The Role of Th17 cells in Juvenile Idiopathic Arthritis

Kiran Nistala

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Rheumatology Unit,
UCL, Institute of Child Health,
London, United Kingdom

Abstract

Autoimmune arthritis in childhood, know as juvenile idiopathic arthritis (JIA), has a heterogeneity, ranging from monoarthritis to recalcitrant polyarthritis, making it a model disease in which to study immuno-regulation. Regulatory T cells, key players in peripheral immune homeostasis are enriched in the joints of JIA patients, particularly those with mild arthritis. To test the factors that lead to this enrichment, Treg trafficking was examined in the context of JIA. Synovial Treg showed enhanced chemotaxis to the inflammatory chemokine CCL5, widely detectable within the joint, when compared to Treg from peripheral blood. The trafficking of a related, but highly inflammatory T cell subset, Th17 cells, was also investigated. Th17 cells play a dominant role in murine models of arthritis, yet their contribution to human disease is unknown. The data presented here, showed that IL-17+ CD4 T cells were enriched in the joints of JIA patients, by a CCR6 dependent mechanism and importantly, their frequency correlated with the severity of disease course.

The majority of synovial IL-17+ CD4 T cells expressed a cytokine and chemokine receptor phenotype intermediate between Th17 and Th1. Here it was shown that these cells (Th17/1) expressed high levels of both Th17 and Th1 specific transcription factors, RORC2 and T-bet. Modelling the generation of Th17/1 *in vitro,* Th17 cells 'converted' to Th17/1 under conditions which mimicked the disease site, namely low TGFβ and high IL-12 levels. Using CD161, a human Th17 marker, it was shown that synovial Th17/1 cells, and unexpectedly, a large proportion of Th1 cells expressed CD161. This study provided evidence to support a Th17 origin for Th1 cells expressing CD161. *In vitro,* Th17 cells which converted to a Th1 phenotype maintained CD161 expression, whilst in the joint CD161+ Th1 cells shared features with Th17 cells, with shared T cell receptor clonality and expression of RORC2, although they were IL-17 negative. We propose that Th17 cells may 'convert' to Th17/1 and Th1 cells in human arthritis. Therefore therapies targeting the induction of Th17 cells could also attenuate the Th17/1 and Th1 effector populations within the inflamed joint.

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Abbreviations

Ab	Antibody
ANA	Anti Nuclear Antibody
APC	Antigen Presenting Cell
BSA	Bovine Serum Albumin
CCL	CC Chemokine Ligand
CCR	CC Chemokine Receptor
CD	Cluster of Differentiation
CDR	Complimentarity Determining Region
CFA	Complete Freud's Adjuvant
CFSE	Carboxyfluorescein Diacetate Succinimidyl Ester
CIA	Collagen Induced Arthritis
CTL	Cytotoxic T Lymphocyte
CTLA-4	Cytotoxic T Lymphocyte Antigen 4
CXCL	CXC Ligand
DC	Dendritic Cell
DMSO	Dimethylsulphoxide
EAE	Experimental Autoimmune Encephalitis
FACS	Fluorescence Activated Cell Sorting
FCS	Foetal Calf Serum
FITC	Fluorescein Isothiocyanate
FOXP3	Forkhead box P3
g	Gravity
G-CSF	Granulocyte Colony Stimulating Factor
GFP	Green Florescent Protein
GITR	Glucocorticoid-Induced TNF-Receptor-Related Protein
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
HLA	Human Leukocyte Antigen

IBD	Inflammatory Bowel Disease
IFNγ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
ILAR	International League Against Rheumatism
IPEX	Immune dysregulation Polyendocrinopathy, Enteropathy X-linked syndrome
JIA	Juvenile Idiopathic Arthritis
LPS	Lipopolysaccharide
mDC	Myeloid Dendritic Cell
MFI	Median Fluorescence Intensity
MHC	Major Histocompatibility Complex
MS	Multiple Sclerosis
NK	Natural Killer
p	Probability
P/S/glu	Penicillin/ Streptomycin/ glutamine
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
pDC	Plasmacytoid Dendritic Cell
PE	R-Phycoerythrin
PeCy5	R-phycoerythrin-cyanine 5
PerCP	Peridinin Chlorophyll Protein
PFA	Paraformaldehyde
PHA	Phytohaemagglutinin
RA	Rheumatoid Arthritis
RF	Rheumatoid Factor
RORC2	Retinoic Acid Related Orphan Receptor C variant 2
RORγt	Retinoic Acid Related Orphan Receptor gamma t
SF	Synovial Fluid
SFMC	Synovial Fluid Mononuclear cells
SJIA	Systemic Juvenile Idiopathic Arthritis

STAT	Signal Transducer and Activator of Transcription
TdT	Terminal deoxynucleotidyl Transferase
TGF-β	Tumour Growth Factor beta
Th	T helper
Th1	T helper type 1 cell
Th2	T helper type 2 cell
Th17	T helper type 17 cell
TNFα	Tumour Necrosis Factor alpha
TR	T Cell Receptor
Treg	T regulatory cell

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Declaration

I, Kiran Nistala, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

1：Introduction

1.1 The immune system

The vertebrate immune system provides the necessary protection against invasion by a vast and unpredictable number of pathogens. The co-ordination of innate and adaptive immune responses gives rise to a highly sophisticated and robust defence network, which can fight new infection and subsequently protect against re-infection throughout life. Innate immunity relies on the function of non-immune cells (e.g. epithelia) and leukocytes such as granulocytes and macrophages, and provides an early but rapid defence against common microorganisms. Innate responses initiate an attack against an invading pathogen by recognition of common cell-surface motifs, resulting in phagocytosis of the pathogen, cytokine release and local inflammation. Encapsulated bacteria, viruses and rapidly evolving microorganisms may escape detection or overwhelm the innate defences, and a further level of protection is then required. Adaptive immunity has evolved to produce an intricate armoury of cells capable of mounting highly specific primary and recall responses. These responses are slower than those of the innate immune system, but are more versatile and specialised. Lymphocytes developing in the bone marrow (B cells) and thymus (T cells) rearrange heterogeneous repertoires of antigen receptors, which provide the exquisite specificity and considerable diversity required for recognition of numerous potential antigens. The cornerstones of adaptive immunity: specificity, adaptability and memory, all rely on the formation and maintenance of functional lymphocyte repertoires.

1.2 T cell subsets

T Lymphocytes make up a significant portion of the immune mechanisms that maintain host defence, acting either directly as effector cells or indirectly through their interaction with other cell lineages. T cell precursors arise in the bone marrow and mature in the thymus (Miller 1961). Different T cell subsets can be defined by the antigen receptor present on the cell surface. T cell receptors (TR, abbreviation nomenclature based on international immunogenetic information system, http://imgt.cines.fr) are glycoprotein heterodimers of αβ or γδ chains. Conventional αβ T cells constitute the largest proportion of the T cell compartment in humans. After maturation, peripheral T cells

express either CD4 or CD8 surface molecules. CD8 T cells, also known as cytotoxic T lymphocytes (CTL), recognize and kill virus-infected or abnormal cells, such as cancerous cells (Colombo *et al*, 1983). CD4+ T cells are known as helper T cells (T_h) which provide T cell "help" largely by secretion of cytokines to drive differentiation of B cells and activate other cells in the immune system. Another subset, γδ T cells, account for around 5% of the T cell compartment, and are thought to bridge the gap between innate and adaptive immunity and regulate the function of conventional T cells (Pennington *et al*, 2005).

1.2.1 T cell recognition of antigen

Conventional T cells recognise protein antigen in the form of short peptides, using the cell surface-expressed TR. Recognition of peptide can only occur if the antigen is correctly processed and presented in a major histocompatibility complex (MHC) molecule (Babbitt *et al*, 1985; Zinkernagel & Doherty 1974). The ligand for the TR is the peptide/MHC complex (p/MHC), and T cells are said to be MHC-restricted following maturation in the thymus. It has long been established that CD8+ T cells recognise antigen in the context of MHC Class I molecules, and CD4+ cells in the context of Class II (Swain 1983). Antigen presenting cells (APC) display MHC molecules, which present antigen to T cells. Most nucleated cells express MHC Class I molecules, but only B cells, dendritic cells (DC), macrophages and thymic epithelial cells (TEC) constitutively express Class II molecules. In addition to being able to efficiently process and present peptide antigens, these "professional" APC express other key costimulatory molecules (see 1.2.8).

1.2.2 TR gene rearrangement

During development in the thymus, T cells rearrange TR α- and β-chain genes (TRAV, TRBV) during V(D)J recombination, a somatic rearrangement of germline gene segments (Tonegawa *et al*, 1981). This process produces the extreme variability required for the generation of a highly diverse lymphocyte repertoire. During gene rearrangement, which occurs in the developing thymocytes, variable (V), diversity (D, TRB locus only), joining (J) and constant (C) gene segments are brought together in a tightly regulated,

ordered manner. There are several tens of V and J segments encoded in the germline of both A and B loci, two BD gene segments, one or two C segments (Table 1.1). The TRA locus is located on chromosome 14q11 in humans. The TRB locus is located on chromosome 7q34 and incorporates 62 BV segments (including pseudo-genes) and two BDJ clusters, each comprising 1 BD gene, 7 BJ genes and 1 BC gene segment.

Table 1.1 Gene segments at TRAV and TRBV loci

TR Locus	Variable	Diversity	Joining	Constant
Alpha	54	0	61	1
Beta	62	2	14	2

Variable, diversity, joining and constant TR gene segments at human TRA and TRB loci (http://imgt.cines.fr, accessed 15/11/2010).

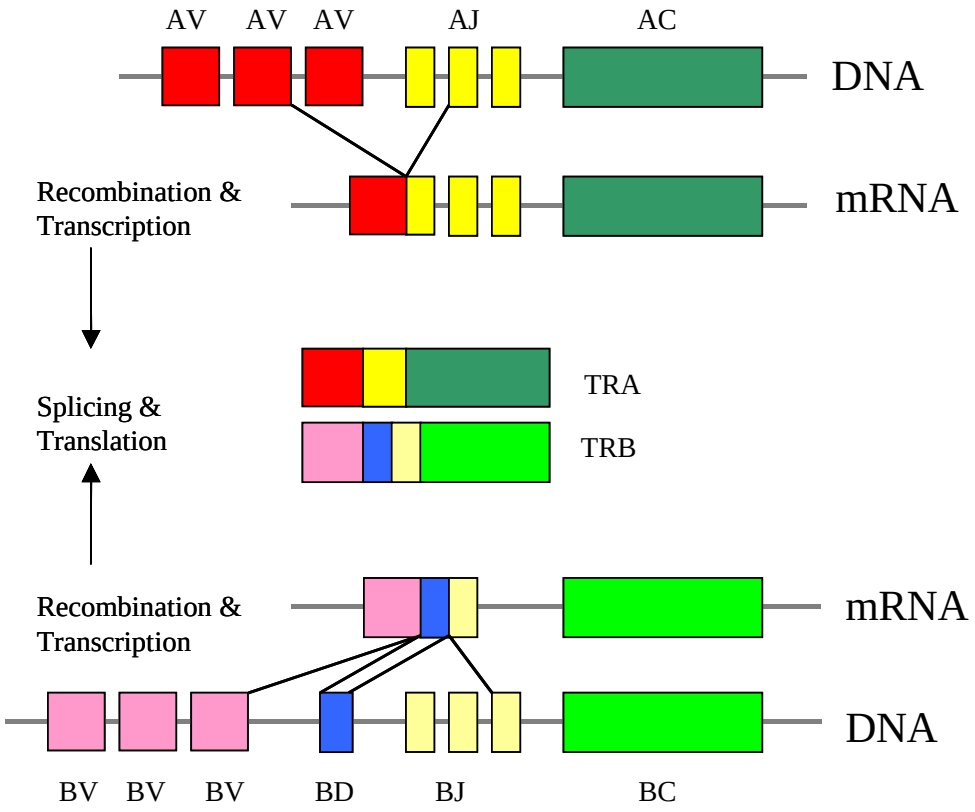


Figure 1.1 Schematic representation of VDJ recombination at TRA and B loci.

The germline TRA and B loci consist of several variable (V) and joining (J) gene segments, and additional diversity (D) segments at the β locus. Only a few gene segments are shown for clarity. At the TRA locus, a V segment to J gene segment recombination occurs. At the TRB locus, initially there is a D segment to J segment rearrangement, followed by a D-J to V segment recombination. The V(D)J product is than transcribed and spliced to a constant (C) segment, which is translated and expressed on the cell surface. Adapted from Janeway (Janeway *et al*, 2005).

1.2.3 V(D)J recombination

V(D)J recombination is catalysed by the recombinase activating gene products, RAG1 and RAG2 (Oettinger *et al*, 1990). The targeted deletion of *RAG* genes caused complete combined immunodeficiency (Shinkai *et al*, 1992), illustrating the necessity for gene rearrangement in developing thymocytes. RAG proteins cause DNA double-strand breaks between the V, (D) and J coding sequences (McBlane *et al*, 1995) which can

cause imprecise joining, resulting in the addition or deletion of template nucleotides. This mechanism is further regulated at the TRB locus, as rearrangement usually operates in a sequential manner. The first recombination is between a D segment and a J gene segment. The D-J join is then recombined to a V segment (Born *et al,* 1985), thus avoiding splicing of V segment to J segment, which would cause omission of a D segment. In many ways V(D)J recombination is a remarkably dangerous process – the generation of double-strand breaks and seemingly random rearrangements of genes could lead to genetic instability and malignant change. The activity of the RAG proteins is therefore under strict regulation and several epigenetic mechanisms have been proposed to control this (Jung & Alt 2004).

1.2.4 Addition of non-template nucleotides

Another enzyme, the DNA polymerase terminal deoxynucleotidyl transferase (TdT) is also important in the formation of the T cell repertoire (Alt *et al,* 1992; Komori *et al,* 1993). Tdt adds non-template nucleotides (N-nucleotides) to the 3' ends of the gene segments to further enhance diversity, contributing particularly toward the sequence which codes for the third complimentarity determining region (CDR3) loop of the TR chain. CDR3 loops are the hypervariable regions of TR and are implicated in the contact of antigenic peptide (Danska *et al,* 1990).

1.2.5 TR structure and complex with p/MHC

The surface T cell receptor enables recognition of antigen by T cells (Davis & Bjorkman 1988). TR is structurally very similar to the Fab fragment of the immunoglobulin molecule, consisting of an α and β chains linked by a disulphide bridge. $\gamma\delta$ T cells bear an alternative receptor made up of γ and δ chains. Both chains of the TR have a amino-terminal variable (V) region and a constant (C) region, each with a carbohydrate moiety attached (Davis & Bjorkman 1988). Adjacent to the transmembrane domain is a short hinge region containing a cysteine residue that forms the inter-chain disulphide bond.

The full cloning and crystallisation of both human and murine TR molecules has facilitated a detailed understanding of the interaction of TR with its ligand, p/MHC (Fossiez *et al,* 1996).

TR interacts with its ligand (p/MHC) via the V domains, and the 3 CDR in each variable domain give rise to “loops” by which the TR primarily contacts the p/MHC complex. The CDR1 and CDR2 loops are encoded in the variable gene segment, whereas the CDR3 loop derives from the junction of the V, (D) and J gene segments, contain N-nucleotides and are therefore hypervariable (Davis *et al,* 1998). Both CDR3α and β loops contact peptide antigen, and as such control the specificity of the TR. At the cell membrane, the αβTR is associated with four other protein subunits, collectively named CD3. CD3ε, δ and γ chains are membrane bound in close proximity to the TR, whereas the CD3ζ homodimer is largely located in the cytosol near the intracellular tail of the TR (Malissen *et al,* 1999). Expression of the CD3 complex is required for TR expression and signalling. (Kuhns *et al,* 2006).

1.2.6 T cell selection and maturation in the thymus

The thymus is required for the production of a functional and appropriately restricted T cell repertoire. This process, known as T cell selection, is vital for ensuring that the T cell repertoire can both bind to self-MHC molecules whilst still maintaining tolerance to self antigens (central tolerance) (Zinkernagel *et al,* 1978). T cell selection in the thymus has two stages, firstly positive selection, by which anti-apoptotic signals are delivered by the thymic epithelium to the T cells that bind to self-MHC molecules, preventing T cell death. The subsequent stages allow negative selection, which eliminates thymocytes bearing high affinity self-reactive TR, so called clonal deletion of self reactive T cells (von Boehmer *et al,* 2003). Fewer than 5% of thymocytes survive selection and exit the thymus to join the circulating naive T cell repertoire (Surh & Sprent 1994).

1.2.7 Naïve T cells

T cells leave the thymus after completing their development and enter the bloodstream to circulate to the peripheral lymphoid organs. T cells that have not met their specific antigen target are referred to as naïve T cells. For activation, a naïve T cell must recognise a foreign protein bound to self-MHC molecule, with simultaneous co-stimulatory signals. Typically naïve T cells do not get activated in the inflamed or infected organ but in peripheral lymphoid structures (von Andrian & Mempel 2003).

Lymph drains the infected organ to the lymphoid tissue containing mature APC which capture, process and then present the antigen bound to self-MHC. Naïve T cells continuously circulate from the bloodstream to lymphoid organs, guided by expression of L-selectin which binds vascular addressins, CD34 and GlyCAM-1 present on high endothelial venules in lymph nodes (Spertini *et al,* 1991). Cells then circulate back to the bloodstream, allowing contact with thousands of APC every day.

1.2.8 T cell activation

As naïve T cells migrate through the secondary lymphoid organs they bind transiently to APC, particularly DCs. Binding between TR and MHC-antigen complex, in association with the CD4 co-receptor (for Th cells) is referred to as signal 1, but on its own is insufficient to activate naïve T cells to proliferate and differentiate. Antigen-specific clonal expansion requires a 2^{nd} signal, referred to as co-stimulation, of which the best characterised are the B7 glycoproteins. B7.1 (CD80) and B7.2 (CD86), members of the immunoglobulin superfamily are highly expressed on DC, macrophages and B cells (Yang *et al*, 1988). Binding of the B7 molecules to their receptor, CD28 (expressed on naïve T cells), is essential for clonal expansion (Jenkins *et al,* 1991). Once activated, naïve T cells can sustain the co-stimulatory signal by upregulating a protein, CD40 ligand (CD154) which binds CD40 (expressed on APC) (Gondek *et al,* 2005), leading to increased expression of B7 molecules on the APC. Immediately following recognition of antigen, gross changes occur in T cell morphology which leads to flattening of the T cell against the APC (Delon *et al,* 1998) and the organisation of a peripheral ring of adhesion molecules surrounding a central TR-p/MHC interaction (Grakoui *et al,* 1999). This structure is termed the "immunological synapse" and facilitates adhesion and communication between APC and T cell. Ligation of the TR, leads to signalling molecule recruitment via CD3 (Samelson & Klausner 1992) and ultimately to induction of transcription factors including Nuclear factor of activated T-cells (NFAT) and transcription of interleukin-2 (IL-2) (Ruff & Leach 1995). Following co-stimulation, the alpha chain of the IL-2 receptor (IL-2Rα or CD25) is synthesised. Although resting T cells express the β and γ chain of the IL-2R, these only bind IL-2 with a moderate affinity. The association with CD25 creates a IL-2 receptor with higher affinity, allowing

T cells to proliferate in response to much lower concentrations of IL-2 (Grant *et al,* 1992). CD28 ligation leads to activation of the transcription factors AP-1, and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) which also increase IL-2 secretion (Piccolella *et al,* 2003; Raab *et al,* 2001). The proliferation and differentiation of naïve T cells is dependent on IL-2, which highlights the importance of the 2nd signal in promoting adaptive immunity. TR signals, in the absence of co-stimulation, may lead naïve T cells to become inactivated and fails to produce IL-2, a state known as "anergy" (Schwartz 1990). There is one additional receptor for the B7 molecules, Cytotoxic T-Lymphocyte Antigen-4 (CTLA-4), which closely resembles the structure of CD28, but binds B7 molecules with a 20 fold greater avidity (Linsley *et al,* 1991). Binding of CTLA-4 to B7 delivers an inhibitory signal to the activated T cells, making them less responsive to stimulation and also limiting secretion of the autocrine growth factor IL-2 (Walunas *et al,* 1994).

1.2.9 Memory T cells

Following the proliferative phase induced by IL-2, activated T cells differentiate into effector T cells, which then have a lower threshold to respond to antigen-MHC complex without a second signal. Maturation to memory T cells, involves loss of L-selectin expression, preventing re-circulation through lymph nodes. Instead, cells upregulate the integrin $\alpha_4\beta_1$ which allows binding to vascular cell adhesion molecule-4 (VCAM-4) expressed on vascular endothelium at sites of inflammation (Bargatze *et al,* 1995). Finally, mRNA expression of the surface phosphatase CD45 undergoes alternate splicing. Naïve CD4+ T cells express the CD45RA form, whilst memory T cells express the RO isoform which associates with TR and CD4 (Birkeland *et al,* 1989). Sallusto and colleagues proposed distinct memory subsets (Sallusto *et al,* 1999). Central memory T cells express CCR7, and are sensitive to cross linking of TR, rapidly upregulating CD40, but do not secrete large amount of cytokines. Whilst effector memory cells rapidly mature into an effector T cell and secrete large amounts of IFNγ, IL-4 and IL-5 early after re-stimulation. These cells lack CCR7 but express inflammatory chemokine receptors allowing them to rapidly enter inflamed tissues. Neither effector nor central memory T cells are fully committed to particular effector lineages, but following further

stimulation with antigen, changes in the transcriptional programming occure which dictates the specific effector phenotype of the cell.

1.2.10 CD4+ T cell subsets

Early experiments with CD4+ T cells discovered two apparently distinct classes of CD4+ T cells: those that activate macrophages and a separate population that promote B cell activation (Mosmann *et al,* 1986; Street & Mosmann 1991). Cloning of T cells and analysis of secreted cytokines confirmed 2 populations with distinct cytokine secretion profiles, leading Mosmann and colleagues to propose the T helper 1 (Th1) vs. Th2 hypothesis. They postulated that T cells produced distinct patterns of immunity as a result of secreting specific cytokines, in the case of Th1 cells, secreting interferon gamma (IFNγ) leading to delayed type hypersensitivity (Mosmann & Coffman 1989) whilst Th2 cells secreted IL-4 and IL-5 leading to humoral immunity (Mosmann *et al,* 1986). Although committed Th1 and Th2 cells are relatively small populations in normal murine or human peripheral lymphocytes, the use of T cell receptor transgenic murine models provided a large source of naïve T cells with identical antigen specificity that allowed the conditions for T cell differentiation to be studied.

1.2.11 Th1 cell differentiation

Murphy and colleagues studied the induction of Th1 cells by the pathogen *Listeria monocytogenes* and first showed that Th1 polarisation could be partially blocked by neutralisation of IFNγ (Hsieh *et al,* 1993). However IFNγ was not able to induce Th1 cells as efficiently as a macrophage derived factor, later confirmed to be the cytokine IL-12. IL-12 exists a heterodimer of 2 subunits, p40 and p35 (Hsieh *et al,* 1993). Th1 differentiation is initiated by TR signalling in combination with IFNγ mediated Signal transducer and activator of transcription 1 (STAT-1) activation. This leads to the induction of T-bet (Tbx-21), the master transcription factor for Th1 cell differentiation (Szabo *et al,* 2000). T-bet increases expression of the IFNγ gene and upregulates the inducible chain of the IL-12 receptor (IL-12Rβ2), which in concert with constituent IL-12Rβ1 allows IL-12 to signal through STAT-4. Once mature, Th1 cells can then produce

IFNγ through either TR-dependent pathways or in response to cytokines alone (Yang *et al,* 1999), which creates a positive feedback loop for Th1 differentiation.

1.2.12 Th2 differentiation

Th2 differentiation is also initiated with TR signalling but in concert with IL-4 receptor signalling via STAT6. This leads to low level expression of GATA3, the master regulator of Th2 differentiation, which auto-activates its own expression driving epigenetic changes allowing expression of the Th2 cytokine cluster (IL4, IL5 and IL-13) and suppressing expression of Th1 related genes, STAT4 and the IL-12β2 chain (Ouyang *et al,* 1998; Zheng & Flavell 1997; Zhu *et al,* 2004). Therefore early IL-4 signalling serves to stabilise the commitment to a Th2 phenotype whilst reciprocally inhibiting the Th1 pathway.

1.3 Autoimmunity

Autoimmunity may be defined as a response of the immune system to self antigens which leads to tissue damage and disease. As discussed previously, central tolerance of T cells to self antigens is generated within the thymus, however several mechanisms can still bypass central tolerance and lead to autoimmunity: alteration of self antigen (by chemicals or viruses), molecular mimicry, release of sequestered antigens, enhanced Th cell function, inappropriate MHC molecule expression on non-antigen presenting cells, and stimulation of autoreactive B cells. Many of these insults end in a final common pathway that leads to uncontrolled T cell activation and consequent tissue damage.

1.3.1 Models of Th1 mediated pathology

The prevailing paradigm for autoimmune diseases till the turn of the millennium implicated pathogenic Th1 cells as the cause of many autoimmune diseases such as autoimmune arthritis, Inflammatory Bowel Disease (IBD) and Multiple Sclerosis (MS), while Th2 cells were thought to drive atopic diseases such as eczema and asthma (De Carli *et al,* 1994). Collagen induced arthritis (CIA) is a murine model of human autoimmune arthritis, generated by injecting animals with type II collagen mixed with complete Freud's adjuvant (CFA, inactivated mycobacterium emulsified in mineral oil) (Courtenay *et al,* 1980). Transfer of T cells from arthritic animals to syngeneic hosts induces disease, but interestingly so does passive transfer of collagen autoantibodies, implicating both T and B cell components of the adaptive immune response in pathology. When IL-12 is genetically deleted, mice with CIA develop milder disease than their wild type littermates (McIntyre *et al,* 1996). Similar results from a murine model of MS, experimental autoimmune encephalitis were found using an anti-IL-12 antibody (Leonard *et al,* 1995). As IL-12 is critical to the differentiation of Th1 cells, these results were taken as evidence for a Th1 aetiology in CIA as well as EAE. However other data from mice with a genetic deletion of the Th1 cytokine, IFNγ, or the IFNγR demonstrated more severe EAE and CIA than in wild type animals, raising doubts over the Th1 based hypothesis (Ferber *et al,* 1996; Kageyama *et al,* 1998).

1.3.2 Th17 cells – a new T cell subset

The contradictory results between the IL-12 deficient mice with attenuated autoimmune disease and the IFNγ-/- mice with exaggerated pathology, were in part explained by the discovery of an additional IL-12 family member, IL-23. IL-23 shares the p40 subunit with IL-12, but has a unique p19 subunit (Cush *et al,* 1992). Cua and colleagues used mice with the specific IL-23p19 and IL12-p35 genes "knocked out" and tested the mice using the CIA and EAE models. As previously noted, the p40 -/- mice had attenuated disease but surprisingly the IL-12 specific p35 knockout had exaggerated disease (Langrish *et al,* 2005; Murphy *et al,* 2003). Instead it was the p19-/- mice which matched the phenotype of the p40-/- mice. When the cytokine profile of T cells from affected animals was analysed, it was clear that attenuation and accentuation of disease correlated with changes in a population of T cells expressing the cytokine IL-17, so called Th17 cells. IFNγ inhibits the differentiation of Th17 cells (Park *et al*, 2005), and thus removal of this inhibition in p35-/- mice depleted of Th1 cells leads to the secondary expansion of Th17 cells. This results in the exacerbation of arthritis seen in p35-/- mice. Although IL-17 had been known to be a product of memory CD4+ T cells for over 10 years, the novel work by Cua's group and others now identified these cells as a lineage, distinct from Th1 and Th2 cells (Aggarwal *et al,* 2003; Park *et al,* 2005) with major ramifications to our understanding of murine models of autoimmunity.

1.3.3 Interleukin 17 family

IL-17A (referred to as IL-17), originally cloned in 1993, is part of a family of cytokines, designated IL-17A-F. IL-17A is a disulfide-linked homodimeric glycoprotein of 155 amino acids (Rouvier 1993). All members of the IL-17 family show conservation in their C-termini with five spatially conserved cysteine residues. IL-17 shares greatest sequence homology with IL-17F (55%) and both cytokines are secreted by Th17 cells, whilst the remainder of the family are secreted by non-T cells. Comparatively little is known about IL-17B-E. Intranasal administration of adenoviruses expressing IL-17C, triggers comparable neutrophilic responses to IL-17A and IL-17F, suggesting that these cytokines mediate common biological effects, probably through shared signalling pathways (Hurst *et al,* 2002). IL-17E, also known as IL-25, is produced by mucosal epithelial cells, as

well as by multiple immune cell types, including NK-T cells, and Th2 cells (Fort *et al,* 2001). Over-expression of IL-25 promotes eosinophilia and stimulates the production of Th2 cell-specific cytokines. Moreover, IL-25 limits Th17 cell development by inducing the expression of IL-13 by DCs or by inhibiting IL-23 production (Kleinschek *et al,* 2007). IL-17A and F can exist as homodimers or a heterodimers (Liang *et al,* 2007). The contribution of each isoform to autoimmune disease is still to be determined.

1.3.4 Cellular sources of IL-17

Although IL-17A and F have been linked to CD4+ memory T cells, namely Th17 cells, secretion is not limited to this population, and γδ T cells, CD8+ T cells, and iNK cells have all been associated with secretion of this cytokine (Happel *et al,* 2003; Lockhart *et al,* 2006; Michel *et al,* 2007; Yao *et al,* 1995a). Eosinophils and neutrophils also express IL-17, detected either as mRNA or intracellular protein but its secretory nature from these cell-types has yet to be confirmed (Ferretti *et al,* 2003; Molet *et al,* 2001).

1.3.5 IL-17 receptors and signalling pathway(s)

The IL-17 receptor (IL-17RA) is a type I transmembrane receptor, widely expressed including on haematopoietic tissue, although most responses to date have been reported in epithelial, endothelial and fibroblast cells, as well as macrophages and DCs (Yao *et al,* 1995b). Human IL-17RA binds IL-17A with relatively low affinity, which led to the search for other receptors. A further transmembrane receptor, IL-17RC was identified which like IL-17RA, can homodimerise but also forms heterodimers with IL-17RA. The precise stoichiometry of the IL-17A-binding complex has not been determined, but limited data suggests that the IL-17RA-RC heterodimer may have greater affinity for IL-17A and F than IL-17RA complexes alone (Tischner *et al,* 2006). In contrast to IL-17RA, IL-17RC expression is low in haematopoietic tissues and high in non-immune cells of the prostate, liver, kidney, thyroid and joints (Haudenschild *et al,* 2002). IL-17RC is critical for the binding and signalling in response to IL-17F which may lead to different tissue specificities for IL-17A and IL-17F (Kuestner *et al,* 2007).

Studies defining IL-17A-induced genes showed that IL-17A activates a highly pro-inflammatory programme of gene expression, typical of that induced by tumour necrosis

alpha (TNFα) and innate immune receptors such as IL-1R and TLRs (Park *et al,* 2005; Shen *et al,* 2005). IL-17A activates NF-κB, a hallmark transcription factor associated with inflammation. Actin related gene 1 (ACT1), a signalling adaptor that was previously linked to NF-κB activation in response to B cell activating factor (BAFF) has been shown to be the signalling intermediate between IL-17RA and NF-κB (Yao *et al,* 1995a). Indeed, ACT1 deficiency in knockout mice impairs IL-17A- and IL-17F-induced activation of NF-κB (Qian *et al,* 2007).

Another pathway which is activated by IL-17 involves the CCAAT/enhancer binding protein transcription factors C/EBPβ and C/EBPδ. Promoter sites for C/EBP binding are found in many genes upregulated by IL-17A, and in the case of the IL-6 response following IL-17A stimulation, there is an absolute requirement for C/EBPβ/γ (Ruddy *et al,* 2004). Interestingly the repeated observation of synergy between TNFα and IL-17 (section 1.3.6.1 and 1.6.2.4), maybe be explained, in part, by the cooperation of both cytokines in activating the C/EBP pathway.

1.3.6 The physiological role of IL-17

IL-17 plays an important role in the host's defence against extracellular bacteria, mobilising and recruiting polymorphonuclear leucocytes (PMNs) to the site of inflammation (Fossiez *et al,* 1996; Gaffen 2009). Consequently mice deficient in IL-17 or the receptor IL-17RA are susceptible to a range of organisms including *K .pneumoniae* pulmonary infection, systemic *Candida albicans*, and *Toxoplasma gondii* (Huang *et al,* 2004; Kelly *et al,* 2005; Ye *et al,* 2001a).

1.3.6.1 Granulopoeisis and neutrophil recruitment

IL-17 does not act directly on PMN to drive recruitment, but indirectly via its actions on target tissues. As discussed in section 1.3.5, IL-17 receptors RA and RC are enriched at tissue sites which provide a physical barrier to external pathogens. IL-17 drives granulopoeisis by inducing G-CSF and GM-CSF from human airway epithelium, endothelial and fibroblast cells (Fossiez *et al,* 1996; Jones & Chan 2002). This effect is enhanced by co-stimulation with TNFα which as noted above, is true for many of the downstream effects of IL-17. Stimulation with recombinant IL-17 leads to secretion of the chemokines CXCL1 and CXCL8 (IL-8), neutrophilic chemoattractants from

bronchial epithelium and keratinocytes (Jones & Chan 2002; Teunissen *et al,* 1998). *In vivo*, IL-17RA deficient mice have reduced G-CSF and macrophage inhibitory protein (MIP)-2 in the lung, delayed recruitment of neutrophils to the alveolar space and are susceptible to *K .pneumoniae* (Ye *et al,* 2001b).

1.3.6.2 Defensins

IL-17 and IL-22 contribute to the maintenance of host defence by inducing the secretion of anti-microbial peptides, Human β-defensin-2 (hBD-2) and S100 proteins A7, A8 and A9 from keratinocytes and human airway epithelia (Kao *et al,* 2004; Liang *et al,* 2006). These peptides function to disrupt microbial membranes and stimulate immune cell recruitment (Harder & Schroder 2005).

1.3.7 Th17 cell differentiation in humans

Early reports suggested that IL-23 was required for the generation of Th17 cells (Aggarwal *et al,* 2003; Langrish *et al,* 2005). However naïve T cells do not express the IL-23R which suggests that other cytokines are required to drive the Th17 program. In 2006, three groups confirmed that IL-6 in combination with TGFβ can drive naïve murine T cells to differentiate into Th17 cells (Bettelli *et al,* 2006; Mangan *et al,* 2006; Veldhoen *et al,* 2006). In humans, IL-1β and IL-23 are required for Th17 differentiation, but the induction of Th17 cells from naïve T cells is modest when compared to the murine system, suggesting subtle differences in Th17 biology between the two species (Acosta-Rodriguez *et al,* 2007a; Wilson *et al,* 2007). Some of these differences were realigned when Volpe and colleagues, using a computational analysis, found that TGFβ was a requirement in the human system (Volpe *et al,* 2008). This was confirmed by the use of serum free media. Fetal calf serum (FCS) has high levels of bovine TGFβ which had masked the requirement for exogenous TGFβ in earlier studies (Manel *et al,* 2008).

1.3.8 The transcriptional control of Th17 cells

Th17 cells harvested *ex vivo* do not express high levels of Th1 and Th2 transcription factors (Mangan *et al,* 2006; Park *et al,* 2005). To investigate the transcriptional control of Th17 cells, Litmman and co-workers analysed mice with the green florescent protein

(GFP) knocked into the initiation site of the transcription factor retinoic acid orphan receptor gamma t (RORγt) (Ivanov *et al*, 2006). Mice heterozygous for this "knockin" mutation ($ROR\gamma t^{GPF+/-}$) simultaneously express GFP and RORγt whilst the homozygous mice ($ROR\gamma t^{GPF+/+}$) are deficient of RORγt. By sorting GFP+ cells from the intestine of $ROR\gamma t^{GPF+/-}$ mice they found that nearly 60% of cells expressed IL-17, whilst none of the GFP- mice did. In the $ROR\gamma t^{GPF+/+}$ mice, with RORγt knocked out, there was a significant reduction, but not complete absence of IL-17 expression in CD4+ T cells. This suggested that RORγt played a role in murine Th17 development, but was not an absolute requirement.

Annunziato and colleagues, using human T cell clones confirmed that Th17 cells but not Th1 or Th2 expressed the human orthologue of RORγt, RORC2 (Annunziato *et al*, 2007). Lentiviral over-expression of RORC2 upregulates IL-17 production in CD4+ T cells, but only in up to 30% of cells (Crome *et al*, 2009; Manel *et al*, 2008) The ligand for the nuclear receptor RORγt remains unknown, and it is possible that presence or absence of this ligand accounts for the limited correlation between RORγt and IL-17 expression in the studies described. Alternatively other transcription factors may modulate Th17 differentiation. Indeed, interferon regulatory 4 (IRF-4) and RORα, which is closely related to RORγt, augment Th17 differentiation (Brustle *et al*, 2007; Yang *et al*, 2008b). Microarray analysis of murine T cells differentiated *in vitro*, indicated that expression of another transcription factor, aryl hydrocarbon receptor (AHR), is upregulated in Th17 cells but not Th1 cells (Veldhoen *et al*, 2008). Mice with a gene deletion of AHR are resistant to EAE, and as well as being deficient in Th17 cells also show significant abrogation of IL-22 expression. Several environmental toxins, such as dioxin or 6-formylindolo(3,2-b)carbazole (FICZ), possibly an endogenous ligand, bind AHR and upregulate IL-17 production. These data were the first to link environmental stimuli to the induction of autoimmune disease as a result of the expansion of Th17 cells.

Several factors can also inhibit Th17 differentiation and are therefore important targets for future therapies. IL-27, a heterodimeric cytokine composed of Epstein-Barr virus–induced gene 3 (EBI3) and p28, a novel protein subunit related to p35, has been shown to suppress the differentiation of Th17 cells. This effect is exerted by two mechanisms, firstly by inhibiting the differentiation of naïve T cell towards Th17 cells in a STAT-1

dependent manner and secondly by the generation of Foxp3 negative IL-10-secreting cells which suppress IL-17 production and limit central nervous system (CNS) inflammation in mouse models (Fitzgerald *et al,* 2007; Stumhofer *et al,* 2006).

1.3.9 C-type lectin CD161

CD161, the human equivalent of murine NKR-P1A, a lectin-like receptor (Giorda *et al,* 1990) is a potential lineage marker for Th17 cells in humans. Expression of CD161 on Th17 cells was discovered by microarray analysis of T cells clones from the inflamed gut (Cosmi *et al,* 2008). CD161+ T cells from cord blood have increased expression of RORC2 compared to CD161- cells, but neither population expresses IL-17 (Cosmi *et al,* 2008). Following the differentiation of naïve cord CD4+ T cells *in vitro,* in the presence of IL-1β and IL-23, only CD161+ cells are able to upregulate IL-17 expression. All memory CD4+ T cells expressing IL-17 from the inflamed gut are within the CD161+ compartment (Kleinschek *et al,* 2009). At the outset of this thesis it was unclear if CD161 expression predetermined the Th17 program directly, or if it was linked with the Th17 phenotype as a downstream target of RORC2.

CD161 has two known ligands, one of which, proliferation-induced lymphocyte-associated receptor (PILAR) is highly enriched within the joint (Huarte *et al,* 2008), and stabilises the effector T cell population through increased expression of the anti-apoptotic Bcl-xL. Ligation of PILAR to CD161 enhances the secretion of inflammatory chemokines and IFNγ but surprisingly not IL-17. Another ligand is lectin-like transcript 1, which belongs to the C-type lectin domain family 2 (Rosen *et al,* 2005) and also increases IFNγ secretion after binding to CD161.

1.4 Regulatory T cells and peripheral tolerance

In the thymus, central tolerance plays a key role in preventing immune responses to self antigens. However this process is imperfect and auto-reactive T cells may escape to the periphery, leading to autoimmune disease. One mechanism of tolerance that has evolved to circumvent this problem is the active suppression of immune responses in the periphery by a regulatory subset of T cells.

"Suppressor" T cells were described many years ago (Nishizuka & Sakakura 1969), but their existence remained controversial for a long period. Mice thymectomized between 2 and 4 days of age develop organ-specific autoimmune disease, which can be prevented, by adding back syngeneic T cells obtained from an adult thymus or spleen. These suppressive cells were later characterized by Sakaguchi and colleagues as CD4+ T cells expressing the alpha chain of the IL-2 receptor (CD25) and referred to as regulatory T cells (Treg) (Sakaguchi 1995; Takahashi *et al*, 1998). In 1998, Treg were shown to suppress the proliferation of CD4+ and CD8+ T cells *in vitro* when stimulated through the TR in the presence of APC (Thornton & Shevach 1998). This assay, allowed Treg to be identified in humans. Several studies showed that the fraction (5-10%) of human CD4+ T cells expressing the highest level of CD25 have suppressive function (Baecher-Allan *et al*, 2001; Jonuleit *et al*, 2002; Taams *et al*, 2001).

1.4.1 Foxp3 a regulatory T cell transcription factor

In 2003, a member of the forkhead family of transcription factors, Foxp3, was identified as a key regulator of Treg cell development and function (Fontenot *et al*, 2003; Hori *et al*, 2003). The importance of Foxp3 has been emphasised by the fact that both animals (scurfy mice) and humans (males with the Immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome, IPEX) with mutations of FOXP3 do not develop CD4+CD25+ T cells in the thymus and succumb to autoimmune disease early in life (Baecher-Allan *et al*, 2004; Bennett *et al*, 2001). Indeed, the key function of Foxp3 in Treg development was confirmed when transfection of Foxp3 in murine $CD4+CD25^-$ cells converted them into functional suppressors (Fontenot *et al*, 2003; Shevach 2004), and upregulated cytotoxic T Lymphocyte antigen 4 (CTLA-4) and glucocorticoid-

induced tumour-necrosis factor (TNF)-receptor-related protein (GITR), proteins that are highly expressed on Treg.

In mice, expression of Foxp3 is necessary and sufficient to induce a Treg phenotype, but in humans the data are conflicting. In experiments similar to the murine studies quoted, human cells did *not* develop a suppressive phenotype by the transduction of FOXP3 using a CMV promoter (Allan *et al*, 2005). However the same group, went on to generate FOXP3+ cells with suppressive function using a lentiviral construct with the human elongation factor 1 alpha (EF1α) promoter (Allan *et al,* 2008). When compared to transduced cells with the CMV promoter, EF1α+ cells had more stable expression of FOXP3 and this correlated with greater suppressive function. The stability of FOXP3 expression by regulatory elements may be central to the phenotype of human FOXP3+ Treg and will be discussed again here and in more detail in Chapter 3.

1.4.2 Thymic and induced Treg

The development of "natural" Treg in the thymus is confirmed by the experiments on thymectomised mice discussed earlier (Sakaguchi 1995). In mice, the development of natural Treg requires thymic stroma, high affinity interactions between Treg TR and self peptide-MHC complexes presented by thymic stromal cells, co-stimulatory signals and the growth factors IL-2 and IL-7 (Wing & Sakaguchi 2010). In contrast to Treg derived from the thymus, Treg generated in the periphery from CD4+Foxp3- (or CD25-) cells are referred to as "peripheral" or "induced" Treg. In mice, Foxp3+ Treg can induce Foxp3 expression and a suppressive capacity, in Foxp3-ve cells, by a mechanism involving surface bound TGFβ (Andersson *et al,* 2008; Chen *et al,* 2003). In humans the data are conflicting. FOXP3 is upregulated at the protein level in CD4+CD25- cells following activation with anti-CD3 and anti-CD28 alone or in the presence of TGFβ and in an early study this appeared to confer a suppressive phenotype (Walker *et al,* 2003). More recent data suggest that activated cells express FOXP3 protein at lower levels than natural Treg and do not have a suppressive phenotype (Allan *et al,* 2007; Tran *et al,* 2007). However in Rheumatoid Arthritis (RA) patients the anti-TNFα antibody, Infliximab, does generate CD62L- FOXP3+ Treg from CD25- effector T cells *in vitro* (Nadkarni *et al,* 2007). This process is dependent on TGFβ and the resulting Treg have a normal suppressive capacity

that is dependent on TGFβ and IL-10. FOXP3+Treg from RA patients prior to treatment with infliximab are predominantely CD62L+ and fail to suppress effector cytokine secretion leading to the hypothesis that CD62L- Treg are "induced" *in vivo* in response to Infliximab (Nadkarni *et al,* 2007). These results with Infliximab have yet to be replicated in other human autoimmune diseases. As there are no clear markers to distinguish natural and peripheral Treg, these distinctions remain problematic for human research and questions remain over existence of induced FOXP3+ Treg in humans (Tran *et al,* 2007). Ultimately the controversy over FOXP3 expression and Treg phenotype in humans may result from the varying experimental conditions analysed or the intrinsic heterogeneity of human Treg.

