confirmed the ability of 1,25(OH)$_2$D$_3$ to promote IL-10 expression. For example, 1,25(OH)$_2$D$_3$ supplement in glucocorticoid-resistant asthmatic patients increased IL-10 production and enhanced their responsiveness to dexamethasone (Xystrakis et al., 2006) Similarly, 1,25(OH)$_2$D$_3$ potentiated the beneficial effects of allergen specific immunotherapy in an IL-10 and TGFβ dependent manner (Taher et al., 2008). Given the importance of IL-10 and TGFβ in the regulation of immune responses, my finding that 1,25(OH)$_2$D$_3$ permitted TGFβ induced IL-10, suggests a central role for 1,25(OH)$_2$D$_3$ in regulatory responses. It will be of interest to investigate whether other factors can substitute for 1,25(OH)$_2$D$_3$ in this effect.

An additive effect of TGFβ and 1,25(OH)$_2$D$_3$ in CTLA-4 up-regulation was also observed in this study, although, in contrast to its role in IL-10 expression, TGFβ was not essential for CTLA-4 up-regulation by 1,25(OH)$_2$D$_3$. Interestingly, TGFβ enhanced VDR mRNA expression, consistent with the report that phosphorylated Smad3 increased VDR transcription

(Daniel et al., 2007). Thus, TGFβ might enhance CTLA-4 expression in response to 1,25(OH)$_2$D$_3$ by increasing VDR availability. Alternatively, TGFβ could enhance VDR transactivation via Smad3 (Yanagisawa et al., 1999). The latter might have relevance in the regulation of CTLA-4 expression, since a VDR binding site is present within the CTLA-4 locus (Ramagopalan et al., 2010). Interestingly, in addition to TGFβ, I observed that pro-T$_h$17 cytokines (IL-1β, IL-6 and IL-23) could actually enhance the 1,25(OH)$_2$D$_3$-mediated increase in CTLA-4 expression, whilst in the absence of 1,25(OH)$_2$D$_3$ they suppressed CTLA-4. Reduction of CTLA-4 by pro-T$_h$17 cytokines might have relevance *in-vivo*, as it could permit a more sustained T cell response by reducing CTLA-4 facilitated suppression during inflammation. It is possible that elevated levels of T$_h$17 cytokines in autoimmune diseases would also result in reduced CTLA-4 expression by effector T cells or T$_{Regs}$. Indeed, T$_{Regs}$ isolated from the peripheral blood of children with newly diagnosed diabetes had low CTLA-4 transcript expression (Luczynski et al., 2009). If low CTLA-4 leads to reduced T$_{Reg}$ effectiveness this could contribute to disease. The fact that CTLA-4 levels reached their highest following 1,25(OH)$_2$D$_3$ supplementation under T$_h$17-polarising conditions might suggest that rather than inhibiting the regulatory properties of 1,25(OH)$_2$D$_3$, inflammatory conditions might actually support them. However, for this observation to have functional relevance it requires that the level of CTLA-4 expression can influence the regulatory capacity of the cell.

Although many studies point to a major role for CTLA-4 in the function of T$_{Regs}$ (Friedline et al., 2009; Kolar et al., 2009; Onishi et al., 2008; Read et al., 2006; Schmidt et al., 2009; Tivol et al., 1995; Waterhouse et al., 1995; Wing et al., 2008; Zheng et al., 2008b), the mechanism

by which CTLA-4 suppresses effector T cells and how regulation relates to the level of its expression is not clear. Data from my lab suggest that CTLA-4 contributes to regulation by internalising its B7 ligands, in a process termed transendocytosis (Qureshi et al. manuscript in preparation). Thus, to investigate whether the level of CTLA-4 on stimulated cells could affect their regulatory potential, I performed transendocytosis assays. These revealed that the extent of internalisation corresponded with the level of cycling CTLA-4 and was CTLA-4 dependent. Thus, 1,25(OH)$_2$D$_3$-treated cells showed enhanced CD86 acquisition relative to control cells. In further support of this finding and its relevance immunologically, I observed that 1,25(OH)$_2$D$_3$-treated T cells could deplete CD80 and CD86 on co-cultured DCs more extensively than could control T cells. Furthermore, blocking CTLA-4 confirmed that the majority of B7 depletion was CTLA-4 dependent. I also demonstrated that CTLA-4 blockade could reduce 1,25(OH)$_2$D$_3$-mediated suppression of T cell division in DC driven stimulations. Together, these data suggest that CTLA-4 dependent depletion of B7 contributes greatly to the mechanism of suppression by 1,25(OH)$_2$D$_3$-treated T cells. Consistent with this conclusion, others have shown that T_{Regs} down-regulate DC B7 in a CTLA-4 dependent manner leading to reduced stimulatory capacity of the DC (Oderup et al., 2006; Wing et al., 2008).

A number of regulatory mechanisms might converge on B7 depletion. The cytokine profile of 1,25(OH)$_2$D$_3$-treated cells might contribute, since IL-10, which is enhanced in 1,25(OH)$_2$D$_3$ treated cells, can suppress B7 ligands (Moore et al., 2001). Furthermore, in the absence of CTLA-4, IL-10 blockade abrogated T_{Reg} function (Read et al., 2006). By contrast, IFNγ, which is decreased in 1,25(OH)$_2$D$_3$-treated T cells, enhances B7 (Fujihara et al., 1996; Moore et al., 2001). Although not measured in this study, 1,25(OH)$_2$D$_3$ can increase TGFβ (Cao et

al., 2003b) and TGFβ can suppress B7 (Misra et al., 2004). Others have shown that CTLA-4 engagement leads to enhanced TGFβ and suppressed IFNγ expression by $CD4^+$ T cells (Gomes et al., 2000). It is therefore possible that TGFβ, IL-10, IFNγ and CTLA-4 exist in a suppressive feedback loop, which can be favoured by the action of 1,25(OH)$_2$D$_3$. In the future, it will be of interest and potentially of therapeutic use, to investigate the nature and relative contribution of CTLA-4 dependent and independent suppression mechanisms employed by 1,25(OH)$_2$D$_3$-conditioned T cells.

If 1,25(OH)$_2$D$_3$ is to be useful therapeutically, it would be important that it not only influences the differentiation of T cells undergoing primary stimulation but that it can also affect previously stimulated T cells. To test this, T_h17 polarised cultures were re-stimulated in the presence of 1,25(OH)$_2$D$_3$ and their phenotype monitored over time. CTLA-4 was up-regulated within six hours. The fast kinetics of this response might suggest that 1,25(OH)$_2$D$_3$ can switch the phenotype of previously stimulated T cells. However, the kinetics of IL-17 and IL-21 suppression in response to 1,25(OH)$_2$D$_3$ were slower and suppression of IFNγ was minimal over the time studied. These distinctions between CTLA-4 and cytokine expressions might be indicative of direct versus indirect signalling mechanisms respectively, or the use of slower, non-VDR mediated signalling for cytokine regulation. However, the presence of a VDRE in both the CTLA-4 (Ramagopalan et al., 2010) and IFNγ (Cippitelli and Santoni, 1998) gene promoters implies that both loci are potential VDR binding sites.

The lack of response by IFNγ compared to CTLA-4 might alternatively reflect differences in the chromatin state around their loci. Indeed, previous studies suggest that T_h1 and T_h2 cells

lose their ability to acquire the opposite cytokine program after only three to four divisions (Grogan et al., 2001). Epigenetic analysis further revealed the enrichment of permissive histone H3 lysine 4 trimethylation (H3K4me3) at the IFNγ locus compared to suppressive histone H3 lysine 27 trimethylation (H3K27me3) at IL-4 and IL-17 loci in T_h1 cells. By contrast, in T_h2 cells, the IL-4 locus contained permissive H3K4me3 whilst IFNγ and IL-17 loci were marked by suppressive H3K27me3 (reviewed by (Zhou et al., 2009a).

The greater 1,25(OH)$_2$D$_3$ sensitivity of IL-17 compared to IFNγ is consistent with reports that T_h17 cells retain plasticity (Zhou et al., 2009a), failing to maintain IL-17 expression in the absence of TGFβ and IL-6, and becoming converted to T_h1 or T_h2 cells in the presence of IL-12 or IL-4 respectively (Lexberg et al., 2008). Thus, it would seem that the histone modifications that form at the IL-17A/IL-17F locus (Akimzhanov et al., 2007) are not stable. Adoptive transfer of T_h17 cells has further demonstrated the ability of T_h17 cells to convert into T_h1 and $T_h17/1$ populations (Bending et al., 2009; Kurschus et al., 2010; Lee et al., 2009; Martin-Orozco et al., 2009). In addition, plasticity of the T_h17 lineage has been demonstrated as repeat stimulation of T_h17 cells in the presence of TGFβ and IL-6 led to their expression of IL-10 and a switch to regulatory phenotype capable of suppressing EAE (McGeachy et al., 2007). Interestingly, although regarded less plastic than T_h17 cells, T_h1 cells co-expressed IFNγ and IL-10 when re-stimulated in the presence of TGFβ and showed reduced encephalitogenicity in the EAE model (Huss et al., 2010). In addition, whilst most studies suggest that T_h1 cells do not acquire IL-17 expression, Kurschus et al. recently suggested this might be possible (Kurschus et al., 2010). Thus, it would seem that T_h1 and T_h17 cells could both demonstrate plasticity under the right conditions.

1,25(OH)$_2$D$_3$ effects in the absence of serum

To rule out the possibility that 1,25(OH)$_2$D$_3$-mediated IL-17 suppression in previously stimulated cultures arose through selective outgrowth of IL-17$^-$ cells or de-novo commitment of previously un-stimulated cells, an attempt was made to purify live IL-17$^+$ cells from a T$_h$17 polarised culture using a cytokine secretion and capture assay. Although 99% IL-17$^+$ purity was obtained, it is possible that by this method false positives were labelled, as IL-17$^-$ cells captured IL-17 from neighbouring IL-17-secreting cells. The data indicate that 1,25(OH)$_2$D$_3$ could suppress IL-17 expression and similar to its effects upon primary cultures, the T$_h$17/1 population was the most sensitive. However, sensitivity was less than seen when primary stimulations were supplemented with 1,25(OH)$_2$D$_3$. These data might therefore support the initial dogma that T cell phenotypes become increasingly fixed upon stimulation. Whilst resistance to 1,25(OH)$_2$D$_3$ could question the benefit of 1,25(OH)$_2$D$_3$ supplementation in the control of inflammation at sites of established disease, its ability to promote tolerance at the cell population level is arguably of great importance. In support of this, several studies in mouse models of autoimmune disease now report the ability of 1,25(OH)$_2$D$_3$ to suppress T$_h$17 populations and correlate this suppression with reduced disease severity (Arnson et al., 2007; Cantorna and Mahon, 2004; Mullin and Dobs, 2007). Thus, 1,25(OH)$_2$D$_3$ remains an attractive molecule for use in the control of chronic inflammatory disease.

6 IS LOCAL CONVERSION OF 25(OH)D_3 BY DENDRITIC CELLS SUFFICIENT TO SUPPORT A REGULATORY $CD4^+$ T CELL PHENOTYPE?

The finding in **chapter 5** of this thesis that the regulatory effects of 1,25$(OH)_2D_3$ could be maintained and even enhanced under inflammatory conditions, suggests that 1,25$(OH)_2D_3$ might be useful in the treatment of autoimmune disease. However, the hypercalcaemic effect of 1,25$(OH)_2D_3$ questions the safety of such an approach. Furthermore, 1,25$(OH)_2D_3$ has limited stability, and strong homeostatic mechanisms operate to retain 1,25$(OH)_2D_3$ serum levels at approximately 0.1nM (Mullin and Dobs, 2007). Thus, although 1,25$(OH)_2D_3$ supplementation might be immunologically effective, it is likely that any effects would be short lived. Supplementation with 25(OH)D_3 might be a safer and longer lasting option, but to be of benefit 25(OH)D_3 must be sufficiently converted at a site of immune activation. Conversion is carried out by the cytochrome P450 enzyme 25-hydroxyvitamin-D_3-1α-hydroxylase (CYP27B1). Monocyte derived macrophages and dendritic cells (DCs) have been shown to express CYP27B1 and facilitate conversion *in-vitro* (Fritsche et al., 2003; Hewison et al., 2007; Liu et al., 2006a) but whether the 1,25$(OH)_2D_3$ generated can in turn modulate the T cell phenotype has not been studied. This chapter therefore looks at whether 1,25$(OH)_2D_3$-mediated $CD4^+$ T cell responses can be observed when $CD4^+$ T cells are stimulated in the presence of inactive 25(OH)D_3 in place of active 1,25$(OH)_2D_3$.

6.1 25(OH)D_3 promotes a regulatory $CD4^+$ T cell phenotype in the presence of APCs

It was considered that T cells stimulated by APCs in the presence of 25(OH)D_3 might show 1,25$(OH)_2D_3$-mediated responses, since others have demonstrated that APCs can convert 25(OH)D_3 to 1,25$(OH)_2D_3$ *in-vitro* (Fritsche et al., 2003; Hewison et al., 2007; Liu et al., 2006a). Furthermore, because T cells and APCs co-habit immune sites, such stimulations are potentially relevant *in-vivo*. To determine the type of APC to use for T cell stimulation, CYP27B1 and VDR transcripts were compared for monocytes, immature DCs (iDCs) and LPS matured DCs (mDCs) by quantitative real-time PCR (**figure 6.1**). Low CYP27B1 expression was found in monocytes and iDCs but maturation with LPS dramatically up-regulated its expression in DCs. Similarly, mDCs showed the highest VDR expression. Based upon these data, it was hypothesised that of the APCs studied, mDCS would support the greatest conversion of 25(OH)D_3 to 1,25$(OH)_2D_3$ and would be the most sensitive to 1,25$(OH)_2D_3$. Hence, mDCs were used initially to investigate the ability of T cells to respond to locally converted 25(OH)D_3. To determine the sensitivity of the response, 25(OH)D_3 and 1,25$(OH)_2D_3$ were titrated over the range 0-50nM and T cell expression of CTLA-4, FoxP3, IL-17 and IFNγ was monitored by flow cytometry. As seen for 1,25$(OH)_2D_3$, concentration dependent induction of CTLA-4 and the CTLA-4^+FoxP3^+ phenotype (**figure 6.2**) as well as suppression of IL-17 and IFNγ (**figure 6.3**) was observed across the 25(OH)D_3 titration. However, in contrast to 1,25$(OH)_2D_3$, for which effects were evident at 0.1nM, responses to 25(OH)D_3 were not seen below 1nM. For the four T cell markers, responses at 50nM 25(OH)D_3 mirrored those at 10nM 1,25$(OH)_2D_3$. Therefore, 50nM 25(OH)D_3 was chosen for future experiments. Using this concentration, it was confirmed for multiple donors that 25(OH)D_3 enhanced CTLA-4 expression by mDC stimulated T cells to a level equivalent to

that attained with 1,25(OH)$_2$D$_3$ (**figure 6.4**). At the same time, 25(OH)D$_3$ suppressed T cell expression of IL-17, IFNγ and IL-21 (**figure 6.5**).

6.2 Ketoconazole inhibits CTLA-4 induction by 25(OH)D_3 supplementation

The broad spectrum cytochrome p450 inhibitor, ketoconazole (KC), has been used by others to demonstrate enzymatic conversion of 25(OH)D$_3$ *in-vitro* (Fritsche et al., 2003; Hewison et al., 2007). Therefore, to test if the effects of 25(OH)D$_3$ upon CD4$^+$ T cell phenotype were a consequence of its local enzymatic conversion, KC was used. Hewison et al, showed that at 5μM, KC could suppress 1,25(OH)$_2$D$_3$ generation in DC and macrophage cultures treated with 25(OH)D$_3$ concentrations up to 150nM (Hewison et al., 2007). Thus, 5μM KC was used for initial CYP27B1 blocking studies and CTLA-4 was chosen as the response marker for 1,25(OH)$_2$D$_3$ signalling, due to its strong 1,25(OH)$_2$D$_3$ sensitivity. 5μM KC was found to slightly reduce the proliferation of mDC stimulated T cells. Since CTLA-4 expression might have been suppressed by compromised stimulation, the effect of KC upon CTLA-4 expression was only compared for activated T cells expressing CD25. This showed that KC reduced the 25(OH)D$_3$ mediated increase in CTLA-4 expression by mDC stimulated T cells three fold (**figure 6.6**).

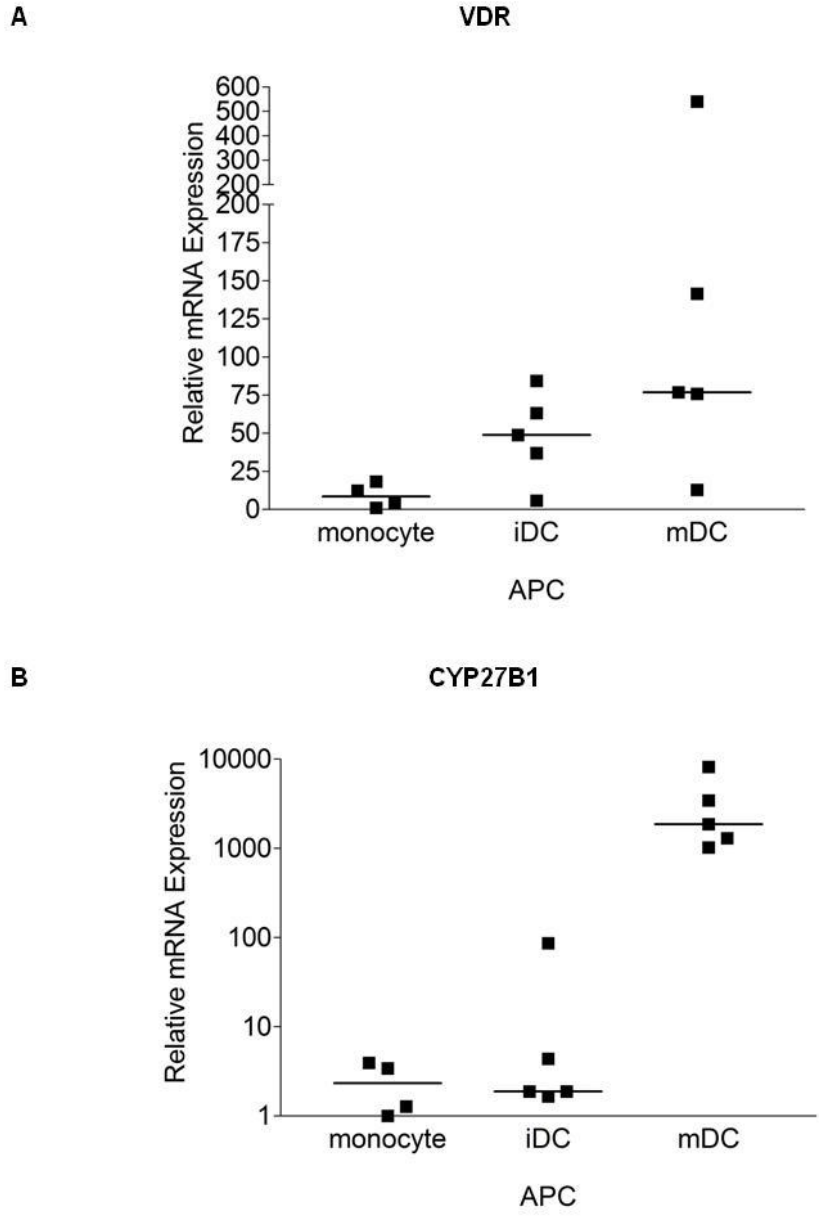

Figure 6.1. VDR and CYP27B1 expression in antigen presenting cells. CYP27B1 **(A)** and VDR **(B)** mRNA expression levels in monocytes, immature DCs (iDCs) and LPS-matured DCs (mDC) were measured by quantitative real-time PCR. Levels were normalised to 18S rRNA and are plotted relative to one other. Horizontal lines indicate median expression.

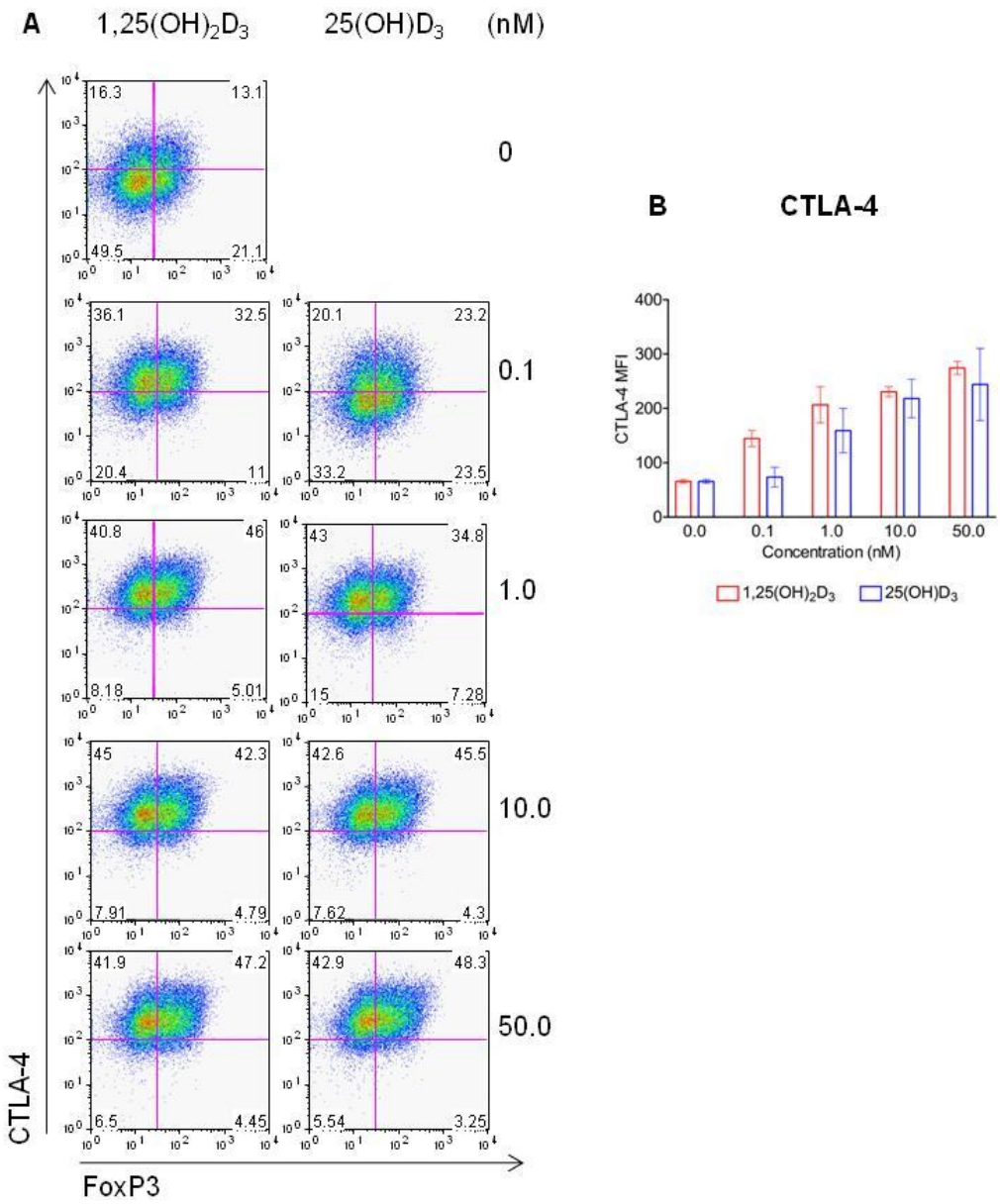

Figure 6.2. $1,25(OH)_2D_3$ and $25(OH)D_3$ affect CTLA-4 and FoxP3 expression. $CD4^+CD25^-$ T cells were stimulated with mature dendritic cells plus anti-CD3 in the presence of varying concentrations of $1,25(OH)_2D_3$, $25(OH)D_3$ or vehicle control. At four days, cells were stained for CD3, CTLA-4 and FoxP3 and analysed by flow cytometry. **A)** FACS plots are from one experiment representative of two performed. Numbers in quadrants refer to percentage of $CD3^+$ cells. **B)** CTLA-4 frequencies for two donors. Mean frequencies are plotted and error bars indicate standard deviation.