1.4.3 Mechanisms of suppression

Regulatory T cells suppress inflammatory responses by a range of mechanisms, which encompass both contact dependent and independent ways. Following cell contact Treg are able to kill cells by utilising perforin and granzyme B pathways (Cao *et al,* 2007; Gondek *et al,* 2005) and inhibit responder T cells by upregulating intracellular cAMP levels which limits IL-2 secretion and proliferation (Bopp *et al,* 2007). Early studies found that Treg express high levels of the inhibitory CD28 ligand, CTLA-4, but it was not clear if this was a major mechanism for Treg suppression. Sakaguchi and co-workers generated a conditional knockout of CTLA-4 linked to the Foxp3 promoter and found that CTLA-4 deficient Treg have a reduced suppressive capacity *in vitro* and *in vivo,* and fail to modulate the activation state of DCs when compared to wild type Treg (Wing *et al,* 2008). Although much of the analysis of human Treg has focused on the suppression of effector T cells *in vitro*, this study clearly demonstrates that Treg act on wider set of targets *in vivo,* including limiting the capacity of DCs to activate other T cells. In mice, Treg express high levels of ectonucleosidase enzymes, CD39 and CD73 which hydrolyse pro-inflammatory adenosine triphosphate (ATP), into adenosine diphosphate (ADP) and ultimately adenosine which has anti-inflammatory actions (Deaglio *et al,* 2007). Interestingly, Wedderburn and co-workers have recently shown that abrogating the function of CD39 or CD73 does not alter the *in vitro* suppressive capacity of Treg in humans (Moncrieffe *et al,* 2010).

Treg secrete the anti-inflammatory cytokines IL-10 and TGFβ, but the role these cytokines play is controversial, and appears to be dependent on the experimental context. *In vitro*, IL-10 and TGFβ do not contribute to suppressive function (Thornton & Shevach 1998), but they do play a role *in vivo*, in the Powrie model of colitis (Asseman *et al*, 1999). As discussed above, TGFβ induces de novo Foxp3+ Treg in the murine system and in some *in vitro* studies in humans, and it is possible that links between TGFβ and Treg function are dependent on the generation of infectious tolerance.

IL-35, a heterodimer of EBI3 and IL-12p35, is also secreted by Treg, and contributes to suppressive function in mice (Collison *et al*, 2007). Using an ingenious experimental design, the authors demonstrated that the suppressive function of IL-35, although secreted, is contact dependent, as activation signals from contact with effector T cells are required for the production of IL-35 from Treg.

1.5 Chemokine mediated T cell trafficking

Chemokines are small proteins that play a role in controlling leukocyte trafficking through the body. Cellular migration mediated by chemokines is regulated by means of a concentration gradient within tissues and also by changes in the expression of chemokine receptors on leukocytes. Interplay between chemokines and their receptors, like other parts of the immune systems, may become dysfunctional in the context of autoimmunity and play a role in maintaining chronic tissue inflammation (Charo & Ransohoff 2006).

1.5.1 CC chemokine family

Human chemokines, of which there are now more than 50 types, are subdivided into families according to structure and function (Zlotnik & Yoshie 2000). The largest family, CC chemokines, is so named because the first two of the four cysteine residues in these molecules are adjacent to each other (several members are described in greater detail in Table 1.2).

1.5.2 CXC and CX3C chemokine families

A second family of chemokines consists of CXC chemokines, which have a single amino acid residue interposed between the first two cysteine residues. CXCL8 is the prototype chemokine of this family, and is a major effector chemokine upregulated epithelial cells in response to IL-17. CXCL8 attracts polymorphonuclear leukocytes to sites of acute inflammation and in combination with G-CSF, is the basis for the neutrophilic response induced by IL-17 (Section 1.3.6.1). The third family is the CX3C family, of which fractalkine (CX3CL1) is the only member.

1.5.3 Chemokine receptors

Chemokines act through specific surface receptors that have a seven-transmembrane–domain coupled to a G-protein (Holmes *et al*, 1991). The binding of the chemokine to the receptor activates signalling cascades that culminate in the rearrangement, change of shape, and movement of cells. As well as directing leukocyte migration, chemokine receptors play a role in T cell activation by being recruited to the immunological synapse

and maintaining T cell-APC binding (Molon *et al,* 2005). There is significant redundancy with the chemokine system that in most cases allows multiple ligands to bind a single chemokine receptor and for some ligands to binds several receptors. In the case of CCR5, there are several ligands including CCL3, CCL4 and CCL5, whilst CCL5 binds to additional receptors CCR1 and CCR5.

Naïve T cells express CCR7 which helps maintain re-circulation of this population within the lymphoid organs, in response to the ligands, CCL19 and CC21, expressed on the high endothelial venule (Mori *et al,* 2001). This increases the chance of naïve T cells meeting their cognate antigen. Following activation, T cells downregulate CCR7 and upregulate a combination of other chemokine receptors, dependent on their master transcriptional program which enables access to the peripheral organs, particularly at sites of inflammation. Historically chemokines and their receptors have been considered as either homeostatic or inflammatory (Schaerli *et al,* 2004). As discussed CCR7 and its ligands play a central role in naïve T cell biology and so play a homeostatic role. In contrast, other pairings, such as the ligand CCL5 and its receptor CCR5 are both upregulated under inflammatory conditions, as found in childhood arthritis (section 1.6.2.6). In some cases, a chemokine can have both homeostatic and inflammatory roles, such as CCL20 which is important in the formation of Peyer's patches and also plays a role in T cell trafficking to inflamed skin (see 1.5.5). A summary of chemokine receptors, CCR1 to CCR7 is shown in Table 1.2.

Receptor	Chemokine ligand and alternative names in parentheses.	Cell types expressing receptor	Disease connection
CCR1	CCL3 (MIP-1α), CCL5 (RANTES), CCL7 (MCP-3), CCL14 (HCC1)	T cells, monocytes, eosinophils, basophils	RA, MS, childhood arthritis
CCR2	CCL2 (MCP-1), CCL8 (MCP-2), CCL7 (MCP-3), CCL13 (MCP-4), CCL16 (HCC4)	Monocytes, DCs (immature), memory T cells	Atherosclerosis, RA MS, resistance to intracellular pathogens, type 2 diabetes mellitus
CCR4	CCL17 (TARC), CCL22 (MDC)	Treg, Th2, DC (mature), basophils, macrophages, platelets	Treg homing to ovarian cancer, parasitic infection, T cell homing to skin
CCR5	CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), CCL11 (eotaxin), CCL14 (HCC1), CCL16 (HCC4)	T cells, monocytes	HIV-1 coreceptor transplant rejection, human autoimmune arthritis
CCR6	CCL20 (MIP-3β)	Memory T cells, Treg, immature DC, B cells	Intestinal T cell homing. Humoral mucosal immunity
CCR7	CCL19, CCL21	Naïve T cells Immature and Mature DC	Normal T cell and DC trafficking

Table 1.2 Summary of selected CC chemokine receptors and their ligands

1.5.4 Regulatory T cell trafficking

Naturally occurring Treg differentiate in the thymus and then exit to the periphery. In humans, Treg make up 5-10% of CD4+ T cells in PBMC and in peripheral lymph nodes, and also an equivalent frequency in murine thymus and spleen (Sakaguchi *et al*, 2007). In humans, where there is limited evidence to support the peripheral induction of Treg, the even distribution of Treg between lymphoid compartments would suggest a homeostatic mechanism that underpins the trafficking of Treg within the body. At the outset of this report it was unclear if Treg migration was distinct from that of conventional T cells. CD62L is important for the homing of naïve T cells to lymph nodes and is expressed on a

subset of Treg (Taylor *et al,* 2004). Although CD62L+ Treg have a similar suppressive capacity to CD62L-, only the CD62L+ subset are efficient at protecting mice from lethal GVHD, which implicates CD62L in the correct trafficking of Treg. CCR7 is also important in the migration of naïve T cell and is expressed on Treg, but the ligand CCL19 fails to induce Treg chemotaxis *in vitro* (Iellem *et al,* 2001). Adoptive transfer experiments with $CCR7^{-/-}$ mice are required to clarify the role this molecule plays in Treg trafficking to the draining lymph nodes.

Is the suppression of naïve T cell activation by Treg in the draining lymph node sufficient to contain autoimmune responses or do some Treg subsets need to migrate directly to the inflamed peripheral organ to suppress local inflammatory reactions? In mouse models of Leishmania, Treg alter immune responses by acting at the local site (Belkaid *et al,* 2002). Several chemokine receptors have been implicated in the trafficking of Treg to inflamed tissue. In murine models Treg expressing CD103 (integrin α_E), migrate efficiently to peripheral tissues and suppress inflammation (Suffia *et al,* 2005). However, in humans this marker is rarely associated with Treg (Rotzschke *et al,* 2009)**.** Iellem and colleagues examined the phenotype of regulatory T cells from human peripheral blood and found them to express high levels of the receptors CCR4 and CCR8 (Iellem *et al,* 2001). This result was unexpected as CCR4 is a chemokine receptor associated with memory T cells that traffic to the skin (Campbell *et al,* 1999) or T cells with a Th2 phenotype (Bonecchi *et al,* 1998). Inspite of expressing both CCR4 and CCR8, *in vitro*, the ligands for CCR4, CCL17 (previously known as thymus and activation regulated chemokine, TARC) and CCL22 (previously known as macrophage derived chemokine, MDC) induced greater chemotaxis that the CCR8 ligand, CCL1. Both CCL17 and CCL22 are produced by mature dendritic cells, which may promote the co-localisation of CCR4+ Treg with DCs and the subsequent suppression of activated DCs (Imai *et al,* 1999). CCL22 has been confirmed as a chemoattractant for Treg *in vivo*, in studies of ovarian carcinoma. Malignant ascitic fluid is enriched for Treg, which are recruited by CCL22 secreted from resident macrophages (Curiel *et al,* 2004).

1.5.5 Th17 cell trafficking

Following reports that Th17 cells were from a lineage distinct from Th1 and Th2 cells, it was predicted that Th17 cells would have a unique profile of chemokine receptors. Acosta-Rodriguez and colleagues sorted CD4+CD25- T cells according expression of CXCR3, CXCR5, CCR4 and CCR6 and after stimulating cells with anti-CD3 and anti-CD28 examined IL-17 secretion by ELISA (Acosta-Rodriguez *et al*, 2007b). Cells expressing CCR4 and CCR6 secreted the highest levels of IL-17 and were enriched for RORC2 mRNA expression. Prior to the link with Th17 cells, CCR6 was notable for its expression on wide range of cells including B cells, immature DCs, Langerhan's cells and subsets of CD8 and NK T cells (Schutyser *et al*, 2003). The ligand for CCR6, CCL20 is expressed constitutively in high levels within mucosal tissue of the gut particularly the colon and appendix, and thought be important in trafficking immature DC, and effector T and B cells to the gut (Kwon *et al*, 2002). CCR6-/- mice, have a relatively normal phenotype, except for under-developed Peyer's patch and the failure to develop humoral responses to oral antigen (Cook *et al*, 2000), suggesting a role in the generation of gut associated lymphoid tissue and mucosal immunity. Under inflammatory conditions CCL20 is upregulated at several sites; the lesional skin of psoriasis, colonic epithelia of IBD patients, and from synovial fibroblasts in response to TNFα and IL-17 (Chabaud *et al*, 2001b; Homey *et al*, 2000).

In the gut of patients with IBD, Th17 cells express CCR6 and undergo a calcium flux response to CCL20, (Annunziato *et al*, 2007). In one study, CCR2 but not CCR5 expression was found on Th17 cells from human PBMC (Katoh *et al*, 2004). None of the studies cited had tested the chemotactic response of Th17 cells to the ligands of CCR2, CCR4 or CCR6.

1.6 Juvenile idiopathic arthritis

Juvenile idiopathic arthritis (JIA) is the most common rheumatic disease of childhood. JIA is defined as arthritis in a child under the age of 16 years, affecting one or more joints, lasting for at least 6 weeks and having no other known aetiology (Petty *et al,* 2004). The incidence of JIA assessed using the ILAR criteria is at a rate of 15/100,000 children/year (Berntson *et al,* 2003), whilst the prevalence of chronic arthritis in childhood has been estimated as 40-80/10,000 children (Manners & Diepeveen 1996; Moe & Rygg 1998).

1.6.1 Clinical spectrum of disease in JIA

Children with JIA can present as many different forms, each of which has a distinct course and severity. In order to facilitate clinical and scientific research, JIA has been classified into several subgroups. The current internationally agreed system of classification was originally set out in 1996, with the aim of producing clearly identifiable homogenous, mutually exclusive groups (Table 1.3) and was last modified in 2004 (Petty *et al,* 2004). Many of the sub-types of childhood arthritis have distinct clinical features, and some of these features are rare in adult inflammatory arthritis. In JIA, in contrast to RA, large joints such as the knees, wrists, and ankles are more prominently involved than small joints. Subcutaneous nodules and rheumatoid factor (RF) seropositivity are unusual, but antinuclear antibody (ANA) seropositivity is frequent in some JIA subtypes. Patients with ANA seropositive arthritis are of a younger age at disease presentation, have a female predilection, asymmetric arthritis, and are at increased risk of chronic anterior uveitis (Ravelli *et al,* 2010).

Some JIA subtypes have a predilection for onset in young childhood. Examples include the systemic onset and the oligoarticular subtypes of JIA. In contrast some JIA subtypes tend to have a slightly older onset in children and have an adult disease counterpart, such as psoriatic arthritis and RF+ polyarticular JIA. Differences in the age of onset of JIA, may have an underlying biological basis, as gene expression profiles from patients with onset <6years of age cluster separately to those with a later onset (Barnes *et al,* 2010). Chronic anterior uveitis is linked with oligoarticular subtypes, and can occur in psoriatic

and polyarticular forms of the disease, although less commonly. Acute anterior uveitis is linked with HLA-B27 associated disease, particularly enthesitis related arthritis and less commonly psoriatic arthritis.

Long term studies have shown that as a whole, JIA is not as benign as previously thought with rates of complete remission off medication still low in many subtypes (Wallace *et al,* 2005), evidence for loss of quality of life in childhood (Gutierrez-Suarez *et al,* 2007), and up to 30–50 % of patients experiencing ongoing inflammation or disability into adulthood (Foster *et al,* 2003; Packham & Hall 2002).

Aims of treatment are to control active inflammation, to allow normal growth and development and function for the child and prevent disability. Patients with oligoarthritis are managed with non steroid anti-inflammatory agents and steroid intra-articular joint injections. Those with polyarthritis or involvement of important joints such as the hip, receive systemic therapy initially with Methotrexate and subsequently “biologic” agents, most commonly targeting TNFα. Targets for more recent biologic therapies include IL-1β, IL-6 and CTLA-4 but there are few studies to inform the choice of treatment in patients who fail anti-TNFα therapies.

Category	Definition	Exclusions
Systemic JIA	Arthritis in one or more joints with, or preceded by, fever of at least 2 weeks duration that is documented to be daily ('quotidian'*) for at least 3 days and accompanied by 1 or more of the following: 1. Evanescent (non-fixed) erythematous rash 2. Generalised lymph node enlargement 3. Hepatomegaly and /or splenomegaly 4. Serositis**	A) Psoriasis or a history of psoriasis in the patient or a first-degree relative. B) Arthritis in an HLA-B27 positive male beginning after the 6th birthday. C) Ankylosing spondylitis, enthesitis related arthritis, sacroiliitis with IBD, Reiter's syndrome, or acute anterior uveitis, or a history of one of these disorders in a first- degree relative. D) The presence of IgM RF on at least 2 occasions at least 3 months apart.
Oligo JIA	Arthritis affecting one to four joints during the first 6 months of disease. Two subcategories are recognised: 1. Persistent oligoarthritis: affecting not more than 4 joints throughout the disease course 2. Extended oligoarthritis: affecting a total of more than 4 joints after the first 6 months of disease	A , B, C, D above, plus E) The presence of systemic JIA in the patient
Polyarthritis (Rheumatoid Factor Negative)	Arthritis affecting 5 or more joints during the first 6 months of disease; a test for Rheumatoid Factor is negative.	A,B,C,D,E
Polyarthritis (Rheumatoid Factor Positive)	Arthritis affecting 5 or more joints during the first 6 months of disease; 2 or more tests for RF at least 3 months apart during the first 6 months of disease are positive	A,B,C,E
Psoriatic Arthritis	Arthritis and psoriasis, or arthritis and at least 2 of the following: 1. Dactylitis^ 2. Nail pitting† and onycholysis 3. Psoriasis in a first- degree relative	B, C, D, E

Enthesitis Related Arthritis	Arthritis and enthesitis ⇑, or arthritis or enthesitis with at least 2 of the following: 1. The presence of or a history of sacroiliac joint tenderness and /or inflammatory lumbosacral pain ∏ 2. The presence of HLA-B27 antigen 3. Onset of arthritis in a male over 6 years of age. 4. Acute (symptomatic) anterior uveitis. 5. History of ankylosing spondylitis, enthesitis related arthritis, sacroiliitis with IBD, Reiter's syndrome or acute anterior uveitis in a first degree-relative	A, D, E
Un-differentiated Arthritis	Arthritis that fulfils criteria in no category or in 2 or more of the above categories.	

Table 1.3 The ILAR Classification of JIA. Adapted from (Petty *et al,* 2004).

* Quotidian fever is defined as a fever that rises to 39°C once a day and returns to 37°C between fever peaks .
**Serositis refers to pericarditis and/or pleuritis and/or peritonitis.
^ Dactylitis is swelling of one or more digits, usually in an asymmetrical distribution, which extends beyond the joint margin.
†A minimum of 2 pits on any one or more nails at any time.
⇑ Enthesitis is defined as tenderness at the insertion of a tendon, ligament, joint capsule, or fascia to bone.
∏ Inflammatory lumbosacral pain refers to lumbosacral pain at rest with morning stiffness that improves on movement.

1.6.2 Aetiopathogenesis of JIA

1.6.2.1 Genetic associations with JIA

The evidence for a genetic contribution to JIA comes from twin studies which show a high concordance of disease in monozygotic twins (Ansell *et al,* 1969; Prahalad *et al,* 2000). A study of 164 affected sibling pairs (ASP) with JIA showed a 70% concordance for gender, 73% for disease onset and 66% for disease course, considerably higher than in a non ASP cohort (Thompson *et al,* 2004). First degree relatives of children with JIA have a higher rate of autoimmune disease than controls (Prahalad *et al,* 2002). Genetic influences on JIA susceptibility and phenotype are polygenic (Prahalad *et al,* 2004) and genome wide scans in JIA ASP families have supported the idea that multiple genetic loci contribute to JIA susceptibility (Thompson *et al,* 2004). The strong association of specific alleles with JIA are within the MHC and were the first to be documented (Edmonds *et al,* 1974). Among the MHC class l loci, HLA-B27 is strongly associated with ERA, while HLA-A*0201 is increased in oligoarthritis (Donn *et al,* 2002). Multiple studies have revealed an increase in HLA-DR alleles, of which the strongest are DRB1*0801 and 1101 with oligoarticular JIA, as well as DRB1*1301, in particular in ANA + cases (Donn *et al,* 1995). DRB1* 0801 and *1401 have been associated with polyarticular JIA. Some associations closely mirror those of the corresponding adult disease, such as the strong association of the HLA-B27 allele with ERA (ankylosing spondlyitis in adults) and the HLA-DRB1*0401 with RF+ polyarticular JIA.

Inflammatory cytokines have been an important target for drug development in JIA over the last decade and similarly their gene polymorphisms have been a key area of scrutiny. There are several HLA – independent TNFα haplotypes that are significantly associated with JIA (Zeggini *et al,* 2002). Patients with a GA/AA genotype at the -308 location have a higher degree of functional impairment, erythrocyte sedimentation rate, 100-mm visual analog scale score for disease activity, and higher serum TNF-α levels when compared to those with the -308 GG genotype. Some of the best characterised genetic associations in JIA have been established in systemic JIA (SJIA). The hypothesis suggesting a link between SJIA and IL-6 was proposed 15 years ago (Rooney *et al,* 1995), with many of the clinical features in SJIA resembling the phenotype of IL-6 over-expression e.g.,

fevers, stunted growth, anaemia (De Benedetti & Martini 1998). A polymorphism (-174G/C) in the regulatory region of the IL-6 gene alters transcription of IL-6 in response to IL-1 and LPS; SJIA patients have a significantly lower frequency of the protective CC genotype (Fishman *et al,* 1998), and the IL-6 -174G allele is a susceptibility gene for SJIA (Ogilvie *et al,* 2003). More recent analysis has confirmed a haplotype association with SJIA (Fife *et al,* 2005). A polymorphism in the promoter region of the Macrophage inhibitory factor (MIF) gene is associated with JIA (Donn *et al,* 2004). This polymorphism (MIF -173*C) results in higher MIF production in the serum and synovium of JIA patients and has been shown to be predictive of outcome in SJIA (De Benedetti *et al,* 2003).

The anti-inflammatory gene IL-10 has been studied in oligoarticular JIA. The 'ATA' haplotype of three linked SNPs at the 5' flanking end of the gene is associated with lower production of IL-10 by PBMC, and was found more frequently in the more severe subtype of JIA, extended oligoarticular JIA than the milder persistent oligoarticular JIA (Crawley *et al,* 1999).

1.6.2.2 Specific T cell related genetic loci

Many of the genes associated with JIA have been linked with the immune system, but more specifically several T cell related loci have come to light over the last few years. A SNP in the STAT4 gene, previously associated with RA and type 1 diabetes, has been linked to JIA in two independent cohorts (Hinks *et al,* 2010; Prahalad *et al,* 2009). PTPN22, a negative regulator of T cell responses is associated with JIA (Hinks *et al,* 2005; Thompson *et al,* 2010). Unlike these studies using a candidate gene approach, future novel genetic associations will be elucidated through whole genome scanning, which requires large multi-centre case control cohorts.

1.6.2.3 Immunopathology

The pathological hallmark of JIA is the inflamed synovium. Histology of this tissue shows thickened synovium which is highly vascular and shows marked hyperplasia of synoviocytes in the lining layer as well as a dense infiltrate of inflammatory cells, comprising T cells, macrophages, and in some cases B cells and NK cells (Bywaters 1977; Gregorio *et al,* 2007; Murray *et al,* 1996). The hypertrophied synovial layer is

highly vascular, with endothelium expressing markers of activation such as HLA-DR and ICAM-1. Many parts of the innate immune system are abnormally activated in JIA, including synovial macrophages, DCs and neutrophils (Foell *et al,* 2004; Gattorno M *et al,* 2003; Jarvis *et al,* 2006; Meazza *et al,* 2002; Varsani *et al,* 2003). The increased vascularity is likely to be related to the increased production of pro-angiogenic factors such as VEGF and the angiogenic chemokines (Barnes *et al,* 2004; Vignola *et al,* 2002).

The strong association of many JIA subtypes with genetic variants at HLA loci, as well as the central role of HLA class I and II proteins in T cell function, and the predominance of T cells in pathological JIA synovial tissue and fluid, led to intense investigation of the role of T cells in the pathology of JIA. T cells within the JIA joint are highly activated memory cells, expressing both rapidly up regulated (CD69) and persistent (DR) activation markers (Black *et al,* 2002; Gattorno *et al,* 2005; Wedderburn *et al,* 2000). These T cells express a restricted set of TR (Wedderburn *et al,* 1999; Wedderburn *et al,* 2001b): the 'clonotypes' are large and long-lived, and the same hierarchy of clones re-expand during a relapse or flare of disease (Wedderburn *et al,* 1999). The finding that this oligoclonality in the intra-articular T cell population is more marked in CD4+ T cells in oligoarthritis (which is associated with class II, HLA-DR genes) yet more marked in CD8+ T cells in enthesitis-related arthritis, (which is associated with the class I allele HLA-B27), supports the concept that recognition of MHC-peptide complexes by T cells plays a role in the pathogenesis of juvenile arthritis (Wedderburn *et al,* 2001b). Early work on inflammatory cytokines produced by synovial T cells suggested that these were heavily skewed towards a 'Th1' IFNγ producing lineage (Gattorno *et al,* 1997; Scola *et al,* 2002; Wedderburn *et al,* 2000).

Many inflammatory cytokines and chemokines are abnormally raised in JIA, and found at the site of destructive synovitis. Subtype differences have emerged, which may allow a 'subtype specific' profiling of serum or synovial fluid in the future although such measurements have to take into account circadian rhythms and the short half lives of these mediators (De Benedetti *et al,* 1997; de Jager *et al,* 2007). SJIA is associated with high levels of TNFα, IL-1, IL-6, MIF and also IL-18. In classical SJIA cases, the levels of IL-6 and IL-1 receptor antagonist (IL-1Ra) rise and fall in parallel with the fever and rash (Rooney *et al,* 1995). IL-6 and MIF levels are each associated with disease activity

in SJIA, and high synovial MIF levels predict a poor response to intrarticular steroid injection (Meazza *et al,* 2002). IL-1β may have a major role in the pathogenesis of SJIA (Pascual *et al,* 2005) and early data from uncontrolled trials of Anakinra (IL-1Ra) support this hypothesis (Nigrovic *et al,* 2010).

1.6.2.4 IL-17 and autoimmune arthritis

At the outset of this thesis there was no published evidence on the role of IL-17 in JIA. Childhood arthritis lacks a specific murine model of autoimmune arthritis and much of the mechanistic understanding of IL-17 in arthritis is derived from generic models of autoimmune arthritis such as CIA. In CIA, mice deficient in IL-17A or IL-23p19 have an attenuated course of arthritis (Murphy *et al,* 2003; Nakae *et al,* 2003a). Administration of antibodies against murine IL-17 reduces the severity of disease after the onset of arthritis (Lubberts *et al,* 2004), again supporting a role for IL-17 in disease pathogenesis. Importantly once arthritis is initiated, IL-17 is able to maintain disease independent of TNFα (Koenders *et al,* 2006).

IL-17 protein and message are readily detectable in the synovial membrane of patients with RA but not osteoarthritis (Chabaud *et al,* 1999). Given the pleiotropic effects of IL-17 on the innate immune system (section 1.3.6) it is easy to comprehend how IL-17 could contribute to the cartilage and bony damage of arthritis (Chabaud *et al,* 2001a). IL-17 acts on synovial fibroblasts stimulating the release of matrix metalloproteinases which directly breakdown cartilage (Koshy *et al,* 2002). RANKL and its receptor, RANK are induced by IL-17 alone and also in synergy with IL-1β, to increase osteoclast differentiation leading to bony erosions (Lubberts *et al,* 2003). Synovial fluid from RA patients has been shown to promote osteoclast differentiation by an IL-17 dependent mechanism (Kotake *et al,* 1999). IL-17 synergises with IL-1β and TNFα to induce pro-inflammatory cytokines (such as IL-6) and chemokines from monocytes and cultured synovial fibroblasts including CXCL8 and G-CSF which stimulate granulopoiesis and neutrophil recruitment to the joint (Chabaud *et al,* 1998; Jovanovic *et al,* 1998; Katz *et al,* 2001). Inspite of the wealth of data from murine models and in vitro assays, there has been little data to correlate levels of IL-17 with clinical measures of disease activity in patients with arthritis. One study to do this examined synovial membrane in RA and

found that IL-17 mRNA expression at onset predicted radiologically defined joint damage (Kirkham *et al*, 2006).

1.6.2.5 Regulatory T cells in JIA

In addition to the pro-inflammatory destructive process within the joint there is strong evidence for ongoing immune regulation in JIA. The existence of a mild form of arthritis in which full blown immunopathology can resolve and disease can enter full remission (persistent oligoarticular JIA), provides a unique model in autoimmunity. Children with persistent oligoarthritis have a higher frequency of CD25+ regulatory T cells in the joint compared to patients with a similar presentation but who subsequently have a more severe clinical course (extended oligoarthritis) (de Kleer *et al*, 2004; Ruprecht *et al*, 2005). Regulatory T cells from the joints of children with arthritis express FOXP3 at the message level and have normal suppressive capacity *in vitro*. Although a defect in Treg function has not been demonstrated in childhood arthritis, the numbers of Treg are severely reduced in sick SJIA patients who are being considered for treatment with autologous stem cell transplant (ASCT) (de Kleer *et al*, 2006). Following transplant, there is homeostatic expansion of CD4+CD25+ Treg which is associated with a change in effector T cell cytokine profile from IFNγ towards IL-10.

In addition, T cells specific for the conserved self antigens, heat shock proteins (HSP), have been shown to be present at significantly higher numbers in children destined to have mild disease course, and these self HSP–specific cells are thought to play a regulatory role (de Kleer *et al*, 2003; Kamphuis *et al*, 2005; Prakken *et al*, 1997).

1.6.2.6 T cell trafficking in JIA

The persistence of a chronic inflammatory infiltrate is a reflection of the summated forces that drive cell recruitment, division, death and retention within the arthritic joint (Buckley *et al*, 2004). Many of these factors have been studied in detail in adult arthritis, in which the contribution of stromal cells is clearly defined (Filer *et al*, 2008a). In childhood arthritis, several groups have examined synovial fluid to assess the enrichment of chemoattractants within the joint. Multiple chemokines are increased in the joints of JIA patients including CCL3, CCL5, CXCL10, CXCL8, CCL21 and CCL2 (De Benedetti *et al*, 1999; Pharoah *et al*, 2006; Singh *et al*, 2007; Yao *et al*, 2006). The

chemokine receptors expressed on synovial T cells, CCR5, CXCR3 and CCR7 closely correspond to the ligands found within the joint, supporting a role for chemokines in attracting and retaining inflammatory T cells within the JIA joint (Gattorno *et al*, 2005; Wedderburn *et al*, 2000).

1.7 Project aims

In this thesis the following key questions/aims were addressed:

1) What are the key influences on the migration of Treg in JIA? (Chapter 3)

2) Characterisation of Th17 cells and their relationship to disease phenotype, in JIA. (Chapter 4)

3) Does the synovial microenvironment drive Th17 plasticity in JIA? (Chapter 5)

4) Can Th1 cells with a Th17 ancestry be successfully identified in the inflamed joint? (Chapter 6)

2：Material and methods

2.1 Sample collection

Full informed consent was obtained from all individuals from whom samples were collected in this study, in accordance with local research ethics committees (Great Ormond Street Hospital/Institute of Child Health Research Ethics Committee). Healthy volunteers were staff from within the Infection and Immunity Theme at the Institute of Child Health, UCL, London. On recruitment, these individuals were each assigned a code number to maintain anonymity. Aged matched healthy children would have been the ideal control, but the lack of access to sufficiently large blood samples led to the use of adult donors as healthy controls in this thesis.

A total of 65 patients, 23 male and 42 female, were included in this work. All patients had a diagnosis of JIA in accordance with ILAR classification (Petty *et al*, 2004). A description of disease subtypes is listed in Table 2.1. All patients are given an anonymous code for sample storage which is used to indicate the patient. All synovial fluid samples were taken at the time of therapeutic joint aspiration which was performed when clinically indicated. Where blood was collected this was a small extra volume taken when clinical blood tests were required. When paired peripheral blood and synovial fluid samples were used from the same patient, this has been indicated in the figure legend.

Disease subtype	**Number of samples**
Oligoarthritis- persistent	18
Oligoarthritis- extended	28
Polyarthritis (RF negative)	7
Polyarthritis (RF positive)	1
Enthesitis related arthritis	3
Psoriatic arthritis	3
Systemic	4

Table 2.1: Summary of JIA patients included in this study.

2.1.1 Isolation of Peripheral Blood Mononuclear Cells (PBMC)

Blood samples were collected into sterile universals containing 35U of preservative free heparin (CP Pharmaceuticals Ltd., Wrexham, UK) and processed within 4 hours. Subsequent processing and cell culture work was performed in a sterile Gelaire BSB 4A Category II fume hood, using endotoxin-free sterile reagents. Blood was diluted with an equal volume of a culture medium (CM), containing RPMI 1640 medium (Invitrogen, Paisley, UK) and 100U/ml Penicillin / 100μg/ml Streptomycin (Invitrogen). Blood-RPMI mix was pipetted onto 15ml of Lymphoprep^{TM} (Axis-Shield, Oslo, Norway). These samples were centrifuged at 800g for 24 minutes. The resulting interface of PBMC was pipetted into a clean falcon tube to which an equal volume of CM was added and centrifuged at 500g for 10 minutes. The cell pellet was resuspended by gentle flicking and made up to the required volume with RPMI mix to count cells (typically the same volume as the original amount of blood taken). Cells were then centrifuged at 300g for 10 minutes and re-suspended in the appropriate volume of either RPMI complete medium (RPMI mix plus 10% v/v foetal calf serum (FCS)), (Invitrogen), or freezing medium.

2.1.2 Isolation of synovial fluid mononuclear cells.

Synovial fluid mononuclear cells (SFMC) were collected and processed in the same way as blood samples. Prior to layering onto lymphoprep, samples were incubated with 10U/ml hyaluronidase (Sigma, Gillingham, UK) at 37^{o}C for 30-40 minutes.

2.1.3 Enumeration of viable cells

Viable cell numbers were counted using 10 μl of cell suspension added to 10μl of 0.4 % trypan blue (Sigma). 10μl of this mix was placed on Neubauer counting chamber (Weber, West Sussex, UK) and all unstained, live cells were counted in the specified 25-box field using a bench top microscope (Euromex, Arnhem, Holland). The counted number was multiplied by 2 (dilution factor) and then by $1x10^4$, to deduce the total number of viable cells per ml of sample. Numbers of blue cells were also noted separately to obtain a measure of cell viability. Cell preparations were typically composed of > 90% viable cells.

2.1.4 Freezing cells

Processed cells were cryo-preserved in liquid nitrogen for long-term storage. After counting, cells were centrifuged and resuspended at a concentration of $1x10^7$ cells/ml in cooled freezing medium that contained filter sterilised FCS supplemented with 10% v/v dimethyl sulphoxide (DMSO; Sigma). 0.5-1ml cell aliquots were transferred into individual cryovials (NUNC, Roskilde, Denmark), which were placed into a freezing pot with isopropanol coolant prior to being placed at -70^{o}C for 24 hours and then into liquid nitrogen storage.

2.1.5 Recovering frozen cells from liquid nitrogen storage

Cryo-preserved cells were removed from liquid nitrogen storage and rapidly thawed in a 37^{o}C water bath. Cells were then transferred to 2ml FCS at 37^{o}C in a drop-wise fashion. The universal container was slowly filled up with warmed CM and centrifuged at 300g for 10 minutes before being counted.

2.2 Fluorescent Activated Cell Sorting (FACS)

2.2.1 Buffers and Solutions

All reagents were from Sigma, unless otherwise stated.

FACS buffer: PBS, 1% FCS and 0.1% sodium azide.

Fix buffer: FACS buffer plus 1% formaldehyde.

Perm buffer: FACS buffer containing 0.1% saponin.

Paraformaldehyde (PFA): PBS with 4% PFA (dissolved by heating in fume hood to 65^{o}C for 45 minutes then cooled and frozen in appropriate aliquots).

Sort buffer : PBS, 2% FCS, 4µM EDTA.

Mini-MACS bead buffer: PBS pH 7.2, 0.5% BSA and 4 mM EDTA (degassed)

2.2.2 Antibodies

Monoclonal antibodies were used as direct conjugates with fluorescein isothiocyanate (FITC), R-phycoerythrin (PE), cy-chrome (PERCP/PECy5), PE-CY7, PC-7,

Allophycocyanin (APC), v450, Alexa Fluor 488, and Alexa Fluor 647. A list of antibodies used is shown in Table 2.1.

Table 2.1 Antibodies used for flow cytometry.

(All antibodies were raised in mouse)

Antibody specificity (or reagent), and fluorochrome	**Company**	**Clone**	**Dilution**
CD3 Pe-Cy5	Becton Dickenson (BD), Oxford, UK	UCHT-1	1/10
CD3 PE	BD	UCHT-1	1/50
CD3 PE-CY7	Southern Biotech, Birmingham, USA	UCHT-1	1/50
CD4 FITC	Sigma	Q4120	1/25
CD4 PERCP	BD	RPA-T4	1/50
CD4 APC	Caltag-Medsystems, Buckingham, UK	S3.5	1/100
CD4 PC7	Beckman Coulter, High Wycombe, UK	SFCI12T4D1	1/100
CD8 PE	DAKO, CA, USA	DK25	1/100
CD14 PerCP	BD	MfP9	1/50
CD25 PE	DAKO	ACT-1	1/25
CD25 PE-CY5	BD	M-A251	1/25
CD45RO-FITC	Serotec, Oxford, UK	UCHL1	1/25
CD161 PE	Ebioscience, Hatfield, UK	HP-3G10	1/25
CD161 PerCP	Ebioscience	HP-3G10	1/25
CCR4 pure	BD	1G1	1/10
CCR5 pure	BD	2D7/CCR5	1/10
CCR6 pure	BD	11A9	1/10
CXCR6 pure	R&D Systems, Abingdon, UK	56811	1/10

CXCR3 PE	R&D Systems	49801	1/10
CXCR6 PE	R&D Systems	56811	1/10
CCR4 PE	BD	1G1	1/10
CCR5 PE	BD	2D7/CCR5	1/5
CCR6 FITC	Biolegend, San Diego, USA	TG7/CCR6	1/50
CCR6 PE	BD	11A9	1/10
TCRγδ FITC	BD	11F2	1/25
Streptavedin FITC	BD	n/a	1/100
Streptavedin PeCy5	BD	n/a	1/300
Streptavedin PerCP	BD	n/a	1/100
IL-4 PE	BD	3010.211	1/10
IFNγ FITC	BD	25723.11	1/25
IFNγ PE	BD	25723.11	1/25
IFNγ v450	BD	B27	1/50
IL-17A Alexa-fluor 488	Ebioscience	Ebio64cap17	1/25
IL-17A Alexa-fluor 647	Ebioscience	Ebio64cap17	1/40
IL-22 PE	R&D Systems	142928	1/10
Foxp3 APC	Ebioscience	PCH101	1/10
RORC2 PE	Ebioscience	AFKJS-9	1/10
T-Bet PE	Ebioscience	Ebio4B10	1/10
CD14 FITC	Sigma	UCHM-1	1/100
CD80 CY	BD	L307.4	1\20
CD83 FITC	BD	HB15e	1\25
CD86 FITC	Serotec	BU63	1\25
HLA DR PE	Sigma	HK14	1\25

2.2.3 Surface marker staining for flow cytometric analysis

To analyse PBMC or SFMC for expression of cell surface proteins, cells were resuspended in cold FACS buffer at a concentration of $1x10^6$ cells/ml. 100µl of this cell suspension was placed in each well of a round-bottomed 96-well plate (Griener Stonehouse, UK). Plates were centrifuged at 300g for 5 minutes at 4°C to obtain a cell pellet, and the solution removed by flicking. The cell pellet was then resuspended in 25µl of FACS buffer containing the relevant antibodies at the specified dilution and incubated on ice in the dark for 30 minutes. After incubation, cells were washed twice with FACS buffer as above, resuspended in 200µl of Fix buffer and transferred to FACS tubes (A1 Lab Supplies, UK) ready to be run on the flow cytometer (Cyan ADP, Dako or LSRII, BD).

2.2.4 Staining for intracellular markers

For analysis of cytokine production by T cells, SFMC or PBMC were incubated in RPMI/5%FCS at 37ºC for 3 hours in the presence of 50ng/ml Phorbol 12-myristate 13-acetate (PMA), 500ng/ml ionomycin and 5µg/ml brefeldin A (all from Sigma). Cells were counted and 1.5 $x10^5$ cells were plated in a 96 well plate. The cells were washed twice in FACS buffer and then fixed in 50µl/well of PFA for 10 minutes at room temperature and washed twice with Perm buffer. Cells were re-suspended in 25µl of Perm buffer containing the appropriate antibody. Cells were incubated in the antibody mix on ice for 30 minutes in the dark before being washed twice more in Perm buffer and once in FACS buffer, then resuspended in 200µl Fix buffer and transferred to LP2 tubes.

2.2.5 Staining for intra-nuclear transcription factors.

Prior to staining, specific buffers were re-constituted according to manufacturers' instructions (Ebioscience) fresh before each experiment. 1 part Fixation/ Permeabilization concentrate was added to 3 parts Fixation/ Permeabilization Diluent to make Fixation/ Permeabilization working solution. The 10**x** stock Permeabilization Buffer was diluted in distilled water to a 1**x** solution prior to use.

Surface molecules were stained as per method 2.2.3. Cells were then washed twice with FACS buffer and resuspended in 100µl of freshly prepared Fixation/ Permeabilization

working buffer. Samples were incubated at 4°**C** for 30 minutes in the dark. Cells were washed twice with 1**x** Permeabilization Buffer and then resuspended in 25µl of 1**x** Permeabilization Buffer made up with the anti-transcription factor antibody (Table 2.1), at the appropriate dilution. Cells were incubated at 4°**C** for 30 minutes in the dark and then washed twice with 1x Permeabilization Buffer and once with FACS buffer and then re-suspended in 200µl of FACS buffer in LP2 tubes.

2.3 Cell sorting

During cell separation of PBMC or SFMC, samples were kept at 4ºC and pre-cooled sort buffer was used unless otherwise indicated. Degassed sort buffer was used for column based sorting.

2.3.1 CD4 T cell enrichment using magnetic beads

2.3.1.1 Column based method

Cells were resuspended in 40µl of buffer per 10^7 cells (for cells numbers less than 10^7 then the stated volumes were used). 10µl of a CD4 negative selection cocktail (Miltenyi Biotec, Gladbach, Germany) was added containing biotin-conjugated monoclonal antibodies against CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCR γ/δ and Glycophorin A. After mixing, the suspension was incubated for 10 minutes at 4°C. A further 30µl of buffer was added followed by 20µl of anti-biotin monoclonal antibodies conjugated to microbeads, as a secondary labelling reagent. After 15 minutes incubation cells were washed by adding 1-2 ml of buffer per 10^7 cells and centrifuged at 300g for 10 minutes. Supernatant was pipetted off completely and 10^8 cells were resuspended in 500 µL of buffer. The column used for depletion was LD (Miltenyi Biotec), in which up to $1x10^8$ of desired cells could be magnetically labelled from a total population of $5x10^8$ cells. The column was then placed in the magnetic field of a MACS Separator (Miltenyi Biotec). The cell suspension was then loaded onto the MACS Column previously rinsed with 3ml of buffer. In total, three washing steps were performed, ensuring complete drainage of the column reservoir between each wash. The magnetically labelled non-$CD4_+$ T cells were depleted by retaining them on a MACS® Column, while the unlabeled

Th cells (CD4+) passed through. Purity of bead sorted cells was compared to unsorted cells by flow cytometry.

2.3.1.2 Column free method

An alternate method of bead based separation was chosen in the latter part of the thesis. This product (Easysep, Stem Cell Technologies, Vancouver, Canada) afforded faster purification times, less frequent centrifugation and was more tolerant of SFMC aggregates. Cells were re-suspended at a concentration of 5 x 10^7 cells/mL in sort buffer. Cells were placed in a 12 x 75 mm polystyrene tube (BD) to properly fit into the EasySep® Magnet. 50µL of negative selection CD4+ T Cell Enrichment Cocktail (containing monoclonal antibodies against CD8, CD14, CD16, CD19, CD20, CD36, CD56, CD123, TCR γ/δ and Glycophorin A, all conjugated to anti-Dextran in a tetrameric antibody complex) was added per mL cells, mixed and incubated for 10 minutes at room temperature. A suspension of magnetic dextran iron particles (magnetic beads) was pipetted vigorously up and down 5 times to ensure adequate mixing and added at 50 µL/mL cells, mixed and incubated for 10 minutes. The cell suspension was brought up to a total volume of 2.5 mL by adding sort buffer. The cells were mixed by gently pipetting and the tube placed into the magnet for five minutes. The magnet was inverted in one continuous motion, pouring off the desired fraction into a new 12 x 75 mm polystyrene tube. The magnetically labelled unwanted cells remain bound inside the original tube, held by the magnetic field of the magnet.

2.3.2 Flow cytometry based purification of cells using cell surface markers

PBMC or SFMC were thawed, counted and resuspended in sort buffer at a concentration of 3x10^7/ml. 150µl of this cell suspension was placed in a 15ml falcon tube. Antibodies were added at the appropriate dilution and incubated for 30 minutes with regular mixing every 10 minutes. Cells were washed twice with sort buffer and resuspended at 2 x 10^7/ml in sort buffer. To purify chemokine expressing sub-populations, SFMC were first gated on CD4+CD25- T cells then sorted into CCR4+CCR6+, CCR4-CCR6+ and CCR4-

CCR6- populations. Cell sorters used were BD FACS Aria Special (BD) and MoFlow (Dako).

2.3.3 Purification of cells using cytokine capture assay

PBMC or SFMC were enriched for CD4+ T cells using negative selection magnetic beads (Easysep, section 2.3.1.2) and then stimulated for 2 hours with 10ng/ml PMA and 1μg/ml ionomycin in RPMI/5%FCS at 37ºC. Cells were washed twice with sort buffer and resuspended in 60μl sort buffer per $15x10^6$ cells. 20μl each of bivalent antibodies with anti-CD45 coupled to anti-IL-17 or anti-IFNγ were added and incubated for 5 minutes. Cells were resuspended in 37ºC RPMI/5%FCS at a concentration of $2.5x10^5$ cells/ml or lower in 50ml falcon tubes. The cell-antibody complexes were incubated at 37ºC for 45 minutes in a MACS rotator (Miltenyi Biotec). Cells were centrifuged and washed twice with sort buffer at 4ºC, before being resuspended in 60μl sort buffer per $15x10^6$ cells. 20μl each of fluorescent conjugated anti-IL-17, anti-IFNγ antibodies were added. In addition antibodies to CD4, and CD161/CCR6 (section 2.2.2) were added in some experiments. Cells were incubated for 10 minutes on ice and washed once with sort buffer, before being resuspended at 20 x 10^6/ml in sort buffer.

Using the flow cytometer, cells were gated on CD4+ lymphocytes before being sorted into IL-17+IFNγ- cells (Th17) and IL-17-IFNγ+ (Th1) populations. Sorted cells were collected in 1.5ml of 10%FCS/RPMI in 5ml polypropylene FACS tubes (BD). Sorted Th1 or Th17 purity was assessed by flow cytometry detecting intracellular cytokines after overnight incubation in brefeldin A (section 2.2.4).

2.3.4 Flow cytometry data analysis

Flow cytometric data were collected on a Cyan ADP or LSR II; 100,000 to 200,000 events were collected for each condition and cells gated by scatter properties. Data were analysed using FlowJo (Treestar Inc, Ashland, USA).

2.4 Migration assay

Chemotaxis assays using CCL17, CCL20, CCL22, and CCL5 (Insight Biotech, Wembley, UK) were performed using 5μm pore polycarbonate filters in a 24 well plate

transwell (Corning Life Sciences, Schiphol-Rijk, The Netherlands). Chemokines were made up to the appropriate concentration in freshly prepared RPMI plus 0.5% BSA. In most experiments, a concentration of 100ng/ml was used for all chemokines based on data from Iellem and colleagues (Iellem *et al,* 2001). 600µl of the chemokine medium was incubated in the lower well for 15 minutes at 37°C. PBMC or SFMC were bead enriched for CD4+ T cells using the column based method (section 2.3.1.1) and reconstituted at 5 x 10^6/ml. 100µl of the CD4 enriched T cells, was placed in the transwell and then transferred into the 24 well plate and the experiment allowed to proceed at 37°C, 5%CO_2 for 90 minutes. Migrated cells were recovered by gently washing off adherent cells from the underside of the transwell into the lower well. Cells in the lower well were retrieved and the well was washed twice with RPMI/0.5%BSA to retrieve any adherent cells. Migrated cells were transferred to a round bottomed 96 well plate. Staining for surface markers or FOXP3 was performed as described above. For analysis of cytokine production by migrated T cells, cells were stimulated and stained with anti-human IL-17, IFNγ, CD3, and CD4 as detailed above. Just prior to acquisition of data by flow cytometry, 20,000 (20µl) FACS counting beads, (Perfect Count, Cytognos, Salamanca, Spain) was added per sample. The number of cells was standardised relative to bead numbers to account for differences in sample volumes lost to cytometer priming. To calculate the chemotactic index, the number of cells migrated in response to chemokine was divided by the number of spontaneously migrated cells (Iellem *et al*, 2001). In some experiments, fold enrichment was calculated by dividing the frequency of FOXP3+ cells (as a % of CD4+ T cells) in cells which migrated in response chemokine, by the frequency of FOXP3+ cells in the population which migrated in response to control medium. An equivalent calculation was carried to assess fold enrichment for IL-17+ cells.

2.5 ELISA and multiplex cytokine assay

2.5.1 IL-17 ELISA

IL-17 ELISA was carried out in accordance with manufacturer's instructions, (Ebioscience). Briefly, a 96 well ELISA plate (Corning Costar, NY, USA) was coated

with 100 µl/well of capture antibody diluted 1 in 250 with coating buffer (made up from powdered sachet diluted in 1L deionised water). The plate was sealed with adhesive plastic cover and incubated overnight at 4°C. An ELISA plate washer (Bio-Tek, Winooski, USA) was used to aspirate wells and wash 5 times with 250 µl/well PBS/1% Tween 20 (Sigma). The first wash step had a 1 minute soak. The plate was blotted on absorbent paper to remove any residual buffer.

The **5x** concentrated assay diluent was mixed 1 part with 4 parts de-ionised water. Wells were blocked with 200 µl/well of 1x assay diluent and incubated at room temperature for 1 hour. The plate was aspirated and washed once. Using 1x assay diluent, the IL-17 standard (1µg/ml) was diluted to make up a solution of 500pg/ml. 7 further 2-fold serial dilutions of the top standards down to 3.9pg/ml and 2 blank wells of 1x assay diluent allowed generation of a standard curve. 100 µl/well of the diluted standard and patient serum was added to the plate, all in duplicates. If there was insufficient volume of patient serum/plasma, samples were diluted in x1 assay diluent. The plate was covered and incubated at room temperature for 2 hours.

Wells were washed 5 times and 100 µl/well of detection antibody diluted 1 in 250 in 1x assay diluent was added and incubated at room temperature for 1 hour. After 5 washes 100 µl/well of Avidin-HRP diluted 1 in 250 in 1x assay diluent was added and incubated at room temperature for 30 minutes. After 5 washes 100 µl/well of substrate solution was added to each well and incubated at room temperature for 15 minutes. The enzyme reaction was terminated by lowering the pH (less than 1.0); this was achieved by the addition of 50µl of stop solution containing 2N sulphuric acid. The optical density was determined using a microplate reader (Thermo Multiscan-ex, MA, USA) reading at 450nm with a correction at 620nm, to compensate for signal artefacts generated from the plate itself. The duplicate readings for each standard, control, and samples were averaged and average control optical density subtracted from each sample reading. A standard curve was generated using sigmoid logistic regression (Ascent plate reader software vr 2.6, Thermoscan) and allowed estimation of concentration of IL-17 in the sample.

2.5.2 CCL20 ELISA

For the detection of human CCL20 a 96 well ELISA microplate pre-coated with a mouse anti-human CCL20 monoclonal Ab (Quantikine, R&D) was used. All required reagents were supplied with the plates. The standard CCL20 solution was diluted with the corresponding calibrator diluent to produce a 1:2 dilution series from 500pg/ml to 7.8pg/ml. The methods followed were comparable to those for the IL-17 ELISA above.

2.5.3 TGFβ1 ELISA

Latent TGFβ1 was converted to detectable, immunoreactive TGF-β1 by acidification of samples. 20μl 1N HCL (Sigma) was added to 40μl plasma or synovial supernatant and incubated for 10 minutes, followed by neutralisation with 20 μL of 1.2 N NaOH/0.5 M HEPES (Sigma). Samples were assayed to check for neutral pH (pH 7.2- 7.6) and if necessary corrected using HCl or NaOH solutions. The TGFβ1 standard solution was diluted with corresponding calibrator diluent to produce a 1:2 dilution series from 2000pg/ml to 31.2pg/ml (R&D Systems).

2.5.4 Multiplex cytokine analysis (LUMINEX)

The Bio-Plex system employing the Luminex multi-analyte profiling technology (x-MAPk), allows individual and multiplex analysis of up to a hundred different small molecules in a single well containing a sample volume of 50 μl (Vignali, 2000; de Jager *et al.,* 2003). A specific antibody is covalently linked to a microsphere with specific fluorescent colours. This complex is incubated with a sample and the specific analyte is bound. Next a biotinylated detection antibody is added and visualized by binding of streptavidin-PE to this antibody. The PE signal of the samples is correlated with the PE signal of a multiplex standard curve. An ‘in house’ Luminex assay was established by Dr S. Ursu (Institute of Child Health, Wedderburn laboratory, UCL). All antibodies used were purchased from commercial sources (Table 2.2 Antibodies used in Luminex). All recombinant cytokines were reconstituted in PBS, pH 7.4, containing 0.5% bovine serum albumin (BSA; Sigma-Aldrich). All proteins were aliquoted and stored at -80^{o}C. Nine different carboxylated polystyrene microspheres were purchased from Bio Rad (Laboratories, Hercules, California, USA). The capture antibodies were covalently

coupled to the micro-spheres by Dr. Simona Ursu as described by de Jager *et al.,* 2003. The assay was performed with the help of Dr. Simona Ursu.

Samples were first treated to remove heterophile antibodies (e.g., Rheumatoid factor) which can generate a false positive signal (de Jager *et al,* 2005). 100µl of sample (plasma, serum or synovial fluid) was added to a 96 well plate coated with protein L (Pierce, Thermo scientific) and incubated for 1 hour at RT. Sample were then ready to be analysed by Luminex.

Calibration curves from recombinant protein standards were prepared using two-fold dilution steps in serum diluent (R&D Systems). Samples were measured and blank values were subtracted from all readings. All assays were carried out directly in a 96-well 1.2 mm filter plate (Millipore, UK) at room temperature and protected from light. A mix containing 1000 microspheres per mediator (total volume 10µl/well) was incubated together with a standard, sample or blank for 1 hour at room temperature. Next, 10 µl of a cocktail of biotinylated antibodies (16.5 µg/ml each) was added to each well and incubated for an additional 60 minutes. Beads were then washed twice with PBS supplemented with 1% BSA and 0.5% Tween 20 at pH 7.4. Fluorescence intensity of the beads was measured in a final volume of 100µl high-performance ELISA buffer. Measurements and data analyses of all assays were performed using the Bio-Plex system in combination with the Bio-Plex Manager software V.4.0 using five parametric curve fitting (Bio-Rad, USA).

Table 2.2 Antibodies used in Luminex experiments

		CAPTURE ANTIBODY			DETECTION ANTIBODY		
Mediator	Protein Source	Clone	Species	Source	Clone	Species	Source
IL-1β	R&D	2805	Mouse	R&D	Polyclonal	Goat	R&D
IL-6	BD	MQ2-13A5	Rat	BD	MQ2-39C3	Rat	BD
IL-12	R&D	24945	Mouse	R&D	Polyclonal	Goat	R&D
TNFα	BD	MAb1	Mouse	BD	MAb11	Mouse	BD

2.6 Gene expression analysis

Changes in gene expression were measured by quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR).

2.6.1 RNA extraction

750µl of TRIZOL (Invitrogen) was added to the cell pellet and stored at -80º until required. Frozen samples were thawed and 200µl of chloroform was added to each sample. After shaking for a few seconds, samples were left for 5 minutes at room temperature and then centrifuged for 15 minutes at 4^{o}C, 12,000g. The top aqueous layer was removed without getting any white precipitate (DNA/protein) found at the interface, and transferred to a new tube. 500µl of isopropanol was added and the sample was again mixed and left for 10 minutes at room temperature. After centrifugation (15 minutes at 4^{o}C, 12x1000g) the supernatant was removed. 180µl of 75% ethanol was added to the pellet and spun at 4^{o}C, 7.5x1000g for 5 minutes. The supernatant was discarded and the precipitated RNA was dried in a drying cabinet for 10 minutes.

The dried RNA pellet was resuspended in sterile distilled water (Sigma), according to RNA pellet size, typically in 10µl. After mixing, samples were placed in a 55ºC heat block for 10 minutes to fully dissolve the RNA. After spinning down the sample for 5 seconds, 1.5µl of RNA was placed on an accurate spectrophotometer Nanodrop machine (Agilent) to determine concentration by measuring absorbance of light at 260nm.

2.6.2 cDNA synthesis

RT-PCR is performed on a DNA template, so extracted RNA is 'converted' to single-stranded complementary DNA prior to PCR amplification. This was performed using a commercial reverse-transcriptase enzyme, Superscript II (Invitrogen). Reactions were performed according to the manufacturer's general instructions. If sample size allowed, 1μg of extracted total RNA (as determined by spectrophotometric measurement at 260 nm) and 250ng random hexamers (2.5μl of a 100ng/μl working solution) were added to a 1.5ml eppendorf tube, whilst on ice, and the total volume made up to 11μl with sterile distilled water. This was then placed at 70^{o}C for 10 minutes to denature mRNA and re-placed on ice. An 8 μl mix, containing 2μl 0.1 M DTT (Invitrogen), 4μl 5x Superscript II First Strand Buffer (Invitrogen), 1μl 10mM dNTP mix (Promega) and 1μl (24.5 units) RNaseOUT (an RNase inhibitor; Invitrogen), was added and placed at room temperature (25^{o}C) for 2 minutes. 1μl (200 units) Superscript II RT enzyme was added after this incubation step, the contents stirred and incubated for a further 10 minutes at 25^{o}C before the reverse-transcriptase reaction was subsequently allowed to proceed at 42^{o}C for 1 hour. The reaction was terminated by incubation at 70^{o}C for 15 minutes before 80μl sterile distilled water was added to bring the final volume to 100μl. cDNA samples were subsequently stored at -20^{o}C until use.

2.6.3 Real time polymerase chain reaction

SYBR green II (Invitrogen) was used as a minor binding groove dye to quantitate double stranded DNA amplified by PCR. 5μl of cDNA or water ('no-template' control;NTC), was added to 10μl x2 SYBR green master mix, which contained platinum *taq* DNA polymerase, SYBR green dye, Tris-HCL, 6mM $MgCl_2$, 400μM of dGTP, dATP, dUTP, dCTP, uracil DNA glycosylase, 1μM ROX reference dye. 1μl each of 10mM (0.5mM final) forward and reverse primer, and 3μl water were added to make a total volume of 20μl. Real time PCR was performed using the OPTICON PCR instrument (Biorad). Amplification was via an initial denaturation at 95^{o}C for 3 minutes to activate the hot-start Taq, with subsequent three-step cycling of 95^{o}C for 30 seconds, 57-62^{o}C (depending on the primer pair) for 30 seconds and 72^{o}C for 30 seconds. A final melt

curve procedure was also carried out routinely from 65^{o}C to 95^{o}C with 0.3^{o}C steps to help confirm amplification of a single PCR product. In addition to confirm the fidelity of the PCR reaction, 5µl of PCR reaction was electrophoresed on ethidium bromide-containing agarose gels in order to visualise the products and verify their size. In parallel, blank reactions without added cDNA were also performed in each PCR run in order to control for contamination. The number of threshold amplification cycles (CN_t) representing the first discernable amplification cycle (when product fluorescence was above background noise) was determined for each reaction using the Opticon software and the average of the two duplicates from each cDNA used for subsequent analyses. Target gene expression was analysed by the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). A summary of primer sequences and annealing temperature are shown in Table 2.3.

2.6.4 PCR primers

Primers used in this thesis were from the following sources

a) published papers; *b)* in-house design using Primer Select software (Lasergene DNAStar, Wisconsin, USA), initially with assistance from Dr J Diss, Molecular Biology Unit, ICH and *(c)* purchased pre-optimised from Quiagen (IRF-4, IL-23R only).

Primer pairs from the first 2 sources were tested to ensure that they:

1. Were T_m-balanced, i.e. possessed calculated annealing temperatures that were the same for both primers in the pair. Annealing temperatures were estimated by the equation:

   T_m = {2 x (sum of A and T nucleotides)} + {4x (sum of C and G nucleotides)}.

2. Had a GC content of ~50%, i.e. an annealing temperature of approximately 60^{o}C.
3. Yielded no strong primer pair-, self- or hairpin dimers.
4. Possessed “nucleotide runs” (repeated nucleotides of the same type, e.g. AAAA) of no more than four nucleotides.
5. Yielded specific products of 70-250 nt.
6. If possible, yielded products that spanned known intron positions.

7. Did not evidently or strongly amplify non-specific products (verified by checking the primer sequences against all published GenBank sequences using BLAST; Altschul *et al.,* 1997).

Table 2.3 Primer pairs utilised in real-time PCR assays

Target	Primer names	Primer sequences 5'-3'	Annealing temp (^{o}C)	Amplicon size (nt)
Actin	Actin F Actin R	AGA TGA CCC AGA TCA TGT TTG AG AGG TCC AGA CGC AGG ATG	**60**	**187**
β2M	β2M F β2M R	TGC TGT CTC CAT GTT TGA TGT ATC T TCT CTG CTC CCC ACC TCT AAG T	**60**	**80**
RORC2	RORC2 F RORC2 R	GAC CAC CCC CTG CTG AGA A GAC ATG CGG CCG AAC TTG A	**62**	**314**
T-bet	T-bet F T-bet R	CCC CAA GGA ATT GAC AGT TG GGG AAA CTA AAG CTC ACA AAC	**60**	**336**
AHR	AHR F AHR R	GGG TTT CAG CAG TCT GAT GT CCA GTG GCT TCT TCA ATT CC	**62**	**145**
IL-17A	IL-17A F IL-17A R	AAT CTC CAC CGC AAT GAG GA ACG TTC CCA TCA GCG TTG A	**60**	**94**
IFNγ	IFNγ F IFNγ R	TGA CCA GAG CAT CCA AAA GA TGT ATT GCT TTG CGT TGG AC	**60**	**146**

2.7 Recombinant cytokines

All recombinant cytokines and chemokines were purchased as lyophilised powders and were made up with sterile filtered PBS and 1% tissue culture grade bovine serum albumin (BSA, Sigma). IL-4 and GM-CSF (both R&D Systems, Abingdon, UK), CCL17, CCL20, CCL22, and CCL5 (Insight Biotechnology) were made up to a final concentration of 100μg/ml and TGFβ (R&D Systems) to 2μg/ml. IL-1β, IL-12 (both R&D), IL-23 (Ebioscience), IL-6 (BD PharMingen), IL-21 (GIBCO/ Invitrogen), were all made up to a final concentration of 5μg/ml.

2.8 Statistical analysis

Data were analysed using SPSS v14.0 (Chicago, Illinois, USA) and Graphpad Prism (San Diego, CA, USA). For non parametric data, comparison between groups was analysed by the Mann Whitney test if unpaired and Wilcoxon Matched pairs test, if paired. For parametric data, comparisons between groups was analysed by paired and unpaired t test, depending on the experimental conditions. To assess the relationship of IL-17+ cells with regulatory T cells skewed data were log transformed, and correlated by linear regression. Correlation of non-parametrically distributed variables was assessed using Spearman's Rank. Throughout this work, only probability of $p \leq 0.05$ was considered significant.

3：Investigating the migration of regulatory T Cells to the inflamed joint

3.1 Introduction

Regulatory T cells (Treg) expressing the master transcription factor, Foxp3, play a key role in the maintenance of peripheral tolerance in murine models of autoimmunity (Wing & Sakaguchi 2010). In CIA, a model of autoimmune arthritis, mice depleted of Treg with an anti-CD25 monoclonal antibody develop more severe arthritis, whilst adoptive transfer of Treg attenuates joint disease (Morgan *et al,* 2003). A role for Treg in childhood arthritis is supported by the link between the number of Treg detected within the joint and severity of clinical disease (de Kleer *et al,* 2004; Ruprecht *et al,* 2005). In this thesis, two related subtypes of JIA were studied: persistent and extended oligoarthritis. Those with persistent oligoarthritis have few inflamed joints and high rates of spontaneous remission when compared to the extended oligoarthritis subtype. In both of these types of arthritis, there is a marked enrichment of Treg, as a proportion of CD4+ T cells within the joint, compared to the numbers in peripheral blood (de Kleer *et al,* 2004). Remarkably, patients with persistent oligoarthritis had a significantly higher frequency of synovial Treg compared to the more severe extended oligoarthritis subtype, supporting a functional role for Treg in attenuating disease course. Treg numbers at the site of inflammation but not in peripheral blood correlated with clinical phenotype, which is consistent with murine data showing that Treg function *in vivo* is dependent on migration of Treg to the inflamed site (Andersen *et al,* 2008). Furthermore synovial Treg demonstrate potent suppression of effector T cell proliferation and cytokine secretion (de Kleer *et al,* 2004; Vercoulen *et al,* 2009).

The mechanisms that lead to the enrichment of CD25hi Treg within the inflamed joints of JIA patients are unclear. Possible explanations include increased rates of Treg proliferation within the joint, or reduced rates of T cell apoptosis, but data from the Wedderburn group have found no significant differences between SFMC and PBMC in terms of cell division or death (Hunter *et al*, 2010). An alternative consideration is that CD4+CD25- effector T cells at the site of inflammation may upregulate CD25 or FOXP3 expression, without necessarily gaining a regulatory phenotype. Murine CD4+CD25- cells cultured with TGFβ can upregulate Foxp3 and develop a phenotype and function that is akin to “natural” Treg which originate from the thymus (Chen *et al,* 2003). In the

human immune system, it is still not clear if FOXP3 expression generated by this system is sufficient for a regulatory phenotype (Wing & Sakaguchi 2010).

The active trafficking of Treg to the inflammatory site in response to local chemoattractants, is an alternate mechanism by which Treg could become enriched within the joint. In healthy adults, Treg express high levels of the chemokine receptor CCR4 and display specific chemotaxis to the corresponding ligands CCL17 and CCL22 (Iellem *et al,* 2001). CCL22 and CCL17 are detectable within the joints of JIA patients (Thompson *et al,* 2001) but it is not known if their Treg demonstrate chemotaxis to these ligands or if the CCR4 axis plays a role in mediating effector T cell trafficking to the joint. Previous work from the Wedderburn group demonstrated high levels of the ligands CCL3, CCL5 and CXCL10 within synovial fluid (Pharoah *et al,* 2006) and expression of the corresponding receptors, CCR5 and CXCR3 on SFMC T cells, suggesting a potential role for these chemotactic axes in Treg migration (Wedderburn *et al,* 2000).

The aim of this Chapter was to test the hypothesis that the CCR4 and CCR5 ligands CCL17, CCL22 and CCL5 may be instrumental in directing Treg trafficking to the inflamed joint by :

(1) comparing the chemotactic responses of CD25hi Treg taken from PBMC and SFMC of JIA patients to those obtained from PBMC of healthy controls.

(2) testing if CD25hi Treg taken from the joints of patients with JIA express a chemokine receptor profile consistent with their chemotactic properties *in vitro*.

(3) asking if synovial Treg, as defined by the expression of high levels of CD25, have a phenotype that is truly consistent with natural regulatory T cells.

3.2 Results

In several published studies of Treg chemotaxis *in vitro*, cells are first enriched for CD4+ T cells, using a magnetic bead-based method before application to the transwell (Iellem *et al*, 2001). As bead-based methods for CD4+ T cells enrichment have been optimised for PBMC samples, these conditions may not be optimal for SFMC. There are several reasons for this: the composition of SFMC is distinct from PBMC, such as a reversed CD4:8 ratio (Hunter *et al*, 2010; Thompson *et al*, 2001), and SFMC are prone to cell aggregation, perhaps due to high expression levels of adhesion molecules as observed on highly activated cells (Black *et al*, 2002; Thompson *et al*, 2001; Wedderburn *et al*, 2000). To test if magnetic bead based sorting of PBMC and SFMC samples led to comparable purity, CD4+ T cells purity before and after enrichment was assessed by flow cytometry.

3.2.1 Magnetic bead based purification of CD4+ T cells

Figure 3.1A illustrates the gating strategy used to assess the proportion of CD4+ T cells in unsorted PBMC. In this sample, CD4+ T cells made up 49.3% of live cells, which were enriched to 96.3% after bead-based sorting (Figure 3.1B). The enrichment was consistent with the expected purity suggested by the manufacturer.

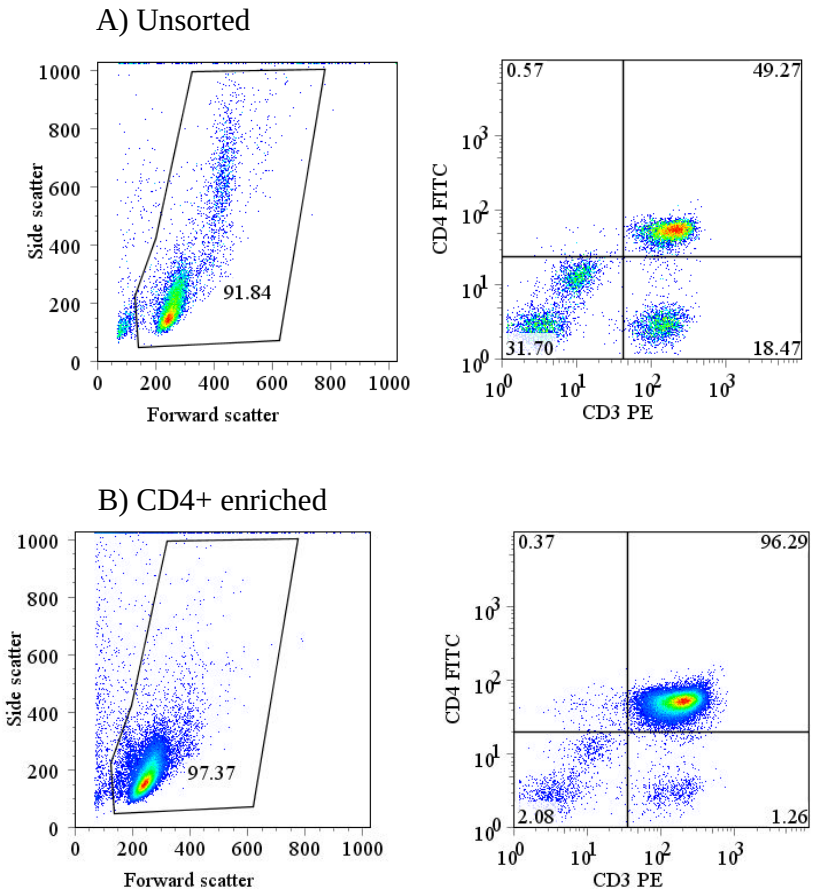


Figure 3.1: CD4+ T cell enrichment by magnetic bead-based negative selection.

Flow cytometry plots from A) unsorted healthy control PBMC, ungated (left) and gated on live cells showing expression of CD3 and CD4 (right). Similar plots for B) bead-enriched CD4+ T cells are shown. Numbers in quadrants represent percentage of parent population. Representative of n=3.

Enrichment of CD4+ T cells from healthy control PBMC showed consistently high purities (mean +/-SEM 94±1.6%, Figure 3.2A). In the case of JIA PBMC the results were less consistent and showed a trend for lower post sort purity than healthy control PBMC (81.6±6%, Figure 3.2B, $p=0.09$, unpaired t test). Sorting of SFMC to enrich for CD4 + T cells led to significantly lower purity than the results of sorted PBMC from healthy controls or patients (purity 56.5±5.3%, $p<0.01$ vs healthy control PBMC, $p=0.016$ vs JIA PBMC, unpaired t test, Figure 3.2C,D). Enrichment of CD4+ T cells from SFMC by flow cytometry would offer a greater purity, but in view of concerns over poor sample yield and to avoid prolonged sample handling, bead based enrichment was accepted as the method of choice for CD4+ T cell isolation for the following experiments on chemotaxis.

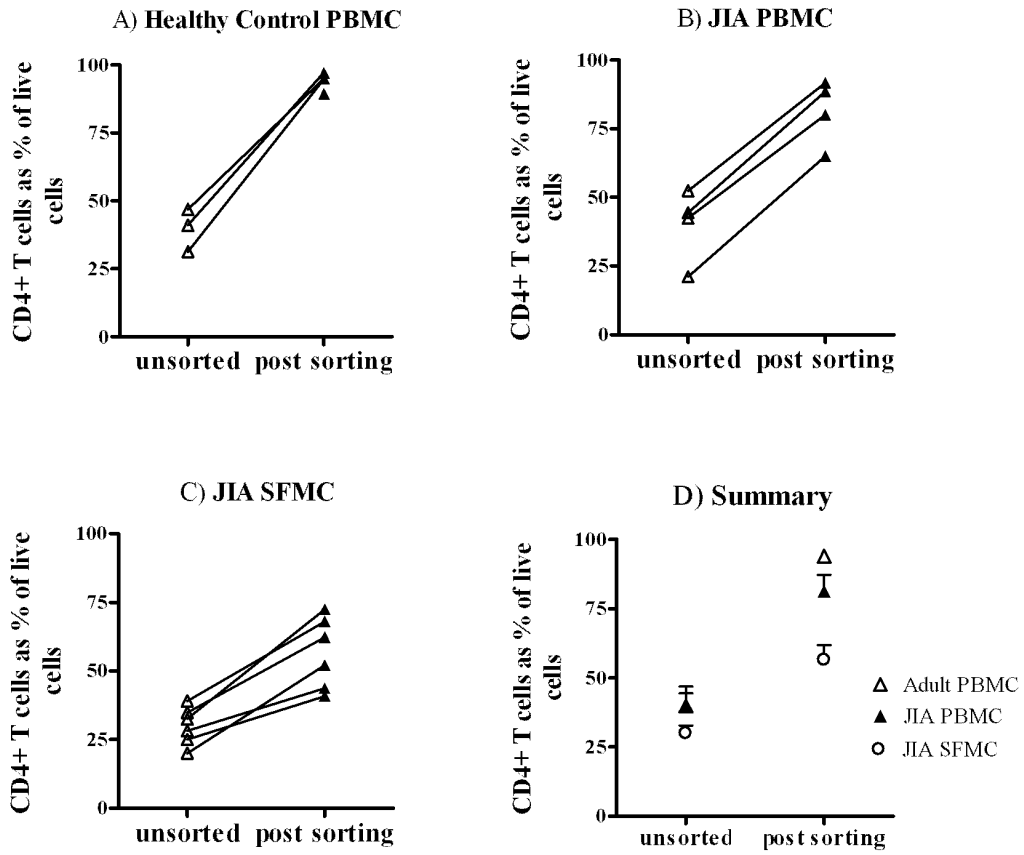


Figure 3.2: SFMC samples have reduced CD4+ T cell purity after magnetic bead-based enrichment.

The percentage of CD4+ T cells gated on live cells in starting population and after enrichment as assessed by flow cytometry as in Figure 3.1 for A) healthy control PBMC samples (n=3) B), JIA PBMC (n=4) C), JIA SFMC (n=6). The results for all 3 sample types are summarised in D, points represent mean + SEM.

3.2.2 Chemotaxis of PBMC to the chemokine ligand CCL22

Initially, healthy control PBMC were used to study the chemotaxis of CD4+ T cells to CCL22, using CD25hi CD4+ T cells as a surrogate for regulatory T cells. Intra-nuclear staining for FOXP3, although more specific for Treg (see Figure 3.8), was not yet in routine use when this work was initiated. The CD4+CD25hi fraction increased as a proportion of the total migrated cells after chemotaxis to CCL22 when compared to control medium (Figure 3.3).

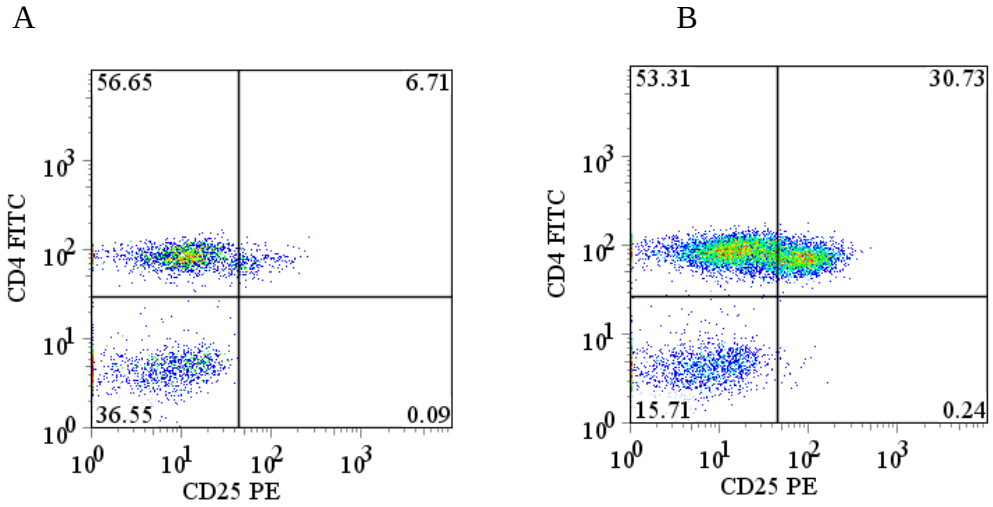


Figure 3.3: Migration of CD4+ T cells from healthy controls in response to the CCR4 ligand, CCL22.

CD4+ T cells from healthy controls were assessed for chemotaxis in response to A) control, 0.5% BSA, and B) 100ng/ml CCL22. Representative plots (1 of 4 independent experiments) showing CD4 and CD25 expression on cells that migrated across the transwell, as analysed by flow cytometry. All plot are gated on live T cells, numbers in quadrants represent percentage of parent population.

Iellem *et al* tested the chemotaxis of Treg from healthy adults to CCL22 at three concentrations, 10, 100 and 1000ng/ml (Iellem *et al*, 2001). There is little Treg chemotaxis at 10ng/ml CCL22 (chemotactic index = 2) whilst high levels of chemotaxis are demonstrated to 100ng/ml (chemotactic index 15) but without a further dose response to the highest concentration of 1000ng/ml. To test if a smaller increase in CCL22 concentration from 100ng/ml would lead to an increase in chemotaxis, 500ng/ml was directly compared with 100ng/ml. Figure 3.4 shows results from three separate healthy controls. Although there was considerable variation in the chemotactic index (defined in Chapter 2) between individuals, for the same donor the higher chemokine concentration did not show a significant enhancement in the chemotactic index. For the remaining experiments therefore, 100ng/ml CCL22 was the concentration of choice.

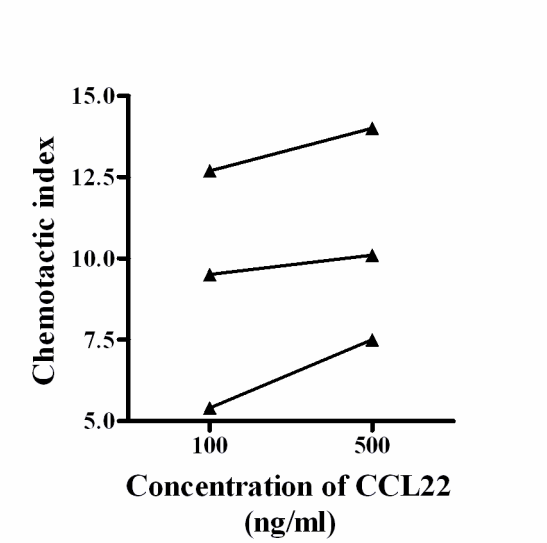


Figure 3.4: Migration of CD4+ T cells from healthy controls to 100ng/ml and 500ng/ml of the CCR4 ligand, CCL22.

Migration of CD4+ T cells from healthy control PBMC in response to 100ng/ml and 500ng/ml CCL22 was quantified by flow cytometry. Chemotactic index was calculated by dividing the number of CD4+CD25hi cells which migrated in response to CCL22 by the number which migrated to control, 0.5% BSA. n=3.

3.2.3 Comparison of Treg chemotaxis in JIA patients with healthy controls

Treg chemotaxis to CCL22 was tested in PBMC and SFMC of JIA patients and compared to healthy controls (Figure 3.5A). Treg from JIA PBMC demonstrated similar levels of chemotaxis to CCL22 as PBMC from healthy controls, but Treg from SFMC showed significantly higher rates of migration compared to Treg from JIA PBMC ($p=0.048$, unpaired t test; only significant differences $p\leq0.05$ are highlighted in this and all subsequent figures). Unexpectedly CCL17, an alternative ligand for CCR4 did not show this pattern. The rates of chemotaxis to CCL17 were similar for Treg from all three sample types, although lower in magnitude than comparable results from CCL22 (Figure 3.5B). Treg from control or JIA PBMC showed minimal chemotaxis to the ligand CCL5, (1.4 ± 0.4, 0.8 ± 0.2 respectively, Figure 3.5C) but there was trend towards higher migration rates in JIA SFMC (3.8 ± 1, vs control PBMC $p=0.17$, vs JIA PBMC $p=0.09$, unpaired t test), although this response was still much lower than SF Treg chemotaxis to CCL22 (Figure 3.5A,C).

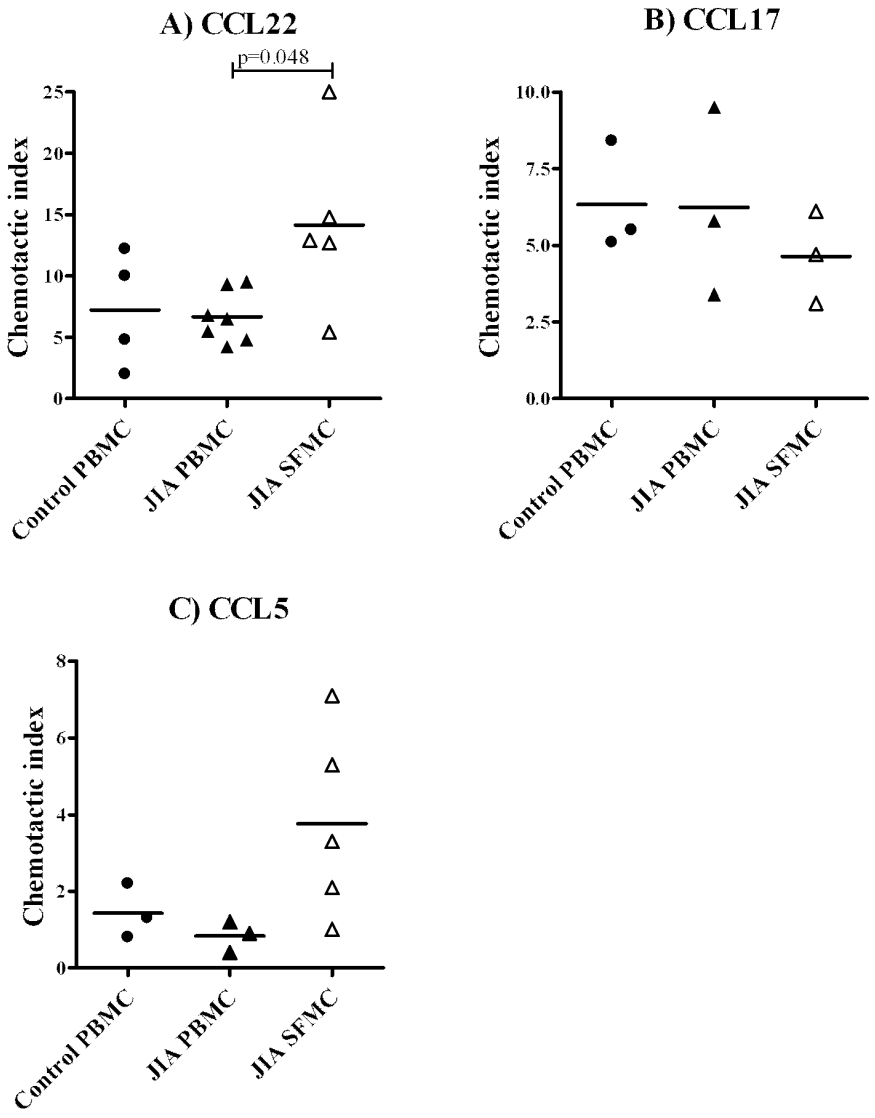


Figure 3.5: Comparison of CD4+CD25hi Treg migration from CD4+ T cell enriched PBMC and SFMC.

CD4+ T cells from healthy control PBMC, JIA PBMC and JIA SFMC (indicated on X axis) were assessed for chemotaxis to A) CCL22 (n=4 healthy controls, 7 JIA PBMC, 5 JIA SFMC), B) CCL17 (n=3 healthy controls, 3 JIA PBMC, 3 JIA SFMC), and C) CCL5 (n=3 healthy controls, 3 JIA PBMC, 5 JIA SFMC) all at 100ng/ml. The chemotactic index was calculated based on the number of CD4+CD25hi cells which migrated to chemokine compared to control medium. Lines represent means.

3.2.4 Chemokine receptor expression on Treg from the inflamed joint

Since synovial Treg displayed enhanced chemotactic responses to CCL22 and CCL5 when compared to PBMC of either controls or patients (Figure 3.5), it was predicted that synovial Treg would express higher levels of the specific chemokine receptors which mediate these responses. Expression of CCR4 and CCR5 (one chemokine receptor for CCL5, known to be highly expressed in SF effector T cells) was studied on CD25hi cells from control PBMC, JIA PBMC and SFMC. In addition two other chemokine receptors known to be involved in autoimmune arthritis, CXCR3, CXCR6 were also examined (Bosco *et al*, 2008; Wedderburn *et al*, 2000). Expression of CCR1 and CCR3, alternate receptors for CCL5 is not found on T cells from RA PBMC or SFMC and was not examined in this thesis (Ruth *et al*, 2001).

The gating strategy used to analyse chemokine expression on CD25- and CD25hi cells is illustrated in Figure 3.6, using CXCR3 expression as an example. Figure 3.7A shows that CCR4 was enriched on CD25hi when compared to CD25- cells in both control and JIA PBMC. In SFMC the differential expression of CCR4 between CD25- and CD25hi was maintained and also CD25hi SFMC had significantly higher expression of CCR4 than CD25hi cells from JIA PBMC. CCR5 expression was much lower in PBMC than SFMC as previously noted (Wedderburn *et al*, 2000). CD25hi cells from SFMC had significantly higher CCR5 expression than CD25- cells (Figure 3.7B). CXCR3 was also enriched within SFMC but there were no differences between Treg and the CD25- pool (Figure 3.7C). CXCR6 showed a similar pattern of results to CCR5, although the overall levels of expression were lower in the case of CXCR6.

In summary, a larger proportion of Treg from SFMC expressed the chemokine receptors CCR4 and CCR5 than from PBMC, results which are consistent with their increased rates of chemotaxis to CCL22 and CCL5 (Figure 3.5).

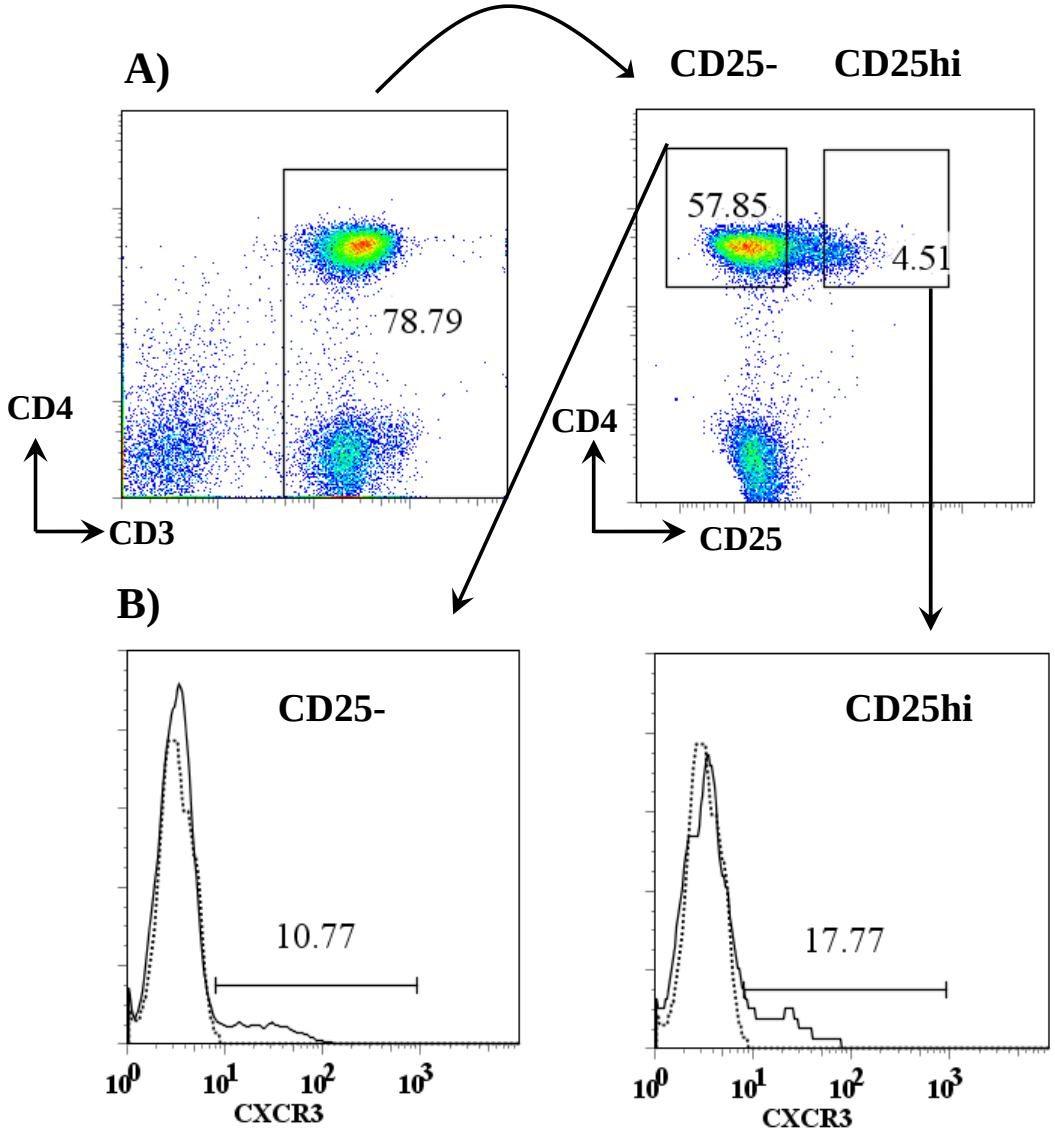


Figure 3.6: Gating strategy utilised to analyse chemokine receptor expression on CD25hi Treg.

PBMC from healthy control were stained with CD3, CD4, CD25 and CXCR3, and analysed by flow cytometry. A) (Left) Dot plot gated on live lymphocytes and (right) gated on T cells showing gates for CD25- and CD25hi cells. B) Histogram of CXCR3 expression on (left) CD25- cells and (right) CD25hi gated as in A. Dotted line represents isotype control. Numbers indicate percentage of parent population expressing CXCR3.