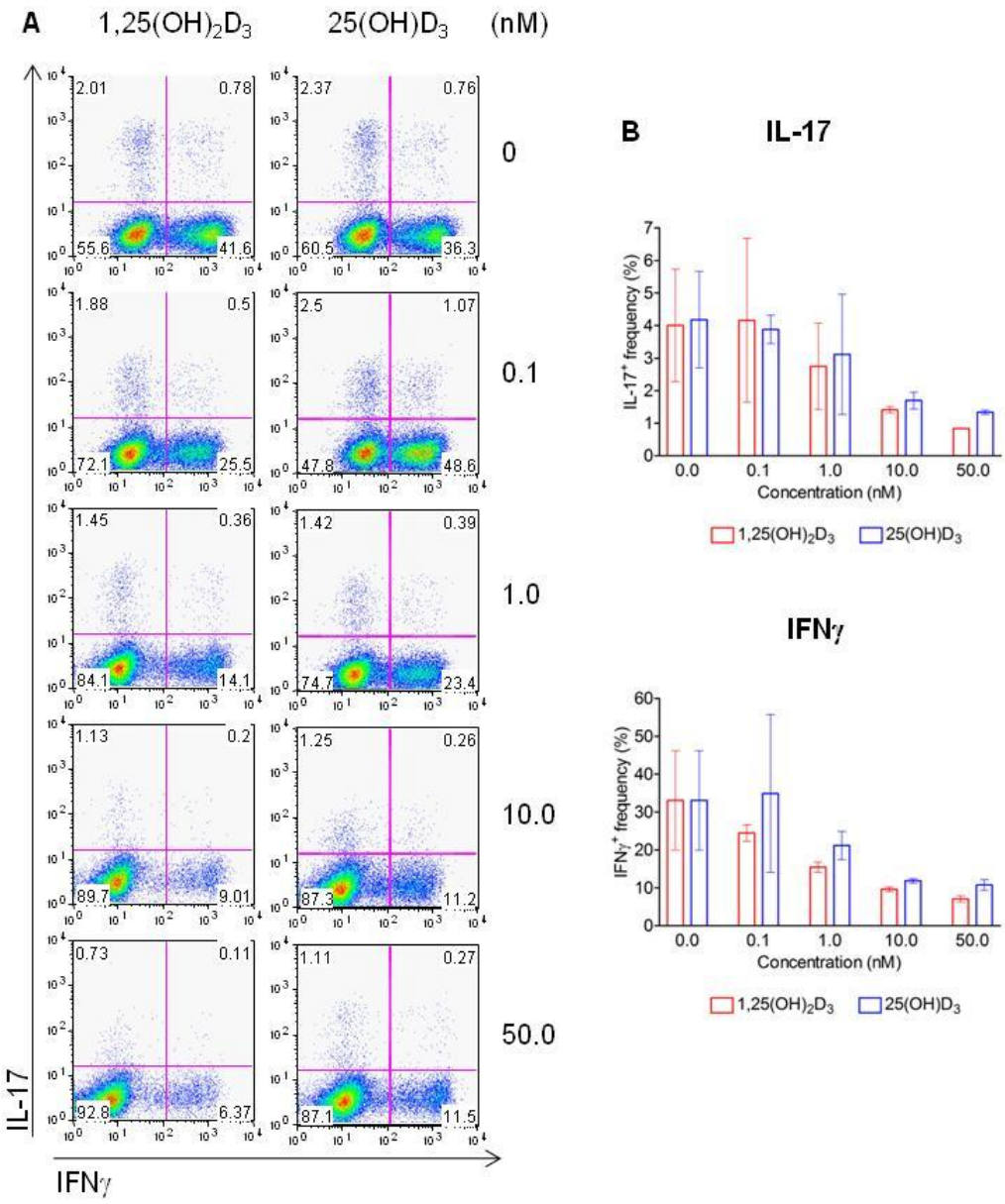

Figure 6.3. 1,25(OH)$_2$D$_3$ and 25(OH)$_2$D$_3$ inhibit IL-17 and IFNγ. CD4$^+$CD25$^-$ T cells were stimulated with mature dendritic cells plus anti-CD3 in the presence of varying concentrations of 1,25(OH)$_2$D$_3$, 25(OH)D$_3$ or vehicle control. At five days, cells were stained for CD3, IL-17 and IFNγ and analysed by flow cytometry. **A)** FACS plots are from one experiment representative of two performed. Numbers in quadrants refer to percentage of CD3$^+$ cells. **B)** IL-17$^+$ and IFNγ$^+$ frequencies for two donors. Mean frequencies are plotted and error bars indicate standard deviation.

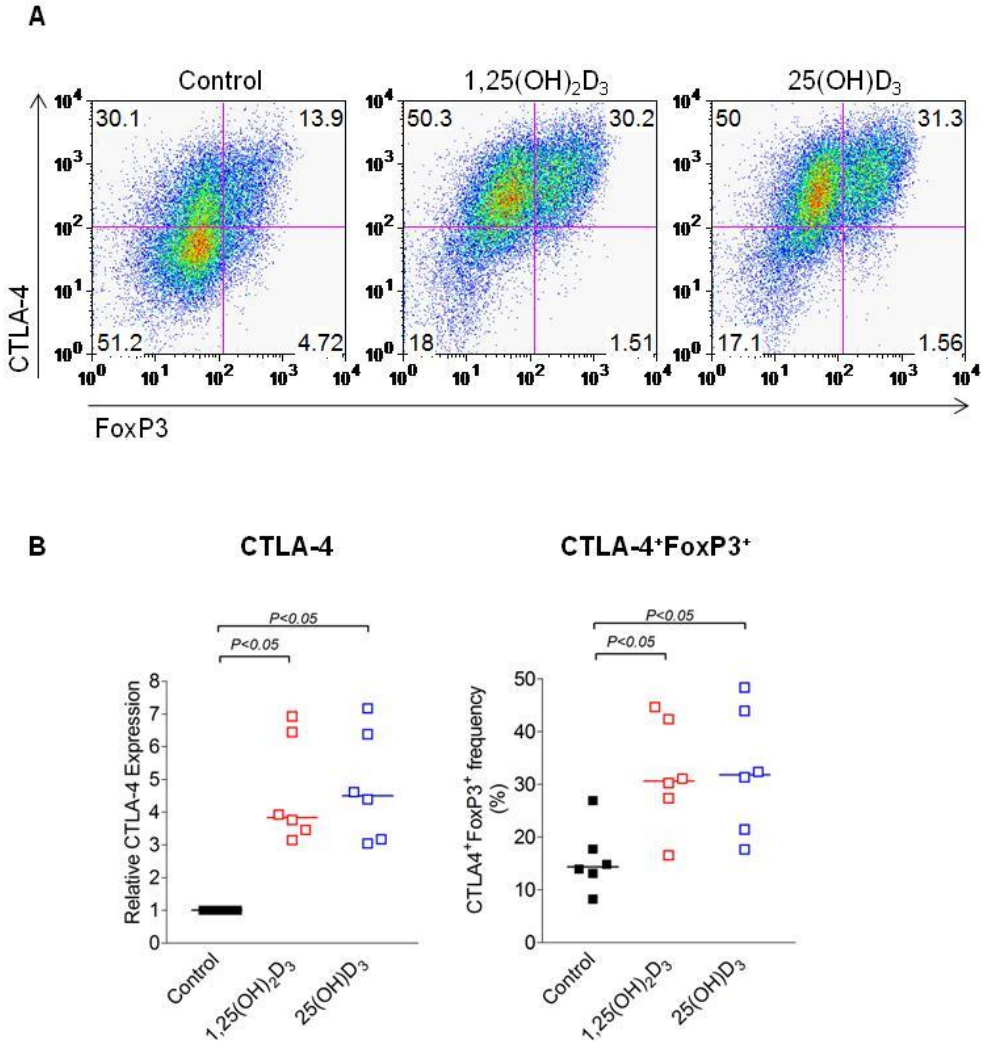

Figure 6.4. Conversion of 25(OH)D_3 during mature dendritic cell-driven stimulations increases CTLA-4 expression and CTLA-4$^+$FoxP3$^+$ frequencies. CD4$^+$CD25$^-$ T cells were stimulated with mature dendritic cells plus anti-CD3 in serum free medium supplemented with IL-2 in the presence of 10nM 1,25(OH)$_2D_3$, 50nM 25(OH)D_3 or vehicle control. At four days, cells were stained for CTLA-4 and FoxP3 and analysed by flow cytometry. **A)** Representative FACS plots are from one experiment. Numbers in quadrants refer to percentage of cells. **B)** Relative CTLA-4 median fluorescence intensities and CTLA-4$^+$FoxP3$^+$ frequencies for multiple donors. Horizontal lines indicate median values. Significance was tested by a two-tailed Wilcoxon matched pairs test.

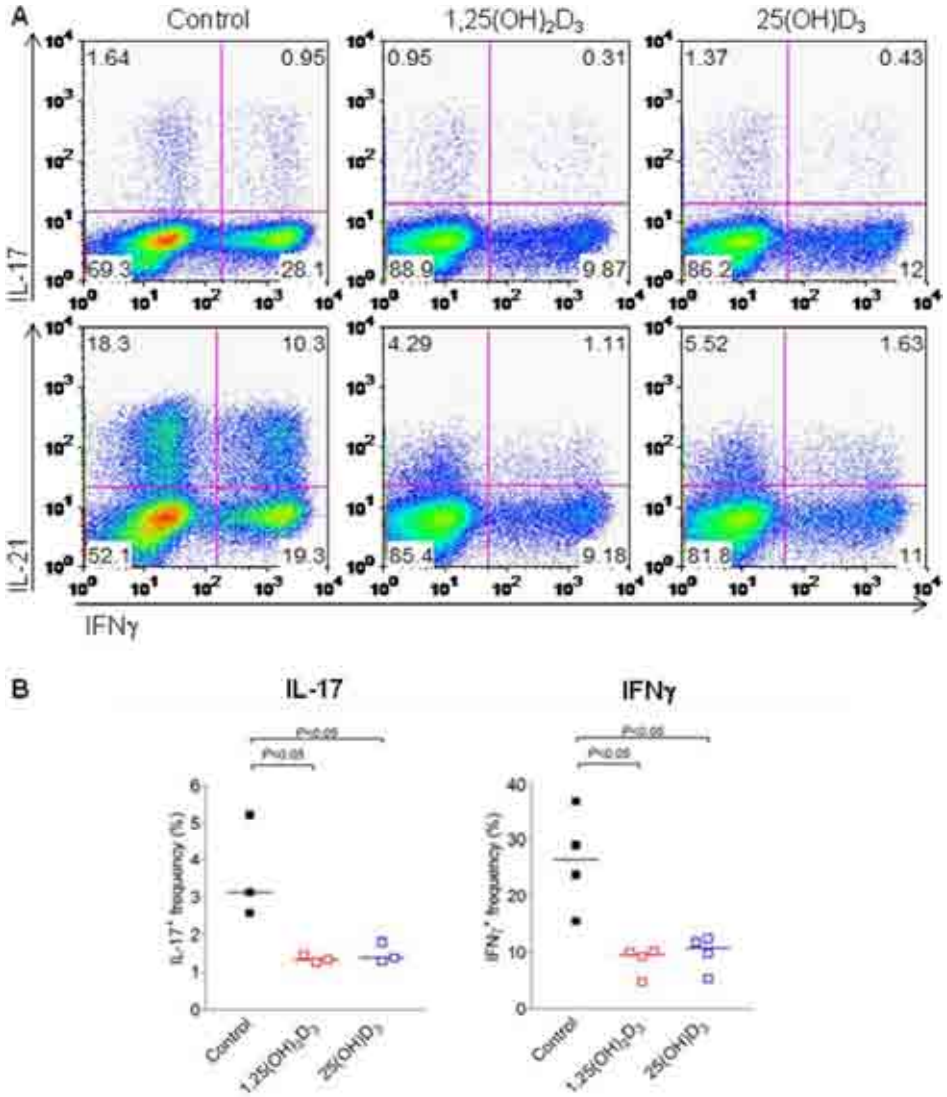

Figure 6.5. 25(OH)D_3 conversion during mature dendritic cell-driven stimulations suppresses IL-17, IFNγ and IL-21 expression. $CD4^+CD25^-$ T cells were stimulated with mature dendritic cells plus anti-CD3 in serum free medium in the presence of 10nM 1,25(OH)$_2D_3$, 50nM 25(OH)D_3 or vehicle control. At five days, cells were intracellular stained for CD3, IL-17, IL-21 and IFNγ and analysed by flow cytometry. **A)** Representative FACS plots are from one experiment. Numbers in quadrants refer to percentage of cells. **B)** IL-17 and IFNγ frequencies for multiple donors. Horizontal lines show median frequencies. Significance was tested by a two-tailed Wilcoxon matched pairs test.

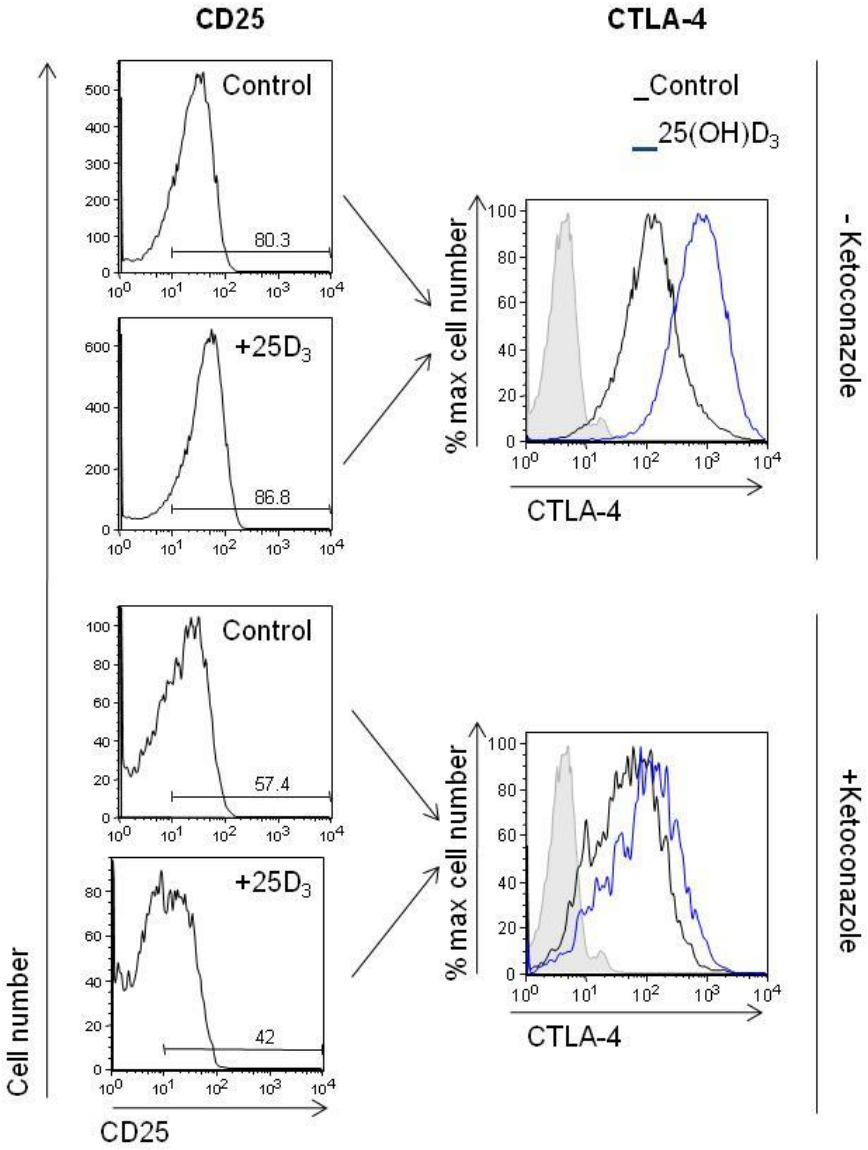

Figure 6.6. Ketoconazole inhibits 25(OH)D_3-mediated CTLA-4 up-regulation during mature dendritic cell-driven stimulations. $CD4^+CD25^-$ T cells were stimulated with mature dendritic cells plus anti-CD3 in serum free medium supplemented with IL-2 in the presence of 50nM 25(OH)D_3 (blue) or vehicle control (black) with or without ketoconazole (5μM). At four days, cells were stained for CD25 and CTLA-4 and analysed by flow cytometry. CTLA-4 expression by $CD25^+$ cells gated is shown. Shaded histogram illustrates isotype control staining. Data are from a single experiment.

In light of the finding that freshly isolated monocytes have very low CYP27B1 expression relative to mDCs (**figure 6.1**), whether 25(OH)D_3 could affect T cell responses in monocyte driven stimulations was also assessed. As illustrated in **figure 6.7**, under these conditions strong induction of CTLA-4 and suppression of inflammatory cytokines, IL-17, IFNγ and IL-21 still occurred, suggesting that CYP27B1 is induced in monocytes upon stimulation or that low CYP27B1 expression is sufficient for conversion.

6.3 25(OH)D_3 is poorly converted by T cells in the absence of APCs

The striking effect of 25(OH)D_3 upon APC stimulated T cells prompted consideration of whether 25(OH)D_3 could also work autonomously upon the T cell. Therefore, CYP27B1 expression was measured in un-stimulated and stimulated T cells by quantitative real-time PCR. Whilst CYP27B1 expression was very low in un-stimulated T cells, it was increased upon stimulation. The maximum level of CYP27B1 transcript was detected at 6-24hr post stimulation. However, relative to 18SrRNA, CYP27B1 was much lower in stimulated T cells compared to mDCs (**figure 6.8**). To test the ability of T cells to convert 25(OH)D_3, its effect upon their expression of CTLA-4 and pro-inflammatory cytokines was measured after stimulation with beads. Consistent with the low expression of CYP27B1 in T cells, induction of CTLA-4 upon 25(OH)D_3 treatment was modest and suppression of cytokines, IL-17, IL-21 and IFNγ was small compared to the effect of 1,25$(OH)_2D_3$ (**figures 6.9-6.10**).

To investigate further whether this limited response of T cells to 25(OH)D_3 in the absence of APCs was a consequence of CYP27B1 mediated 25(OH)D_3 conversion, bead stimulations

were repeated in the presence of KC. Interestingly, KC concentrations above 0.05μM strongly impaired the proliferation of bead stimulated T cells. This was in contrast to its effects upon mDC stimulations. Since both CYP27B1 and VDR are expressed at very low levels in un-stimulated T cells but are increased upon stimulation, whether bead stimulation for three days prior to 25(OH)D_3 and KC treatment would improve T cell tolerance to KC treatment was tested. T_h17 polarising conditions were also used, since these enhance VDR expression and would maximise 1,25$(OH)_2D_3$-mediated CTLA-4 induction. CFSE dilution confirmed division at all KC concentrations across the range 0-2.5μM. Furthermore, KC concentration dependent inhibition of 25(OH)D_3-mediated CTLA-4 induction was observed for dividing cells, with total inhibition occurring at 2.5μM KC (**figures 6.11)**. Therefore, CYP27B1 catalysed conversion of 25(OH)D_3 by the T cell appeared to facilitate its up-regulation of CTLA-4 following 25(OH)D_3 treatment.

Overall, data reported in this chapter suggest that APCs express high levels of CYP27B1 and can efficiently convert 25(OH)D_3 to support 1,25$(OH)_2D_3$-modulated responses in surrounding T cells. By contrast, T cells express low levels of the converting enzyme, thus, in the absence of APCs, 25(OH)D_3 supplementation has modest anti-inflammatory effects upon T cell phenotype.

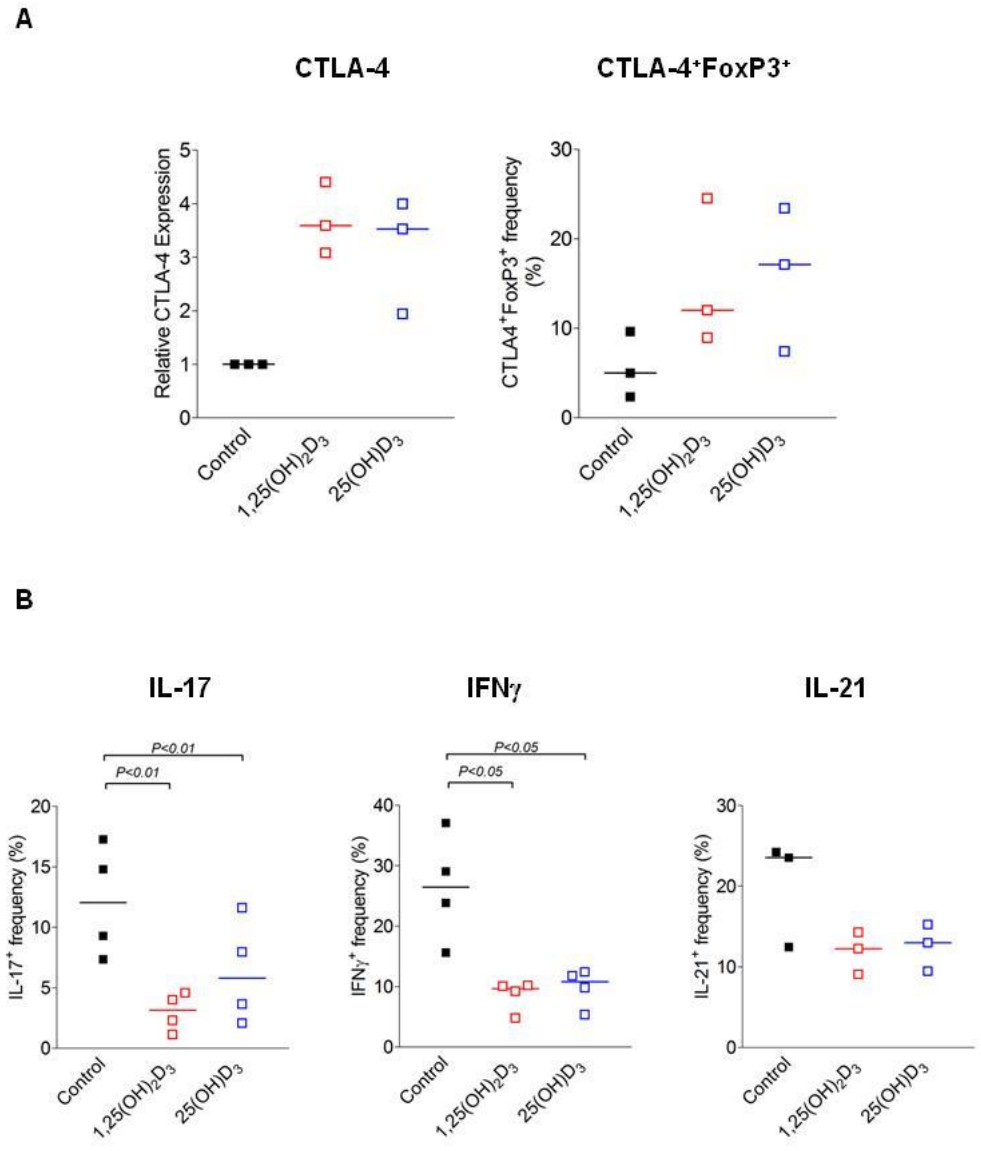

Figure 6.7. *In-vitro* conversion of 25(OH)D_3 during monocyte-driven T cell stimulations suppresses IL-17, IFNγ and IL-21 expression. $CD4^+CD25^-$ T cells were stimulated with monocytes plus anti-CD3 in serum free medium in the presence of 10nM 1,25$(OH)_2D_3$, 50nM 25(OH)D_3 or vehicle control. **A)** At four days, cells were stained for CD3, CTLA-4 and FoxP3. **B)** At five days, cells were intracellular stained for CD3, IL-17, IFNγ and IL-21 and analysed by flow cytometry. Data are shown for several donors. Horizontal lines indicate median values. Significance was tested by a two-tailed Wilcoxon matched pairs test.

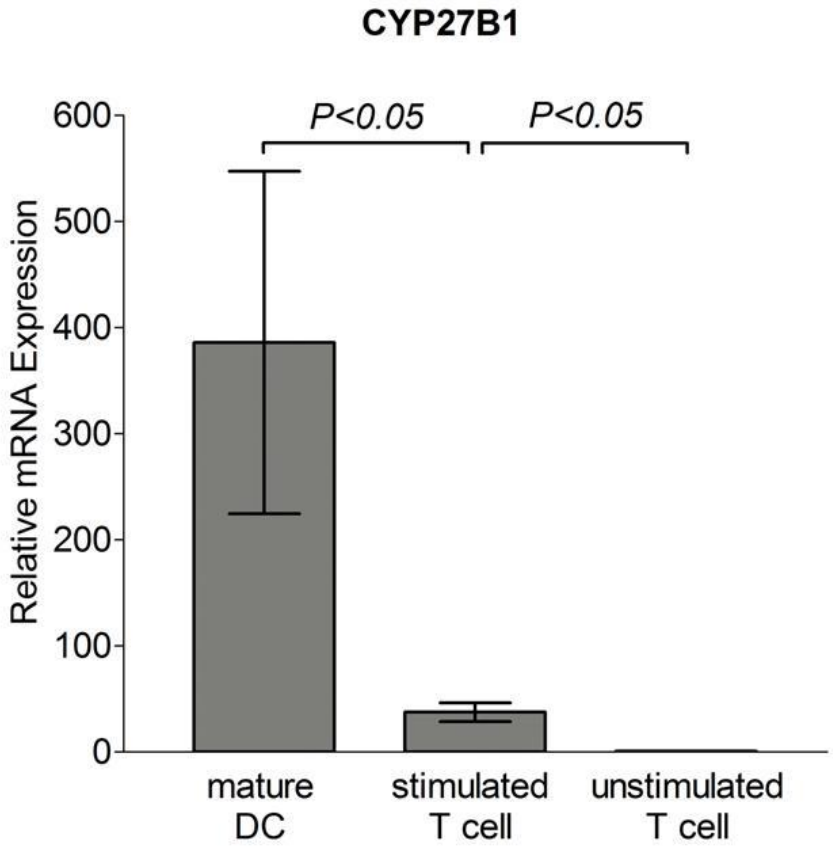

Figure 6.8. Stimulated T cells express CYP27B1. CYP27B1 mRNA expression by un-stimulated and antiCD3/CD28 stimulated T cells was measured by quantitative real-time PCR and compared against expression by mature DCs. Levels were normalised to 18S rRNA and expression relative to that in unstimulated T cells calculated. Data is for 3 donors and error bars indicate standard deviation. Significance was tested by a two-tailed Mann-Whitney U test.