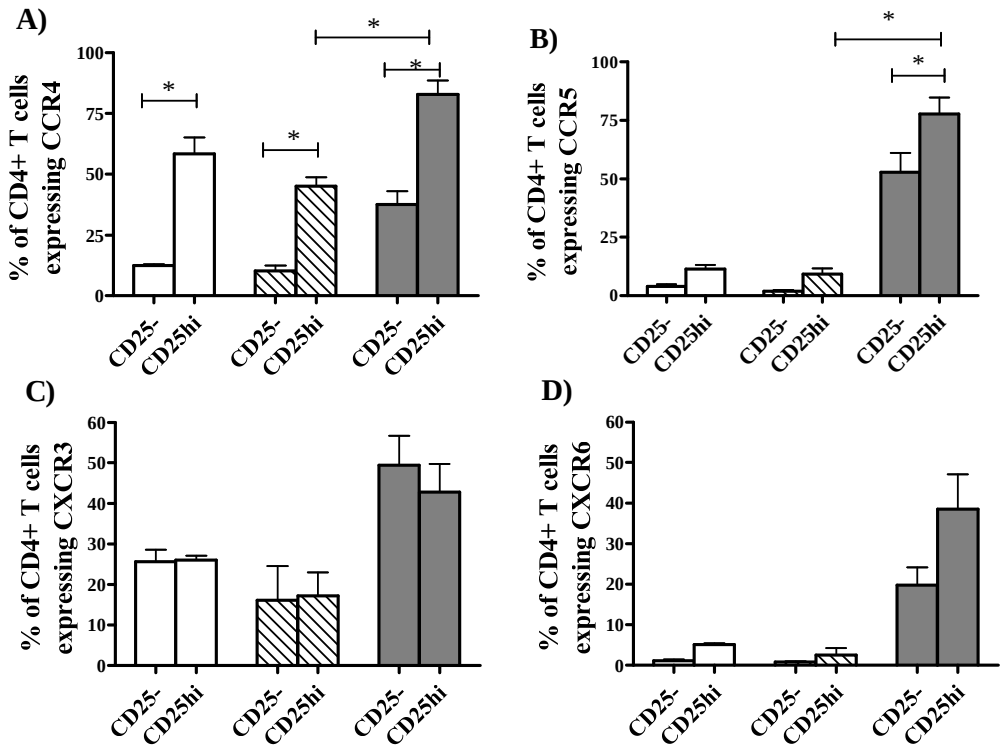


Figure 3.7: High proportions of synovial fluid regulatory T cells express inflammatory chemokine receptors.

Healthy control PBMC (open columns), JIA PBMC (hashed columns) and JIA SFMC (grey columns) were stained with CD3, CD4, CD25 and the indicated chemokine receptor, and analysed by 4 colour flow cytometry. The proportion of CD3+CD4+CD25- or CD3+CD4+CD25hi cells expressing the chemokine receptor (A) CCR4 (B) CCR5 (C) CXCR3 (D) CXCR6 is displayed. Columns represent mean and bars SEM. Control PBMC n=3, JIA PBMC n=5, SFMC n=5.*, $p<0.01$

3.2.5 FOXP3 expression is dissociated from CD25 within the joint

In the work presented to date, high levels of CD25 expression were used as a surrogate marker for the identification of FOXP3+ Treg. The CD25hi fraction of CD4+ T cells from the joint has been shown to express high FOXP3 mRNA levels (de Kleer *et al*, 2004). However CD25 intermediate and CD25 lo cells also express FOXP3, albeit at lower levels. The availability of a highly specific monoclonal antibody to FOXP3, allowed the relationship between CD25 and FOXP3 to be examined in more detail. In peripheral blood it was clear that FOXP3 protein expression co-localised with high levels of CD25, but in the joint the link between these two proteins was more complex. In SFMC (Figure 3.8), some cells expressed high levels of CD25 without FOXP3, and the converse was also true, CD25 negative cells that were FOXP3+.

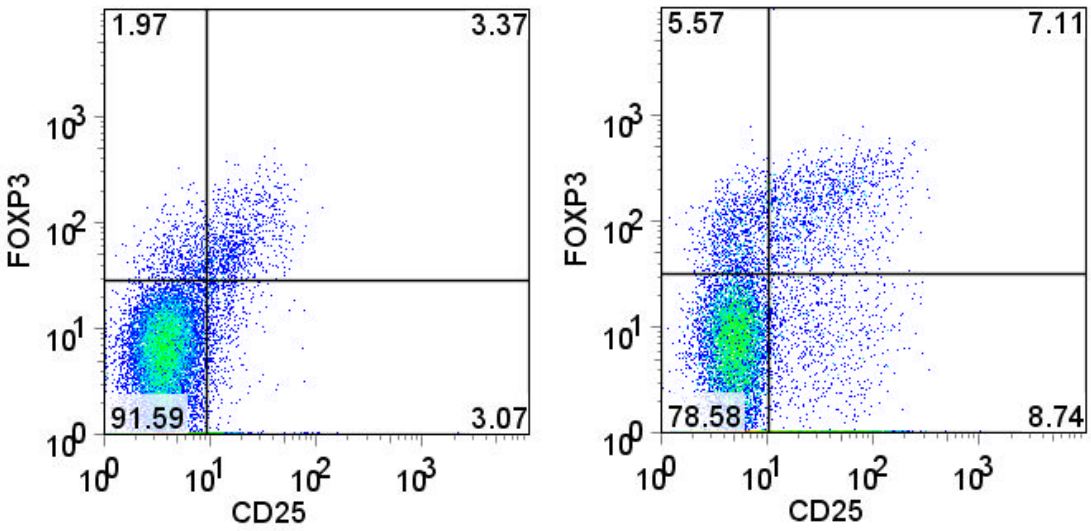


Figure 3.8: FOXP3 expression is dissociated from CD25 expression in SFMC.

Paired PBMC (left) and SFMC (right) from a JIA patient were stained for CD25 and FOXP3. Flow cytometry plots are gated on CD4+ T cells, percentages indicated proportion of parent population, representative of n=4.

The relationship between CD25 and FOXP3 in SFMC was highly variable between individuals and so a large number of SFMC samples were analysed for CD25 and FOXP3 expression. The correlation of Treg frequency as assessed by FOXP3 and CD25 is displayed in Figure 3.9A. A weak positive correlation between the two Treg markers was noted. To further analyse these data, the results were displayed on a Bland Altman plot (Figure 3.9B). This plot defines the difference in Treg frequency as assessed by FOXP3 and CD25hi on the vertical axis against the actual frequency of CD25hi cells as a percentage of CD4+ T cells, on the horizontal axis. A positive value on the y axis, indicates a higher frequency of Treg as defined by FOXP3 when compared to CD25hi cells. The graph shows that Treg frequency as assessed by CD25hi underestimated the number of Treg when defined by FOXP3 expression, particularly in samples with low frequencies of CD25hi cells (<10%).

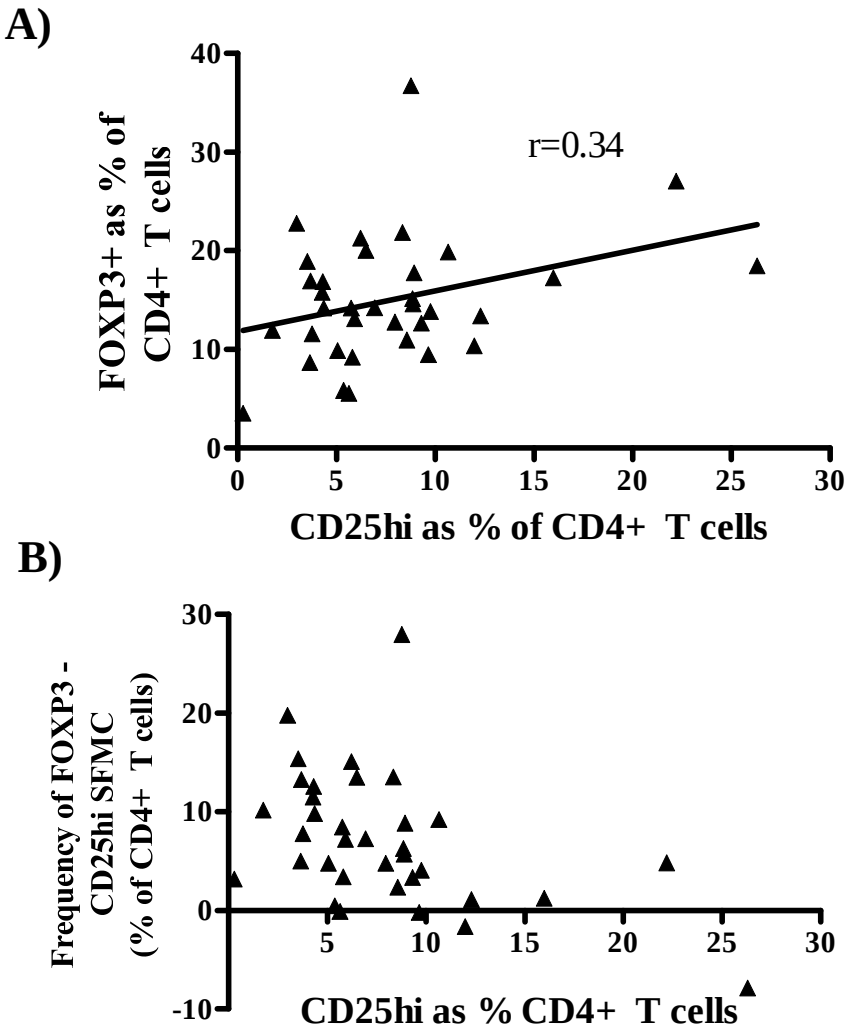


Figure 3.9: The enumeration of Treg in SFMC samples by FOXP3 and CD25 expression.

SFMC from JIA patients were analysed for FOXP3 and CD25 expression on CD4+ T cells by flow cytometry and comparison of the 2 variables presented as A) scattergraph and B) Bland-Altman plot, n=35, with assistance from Dr P Hunter, Wedderburn Laboratory, UCL, ICH.

3.2.6 Expression of CD127 on synovial Treg

The discrepancy between CD25 and FOXP3 expression within the joint, raises concerns that the earlier use of CD25hi expression as a surrogate marker of Treg, may not be valid

within the joint. Further reports also questioned the use of FOXP3 expression in marking Treg in the human system (Tran *et al,* 2007). Tran and colleagues demonstrated that human but not murine Treg upregulated FOXP3 expression following activation and this did not correlate with a regulatory function. Synovial CD25hi cells have been shown to suppress effector T cell proliferation *in vitro*, but this was analysed on a population level and does not exclude the possibility that a fraction of CD25hi or FOXP3+ cells within the joint are activated effector cells i.e., without a suppressive capacity (de Kleer *et al,* 2004). Given the presence of FOXP3+ cells within the joint which lack expression of the classical Treg marker CD25, it was important to test if these cells had a general phenotype consistent with bona fida Treg.

IL-7 is growth factor which is important in T cell homeostasis in the periphery (Welch *et al,* 1989). Expression of the alpha chain of the IL-7 receptor (IL-7Rα), CD127, has been shown to distinguish Treg from effector T cells. Following T cell activation, IL-7Rα is upregulated on effector T cells, in contrast to Treg which are IL-7Rα lo (Seddiki *et al,* 2006; Zhu *et al*, 2006). Expression of IL-7Rα was analysed on PBMC and SFMC (Figure 3.10). FOXP3+ CD4+ T cells from SFMC, like FOXP3+ cells from peripheral blood (Figure 3.10A), had an IL-7Rα lo phenotype (Figure 3.10B). Interestingly, SFMC had a population of IL-7Rα lo cells which were FOXP3-ve, and these cells were not present in PBMC. It is possible that activating conditions present within the joint may lead some effector cells to downregulate CD127 (Aerts *et al*, 2008).

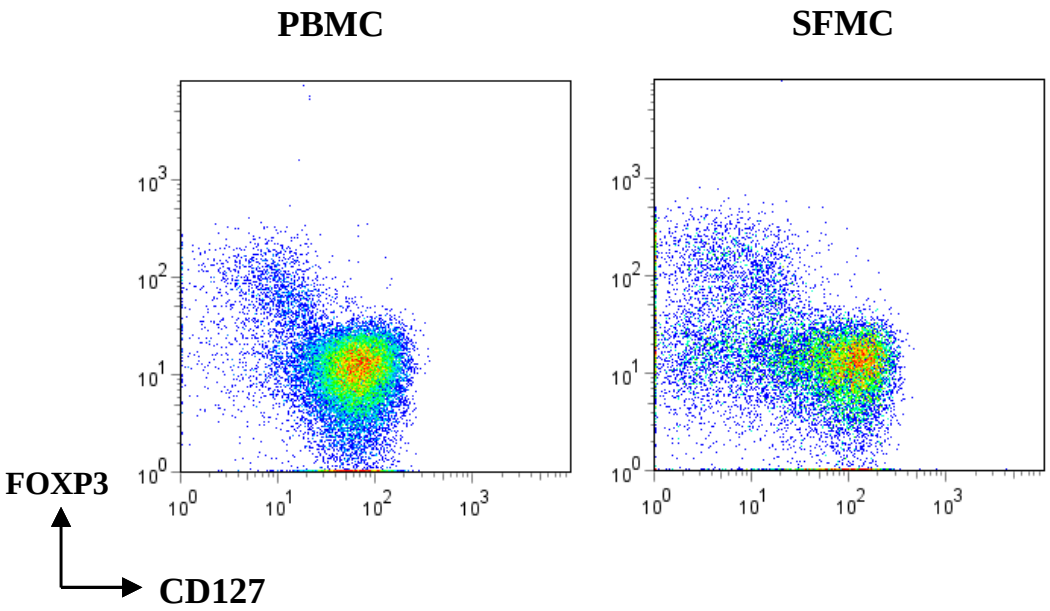


Figure 3.10: FOXP3+ cells within the joint have low expression of CD127.

Representative dot plot (of 3 independent experiments) of FOXP3 and CD127 expression in (left) PBMC from healthy control and (right) JIA SFMC as analysed by flow cytometry, gated on CD4+ T cells.

3.2.7 Cytokine expression in synovial Treg

Tran and colleagues compared the phenotype of natural FOXP3+ Treg with T cells activated *in vitro* that had upregulated FOXP3 expression (Tran *et al*, 2007). Induced FOXP3+ cells but not natural Treg expressed high levels of IL-2. To test if FOXP3+ SFMC were indeed IL-2 negative, PBMC and SFMC were stimulated with PMA and ionomycin in the presence of brefeldin A, prior to staining for FOXP3 and IL-2 (Figure 3.11). As expected, FOXP3+ CD4+ T cells from PBMC did not express IL-2. The results from SFMC confirmed that FOXP3+Treg within the joint matched their counterparts in peripheral blood and did not express IL-2, consistent with a true Treg phenotype.

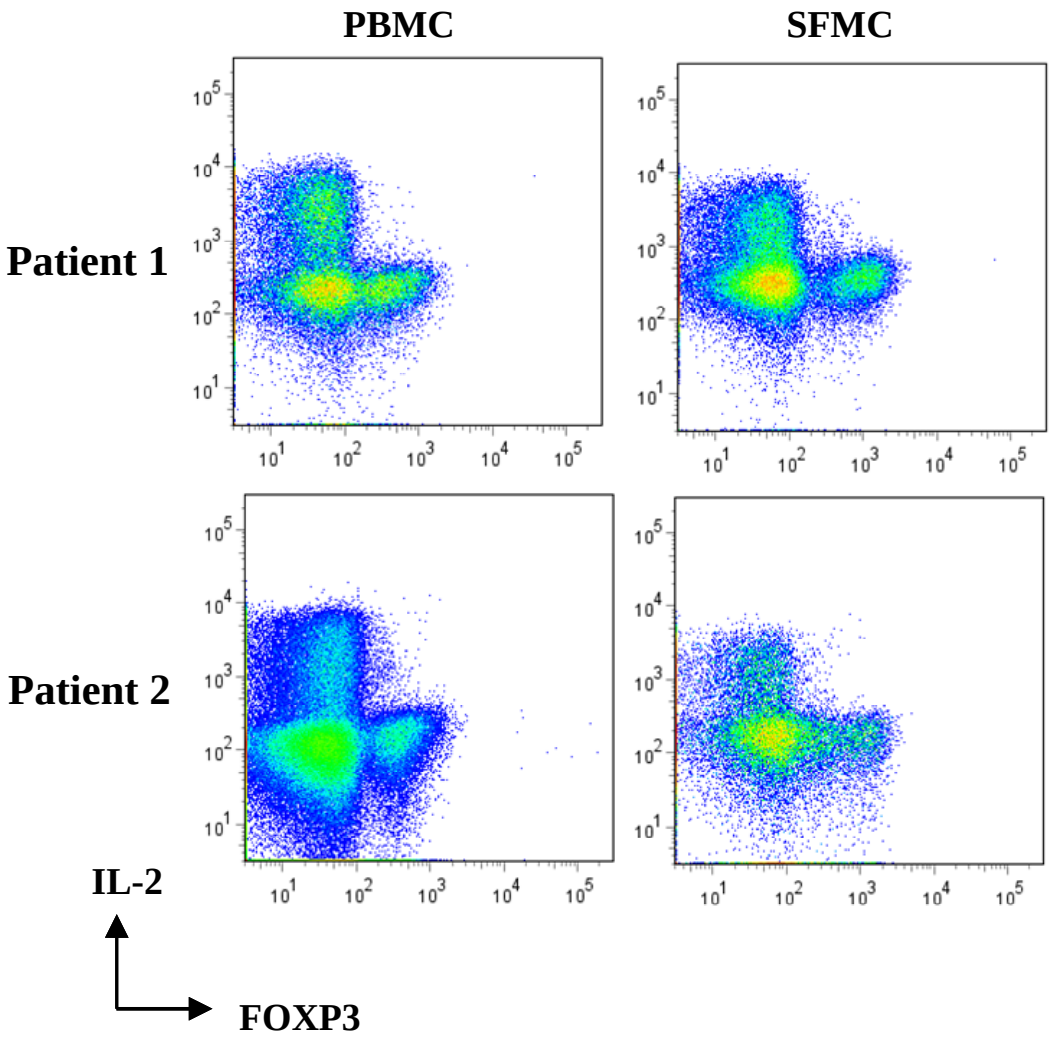


Figure 3.11 FOXP3+ cells from SFMC do not express IL-2.

(Left) PBMC and (right) SFMC from JIA patients were stimulated with PMA and ionomycin in the presence of brefeldin A. Dot plots gated on CD4+ T cells show IL-2 and FOXP3 expression as analysed by flow cytometry, in 2 JIA patients (representative of n=4).

Next, expression of the pro-inflammatory cytokine IFNγ was analysed in FOXP3+ cells from PBMC and SFMC (Figure 3.12). In PBMC samples, there was a clear separation of IFNγ and FOXP3 expression. In SFMC, the majority of cells had either IFNγ or FOXP3 expression. There were a small percentage of cells expressing low levels of both IFNγ and FOXP3.

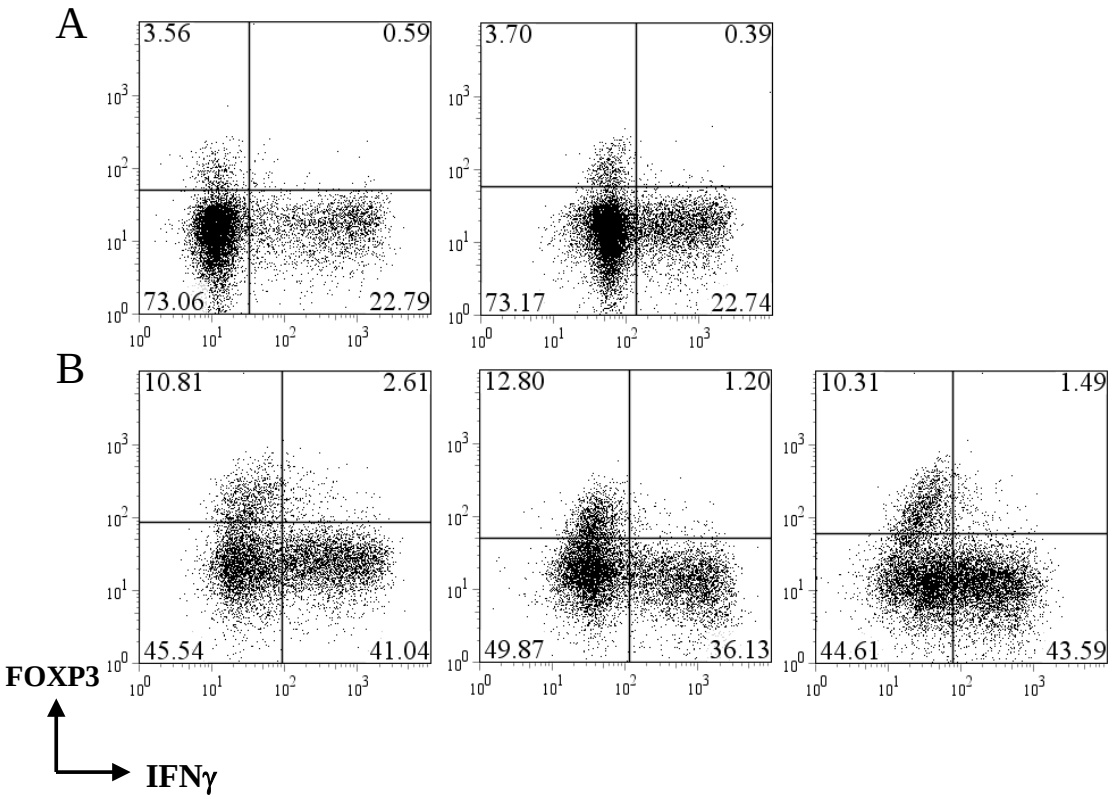


Figure 3.12 Analysis of IFNγ co-expression with FOXP3 in PBMC and SFMC.

A) PBMC from healthy controls and B) SFMC from JIA patients were stimulated with PMA and ionomycin in the presence of brefeldin A, and analysed for IFNγ and FOXP3 expression by flow cytometry. Dot plots are gated on CD4+ T cells and numbers represent percentage of parent population. (n=2 PBMC, n=3 SFMC).

3.2.8 Synovial Treg express CD45RO

FOXP3+ cells from within the joint, were CD127lo, IL-2 negative and the majority of cells IFNγ negative, a phenotype consistent with that of natural FOXP3+ Treg. The confirmation of a true Treg phenotype within the joint, helps validate earlier attempts to compare chemotactic activity between PBMC and SFMC Treg.

However another factor may have confounded comparisons of chemotaxis between SFMC and PBMC Treg. A study by Lim and colleagues, carried out in healthy adults, reported an enrichment of inflammatory chemokine receptors on CD45RO+ FOXP3+ Treg (Lim *et al,* 2006). Although CD45RO- and CD45RO+FOXP3+ Treg both exhibit non specific chemokinesis, chemotaxis is restricted to the CD45RO+ (memory) population. Therefore to test if Treg differed appreciably in the expression of the CD45

isoform between JIA PBMC and SFMC, samples were co-stained for FOXP3 and CD45RO (Figure 3.13). This confirmed that the majority of Treg found within the synovial compartment of patients with JIA, expressed CD45RO, as do the majority of synovial T cells (Wedderburn *et al,* 2000). Whilst in PBMC from patients CD45RO+ and CD45RO- Treg were in similar proportions (Figure 3.13).

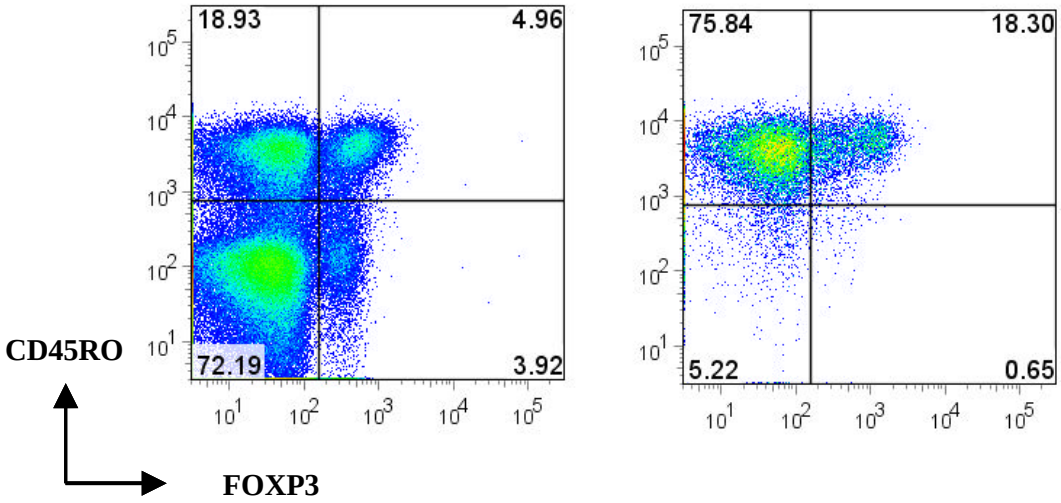


Figure 3.13 Synovial FOXP3+ Treg express CD45RO.

Dot plots of paired PBMC (left) and SFMC (right) from a JIA patient demonstrating FOXP3 expression against CD45RO. Both plots are gated on CD4+ T cells, numbers indicate percentage of parent population. Representative one plot of n=3.

3.3 Discussion

Over the last decade regulatory T cells have emerged as a distinct population of T cells that are central to the peripheral control of autoimmune responses. Important questions have been raised about site of action of Treg: some studies suggest that Treg act by preventing naïve T cell priming in the draining lymph node (Tang *et al,* 2006), while in others Treg have been shown to infiltrate the target organ and suppress T effector function at the site of disease (Belkaid *et al,* 2002). In the context of human arthritis, there is a clear enrichment of Treg within the joint (de Kleer *et al,* 2004), but at the inception of this thesis, little was known about the mechanisms that drive Treg to this site. As discussed in Chapter 1, lymphocyte trafficking is controlled by a range of processes, which include cell chemotaxis, a process largely driven by chemokines and their receptors. Chemokines direct cell movement by means of a concentration gradient towards the tissue. It was the hypothesis of this Chapter, that ligands of CCR4 and CCR5 would play key role in recruiting Treg to the joint.

3.3.1 Chemotaxis of CD25hi Treg to the joints of JIA patients

Firstly, a transwell migration assay was optimised to assess Treg chemotaxis *in vitro*. In accordance with published methods (Iellem *et al,* 2001) PBMC and SFMC were first enriched for CD4+ T cells before chemotaxis was assessed. Bead based sorting of SFMC led to a lower purity of CD4+ T cells when compared to healthy control or JIA PBMC (Figure 3.2). A comparison of the Treg chemotaxis using unsorted PBMC or CD4+ T cells as the starting population, found higher rates of Treg migration in the CD4+ enriched population (data not shown). The explanation for this observation is unclear, but may relate to competition by non-T cells, in the unsorted population. This suggests that the poor enrichment of CD4+ T cells from SFMC, could underestimate true SFMC Treg migration rates, and needs to be considered in the interpretation of the results of this study.

When Treg chemotaxis to CCR4 ligands was compared between sample types, SFMC showed greater rates of migration to CCL22 than PBMC (Figure 3.5). Surprisingly, there was no difference in Treg migration between the synovial and peripheral blood

compartments in response to the CCR4 ligand, CCL17. The discordant responses of SFMC to the CCR4 ligands, CCL22 and CCL17 is unexpected as other studies have shown that these two ligands have similar potency to induce Treg chemotaxis (Iellem *et al,* 2003) and the ligands signal by means of the same pathway (Cronshaw *et al,* 2006). As there was a large variation in the degree of Treg chemotaxis between individuals, a larger cohort is needed to confirm this finding of differential SFMC responses to CCL22 and CCL17. At present there are no published studies examining the migration of synovial T cells to CCR4 ligands.

Thompson and colleagues found that CCL22 and CCL17 proteins are detectable within the joints of patients with JIA (median 105 and 591 pg/ml respectively) (Thompson *et al,* 2001), but at concentrations that are several log lower than those used in the *in vitro* chemotaxis assay. CCL17 is present at a higher concentration than CCL22 within the joint, but given the greater potency of CCL22 to induce Treg chemotaxis *in vitro,* CCL22 may still be the dominant CCR4 ligand involved in the recruitment of Treg to the joint. However there is no enrichment of CCL22 and CCL17 levels in synovial fluid when compared to paired plasma. Although these data suggests the absence of a clear chemokine gradient between blood and synovial fluid, it is possible that the enrichment of CCR4 ligands in the micro-environment adjacent to synovial capillaries is more important than levels in synovial fluid itself.

Treg chemotaxis to the CCR5 ligand, CCL5 was also enhanced in SFMC when compared to PBMC from healthy controls or JIA patients. This result contrasts with the only published report in RA patients which found the chemotactic response of synovial T cells to CCL5 was attenuated when compared PBMC (Hisakawa *et al,* 2002). However Hisakawa and colleagues did not distinguish the specific chemotaxis of synovial Treg from other T cell subsets.

CCL5 is enriched within the joints of JIA patients and also patients' serum (Pharoah *et al,* 2006; Yao *et al,* 2006), when compared to serum from healthy controls. CCL5 is important in the migration of Th1 effector cells to the joint (Shadidi *et al,* 2003) as evidenced by the marked diminution of the chemotactic activity of SF supernatants on mononuclear cells, after treatment with anti-CCL5 antibodies (Yao *et al,* 2006). There is a body of evidence to support a pro-inflammatory role for CCL5 in JIA. Serum CCL5

levels correlate with serological indices of inflammation and high levels are linked with shorter periods of remission (Yao *et al*, 2006). JIA patients with a CCL5 promoter polymorphism (-28G/G) express higher CCL5 and have earlier relapse of disease and shorter responses to intra-articular steroids (Yao *et al*, 2006). Although Treg showed a chemotactic response to CCL5, it is likely that effector cells would show a similar or possibly greater response and may explain the association of CCL5 with disease activity in JIA.

Herein lies a paradox of lymphocyte trafficking, in that ligands responsible for the trafficking of both effector and regulatory T cells are detectable within the joint. From an evolutionary perspective, to successfully respond to infection T effector and Treg recruitment needs to be temporally spaced. If Treg arrive at the site of infection simultaneously with effector cells, the host response will be muted (Yurchenko *et al*, 2006), and yet the failure of the Treg influx could lead to persistence of inflammation and autoimmunity. The complexity of this interplay has been studied in mouse models, examining recruitment mediated through the chemokine receptor CCR2. Early blockade of CCR2, which is expressed on both Treg and T effectors, during days 0-15 after the initiation of collagen induced arthritis ameliorates disease, as the effector T cell influx is prevented. Blockade of CCR2, on days 21-36, leads to a deterioration of arthritis, as the recruitment of CCR2+ Treg is prevented (Bruhl *et al*, 2004). So the timing of T cell migration may be the key to distinct effector and regulatory phases within the target organ. It is unclear if this staged influx also applies in the context of a chronic inflammatory process such as that seen in JIA.

3.3.2 The role of chemokine receptor expression in regulating Treg trafficking

It was hypothesised that the enhanced chemotaxis of synovial Treg was due to the enrichment of chemokine receptors on cells from this compartment. Indeed all CD4+ T cells from the joint expressed higher levels of the chemokine receptors tested, when compared to equivalent populations from control and JIA PBMC. This was most notable for CCR5 (Figure 3.7). More specifically, Treg as defined as CD25hi CD4+ T cells from the joint had higher expression of CCR4 and CCR5, than equivalent Treg from the blood

of JIA patients. A similar pattern of results has been reported in RA patients (Jiao *et al,* 2007). The functional consequence of this increased density of chemokine receptors has been examined using Jurkat cell lines transduced with CCR5 (Desmetz *et al,* 2007). Cells expressing CCR5 at an increased density had greater chemotactic responses to synoviocyte supernatants. Multiple murine models of autoimmune disease have confirmed a critical role for CCR4 and CCR5 in recruiting Treg cells to inflamed tissues (Lee *et al,* 2005; Yuan *et al,* 2007). However, as discussed earlier, other models have suggested that Treg homing to lymph nodes (by means of CCR7) is important to mediate suppression of autoimmunity (Menning *et al,* 2007). A recent study using the islet allograft diabetes model helped resolve this apparent paradox by tracking the migration of adoptively transferred GFP-labelled Foxp3+ Treg (Zhang *et al,* 2009). CCR2, CCR4, CCR5 and P- and E-Selectin ligand expression on Treg was found to be essential for Treg trafficking to the inflamed site, in order to suppress alloimmunity. In the allograft, Treg were activated and then migrated to the draining lymph node in a CCR2, CCR5 and CCR7 dependent manner to inhibit DC and effector T cell migration to the site of inflammation. Consistent with these studies, the data in this chapter supports a role for CCR4 and CCR5 and their ligands, in driving Treg recruitment to the joints of children with JIA.

3.3.3 Successfully identifying Treg in the synovial compartment

The discussion of the data presented has assumed that CD25hi cells within the joint correspond to the Treg population found in PBMC. In mice the IL-2Rα, CD25, successfully identifies Foxp3+ Treg, however in humans up to 30% of CD4+ T cells express CD25 (Baecher-Allan *et al,* 2001). The CD25hi fraction of CD4+ T cells in the peripheral blood of healthy controls contains the majority of FOXP3+ suppressive Treg in humans (Baecher-Allan *et al,* 2001). However in this thesis it was clear that even CD25hi expression did not closely correlate with FOXP3 expression in the joint, and indeed underestimated the proportion of FOXP3+ Treg. In humans, FOXP3 expression is upregulated following activation of T cells and can be dissociated from regulatory function (Allan *et al,* 2007; Tran *et al,* 2007). These data raise questions about the role of FOXP3 as a marker of Treg in SFMC. Tran and colleagues suggested that a particular

anti-FOXP3 monoclonal antibody clone, PCH101 was responsible for the above discrepancy between FOXP3 expression and function, but this suggestion has been disputed by others (Pillai & Karandikar 2008). In this study all staining for FOXP3 protein was carried with the anti-FOXP3 clone, PCH101. The absence of IL-2 expression is a consistent feature of Treg in mice and humans. So to test whether FOXP3+ cells within the joint had a phenotype consistent with Treg, SFMC were co-stained for IL-2 and FOXP3. Indeed all synovial FOXP3+ cells were IL-2 negative, and expressed low levels of CD127, both consistent with a true Treg phenotype.

More recently it has been suggested that natural or thymic Treg can be divided into functionally distinct subsets. Markers have included the ectonucleosidases CD39 and CD73 (Moncrieffe *et al*, 2010), the class 1 antigen, HLA-DR (Beriou *et al*, 2009) and isoforms of CD45. Detailed analysis of CD45 expression on Treg subsets indicates that naïve CD45RA+FOXP3lo Treg transition *in vivo* to a highly suppressive effector Treg phenotype, expressing CD45RO and high levels of FOXP3 (Miyara *et al*, 2009). Both populations are functional in a standard *in vitro* suppression assay. Further proof of this "true" Treg phenotype comes from the examination of the methylation status of the FOXP3 gene. It has been recently shown that the 5′ flanking region and a STAT5-responsive region in the intron 1 of FOXP3 are critical for the induction and enhancement of FOXP3 expression (Floess *et al*, 2007). Both of the Treg populations described above are almost completely demethylated at the loci described, which maintains FOXP3 in a transcriptionally active state. In contrast to these populations, a further population of FOXP3lo CD45RA- cells is expanded in the peripheral blood of patients with autoimmune disease. These cells express relatively high levels of IL-2 and IFNγ, are poorly suppressive *in vitro*, and are partially methylated at the FOXP3 locus. Taken together, this last population shares features with Treg "induced" *in vitro* in response to TGFβ described by Tran and colleagues rather than bona fida, or "natural" Treg. It is interesting that a small population of FOXP3+ cells within the joint did express IFNγ, and these cells typically had lower levels of FOXP3 protein. It is possible that a proportion of synovial Treg are similar to the FOXP3lo CD45RA- cells described by Miyara and colleagues. The methylation status of Treg within the joint will help answer this important question.

Returning to the earlier comparison of chemotaxis of CD25hi Treg from the joint and blood of JIA patients it is now clear that several confounders need to be considered. Firstly, CD25hi cells from the joint only make a proportion of FOXP3+ Treg present at the inflammatory site. Secondly, the majority of Treg within the joint are CD45RO+FOXP3hi which is linked with higher chemokine receptor expression and greater rates of chemotaxis than CD45RO-FOXP3int cells. This latter population are more numerous in PBMC. As a result, the chemotactic receptor expression and rates of chemotaxis for CD45RO+FOXP3hi cells needs to be assessed in paired PBMC and SFMC. This would enable a true "like for like" comparison between compartments and help test if Treg from SFMC exhibit greater chemotaxis than PBMC.

3.3.4 Summary

CD25hi Treg that are detectable within the joints of children with inflammatory arthritis have enhanced expression of the inflammatory chemokine receptors, CCR4 and CCR5, and demonstrate greater chemotaxis to their ligands than Treg from the peripheral blood of JIA patients. These data suggest the CCR4 and CCR5 axes may be important in the enrichment of Treg within the JIA joint. Given the enhanced suppressive capacity of synovial Treg when compared to Treg from peripheral blood of JIA patients (de Kleer *et al,* 2004; Vercoulen *et al,* 2009) it remains unclear why arthritis continues unchecked. Some studies have indicated that Treg are defective at suppressing pro-inflammatory cytokine production in RA (Ehrenstein *et al,* 2004; Sugiyama *et al,* 2005), but this has not been found in JIA (Vercoulen *et al,* 2009). It is possible that Treg function is attenuated by local dendritic cell derived cytokines such as IL-6 (Pasare & Medzhitov 2003), or other inflammatory ligands (Peng *et al,* 2005) that may be present in the joint. Finally the lack of suppression may not be related to an intrinsic Treg defect but to the potency of effector T cells at the inflammatory site, which resist suppression (Korn *et al,* 2007; Zhu *et al,* 2006). The emergence of Th17 cells as a novel pro-inflammatory population of T cells, important in murine models of arthritis, led attention to be turned to these cells and their role in JIA.

4：The frequency of Th17 cells in JIA joints correlates with clinical severity

4.1 Introduction

IL-17, a pro-inflammatory cytokine first identified in 1993 (Rouvier 1993) acts on a wide range of cells including cells found within the joint lining and stroma, contributing to the cartilage and bone damage seen in autoimmune arthritis (Chabaud *et al,* 2001a). IL-17 stimulates synovial fibroblasts to release matrix metalloproteinases (MMP) which directly breakdown cartilage (Koshy *et al,* 2002). IL-17 synergises with IL-1β by inducing RANKL and its receptor, RANK which increases osteoclast differentiation leading to bony erosions (Lubberts *et al,* 2003). IL-17 also synergises with TNFα to induce pro-inflammatory cytokines and chemokines from monocytes and cultured synovial fibroblasts (Chabaud *et al,* 1998; Jovanovic *et al,* 1998; Katz *et al,* 2001).

The CD4+ T cell subset (Th17) that secrete IL-17 was identified in mice in 2005 (Park *et al,* 2005). Consistent with data on the cytokine IL-17, Th17 cells are thought to be critical to the pathogenesis of CIA, a murine model of autoimmune arthritis (Murphy *et al,* 2003). Although Th17 cells are the dominant pathogenic population in the CIA model, the prevalence and characteristics of these cells in human inflammatory arthritis had not been investigated at the time this thesis started (Joosten *et al,* 2008; Nakae *et al,* 2003b).

The aims of the studies described in this Chapter were to:

(1) Identify IL-17 secreting CD4+ T cells within the synovial membrane and fluid of patients with JIA.

(2) Investigate any potential relationship between Th17 cell frequency and clinical phenotype in childhood arthritis.

(3) Understand mechanism(s) of recruitment of Th17 cells to the joint, by studying their chemotactic responses *in vitro.*

(4) Characterise the expression of key transcription factors within the synovial Th17 population.

4.2 Specific method

4.2.1 Immunohistochemistry

Synovial tissue samples were collected from JIA patients undergoing surgery for joint replacement, snap frozen in liquid nitrogen and stored at -80ºC. 7µm tissue sections were cut using a cryostat at –20ºC and collected on poly-l-lysine coated slides (BDH, VWR, Leicester, UK). Sections were air dried at room temperature for a minimum of 2 hours (or overnight) and fixed in 100% acetone (BDH) at room temperature. Slides were wrapped in foil and stored at -80ºC. Slides were thawed and sections circled with a wax pen (DAKO) to create a water-resistant well for staining. Slides were washed in PBS for 3 minutes. Excess PBS was shaken off, and to quench any endogenous peroxidise activity, 100-150µl of 3% H_2O_2 (BDH, 250µl H_2O_2 in 2.5ml PBS) was applied for 7 minutes. Slides were washed a further three times in PBS and non-specific binding was blocked using a serum free protein block (DAKO) for 10 minutes at room temperature. The block was shaken off and primary antibodies (Table 4.1), made up in 100µl 0.1%BSA (Sigma) in PBS at the specified dilution, were added and incubated for 30 minutes at room temperature or overnight at 4ºC. Slides were washed three times with PBS and the secondary antibody (Table 4.1) added at the appropriate concentration for 30 minutes at room temperature. Sections were washed three times with PBS.

A streptavidin-biotinylated horseradish peroxidise (HRP) complex (DAKO) was prepared in accordance with manufacturer's instructions. Briefly, to 5mls Tris-buffered saline (0.05mol/L Tris/HCL, 0.15mol/L NaCl pH 7.6, Sigma) 1 drop each of streptavidin and HRP reagents were added and mixed. 100µl of the complex was added and sections incubated for 30 minutes. Slides were washed three times in PBS and incubated in a chromogen substrate (3,3'-diaminobenzidine, DAB, Sigma) which reacts with HRP and was visualised as brown staining. Sections were washed in running water and counterstained with Harris haemotoxylin (BDH) for 1 minute, washed in running water, then dipped briefly in acid alcohol (1% HCL in 70% Ethanol (Sigma)). After washing in running water, sections were dehydrated progressively in 70% Ethanol, 100% ethanol and in 100% Zylene (all BDH) and then mounted in a synthetic resin (DPX, BDH) and inspected by light microscopy.

Antibody specificity (or reagent)	Species in which antibody is raised	Company	Clone	Working dilution
CD4	Mouse	Novacastro, Newcastle upon Tyne	4B12	1/50
IL-17	Goat	R&D	AF-317-NA	1/20
Biotinylated anti-goat Ig	Rabbit	Dako	n/a	1/200
Biotinylated anti-mouse-Ig	Donkey	Chemicon, Billerica, MA	n/a	1/500

Table 4.1 Antibodies used for Immunohistochemistal analysis.

4.3 Results

4.3.1 IL-17 is present in high levels in synovial fluid and membrane

Early data from the Prakken group, generated by the measurement of IL-17 using a bead based multiplex cytokine assay, suggested that IL-17 was higher in the plasma of JIA patients compared to SF (de Jager *et al,* 2007). This was unexpected and contrasts with data from patients with RA (Kotake *et al,* 1999). To further clarify this, IL-17 was measured by ELISA in serum and SF from JIA patients. Using this detection method, a significant elevation ($p<0.01$, unpaired t test) of IL-17 in JIA SF compared to serum was observed (Figure 4.1).

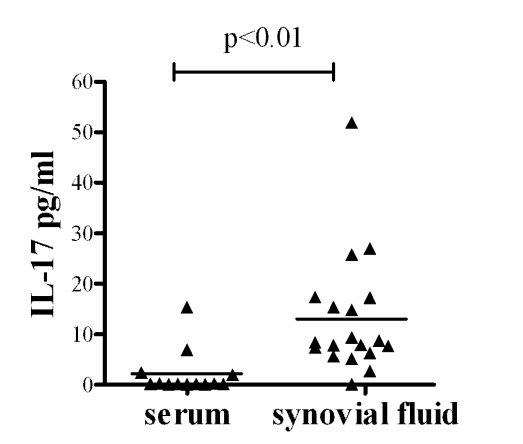


Figure 4.1: IL-17 levels in patients with JIA are higher in synovial fluid and than in serum.
Serum (n=13) and SF (n=19) samples from JIA patients were analysed for IL-17 protein levels by ELISA. Lines represent median levels.

In the pathophysiology of JIA, the normal synovial membrane, undergoes marked hyperplasia due to an accumulation of macrophages and fibroblast-like synoviocytes in the lining layer, and in the sub-lining layer, there is infiltration of T cells, plasma cells, mast cells and natural killer (NK) cells (Nistala *et al*, 2009). In order to identify CD4+ T cells that secrete IL-17 within the synovial membrane, serial sections of synovial tissue taken from the joints of JIA patients were stained for IL-17 and CD4 expression. A high frequency of IL-17+ cells co-localised to areas infiltrated with CD4+ T cells (Figure 4.2).