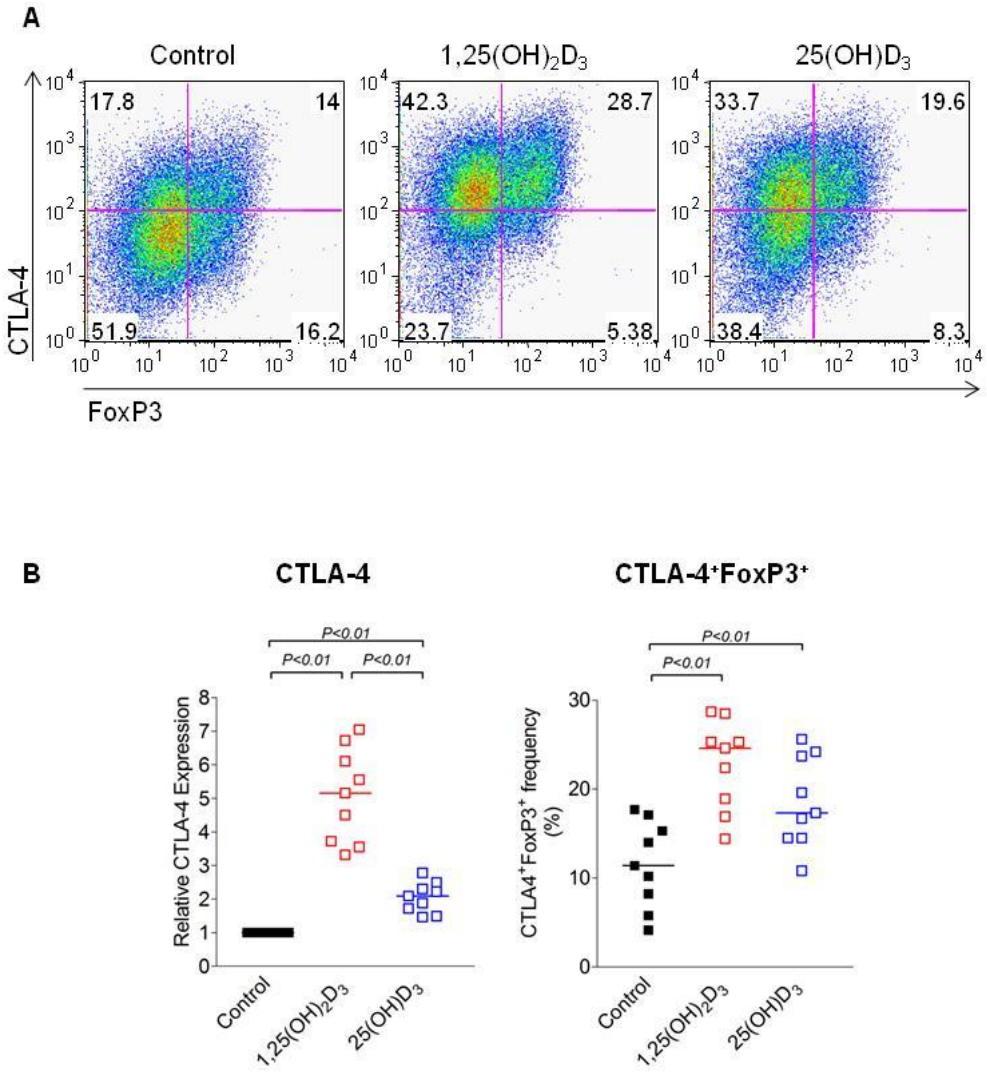

Figure 6.9. *In-vitro* activation of 25(OH)D_3 during bead-driven stimulations modestly increases CTLA-4 expression and the frequency of CTLA-4$^+$FoxP3$^+$ T cells. CD4$^+$CD25$^-$ T cells were stimulated with anti-CD3/CD28 beads in serum free medium supplemented with TGFβ in the presence of 10nM 1,25(OH)$_2$D$_3$, 50nM 25(OH)D_3 or vehicle control. At four days, cells were stained for CTLA-4 and FoxP3 and analysed by flow cytometry. **A)** Representative FACS plots from one experiment. Numbers in quadrants refer to percentage of cells. **B)** Relative CTLA-4 median fluorescence intensities and CTLA-4$^+$FoxP3$^+$ frequencies for multiple donors. Horizontal lines show median values. Significance was tested by a two-tailed Wilcoxon matched pairs test.

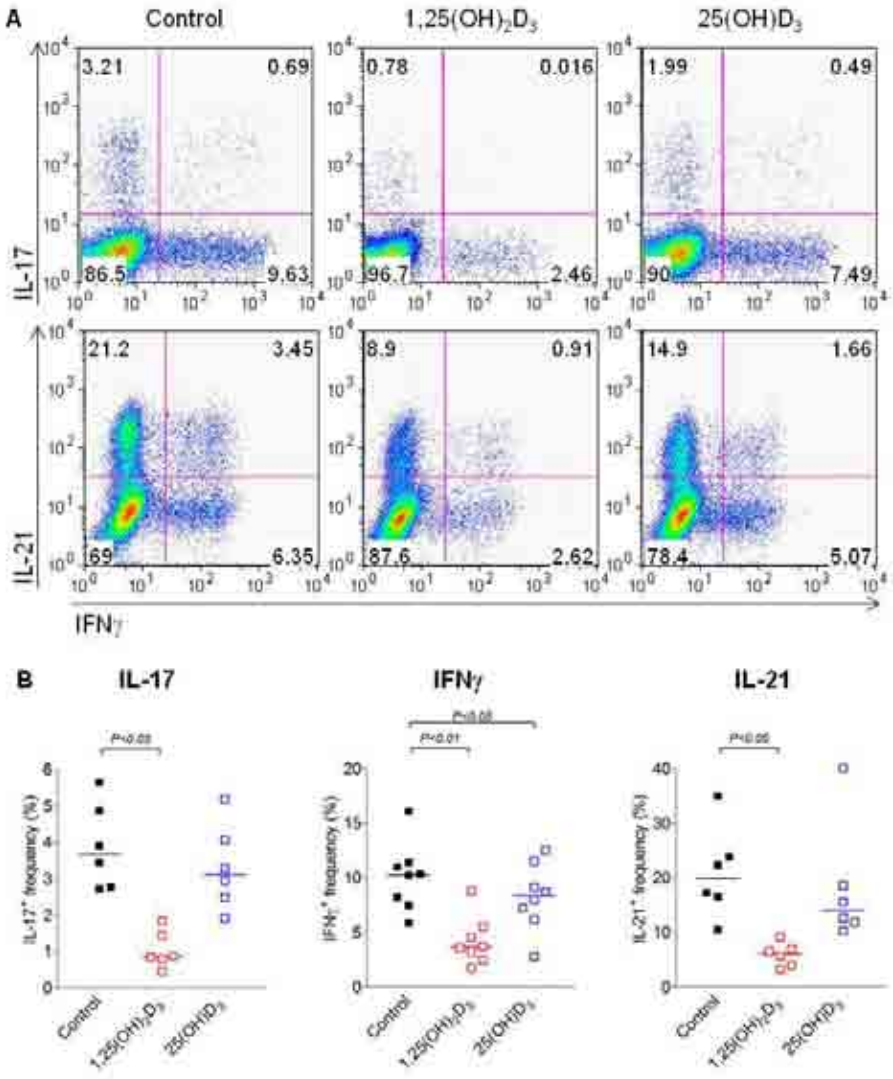

Figure 6.10. Inflammatory cytokines are modestly reduced following conversion of 25(OH)D_3 during bead-driven T cell stimulations. $CD4^+CD25^-$ T cells were stimulated with anti-CD3/CD28 beads in serum free medium supplemented with TGFβ, IL-1β, IL-6 and IL-23 in the presence of 10nM $1,25(OH)_2D_3$, 50nM 25(OH)D_3 or vehicle control. At five days, cells were intracellular stained for IL-17, IL-21 and IFNγ and analysed by flow cytometry. **A)** Representative FACS plots from one experiment. Numbers in quadrants refer to percentage of cells. **B)** IL-17, IFNγ and IL-21 frequencies for multiple donors. Horizontal lines indicate median frequencies. Significance was tested by a two-tailed Wilcoxon matched pairs test.

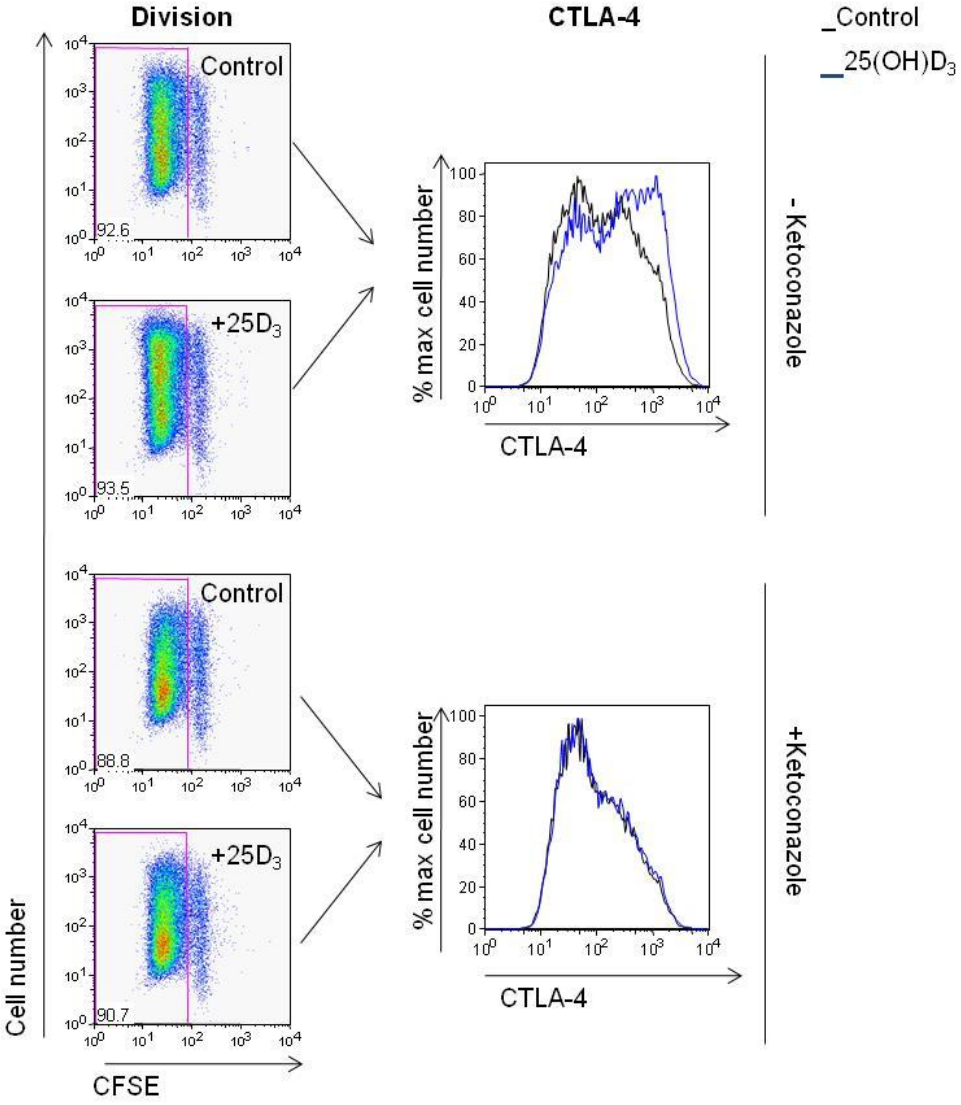

Figure 6.11. Ketoconazole inhibits 25(OH)D_3-mediated CTLA-4 up-regulation during bead-driven T cell stimulations. CFSE labelled $CD4^+CD25^-$ T cells were stimulated for three days with beads in serum free medium supplemented with TGFβ and IL-2. Cells were then treated with 50nM 25(OH)D_3 (blue) or vehicle control (black) and ketoconazole (2.5μM) or corresponding control. At five days, cells were stained for CTLA-4 and analysed by flow cytometry. CTLA-4 expression was compared for divided cells (pink quadrant). Data are from a single experiment.

6.4 Discussion

Physiologically, it is important that serum 1,25$(OH)_2D_3$ is tightly maintained at a low level of approximately 0.1nM (Mullin and Dobs, 2007). Although I observed that CTLA-4 could be enhanced at this concentration, effects upon inflammatory cytokines were minimal. Others have shown that APCs express the converting enzyme 25-hydroxyvitamin-D_3-1α-hydroxylase (CYP27B1) and can generate 1,25$(OH)_2D_3$ *in-vitro* (Fritsche et al., 2003; Hewison et al., 2007; Liu et al., 2006a) but whether this production permits regulation of the T cell phenotype has not been addressed. It was therefore important to determine whether immune cells can sufficiently convert 25$(OH)D_3$ to 1,25$(OH)_2D_3$ for 1,25$(OH)_2D_3$-mediated effects upon T cell phenotype to be observed.

It was demonstrated in this chapter that 25$(OH)D_3$ treatment induced CTLA-4 up-regulation and suppressed IL-17, IFNγ and IL-21 production by $CD4^+$ T cells when they were stimulated with mDCs or monocytes. The efficiency of 25$(OH)D_3$ conversion in these stimulations was clear, as the magnitude of the T cell response was comparable to that obtained upon 1,25$(OH)_2D_3$ supplementation. Consistent with these observations, APCs were found to express CYP27B1 and in keeping with others' reports, strong induction of CYP27B1 occurred upon DC maturation with LPS (Fritsche et al., 2003). The finding that KC, a cytochrome P450 enzyme inhibitor, greatly reduced CTLA-4 up-regulation in mDC driven stimulations also supported conversion of 25$(OH)D_3$ by CYP27B1 in these assays. The fact that incomplete blockade was observed might be explained by the greater affinity of KC for 24-hydroxylase (CYP24) than for CYP27B1 (Yee et al., 2006). CYP24 converts 25$(OH)D_3$

and 1,25$(OH)_2D_3$ into biologically inactive products, thus such differing affinities could result in the accumulation of 1,25$(OH)_2D_3$ at low level and therefore weak induction of CTLA-4.

The observation that freshly isolated monocytes had very low levels of CYP27B1 relative to mDCs but permitted equivalent 1,25$(OH)_2D_3$-mediated T cell responses following 25$(OH)D_3$ supplementation, could suggest that cross-talk between the monocyte and the T cell leads to maturation of the monocyte and its induction of CYP27B1. By this model, it would seem that the activated immune system is armed for efficient use of 25$(OH)D_3$, which could be particularly important for restoring immune homeostasis.

It was further shown in this chapter that T cells can respond to 25$(OH)D_3$ in the absence of APCs. However, CTLA-4 up-regulation upon 25$(OH)D_3$ supplementation was weak compared to the effect of 1,25$(OH)_2D_3$, as was suppression of IL-17, IFNγ and IL-21. This low level of response correlated with the comparatively low level of CYP27B1 detected in stimulated T cells. Nonetheless, the finding that KC could block 25$(OH)D_3$ mediated CTLA-4 up-regulation supports a functional role for CYP27B1 in T cells. Presently, literature describing the expression of CYP27B1 in T cells is limited: Adams and Hewison mentioned their observation of it (Adams and Hewison, 2008) and Von Essen et al. alluded to CYP27B1 activity in their T cell assays (von Essen et al., 2010). Correale et al. also reported CYP27B1 expression and activity by stimulated T cells, however their assays imply the presence of contaminating $CD4^+$ APCs (Correale et al., 2009). My observations therefore strengthen suggestions that CYP27B1 is expressed by stimulated T cells and demonstrate its potential functional importance there. Identifying factors that regulate CYP27B1 activity in T cells

might shed light on the types of immune response that are strongly regulated by vitamin D. In addition, such factors could contribute to individual variability in vitamin D responses.

Studies of 25(OH)D_3 effects in the innate immune system have highlighted the importance of Toll Like Receptor (TLR) 2 and 4 signalling for CYP27B1 induction in macrophages (Liu et al., 2006a; Reichel et al., 1987a) and DCs (Fritsche et al., 2003) as well as in keratinocytes (Schauber et al., 2007). However, TLR mRNAs have also been detected in purified T cells (Hornung et al., 2002; Urry et al., 2009; Zarember and Godowski, 2002) and their ability to respond to TLR2, 5, 7, 8 and 9 ligands shown (Caron et al., 2005; Komai-Koma et al., 2004; Urry et al., 2009). It is therefore tempting to speculate that signalling through TLRs might influence CYP27B1 expression in T cells. Indeed, the observation that 1,25$(OH)_2D_3$ could regulate T cell expression of TLRs 2,3, 5, 7 and 9 (Urry et al., 2009) might support their role in the control of vitamin D metabolism.

Overall, the data presented in this chapter strongly suggest that local 25(OH)D_3 conversion by APCs and T cells could be sufficient to support the anti-inflammatory effects of 1,25$(OH)_2D_3$ described in earlier chapters of this thesis. It should also be considered that local 1,25$(OH)_2D_3$ levels could be topped up by CYP27B1 activity in non-immune cells, as its expression in a diverse range of tissues and cell types is described {Zehnder, 2001 #506}. The question that then remains is, what serum 25(OH)D_3 concentration is sufficient to support 1,25$(OH)_2D_3$ mediated immune modulation?

50nM 25(OH)D_3 was used for most experiments in this chapter but in mDC stimulations, concentrations as low as 1nM gave mild anti-inflammatory effects. Thus, if serum level

directly related to the level within the tissues, even vitamin D deficient individuals who have a 25(OH)D_3 level of below 25nM might be expected to have sufficient vitamin D for immune homeostasis. However, the finding that T cell responses to 25(OH)D_3 were concentration dependent across typical, even insufficient concentrations (Holick, 2007; Hollis, 2005), is consistent with epidemiological data inversely correlating vitamin D status with disease severity for a number of inflammatory diseases (reviewed by (Arnson et al., 2007; Cantorna and Mahon, 2004; Mullin and Dobs, 2007)). The fact that immunomodulatory effects of 25(OH)D_3 were evident in my assays at arguably insufficient concentrations might be explained by the existence of CYP27B1 regulation *in-vivo*, which is mediated by cytokines and growth factors (Leventis and Patel, 2008). Although serum 25(OH)D_3 levels necessary for efficient immune function need further investigation, the data presented in this chapter support the concept that 25(OH)D_3 supplementation could reduce the risk or severity of inflammatory diseases.

7 CAN THE T_{REG}/T17 RATIO PREDICT DISEASE OUTCOME IN EARLY SYNOVITIS PATIENTS?

7.1 Introduction

A number of inflammatory diseases are now associated with IL-17 overproduction. However, in most instances, the direct contribution of IL-17 to the disease pathogenesis is not known (reviewed by (Miossec, 2009). Neither is it always clear that T cells are the responsible producer, since studies have often reported IL-17 protein levels in body fluids or mRNA levels in tissues (reviewed in (Korn et al., 2009; Park et al., 2005; Steinman, 2007; van Beelen et al., 2007)). Rheumatoid arthritis (RA) is one of the most studied inflammatory diseases with regard to a role for IL-17. It is a painful and disabling condition characterised by the destruction of articular cartilage and bone, although multiple organs are often affected, including the skin, blood vessels, heart, lungs and muscles. It affects approximately 1% of the population with strong female bias and has a peak age of incidence of 40 to 60 years (Firestein, 2003).

Studies into the pathogenic mechanisms underlying RA began in the mid 20th century. Initially it was characterised as an immune complex-mediated disease, since rheumatoid factor (RF) was identified in the blood of affected patients, complement and RF were present in RA synovium and immune complexes were found in their cartilage (Firestein, 2003). However, the presence of a large number of T cells in the RA synovium suggested that they too could participate in the pathology of RA (Firestein, 2003).

Consistent with T cell involvement, the strongest genetic association in RA maps to the third hypervariable region of the HLA-DRβ chain, which is a component of the MHC class II complex that presents antigen to $CD4^+$ T cells (Firestein, 2003; Nepom et al., 1989). Other genetic polymorphisms associated with RA also involve T cell related genes including those encoding the co-stimulation molecules CD28, ICOS, CTLA-4 and CD40 and the lymphoid specific phosphatase, *PTPN22* (Kim et al., 2010; Plant et al., 2010). T cells might also support TNFα production which has been measured at high concentration in synovial tissue cultures (Elliott et al., 1994). Furthermore, CTLA4-Ig and anti-TNFα therapies that target T cell activities are both effective in RA (Firestein, 2003; Kremer et al., 2003). Lastly, inhibition of lymphocyte apoptosis and lymphocyte retention in the joint is reported. This occurs in response to factors released from stromal cells, including the hyperplastic fibroblasts that are a hallmark of chronic inflammation in RA (reviewed in (Raza, 2010)). Despite these associations, the role of the T cell in RA pathology met with opposition as cytokines, such as IL-2 and IFNγ, believed to be markers of T cell activation, were present at low concentrations in the RA synovium and synovial fluid. Conversely, macrophage and fibroblast products, including IL-1, IL-6, IL-15, IL-18, TNFα, GM-CSF and various chemokines were abundant (Firestein, 2003; Firestein et al., 1990; Smeets et al., 1998).

An indication of the potential involvement of IL-17 in RA came from the discovery that RA synovial tissue contained IL-17 mRNA whilst that from osteoarthritis (OA) patients did not (Chabaud et al., 1999). In addition, RA but not OA synovial tissue generated IL-17 when cultured *in-vitro* and immuno-staining of synovial tissue demonstrated the presence of $IL\text{-}17^+$ cells (Chabaud et al., 1999). A mechanism of IL-17-mediated pathogenesis has been

described for RA following the discovery that IL-17 can act upon most parenchymal cells, including macrophages and fibroblasts, that are present at high frequency in the synovium, to induce a pro-inflammatory pattern similar to that found in an inflamed joint (Firestein, 2003; Firestein and Zvaifler, 1987; Miossec et al., 2009). IL-17 could also contribute to bone erosion as it induces RANKL expression by osteoblasts. The engagement of RANKL with RANK expressed by osteoclasts leads to their activation and consequentially bone erosion (Miossec et al., 2009; Udagawa et al., 2002).

Given the potential involvement of T cells in RA and the detection of IL-17 in RA tissues and synovial fluid (SF) (Chabaud et al., 1999; Hirota et al., 2007; Kotake et al., 1999; Ziolkowska et al., 2000), it seems reasonable to speculate that IL-17^+ T cells could contribute to the pathogenesis of RA. Consistent with this, in the collagen induced arthritis model of autoimmune arthritis, IL-23 deficient mice did not have IL-17^+ $CD4^+$ T cells and completely lacked disease even though T_h1 cells were present (Murphy et al., 2003). Before the start of this study, reports on the frequencies of IL-17^+ T cells in peripheral blood (PB) or SF of RA patients had not been made, although immuno-staining had implied the presence of IL-17^+ cells amongst T cell populations (Chabaud et al., 1999). A few studies have now been published describing the frequencies of T_h17 cells in the PB and SF of RA patients but data are conflicting (Church et al., 2010; Colin et al., 2010; Leipe et al., 2010; Yamada et al., 2008), thus the role of IL-17^+ T cells in RA remains to be established. There is also evidence to suggest that the molecular and cellular mechanisms operating in early versus established stages of RA might differ and that the features of early synovitis could relate to disease outcome. In support of this, within three months of symptom onset a number of cytokines were found to be elevated in the SF of individuals that went on to develop RA relative to

those who did not. Furthermore, the pattern was not present in established disease. Interestingly IL-17 was amongst this group of elevated early cytokines (Raza et al., 2005). For many years, it has been recognised that treatment, during the early, so-called 'therapeutic window of opportunity,' can reduce the cumulative inflammatory burden of the disease and thereby improve outcome. For RA, this therapeutic window might be only three to four months (Raza, 2010). To distinguish within this period individuals that are destined for RA from those that are not, an understanding of the molecular mechanisms that operate at early stages of disease, is required.

If T_h17 cells contribute to RA, dysregulation of the T_{Reg}/T_h17 balance might be predicted, given that a reciprocal relationship between T_h17 and T_{Reg} cells has been observed (Bettelli et al., 2006; Quintana et al., 2008; Schambach et al., 2007; Veldhoen et al., 2008a). The studies in this chapter therefore set out to address whether dysregulation of the T_{Reg} /T17 balance is evident in early synovitis patients and whether it might be used to predict their disease outcome.

In previous chapters in this thesis, pro-regulatory effects of vitamin D even under inflammatory conditions have been clearly shown. The second aim of work in this chapter was therefore to see if cells from synovitis patients could respond to $1,25(OH)_2D_3$ *in-vitro* and if the response related to disease outcome.

Results

Raw data from studies in this chapter are tabulated in **appendix tables 10.1 to 10.5**.