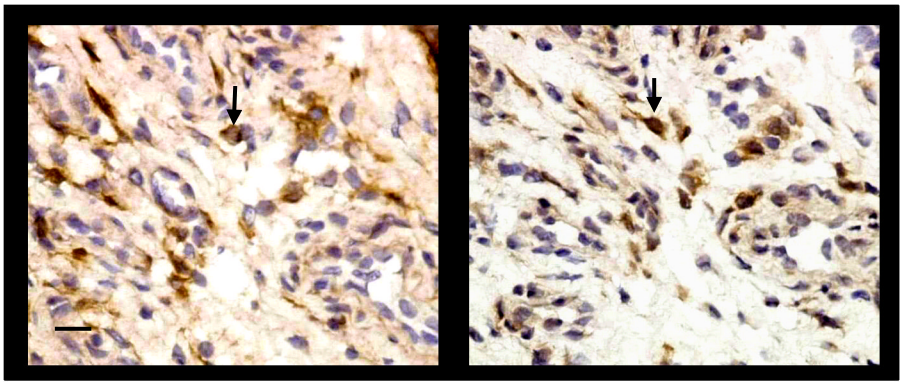
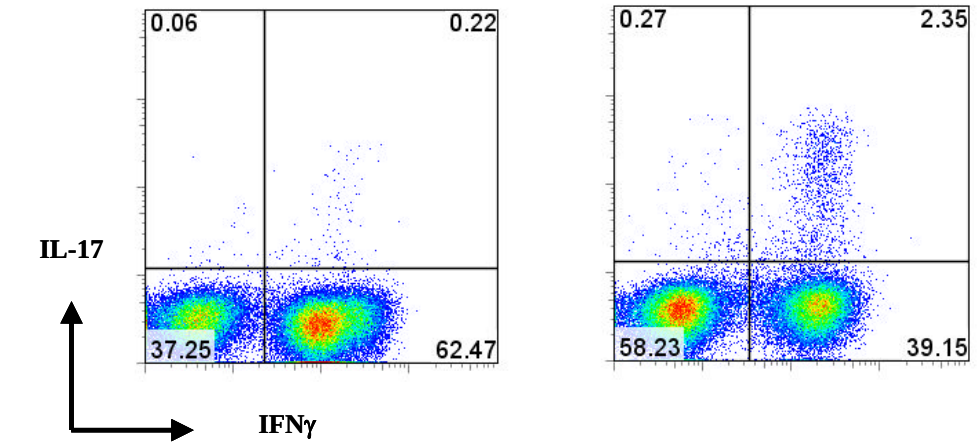


Figure 4.2: IL-17 detection in the synovial membrane of JIA patients.
Immunohistological localisation of IL-17 in JIA synovial tissue. Immunohistochemical staining for CD4 (left panel), IL-17 (right panel) on frozen synovial biopsy tissue from a child with polyarticular JIA. Panels show sections with typical hypertrophied synovium and dense inflammatory infiltrate. Positive cells which express the marker of interest are stained brown and are highlighted by an arrow in each panel. Scale bar represents 20 μm. One representative of 3 synovial biopsies is shown. (x40 magnification, performed with the assistance of H Varsani, ICH, UCL).

In parallel with the detection of T cells in synovial tissue, T cells expressing IL-17 after stimulation with PMA and ionomycin in the presence of brefeldin A were also enumerated in PBMC and SFMC. Cells were stained for CD3, CD4 and IL-17 and analysed using flow cytometry. Complementary to the IL-17 protein expression by ELISA and histology results, a clear enrichment of IL-17-producing T cells within SF compared to peripheral blood of JIA was demonstrated (Figure 4.3). The large majority of IL-17 positive T cells were within the CD4+CD3+ population (Figure 4.3A). Within the peripheral blood of JIA, the median frequency of IL-17 producing cells was 0.43% of total CD4+ T cells (inter-quartile range, IQR, 0.3-0.9) and this proportion did not differ significantly from those in healthy controls (median 0.67%, IQR 0.5-0.8). In contrast the frequency of synovial IL-17+ CD4+ T cells was significantly increased (Figure 4.3B left hand plot, median 1.2%, IQR 0.7-2.6) compared to the peripheral blood of both JIA and controls ($p<0.01$, $p=0.018$ respectively, Mann-Whitney) Although this difference was seen across all subtypes of JIA, the degree of enrichment varied between clinical phenotypes. Analysis of the frequency of IL-17+ CD4+ T cells by subtype revealed a relationship with disease severity, i.e., greater enrichment of IL-17+ T cells with the

more severe form of JIA was observed. Thus samples from children with extended oligoarticular JIA (more severe form) had a significantly higher frequency of synovial CD4+ IL-17+ cells (Figure 4.3B, right hand plot, median 2.1, IQR 1.3-4.8) compared to the milder form of disease, persistent oligoarticular JIA (median 0.91, IQR 0.6-2.8, p=0.046, Mann Whitney).

A)



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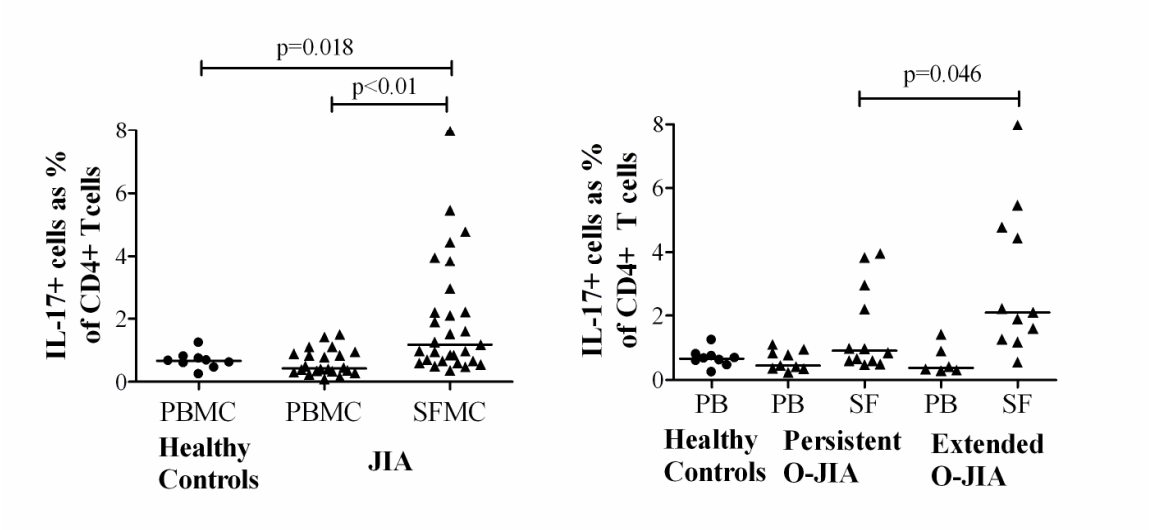


Figure 4.3: Th17 cells are enriched in JIA synovial fluid and synovial frequency correlates with clinical subtype of arthritis.
A) Representative dot plots of paired (left) JIA PBMC and (right) SFMC stained for CD3, CD4 and IL-17 and analysed by flow cytometry. Plots are gated on live lymphocyte gate and CD3+ cells. B) (Left) Scatter plot of the number of IL-17+ CD4+ T cells in JIA SFMC (n=28) and JIA PBMC (n=22) and healthy controls (n=9). Bars represent median values. (Right) Scatter plot of the number of IL-17+ CD4+ T cells in PBMC and SFMC of persistent (PBMC n=9, SFMC n=12) and extended (PBMC n=6, SFMC n=13) oligoarticular JIA patients and PBMC (n=9) of healthy controls. Bars represent median values.

The frequency of Th17 cells in other JIA subtypes was analysed by flow cytometry and compared to oligoarthritis subtypes (extended and persistent, Figure 4.4). There were no significant differences between the frequency of Th17 cells in the joints of polyarthritis, psoriatic, ERA and systemic subtypes when compared to the oligoarthritis subtypes. There was a high outlier in both the polyarthritis and psoriatic patient subgroups. These two patients had no identifiable clinical feature or disease correlate, such as ANA or RF status, or history of uveitis that was linked to the high Th17 levels seen.

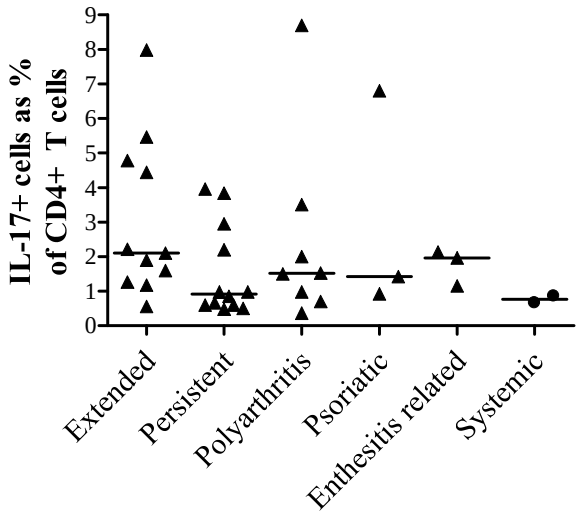


Figure 4.4: The frequency of synovial Th17 cells in specific subtypes of JIA.

Scatter plot of the number of IL-17+ CD4+ T cells in JIA SFMC (extended n=13 and persistent n=12 as in Figure 4.3, polyarthritis n=8, psoriatic n=3, enthesitis related arthritis n=3 and systemic n=2). Bars represent median values.

4.3.2 Frequency of FOXP3+ regulatory T cells is inversely related to Th17 within the joint

The Wedderburn group and others have previously shown that frequency of Treg in the joint may correlate with different clinical phenotypes in JIA (de Kleer *et al*, 2004; Ruprecht *et al*, 2005). The relationship between Treg and Th17 cells in the context of this autoimmune disease was therefore examined. Previous work using high surface expression of CD25 (the IL-2Rα chain) to identify Treg within the synovial compartment, showed that these cells expressed high levels of FOXP3 mRNA, and

confirmed their suppressive function *in vitro* (de Kleer *et al,* 2004). It should be noted however that in some SFMC samples, a proportion of CD25+ T cells were clearly FOXP3 negative (see Chapter 3) presumably representing activated effector cells. This suggests that FOXP3 expression may be a better marker for the enumeration of Treg within the joint. Treg were identified following staining for surface CD3, CD4, CD25 and intra nuclear staining for FOXP3 protein followed by flow cytometric analysis. Analysis of FOXP3+ Treg frequency in SF from only 14 patients demonstrated a significant difference in the frequency of Treg in the joint between persistent and extended oligoarticular subtypes of JIA, (Figure 4.5A, p=0.018, Mann Whitney). Remarkably the simultaneous analysis for IL-17+ CD4+ T cells and FOXP3+ Treg in the same samples showed that these two populations existed in an inverse relationship at the inflammatory site (Figure 4.5B, p=0.016, data were log transformed to allow linear relationship to be assessed by Pearson's correlation). No analogous relationship was observed between Treg and Th17 cells within the PBMC of patients (Figure 4.5C).

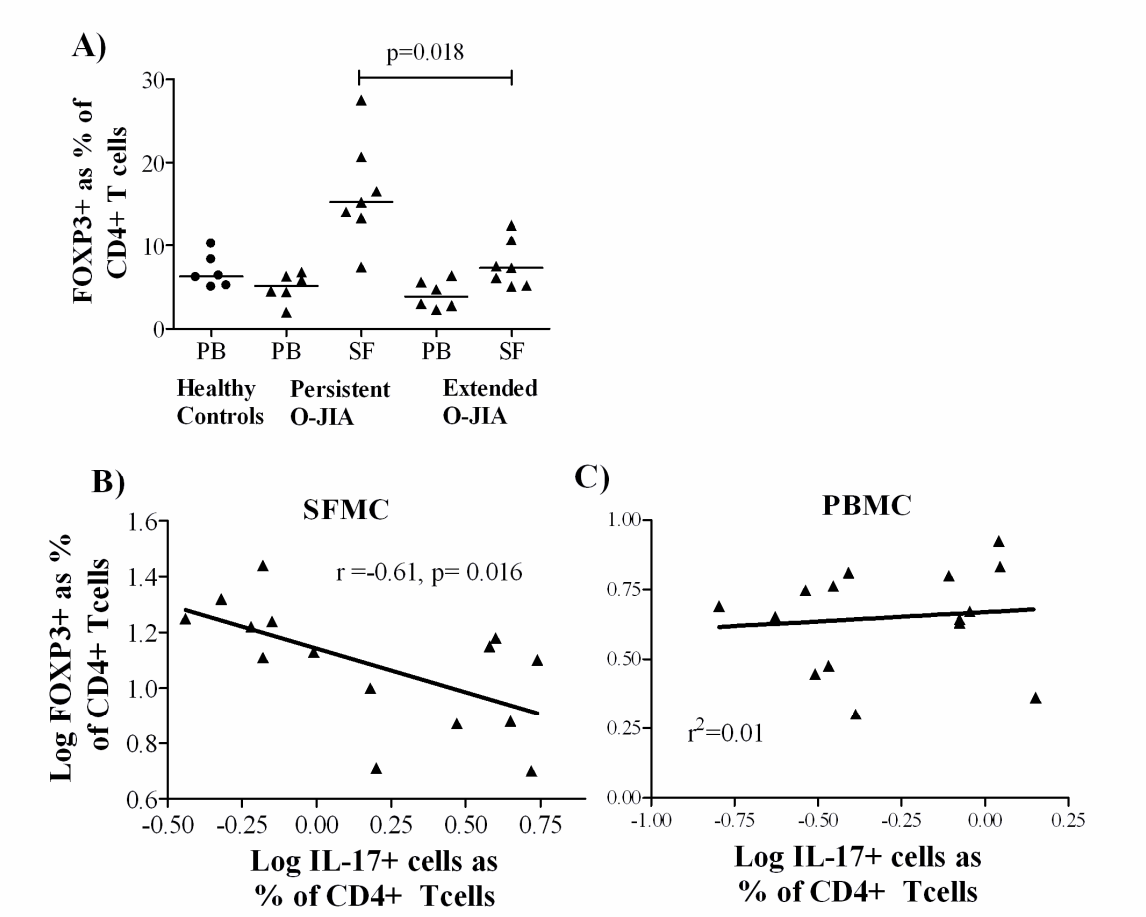


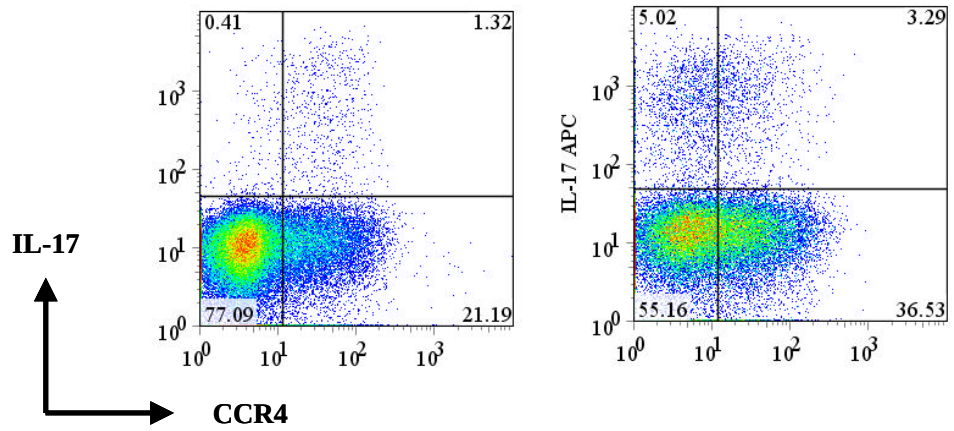
Figure 4.5: The frequency of synovial FOXP3+ T cells is inversely related to IL-17+ CD4+ T cells.

A) The frequency of FOXP3+ CD4+ T cells in PBMC and SFMC of persistent (n=6 PBMC, 7 SFMC) and extended oligoarthritis JIA patients (n=6 PBMC, 7 SFMC) and PBMC of healthy controls (n=6). Lines show the median value for each group. In B) SFMC (n=15) and C) PBMC (n=15), IL-17+ (horizontal axis) and FOXP3+ CD4+ T cells numbers (vertical axis) from JIA patients were compared, data were log-transformed.

4.3.3 Mechanism(s) that may contribute to enrichment of Th17 cells within the joint; a potential role for chemotaxis

Although the frequency of Th17 cells in the joint had a reciprocal association with Treg, both cell populations are enriched in the joint relative to peripheral blood in patients with JIA. Given the close links between the differentiation of Treg and Th17 cells in murine models (Bettelli *et al*, 2006), and evidence for shared expression of chemokine receptors between these two cell types (Acosta-Rodriguez *et al*, 2007b), it was hypothesised that shared chemotactic influences lead to their enrichment within the joint. Earlier, the functional role of the chemokine receptor CCR4 on Treg chemotaxis was examined (Chapter 3). This work was further extended by studying the expression of this receptor on Th17 cells in JIA.

A)



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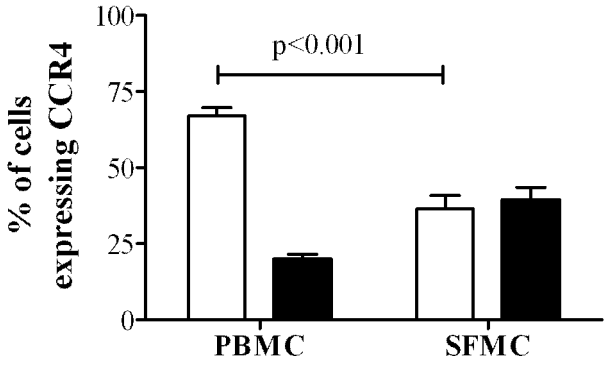


Figure 4.6: IL-17+ CD4+T cells show divergent patterns of chemokine receptor CCR4 expression in PBMC and SFMC of JIA patients.
A) Representative dot plots from (left) PBMC and (right) SFMC of JIA patients showing CCR4 expression on IL-17+ CD4+ T cells. Plots are gated on live lymphocyte gate and CD4+ T cells. B) Percentage of IL-17+CD4+ T cells (open bars) and all CD4+ T cells (filled bars) expressing CCR4 in PBMC and SFMC of JIA patients (n=5). Bars represent mean values, error bars represent 1 SEM.

CCR4 expression in a paired PBMC and SFMC JIA sample showed that the majority of IL-17+ CD4+ T cells in peripheral blood expressed CCR4 (Figure 4.6A, left panel). In contrast IL-17+ CD4+ T cells from the same patient's joint expressed lower levels of CCR4 (Figure 4.6A, right panel). This distinct pattern of CCR4 expression within the joint was confirmed in a larger sample (Figure 4.6B, n=5, $p<0.001$, unpaired t test). The underlying basis for this difference was explored in greater detail later in this study (section 4.3.7).

Two chemokines CCL17 and CCL22 bind the chemokine receptor CCR4. To test if Th17 cells from peripheral blood demonstrated a functional chemotactic response commensurate with their expression of CCR4, CD4+ T cells from healthy controls were assessed for chemotaxis to CCL22. The transwell assay was adapted to include the detection of cytokine expressing cells, by stimulating cells that had migrated into the lower chamber, with PMA and ionomycin in the presence of brefeldin A, prior to intracellular staining. Migration of Th17 cells was compared to Treg by co-staining migrated cells for FOXP3. Calculations of chemotactic index are dependent on an accurate quantification of Th17 and Treg in the migrated population. As IL-17+ cells make up <1% of CD4+ T cells in healthy PBMC, unless there is a significant enrichment after migration, very few IL-17+ cells are detectable in the migrated population (total migrated cell numbers were $5\text{-}10x10^3$), typically less than 50 cells. To prevent stochastic fluctuations in these small numbers skewing chemotactic results, the enrichment of Th17 as a proportion of all CD4+ T cells was compared in control and chemokine migrated cells. These data are therefore presented as fold enrichment. As expected, FOXP3+ Treg from healthy controls demonstrated chemotaxis towards CCL22, but surprisingly this was not seen in the case of Th17 cells (Figure 4.7, $p=0.028$, paired t test).

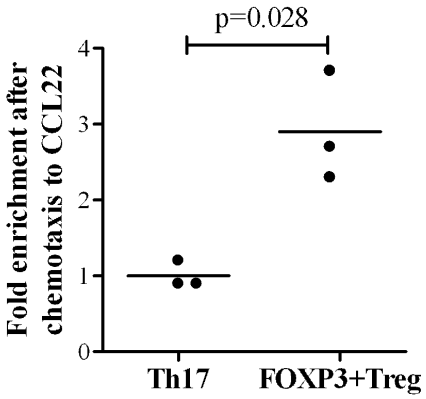


Figure 4.7: FOXP3+Treg but not Th17 cells preferentially migrate to CCL22.
CD4+ T cells from healthy control PBMC (n=3) were assessed for chemotaxis to 100ng/ml CCL22. Cells which migrated across the transwell were analysed for IL-17 and FOXP3 expression by flow cytometry. The percentage of IL-17+ or FOXP3+ cells in the population which migrated to CCL22 was compared to control medium to calculate fold enrichment. Lines represent mean levels.

This difference in chemotaxis, could reflect greater expression of CCR4 on Treg compared to Th17. To test this hypothesis, CCR4 expression in both populations was assessed across several healthy donors and compared with total CD4+ T cells (Figure 4.8). Indeed healthy peripheral blood Treg had significantly higher levels of CCR4 expression than Th17 cells, consistent with their greater chemotaxis to CCL22. Th17 cells, did show higher CCR4 expression than total CD4+ T cells, and so it remains unclear why Th17 cells failed to show a chemotactic response to CCL22.

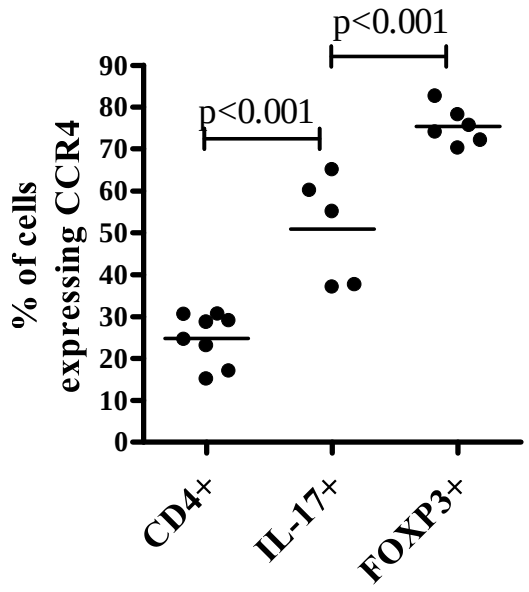
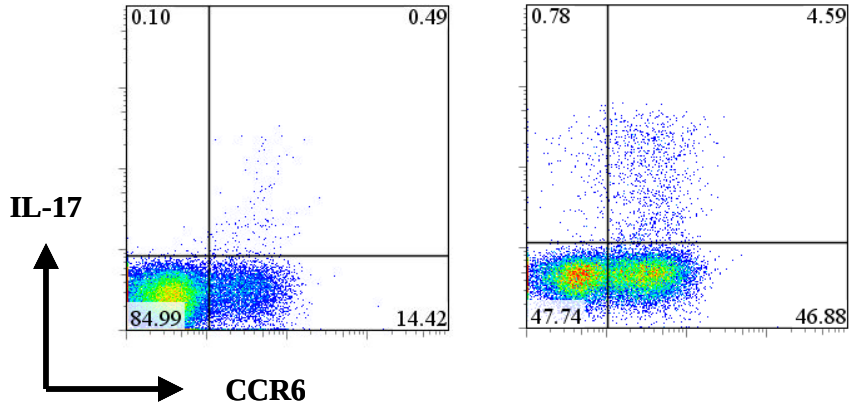


Figure 4.8: FOXP3+ Treg express greater levels of the chemokine receptor CCR4 than Th17 cells.
Percentage of CD4+ (n=8), IL-17+ (n=5) and FOXP3+ CD4+ T cells (n=6) expressing CCR4 in PBMC of healthy controls as analysed by flow cytometry. Lines represent mean levels.

4.3.4 Th17 cells express high levels of the chemokine receptor CCR6

Recent data suggests that Th17 cells from healthy humans also express high levels of the chemokine receptor CCR6 (Acosta-Rodriguez *et al*, 2007b). CCR6 expression was analysed in a paired PBMC and SFMC JIA sample after stimulation, using flow cytometry. In these samples the majority of IL-17+ CD4+ T cells in both peripheral blood (Figure 4.9A, left panel) and SF (right panel) expressed CCR6. This pattern was confirmed in a larger set of samples, where a larger proportion of IL-17+ CD4+ T cells were found to express CCR6 than the CD4+ T cell population as a whole (Figure 4.9B).

A)



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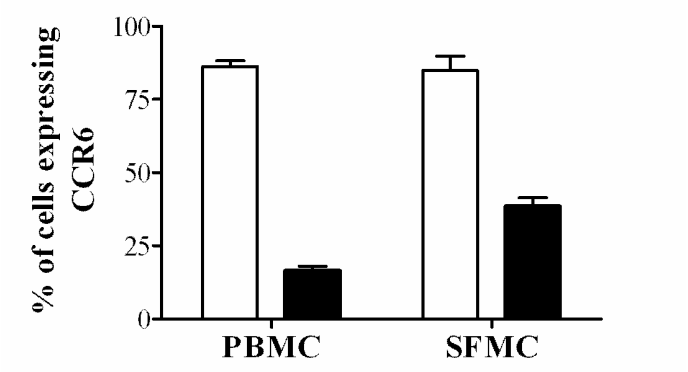
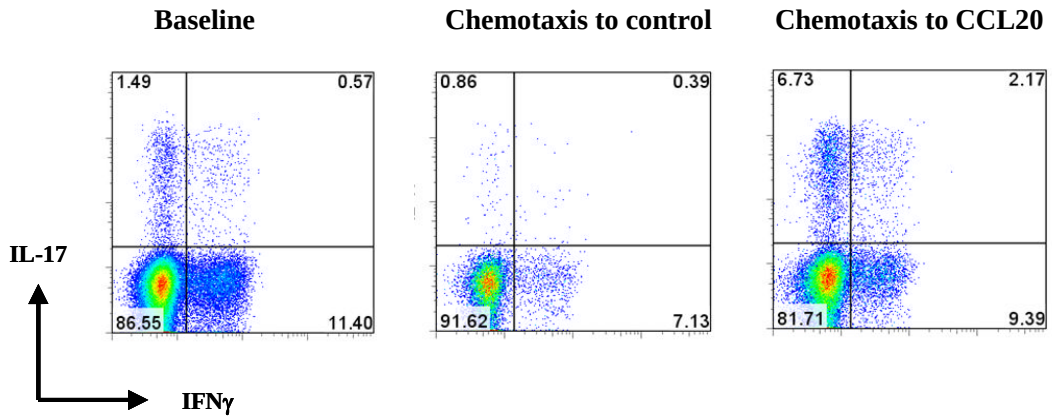


Figure 4.9: Th17 from JIA patients express high levels of CCR6.

A) Representative dot plots from paired (left) PBMC and (right) SFMC of JIA patient (representative 1 of 5 experiments) showing CCR6 expression on IL-17+ CD4+ T cells. Plots are gated on live lymphocyte gate and CD4+ T cells. B**)** Percentage of all CD4+ T cells (filled bars) and IL-17+ CD4+ T cells (open bars) expressing CCR6 in the peripheral blood and the SF of JIA patients (n=5). Bars represent mean values. Error bars represent 1 SEM.

The chemotactic response of Th17 cells to the only known CCR6 ligand, CCL20, was assessed using the transwell assay. As before, CD4+ T cells which exhibited chemotaxis were stained for IL-17 and IFNγ after stimulation. In the majority of experiments a proportion of IL-17+ were noted to be also IFNγ+ (Figure 4.10A, and section 4.3.6) both before and after migration. Th17 cells showed a marked enhancement of migration to increasing doses of CCL20, unlike CD4+ T cells as a whole (Figure 4.10B).

A)



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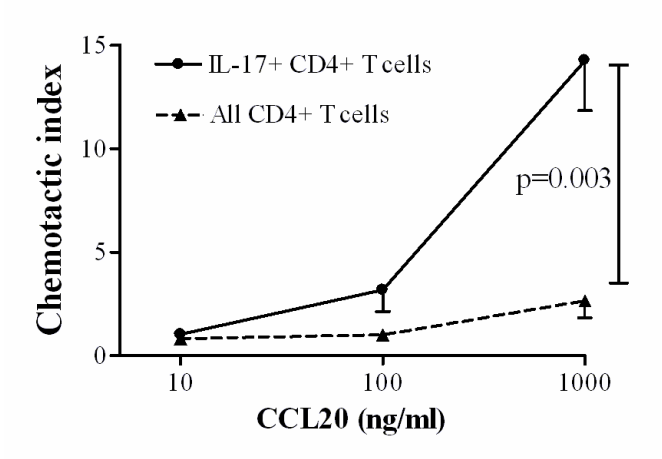


Figure 4.10: Th17 cells show enhanced migration to CCR6 ligand, CCL20.

A) Migration of CD4+ T cells from healthy control PBMC to 10, 100 or 1000ng/ml CCL20. Intracellular IL-17 and IFNγ was assessed by flow cytometry (left plot) before and (middle plot) after migration to control medium (RPMI/0.5%BSA) or (right plot) 1000ng/ml CCL20. Data are gated on live CD3+CD4+ cells. One representative experiment of 5. B) Migration of IL-17+ CD4+ T cells compared to all CD4+ T cells, in response to a titration of the CCL20 gradient. Data expressed as mean (bars 1SEM) chemotactic index of 5 independent experiments.

Under standard conditions using 100ng/ml CCL20, the chemotaxis of IL-17+ CD4+ cells from JIA PBMC did not differ significantly from healthy controls (3.2+/-1.0 vs 3.8+/-0.5, unpaired t test) but had significantly higher migration than total CD4+ T cells (Figure 4.11, $p<0.001$, paired t test).

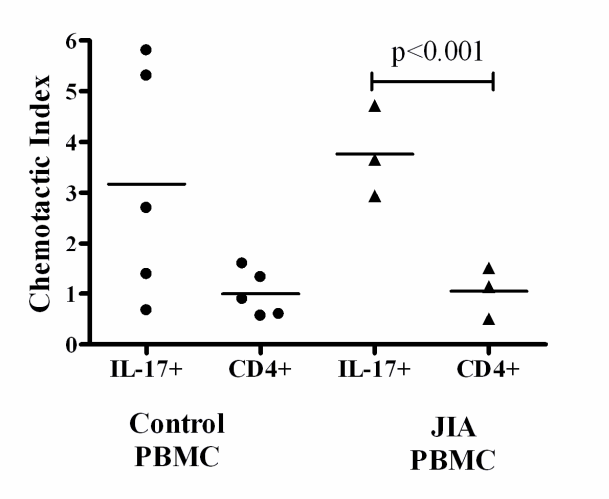


Figure 4.11: Comparison of Th17 chemotaxis to CCL20, in control and JIA PBMC samples.
Migration of IL-17+ CD4+ T cells compared to all CD4+ T cells from healthy control (n=5) and JIA PBMC (n=3), in response to 100ng/ml CCL20. Cytokine expressing cells were detected by flow cytometry. Lines represent mean values.

4.3.5 Elevated CCL20 levels within the joint may account for Th17 enrichment

Th17 cells from JIA PBMC and SFMC demonstrated high surface CCR6 expression, and this correlated with a strong chemotactic response to the corresponding ligand, CCL20, *in vitro*. To test if CCL20 was important to the enrichment of Th17 in SFMC *in vivo*, CCL20 levels were assessed by ELISA in JIA serum and SF. A significant elevation of CCL20 within the joints of JIA patients (Figure 4.12A) was observed (median 22.8pg/ml, inter-quartile range (IQR) 0.2-87.0), compared to serum (median 0.2pg/ml, IQR 0.15-0.3pg/ml, p=0.03 Mann Whitney). A significant positive correlation between SF CCL20 levels and Th17 frequency within the joint was also noted (Figure 4.12B, Spearman's Rank 0.74, p=0.02). Taken together, these results provide evidence to support a role for CCL20 in driving Th17 infiltration to the inflamed site in JIA.

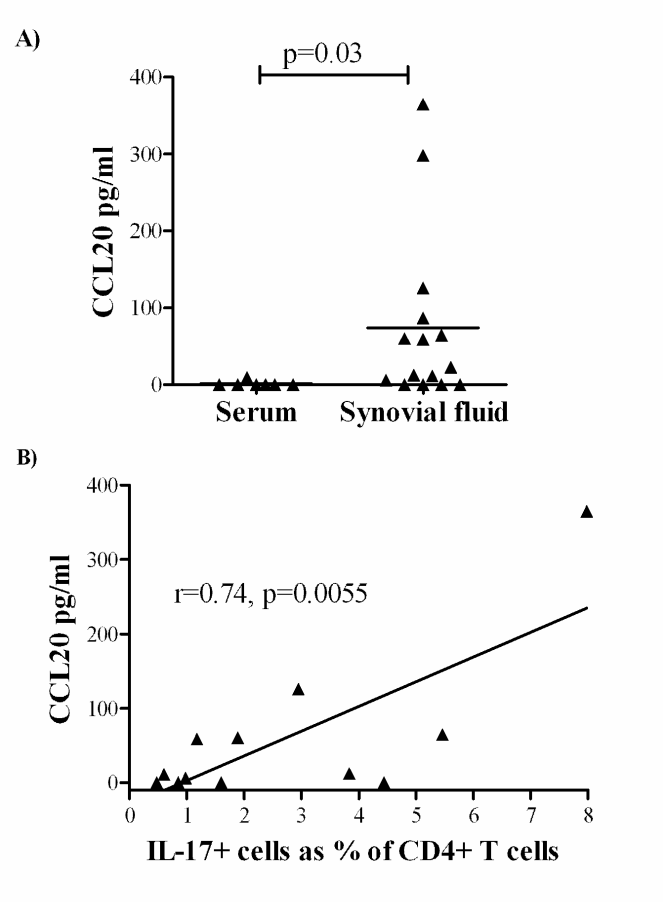


Figure 4.12: CCR6 ligand, CCL20 levels are elevated in SF and correlate with the frequency of Th17 cells within the joint.
A) CCL20 was measured in JIA serum (n=7) and SF supernatants (n=15) by ELISA. Lines represent median levels. B) The frequency of IL-17+ CD4+ T cells in corresponding SFMC samples was assessed by flow cytometry. Scatter plot of Th17 cell frequency in the joint against CCL20 concentration as detected by ELISA.

4.3.6 Co-expression of inflammatory cytokines by IL-17+CD4+ T cells from JIA synovial fluid

Given the apparent discrepancy in the expression of CCR4 on IL-17+ synovial T cells compared to those in peripheral blood (Figure 4.6) and the recent demonstration that cells with a CCR6+CCR4- phenotype may include Th1 cells that also produce IL-17 (Acosta-Rodriguez *et al*, 2007b), it was hypothesised that synovial IL-17+ T cells in JIA may include a sub-population of cells which secrete both IL-17 and IFNγ. The functional diversity of IL-17+ cells within the joint was investigated with respect to IFNγ but also IL-22 which is produced by Th17 cells (Liang *et al*, 2006). A representative dot plot of paired JIA PBMC and SFMC, stimulated and then stained for CD3, CD4 and intracellular cytokine demonstrated distinct IL-17+ and IFNγ producing CD4+ T cells in JIA PBMC (Figure 4.13A, left). However in the SFMC sample (Figure 4.13A, right), as well as a large IFNγ+ population, the majority of IL-17+ cells also co-expressed IFNγ. IL-17+ cells were also found to co-express IL-22 in both PBMC and SFMC (Figure 4.13B). In contrast the production of the Th2 cytokine IL-4, was mutually exclusive with IL-17, with expression of these cytokines in discrete and separate populations in both blood and joint (Figure 4.13C).

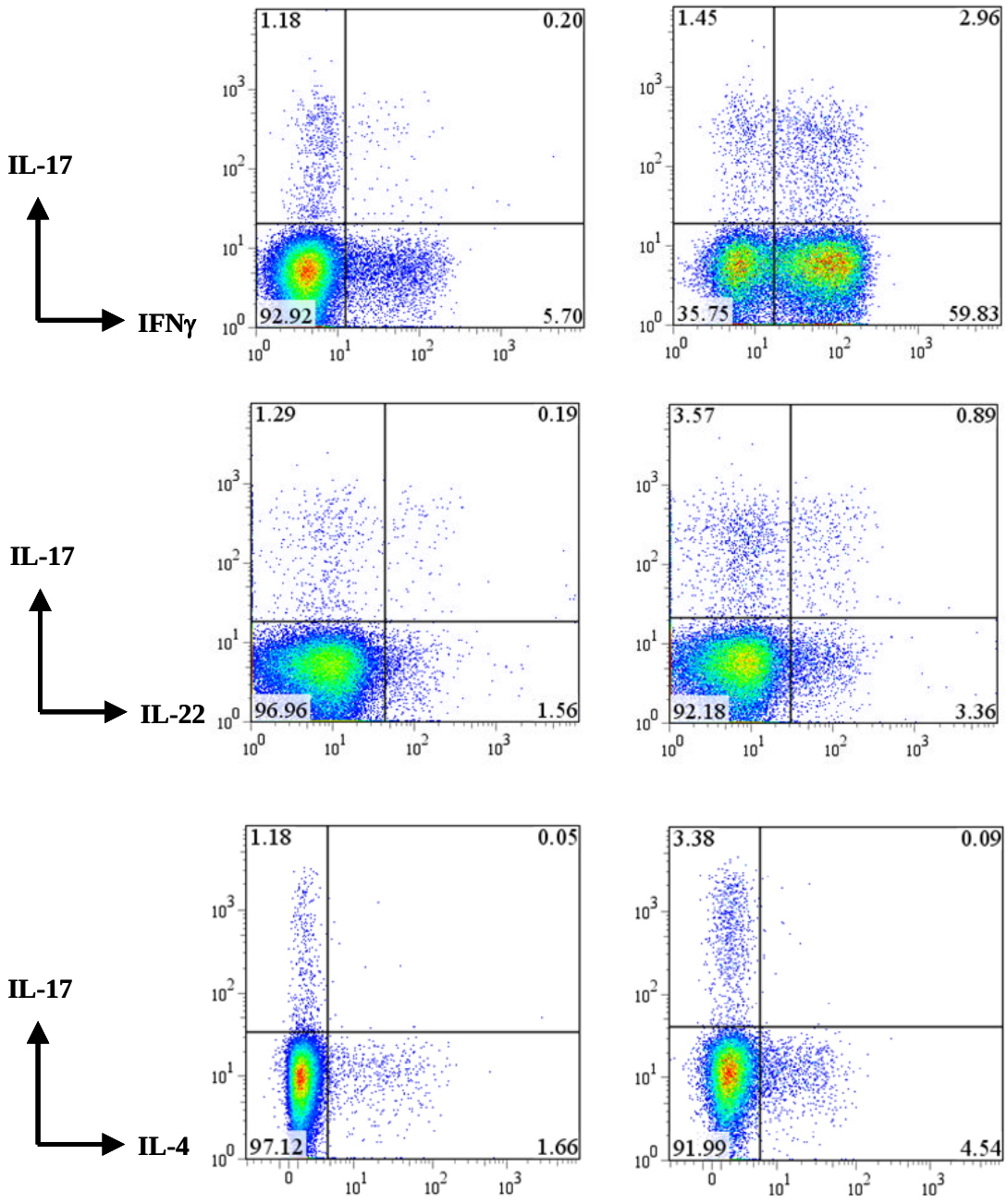


Figure 4.13: IL-17+ CD4+ T cells from JIA SFMC demonstrate polyfunctional cytokine expression

Dot plots of paired PBMC (left) and SFMC (right) from JIA patients stained for IL-17 and A) IFNγ, B) IL-22, C) IL-4 as analysed by flow cytometry. Plots are gated on live lymphocyte gate and CD3+CD4+ cells, numbers indicate percentage of parent population. Representative of n=3.

4.3.7 IL-17+ IFNγ+ CD4+ T cells are enriched within the joint

The presence of poly-functional IL-17+ cells secreting IFNγ within the joint, is in contrast to early reports of Th17 cells as a lineage distinct from Th1 cells (Park *et al*, 2005; Veldhoen *et al*, 2006). Although a small proportion of Th17 cells co-express the Th1 cytokine IFNγ in the peripheral blood of healthy adults (Acosta-Rodriguez *et al*, 2007b), within the inflamed joints of JIA patients, this proportion is greatly increased, with 50% of all IL-17+ CD4+ T cells co-expressing IFNγ (from here on referred to as Th17/1 cells) (Figure 4.14).

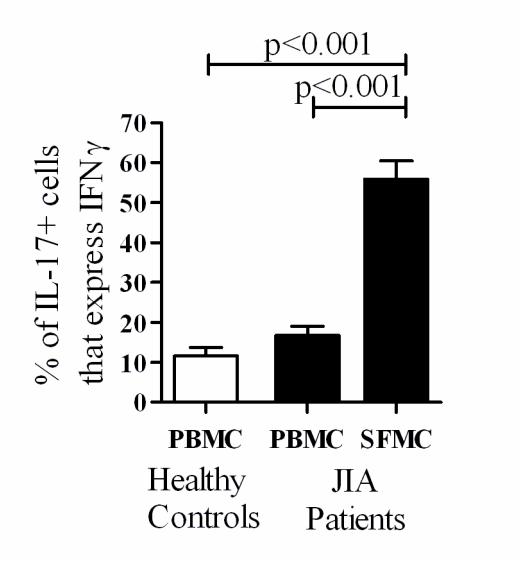


Figure 4.14: IL-17+ IFNγ+ CD4+ T cells are enriched within the joint

Mean co-expression of IFNγ in IL-17+ CD4+ T cells taken from healthy control PBMC (*n*=9), JIA PBMC (*n*=17), SFMC (*n*=21) as analysed by flow cytometry. Bars represent mean values, error bars 1SEM.

To confirm that the enrichment of these Th17/1 cells within the joint was directly correlated with the CCR4lo phenotype of Th17 found at the inflammatory site (Figure 4.6), synovial CD4+CCR6+ T cells were sorted into CCR4-CCR6-, CCR4-CCR6+ and CCR4+CCR6+ fractions by flow cytometry. The sorted cells from the CCR4-CCR6+ population were significantly enriched with Th17/1 cells when compared to the CCR4+CCR6+ fraction (Figure 4.15A, p=0.007, paired t-test). Consistent with this, after *in vitro* stimulation of these sorted SF populations, the cytokines released correlated with intracellular cytokine detected by flow cytometry (Figure 4.15B).

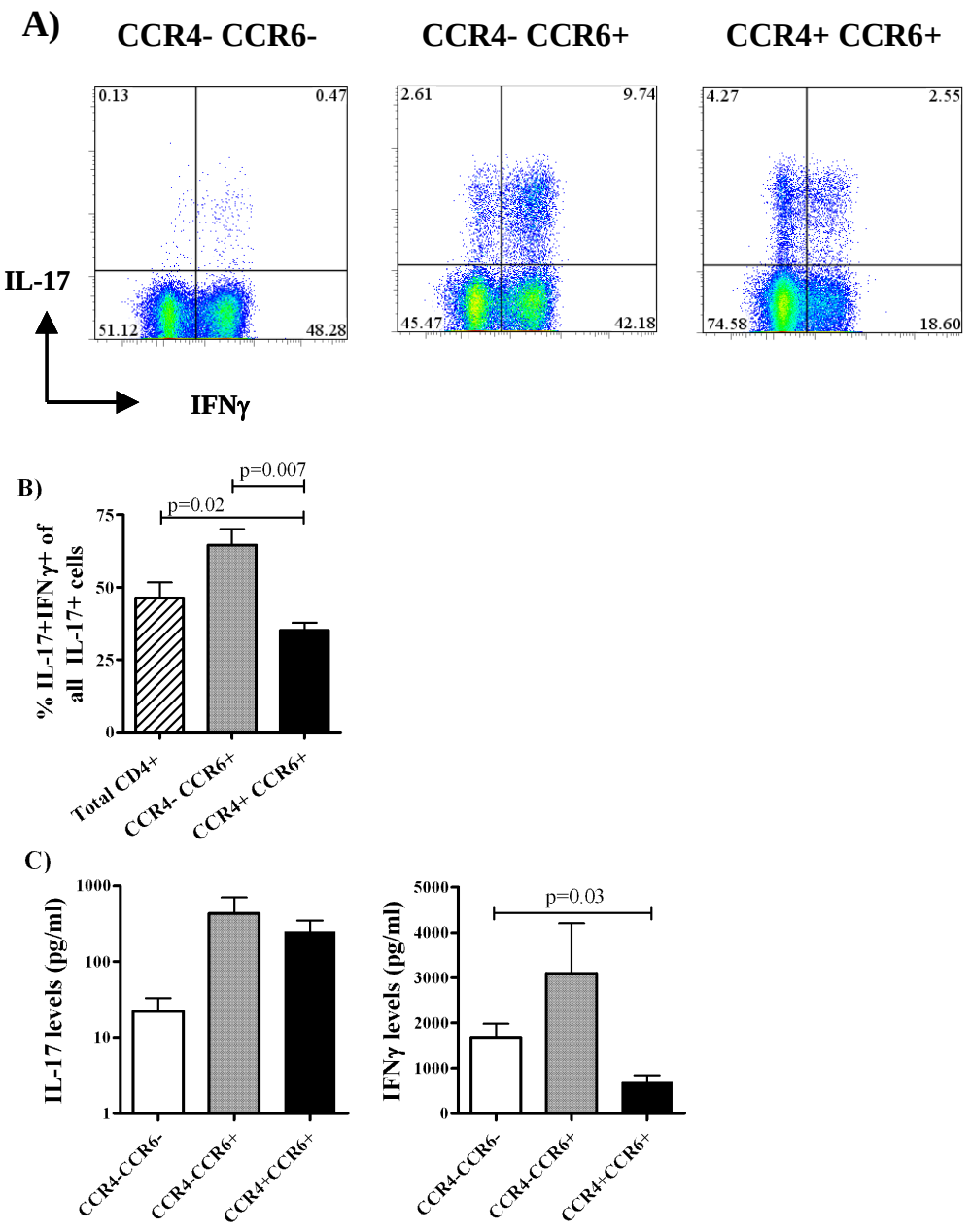


Figure 4.15: IL-17+IFNγ+ co-expressing CD4+T cells are enriched in the CCR4-CCR6+ fraction of synovial mononuclear cells.