7.2 Early synovitis disease outcome is not related to T_{Reg} or T17 frequencies

To test the hypothesis that dysregulated frequencies of T_{Regs} or T17 cells might be involved in the development of RA, mononuclear cells were isolated from the peripheral blood (PB) and synovial fluid (SF) of early synovitis patients who were recruited according to stated criteria (**chapter 2.10**). Frequencies of $CD4^+CD25^+FoxP3^+$ (T_{Reg}) and IL-17^+ (T17) T cells were measured *ex-vivo.* As shown by **figures 7.1 and 7.2**, low frequencies of T17 cells were present in the PB of all patients and one-way ANOVA against outcome indicated no difference between the frequencies of T17 cells in the PB of patients diagnosed at eighteen months to have RA, versus those whose disease resolved (**figure 7.3A**). In addition, no difference in T17 frequency was observed between those who developed RA and those who showed non-RA forms of persistent synovitis (**figure 7.3A**). Furthermore, the peripheral blood T17 frequencies of early synovitis patients were not different to those of healthy controls or established RA patients. Thus, an alteration of the T17 frequency in PB does not appear to occur during the progression of RA or contribute to the disease outcome.

IL-17 production by T cells within the joint might be of greater influence in the development of RA. Interestingly, very few T17 cells were likewise identified in SF for most patients, although the frequency in SF was significantly greater than the frequency in PB when analysing all data (**figure 7.2**) and paired samples only (**figure 7.5B**). Interestingly, the

highest T17 frequencies in SF were measured in samples from self-limiting and non-RA persistent patients (**figure 7.3B**). However, more SF samples are required before a breakdown analysis against outcome can be performed for T17 frequencies in SF.

T_{Reg} frequencies were generally low (median = 4.6) in the PB of early synovitis patients (**figure 7.1 and figure 7.2**), although one-way ANOVA suggests that they might be elevated in non-RA persistent cases relative to RA or self-limiting disease (**figure 7.4A**). More non-RA persistent samples are required, however, to test this finding more strongly. T_{Reg} frequencies in early synovitis patients did not differ from those of healthy controls or established RA patients according to one-way ANOVA, however, post-hoc analysis carried out specifically for established RA patients compared to early synovitis patients who progressed to RA, showed a significantly higher percentage of T_{Regs} in established disease. A clear enrichment of T_{Reg} cells in SF compared to PB was also seen when considering all data (**figure 7.2)** and paired samples only (**figure 7.5B**). However, the ratio of T_{Reg} vs. T17 cells was not altered between PB and SF (**figure 7.5B**), possibly suggesting that the balance between pro and anti-inflammatory activities is maintained in the two compartments. Furthermore, the T_{Reg}/T17 ratio in PB did not appear to stratify with disease classification (**figure 7.5A**) implying that T_{Reg}/T17 dysregulation in PB might not influence disease outcome. In addition, contrary to the concept of a reciprocal relationship between T_h17 and T_{Reg} cells (Bettelli et al., 2006), no correlation was observed between T17 and T_{Reg} frequencies in PB or SF (**figure 7.6**). Notably, all SF T cells displayed a memory CD45-RO phenotype, whilst only 51.4±14.36% of $CD4^+$ T cells from PB were CD45-RO^+ (**figure 7.2**).

Interestingly, established RA has been associated with a low level of IFNγ in SF (Firestein and Zvaifler, 1987). Low IFNγ concentrations were also measured in the SF of early synovitis patients who developed RA, whilst higher levels were detected in those who progressed to non-RA persistent synovitis (Raza et al., 2005). Therefore, the frequency of $IFN\gamma^+$ T cells was also compared across outcome groups. As for T17 frequencies, no differences in $IFN\gamma^+$ frequencies were detected for early synovitis patient outcome groups or for the combined one-way ANOVA of early synovitis patients, established RA and healthy controls (**figure 7.7A**). However, post-hoc analysis performed specifically for established RA patients compared to early synovitis patients that progressed to RA, showed a significantly higher expression of IFNγ by patients with established disease. Strikingly, T cells isolated from SF showed very high IFNγ expression compared to those from PB (**figure 7.2**). In addition, T17 cells in PB were primarily $IFN\gamma^-$ whilst T17 cells from SF included both $IFN\gamma^-$ and $IFN\gamma^+$ subsets (**figure 7.8**).

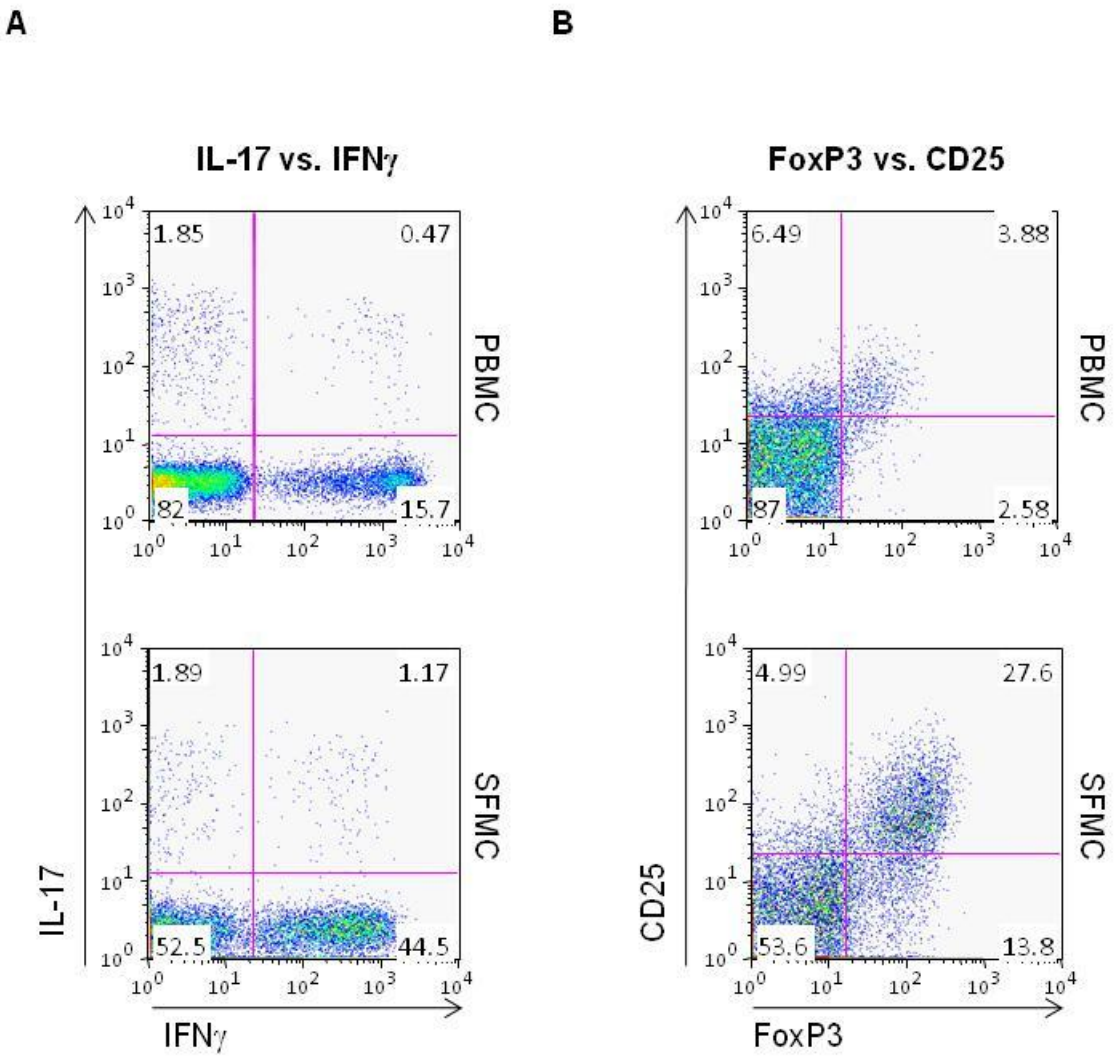

Figure 7.1. *Ex-vivo* analysis of T17 and T_{Reg} frequencies in the blood and synovial fluid of synovitis patients. PBMCs and SFMCs were isolated from synovitis patients and T cells were analysed for IL-17, IFNγ, FoxP3 and CD25 expression by flow cytometry. Representative FACS plots from one patient are shown. Numbers in quadrants indicate percentages of $CD3^+$ T cells **(A)** and $CD3^+CD4^+$ T cells **(B)**.

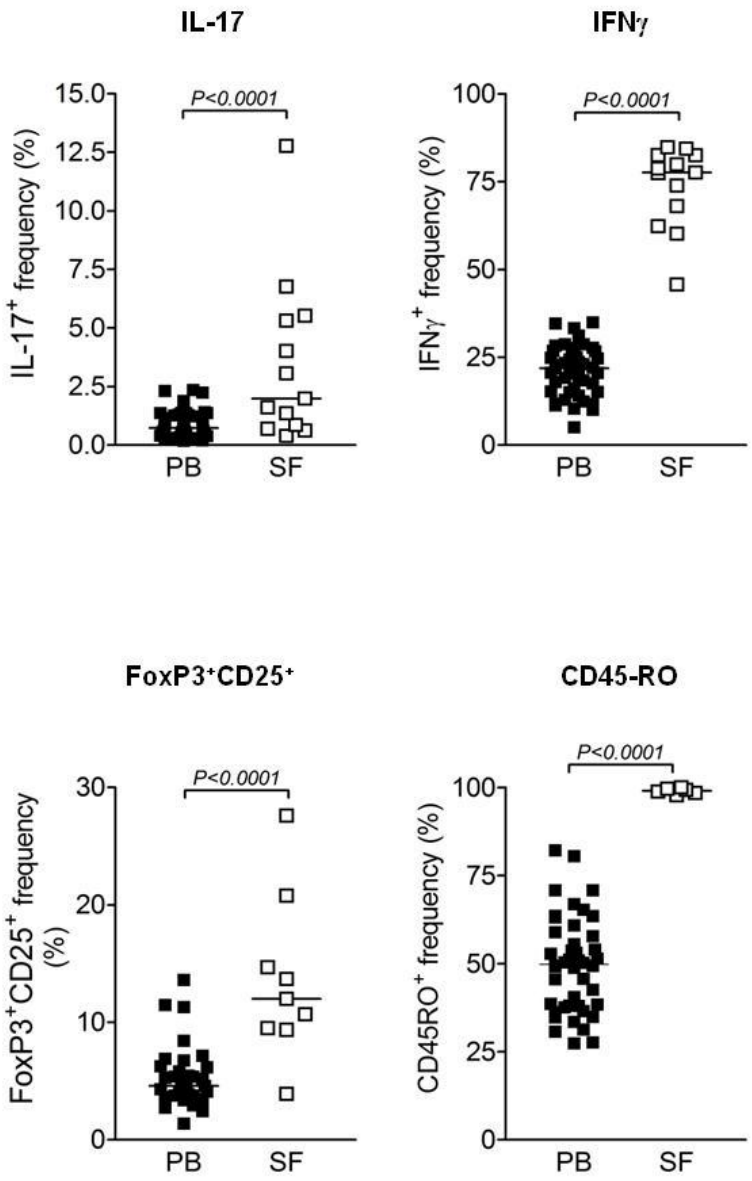

Figure 7.2. Comparison of *ex-vivo* IL-17$^+$, IFNγ$^+$, FoxP3$^+$CD25$^+$ and CD45-RO$^+$ T cell frequencies in the blood versus the synovial fluid of early synovitis patients. PBMCs and SFMCs were isolated from synovitis patients and T cells analysed for IL-17, IFNγ, FoxP3, CD25 and CD45-RO expression by flow cytometry. Horizontal lines indicate median frequencies. Significance was tested by a two-tailed Mann Whitney U test.

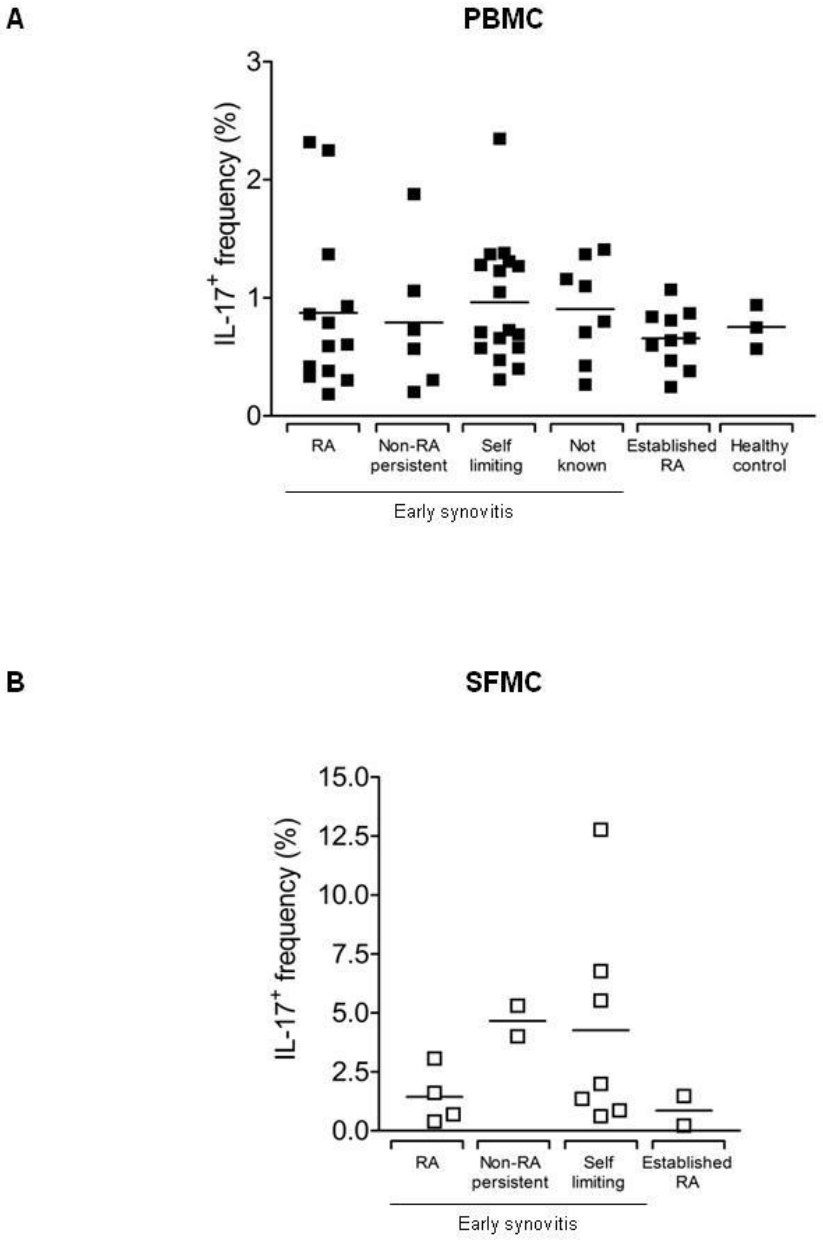

Figure 7.3. IL-17 expression by T cells from the blood and synovial fluid of synovitis patients. PBMCs **(A)** and SFMCs **(B)** were isolated from early synovitis patients (classified at follow up as RA, non-RA-persistent, self-limiting or not known), established RA patients and healthy controls. Frequencies of IL-17$^+$ T cells were analysed directly *ex-vivo* by flow cytometry. Horizontal lines indicate mean frequencies. Two-tailed, one-way ANOVA was performed for T cells from PB. P>0.05 for early synovitis outcomes alone and all known outcomes combined.

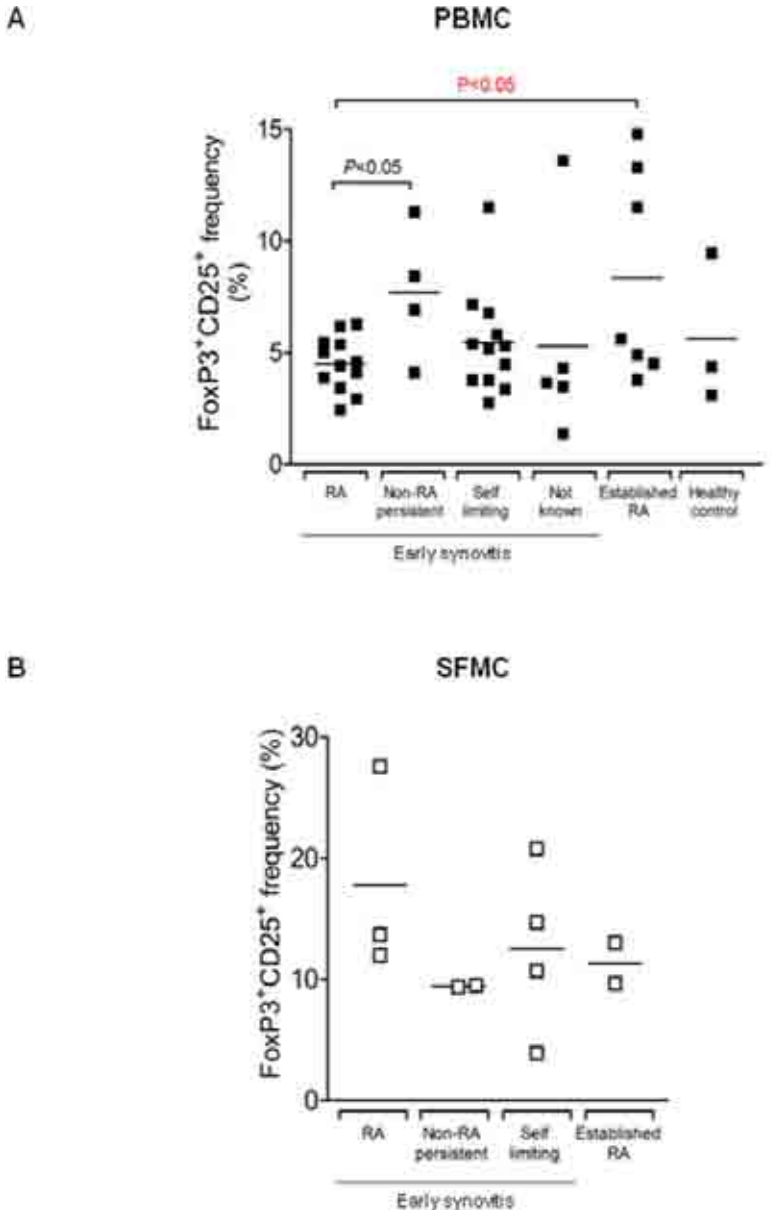

Figure 7.4 FoxP3$^+$CD25$^+$ (T_{Reg}) frequencies in the blood and synovial fluid of synovitis patients. PBMCs **(A)** and SFMCs **(B)** were isolated from early synovitis patients (classified at follow up as RA, non-RA-persistent, self-limiting or not known), established RA patients and healthy controls. Frequencies of FoxP3$^+$CD25$^+$ T cells were analysed directly *ex-vivo* by flow cytometry. Horizontal lines indicate mean frequencies. Two-tailed one-way ANOVA was performed for T cells from PB. Significant differences for early synovitis patients alone (black) and all known outcomes combined (red) are shown.

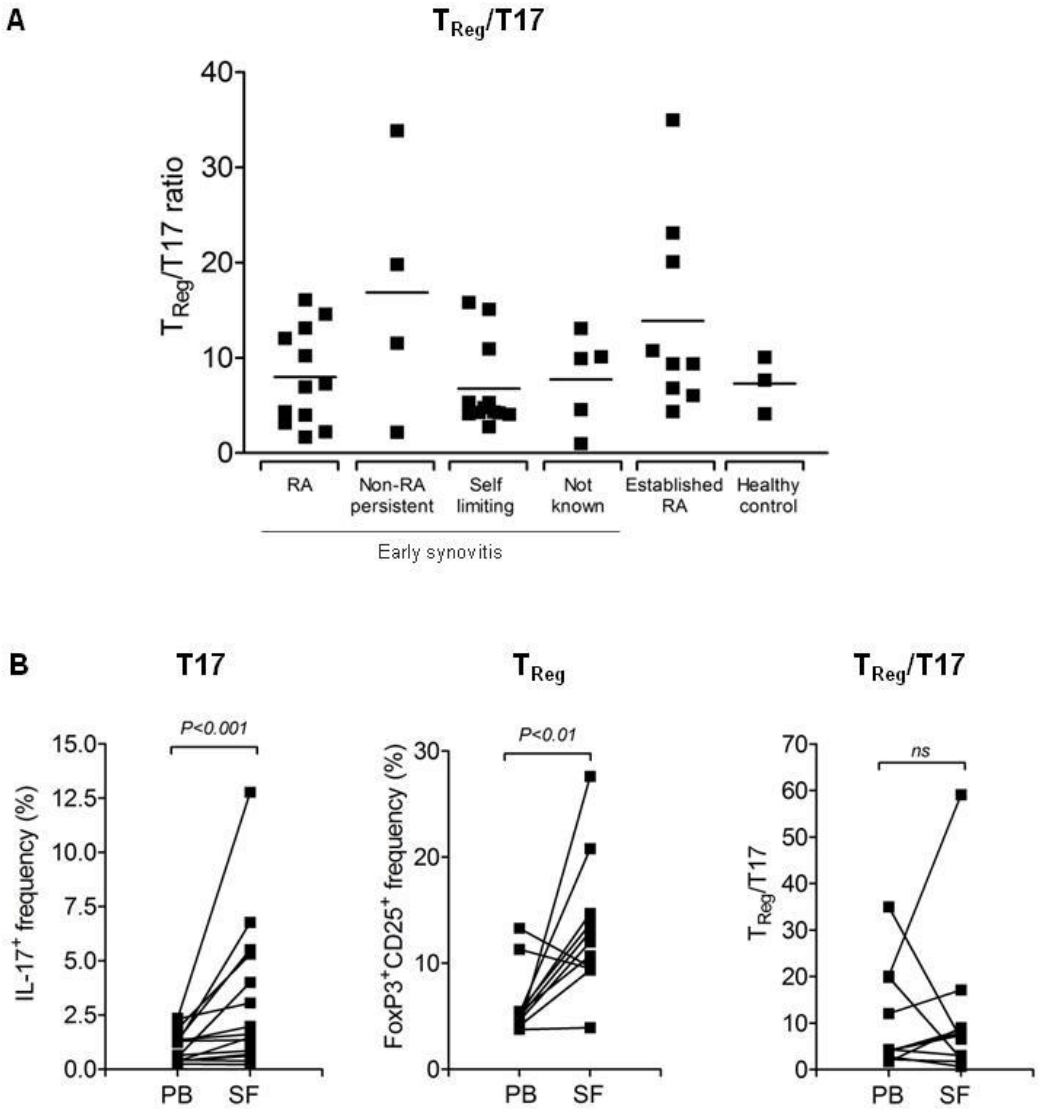

Figure 7.5. The T_{Reg}/T17 ratio is maintained in blood and synovial fluid. PBMCs and SFMCs were isolated from synovitis patients and the *ex-vivo* frequencies of IL-17$^+$ and CD4$^+$FoxP3$^+$CD25$^+$ T cells determined by flow cytometry. **A)** T_{Reg}/T17 ratio for PB T cells from early synovitis patients (classified at follow up as RA, non-RA-persistent, self-limiting or not known), established RA patients and healthy controls. Horizontal lines indicate mean ratios. Two-tailed one-way ANOVA was performed. *P*>0.05 for early synovitis patient outcomes alone and for all known outcomes combined. **B)** T17, T_{Reg} and T_{Reg}/T17 ratio for PB T cells versus SF T cells from early synovitis patients. Significance was tested by a two-tailed Wilcoxon matched pairs test.

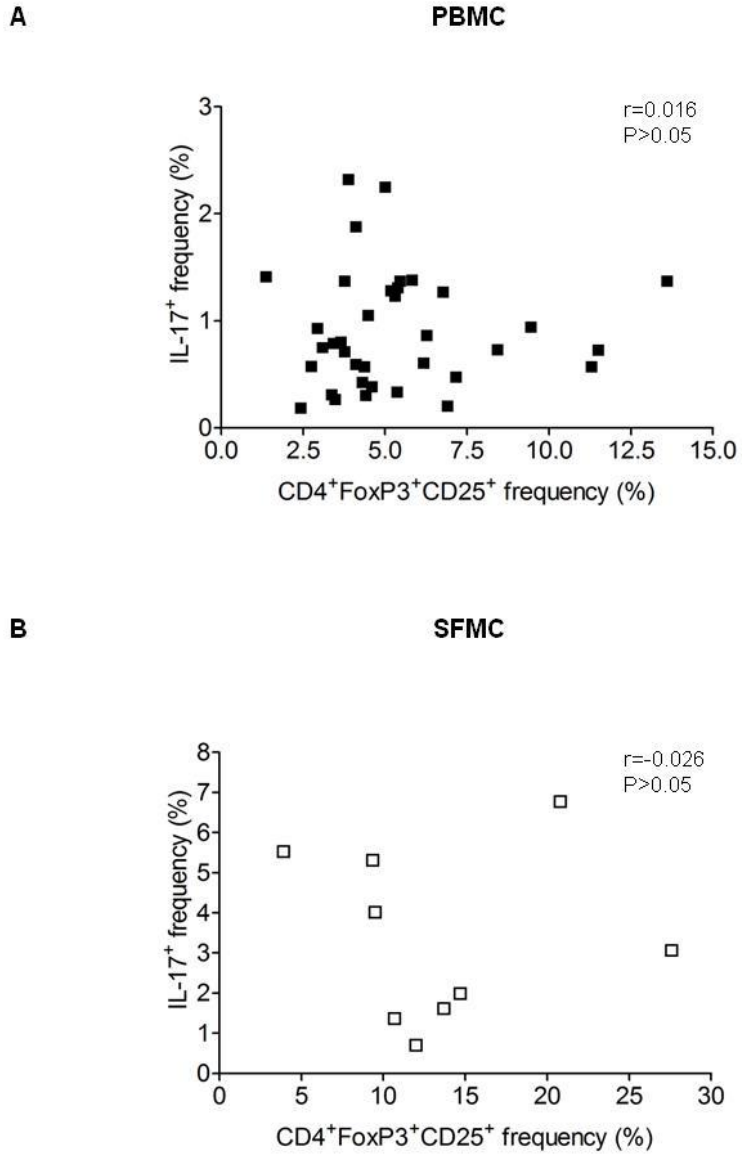

Figure 7.6. T_{Reg} and T17 relationships in blood and synovial fluid. PBMCs **(A)** and SFMCs **(B)** were isolated from early synovitis patients and *ex-vivo* frequencies of IL-17$^+$ and CD4$^+$FoxP3$^+$CD25$^+$ T cells determined by flow cytometry. Correlation of IL-17$^+$ (T17) versus CD4$^+$FoxP3$^+$CD25$^+$ (T_{Reg}) frequencies for PBMCs and SFMCs is shown. Pearson's correlation coefficient (r) was calculated and used to determine the level of significance (*P*).