A) Synovial CD4+ T cells were sorted according to expression of CCR4 and CCR6. Dot plots of IL-17 and IFNγ production in CCR4-CCR6, CCR4-CCR6+ and CCR4+CCR6+ sorted populations, 1 representative experiment of 4. B) IL-17+IFNγ+ cells as a proportion of IL-17+ cells in sorted populations as in A, detected by intracellular cytokine staining n=4. C) (left) IL-17, and (right) IFNγ protein detected by Luminex in supernatants from sorted populations as in A, after stimulation with PMA and ionomycin for 5 hours, n=4.

4.3.8 Th17 and Th17/1 cells from synovial fluid express the "master" Th17 transcription factor, RORC2

As Th17/1 cells have an intermediate cytokine profile between Th17 and Th1 cells, and intermediate chemokine receptor profile (Th17 cells were enriched in CCR4+ population, whilst Th1 cells were enriched in the CCR4- populations, Figure 4.15), this "intermediate" status was also examined in relation to their transcriptional programming. Transcription factor mRNA expression was analysed from synovial Th17 and Th17/1 cells after sorting according to chemokine receptor expression (Figure 4.15). RORC2 mRNA expression was found to be elevated in both Th17 and Th17/1 enriched populations when compared to Th1 cells suggesting overlapping transcriptional control in both Th17 subsets (Figure 4.16A, left). There were no differences in an alternative Th17-related transcription factor, aryl hydrocarbon receptor (AHR) (Figure 4.16B right), or T-bet expression (Figure 4.16C) between sorted synovial populations.

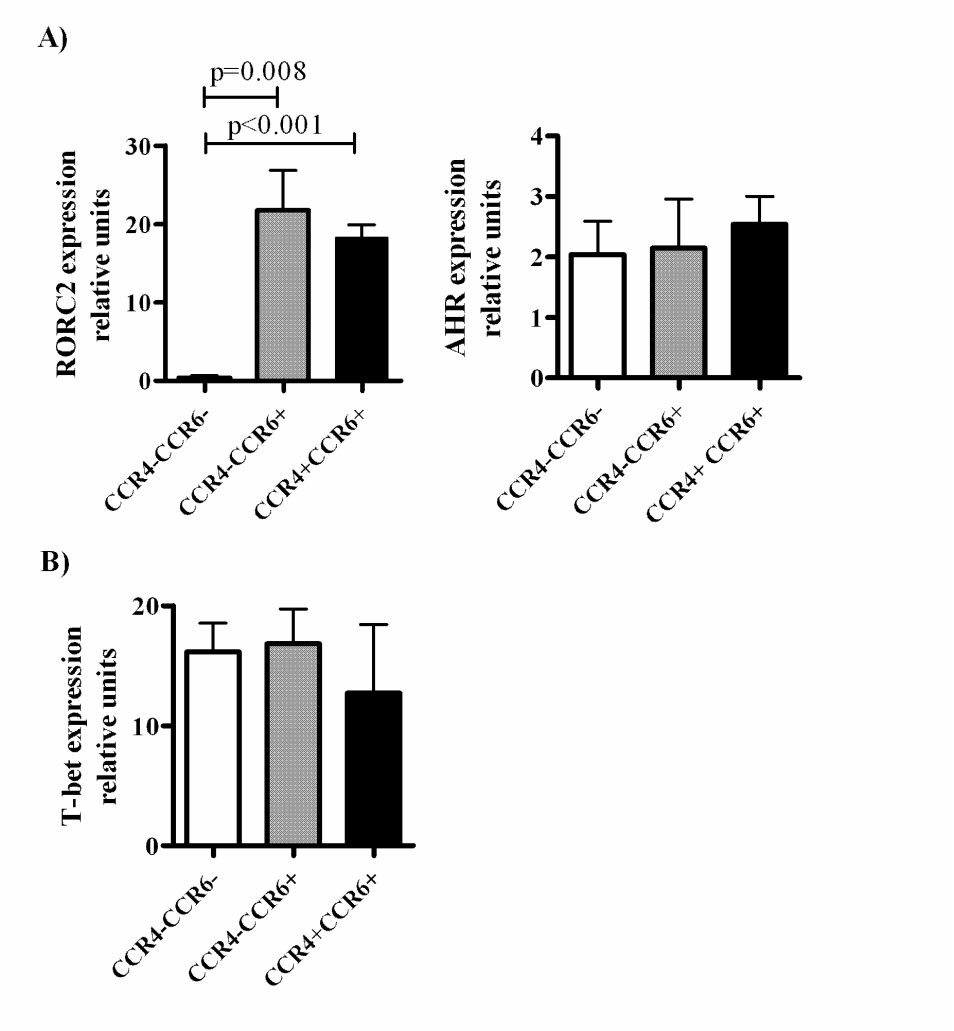


Figure 4.16: SFMC enriched for Th17 and Th17/1 cells, express the transcription factor, RORC2

mRNA expression of A) (left) RORC2 (n=6) and (right) AHR (n=4) and B) T-bet (n=4) in SFMC sorted for chemokine expressing populations as in Figure 4.15, normalised to β2M levels. Bars represent mean values, error bars 1SEM.

4.3.9 IL-17 secreting cells can be isolated by a cytokine capture assay

Although Th17 cells were enriched after sorting by chemokine receptor expression, the resulting sorted populations still contained a mixed population of Th17, Th17/1 and Th1 cells. In order to clearly separate cytokine expressing cells, IL-17 and IFNγ-specific capture assays were optimised (Chapter 2, section 2.3.3). Cytokine secreting cells were detected by "capture assay" and purified by flow cytometry (Figure 4.17A). The cytokine expressing phenotype of sorted cells was confirmed by further intracellular cytokine staining after incubation with brefeldin A (Figure 4.17B,C).

Using this system, the mean purity of Th1 cells after sorting from healthy control PBMC was 91.7% IL-17-IFNγ+, 0.2% IL-17+IFNγ+, <0.1% IL-17+IFNγ-, and of Th17 cells was 92.8% IL-17+IFNγ-, 2.1% IL-17+IFNγ+, and <0.1% IL-17-IFNγ+. IL-17+IFNγ+ cells contaminated Th17 cells more than sorted Th1 cells. This maybe because IL-17+IFNγ+ cells were a very small proportion of the total IFNγ population, but a relatively large proportion of all IL-17+ cells, before sorting.

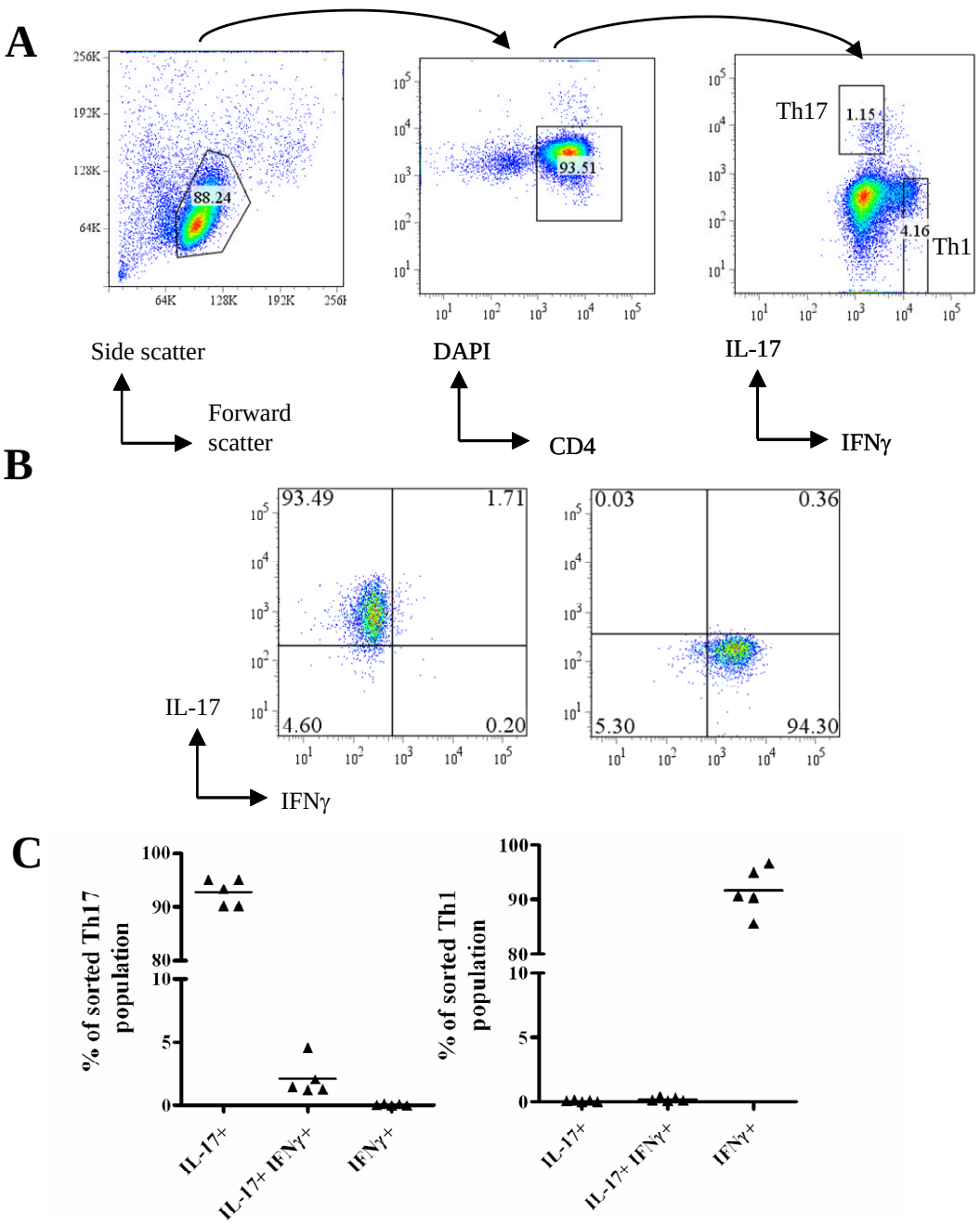


Figure 4.17: Cytokine capture assay allows purification of IL-17 and IFNγ secreting CD4+ T cells.

A) Sorting strategy to purify Th17 and Th1 cells. IL-17 and IFNγ secreting T cells were detected by flow cytometry using a capture assay. (Left plot) Cells were gated (indicated by arrows) on cell scatter and then (middle plot) DAPI-CD4+ and (right plot) high expression of IL-17 or IFNγ as indicated. Numbers in all plots indicate the percentages of parent population. B) Captured Th17 and Th1 cells from healthy controls were sorted as in A) then re-incubated overnight in brefeldin A. Representative dot plot of intracellular IL-17 and IFNγ expression detected by flow cytometry in purified population. C) Summary of 5 donors analysed for cell purity as in B. Lines represent mean levels.

This assay was next adapted to detect Th17, Th17/1 and Th1 cells from SFMC, based on surface captured cytokines and cells were sorted by flow cytometry into distinct populations (Figure 4.18A). Transcription factor mRNA expression analyses of these cell populations, confirmed the greater expression of RORC2 mRNA in both Th17 and Th17/1 than in Th1 cells, but no differences in interferon regulatory 4 (IRF4) which has also been linked to Th17 differentiation (Schubert *et al,* 2001), (Figure 4.18B). Interestingly, pure Th17/1 cells have an intermediate expression of T-bet when compared to Th17 and Th1 (Figure 4.18C), a finding that was originally masked when studying the Th17/1 enriched CCR4-CCR6+ population (see Figure 4.16). In contrast the levels of RORC2 expression were typically higher in Th17/Th1 than Th17 cells (Figure 4.18B).

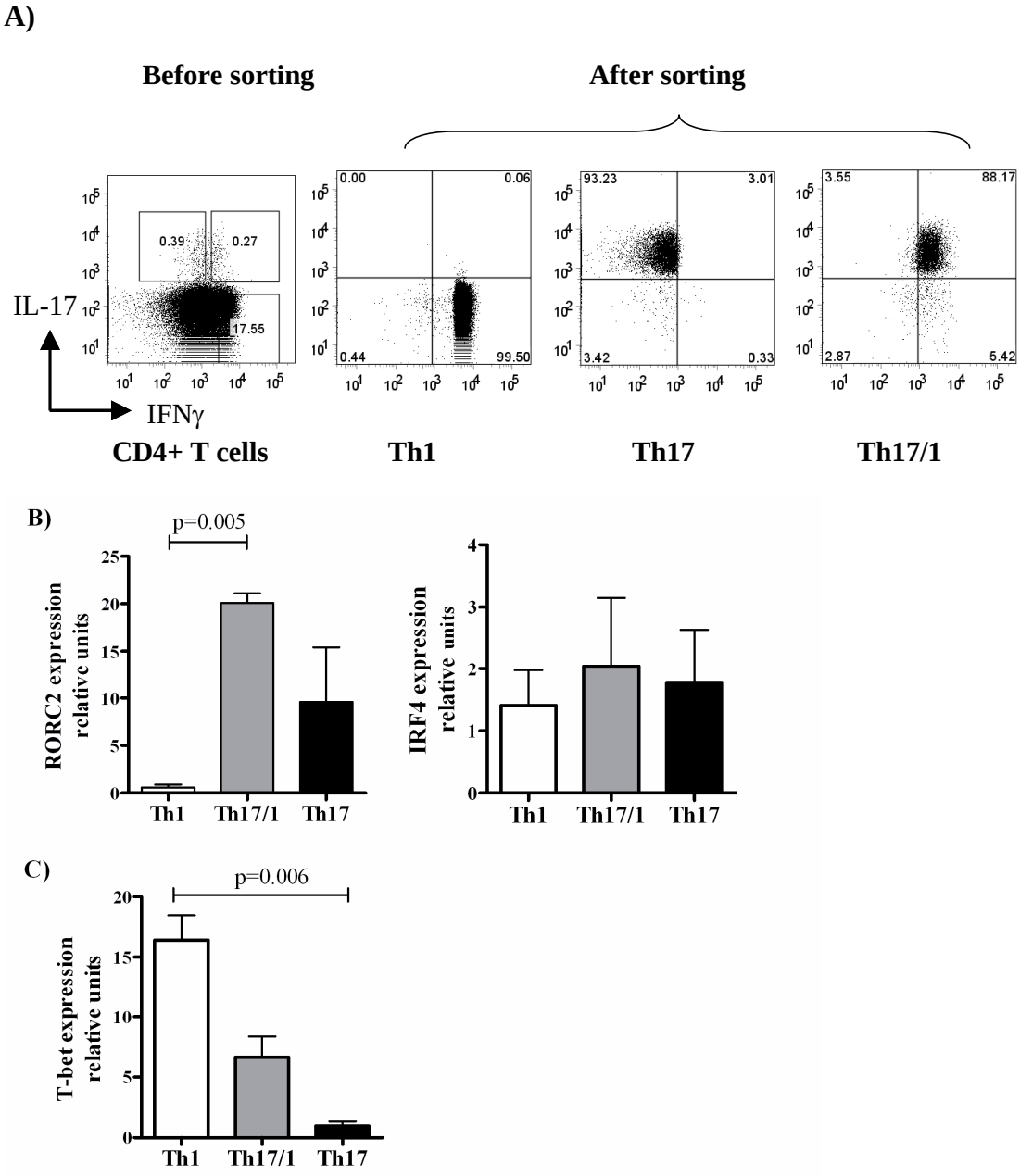
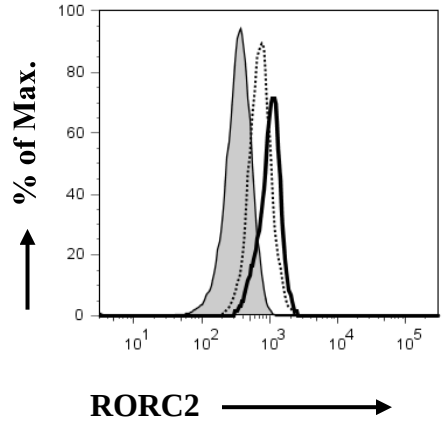


Figure 4.18: Th17/1 cells purified from SFMC share transcription factor profiles with Th1 and Th17 cells.

A) IFNγ and IL-17 secreting CD4+CD3+ SFMC were detected by flow cytometry using a cytokine capture assay. Representative dot plot of unsorted SFMC (left) gated on CD4+ T cells demonstrating surface capture of IL-17 and IFNγ, and sorted by flow cytometry into Th1, Th17 and Th17/1 populations (right 3 plots). Numbers in plots indicate the percentages of cells secreting cytokines. B) (left) RORC2, (right) IRF-4, and C) T-bet mRNA expression in synovial CD4+ T cells populations sorted into cytokine expressing subsets as above. Bars represent mean values (error bars 1SEM) of 3 independent experiments.

It was next confirmed that RORC2 is also detectable at the protein level by flow cytometry in both Th17 and Th17/1 cells. A typical histogram showed expression of RORC2 to be higher in Th17 cells (bold line) when compared to Th1 cells (dotted line), and isotype control (grey histogram), in a healthy control PBMC sample (Figure 4.19A). A summary of a larger sample is shown in Figure 4.17B. RORC2 protein expression closely mirrored its mRNA expression, with significantly higher levels of RORC2 protein in Th17 and Th17/1 cells from both PBMC of healthy controls (left) and SFMC from JIA patients (right).

A)



B)

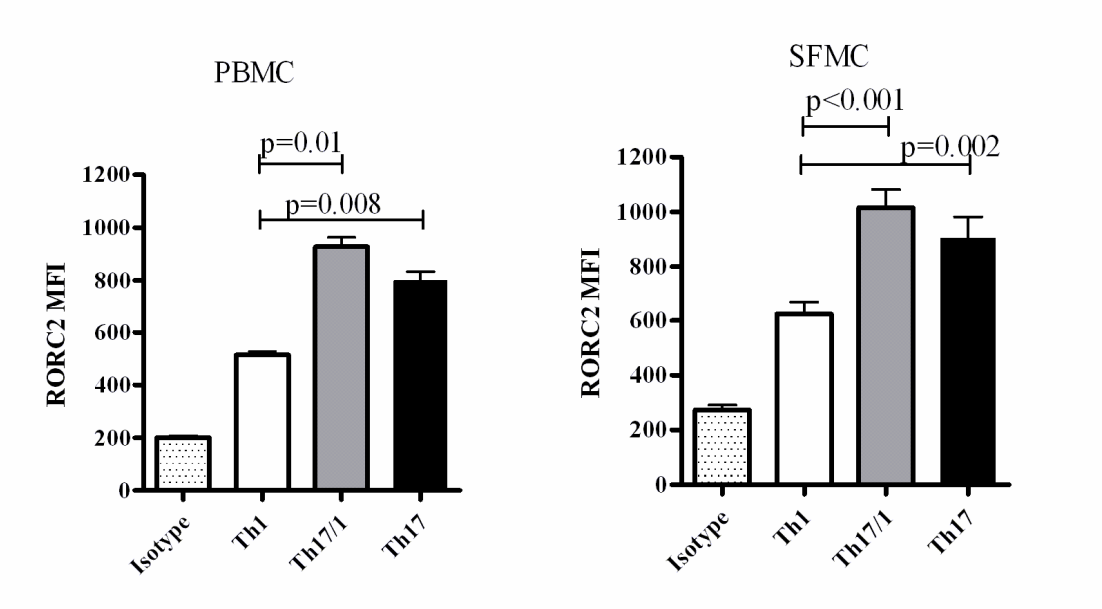


Figure 4.19: Th17/1 and Th17 cells express high levels of RORC2 protein.
RORC2 protein expression in T cells analysed by flow cytometry. A) Histogram of RORC2 expression in healthy control PBMC, gated on CD4+ T cells; isotype control (grey histogram), Th1 cells (dotted line) and Th17 cells (bold line), representative of *n*=3. B) Summary of RORC2 protein expression (MFI) in cytokine expressing sub-populations from healthy control PBMC (left, n=3) and JIA synovial CD4+ T cells (right, n=5). Bars represent mean values, error bars 1SEM.

4.4 Discussion

4.4.1 Th17 frequency in JIA joints correlates with severity of clinical course

Although the emergence of TNFα target therapy has been a major step forward in the clinical management of inflammatory arthritis, including JIA, there remains a significant minority of patients who fail to respond to methotrexate or the first line anti-TNF agent, Etanercept (Giannini *et al,* 2009). In addition to TNFα blockade, a range of new biologic agents has emerged but thus far none has generated high levels of remission across the clinical spectrum of JIA. The emergence of a novel population of pro-inflammatory cells, Th17, found to be critical for disease in mouse models, led several groups to ask if these cells were important in human autoimmune arthritis. JIA offers an excellent disease model in which to ask questions about the immunopathogenicity of this new population. JIA is a heterogeneous disease with multiple subtypes which although defined by their clinical features, have a clear basis in immunopathology and genetics (Nistala *et al,* 2009). By analysing patients from two closely linked JIA subtypes, which present in a similar fashion but go on to very distinct disease courses (one mild and self-remitting, the other more severe and requiring aggressive treatment), this study aimed to define whether Th17 infiltration into the joint correlated with clinical phenotype.

Firstly, IL-17 protein levels were assessed within SF. Surprisingly, prior reports analysing IL-17 by Luminex had suggested that levels were lower in the joints of JIA patients compared to their serum (de Jager *et al,* 2007). Results from this study showed a clear enrichment of IL-17 protein within the joint, when assessed by ELISA. These data are consistent with other published studies (Agarwal *et al,* 2008) and results from RA (Chabaud *et al,* 1999). A discrepancy between the data generated by Luminex and ELISA, could reflect the greater tendency for cross-reactivity between capture antibodies in the multiplex system which uses monoclonal antibodies generated in multiple species. It was important to also show the presence of IL-17 protein in synovial membrane which is the main site of inflammation in JIA, whilst SF represents the exudate from this tissue. IL-17 protein was detected in synovial membrane and appeared to localise to areas rich

in CD4+ T cells. However it would be important to formally confirm co-localisation using fluorescent immunohistochemistry, as recent data have suggested multiple alternative sources for IL-17 in human arthritis, including mast cells (Hueber *et al,* 2010) but not gamma delta cells (Ito *et al,* 2009). In parallel with IL-17 protein levels, the proporsion of Th17 cells was also enriched in SF when compared to the peripheral blood of JIA patients. It is worth noting that this is not the case in all forms of human autoimmune arthritis. A large study of patients with RA failed to shown an enrichment of Th17 cells within the patients' joints (Yamada *et al,* 2008).

To test the original hypothesis of this part of the study, i.e., that Th17 numbers may be associated with a more severe clinical phenotype in JIA, the frequency of Th17 cells was analysed across different JIA subtypes. This showed a remarkable increase in the frequency of Th17 cells within the joints of patients with the oligoarticular sub-type and in particular the more severe form extended oligoarthritis (Figure 4.3). Interestingly this subgroup of patients is at risk of autoimmune chronic anterior uveitis, another disease that has been linked to Th17 cells in murine models (Luger *et al,* 2008) and human studies (Amadi-Obi *et al,* 2007). Patients with polyarthritis had a heterogeneous distribution of Th17 frequencies in their joints. If a larger number of RF+ and RF- synovial samples were available it would be interesting to compare the two groups and see if RF+ JIA patients are similar to their adult counterparts with low numbers of synovial Th17 (Yamada *et al,* 2008). In adult forms of ankylosing spondylitis and psoriatic arthritis the frequency of Th17 cells is increased in PBMC when compared to healthy controls (Shen *et al,* 2009). Not enough samples were available to test if this was true of the synovial compartment in JIA patients with equivalent disease subtypes.

4.4.2 Th17 frequency is reciprocal to regulatory T cells within the inflamed joint

As discussed in Chapter 1, there are close developmental links between Treg and Th17 cells, which led to the comparison of Th17 and Treg frequencies in JIA samples. Treg showed a clear inverse relationship with Th17 frequency, but only in SFMC samples (Figure 4.5). This reciprocal relationship between proporsions of Th17 and Treg cells in the joint has since been confirmed in early arthritis (Hunter *et al,* 2010) and at a

molecular level through analysis of the relevant transcription factors, FOXP3 and RORC2 (Olivito *et al,* 2009). This inverse relationship is the first to be documented in human studies but this pattern has been described in several murine studies and appears to be a fundamental signature of the Treg-Th17 relationship (Littman & Rudensky 2010). Several mechanisms have come to light which all, either promote Treg numbers whilst restraining Th17, or have the opposite effect, but in either case lead to an inverse relationship between Treg and Th17 numbers. The first mechanism found to reciprocally influence Treg and Th17 was the balance of IL-6 and TGFβ during T cell differentiation (Bettelli *et al,* 2006), whilst subsequent studies have examined the action of IL-2 (Laurence *et al,* 2007), retinoic acid (Mucida *et al,* 2007) and IL-35 (Collison *et al,* 2007; Niedbala *et al,* 2007). At a signalling level, the phosphatase SHIP which regulates activity of PI3kinase, has been shown to be pivotal to its reciprocal development (Locke *et al,* 2009). From the organism's perspective it likely that the immune system evolved to promote immunity in the face of infection or regulation to resolve the inflammatory process, but not both processes simultaneously at the same location. Weaver and colleagues have suggested that co-evolution of Th17 and Treg cells has occurred as a mechanism which enables vertebrates to benefit from commensal microflora in the gut (Weaver & Hatton 2009). In the context of arthritis, it is possible that the immune microenvironment promotes either predominantly Treg development or Th17 but not both.

A novel hypothesis to account for the inverse relationship in JIA arises from reports of a remarkable plasticity of T cell lineages (Murphy & Stockinger 2010). Rather than being terminally differentiated, Treg can convert to Th17 in the presence of the inflammatory cytokines IL-1β and IL-23, whilst TGFβ alone in high levels appears to stabilise Treg (Koenen *et al,* 2008; Manel *et al,* 2008). FOXP3 and RORC2 have a close inter-relationship; FOXP3 directly binds the RORC2 protein and represses downstream targets, including IL-17 (Ichiyama *et al,* 2008). However inflammatory conditions through the production of pro-inflammatory cytokines (e.g., IL-1β and IL-23) may attenuate FOXP3 expression, releasing RORC2 to upregulate IL-17, so converting Treg into Th17 cells. This leads to the hypothesis that this same conversion of Treg to Th17 occurs within the joints of JIA patients. Moreover, the inverse relationship could be

explained if synovial Treg and Th17 arise from a common pool and depending on the inflammatory microenvironment Treg either retain FOXP3 expression or convert to Th17. Future studies are planned to test this hypothesis by analysing shared TRBV CDR3 sequences in the two populations.

4.4.3 Recruitment of Th17 cells to the inflamed joint

Although discussion has focused on the inverse relationship between Treg and Th17, it is important to recognise that both populations are enriched in the joint relative to paired samples from the patient's PBMC. To understand mechanisms important in the recruitment of Th17 to the inflamed joint, chemokine receptor expression was analysed on Th17 cells from JIA samples. CCR4 expression had been reported on Th17 cells from healthy adults (Acosta-Rodriguez *et al,* 2007b; Annunziato *et al*, 2007; Lim *et al,* 2008), and was also found to be expressed on Th17 from the PBMC of JIA patients. CCR4 ligands, CCL17 and CCL21 have been detected within the joints of JIA patients and may play a role in recruiting Treg (Chapter 3). However Th17 cells showed little evidence of chemotaxis to CCL22 when compared to Treg, which may reflect much lower expression of CCR4 on Th17 cells. It is possible that specialised skin specific sub-sets of T cells, expressing high levels of IL-22 and CCR4 may show greater migration to CCR4 ligands (Trifari *et al,* 2009). To date the functional role or potential redundancy of CCR4 in the tracking of Th17 cells has not been explored in animal models. It is interesting to note that CCR4 does not appear to be downstream of RORC2, as forced expression of the transcription factor failed to upregulate CCR4 (Manel *et al,* 2008). In contrast, CCR6 was strongly upregulated following over-expression of RORC2 (Crome *et al,* 2009; Manel *et al,* 2008) and found to be expressed on Th17 cells from all JIA samples. Th17 cells showed a strong chemotactic response to the CCR6 ligand, CCL20. Given the elevation of CCL20 within the inflamed joint and a clear correlation between Th17 frequency in the joint and corresponding CCL20 levels, there is a strong case for the CCR6-CCL20 axis driving Th17 recruitment to the joint in JIA. Furthermore two animal models have supported a dominant role for CCR6 in permitting the initial inflammatory influx to the inflammatory site (Hirota *et al*, 2007; Reboldi *et al,* 2009). What is the source of synovial CCL20? Th17 cells may secrete CCL20, but studies of RA have

identified synovial fibroblasts as the dominant producer of CCL20 (Hirota *et al,* 2007). In JIA, synovial monocytes produce large amounts of CCL20 in response to local hypoxic conditions (Bosco *et al,* 2008). This raises the possibility that the initial innate immune response may dictate further adaptive Th17 responses. Further to this, a recent study has shown a positive feedback loop between neutrophils and Th17 cells, whereby activated neutrophils at the inflammatory site release CCL20 which results in the recruitment of Th17 cells. Once recruited Th17 cells attract and activate neutrophils through the secretion of CXCL8 and GM-CSF, TNFα and IFNγ (Pelletier *et al,* 2010).

4.4.4 Synovial Th17 cells have polyfunctional cytokine secretion

In contrast to early murine reports of Th17 cells as a pure IL-17-secreting lineage (Park *et al,* 2005; Veldhoen *et al,* 2006), IL-17+ expressing cells enriched in the inflamed joint frequently co-express IFNγ (Th17/1 cells). This feature of Th17 cells appears to be more closely linked to the human inflammatory site (Aarvak *et al,* 1999; Annunziato *et al,* 2007), than their murine counterparts (Bending *et al,* 2009; Murphy *et al,* 2003; Park *et al,* 2005). The origins and mechanisms of induction of Th17/1 cells in arthritis are explored further in Chapter 5. A subset of Th17 cells have now been shown to co-express the pro-inflammatory cytokine IL-22 (Wilson *et al,* 2007; Zheng *et al,* 2008), and this was also true of Th17 cells from the peripheral blood and joints of JIA patients and may contribute to the bony damage seen in inflamed joints (Ikeuchi *et al,* 2005; Kelchtermans *et al,* 2009). However SFMC did not have cells with overlapping IL-4 and IL-17 expression, which have been detected in patients with atopic diseases (Cosmi *et al,* 2010). Although not examined in this study, Th17 cells have been shown to express TNFα (Yamada *et al,* 2008) and IL-9 (Beriou *et al,* 2010). Given the polyfunctional nature of cytokine production from Th17 cells, it would be interesting to speculate on the relative contributions of IL-17 versus other Th17-related actions in driving autoimmune arthritis. IL-17 knockout mice appear to develop CIA of a greater severity (Nakae *et al,* 2003a) than IL-23p19 deficient mice (Murphy *et al,* 2003). Although T cells from the latter model are still able to secrete IL-17, the lack of IL-23 almost completely attenuates Th17 differentiation (also see Chapter 6 discussion). These data supports a pathogenic role for Th17 cells independent of IL-17. Although recent evidence suggests important

role for IL-23 that is independent of Th17 cells, driving pathogenic gamma delta and innate lymphoid cells (Buonocore *et al*, 2010; Sutton *et al*, 2009)

The discovery that Th17 cells from the joint had a cytokine expression phenotype intermediate between Th17 and Th1 cells, led to the re-examination of the chemokine receptor expression data from SFMC. CCR4 expression on synovial IL-17+ cells was also found to be skewed towards a Th1 phenotype, in being CCR4 lo. To test if this could be linked to the predominance of Th17/1 cells within the joint, SFMC were sorted into CCR4+ and CCR4- populations, after being gated on CCR6. This showed a clear enrichment of Th17/1 cells within the CCR4- fraction (Figure 4.15), explaining the synovial CCR4lo phenotype detected in earlier experiments (Figure 4.6). To further understand the relationship between Th17/1 and Th17 cells, the transcriptional profile of Th17 subsets was examined within the joint.

4.4.5 Transcriptional programming in synovial Th17 cells

The enrichment of Th17 subpopulations using surface chemokine receptor expression, offered a valuable opportunity to examine the transcriptional profile of T cell subsets within the joint. As discussed above, all IL-17+ cells were limited to the CCR6+ T cell population. Interestingly both Th17/1 and Th17 enriched populations expressed high levels of RORC2 relative to the CCR6- population, which was IL-17-. When expression of T-bet was examined in the three sorted populations (Figure 4.15, CCR4-CCR6-, CCR4-CCR6+, CCR4+CCR6+), there were no significant differences in spite of there being a greater proportion of IFNγ+ cells within the CCR4- populations. It is possible that there is a non-linear relationship between T-bet mRNA expression and IFNγ protein expression. The large numbers of Th1 cells "contaminating" all three populations may also limit the potential to detect differences in T-bet expression. To overcome this problem, and clearly distinguish viable Th17 and Th1 cells *ex-vivo* a cytokine capture technique was utilised, avoiding the potential for epigenetic modification or phenotype plasticity that may accrue during long-term *in vitro* culture (Wei *et al*, 2009). Purified synovial Th1 cells had significantly higher T-bet mRNA expression than Th17 cells, with Th17/1 cells being intermediate between Th17 and Th1. T-bet expression has been linked to autoimmune pathology, independent of IFNγ, and may confer a greater pathogenicity

to the synovial Th17/1 population when compared to Th17 cells (Yang *et al,* 2009). It should be noted that contamination of Th1 cells in the Th17/1 population (Figure 4.18) may contribute to higher levels of T-bet found in the latter population. Interestingly, Th17 cell clones derived from the gut of patients with IBD had equivalent expression of T-bet to Th17/1 and Th1 cells (Annunziato *et al,* 2007). It is not clear if this represents a difference in Th17 phenotype between gut and joint, rather than an artefact of single cell cloning.

With respect to Th17 transcription factors, the results presented in this study showed that RORC2 expression was more specifically linked to Th17 and Th17/1 cells than either IRF4 or AHR. This may reflect a role for IRF4 and AHR that is permissive but not critical to Th17 differentiation (Brustle *et al,* 2007; Veldhoen *et al,* 2008). In adult arthritis, AHR expression was elevated in the synovial tissue of RA patients when compared to patients with osteoarthritis, but AHR was not co-stained with IL-17(Kobayashi *et al,* 2008). As AHR expression has been linked to other T cell sub-sets including IL-22+ cells, Treg and Tr1 like cells, (Gandhi *et al,* 2010; Quintana *et al,* 2008; Trifari *et al,* 2009) the detection of AHR in the joints of RA patients may not be tied to IL-17 producing cells.

4.4.6 Summary

In this Chapter a novel population of pro-inflammatory cells, Th17 cells, critical to murine models of arthritis, was shown to be enriched within the inflamed joints of patients with JIA. Moreover, the frequency of Th17 cells within the joint was closely linked to the severity of disease course in these patients. Here it was shown that Th17 cells are recruited in a CCR6-dependent manner, and that the frequency found within the joint directly correlated with the corresponding levels of the ligand for CCR6, CCL20. The phenotype of Th17 cells within the joint was distinct from that found in peripheral blood, in that a large proportion of synovial cells co-expressed IFNγ. The mechanisms that underlie this enrichment of Th17/1 cells within the joint are explored in Chapter 5.

5 The synovial milieu drives Th17 cell plasticity in JIA

5.1 Introduction

Evidence provided in the previous chapters suggests a link between the frequency of IL-17 expressing T cells within the joint and the severity of clinical course in oligoarthritis JIA. IL-17+ CD4+ T cells, as well as being enriched within the joint, bear a distinct phenotype from those in peripheral blood. The majority of synovial IL-17+ CD4+ T cells are intermediate between Th17 and Th1 cells in terms of cytokine production, expressing IL-17 as well as IFNγ (referred to as Th17/1 cells). This raises questions about the underlying mechanism(s) that lead to an increased frequency of Th17/1 cells relative to Th17 cells within the joint (Chapter 4). Th17/1 and Th17 cells share expression of CCR6, a chemokine receptor which is central to the recruitment of Th17 to inflamed organs (Hirota *et al,* 2007). If the CCR6 pathway is important in JIA, then Th17 and Th17/1 cells should be recruited to the joint in the same proportions as in blood, rather that the predominance of Th17/1 seen in SFMC. An alternative hypothesis to account for the dominance of Th17/1 cells within the joint, is that Th17 cells, once recruited to the joint, undergo plasticity towards a Th1 phenotype. Data from murine studies have demonstrated a role for IL-12 and the related cytokine IL-23 in driving Th17 plasticity towards a Th1 phenotype (Lee *et al,* 2009). This conversion was opposed *in vitro* by high levels of TGFβ or signalling through the IL-6 receptor (Jones *et al,* 2010). These data and the observations from JIA SFMC led to the hypothesis that the cytokine environment within the joints of JIA patients could drive plasticity of Th17 cells towards a Th17/1 phenotype.

In this Chapter this hypothesis was tested by:

(1) Measuring the levels of IL-6, IL-12, IL-23 and TGFβ in SF from patients with JIA.

(2) Asking if resident dendritic cells accounted for the cytokine milieu found within the joint.

(3) Testing if Th17 cells purified directly *ex-vivo* undergo plasticity when cultured in conditions that model the synovial microenvironment.

(4) Testing if Th1 cells purified *ex-vivo* are able to revert to a Th17 phenotype when cultured *in vitro* under Th17 polarising conditions.

5.2 Specific Methods

5.2.1 Cell culture of purified cytokine expressing cells

Cytokine secreting cells were purified by flow cytometry (Chapter 4) and cultured at 37ºC in 5% CO_2 in serum free "Ex-vivo15" medium (Lonza, Slough, UK) in the presence of IL-2 (50iu/ml, Roche) alone or with a combination of the following human recombinant cytokines: TGFβ (5ng/ml,) IL-1β (20ng/ml), IL-12 (10ng/ml, all R&D), IL-23 (10ng/ml, Ebioscience), IL-6 (10ng/ml, BD PharMingen), IL-21 (10ng/ml, GIBCO/ Invitrogen). Serum free medium was used to avoid contamination with FCS which has been shown to inhibit Th17 generation *in vitro*, though the mechanism for this effect is still unclear (Manel *et al*, 2008). Additionally the impact of exogenous TGFβ on Th17 stability could not be studied in the presence of FCS which may contain high levels of bovine TGFβ (Kyrtsonis *et al*, 1998). On day 6 of culture, cells were harvested and re-stimulated with PMA and ionomycin in the presence of brefeldin A for 3 hours. Cells were washed with PBS and resuspended in a live/dead discriminant (Live/dead®, Invitrogen) dye diluted at 1 in 200 with PBS. Cells were stored for 30 minutes at 4ºC in the dark and washed once with FACS buffer before detection of intracellular cytokines.

5.2.2 Culturing Monocyte derived dendritic cells (MoDC)

MoDC were cultured in RPMI mix (Chapter 2) supplemented with 5% AB human serum (Sigma). Monocytes (precursors for myeloid DC) were isolated from blood (healthy control or JIA PBMC) or SF (JIA patients only) using the adherence technique as follows: PBMC or SFMC were resuspended at a concentration of $3.3x10^6$ cells/ml in RPMI supplemented with 5% AB human serum. 10×10^6 cells were added to each well of a 6-well plate and incubated for 2 hours at 37°C in 5% CO_2. After this period, non-adherent cells were removed by three washes with RPMI mix. 2ml of RPMI mix supplemented with 5% AB human serum containing 100ng/ml human recombinant GM-CSF and 50ng/ml human recombinant IL-4 was added to each well and the cells incubated at 37°C in 5%CO_2 for 6 days.

5.2.3 Detection of cytokine secretion from MoDC

MoDC were harvested on day 6, counted and replated in RPMI/5%Human AB serum (Invitrogen) in 96 well plates at a concentration of $1x10^5$/ml. Cells were cultured in medium alone or stimulated with 500ng/ml anti-CD40 monoclonal antibody (Immunotech/Beckman Coulter) or 100ng/ml LPS (Sigma) for 24 hours at 37ºC in 5% CO_2 and supernatant harvested, aliquoted and stored at -80ºC for analysis. Cytokines were detected by multiplex immuno-assay.

5.3 Results

5.3.1 Synovial fluid has a high IL-12/TGFβ ratio

Cytokine levels of IL-12, IL-23 and TGFβ in the joint were compared with levels in patients' blood. The synovial compartment had a remarkably distinct balance when compared to plasma, with significantly higher levels of IL-12 (Figure 5.1, median 143pg/ml vs 23pg/ml, p=0.04, Mann Whitney) and a relative absence of TGFβ (median 158pg/ml vs 6166pg/ml, p<0.01, Mann Whitney). IL-23 was below the threshold for detection in all SF or plasma samples.

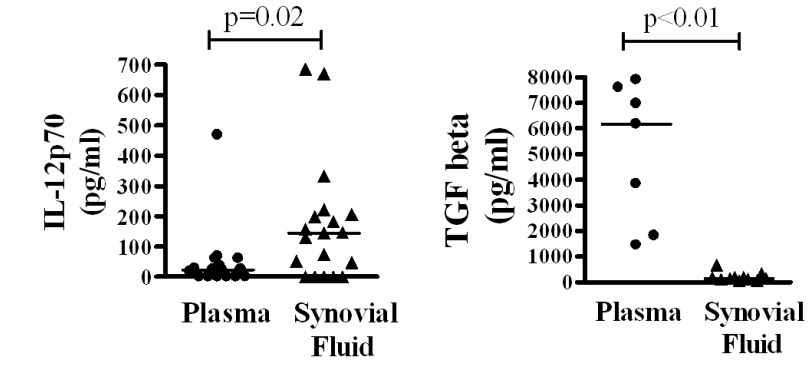


Figure 5.1: Synovial fluid has a high IL-12:TGFβ ratio.

IL-12 and TGFβ protein levels were measured in JIA plasma and synovial supernatants by ELISA (TGFβ) and Luminex (IL-12), (IL-12 *n*=16 plasma, 19 SF and TGFβ *n*= 7 plasma, 11 SF). Lines represent median levels.

The cellular source of IL-12 production within JIA joints is not known. In patients with RA, MoDC from SF have been shown to secrete IL-12 in higher levels than from peripheral blood (Mottonen *et al,* 2002). To confirm elevated IL-12 production from cells retrieved from the joint, unsorted SFMC were stimulated with LPS alone or after pre-incubation with IFNγ, which is known to augment IL-12 secretion (Ma *et al,* 1996), via STAT-1 signalling (Hu *et al,* 2002). As IFNγ secreting cells are present in high levels within the joint (Wedderburn *et al,* 2000) it was possible that SFMC would secrete high levels of IL-12 without IFNγ pre-treatment *in vitro*. Stimulation of PBMC with LPS alone did not stimulate high levels of IL-12 secretion compared to medium alone (Figure 5.2A). Following pre-treatment of PBMC with IFNγ there was an increase in IL-12 secretion in 2 of 4 PBMC samples tested. In view of the large variation in cytokine secretion between individuals, this increase did not achieve statistical significance. LPS stimulated SFMC did not show an increase in IL-12 secretion compared to medium alone. Following pre-treatment of SFMC with IFNγ, there was small non-significant increase in IL-12 (58±39pg/ml vs 4.3±2.1pg/ml, mean±SEM). Other cytokines relevant to the induction and plasticity of Th17 cells, IL-23 and IL-1β were also measured by multiplex immunoassay (Figure 5.2B-D). Again large inter-individual variation made it hard to establish differences in IL-23 secretion between PBMC and SFMC and would require a much greater sample size (Figure 5.2B). Baseline IL-1β secretion was higher in PBMC and showed a greater response to IFNγ pre-incubation than SFMC (Figure 5.2C). Similarly TNFα secretion showed a more consistent response to LPS with and without IFNγ pre-incubation in PBMC. However from this limited number of samples, there was no clear distinction in the production of TNFα between SFMC and PBMC samples.

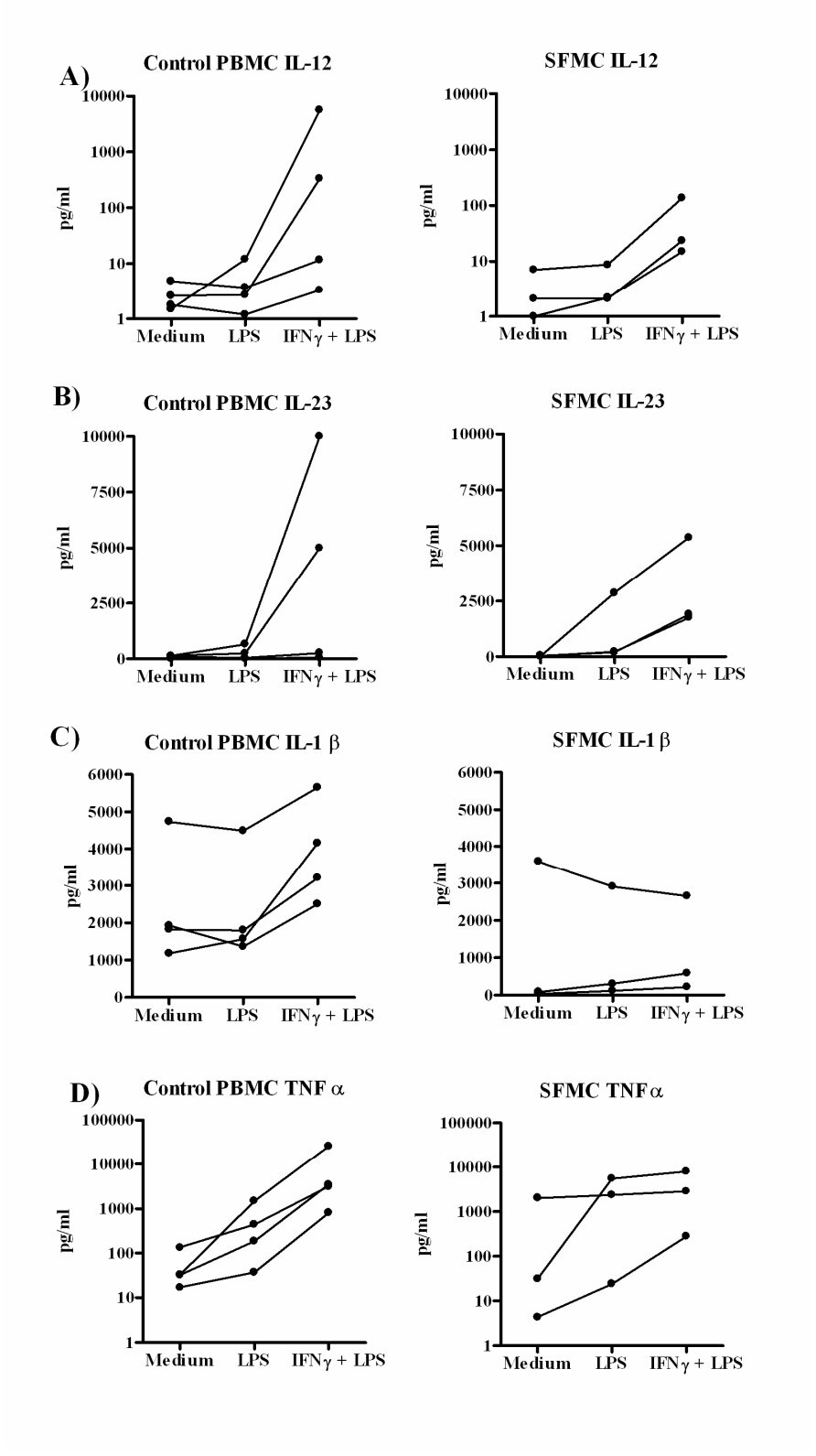


Figure 5.2: Induction of cytokines from Healthy control PBMC and JIA SFMC.

Healthy control PBMC (n=4) and JIA SFMC (n=3) were cultured in medium or 100ng/ml LPS either alone or following pre-incubation with 10u/ml IFNγ for 6 hours. Supernatants were harvested at 24 hours from (left) PBMC and SFMC (right) and A) IL-12, B) IL-23, C) IL-1β and D) TNFα detected by multiplex immunoassay.

5.3.2 IL-12 expression from synovial monocyte derived dendritic cells (MoDC)

As unsorted total SFMC samples failed to show increased secretion of IL-12 compared to PBMC, in response to a TLR4 signal (LPS), with or without IFNγ pre-treatment, an alternate approach was taken to examine IL-12 production from synovial MoDC. In view of the limited numbers of resident DC within the arthritis joints, traditionally DC derived from synovial monocytes (Sallusto & Lanzavecchia 1994) have been studied as a method to model the *in vivo* pathology of the joint (Santiago-Schwarz *et al,* 2001). To test if MoDC from JIA joints were enriched for IL-12 relative to PBMC, MoDC from 3 paired JIA PBMC and SFMC were harvested and analysed by PCR to detect expression of IL-12 family transcripts. IL-12 and IL-23 share expression of the p40 sub-unit which forms a heterodimer with p35 in the case of IL-12, and p19 in the case of IL-23. Analysis of cells from the three JIA patients did not show a significant difference in IL-12p35 (Figure 5.3A) or p19 (Figure 5.3B) expression in moDC derived from SFMC when compared to paired PBMC.

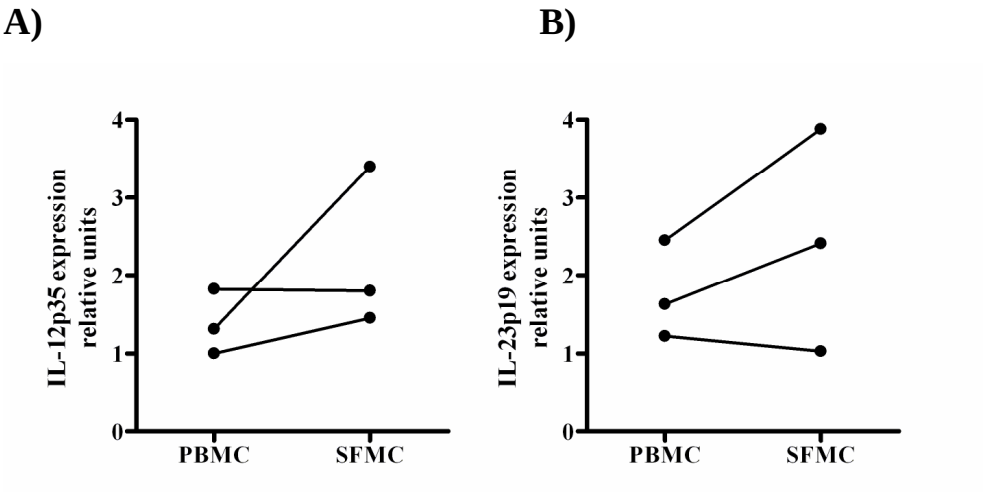


Figure 5.3: Detection of mRNA of IL-12p35 and IL-23p19 subunits in monocyte derived dendritic cells generated from JIA PBMC and SFMC

MoDC were generated from JIA PBMC or SFMC samples after culture with IL-4 and GM-CSF. MoDC were harvested on day 6 without prior stimulation, and RNA extracted. A) IL-12p35 and B) p19 expression was assessed by quantitative RT-PCR using β2m microglobulin as a housekeeping gene. cDNA courtesy of Dr E.Sala-Soriano, ICH, UCL.

Next it was tested whether synovial MoDC had greater IL-12 production at the protein level than those derived from paired PBMC. MoDC were split into 3 aliquots, one of which was left unstimulated, one stimulated with LPS to model TLR4 signals that may be present at the site of inflammation. In the third condition, MoDC were treated with an anti-CD40 antibody with agonistic properties (Chiang *et al*, 2008). This stimulus may be considered to be more physiological than LPS, as it models the co-activation signals found on activated CD40 ligand-positive T cells within the joint during inflammatory arthritis (MacDonald *et al*, 1997). Of the 3 paired JIA samples used to generate MoDC, one SFMC sample yielded too few MoDC to be tested. The remaining results are displayed in Figure 5.4. IL-1β was secreted at very low levels in unstimulated MoDC (Figure 5.4A). Stimulation with anti-CD40 failed to upregulate secretion of any of the measured cytokines either in PBMC or SFMC. As a result of the large variation between samples and the relatively small number of samples analysed, it is difficult to draw conclusions on the level of cytokines secreted by PBMC and draw comparisons with SFMC. Overall there did not appear to be higher levels of secreted cytokines in response to LPS within the SFMC compartment when compared to PBMC.

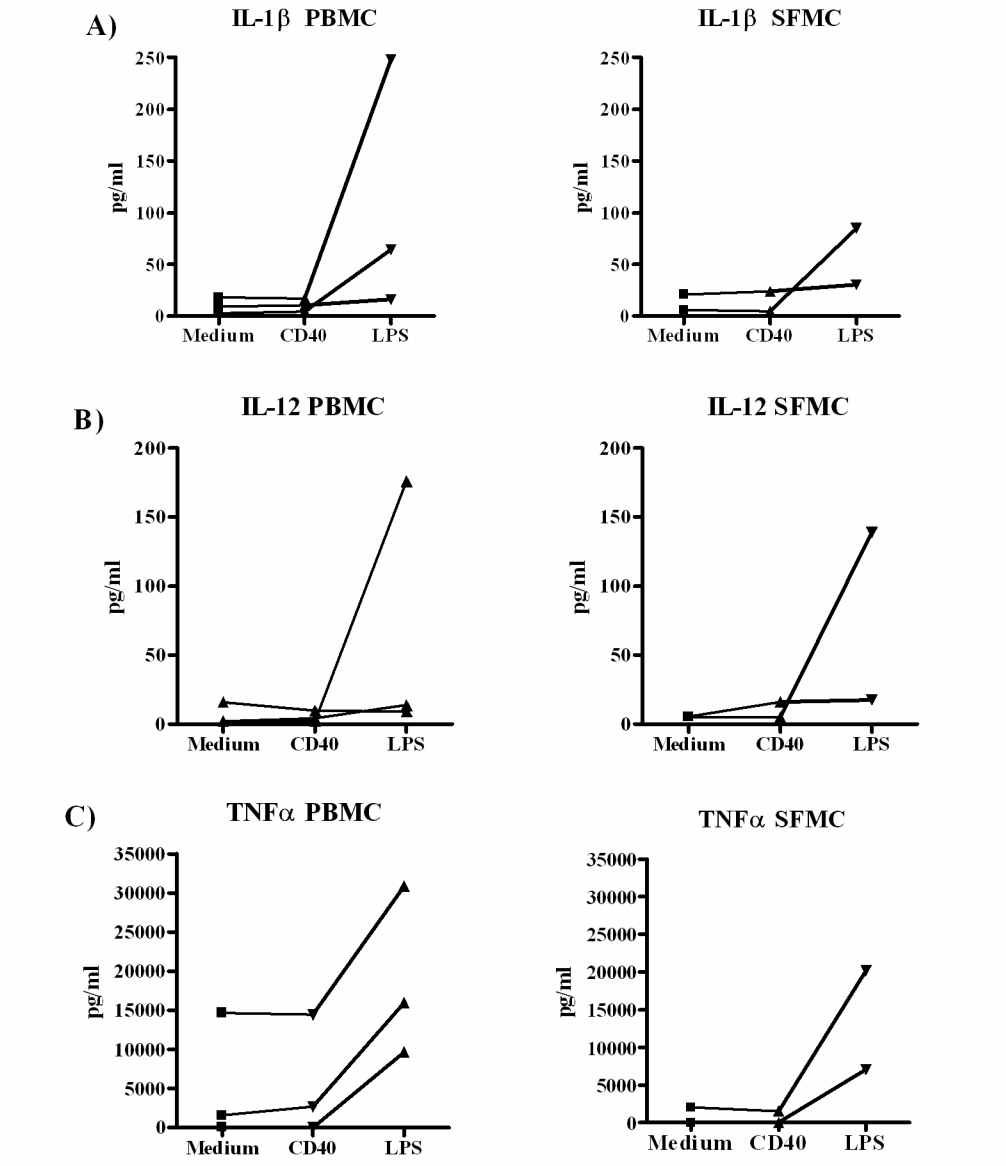


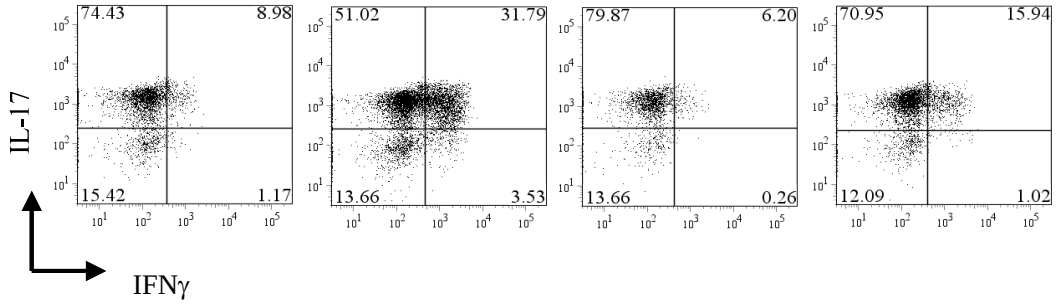
Figure 5.4: Cytokine secretion from JIA PBMC and SFMC monocyte derived dendritic cells.

MoDC were generated from JIA PBMC or SFMC samples by culture with IL-4 and GM-CSF. After 6 days MoDC were harvested and incubated in medium alone or stimulated with 500ng/ml αCD40 monoclonal antibody or 100ng/ml LPS. Supernatants were harvested at 24hrs from (left) PBMC derived MoDC and (right) SFMC derived MoDC and A) IL-1β, B) IL-12 and C) TNFα proteins were detected by multiplex immunoassay.

5.3.3 The synovial microenvironment drives Th17 plasticity

To model the effects of the synovial microenvironment on Th17 plasticity the cytokine capture assay was used to purify Th17 cells from healthy control PBMC (average purity Th17 cells 93%, Th17/1 <2%, Th1<0.1%, Chapter 4). Purified peripheral blood Th17 cells were cultured in serum free medium in the presence of IL-12 to reflect the synovial compartment, or TGFβ to mimic plasma, or both cytokines. Th17 cells rapidly upregulated IFNγ production and showed a significant increase in proportion of Th17/1 cells when cultured with IL-12 (Figure 5.5A, 2nd plot, p=0.03, paired t-test). In contrast the presence of TGFβ stabilised the Th17 phenotype (3rd plot), but failed to overcome the effects of IL-12 when cells were cultured with both cytokines together (last plot). A summary of 3 independent experiments showed that IL-12 also promoted a small proportion of cells to develop a Th1 phenotype (5.2% ±1.9%, Figure 5.8B).

A)



TGFβ	-	-	+	+
IL-12	-	+	-	+

B)

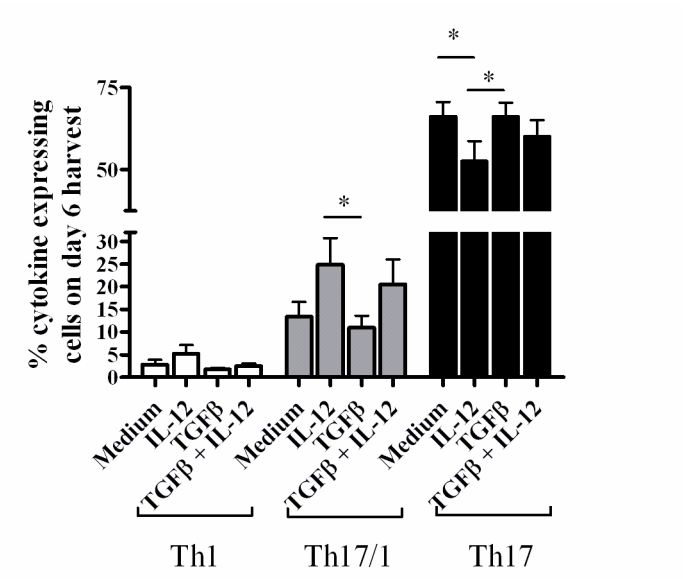


Figure 5.5: High ratio of IL-12: TGFβ promotes the generation of Th17/1 cells *in vitro.*

A) Th17 cells were purified by capture assay from healthy controls and cultured in serum free medium in the presence of IL-2 or additionally with recombinant IL-12, TGFβ or both as shown. Dot plots of cytokine production in Th17 cells after culture for 6 days. Numbers in all plots indicate the percentages of cells secreting cytokines.
B) Summary of 3 independent experiments culturing purified Th17 cells as in A. Bars represent mean number (error bars 1SEM) of cells as a percentage of live cells harvested on day 6 with resultant Th1 cells (unfilled bars), Th17/1 cells (grey bars) or Th17 cells (black bars) * $p<0.05$.

5.3.4 IL-6 is elevated within the joint but does not modulate Th17 plasticity *in vitro*

Initial murine studies established a critical role for IL-6 in the induction of Th17 *in vitro*, when combined with TGFβ (Veldhoen *et al,* 2006). More recently IL-6 has also been shown to modulate Th17 plasticity in murine models. IL-6, once bound to the soluble form of its receptor (sIL-6R) can bind gp130, a widely expressed IL-6 co-receptor (Taga *et al,* 1989), leading to activation of the IL-6 signalling pathway. This mechanism is known as *trans*-signalling. IL-6 *trans*-signalling promoted the stability of murine Th17 cells both *in vitro* and *in vivo* (Jones *et al,* 2010) and prevented conversion to Th1 cells. The role of IL-6 in the development and stability of human Th17 cells is still controversial (Chapter 1). IL-6 has been identified as a key player in the pathophysiology of autoimmune arthritis, in particular systemic forms of JIA (de Benedetti F. *et al,* 1991). To test if IL-6 may influence the plasticity of Th17 cells within the joint, firstly IL-6 levels were measured in JIA plasma and SF, by the multiplex cytokine assay.

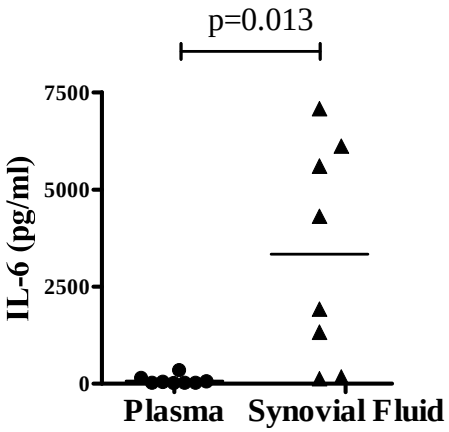


Figure 5.6: IL-6 is elevated within the joint.

IL-6 protein levels were measured in JIA plasma and synovial supernatants by Luminex (*n*=8). Lines represent mean levels.

Figure 5.6 shows that IL-6 is significantly elevated within synovial fluid compared to plasma (p=0.013, paired t-test). It was possible that IL-6 would oppose the action of IL-

12 *in vitro* and stabilise the Th17 phenotype. To test this, Th17 cells purified from peripheral blood using the capture assay were again cultured in the presence of IL-12 but additionally with or without IL-6. IL-6 alone did not significantly change the proportions of Th1, Th17/1 and Th17 cells (Figure 5.7). As noted previously (Figure 5.5), IL-12 led to an increase in the proportion of Th1 and Th17/1 cells with loss of the Th17 phenotype. The addition of IL-6 did not alter the action of IL-12 in promoting Th17 plasticity.

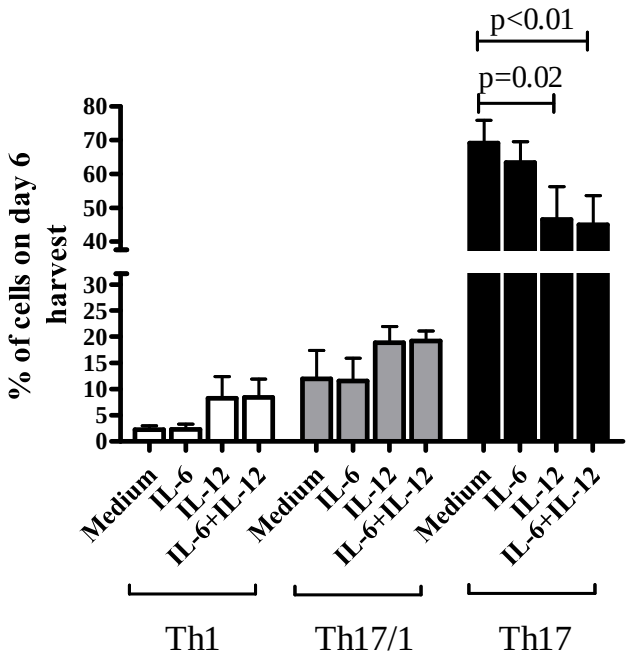


Figure 5.7: IL-6 fails to stabilise the Th17 phenotype *in vitro*.

Th17 cells were captured from healthy controls and cultured in serum free medium in the presence of IL-2 or additionally with recombinant IL-12, IL-6 or both as shown. Bars represent mean number (error bars 1SEM) of cells as a percentage of cells harvested on day 6 with a Th1 (unfilled bars), Th17/1 (grey bars) or Th17 phenotype (black bars) as detected by flow cytometry. (n=3).

5.3.5 Th1 cells fail to revert to a Th17 phenotype *in vitro*

If synovial Th1 cells upregulated IL-17 expression this could explain the dominance of Th17/1 within the joint. To test this hypothesis, the plasticity of Th1 cells from peripheral blood was tested directly *ex-vivo* (average sorted Th1 purity 91.7%, Th17/1 and Th17 <0.2%) by culturing cells with a range of cytokines known to promote a Th17 phenotype in naïve T cells (Figure 5.8). Although none of the conditions led to the emergence of a large population of Th17 or Th17/1 cells, IL-1β and IL-23 in the presence of TGFβ (bottom right plot) led to a small population of cells co-expressing IL-17 and IFNγ. None of the conditions tested led to the generation of Th17 cells.

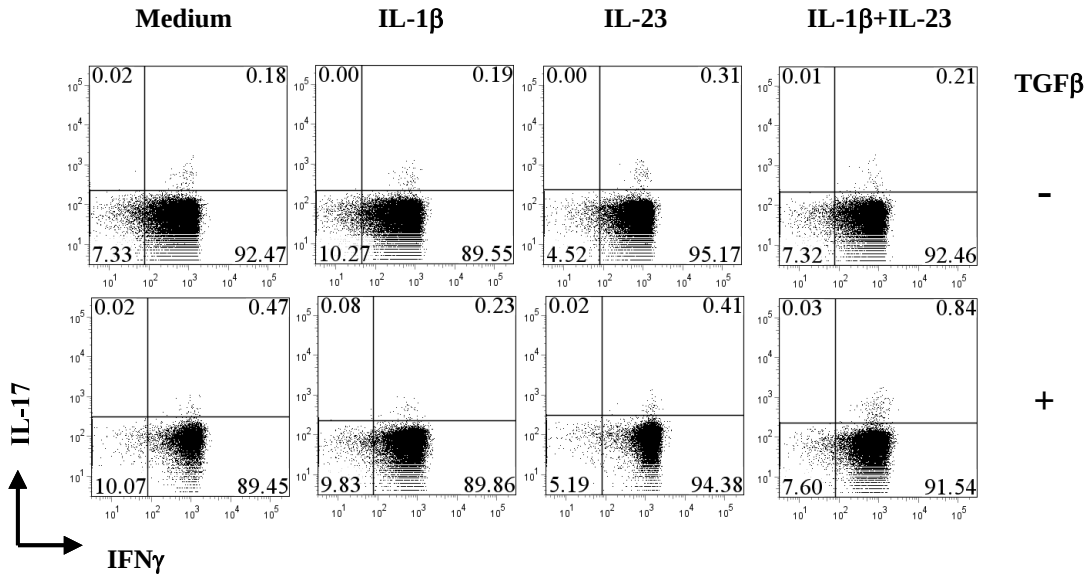


Figure 5.8: Th1 cells fail to revert to a Th17 phenotype

Th1 cells from healthy control donors were detected using a cytokine capture assay and sorted to high purity. Cells were cultured in serum free medium with IL-2 alone or with recombinant IL-1β, IL-23, or both and (bottom row) additionally in the presence of TGFβ as shown. One representative dot plot (of 3 independent experiments) of intracellular IL-17 and IFNγ detected by flow cytometry on day 6 of culture.

5.3.6 IL-21 is present within the inflamed joint but fails to induce Th1 plasticity

The gamma chain cytokine IL-21, has recently been shown to induce both human and murine Th17 cells in an IL-6 independent pathway (Korn *et al*, 2007; Yang *et al*, 2008a). Given the putative role for IL-21 in the pathophysiology of autoimmune arthritis (Young *et al*, 2007) it was important to test the role of IL-21 on Th17 plasticity. IL-21 protein levels were measured in JIA plasma and SF. Although detectable within the joint, IL-21 levels were not enriched at the inflammatory site (Figure 5.9).

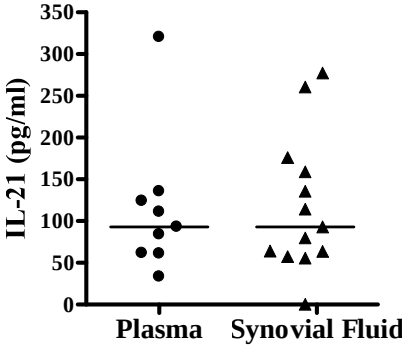


Figure 5.9: IL-21 is present in JIA plasma and synovial fluid

IL-21 protein levels were measured in JIA plasma and SF by ELISA. Lines represent median values (n=9 plasma, n=13 SF).

Although IL-21 was not increased in synovial fluid, IL-21 receptor levels have been shown to be enriched within the inflamed joints of RA patients (Jungel *et al*, 2004), and thus sensitivity to IL-21 could be altered at the inflammatory site. To test if IL-21 may promote Th1 plasticity *in vitro*, purified Th1 cells were cultured in the presence of IL-21, with or without additional TGFβ (Figure 5.10). IL-21 did not induce a Th17/1 population when in culture alone. The addition of TGFβ led to an expansion of IL-17-IFNγ- cells but did not potentiate the generation of Th17/1 cells.

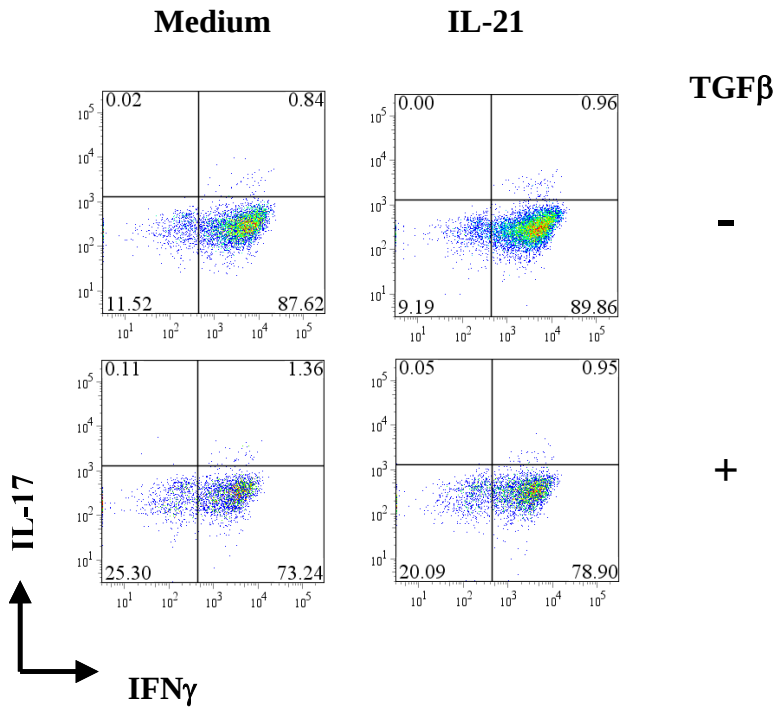


Figure 5.10: Th1 cells fails to revert to Th17 phenotype after culture in the presence of IL-21.

Th1 cells from healthy control donors were detected using a cytokine capture assay and sorted to high purity. Cells were cultured in serum free medium with IL-2 alone or with recombinant IL-21 and (bottom row) additionally in the presence of TGFβ as shown. One representative dot plot (of 3 independent experiments) of intracellular IL-17 and IFNγ detected by flow cytometry on day 6 of culture.

5.4 Discussion

5.4.1 Defining the cytokine microenvironment with the joint

In this study, Th17 cells from the joints of patients with inflammatory arthritis had a phenotype distinct from Th17 cells in PBMC, and shared many features of Th1 cells. Following reports of plasticity in murine Th17 cells (Bending *et al,* 2009; Lee *et al,* 2009), it was predicted that Th17/1 cells within the joint develop from a Th17 pool in response to local cytokine cues. After examining the key cytokines implicated in murine studies, it was clear that the joint could provide a microenvironment which contributes to Th17 plasticity, by being rich in IL-12 but with relatively low levels of TGFβ. Before identifying the source of IL-12, mononuclear cells from peripheral blood and the joint were compared, to assess the extent of IL-12 secretion in response to inflammatory signals. In LPS stimulated PBMC there was a marked increase in IL-12 secretion following pre-incubation with IFNγ. This is a recognised finding, and results from the increased transcription of IL-12p35 and p40 in response to IFNγ (Ma *et al,* 1996) and is mediated via STAT-1 (Hu *et al,* 2002). SFMC stimulated with LPS and IFNγ showed a much smaller response than PBMC, which is surprising given the enrichment of IL-12 within the joint. This result may be explained by the inhibitory effect of TNFα, itself enriched within the joint, on IL-12p40 mRNA expression and therefore IL-12 secretion (Ma *et al,* 2000). There is evidence that IL-10 (also present in the inflamed joint) can mediate a suppression of the monocyte response to IFNγ, via STAT-3 signalling (Williams *et al,* 2004) Alternatively, the high frequency of Treg within the SFMC compartment may attenuate macrophage and dendritic cell function (Grossman *et al,* 2004), and so limit IL-12 secretion by SFMC.

To examine IL-12 expression by synovial DC without the confounding influence of synovial Treg, monocyte derived DC were generated using standard methods. Neither the IL-12 or IL-23 specific sub-units were enhanced at the level of messenger RNA in unstimulated synovial MoDC when compared to MoDC from peripheral blood. Expression of the common IL-12p40 subunit was not examined but this may be important, as the p40 subunit plays a greater role in regulating secretion of IL-12p70 than

p35 which is much more widely expressed (Trinchieri *et al,* 2003). At the level of protein, MoDC from SFMC did not secrete greater IL-12p70 in response to LPS, than those derived from paired PBMC. Although previously optimised in the laboratory of Prof. B.Chain (UCL Infection and Immunity), it is not clear that the anti-CD40 antibody used in this study displayed agonistic properties. In a study of RA, ligation of CD40 using a CD154 (CD40 ligand) transfected cell line, showed a clear enhancement of IL-12p40 and p70 secretion from MoDC derived from SFMC when compared to PBMC (Mottonen *et al,* 2002). Further experiments testing the synergy of CD40 ligation with LPS would have been valuable, as previous studies have indicated that this dual signal offers a greater enhancement of IL-12 secretion (Snijders *et al,* 1998). Further work should also examine synovial neutrophils (Cassatella *et al,* 1995) or cells specific to the synovial lining to see if these populations are responsible for the high levels of IL-12 found within the joint (Sakkas *et al,* 1998).

5.4.2 Modelling the effect of the synovial microenvironment on Th17 plasticity

It was hypothesised that the distinct balance of IL-12 and TGFβ found in SF would drive Th17 plasticity. When modelled *in vitro,* highly purified Th17 cells rapidly upregulated IFNγ developing a Th17/1 phenotype after being cultured in the presence of IL-12 without TGFβ. Annunziato and colleagues had previously demonstrated similar results using human Th17 clones (Annunziato *et al,* 2007). However long term culture, such as that involved in single cell cloning may incur epigenetic modifications and so alter the phenotype of T cells *in vitro*. As T cell plasticity is critically dependent on the epigenetic control of cytokine and transcription factor loci (Lee *et al,* 2009; Mukasa *et al,* 2010), the data presented in this Chapter, using Th17 directly *ex vivo,* are an important validation of previously published results. In the case of other human autoimmune diseases it is still unclear if local IL-12 production drives the enrichment of Th17/1 frequently found at other inflammatory sites (Aarvak *et al,* 1999; Annunziato *et al,* 2007). Data from murine models of autoimmune disease now confirm the importance of Th17 plasticity *in vivo* and will be discussed further in Chapter 6.

In this study, the addition of TGFβ to IL-12 partially stabilised the Th17 phenotype. This was consistent with results from Lee and colleagues (Lee *et al,* 2009) who found that IL-12 led murine Th17 to convert to Th17/1 or Th1 cells, but the production of IL-17 was maintained when TGFβ was present. This results from TGFβ stabilising RORC2 expression (Manel *et al,* 2008) and amplifying permissive epigenetic modifications across the IL-17A/IL-17F locus thus maintaining the Th17 phenotype (Mukasa *et al,* 2010). It is also likely that TGFβ indirectly supports Th17 cells by preferentially suppressing the proliferation of Th1 cells (Santarlasci *et al,* 2009). Higher levels of TGFβ in plasma than SF would support the maintenance of a Th17 phenotype in PBMC. However it is important to note that TGFβ measured by ELISA, represents total TGFβ, most of which is "latent", being bound to a family of carrier proteins (Li & Flavell 2008). In addition, platelets, which are present in much higher levels in plasma than SF, can secrete high levels of TGFβ once activated (Assoian & Marcantonio 1996). A bio-assay of plasma and SF TGFβ levels may offer a more physiological comparison between the two sites.

Modelling the synovial microenvironment *in vitro* is difficult, in view of the vast range of inflammatory mediators present at the inflammatory site (Pharoah *et al,* 2006). It is known unfractionated SF supernatants from RA patients can strongly promote the differentiation of naïve T cells towards a Th1 pathway, partly dependent on soluble TNF receptor (Santiago-Schwarz *et al,* 2001). However these studies were performed before the Th17 pathway was identified so skewing towards Th17 cells was not examined. Other candidates that may be important in JIA include IL-6, which together with its soluble receptor is present in high levels within the inflamed joint (Peake *et al,* 2006). Given that ligation of the IL-6/sIL-6R-complex to the signalling receptor gp130 (*trans* signalling) plays an important role in animal models of arthritis (Nowell *et al,* 2009), it was important to test the effects of IL-6 on human Th17 plasticity. In this study, IL-6 alone, failed to modulate Th17 plasticity *in vitro,* although the effects of *trans* signalling were not directly tested and this may be more relevant to the physiological system (Jones *et al,* 2010).

This study has focused on the role of soluble mediators in regulating Th17 plasticity within the joint during inflammatory arthritis. Although synovial DC were discussed

earlier in the context of IL-12 secretion, the direct contact dependent effects of this population on T cell priming have not been assessed in this study. Early data that preceded the discovery of Th17 cells, showed that DC from joints of patients with RA were potent stimulator of autologous T cells when compared to PB MoDC (Thomas *et al,* 1994). More recently two groups have shown that activated antigen presenting cells from the inflammatory site can promote local Th17 responses, partly through a cell contact dependent manner, but these studies have not always distinguished between the generation of Th17 or Th17/1 cells (Dhodapkar *et al,* 2008; Evans *et al,* 2009). Fibroblasts from the inflamed joint may also contribute to the local generation of Th17 cells. Human fibroblasts have been shown to secrete Prostaglandin E2 which acts on its receptor EP4 on DC to increase IL-23 secretion and drive Th17 differentiation (Schirmer *et al,* 2010; Yao *et al,* 2009). As IL-17 activates fibroblasts, this suggests a cross-talk between Th17 cells and synovial fibroblasts which drives inflammation (Cho *et al,* 2004; Yamamura *et al,* 2001).

It is likely that the generation and plasticity of Th17 cells within the joint is dependent on a synthesis of signals, both from soluble mediators and direct cell contact with DC and other APC.

5.4.3 Testing the plasticity of Th1 cells

The discovery of Th17 plasticity challenged the notion of T cells subsets as terminally differentiated cell lineages. This also questioned if all T cell subsets have interchangeable cytokine phenotype, and in particular, if Th1 cells could upregulate IL-17 and develop a Th17/1 phenotype. To test this, high purified Th1 cells were cultured with a range of cytokines, either alone and in combinations. None of the conditions tested, including IL-21 or IL-23 led to the development of a significant Th17 or Th17/1 population. After culture with TGFβ, IL-1β and IL-23 (Figure 5.8), a very small population of Th17/1 cells (<1%) was detectable. From this experiment it is not possible to exclude the contamination of a small number of Th17 or Th17/1 cells in the sorted Th1 population (Chapter 4, Figure 4.17). As Th17 and Th17/1 populations both express high levels of IL-1β and IL-23 receptors (Annunziato *et al,* 2007; Lee *et al,* 2010) these cells may preferentially expand to the added cytokines. Annunziato and colleagues also found that

Th1 clones could not revert to a Th17 or Th17/1 phenotype and similar results were found in the murine system (Annunziato *et al,* 2007; Lee *et al*, 2009; Shi *et al,* 2008). The failure of Th1 cells to revert to a Th17 phenotype may be due to the strong epigenetic repression (as defined by histone lysine methylation profiling) of the IL-17 and RORγt loci in this population (Wei *et al,* 2009). Wei and colleagues contrasted this result with that of the Th17 population, where the T-bet locus is not fully repressed, facilitating the expression of T-bet after exposure to IL-12.

Taken together, data on the plasticity Th17 and Th1 cells suggest a linear direction of conversion from Th17 to Th1 cells and not back. Why has the immune system evolved such a system of T cell plasticity? One explanation can be sought from the immunological response to pathogens. The first contact of the immune system with pathogens is typically at breached epithelial surfaces and requires a vigorous neutrophilic response to neutralise extracellular bacteria. IL-17 secreting cells, enriched at the mucosal site, are ideally placed to recruit multiple effector responses from the innate immune system, including a rapid neutrophil influx to serve this function. However, if the pathogen evades this response and is able to establish an intracellular niche, a different effector arm is required. Following the initial inflammatory response, recruited myeloid cells capable of secreting IL-12 will promote phenotype switching from Th17 to a Th1 response, leading to the secretion of IFNγ which is critical to the clearance of intracellular pathogens. This switch is a highly efficient mechanism by which T cells, already recruited to the site of infection, can offer two sequential methods to neutralise pathogens. Data tracking antigen specific Th1 and Th17 cells using tetramer reagents in wild type mice, support this model of a short lived Th17 population which is driven by bacteria at the mucosal site, and a Th1 response which is much more long lived (Pepper *et al,* 2010).

An alternate explanation for the Th17 to Th1 transition has been proposed by Weaver and others, in which IFNγ plays a regulatory role, inhibiting potentially destructive IL-17 responses and also contributing to the contraction phase of the acute inflammatory responses (Kelchtermans *et al,* 2008; Lee *et al,* 2010). This hypothesis will be considered in greater detail in Chapter 7, when discussing the relative contribution of IL-17 and IFNγ to the inflammatory process in JIA.

5.4.4 Summary

The work presented in this Chapter set out to explore the mechanisms underlying the predominance of Th17/1 cells found in the CD4+ T cell population within the joints of patients with JIA. SF was found to be enriched for IL-12 and to have relatively low levels of TGFβ. When modelled *in vitro*, this particular cytokine milieu led to the transition of Th17 cells towards a Th17/1 phenotype. However Th1 cells were resistant to conversion to a Th17 or Th17/1 phenotype. To confirm that these findings were relevant to the plasticity of Th17 cells within the inflamed joint, the clonal relationship between synovial Th17, Th17/1 and Th1 cells was examined next.

6：Th17 cells share clonal links with CD161+ Th1 cells from the inflamed joint

6.1 Introduction

The results presented in Chapter 5 demonstrated the plasticity of Th17 cells towards a Th17/1 phenotype in response to IL-12. In contrast, Th1 cells failed to upregulate IL-17 production under any conditions tested. Together, these results led to the hypothesis that at the inflammatory site in autoimmune arthritis, Th17 cells expressing IFNγ (Th17/1) may originate from the Th17 pool. This hypothesis was tested by analysing the clonality of Th17/1, Th17 and Th1 cells from inflamed joints by analysis of T cell receptor (TR) complementarity determining region-3 (CDR3) by spectratyping and sequencing. During thymic selection, T cells undergo rearrangement of genes defining the CDR3 of the TR variable β chain (TRBV), which remains unique to each subsequent progeny irrespective of any changes in cell phenotype or cytokine expression. Analysis of CDR3 length by spectratyping offers a method of assessing clonal expansion, particularly if the clonal length is non-modal. Furthermore, the CDR3 sequence at the nucleotide level identifies unique T cell clones, allowing comparisons to be made between distinct T cell subsets to detect shared ancestry. In the present study Th17, Th17/1 and Th1 cells purified from SFMC were assessed for CDR3 length and sequence, to test the proposed hypothesis.

As well as Th17 undergoing plasticity to a Th17/1 phenotype, a small proportion of cells lost IL-17 expression and transitioned to a Th1 phenotype *in vitro* (Chapter 5). To seek evidence for this conversion *in vivo*, TR clonality of Th17 was compared to Th1 cells, either as "all" Th1 cells, by cytokine capture or with the latter population divided into 2 distinct sub-sets according to expression of CD161. The lectin-like receptor, CD161 has been proposed as a potential surface marker of Th17 cells, identifying naïve cells which show a pre-commitment towards Th17 differentiation as well as mature Th17 cells (Cosmi *et al*, 2008). The hypothesis that CD161 may mark Th1 cells which originate from a Th17 pool was first explored by comparing expression of "Th17-signature" molecules between CD161+ and CD161- Th1 cells. Further evidence for a shared origin was sought by sorting CD161+ Th1 cells and IL-17+ CD4+ populations and comparing TR clonality and CDR3 sequence.

6.2 Specific Methods

6.2.1 Spectratyping of TR Variable β chain

6.2.1.1 CDR3 RT-PCR

CDR3 TR spectratyping was performed by preparing cDNA from cytokine-captured purified T cell subsets. TR Variable β chain (TRBV)-specific primers (Table 6.1) for 12 sub-families that make up a high proportion of CD4+ T cells in the healthy repertoire (Wedderburn *et al,* 2001b) and are known to be expressed at a high frequency in SFMC (Wedderburn *et al,* 2001a), were used in pairs with a β constant region (BC)-specific primer to amplify the CDR3 region of the TRBV chain, and products being amplified using a secondary (internal) BC-primer.

TR primer	Sequence (5' to 3')	FAM labelled TRBV Product length (bp)
Primary BC primer	GGGTGTGGGAGATCTCTGC	n/a
Secondary BC primer*	ACACAGCAGCCTCGGGTGGG	n/a
BV3	CGCTTCTCCCTGATTCTGGAGTCC	166
BV6b1	CAGGGSCCAGAGTTTCTGAC	266
BV6b2	CAGGGCTCAGAGGTTCTGAC	266
BV8	GGTACAGACAGACCATGATGC	285
BV9	TTCCCTGGAGCTTGGTGACTCTGC	146
BV12	TCCYCCTCACTCTGGAGTC	162
BV13a	GGTATCGACAAGACCCAGGCA	282
BV14	GGGCTGGGCTTAAGGCAGATCTAC	265
BV18	CTGCTGAATTTCCCAAAGAGGGCC	189
BV20	TGCCCAGAATCTCTCAGCCTCCA	204
BV21	GGAGTAGACTCCACTCTCAAG	172
BV23	ATTCTGAACTGAACATGAGCTCCT	165
BV24	GACATCCGCTCACCAGGCCTG	154

* labelled with fluorescent dye, FAM

Table 6.1 Primers used for CDR3 RT-PCR.

BV6b1 and BV6b2 primers were mixed together in a 1:1 ratio and referred to as primer BV6b. All primers were made up to a final concentration of 10pmol/µl. TRBV nomenclature as in (Arden *et al,* 1995).

BV primers were paired as follows so that product sizes did not overlap when spectratyped ; BV9 and 18, 23 and 6b, 3 and 13a, 21 and 8, 24 and 20, 12 and 14. 1µl of each the corresponding primer pairs were aliquoted into a 0.5ml eppendorf and 23µl of the master mix (Table 6.2) was added.

Reagent* (stock concentration)	**N=1**	**N=6.5**
Buffer (x10)	2.5µl	16.25µl
$MgCl_2$ (50mM)	1µl	6.5µl
Taq DNA polymerase	0.25µl	1.6µl
BC primer (10pmols/µl)	2µl	13µl
dNTPs (10mM)	0.5µl	3.3µl
cDNA	5µl	32.5µl
Water	11.75µl	76.4µl
Total	23µl	149.5µl

Table 6.2 Master mix for CDR3 RT-PCR reaction

*reagents all from Bioline.

RT-PCR amplification conditions were as follows: 95°C for 25 seconds, 40 cycles of 95°C for 25 seconds, 60°C for 45 seconds, 72°C for 45 seconds followed by 72°C for 5 minutes and then stored at 4°C.

6.2.1.2 Run-off labelling reaction

2µl of the RT-PCR reaction was pipetted into a 0.2ml thin-walled PCR tube with 8µl of the master mix for the run-off reaction (Table 6.3). Products were amplified as follows: 95°C for 25 seconds, 15 cycles of 95°C for 25 seconds, 60°C for 45 seconds, 72°C for 45 seconds, followed by 72°C for 5 minutes. Products were stored in the dark at -20°C until ready to run for spectratyping.

Reagent* (stock concentration)	**N=1**	**N=6.5**
Buffer (x10)	1µl	6.5µl
$MgCl_2$ (50mM)	0.5µl	3.25µl
Taq DNA polymerase (5U/µl)	0.033µl	0.2µl
BC FAM primer (1pmol/µl)	1µl	6.5µl
dNTPs (10mM)	0.7µl	4.6µl
Water	4.8µl	31.2µl
Total	8µl	52.3µl

Table 6.3 Master mix for CDR3 run-off PCR

*reagents all from Bioline.