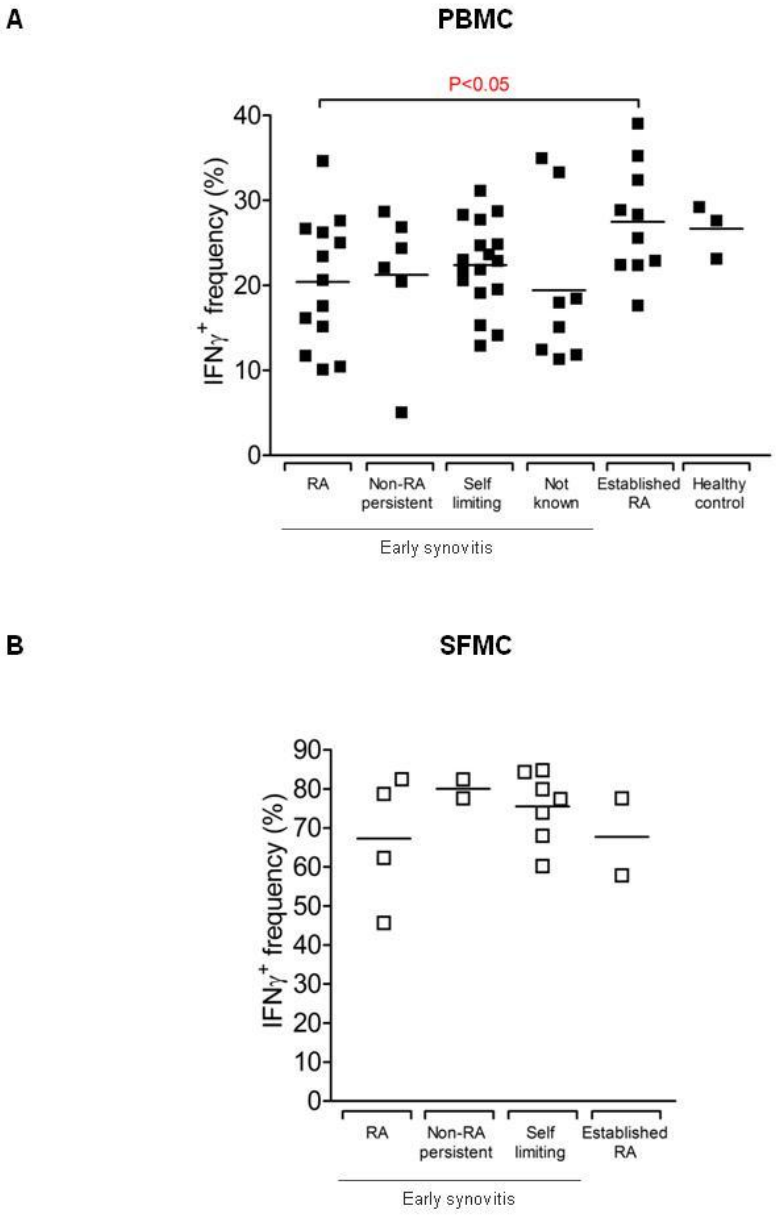

Figure 7.7. IFNγ expression by T cells in the blood and synovial fluid of synovitis patients. PBMCs **(A)** and SFMCs **(B)** were isolated from early synovitis patients (classified at follow up as RA, non-RA-persistent, self-limiting or not known), established RA patients and healthy controls. Frequencies of IFNγ$^+$ T cells were analysed directly *ex-vivo* by flow cytometry. Horizontal lines indicate mean frequencies. Two-tailed one-way ANOVA was performed for T cells from PB. P>0.05 for early synovitis patient outcomes alone but P<0.05 for early versus established RA patients when all known outcomes were combined.

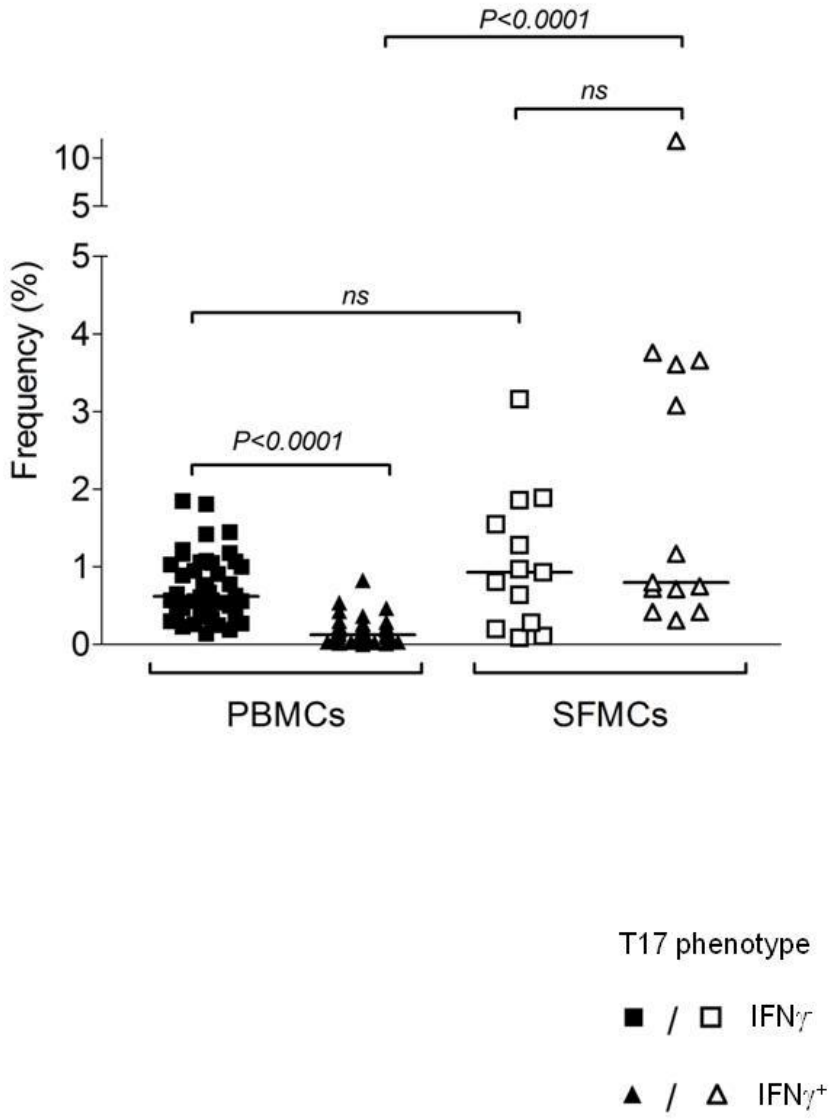

Figure 7.8. T cells isolated from the blood and synovial fluid of early synovitis patients show different IL-17 and IFNγ expression profiles. PBMCs and SFMCs were isolated from early synovitis patients and IL-17 and IFNγ expression by T cells measured by flow cytometry. Frequencies of T17 cells classified as $IFN\gamma^-$ (square) or $IFN\gamma^+$ (triangle) are shown. Horizontal lines indicate median frequencies. Significance was tested by a two-tailed Mann Whitney U test.

7.3 25(OH)D_3 status does not reflect disease outcome or the frequency of T cells expressing IL-17, IFNγ or FoxP3 and CD25

Since epidemiological studies associate low vitamin D status with increased risk of autoimmune diseases, including RA (Arnson et al., 2007; Cantorna and Mahon, 2004; Merlino et al., 2004), whether serum 25(OH)D_3 level varied across early synovitis disease outcome was examined but no differences were observed (**figure 7.9A**). Since *ex-vivo* IL-17^+, IFNγ^+ and T_{Reg} frequencies were variable, whether they correlated with 25(OH)D_3 status was also tested. No correlation was observed for IL-17^+ or IFNγ^+ frequencies but surprisingly negative correlation was detected for PBMC T_{Reg} frequencies (**figure 7.9B**). In agreement with this trend, CTLA-4 expression by $CD4^+FoxP3^+$ T cells from blood appeared to correlate inversely with 25(OH)D_3 level, although more samples are needed to qualify this statistically (**figure 7.9B**).

Arguably, vitamin D might have its greatest effect at the inflammatory site where matured APCs convert 25(OH)D_3 to 1,25$(OH)_2D_3$. Whether serum 25(OH)D_3 correlated with IL-17^+, IFNγ^+ or T_{Reg} frequencies in SF was therefore examined but no correlation was seen for any marker (figure 7.10).

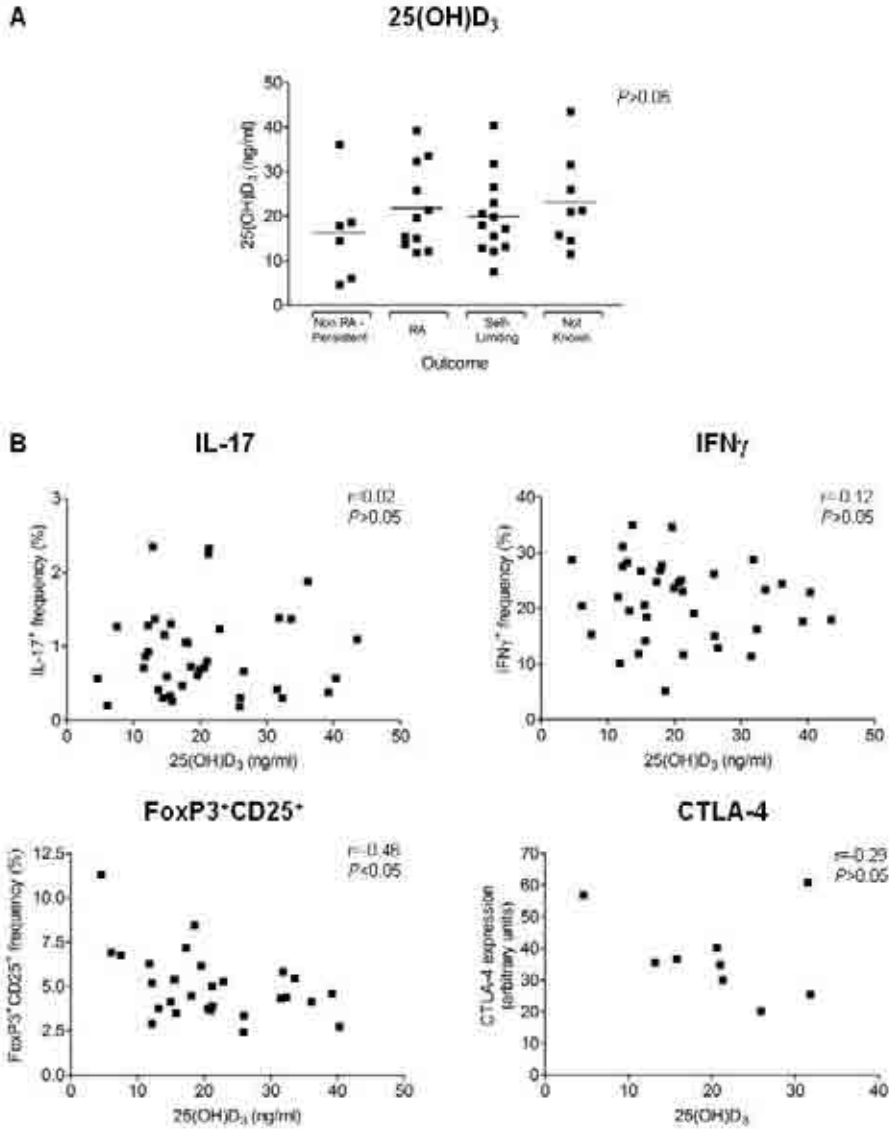

Figure 7.9. 25(OH)D_3 level does not influence disease outcome or the expression of inflammatory and regulatory markers by peripheral blood T cells. A) Serum 25(OH)D_3 level of early synovitis patients grouped according to disease outcome. Horizontal lines indicate mean frequencies. Significance was tested by two-tailed one-way ANOVA. **B)** Relationships between serum 25(OH)D_3 and ex-vivo IL-17$^+$, IFNγ^+ and FoxP3$^+$CD25$^+$ frequencies and CTLA-4 expression level. Pearson's correlation coefficient (r) was calculated and used to determine the level of significance (*P*).

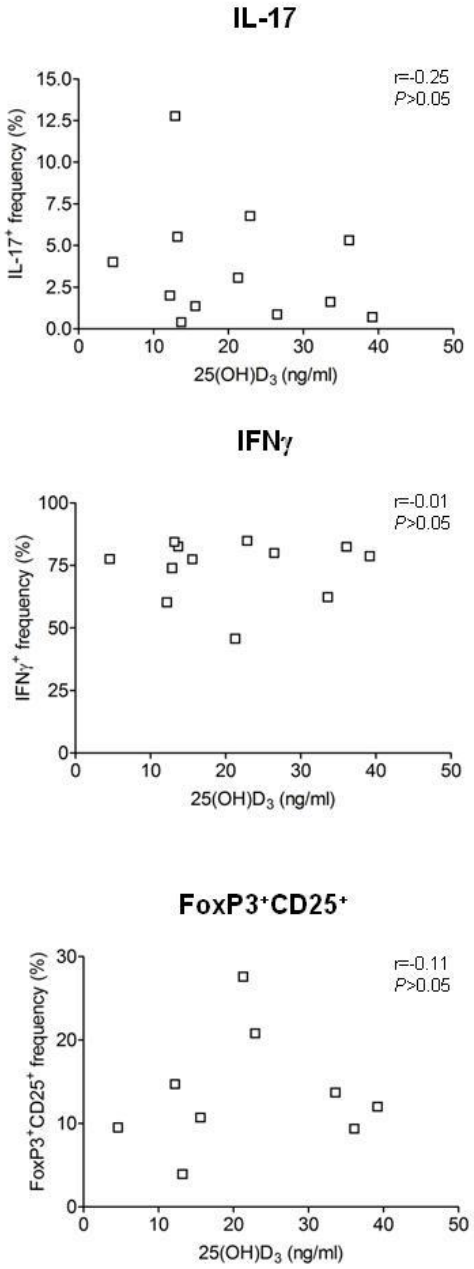

Figure 7.10. Expression of inflammatory and regulatory markers by synovial fluid T cells is not affected by serum 25(OH)D_3 status. Relationships between serum 25(OH)D_3 status, and *ex-vivo* T cell IL-17, IFNγ and FoxP3$^+$CD25$^+$ frequencies are shown. Pearson's correlation coefficient (r) was calculated and used to determine the level of significance (*P*).

To investigate whether 25(OH)D_3 status might relate to the level of disease activity, correlation between 25(OH)D_3 status and disease activity score 28 (DAS28) was tested. **Figure 7.11** shows an inverse correlation. Because the DAS28 has not yet been validated as a marker of disease activity in synovitis conditions other than RA, serum 25(OH)D_3 was also analysed against the erythrocyte sedimentation rate (ESR), which is used as a measure of inflammation. ESR was chosen over C Reactive Protein (CRP), because CRP was included in the DAS28 assessment. Inverse correlation of ESR with 25(OH)D_3 was again observed, thus implying that 25(OH)D_3 could influence disease activity.

7.4 Induced IL-17 and IFNγ expression and 1,25(OH)$_2$D$_3$ response do not predict disease outcome

Although no association was found between *ex-vivo* T17 frequencies and disease outcome, it is possible that individuals may differ in their propensity to express IL-17 or IFNγ upon stimulation and that this might relate to disease outcome. Similarly, an individual's sensitivity to 1,25(OH)$_2$D$_3$ during stimulation could influence disease outcome. These questions were addressed by stimulating PBMCs and SFMCs in the presence or absence of 1,25(OH)$_2$D$_3$ before measuring IL-17 and IFNγ frequencies (**figure 7.12, 7.13** and **7.14**). As shown by **figure 7.13**, IL-17 and IFNγ expression by stimulated T cells from PB did not differ across early synovitis outcome groups. Neither were IL-17$^+$ and IFNγ$^+$ frequencies different when also considering established RA and healthy individuals. 1,25(OH)$_2$D$_3$ suppressed IL-17$^+$ and IFNγ$^+$ frequencies in all groups and ANOVA analysis implied no difference between any groups in the sensitivity of IL-17 and IFNγ expression to 1,25(OH)$_2$D$_3$ treatment (P>0.05).

Thus, T cells from synovitis patients, including established RA patients, were sensitive to $1,25(OH)_2D_3$.

For patients combined, a wide range of IL-17^+ and IFNγ^+ frequencies at seven days were measured in the absence of $1,25(OH)_2D_3$. Since the stimulations had been conducted in medium supplemented with self-serum, whether serum $25(OH)D_3$ might have influenced IL-17^+ and IFNγ^+ frequencies was also examined but no correlation was found (**figure 7.15**). Although $1,25(OH)_2D_3$ strongly suppressed IL-17 and IFNγ production by PB T cells, it had limited effect upon T cells from SF: IL-17 expression was only slightly decreased and IFNγ was not significantly suppressed (**figure 7.14**). However, for both PBMCs and SFMCs, IFNγ^+ T17 cells were the most sensitive to suppression by $1,25(OH)_2D_3$ as determined from the ratio of expressing cells under control versus treatment conditions (**figure 7.16**). This might imply that T cells in synovial fluid are more permanently polarised than $CD4^+$ T cells in PB.

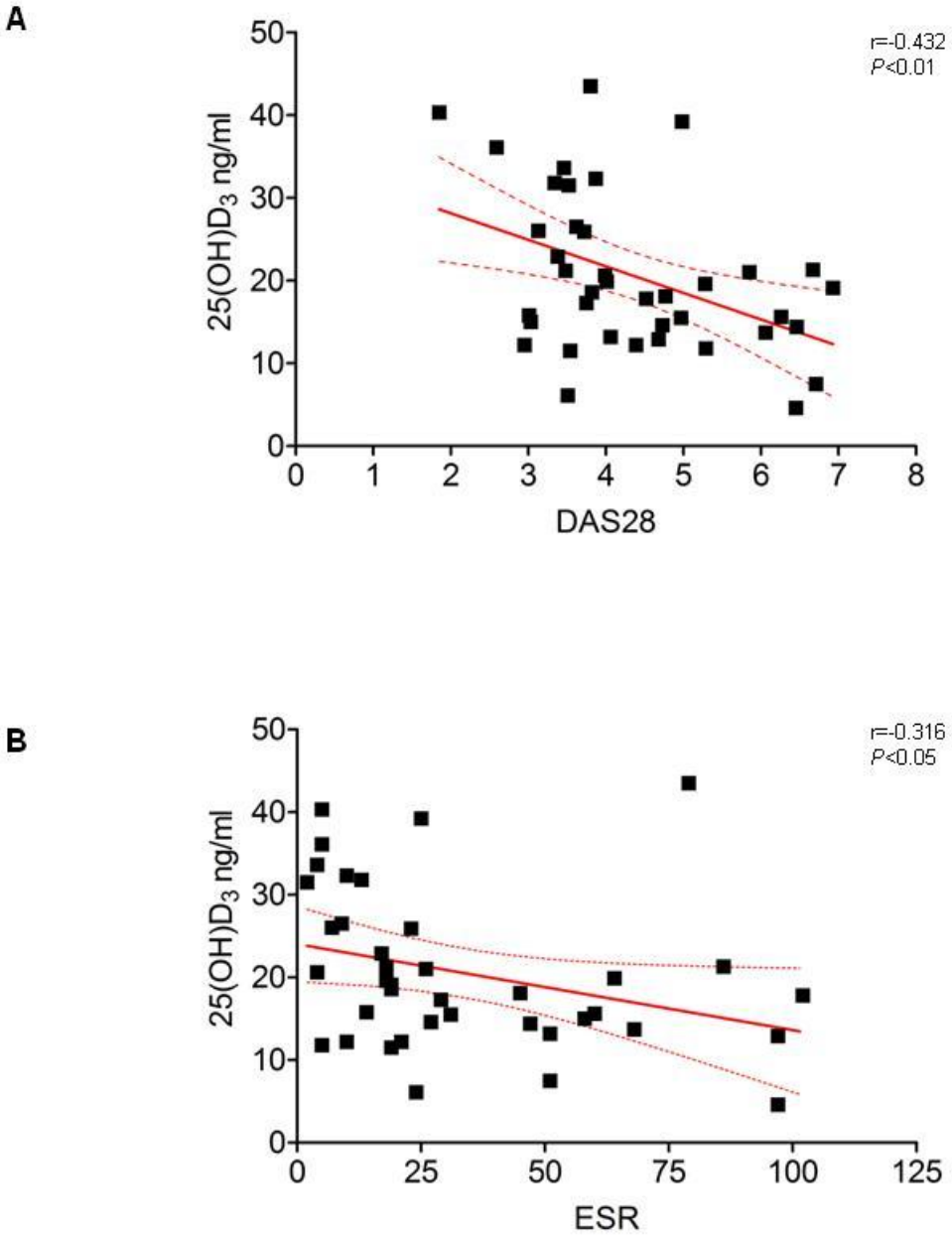

Figure 7.11. 25(OH)D_3 inversely correlates with disease activity in early synovitis patients. Serum 25(OH)D_3 concentration was plotted against **A)** disease activity score 28 (DAS28) including C reactive Protein level (CRP) and **B)** erythrocyte sedimentation rate (ESR). Pearson's correlation coefficient (r) was calculated and used to determine the level of significance (*P*). The linear regression line is shown in red (solid line) and 95% confidence interval residuals shown (dotted lines).

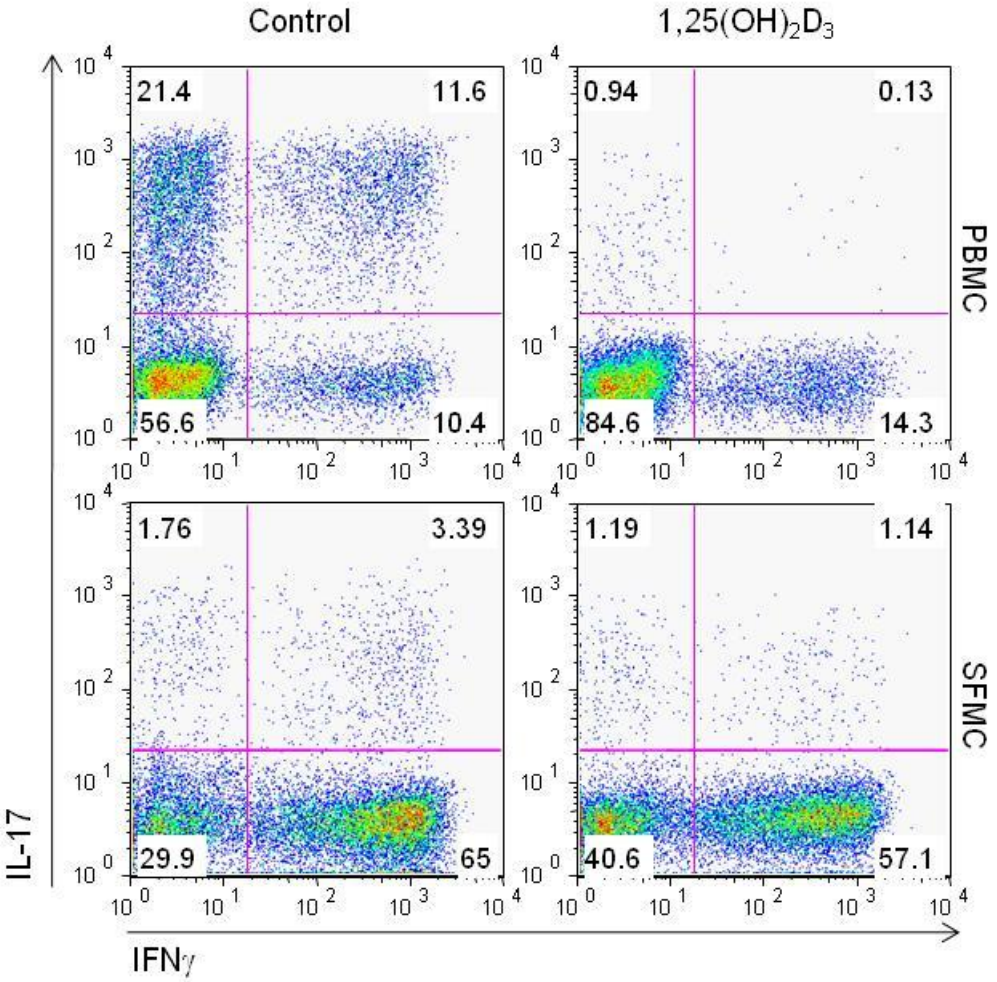

Figure 7.12. Effect of $1,25(OH)_2D_3$ upon IL-17 and IFNγ expression by T cells from the blood and synovial fluid of synovitis patients. PBMCs and SFMCs from synovitis patients were stimulated for seven days with anti-CD3 in the presence or absence of $1,25(OH)_2D_3$ (100nM) and T cells analysed for IL-17 and IFNγ expression by flow cytometry. Representative FACS plots for one patient are shown.

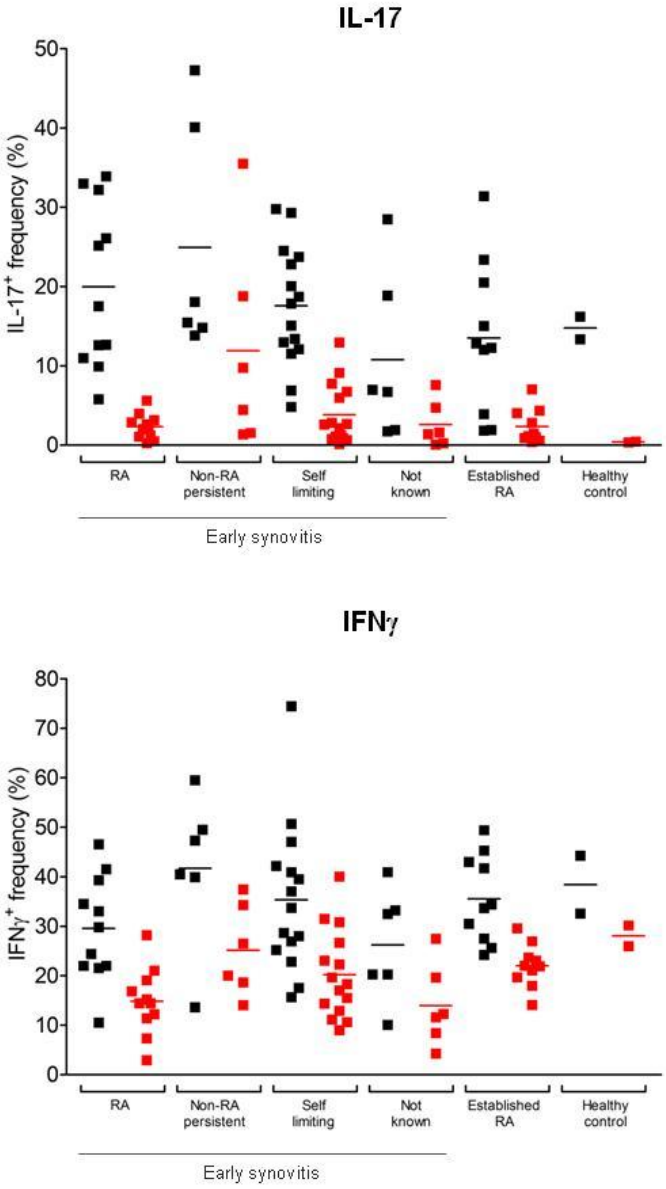

Figure 7.13. IL-17$^+$ and IFNγ$^+$ post-culture frequencies do not differ across outcome groups. PBMCs from early synovitis patients (classified at follow up as non-RA-Persistent, RA, self-limiting or not known), established RA patients and healthy controls were stimulated with anti-CD3 in the presence of 100nM $1,25(OH)_2D_3$ (red) or vehicle control (black). At seven days, IL-17 and IFNγ expression by T cells was determined by intracellular staining and flow cytometry. Horizontal lines indicate mean frequencies. Two-tailed, one-way ANOVA was performed for IL-17 and IFNγ expression by control cultures. *P*>0.05 for early synovitis outcomes alone and for all known outcomes combined.

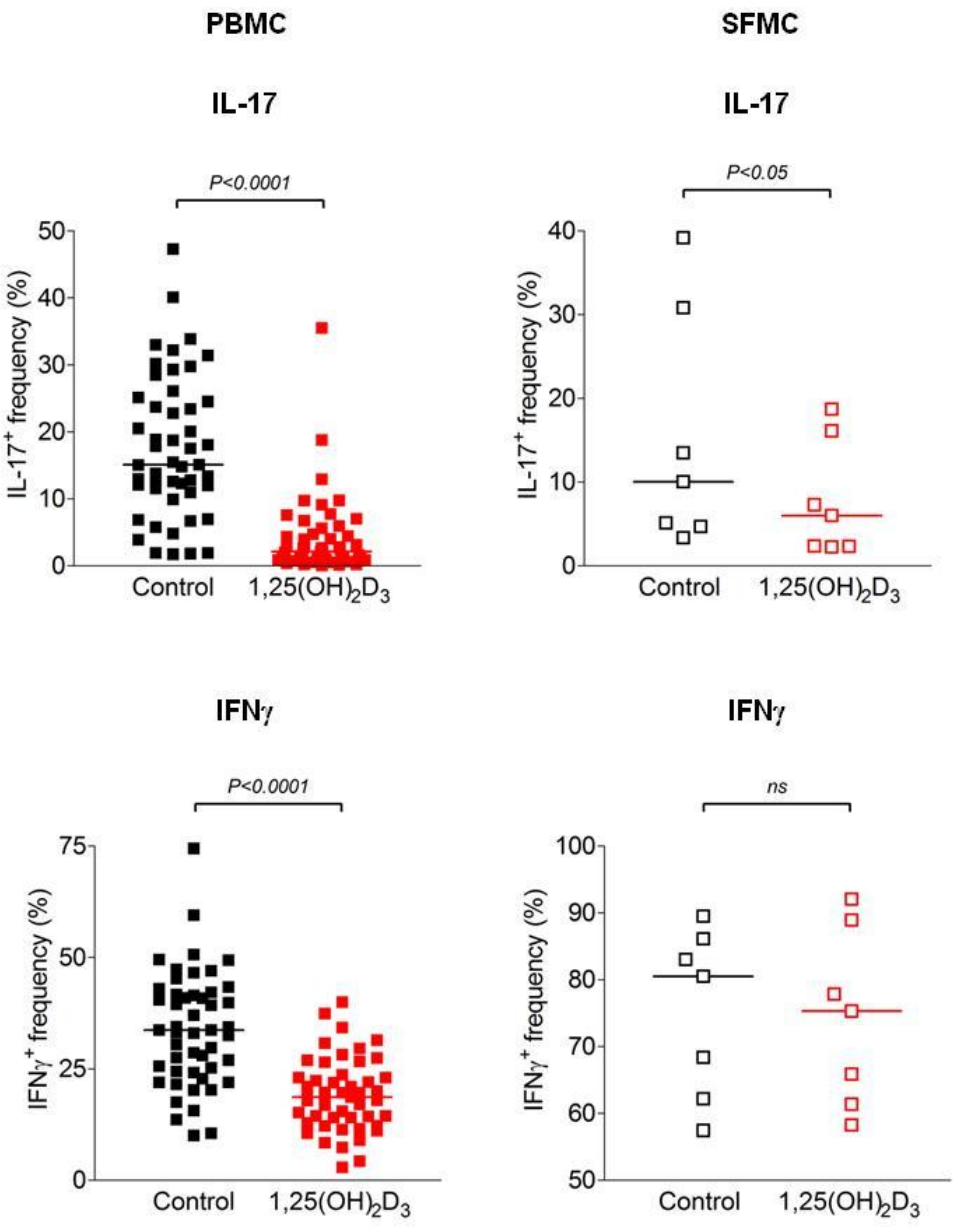

Figure 7.14. Synovial fluid T cells are less sensitive to $1,25(OH)_2D_3$ than T cells from blood. PBMCs and SFMCs from synovitis patients were stimulated with anti-CD3 in the presence of 100nM $1,25(OH)_2D_3$ (red) or vehicle control (black). At seven days, frequencies of T cells expressing IL-17 or IFNγ were determined by intracellular staining and flow cytometry. Horizontal lines indicate median frequencies. Significance was tested by a two-tailed Mann Whitney U test.

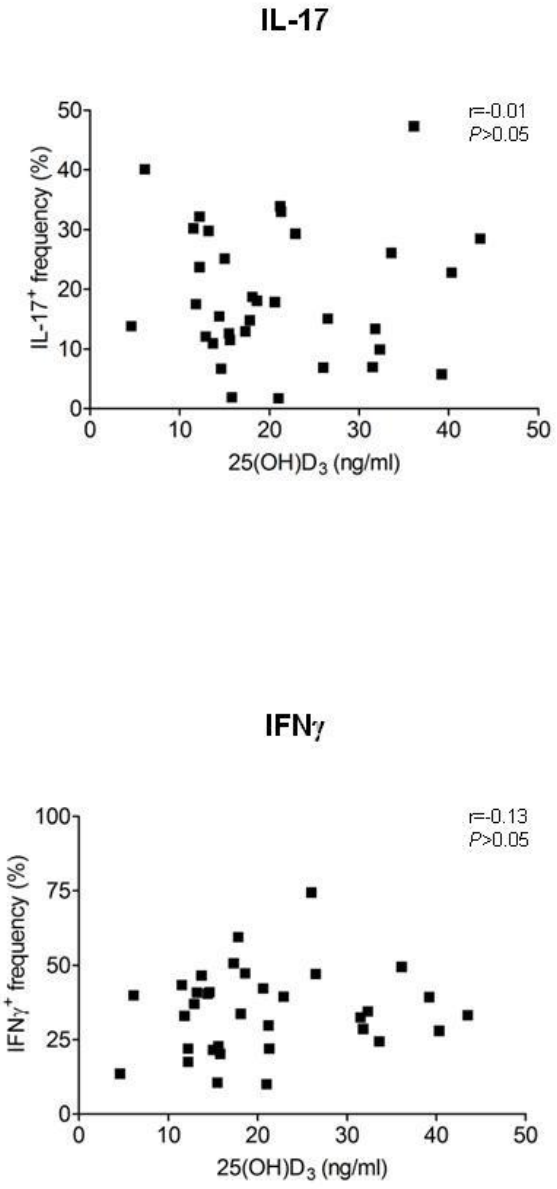

Figure 7.15. Serum 25(OH)D_3 does not influence IL-17 or IFNγ expression by peripheral blood T cells in response to stimulation. Relationship between serum 25(OH)D_3 and the frequencies of IL-17$^+$ and IFNγ$^+$ T cells following seven day anti-CD3 stimulation of PBMCs from early synovitis patients. Pearson's correlation coefficient was calculated (r) and used to determine the level of significance (*P*).

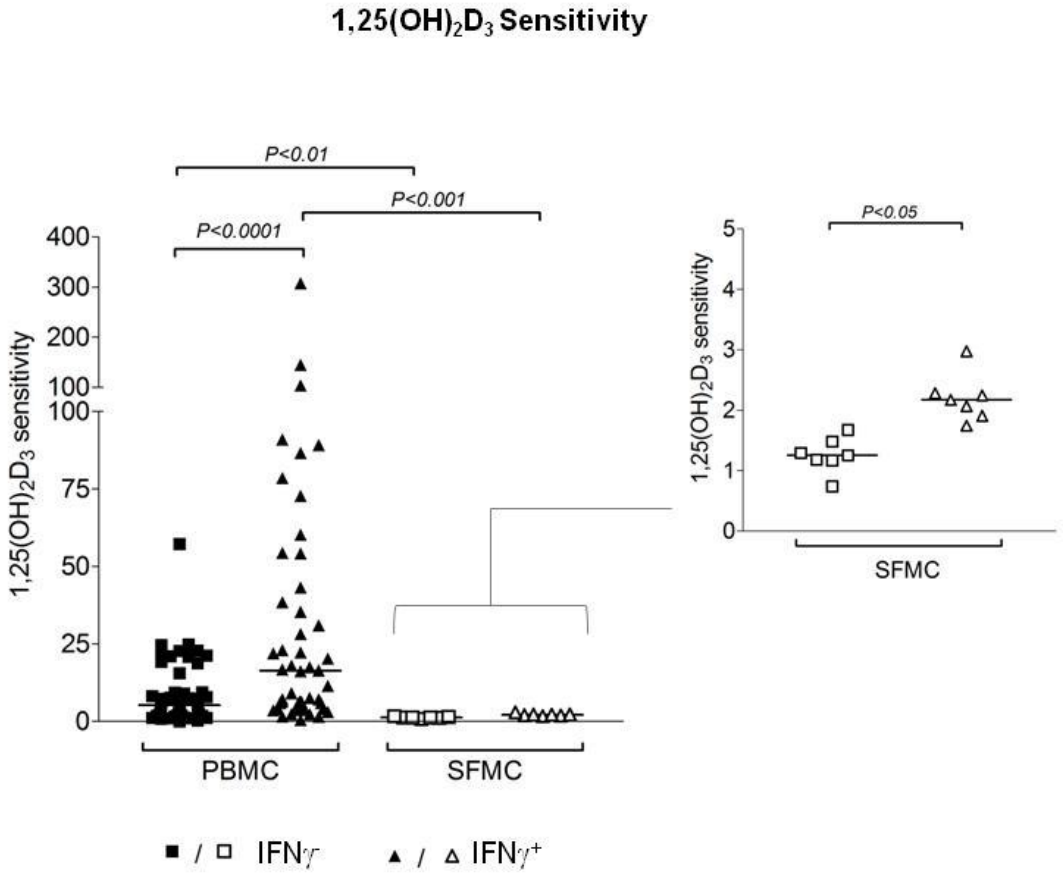

Figure 7.16. The IFNγ^+ subset of T17 cells is more sensitive to $1,25(OH)_2D_3$ than the IFNγ^- subset. PBMCs and SFMCs from synovitis patients were stimulated with anti-CD3 in the presence of 100nM $1,25(OH)_2D_3$ or vehicle control. At seven days, frequencies of T cells expressing IL-17 or IFNγ were determined by intracellular staining and flow cytometry. $1,25(OH)_2D_3$ sensitivity was calculated for each subset by $frequency_{control}/frequency_{1,25(OH)2D3}$. The higher the value, the greater the suppression by $1,25(OH)_2D_3$. Significance was tested by a two-tailed Mann Whitney U test. Horizontal lines indicate median values.

7.5 Discussion

In this chapter, I have presented data from an ongoing study designed to investigate whether dysregulation of the T_{Reg}/T17 balance in the synovial fluid (SF) or peripheral blood (PB) of early synovitis patients relates to their disease outcome. *Ex-vivo* frequencies of IL-17^+ (T17) and $CD4^+FoxP3^+CD25^+$ (T_{Reg}) T cells from the PB and SF of early synovitis patients have been measured and analysed against outcome as determined at 18-months. Presently, for T cells from PB, no difference is evident between T17 or T_{Reg} frequencies across early synovitis outcome groups, although for T_{Regs}, increased numbers in established RA relative to early RA are seen. Interestingly, for T17, T_{Reg} and IFNγ^+ frequencies in PB, no difference was observed between synovitis patients and healthy controls, although, more controls are needed to strengthen this analysis. Others have likewise observed no difference between T_h17 frequencies in the blood of healthy controls and established RA patients (Church et al., 2010; Yamada et al., 2008). However, two groups recently reported higher T_h17 frequencies in PBMCs from RA patients relative to healthy or OA controls (Colin et al., 2010; Leipe et al., 2010). Furthermore, Leipe et al. found that T_h17 frequencies positively correlated with disease activity scores (DAS28 and CRP) but Yamada et al. did not observe this. However, in the study by Leipe et al., T_h17 frequencies were at the limit of detection for controls and were very low even for RA patients (~0.5%). By comparison, in my study and those by Church et al., Colin et al., and Yamada et al., T_h17 frequencies of ~1.0%, were measured. Therefore, it would seem that the conditions in the study by Leipe et al. were different to those in my study and others, and might account for their different conclusions.

My observation that PB IL-17$^+$ T cell frequencies do not vary between RA patients and controls agrees with observations in other autoimmune conditions (Annunziato et al., 2007; Brucklacher-Waldert et al., 2009). Furthermore, for conditions where increased PB frequencies have been detected the levels in PB were generally still low (~1.0%) (Chen et al., 2010; Shen et al., 2009). It is possible that chemokine signalling keeps reactive cells at the inflammatory site. In this regard, CCL20, the ligand for CCR6, which is expressed at high levels on both T_h17 and T_{Regs}, is elevated in synovial fluid (Hirota et al., 2007; Lim et al., 2008; Nistala et al., 2008). Consistent with T_h17 accumulation in the joint, I found enhanced IL-17$^+$ T cell frequencies in SF relative to PB. Church et al. and Leipe et al. supported this finding although Yamada et al. showed a decline in total T_h17 cells in SF relative to blood. In further support of increased T_h17 frequencies at the inflammatory site, elevated T_h17 frequencies were found in the gut of Crohns patients and the CNS of MS patients when compared to PB (Annunziato et al., 2007; Brucklacher-Waldert et al., 2009). Strikingly, I observed that nearly all SF T cells expressed IFNγ. Although this finding contradicts the low IFNγ protein levels detected in RA SF (Firestein et al., 1990; Raza et al., 2005), most studies that analysed IFNγ expression at the single cell level observed significantly higher IFNγ$^+$ T cell frequencies in SF or synovial tissue relative to PB (Church et al., 2010; Dolhain et al., 1996; Leipe et al., 2010; Morita et al., 1998; Yamada et al., 2008). However, this discrepancy may be a consequence of the analysis method, as PMA and ionomycin re-stimulation used for intracellular cytokine staining could reveal 'past histories' of gene expression recorded by chromatin remodelling. Indeed, IL-17 and IFNγ were not detected by intracellular staining in the absence of PMA and calcium signalling (Dolhain et al., 1996; Yamada et al., 2008). The fact that 99% of SF T cells were of the CD45-RO memory phenotype would support a previously established expression profile, which, although no longer active, could be re-

instated by PMA and calcium signalling. Whether, re-stimulation through the T cell receptor would similarly drive IFNγ in T cells from the joint would be of interest and could address more physiologically the role of IFNγ in RA pathology. The possibility that IFNγ is expressed during the initial stages of disease is in keeping with the relatively low frequencies of T17 cells that I and others detected in SFMCs (Church et al., 2010; Leipe et al., 2010; Yamada et al., 2008), since IFNγ is inhibitory to T_h17 differentiation (Harrington et al., 2005; Park et al., 2005). However, additional inhibitory signals could contribute, such as the strength of co-stimulation (Bouguermouh et al., 2009; Gomez-Rodriguez et al., 2009; Purvis et al., 2010) and IL-2 (Acosta-Rodriguez et al., 2007a; Laurence et al., 2007) although low IL-2 levels are reported in the RA joint (Firestein et al., 1990).

My observation that T_{Reg} frequencies are not increased in the blood of synovitis patients compared to controls is likewise consistent with others findings (Cao et al., 2003a; Flores-Borja et al., 2008; van Amelsfort et al., 2004). It is important to note, however, that not all studies included FoxP3 as a marker. Indeed, in one study where FoxP3 expression was assessed, fewer T_{Regs} were found in the PB of RA patients versus controls and FoxP3 expression was lower (Valencia et al., 2006). Decreased PB T_{Reg} frequencies are similarly described in other autoimmune conditions (Kukreja et al., 2002). Interestingly, my data suggest that T_{Reg} frequencies might increase as RA progresses. This could represent an attempt to restore regulation. More samples are needed however to strengthen these conclusions. I also observed a clear increase in the frequency of T_{Regs} in SF compared to PB across disease outcomes. Such T_{Reg} enrichment in SF relative to PB has been reported by

others studying RA (Cao et al., 2003a; Jiao et al., 2007; van Amelsfort et al., 2004) and is described for childhood arthritis (de Kleer et al., 2004; Ruprecht et al., 2005).

Given that both T_{Regs} and T17 cells were enriched in SF, I was interested to compare the T_{Reg}/T17 ratio for SF and PB. Surprisingly, I found that the T_{Reg}/T17 ratio was balanced for the two sites. This could imply that inflammatory versus regulatory balance was equivalent in the two compartments. In contrast to the concept that T_h17 and T_{Reg} are reciprocal fates (Bettelli et al., 2006; Quintana et al., 2008; Schambach et al., 2007; Veldhoen et al., 2008a), I did not observe an inverse relationship between T17 and T_{Reg} frequencies from PB or SF. This correlation has not been directly measured previously in RA but an inverse relationship between T_h17 and T_{Reg} cells in SF was found in childhood arthritis (Nistala et al., 2008). It is possible that different mechanisms operate in the two conditions but data from other cohorts are needed to clarify my findings.

Even if dysregulation of T_{Reg} and T17 frequencies does not contribute to RA pathogenesis, it is possible that excessive T_h17 activity or loss of T_{Reg} function is responsible for the progression of RA. In this regard, Leipe et al. suggested that T_h17 cell activity might become uncontrolled in RA pathogenesis through elevated RORC expression and acquired resistance to natural antagonists of their development (Leipe et al., 2010). However, in contrast to their findings and those by Colin et al. (2010), I did not observe enhanced propensity for T17 development by PBMCs from synovitis patients. The possibility that $FoxP3^+CD25^+$ T cells in the joint are not effective T_{Regs} might be supported by the need for multiple rounds of stimulation to induce regulatory activity within iT_{Regs} (Horwitz et al., 2008b; Shevach et al.,

2008). Possibly low CTLA-4 expression might contribute to this effect, since I showed in **chapter five** of this thesis that inflammatory conditions could suppress CTLA-4 and reduce the ability of T cells to down-regulate B7 expression by DCs. Thus, within an inflamed joint, T_{Reg} function could be compromised by low CTLA-4 expression. In agreement with defective T_{Reg} activity, Valencia et al. demonstrated that PB T_{Regs} from RA patients had reduced ability to suppress responder T cell proliferation and IFNγ expression (Valencia et al., 2006). Counter to this, however, others showed that $CD4^+CD25^+$ cells from RA patients were anergic and able to suppress responder cell proliferation (Ehrenstein et al., 2004; van Amelsfort et al., 2004), although reduced ability to suppress TNFα and IFNγ was observed. The latter might relate to low CTLA-4 expression by RA T_{Regs} and abnormal CTLA-4 biology, although how CTLA-4 contributes to such responses remains to be established (Flores-Borja et al., 2008). Loss of regulation within the joint could equally arise if $CD4^+CD25^-$ T cells are less responsive to suppression. Consistent with this, $CD4^+CD25^-$ T cells from SF were less responsive to suppression than those from PB (van Amelsfort et al., 2004). Interestingly, T_h17 cells in particular might be resistant to T_{Regs}, since both healthy and RA T_{Regs} did not suppress IL-17 production (Flores-Borja et al., 2008). Also, in the presence of T_{Regs}, $IL\text{-}17^+$ cells were increased even though $IFN\gamma^+$ frequencies decreased (Evans et al., 2007). These effects might relate to high CTLA-4 expression by T_{Regs}, since CTLA-4-Ig treatment could increase T_h17 frequencies (Bouguermouh et al., 2009).

In light of my finding that $1,25(OH)_2D_3$ strongly promotes a T_{Reg} phenotype, whether disease outcome or T_{Reg} and T17 frequencies related to serum $25(OH)D_3$ level was also addressed in this study. No correlation of $25(OH)D_3$ level with disease outcome or T17 frequencies was

observed and in contrast to expectation, T_{Reg} frequencies correlated inversely with 25(OH)D_3 level. In support of this trend, early data suggests that CTLA-4 expression by PB $CD4^+FoxP3^+$ cells might also vary inversely with 25(OH)D_3 level. However, more samples are needed to verify this trend statistically. It will also be important to perform the same analysis on SF samples once a sufficient sample size is available. In addition to analysing SF T17 and T_{Reg} frequencies against serum 25(OH)D_3 levels it would be interesting to study their correlation against SF 25(OH)D_3, since such data might provide a better indication of the relevance of vitamin D in the control of inflammation at a disease locus.

My study has confirmed, that PBMCs and SFMCs from early synovitis patients are responsive to 1,25$(OH)_2D_3$ when cultured *in-vitro,* as IL-17 and IFNγ frequencies were suppressed when PBMC stimulations were supplemented with 1,25$(OH)_2D_3$. Consistent with observations throughout this thesis, the $IFN\gamma^+$ subset of T17 cells was the most sensitive to 1,25$(OH)_2D_3$ for both PBMCs and SFMCs. Interestingly though, PBMCs were more sensitive to 1,25$(OH)_2D_3$ than SFMCs. Given that all SFMC T cells, including $CD4^+CD25^-$ cells, displayed an activated surface marker profile, these data might support the hypothesis that repetitive activation reduces T cell plasticity (Grogan et al., 2001). Furthermore, the fact that 1,25$(OH)_2D_3$ suppressed IL-17 but not IFNγ in SFMCs supports the concept that IL-17 is a more plastic cytokine than IFNγ (Zhou et al., 2009a). Whilst the lack of IFNγ suppression by SF T cells may question the potential effectiveness of 1,25$(OH)_2D_3$ supplementation in the treatment of RA, the fact that all patients could respond to 1,25$(OH)_2D_3$ implies that synovitis patients are not generally defective in VDR responses. Furthermore, given the associations of IL-17 with RA pathology (Firestein, 2003; Miossec et al., 2009), the ability of 1,25$(OH)_2D_3$ to

suppress IL-17 even in cells from SF, is encouraging. In support of the potential use of vitamin D in the control of RA, I observed negative correlations between 25(OH)D_3 status and two measures of disease activity: DAS28 and ESR. However, rather than low 25(OH)D_3 being the cause of increased joint inflammation, it could be a consequence, since as disease severity increases, patients might spend less time outside, thus causing their 25(OH)D_3 status to fall. Nonetheless, the involvement of vitamin D in the control of RA is supported by the finding that greater vitamin D intake through diet or supplement was inversely associated with RA risk (Merlino et al., 2004) and that 1α-(OH)-D_3, a vitamin D analogue could improve responses to standard DMARD therapy in active RA (Andjelkovic et al., 1999). However, in contrast to my observations, two other studies in RA did not show an association between 25(OH)D_3 and disease activity scores (Kroger et al., 1993; Oelzner et al., 1998). Since these studies involved patients with established disease, it is possible that other medications had confounding influence.

As described above, the results presented in this chapter, as well as those of others, do not clearly demonstrate an association between aberrant T17 or T_{Reg} frequencies and RA pathogenesis. This is in contrast to the finding that RA is T_h17 driven in the CIA mouse model. However, it is possible that the method of immunisation contributes to the pathogenic process in these mice as *Mycobacterium tuberculosis*, a potent T_h17 inducer, is a component of Freund complete adjuvant, which is typically used in the immunisation procedure (Yamada et al., 2008). Further support for a non-T_h17 dependent pathology in RA is the observation by FACS (Colin et al., 2010; Leipe et al., 2010; Yamada et al., 2008) and immunohistochemistry (Chabaud et al., 1999), that very few T cells within the synovium are IL-17^+ (Chabaud et al., 1999). Thus, even though IL-17 is a potent cytokine, it is difficult to imagine that T cell

derived IL-17 alone is sufficient to drive the inflammatory processes within the rheumatoid joint. It is now apparent that a variety of cells, besides $CD4^+$ T cells express IL-17, including γδ T cells, NKT cells, NK cells, neutrophils, eosinophils, Lymphoid tissue inducer cells (LTis) and mast cells (Hueber et al., 2010). Interestingly, a very recent study revealed that mast cells might be a major source of IL-17 within the synovium, as the majority of IL-17A staining on RA synovium sections localised to mast cells. Furthermore, a range of factors, which could feature in the joint, were found to promote their IL-17 expression (Hueber et al., 2010).

Irrespective of the cellular source of IL-17, its ability to drive inflammation and bone erosion makes it an attractive candidate for biological therapy in RA. Clinical trials assessing the safety, tolerability and efficacy of monoclonal antibodies to IL-17 are promising (Genovese et al., 2010; Tak, 2009). In addition, the success of anti-IL-6 and anti-IL-1β therapies in childhood arthritis (Allantaz et al., 2007; Yokota et al., 2008) could involve their ability to suppress IL-17 expression. However, because IL-17 plays a major role in protection from certain pathogens (reviewed by (Miossec et al., 2009)), such therapies could leave patients at risk of infection. Thus, it will be important to characterise the dependency of RA pathology upon IL-17 and appropriately balance the benefits and disadvantages of its availability.

8 GENERAL DISCUSSION

The frequency of autoimmune diseases is increasing (Black, 2001), thus the identification of predisposing environmental and genetic factors is crucial. Dysregulation of the T_{Reg}/T_h17 balance is implicated in a number of autoimmune conditions and disease models. Therefore, factors that modulate this balance are of potential therapeutic and prophylactic benefit. Over the past 30 years, the importance of vitamin D in immune modulation has become increasingly recognised. Whilst *in-vitro* and *in-vivo* studies have pointed to its ability to suppress DC maturation and modulate $CD4^+$ T cell differentiation away from T_h1 and towards T_h2 and T_{Reg} (reviewed in (Adams and Hewison, 2008; Adorini, 2002)) our understanding of the mechanisms by which vitamin D protects against autoimmunity is not complete. Indeed, the effect of $1,25(OH)_2D_3$ upon novel $CD4^+$ T cell subsets, such as T_h17, T_{fh} and T_h22 cells had not been published prior to the start of work for this thesis. In addition, most studies that described an effect of $1,25(OH)_2D_3$ upon T cell differentiation had used APC-containing systems (Adorini, 2002; Adorini et al., 2003; Penna and Adorini, 2000) or enriched or impure lymphocyte populations (Reichel et al., 1987b). Thus, the direct influence of $1,25(OH)_2D_3$ upon T cell differentiation was not well established. This thesis therefore set out to examine the influence of $1,25(OH)_2D_3$ upon $CD4^+$ T cell differentiation and to investigate whether its effects were by direct action upon the T cell or indirect via the APC.

A striking ability of $1,25(OH)_2D_3$ to suppress $CD4^+$ T cell expression of pro-inflammatory cytokines IL-17, IFNγ, IL-21 and IL-22, even in the absence of APCs, was found. This indicates that $1,25(OH)_2D_3$ can act directly upon T cells to modify their phenotype. Since these cytokines are the hallmark cytokines of T_h17 (Harrington et al., 2005), T_h1 (Mosmann

and Coffman, 1989), T_{fh} (Vogelzang et al., 2008) and T_h22 (Trifari et al., 2009) effector classes respectively, it might further be concluded that $1,25(OH)_2D_3$ has a broad ability to suppress pro-inflammatory effector classes. $1,25(OH)_2D_3$ therefore has relevance in the control of immune responses to a wide range of bacterial and fungal pathogens as well as in autoimmune diseases and cancer.

The work in this thesis also confirmed that $1,25(OH)_2D_3$ can promote iT_{Reg} development as suggested by earlier studies (Barrat et al., 2002; Ghoreishi et al., 2009; Gregori et al., 2002; Urry et al., 2009), since its ability to dramatically increase CTLA-4 expression, and synergise with TGFβ to favour a CTLA-4^+FoxP3^+ population and IL-10 expression was striking. In addition, IL-2 was found to co-operate with $1,25(OH)_2D_3$ for the induction of a T_{Reg} phenotype and suppression of IL-17.

Studies within this thesis suggest that $1,25(OH)_2D_3$ might be valuable in the treatment of active inflammatory diseases, especially those driven by an imbalance between T_{Reg} and T_h17 frequencies or activities. Firstly, anti-inflammatory effects of $1,25(OH)_2D_3$ were maintained under T_h17 polarising conditions, secondly stimulation of T_h17 polarised cultures in the presence of $1,25(OH)_2D_3$ and IL-2 shifted the bulk phenotype towards T_{Reg}. Thirdly $1,25(OH)_2D_3$ could reduce IL-17^+ frequencies or the level of IL-17 expression by purified IL-17^+ cells, thus suggesting that $1,25(OH)_2D_3$ can act upon previously committed T_h17 cells as would be found at an inflammatory site.

To be effective therapeutically, it would be necessary, however, that patients can respond to $1,25(OH)_2D_3$. Response is primarily through the vitamin D Receptor (VDR). Allelic variations in VDR exist and associations have been made with susceptibility to autoimmune disease, although, in the main, results are conflicting and no functional consequence of the mutations has been found (reviewed by (Arnson et al., 2007; Mullin and Dobs, 2007)). In this study it was demonstrated that T cells from established and early synovitis patients respond efficiently to $1,25(OH)_2D_3$, suggesting that response to $1,25(OH)_2D_3$ is not impaired in RA. However, the highly activated T cells from SF were considerably less sensitive to $1,25(OH)_2D_3$ than those from PB, implying that repeat stimulation might 'fix' a cytokine profile even in T_h17 cells, which are generally regarded more plastic than T_h1 or T_h2 cells (Zhou et al., 2009a). This finding was in keeping with the seemingly lower sensitivity of purified IL-17^+ cells to $1,25(OH)_2D_3$ compared to $CD4^+CD25^-$ T cells undergoing primary stimulation.

Across all studies in this thesis, the suppressive effect of $1,25(OH)_2D_3$ was most profound upon the IFNγ^+ subset of IL-17^+ cells. Interestingly, few cells in PB possessed this phenotype *ex-vivo* but it was detected in T_h17 cells isolated from SF. Consistent with this finding, others have reported enrichment of IL-17^+/IFNγ^+ T cells at disease sites (Annunziato et al., 2008; Nistala et al., 2010; Nistala et al., 2008). Such enrichment suggests that they could be the most pathogenic species. Thus, the finding that $1,25(OH)_2D_3$ most efficiently suppressed this population is encouraging when considering its therapeutic value.

In support of a therapeutic or prophylactic use for $1,25(OH)_2D_3$ in the control of autoimmune conditions, $1,25(OH)_2D_3$ and its analogues have been able to suppress and prevent disease symptoms in mouse models of multiple sclerosis (MS), type 1 diabetes (TID), inflammatory bowel disease (IBD), uveitis, systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) (reviewed in (Adorini, 2002; Arnson et al., 2007; Mullin and Dobs, 2007; Smolders et al., 2008)). In addition, $1,25(OH)_2D_3$ analogues are widely used topically in the treatment of human psoriasis (reviewed by (Adorini, 2002). Small-scale studies also show their possible benefit in RA (Andjelkovic et al., 1999) and steroid resistant asthma (Xystrakis et al., 2006). The primary concern with the systemic use of $1,25(OH)_2D_3$ is its hypercalcaemic effect, although a trial in MS demonstrated overall safety at the dose used (Wingerchuk et al., 2005). Nonetheless, treatment with $1,25(OH)_2D_3$ analogues that have reduced calcaemic effect and are more metabolically stable (Adorini et al., 2003), should improve the efficiency and reduce the risk of complications associated with systemic $1,25(OH)_2D_3$ treatment.

Given that the $25(OH)D_3$ converting enzyme, 25-hydroxyvitamin-D_3-1α-hydroxylase (CYP27B1), is expressed broadly throughout the body, including in immune cells (Hewison et al., 2007), $25(OH)D_3$ supplements could be given instead of $1,25(OH)_2D_3$ for anti-inflammatory therapy. In this thesis, whether locally generated $1,25(OH)_2D_3$ could affect T cell phenotype was therefore addressed. It was found that in the presence of APCs, $25(OH)D_3$ strongly induced CTLA-4 in a CYP27B1 dependent manner and suppressed IL-17 and IFNγ. Low CYP27B1 expression in T cells was also observed and was sufficient to support weak CTLA-4 induction and suppression of inflammatory cytokines in the presence of $25(OH)D_3$ and absence of APC. By confirming that local $25(OH)D_3$ conversion can modulate T cell

phenotype, these data help to explain why low serum levels of $25(OH)D_3$ have been associated with autoimmune conditions even though $1,25(OH)_2D_3$ levels remain relatively constant (Als et al., 1987; Andreassen et al., 1998; Andreassen et al., 1997; Holick, 2007; Kamen et al., 2006; Munger et al., 2006). These data also provide a cellular and molecular rationale for the use of inactive $25(OH)D_3$ in the treatment of autoimmune disease. Most studies to investigate the effect of $25(OH)D_3$ upon autoimmunity have centred around MS. In the main they have shown clinical benefit (reviewed by (Cantorna and Mahon, 2004). The success could, in part, be due to the effect of vitamin D within the nervous system, since VDR is expressed in the CNS and vitamin D derivatives can promote the expression of neurotrophins (Riaz et al., 1999) and suppress neurotoxic reactions (Kalueff et al., 2004). However, tolerogenic immune responses, including an increase in serum TGFβ, were observed in patients after $25(OH)D_3$ treatment (Mahon et al., 2003). The effect of vitamin D supplementation upon T1D risk and IBD has also been studied. However, whilst reduced risk or clinical benefit was observed, immunological effects were not assessed in these interventions (reviewed in (Cantorna and Mahon, 2004) and (Zella and DeLuca, 2003)). Therefore, more clinical studies are needed to assess the use of $25(OH)D_3$ for the control of a wider range of inflammatory diseases and to determine more definitively whether effects observed involve modulation of immune parameters. As such, trials are planned to assess the therapeutic and immune-modulatory effects of $25(OH)D_3$ upon patients with steroid resistant asthma, MS, RA and Crohn's disease (NIH, 2010).

With reference to the above, it is important to consider, that $25(OH)D_3$ levels do not always correlate with disease risk or severity (Huisman et al., 2001; Kroger et al., 1993; Munger et al., 2006; Oelzner et al., 1998). Whilst this might relate to confounding factors such as the use

of other treatments, it could point to defects in the conversion and/or distribution of 25(OH)D_3 mediated by CYP27B1 and the vitamin D binding protein (VDBP) respectively. Indeed, polymorphisms in CYP27B1 (Bailey et al., 2007; Lopez et al., 2004) and VDBP (Papiha and Pal, 1985; Speeckaert et al., 2006) are associated with a range of autoimmune conditions, although, as for VDR (Arnson et al., 2007; Mullin and Dobs, 2007), results in different populations are conflicting. At extra-renal sites, a range of factors regulate the activity of CYP27B1, such as cytokines and growth factors (Leventis and Patel, 2008), and TLR ligand signalling appears to control CYP27B1 expression in APCs (Adams and Hewison, 2008). A defect in any of these pathways could reduce 25(OH)D_3 sensitivity and might account for the lack of correlation between 25(OH)D_3 level and disease severity.

The array of factors that contribute to extra-renal 25(OH)D_3 conversion could also set the level of 25(OH)D_3 required for optimal immune function much higher than the 80nM, currently recommended for optimal calcium regulation and bone strength (Hollis, 2005). Thus, the apparent lack of association between 25(OH)D_3 and disease severity could be because most populations were deficient as far as immune regulation is concerned. In support of different serum 25(OH)D_3 optimums for different body systems, it has been shown that insulin sensitivity in non-diabetic patients correlates positively with vitamin D status and that optimal glycaemic control requires a serum 25(OH)D_3 level of 114nM (Heaney, 2005). This far exceeds the 'normal' 25(OH)D_3 level but might be reached by individuals who lead an outdoor lifestyle and are not overly clothed (Haddad and Chyu, 1971). Studies to determine tolerable 25(OH)D_3 doses are ongoing but estimating the required level of 25(OH)D_3 supplement for efficient immune function necessitates the identification of efficient biomarkers of immune response. The recently published genome wide map of VDR binding

sites in lymphoblastic cell lines could aid identification of potential markers (Ramagopalan et al., 2010).

In this thesis, CTLA-4 stood out as a very sensitive $1,25(OH)_2D_3$ immune target. However, for PBMCs from early synovitis patients CTLA-4 expression by $CD4^+FoxP3^+CTLA\text{-}4^+$ T cells did not appear to correlate positively with serum $25(OH)D_3$ level, although further samples would be needed to confirm this trend. A potential problem with CTLA-4 as a marker of vitamin D status might be its sensitivity to other factors. For example, suppression of CTLA-4 by T_h17 polarising cytokines in the absence of $1,25(OH)_2D_3$ was observed, whilst $1,25(OH)_2D_3$ synergised with these cytokines to promote very high levels of CTLA-4. IL-2 was also found to synergise with $1,25(OH)_2D_3$ to enhance CTLA-4. Data from ongoing work (not shown) further suggests that although $1,25(OH)_2D_3$ is quite unique in its ability to greatly enhance CTLA-4 expression, the combined action of $1,25(OH)_2D_3$ with other anti-inflammatory agents can also influence levels. Therefore, particularly for individuals receiving anti-inflammatory treatment, CTLA-4 expression might not correlate closely with serum $25(OH)D_3$ level.

CTLA-4 is highly expressed on T_{Regs} and is induced in effector T cells upon activation. Mounting evidence supports its major role in the suppressive activity of T_{Regs} (Friedline et al., 2009; Kolar et al., 2009; Onishi et al., 2008; Read et al., 2006; Schmidt et al., 2009; Tivol et al., 1995; Waterhouse et al., 1995; Wing et al., 2008; Zheng et al., 2008b). Work in this thesis has demonstrated that the level of CTLA-4 is functionally important, at least as far as extrinsic regulation mechanisms are concerned. By its ability to dramatically up-regulate

CTLA-4 expression, $1,25(OH)_2D_3$-enhanced CTLA-4-dependent removal of co-stimulatory ligands from APCs. This effect contributed greatly to $1,25(OH)_2D_3$-mediated suppression of T cell proliferation observed when T cells were stimulated by APCs. However, it might also underlie the ability of $1,25(OH)_2D_3$-conditioned stimulated T cells to suppress the division of $CD4^+CD25^-$ responder T cells in DC driven stimulations. The fact that CTLA-4 blockade could not completely overcome CD86 down-regulation on primary APC driven responses indicates, however, that additional, non-CTLA-4 dependent mechanisms of suppression might be employed by $1,25(OH)_2D_3$-conditioned T_{Regs}. It is likely that their cytokine profile is involved. This includes enhanced production of IL-10 and reduced IFNγ, both of which could decrease co-stimulatory molecule expression by DCs (Fujihara et al., 1996; Moore et al., 2001). Future studies will examine in more detail the nature of the CTLA-4 dependent and independent mechanisms of suppression employed by $1,25(OH)_2D_3$-conditioned T cells.

In addition to its potential use as a medicinal supplement, $1,25(OH)_2D_3$ in consort with TGFβ and IL-2 could be useful for the *ex-vivo* generation of T_{Regs} for adoptive transfer to enhance tolerance. This approach has application in the treatment of autoimmune diseases, such as diabetes but also in short term immune suppression to prevent graft versus host disease or graft rejection in sold organ transplantation (Pahwa et al., 2010). However, because both FoxP3 and CTLA-4 showed transient patterns of expression in this study, even following 5-aza-2-deoxycytidene treatment, the cells might not retain suppressive function long-term. Further work in this area is warranted to determine if altered culture conditions would improve stability of CTLA-4 and FoxP3 expression.

Others are using protocols to expand natural T_{Regs} instead of generating iT_{Regs} for transplant (Pahwa et al., 2010; Sheng et al., 2010). If $1,25(OH)_2D_3$ could further enhance CTLA-4 expression by nT_{Regs} its supplementation might generate highly efficient nT_{Regs}. Enhanced expression of CTLA-4 on T_{Regs} in the skin following topical treatment with a $1,25(OH)_2D_3$ analogue, suggests this will be possible (Ghoreishi et al., 2009). One concern surrounding adoptive T_{Reg} treatment strategies is the possibility that $FoxP3^+$ cells might differentiate into T_h17 or T_{fh} cells *in-vivo* under appropriate pro-inflammatory conditions (Tsuji et al., 2009; Zhou et al., 2009b). Nonetheless, the report that TGFβ and IL-2 induced T_{Regs} are resistant to T_h17 conversion by IL-6 (Zheng et al., 2008a) maintains an element of doubt over the potential for iT_{Regs} to switch phenotype. For nT_{Regs}, there is suggestion that culturing under T_h17 polarising conditions permits IL-17 expression whilst maintaining FoxP3 (Wei et al., 2009). Data from this thesis suggest however, that if $1,25(OH)_2D_3$ levels at the site of active disease can be sufficiently maintained, then the presence of pro-inflammatory cytokines might actually enhance the suppressive phenotype of the T_{Regs} by favouring high levels of CTLA-4 and permitting IL-10, whilst suppressing inflammatory cytokines. Since IL-2 supplementation was also found to enhance FoxP3 expression in this thesis, combined treatment with $25(OH)D_3$ and IL-2 at the time of transfer could be particularly effective at maintaining the T_{Reg} phenotype *in-vivo*. In support of the beneficial effect of IL-2 in the treatment of autoimmune conditions, it was recently shown that low dose IL-2 can selectively promote T_{Reg} expansion in the Non-Obese Diabetic (NOD) mouse model of diabetes when administered at disease onset, leading to long-lasting remission of disease (Grinberg-Bleyer et al., 2010). In addition, a clinical trial involving IL-2 and rapamycin is currently ongoing for type 1 diabetes (NCT00525889).

In summary, the work in this thesis has provided further insight into the cellular and molecular basis of the association between low vitamin D status and autoimmune conditions, demonstrating in particular its strong ability to directly promote an anti-inflammatory phenotype in T cells. The findings are exciting, since they suggest that vitamin D supplementation could offer a simple and relatively inexpensive therapeutic method for the control of autoimmune conditions. Furthermore, the antimicrobial properties of $1,25(OH)_2D_3$ in the innate immune system (Adams and Hewison, 2008) set it apart from other immunosuppressant strategies that compromise this line of defence.

Work in this thesis has also investigated the balance of T17 and T_{Reg} cells in synovitis patients and analysed it with respect to outcome group. Since for PB no differences were observed between groups or with respect to healthy controls or established RA suffers, it is concluded that dysregulation of the T_{Reg}/T_h17 balance is unlikely to contribute to disease outcome or play a major role in its pathogenesis. To strengthen conclusions for SF, more samples are needed and collection is ongoing. Nonetheless, the relatively low number of T17 cells measured in SF suggests that they are not central players. The observation that IL-17^+ and IFNγ^+ T cell frequencies did not correlate with serum $25(OH)D_3$ level might suggest a general $25(OH)D_3$ deficiency as far as immune function is concerned. Nevertheless, the fact that all patients responded to $1,25(OH)_2D_3$ *in-vitro* would support the use of $1,25(OH)_2D_3$ analogues in RA therapy. In particular, it would be worth investigating whether vitamin D treatment within the therapeutic window of three months (Raza, 2010) could prevent RA onset or reduce disease severity.

9 APPENDIX

Table 9-1. Clinical data for synovitis patients. (Abbreviations: Disease activity score 28 (DAS28), C Reactive Protein (CRP), Erythrocyte sedimentation rate (ESR), rheumatoid factor (RF), Cyclic Citrullinated Peptide Antibody (CCP), Disease modifying antirheumatic drug (DMARD), - indicates that data could not be collected or was not available).

Patient code	Disease outcome	Gender	Age	Symptom Duration (wks)	DAS28 (CRP) score	CRP	ESR	RF	CCP	DMARD / Biological drugs	$25(OH)D_3$ ng/ml
T08.109	RA	m	55	6	6.93	11	19	0	-	-	19.1
T08.126	RA	f	19	12	2.87	0	4	0	-	-	-
T08.187	RA	f	69	6	6.06	26	68	0	-	-	13.7
T08.200	RA	f	51	8	4.39	6	21	21	-	-	12.2
T09.064	RA	m	67	7	4.98	83	25	40	-	-	39.2
T09.090	RA	f	48	2	3.46	102	4	0	-	-	33.6
T09.108	RA	f	66	6	3.89	5	21	84	-	-	-
T09.124	RA	m	76	7	5.28	14	18	0	-	-	19.6
T09.145	RA	m	66	8	4.97	20	31	0	-	-	15.5
T10.049	RA	m	55	12	5.29	0	5	21	-	-	11.8
T10.058	RA	m	54	4	3.03	45	58	0	-	-	15.0
T10.084	RA	f	63	4	6.67	124	86	0	-	-	21.3
T10.118	RA	f	48	12	3.87	10	10	339	-	-	32.3
T10.131	RA	f	27	10	3.72	13	23	203	-	-	25.9
T08.179	Non-RA persistent	f	67	5	6.46	20	47	20	-	-	14.4
T08.182	Non-RA persistent	m	43	3	4.52	188	102	0	-	-	17.8
T08.203	Non-RA persistent	m	24	3	2.59	0	5	0	-	-	36.1
T09.060	Non-RA persistent	f	45	9	3.51	12	24	0	-	-	6.1
T09.136	Non-RA persistent	f	55	4	3.82	16	19	30	-	-	18.6
T10.126	Non-RA persistent	f	43	2	6.45	70	97	0	-	-	4.6

Patient code	Disease outcome	Gender	Age	Symptom Duration (wks)	DAS28 (CRP) score	CRP	ESR	RF	CCP	DMARD / Biological drugs	25(OH)D_3 ng/ml
T08.147	Self-limiting	f	40	4	4.74	51	36	0	-	-	-
T08.162	Self-limiting	m	35	3	5.76	30	58	0	-	-	-
T08.170	Self-limiting	m	33	4	3.62	6	9	0	-	-	26.5
T08.183	Self-limiting	m	45	2	4.68	103	97	0	-	-	12.9
T08.186	Self-limiting	f	35	6	4.01	29	64	0	-	-	19.9
T09.103	Self-limiting	m	74	4	3.38	0	17	0	-	-	22.9
T09.114	Self-limiting	f	33	4	1.85	0	5	0	-	-	40.3
T09.133	Self-limiting	m	57	4	NA	NA	NA	0	-	-	-
T09.141	Self-limiting	f	54	5	3.75	14	29	0	-	-	17.3
T09.146	Self-limiting	m	32	8	2.95	10	10	0	-	-	12.2
T09.153	Self-limiting	m	32	4	6.71	14	51	0	-	-	7.5
T09.160	Self-limiting	m	74	6	4.77	13	45	0	-	-	18.1
T10.007	Self-limiting	f	80	7	6.26	52	60	0	-	-	15.6
T10.027	Self-limiting	f	53	4	3.34	0	13	0	-	-	31.8
T10.043	Self-limiting	f	44	1	3.99	0	4	0	-	-	20.6
T10.113	Self-limiting	m	34	2	4.06	7	51	0	-	-	13.2
T08.169	Not known	m	27	7	3.54	9	19	0	-	-	11.5
T09.067	Not known	-	-	-	-	-	-	-	-	-	-
T09.093	Not known	m	62	4	3.13	12	7	85	-	-	26.0
T09.142	Not known	f	43	5	3.48	6	18	0	-	-	21.2
T10.112	Not known	f	23	9	5.85	40	26	0	-	-	21.0
T10.127	Not known	f	44	2	3.01	0	14	0	-	-	15.8
T10.138	Not known	m	55	11	3.52	6	2	0	-	-	31.5
T10.140	Not known	-	-	-	-	-	-	-	-	-	-
T10.141	Not known	f	24	11	3.80	66	79	0	-	-	43.5
T10.142	Not known	f	39	8	4.73	15	27	0	-	-	14.6
T09.104	Established RA	m	46	3	5.90	7	34	positive	positive	nil	-

Patient code	Disease outcome	Gender	Age	Symptom Duration (wks)	DAS28 (CRP) score	CRP	ESR	RF	CCP	DMARD / Biological drugs	25(OH)D_3 ng/ml
T09.132	Established RA	m	57	5	3.17	0	7	positive	positive	nil	-
T09.166	Established RA	f	56		5.59	5	35	positive	-	nil	-
T10.010	Established RA	f	35	2	1.67	0	9	positive	positive	Nil	-
T10.029	Established RA	f	61	20	5.87	0	18	positive	positive	Nil	-
T10.033	Established RA	m	21	<1 year	3.67	0	5	negative	negative	Nil	-
T10.114	Established RA	f	75	40	4.49	49	55	negative	positive	methotrexate, sulfasalazine	-
T10.128	Established RA	f	43	1	3.91	0	4	positive	positive	Nil	-
T10.139	Established RA	f	57	1	7.91	59	91	positive	positive	Nil	-
T10.146	Established RA	f	55	5	7.08	221	124	positive	positive	methotrexate	-

Table 9-2. ***Ex-vivo*** **data for peripheral blood T cells from synovitis patients and controls. (- indicates that data could not be collected).**

Patient code	Disease outcome	$CD3^+$ (%)	$CD4^+$ (% of $CD3^+$)	CD45-RO^+ (% of $CD3^+$)	$IL17^+$ $IFN\gamma^-$ (%)	IL-17^- $IFN\gamma^+$ (%)	IL-17^- $IFN\gamma^+$ (%)	IL-17^+ total (%)	$IFN\gamma^+$ total (%)	T_{Reg} (%)	T_{Reg} /T17 ratio	CTLA4 MFI
T08.187	RA	63.0	71.0	34.8	0.4	0.0	27.6	0.4	27.6	-	-	-
T08.200	RA	59.6	70.9	36.3	0.8	0.2	17.4	0.9	17.6	2.9	3.2	-
T09.064	RA	60.3	52.9	51.2	0.4	0.0	23.4	0.4	23.4	4.6	12.0	-
T09.090	RA	63.2	74.8	49.2	1.0	0.4	14.8	1.4	15.2	5.5	4.0	-
T09.108	RA	61.7	81.5	70.8	0.7	0.1	34.5	0.8	34.6	3.4	4.3	-
T09.124	RA	55.1	72.3	60.8	0.6	0.0	20.6	0.6	20.6	6.2	10.2	-
T09.145	RA	63.0	77.8	31.3	0.3	0.0	10.1	0.3	10.1	5.4	16.1	-
T10.049	RA	54.1	73.5	63.6	0.8	0.1	26.6	0.9	26.7	6.3	7.3	-
T10.058	RA	57.7	86.0	51.4	0.6	0.0	11.7	0.6	11.7	4.1	6.9	-
T10.084	RA	58.2	85.1	45.6	1.9	0.5	15.7	2.3	16.2	3.9	1.7	29.9
T10.118	RA	61.5	60.5	63.1	0.3	0.0	26.2	0.3	26.2	4.4	14.6	-
T10.131	RA	67.5	60.9	36.5	0.1	0.0	10.4	0.2	10.4	2.4	13.1	20.2
T08.179	Non-RA persistent	59.5	80.6	42.6	0.3	0.1	26.8	0.3	26.9	-	-	-
T08.182	Non-RA persistent	44.8	54.9	50.4	0.8	0.3	24.1	1.1	24.4	-	-	-
T08.203	Non-RA persistent	68.1	66.4	38.4	1.5	0.4	20.0	1.9	20.4	4.1	2.2	-
T09.060	Non-RA persistent	63.7	63.6	58.9	0.2	0.0	5.1	0.2	5.1	6.9	33.9	-
T09.136	Non-RA persistent	52.0	73.9	63.5	0.6	0.1	28.6	0.7	28.7	8.4	11.5	-
T10.126	Non-RA persistent	38.6	58.8	49.4	0.5	0.1	22.0	0.6	22.1	11.3	19.8	56.8
T08.147	Self-limiting	67.3	62.9	37.6	0.5	0.1	20.5	0.6	20.6	-	-	-
T08.162	Self-limiting	66.8	66.5	33.5	0.3	0.1	12.8	0.4	12.9	-	-	-
T08.170	Self-limiting	74.3	56.3	55.5	0.5	0.1	28.2	0.7	28.3	-	-	-
T08.183	Self-limiting	58.5	71.4	50.2	1.8	0.5	23.1	2.4	23.6	-	-	-

Patient code	Disease outcome	$CD3^+$ (%)	$CD4^+$ (% of $CD3^+$)	$CD45\text{-}RO^+$ (% of $CD3^+$)	$IL17^+$ $IFN\gamma^-$ (%)	$IL\text{-}17^-$ $IFN\gamma^+$ (%)	$IL\text{-}17^-$ $IFN\gamma^+$ (%)	$IL\text{-}17^+$ total (%)	$IFN\gamma^+$ total (%)	T_{Reg} (%)	T_{Reg} /T17 ratio	CTLA4 MFI
T08.186	Self-limiting	82.2	77.2	38.0	0.6	0.1	19.0	0.7	19.1	-	-	-
T09.103	Self-limiting	57.1	69.7	70.8	1.1	0.2	22.7	1.2	22.9	5.3	4.3	-
T09.114	Self-limiting	56.6	67.2	52.8	0.5	0.1	21.8	0.6	21.9	2.8	4.8	-
T09.133	Self-limiting	57.7	58.7	57.8	0.7	0.1	24.8	0.7	24.9	11.5	15.8	-
T09.141	Self-limiting	56.5	66.5	82.2	0.4	0.0	31.1	0.5	31.1	7.2	15.1	-
T09.146	Self-limiting	59.3	65.4	40.5	1.1	0.2	15.1	1.3	15.3	5.2	4.0	-
T09.153	Self-limiting	69.0	57.0	66.9	1.0	0.2	27.5	1.3	27.7	6.8	5.3	-
T09.160	Self-limiting	76.0	85.2	14.8	0.9	0.1	14.0	1.1	14.1	4.5	4.3	-
T10.007	Self-limiting	56.7	55.6	53.7	1.1	0.2	28.5	1.3	28.7	5.4	4.1	-
T10.027	Self-limiting	72.2	62.1	53.1	1.2	0.2	24.5	1.4	24.7	5.8	4.2	25.3
T10.043	Self-limiting	61.3	79.5	50.9	0.6	0.1	19.4	0.7	19.5	3.8	5.3	40.1
T10.113	Self-limiting	53.4	72.8	49.4	1.1	0.3	21.6	1.4	21.9	3.8	2.8	35.7
T08.169	Not known	66.1	53.2	38.1	0.6	0.1	33.2	0.7	33.3	-	-	-
T09.067	Not known	80.5	78.9	34.9	1.2	0.2	14.9	1.4	15.1	1.4	1.0	-
T09.093	Not known	48.2	68.1	45.7	0.3	0.0	23.0	0.3	23.0	3.4	10.9	-
T09.142	Not known	58.3	59.6	65.3	1.4	0.8	24.2	2.3	25.0	5.0	2.2	-
T10.112	Not known	55.1	66.7	38.6	0.5	0.3	18.2	0.8	18.5	3.7	4.6	34.8
T10.127	Not known	70.9	72.2	27.4	0.2	0.0	11.3	0.3	11.3	3.5	13.1	36.7
T10.138	Not known	60.8	69.4	48.8	0.4	0.0	12.4	0.4	12.4	4.3	10.1	60.7
T10.140	Not known	29.6	74.9	54.0	1.2	0.2	17.8	1.4	18.0	13.6	9.9	37.0
T10.141	Not known	64.2	77.7	27.6	1.0	0.2	11.7	1.1	11.9	-	-	-
T10.142	Not known	68.1	74.0	80.5	0.9	0.3	34.7	1.2	35.0	-	-	-
T09.104	Established RA	50.7	67.2	89.1	0.8	0.3	28.6	1.1	28.9	11.5	10.7	-
T09.132	Established RA	55.7	53.7	85.7	0.5	0.1	32.3	0.6	32.4	14.8	23.1	-
T09.166	Established RA	67.3	68.8	44.1	0.5	0.1	25.5	0.6	25.6	5.6	9.4	-
T10.010	Established RA	50.6	73.0	52.6	0.5	0.2	17.5	0.7	17.7	4.5	6.8	-
T10.029	Established RA	63.7	63.8	62.4	0.7	0.1	28.2	0.8	28.3	4.9	6.0	31.7

Patient code	Disease outcome	$CD3^+$ (%)	$CD4^+$ (% of $CD3^+$)	$CD45\text{-}RO^+$ (% of $CD3^+$)	$IL17^+$ $IFN\gamma^-$ (%)	$IL\text{-}17^-$ $IFN\gamma^+$ (%)	$IL\text{-}17^-$ $IFN\gamma^+$ (%)	$IL\text{-}17^+$ total (%)	$IFN\gamma^+$ total (%)	T_{Reg} (%)	T_{Reg} /T17 ratio	CTLA4 MFI
T10.033	Established RA	68.9	58.5	48.9	0.6	0.3	22.1	0.9	22.4	3.8	4.3	41.0
T10.114	Established RA	52.0	70.1	54.0	0.2	0.0	22.9	0.2	22.9	4.9	20.1	28.4
T10.128	Established RA	78.6	64.9	45.8	0.4	0.0	22.4	0.5	22.4	4.4	9.4	41.3
T10.139	Established RA	72.4	79.8	35.7	0.7	0.2	35.1	0.8	35.3	-	-	-
T10.146	Established RA	49.9	50.0	68.4	0.3	0.1	38.9	0.4	39.0	13.3	35.0	24.8
T09.137	Healthy control	55.9	48.9	59.3	0.7	0.2	29.0	0.9	29.2	9.5	10.1	-
T09.138	Healthy control	60.7	67.0	58.0	0.6	0.1	27.5	0.8	27.6	3.1	4.1	-
T09.139	Healthy control	56.1	56.0	54.2	0.4	0.1	23.0	0.6	23.1	4.4	7.7	-

Table 9-3. IL-17 and IFNγ expression by peripheral blood T cells from synovitis patients and controls post seven-day stimulation in the presence or absence of $1,25(OH)_2D_3$ (D_3). (- indicates that data could not be collected).

Patient code	Disease outcome	IL-17$^+$ IFNγ$^-$ (%)	IL-17$^+$ IFNγ$^-$ (D_3) (%)	IL-17$^+$ IFNγ$^+$ (%)	IL-17$^+$ IFNγ$^+$ (D_3) (%)	IL-17 IFNγ$^+$ (%)	IL-17$^-$ IFNγ$^+$ (D_3) (%)	IL-17$^+$ total (%)	IL-17$^+$ total (D_3) (%)	IFNγ$^+$ total (%)	IFNγ$^+$ total (D_3) (%)
T08.187	RA	4.0	2.0	7.0	1.9	39.6	19.1	11.0	4.0	46.6	21.0
T08.200	RA	22.3	4.1	9.9	1.6	12.1	12.9	32.2	5.6	22.0	14.5
T09.064	RA	3.7	1.9	2.1	0.1	37.2	11.3	5.8	2.0	39.3	11.4
T09.090	RA	14.6	2.4	11.5	0.7	12.9	11.5	26.1	3.2	24.4	12.2
T09.108	RA	6.5	1.0	6.2	0.2	35.3	18.9	12.7	1.2	41.5	19.1
T09.124	RA	-	-	-	-	-	-	-	-	-	-
T09.145	RA	10.0	0.5	2.6	0.0	7.9	2.9	12.6	0.5	10.5	2.9
T10.049	RA	12.6	0.2	4.9	0.0	28.1	15.2	17.5	0.2	33.0	15.2
T10.058	RA	18.1	2.0	7.1	0.1	14.5	7.2	25.2	2.1	21.6	7.3
T10.084	RA	21.4	0.9	11.6	0.1	10.4	14.3	33.0	1.1	22.0	14.4
T10.118	RA	4.9	1.8	5.0	0.8	29.5	27.4	9.9	2.6	34.5	28.2
T10.131	RA	-	-	-	-	-	-	-	-	-	-
T08.179	Non-RA persistent	8.7	1.1	6.8	0.2	33.7	19.8	15.5	1.4	40.5	20.0
T08.182	Non-RA persistent	4.2	14.4	10.6	21.1	48.9	16.3	14.8	35.5	59.5	37.4
T08.203	Non-RA persistent	21.1	11.7	26.2	7.1	23.3	19.4	47.3	18.8	49.5	26.5
T09.060	Non-RA persistent	22.3	7.3	17.8	2.5	22.1	16.2	40.1	9.8	39.9	18.7
T09.136	Non-RA persistent	7.5	3.0	10.6	1.5	36.7	32.8	18.1	4.5	47.3	34.3
T10.126	Non-RA persistent	11.3	1.4	2.5	0.2	11.1	13.9	13.8	1.5	13.6	14.1
T08.147	Self-limiting	2.7	0.1	2.1	0.0	13.6	17.0	4.8	0.1	15.7	17.0
T08.162	Self-limiting	10.1	0.7	10.0	0.2	17.0	8.8	20.1	0.9	27.0	9.0
T08.170	Self-limiting	8.7	8.7	6.4	4.3	40.6	27.2	15.1	12.9	47.0	31.5
T08.183	Self-limiting	5.5	5.0	6.6	4.1	30.4	26.7	12.1	9.1	37.0	30.8

Patient code	Disease outcome	IL-17$^+$ IFNγ$^-$ (%)	IL-17$^+$ IFNγ$^-$ (D_3) (%)	IL-17$^+$ IFNγ$^+$ (%)	IL-17$^+$ IFNγ$^+$ (D_3) (%)	IL-17 IFNγ$^+$ (%)	IL-17$^-$ IFNγ$^+$ (D_3) (%)	IL-17$^+$ total (%)	IL-17$^+$ total (D_3) (%)	IFNγ$^+$ total (%)	IFNγ$^+$ total (D_3) (%)
T08.186	Self-limiting	-	-	-	-	-	-	-	-	-	-
T09.103	Self-limiting	16.2	2.2	13.1	0.4	26.4	19.3	29.3	2.6	39.5	19.7
T09.114	Self-limiting	12.6	0.5	10.2	0.1	17.8	22.9	22.8	0.7	28.0	23.0
T09.133	Self-limiting	16.2	1.7	8.3	0.4	16.9	10.2	24.5	2.2	25.2	10.6
T09.141	Self-limiting	6.1	4.9	6.9	1.9	43.8	24.8	13.0	6.7	50.7	26.7
T09.146	Self-limiting	15.9	0.6	7.8	0.1	9.7	11.0	23.7	0.8	17.5	11.1
T09.153	Self-limiting	-	-	-	-	-	-	-	-	-	-
T09.160	Self-limiting	10.2	5.7	8.5	2.1	25.2	13.4	18.7	7.8	33.7	15.5
T10.007	Self-limiting	8.9	4.9	2.6	1.1	20.2	17.2	11.5	6.0	22.8	18.3
T10.027	Self-limiting	7.3	1.9	6.1	0.9	22.5	13.4	13.4	2.8	28.6	14.3
T10.043	Self-limiting	7.5	1.0	10.4	0.1	31.8	12.8	17.9	1.1	42.2	12.9
T10.113	Self-limiting	11.4	1.6	18.4	1.1	22.5	21.2	29.8	2.7	40.9	22.3
T08.169	Not known	-	-	-	-	-	-	-	-	-	-
T09.067	Not known	-	-	-	-	-	-	-	-	-	-
T09.093	Not known	2.1	0.8	4.8	0.4	69.6	39.6	6.9	1.2	74.4	40.0
T09.142	Not known	16.6	2.1	17.3	0.8	12.5	16.1	33.9	2.9	29.8	16.9
T10.112	Not known	1.0	0.0	0.8	0.0	9.3	8.4	1.8	0.0	10.1	8.4
T10.127	Not known	1.0	0.2	0.9	0.0	19.4	4.3	1.9	0.2	20.3	4.3
T10.138	Not known	2.5	0.9	4.5	0.5	28.0	11.8	7.0	1.4	32.5	12.3
T10.140	Not known	12.2	1.3	6.7	0.3	13.6	11.3	18.9	1.6	20.3	11.6
T10.141	Not known	13.0	5.0	15.5	2.6	17.7	17.0	28.5	7.6	33.2	19.6
T10.142	Not known	2.4	2.9	4.3	1.8	36.6	25.6	6.7	4.7	40.9	27.4
T09.104	Established RA	8.2	0.4	3.8	0.0	23.7	21.9	12.0	0.4	27.5	21.9
T09.132	Established RA	18.2	3.3	13.2	0.8	28.5	28.8	31.4	4.0	41.7	29.6
T09.166	Established RA	8.1	4.0	12.4	3.0	21.3	18.0	20.5	7.0	33.7	21.0
T10.010	Established RA	10.7	0.6	12.7	0.3	17.8	21.7	23.4	0.9	30.5	22.0

Patient code	Disease outcome	IL-17^{+} IFNγ^{-} (%)	IL-17^{+} IFNγ^{-} (D_3) (%)	IL-17^{+} IFNγ^{+} (%)	IL-17^{+} IFNγ^{+} (D_3) (%)	IL-17 IFNγ^{+} (%)	IL-17^{-} IFNγ^{+} (D_3) (%)	IL-17^{+} total (%)	IL-17^{+} total (D_3) (%)	IFNγ^{+} total (%)	IFNγ^{+} total (D_3) (%)
T10.029	Established RA	0.4	0.3	1.6	0.3	43.7	23.4	1.9	0.6	45.3	23.7
T10.033	Established RA	3.1	1.7	12.0	2.6	37.4	24.3	15.1	4.4	49.4	26.9
T10.114	Established RA	-	-	-	-	-	-	-	-	-	-
T10.128	Established RA	0.9	0.5	3.0	0.5	22.6	17.5	3.9	1.0	25.6	18.0
T10.139	Established RA	6.1	1.1	6.7	0.4	27.7	13.7	12.8	1.5	34.4	14.1
T10.146	Established RA	0.9	0.8	0.9	0.3	23.3	19.4	1.8	1.1	24.2	19.7
T09.137	Healthy control	7.3	0.4	6.1	0.1	26.5	25.9	13.4	0.5	32.6	26.0
T09.138	Healthy control	6.4	0.3	9.8	0.1	34.4	30.1	16.2	0.3	44.2	30.2
T09.139	Healthy control	-	-	-	-	-	-	-	-	-	-

Table 9-4. ***Ex-vivo*** **data for synovial fluid T cells from the joints of synovitis patients. (- indicates that data could not be collected).**

Patient code	Disease outcome	$CD3^+$ (%)	$CD4^+$ (% of $CD3^+$)	$CD45\text{-}RO^+$ (% of $CD3^+$)	$IL\text{-}17^+$ $IFN\gamma^-$ (%)	$IL\text{-}17^-$ $IFN\gamma^+$ (%)	$IL\text{-}17^-$ $IFN\gamma^+$ (%)	$IL\text{-}17^+$ total (%)	$IFN\gamma^+$ total (%)	T_{Reg} (%)	T_{Reg}/T17
T08.187	RA	-	-	-	0.1	0.3	82.2	0.4	82.5	-	-
T09.065	RA	-	-	-	0.3	0.4	78.3	0.7	78.7	12.0	17.1
T09.092	RA	64.4	62.6	98.4	0.8	0.8	61.5	1.6	62.3	13.7	8.5
T10.084	RA	-	-	-	1.9	1.2	44.5	3.1	45.7	27.6	9.0
T08.203	Non-RA persistent	87.3	54.4	100.0	1.6	3.8	78.7	5.3	82.5	9.4	1.8
T10.126	Non-RA persistent	58.7	55.3	99.3	0.9	3.1	74.5	4.0	77.6	9.5	2.4
T08.162	Self-limiting	-	-	-	0.2	0.4	67.6	0.6	68.0	-	-
T08.170	Self-limiting	73.3	33.8	97.8	0.1	0.8	79.2	0.9	80.0	-	-
T08.183	Self-limiting	-	-	-	1.0	11.8	62.1	12.8	73.9	-	-
T09.106	Self-limiting	-	-	-	3.2	3.6	81.2	6.8	84.8	20.8	3.1
T09.141	Self-limiting	-	-	-	-	-	-	-	-	-	-
T09.146	Self-limiting	-	-	-	1.3	0.7	59.5	2.0	60.2	14.7	7.4
T10.007	Self-limiting	79.6	42.5	99.6	0.6	0.7	76.7	1.4	77.4	10.7	7.9
T10.113	Self-limiting	52.8	41.7	98.8	1.9	3.7	80.7	5.5	84.4	3.9	0.7
T10.114	Established RA	45.7	54.6	97.7	0.2	0.0	57.8	0.2	57.8	13.0	59.1
T10.146	Established RA	86.7	48.5	99.6	0.5	1.0	76.6	1.5	77.6	9.7	6.5

Table 9-5. IL-17 and IFNγ expression by synovial fluid T cells from the joints of synovitis patients post seven-day stimulation in the presence or absence of $1,25(OH)_2D_3$ (D_3). (- indicates that data could not be collected)

Patient code	Disease outcome	$IL\text{-}17^+$ $IFN\gamma^-$ (%)	$IL\text{-}17^+$ $IFN\gamma^-$ (D_3) (%)	$IL\text{-}17^+$ $IFN\gamma^+$ (%)	$IL\text{-}17^+$ $IFN\gamma^+$ (D_3) (%)	$IL\text{-}17^-$ $IFN\gamma^+$ (%)	$IL\text{-}17^-$ $IFN\gamma^+$ (D_3) (%)	$IL\text{-}17^+$ Total (%)	$IL\text{-}17^+$ total (D_3) (%)	$IFN\gamma^+$ total (%)	$IFN\gamma^+$ total (D_3) (%)
T08.187	RA	-	-	-	-	-	-	-	-	-	-
T09.065	RA	-	-	-	-	-	-	-	-	-	-
T09.092	RA	2.5	2.0	11.0	5.3	75.1	72.5	13.5	7.3	86.1	77.8
T10.084	RA	1.8	1.2	3.4	1.1	65.0	57.1	5.2	2.3	68.4	58.2
T08.203	Non-RA persistent	4.7	2.8	34.5	15.9	55.0	73.0	39.2	18.7	89.5	88.9
T10.126	Non-RA persistent	1.1	1.0	2.2	1.3	55.2	60.1	3.4	2.3	57.4	61.4
T08.162	Self-limiting	-	-	-	-	-	-	-	-	-	-
T08.170	Self-limiting	0.3	0.4	4.4	1.9	78.6	90.1	4.7	2.4	83.0	92.0
T08.183	Self-limiting										
T09.106	Self-limiting	11.4		19.3		55.9		30.7		75.2	
T09.141	Self-limiting	-	-	-	-	-	-	-	-	-	-
T09.146	Self-limiting	-	-	-	-	-	-	-	-	-	-
T10.007	Self-limiting	-	-	-	-	-	-	-	-	-	-
T10.113	Self-limiting	5.9	5.0	24.9	11.1	55.6	64.2	30.8	16.1	80.5	75.3
T10.114	Established RA	-	-	-	-	-	-	-	-	-	-
T10.146	Established RA	2.9	2.2	7.2	3.8	55.0	62.1	10.0	6.0	62.2	65.9

10 REFERENCES

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11 PUBLICATIONS

Papers arising directly from this thesis and published prior to its completion include:

Jeffery, L.E., Burke, F., Mura, M., Zheng, Y., Qureshi, O.S., Hewison, M., Walker, L.S., Lammas, D.A., Raza, K., and Sansom, D.M. (2009). 1,25-Dihydroxyvitamin D(3) and IL-2 combine to inhibit T cell production of inflammatory cytokines and promote development of regulatory T cells expressing CTLA-4 and FoxP3. JImmunol *183*, 5458-5467