6.2.1.3 Spectratyping

A mixture of 1.75µl GeneScan-500 ROX fluorescent ladder (Applied Biosystems) and 54.25µl Hi-Di Formamide (Applied Biosystems) was made and 8µl aliquoted in an optical reaction 96 well plate (Applied Biosystems). Run-off PCR products (Run-off labelling reaction, 6.2.1.2) were diluted 1:2 with molecular grade water and 2µl placed into the corresponding wells. An optical reaction septum (Applied Biosystems) was placed over the plate, ensuring the septum was positioned correctly to prevent damage to the capillaries. The plate was placed onto a PCR block with the lid open and incubated for 3 minutes at 95ºC to denature samples. Plates were run on an AB3130 Genetic Analyzer (Applied Biosystems), courtesy of Dr S Adams, Bone Marrow Laboratory, Great Ormond Street Hospital, London, UK.

6.2.2 Sequencing of TR Variable β chain

6.2.2.1 Luria Bertani (LB) Agar

1 litre of LB agar was made by adding 35g of LB agar powder (Tryptone 10g/L, Yeast extract 5g/L, NaCl 5g/L, Agar 15g/L, Sigma) to 1 litre of distilled water and dissolving by heating to boiling whilst stirring. The solution was autoclaved for 15 minutes at 121°C and after cooling, 100µg/ml ampicillin (Sigma) was added.

6.2.2.2 Transformation of competent cells

TRBV PCR products were cloned using a TOPO-TA cloning strategy (all products from Invitrogen). 2µl of primary CDR3 PCR product (6.2.1.1) was mixed with 1µl salt solution (1.2M NaCl, 0.06 M $MgCl_2$), 2µl of water and 1µl of TOPO vector. The reaction was mixed gently and incubated for 5 minutes at room temperature. 2µl of the mixture was added to competent *E.coli* and then incubated on ice for 5 minutes. Cells were heat shocked for 30 seconds at 42ºC and then transferred to ice. 250µl of super optimal broth with catabolite suppression (S.O.C) medium (2% tryptone, 0.5% yeast extract, 10mM NaCl, 2.5mM KCL, 10mM MgCL2, 10mMgSO4, 20mM glucose) was added to the cells and shaken at 200 rpm for 1 hour at 37ºC. 10-30µl of competent cells was spread on pre-warmed LB agar plates and incubated overnight at 37ºC.

6.2.2.3 Analysing transformants by PCR

Individual colonies were selected and amplified by PCR using M13 primers. Each 25µl reaction consisted of 20.5µl water, 2.5µl x10 buffer, 0.75µl 50mM $MgCl_2$, 0.5µl each of 1pmol/µl M13 forward (5'GTAAAACGACGGCCAG 3') and reverse (5'CAGGAAA CAGCTATGAC) primers, 0.2µl 25mM dNTPs, 0.1µl 5U/µl *Taq* polymerase. Figure 6.1 shows that the majority of the resulting colonies had a single PCR product of the expected length (400-500bp). Following sequencing, 9 of the 11 colonies displayed were confirmed to have correctly cloned the BV12 gene.

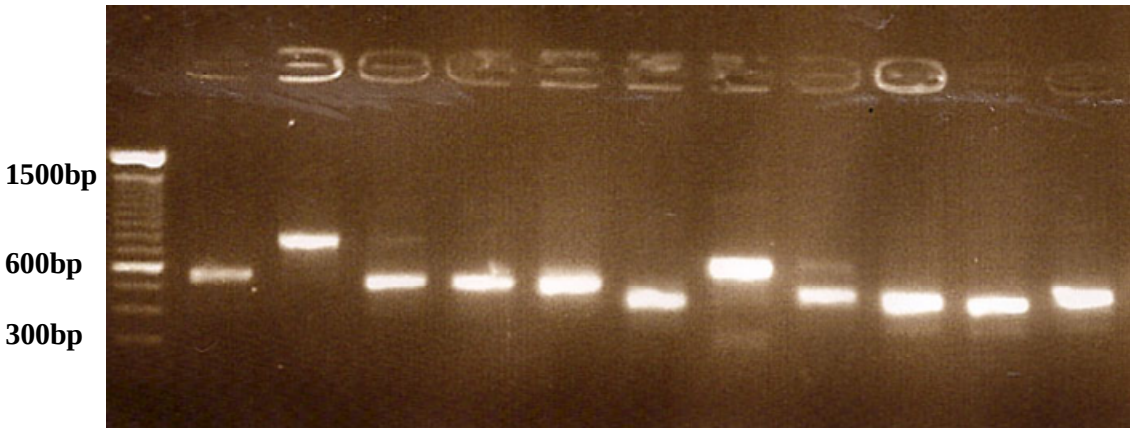


Figure 6.1: Amplification of CDR3 TR sequences by TOPO-TA cloning.

BV12 PCR products were cloned using a TOPO-TA vector and individual colonies amplified by M13 primers. PCR products were analysed by gel electrophoresis (lanes 2-12) and correct BV12 products confirmed by sequencing for lanes 2,4-7,9-12. Numbers indicate product lengths of DNA ladder (lane 1).

Sequencing was performed by the UCL Sequencing service on 3730xl capillary sequencer with BigDye 3.1 sequencing chemistry (Applied Biosystems) and analysed using Sequencher (Gene codes).

6.2.3 Sorting strategy

6.2.3.1 Purifying Th17 and Th1 cells

IL-17 secreting cells were identified from healthy control PBMC and SFMC using the cytokine capture assay (Chapter 2). CD161+ Th17 cells were sorted from healthy control PBMC by FACSaria flow cytometer, gating on CD4+ cells expressing high levels of IL-17 and not IFNγ, and then on CD161+ cells. Th1 cells were sorted from SFMC by, gating on CD4+ cells expressing high levels of IFNγ (IL-17+ cells were excluded) and then on CD161+ or CD161-cells. IL-17+ SFMC were sorted by gating on Th17 and Th17/1 cells separately in some experiments and with both populations together in other experiments (see figure legends for details).

6.2.4 Culture systems used in this chapter

6.2.4.1 Comparison of IMDM and RPMI media

Healthy control PBMC were enriched for CD4+ T cells using magnetic beads and stimulated with plate bound anti-CD3 (1μg/ml, R&D) and soluble anti-CD28 (1μg/ml, BD PharMingen). Cells were cultured in IMDM/10% FCS (Invitrogen) or RPMI/10% FCS in medium alone or in the presence of 10ng/ml IL-12 (R&D) at a concentration ranging from 10-1000pg/ml in 4%CO_2 at 37ºC for 4 days. Cells were then re-stimulated for 3 hours in the presence of 50ng/ml PMA, 500ng/ml ionomycin and 5μg/ml brefeldin A (all from Sigma) and intracellular cytokines detected by flow cytometry.

6.2.4.2 Assessment of CD161 expression in vitro, following stimulation with PMA and ionomycin

Healthy control PBMC were sorted into CD4+ T cells CD161+ or CD161- fractions. Cells were left unstimulated or stimulated with 10ng/ml PMA and 1μg/ml ionomycin for 2 hours at 37ºC. 10^5 cells were cultured in IMDM/10%FCS at a concentration of $5x10^5$/ml for 6 days. Sorted cells were analysed for CD161 expression by flow cytometry on day 0 or on day 6 of culture after re-staining with anti-CD161 antibody.

6.2.4.3 Culture of CD161+ Th17 cells under "Th1 conditions"

CD161+Th17 cells purified by cytokine capture assay were cultured in IMDM 10% FCS in the presence of 10ng/ml IL-12 (R&D) to track CD161 expression. On day 6 CD161, and intracellular IL-17 and IFNγ expression was detected by flow cytometry after re-stimulation as above. Live/dead® discriminant dye was used in accordance with manufacturer's instructions (Invitrogen).

6.2.4.4 Culture of CD161 sorted SFMC populations.

SFMC were gated on CD4+CD25- T cells and then sorted into a CCR6+CD161+ fraction, and after gating on CCR6- cells into CD161+ and CD161- populations. Sorted cells were stimulated with anti-CD3 and CD28 (as above) in medium alone or in the presence of 10ng/ml IL-23. An aliquot of sorted cells was transferred into Trizol (Invitrogen) for RNA extraction, and/or intracellular IL-17 and IFNγ expression was detected by flow cytometry after re-stimulation (as above).

6.3 Results

6.3.1 Th17 cells share clonal ancestry with Th17/1 cells and Th1 cells within the joint

Synovial T cells were separated into the three populations (Th1, Th17/1 and Th17 cells) by cytokine capture directly *ex vivo* (Chapter 5). TRBV analysis for 12 sub-families across the CDR3 junction was performed. Figure 6.2 shows spectra from 2 patients. In both patients, only 3 of the 12 TRBV sub-families tested produced clear spectra in all three T cell subpopulations. The failure of other families to successfully generate spectra may reflect the limitations of the small number of cells obtained from cytokine capture. As previously noted synovial T cells were highly oligoclonal (Wedderburn *et al,* 1999). In the case of patient JAS19, families BV18 and 3 showed oligoclonal patterns shared between Th17 and Th17/1 cells, which were distinct from the Th1 oligoclonal patterns. For patient PA284, BV18 and BV20 replicated this pattern, suggesting closer clonal links between Th17 and Th17/1 than Th1 cells.

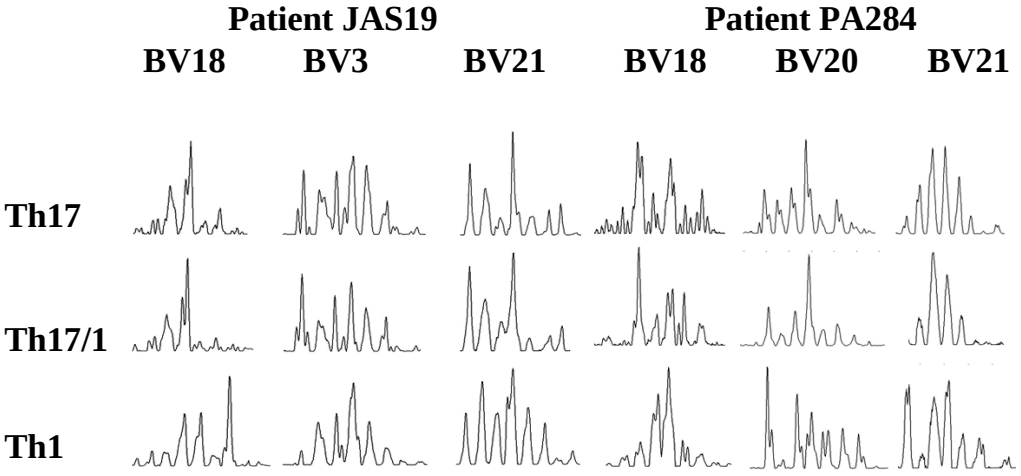


Figure 6.2: Th17/1 from the inflamed joint share clonality with Th17 cells.

Th17 (Top), Th17/1 (middle) and Th1 cells (bottom) were sorted from SFMC by flow cytometry using the cytokine capture assay and TRBV spectratyping was performed on sorted populations, *n*=3. 3 TRBV families are shown, from 2 separate SFMC samples.

To confirm links between Th17 and Th17/1 cells, TRBV products were cloned using the TOPO-TA vector and analysed for CDR3 nucleotide sequences. The resulting sequences for TRBV21 from patient JAS19 (Figure 6.2) are summarised in Figure 6.3. A large number of sequences were shared between Th17 cells (left) and Th17/1 ((middle), red and blue segments combined, 20% and 31.6% respectively) and also between Th17/1 and Th1 populations ((right) red, blue, and green segments combined, 34.2% and 10.8% respectively). However sequences common to all three subpopulations were less frequent (blue segment only, 5% Th17, 7.9% Th17/1 and 5.4% Th1), and the majority of TR sequences were unique to each T cell population (white segments). For sequence details see Table 6.4.

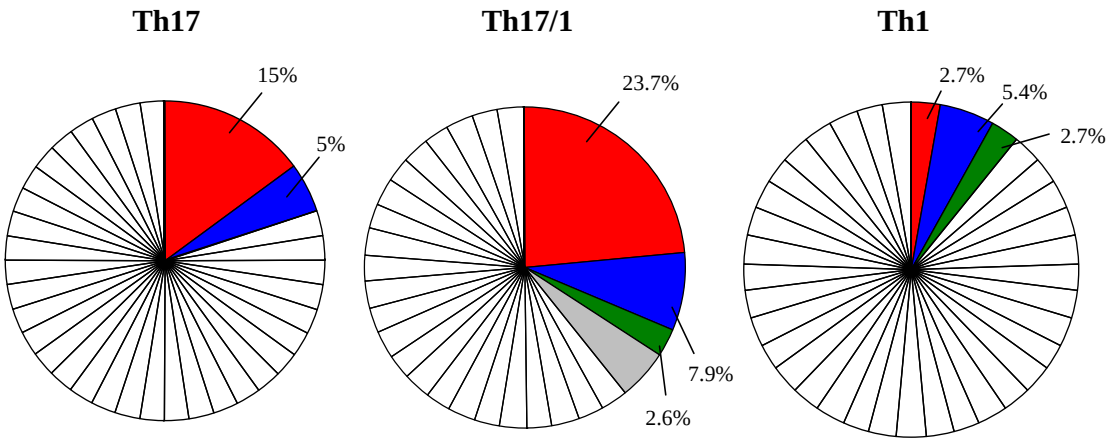


Figure 6.3: Summary of shared clones between cytokine expressing T cell subsets.

PCR products for TRBV21 from patient JAS19 (Figure 6.2) were cloned and sequenced across the CDR3. Sequence results are illustrated as pie charts with coloured segments to indicate clones that overlapped between cell populations. Numbers indicates clone size as a percentage of total colonies sequenced for that cell population. Non-overlapping clones are indicated in grey, and unique sequences in white for all 3 populations.

TRBV Sequence across CDR3 region **TRBV21** N region **TRBJ** TRBC	**Population**		
	Th17	**Th17 /1**	**Th1**
BJ2.6 TRBC2 **C A S S** P T D **S G A N V L T F G A G S R L T V L** E D L K N **TGT GCC AGC AGC** CCC ACG GAC **TCT GGG GCC AAC GTC CTG ACT TTC GGG GCC GGC AGC AGG CTG ACC GTG CTG** GAG GAC CTG AAA AAC	**6/40** 15%	**9/38** 23.7%	**1/37** 2.7%
BJ1.3 TRBC1 **C A** T Q G T **G N T I Y F G E G S W L T V V** E D L N K **TGT GCC** ACC CAG GGG ACG **GGA AAC ACC ATA TAT TTT GGA GAG GGA AGT TGG CTC ACT GTT GTA** GAG GAC CTG AAC AAG	**2/40** 5%	**3/38** 7.9%	**2/37** 5.4%
BJ1.1 TRBC1 **C A S S** P T G G G **T E A F F G Q G T R L T V L** E D L N K **TGT GCC AGC AGT** CCT ACG GGG GGT GGC **ACT GAA GCT TTC TTT GGA CAA GGC ACC AGA CTC ACA GTT GTA** GAG GAC CTG AAC AAG	**0**	**1/38** 2.6%	**1/37** 2.7%
BJ2.1 TRBC2 **C A S S** S M G A S S Y **N E Q F F G P G T R L T V L** E D L K N **TGT GCC AGC TCC** TCG ATG GGG GCG AGC TCC TAC **AAT GAG CAG TTC TTC GGG CCT GGG ACA CGG CTC ACC GTG CTA** GAG GAT CTG AAA AAC	**0**	**2/38** 5.2%	**0**
Unique sequences	**32/40** 80%	**23/38** 60.5%	**33/37** 89%

Table 6.4: Unique TRBV21 sequences for clones from patient JAS19 (Figure 6.2).

Th17, Th17/1 and Th1 cells were sorted from SFMC using cytokine capture assay. A total of 115 PCR products for TRBV21 were cloned and sequenced (40 from Th17 cells, 38 from Th17/1 cells and 37 from Th1 cells). TRBV sequence across the CDR3 region is displayed and frequency of clones in each of the three populations and percentage of total sequences. TRBV21 and J region sequence are displayed in bold. TRBJ and TRBC segments are indicated. TRBC1 AND TRBC2 refer to the two TR constant genes. Nomenclature see (Arden *et al*, 1995).

6.3.2 Th1 cells have a bi-modal distribution of CD161 expression.

Overlapping TR sequence between IL-17 expressing cells and Th1 cells could indicate that Th17 cells convert to a Th1 phenotype *in vivo*. It was hypothesised that Th1 cells with a Th17 ancestry may be identified by the cell surface marker, CD161, the human equivalent of the murine NKR-P1A protein (Giorda *et al,* 1990), expressed on a range of cells types including NK cells, CD4+ and CD8+ T cells (Takahashi *et al,* 2006). CD161 has emerged as a potential lineage marker for Th17 cells in humans. Forced over-expression of RORC2 leads to CD161 expression (Crome *et al,* 2009; Maggi *et al,* 2010), and only CD161+ cells taken from cord blood have the capacity to differentiate into Th17 cells (Cosmi *et al,* 2008).

To examine CD161 expression in the joint, SFMC samples from JIA patients were stimulated with PMA and ionomycin in the presence of brefeldin and then stained for cytokine and CD161 expression. Figure 6.4A shows that the majority of IL-17+ cells were CD161+ (left plot), whilst IFNγ+ cells showed distinct CD161+ and CD161- populations (right plot). The pattern of CD161 expression in Th1 cells was confirmed by analysing the same data as histograms (Figure 6.4B, right plot). Histograms also allowed synovial IL-17+ cells to be distinguished as (left plot) Th17 and (middle plot) Th17/1 cells which showed the majority (86.7%, 90.5% respectively) of both populations were CD161+.

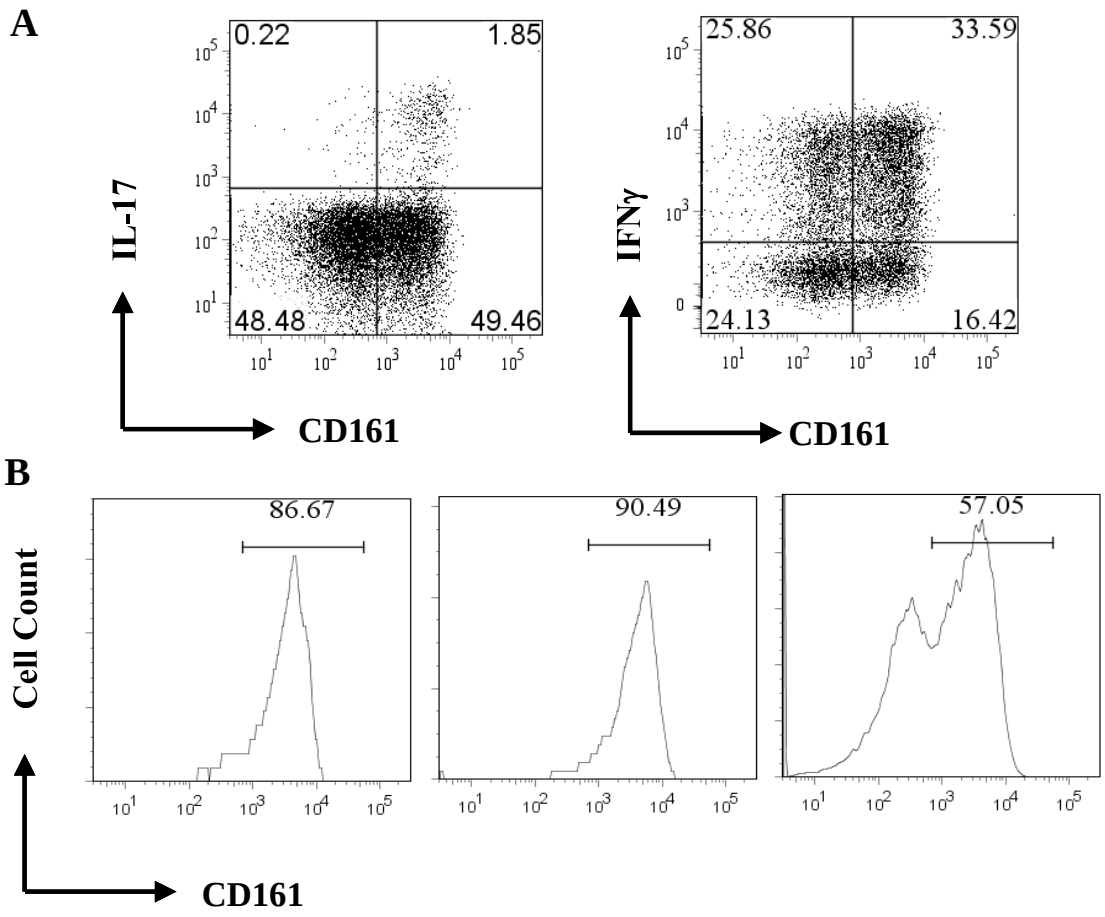


Figure 6.4: Th1 cells from the joint have a bimodal distribution of CD161 expression.
A) Representative flow cytometric analysis of SFMC comparing CD161 expression with IL-17 and IFNγ production, plots gated on CD4+ T cells. Numbers in all plots indicate percentage of parent population. B) CD161 expression in gated SFMC (left) Th17, (middle) Th17/1 and (right) Th1 cells, representative of *n*=4.

6.3.3 Comparison of IMDM and RPMI as the culture for Th17 cells

If CD161 predicts Th17 commitment in naïve cord blood T cells prior to IL-17 expression (Cosmi *et al,* 2008), it follows that expression of CD161 might also be maintained following a switch of Th17 cells to a Th1 phenotype. Prior to testing this hypothesis, the appropriate medium to culture Th17 cells and the dynamics of CD161 expression after T cell stimulation needed investigation.

A recent report suggested Iscove's Modified Dulbecco's Media (IMDM) to be superior to RPMI in the induction of Th17 cells in both murine and human systems (Veldhoen *et al,* 2009), which may be explained by higher tryptophan levels in IMDM. To directly compare the two media, purified CD4+ T cells from healthy donors were stimulated with anti-CD3 and anti-CD28, and cultured for 4 days in either RPMI or IMDM. This was tested with medium alone and also a range of IL-12 concentrations, to model the effect of a synovial microenvironment. Figure 6.5A shows the percentage of CD4+ T cells expressing IL-17 was highest without IL-12 ($p=0.008$, paired t-test). However there was no significant increase in Th17 frequency using IMDM when compared to RPMI ($p=0.3$, 2 way repeated measures ANOVA). This may reflect the culture of cells in neutral or Th1 polarising conditions in this study, whilst the superiority of IMDM may be limited to Th17 inducing conditions as used in Veldhoen et al (Veldhoen *et al,* 2009). Surprisingly, there was no appreciable increase in the proportion of Th17/1 cells following the addition of IL-12, (Figure 6.5B). As expected there was a dramatic increase in Th1 cells in the presence of 10ng/ml IL-12, but the dose response rapidly tailed off with increasing concentration. Again medium type did not impact on the proportion of Th1 cells (Figure 6.5C). IMDM was still selected as the medium of choice for culturing Th17 cells on the basis of published findings (Veldhoen *et al,* 2009). Although IL-12 did not appear to alter Th17/1 generation in these experiments, the increase in Th1 cells was taken as evidence of a biological response to IL-12 and the lowest concentration of 10ng/ml IL-12 was chosen for further studies.

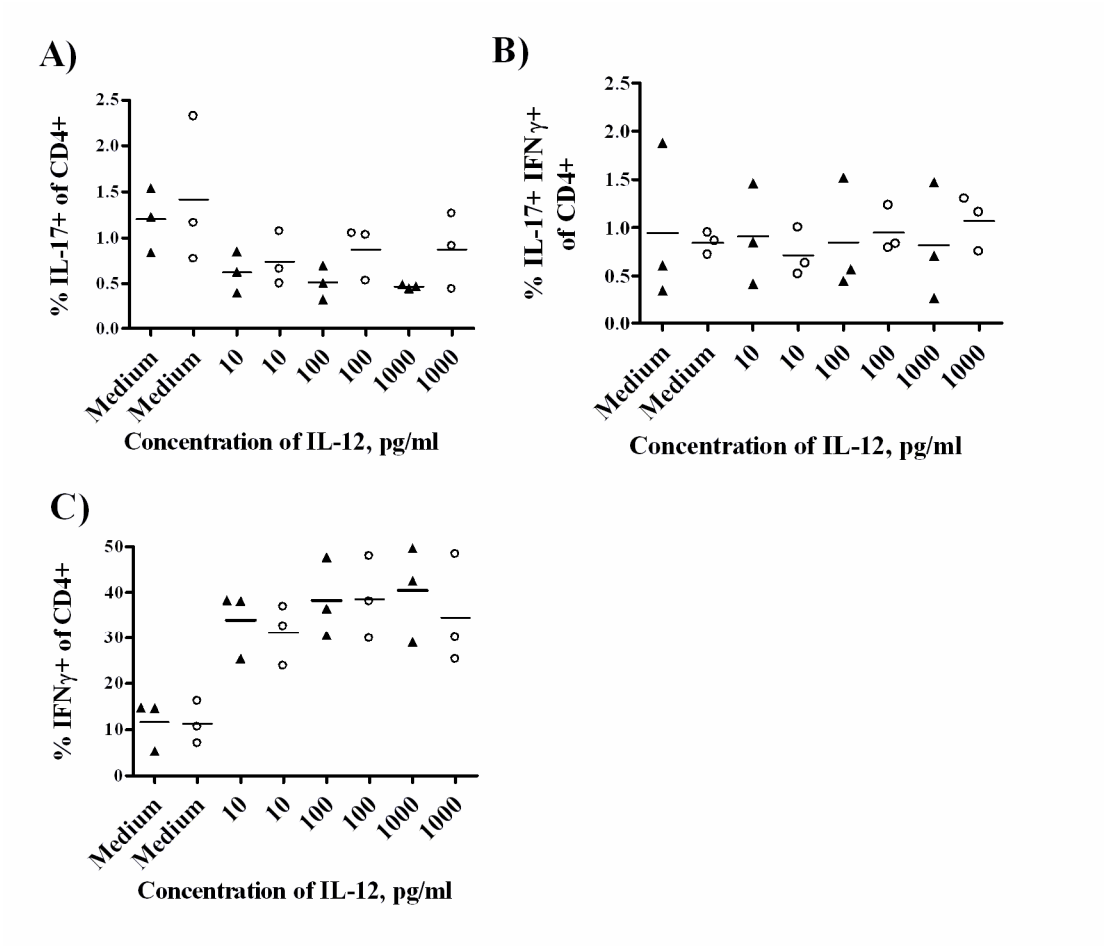


Figure 6.5 Comparison of CD4+ T cell culture in IMDM and RPMI.
CD4+ T cells from healthy donors were stimulated with plate bound anti-CD3 and soluble anti-CD28 in media alone or with increasing concentrations of recombinant IL-12, and harvested after 4 days. Scattergraphs are displayed of the percentage of A) Th17 B) Th17/1 and C) Th1 cells of the total population in RPMI (triangles) or IMDM (circles) conditions, as detected by flow cytometry, n=3. Lines represent mean levels.

6.3.4 Dynamics of CD161 expression *in vitro*

To test the dynamics of CD161 expression after stimulation, CD4+ T cells from a healthy donor were sorted by flow cytometry into CD161+ and CD161- populations and stimulated with PMA and ionomycin for 2 hours to mimic the capture process (Chapter 2), or left unstimulated. Cells were cultured for 6 days and then re-stained for CD161 expression. A small proportion of CD161+ CD4+ T cells became CD161- on day 6 (Figure 6.6) in both unstimulated control and stimulated cells, which was associated with some downregulation of CD161 expression as detected by flow cytometry (MFI). CD161- CD4+ T cells did not acquire CD161 expression after culture, raising the possibility that the surface marker CD161 could be used to track Th17 cells which undergo plasticity *in vitro.*

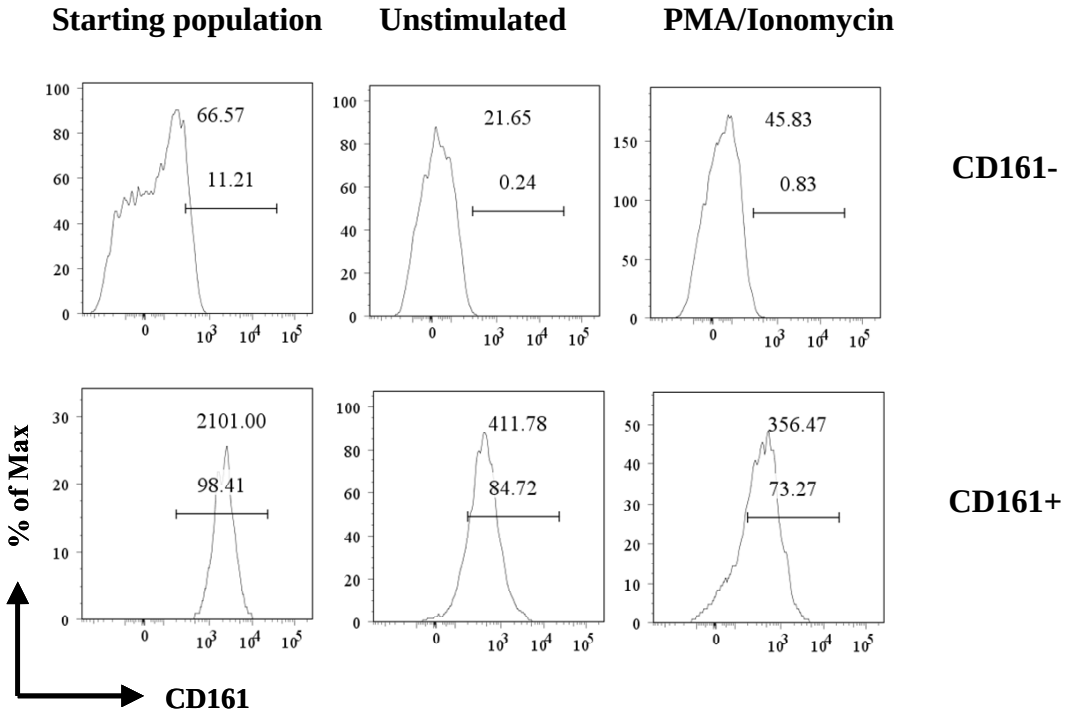


Figure 6.6: CD4+ T cells retain fidelity for CD161 expression in short term culture after stimulation with PMA and ionomycin.
CD4+ T cells from a healthy donor were sorted by flow cytometry into (top row) CD161- and (bottom row) CD161+ fractions. CD161 expression was assessed by flow cytometry in sorted populations at baseline (left plots), and after 6 days of culture in medium (middle plots) without pre-stimulation, or (right plots) with 2 hours of stimulation with PMA and ionomycin on day 0. All plots are gated on live CD4+ T cells, lower numbers indicate percentage of cells expressing CD161 and upper number CD161 MFI of the whole population, n=1.

6.3.5 CD161 expression is maintained on Th17 cells which 'convert' to Th1 cells *in vitro*

To test whether CD161 expression is maintained after conversion of Th17 to a Th1 phenotype, sorted CD161+ Th17 cells were cultured in the presence of IL-12 to promote plasticity towards Th17/1 and Th1 cells. Figure 6.7A shows the mixed cytokine expression profile of Th17 cells cultured for six days in IMDM in the presence of 10ng/ml IL-12. Cells which retained the Th17 phenotype were CD161+ (Figure 6.7B, left plot), as were cells which transitioned to a Th17/1 phenotype (right plot). Cells which developed a Th1 phenotype, upregulating IFNγ and losing IL-17 expression, also maintained CD161 expression (82%±5, middle plot). In contrast sorted CD161- CD4+ T cells cultured under the same conditions failed to upregulate CD161 (Figure 6.8).

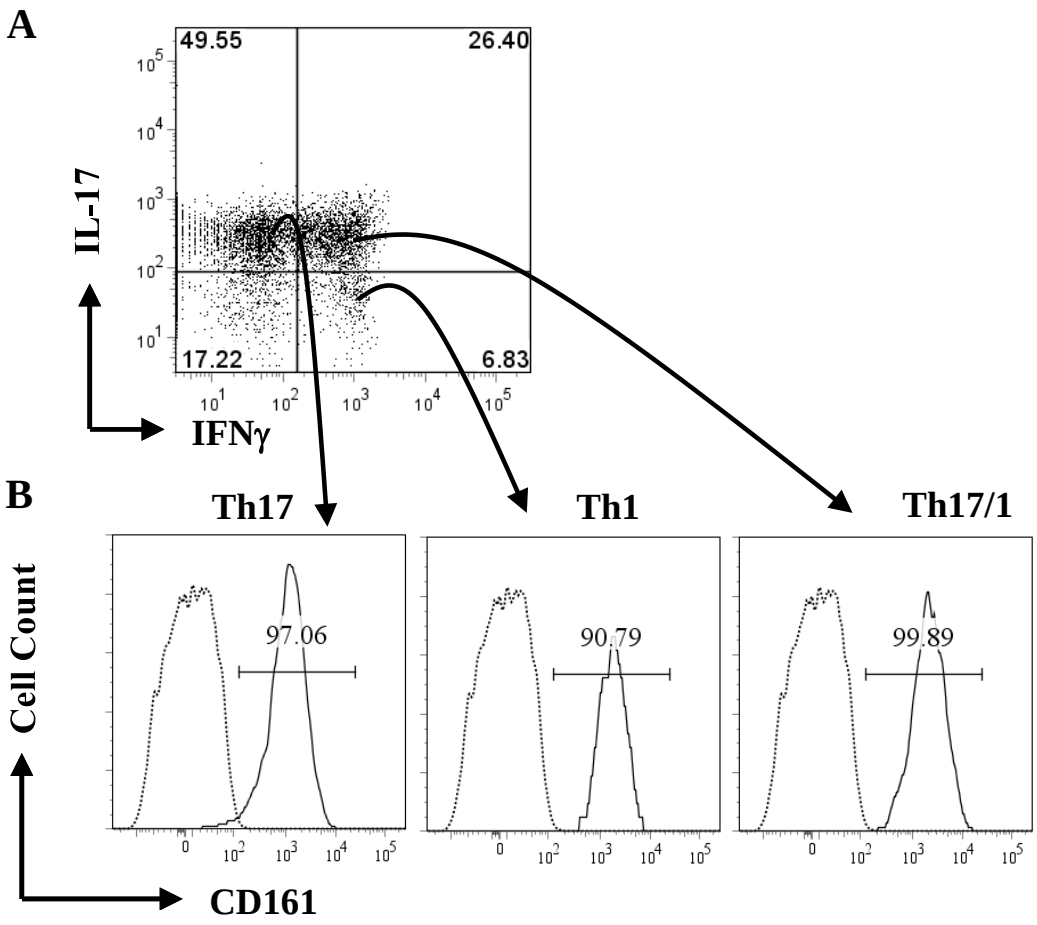


Figure 6.7: Th17 cells maintain CD161 expression after conversion to Th1 cells *in vitro.*

A) IL-17+ cells expressing CD161 from healthy control PBMC were sorted and cultured in the presence of 10ng/ml IL-12. Representative dot plot of intracellular IL-17 and IFNγ detected on day 6 of culture, and B) CD161 expression in same cells (indicated by arrows) gated on (left) Th17, (middle) Th1 and (right) Th17/1 sub-populations, *n*=3. Dotted line represents isotype control and numbers in histograms indicate percentage of parent population expressing CD161.

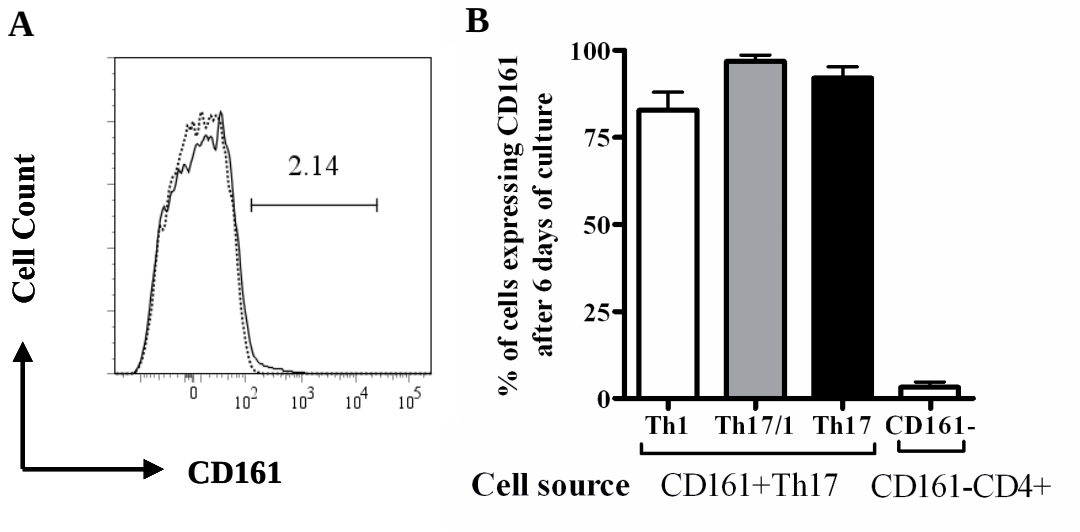


Figure 6.8: CD161 negative CD4+ T cells fail to upregulate CD161.

A) CD4+CD161- T cells from healthy control PBMC were sorted and cultured in the presence of 10ng/ml IL-12 as in Figure 6.7. Dotted line represents isotype control and numbers in histograms indicated percentage of parent population expressing CD161 on day 6 of culture. B) Summary of 3 independent experiments, showing CD161 expression on gated sub-populations as in (A) and Figure 6.7. Bars represent mean values (±SEM).

Consistent with these *in vitro* data suggesting that CD161 may identify cells within the Th1 population that have a Th17 ancestry, CD161+Th1 cells sorted from the joints of JIA patients had significantly higher RORC2 mRNA expression than CD161- Th1 cells (Figure 6.9A). IL-17+ cells, as expected, exhibited the highest expression of RORC2. It is unlikely that CD161+ Th1 cells had higher RORC2 expression because of inadvertent contamination from IL-17+ cells, as IL-17 message levels were equivalent in CD161+ and CD161- Th1 cells (Figure 6.9B).

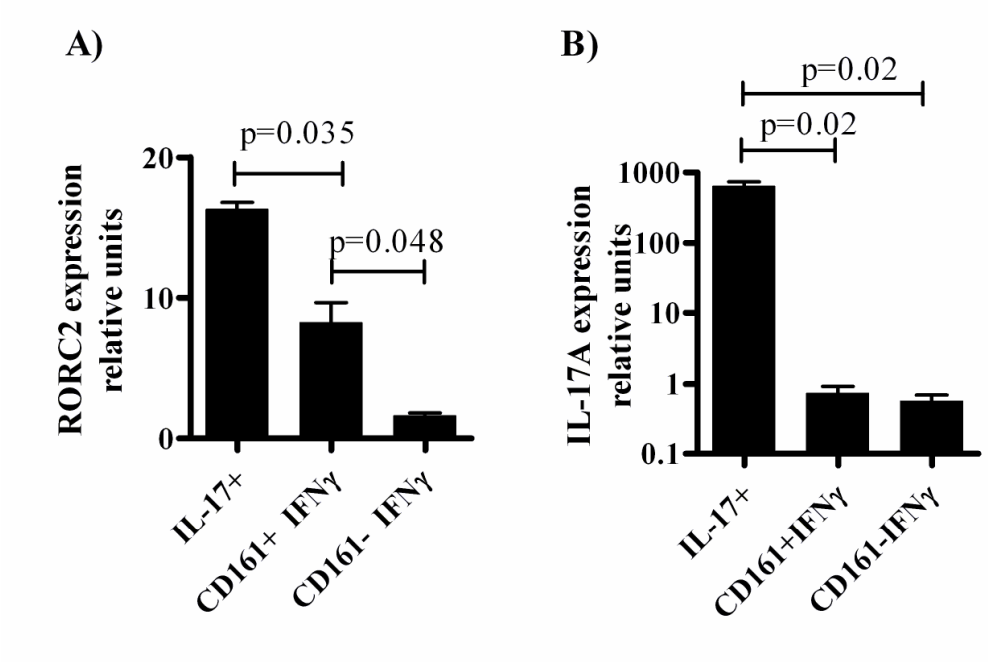


Figure 6.9: CD161 defines a population of Th1 cells expressing high levels of RORC2 mRNA.
Cytokine expressing synovial CD4+ T cells from patients with JIA were detected using the cytokine capture assay, sorted into IL-17+, CD161+IFNγ+ and CD161-IFNγ+ populations and analysed by qPCR. A) RORC2 and B) IL-17 mRNA expression in sorted synovial populations. Bars represent mean values (±SEM) of 3 independent experiments.

6.3.6 IL-23R and CCR6 expression are enhanced on CD161+ Th1 cells

The IL-23 receptor (IL-23R) and CCR6 have been shown to be downstream targets of RORC2 and are upregulated following the over-expression of RORC2 (Manel *et al,* 2008). In view of the increased levels of expression of RORC2 within the CD161+ Th1 population, it was predicted that IL-23R and CCR6 levels would be also be enriched within the CD161+ Th1 population compared to CD161- Th1 cells. To test this, SFMC were co-stained for intracellular IL-17 and IFNγ expression against CD161 and CCR6 and analysed by flow cytometry. The SFMC sample tested in Figure 6.10A showed that IL-17+ cells were almost universally CCR6+ (mean of IL-17+ cells expressing CCR6 was 90%±2.7% SEM, n=6, Figure 6.11), confirming previous results (Chapter 4). Within the Th1 compartment, CCR6 expression was much lower and predominately confined to the CD161+ population (Figure 6.10B).

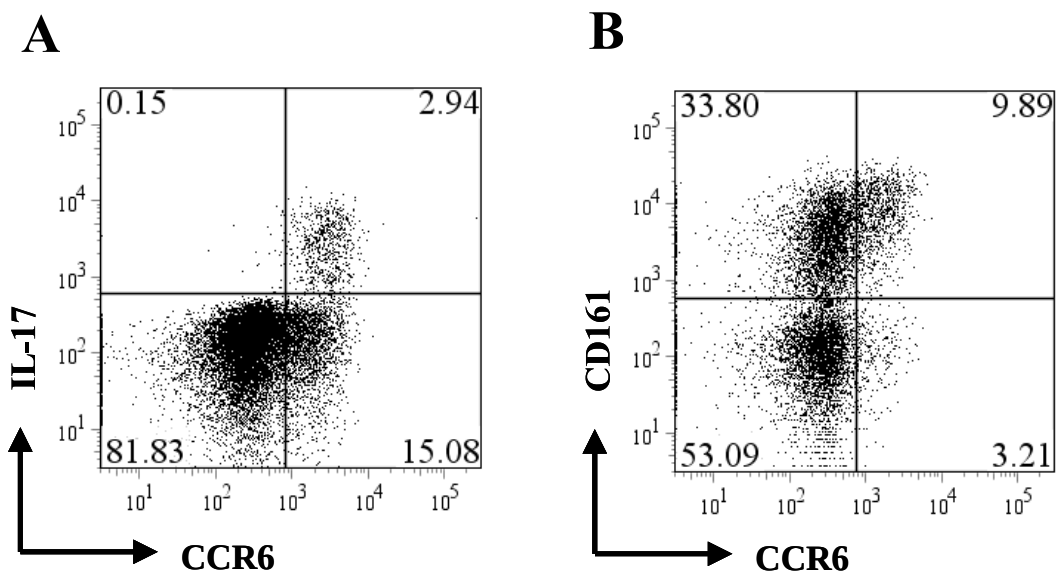


Figure 6.10: Representative dot plot of CCR6 expression in SFMC, detected by flow cytometry.
A) Plot gated on CD4+ T cells, numbers indicate percentage of parent population. (B) Plot gated on Th1 cells, representative of *n*=6.

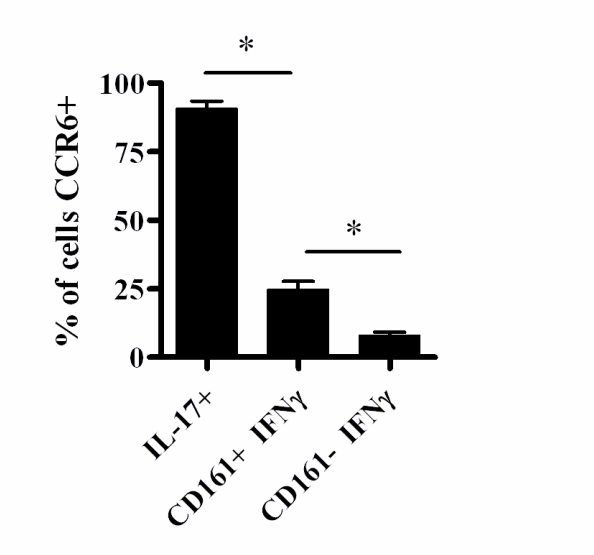


Figure 6.11: CCR6 protein expression is enhanced on CD161+ Th1 cells when compared to CD161- Th1 cells.
Mean percentage (±SEM) of IL-17+, CD161+IFNγ+ and CD161-IFNγ+ SFMC populations expressing CCR6 protein as detected by flow cytometry, (*n*=6).

Analysis of a set of SFMC samples (n=6) confirmed this pattern, with CD161+ Th1 cells having a significantly greater proportion of CCR6+ cells (mean 24% ± 3% SEM) than CD161- Th1 (8% ±1%, Figure 6.11, $p<0.01$, paired t-test).

IL-23R expression was also tested in Th1 cells, purified by cytokine capture and split according to CD161 expression. This showed that CD161+ Th1 cells expressed significantly higher levels of IL-23R mRNA that CD161- cells ($p=0.02$, paired t-test, Figure 6.12).

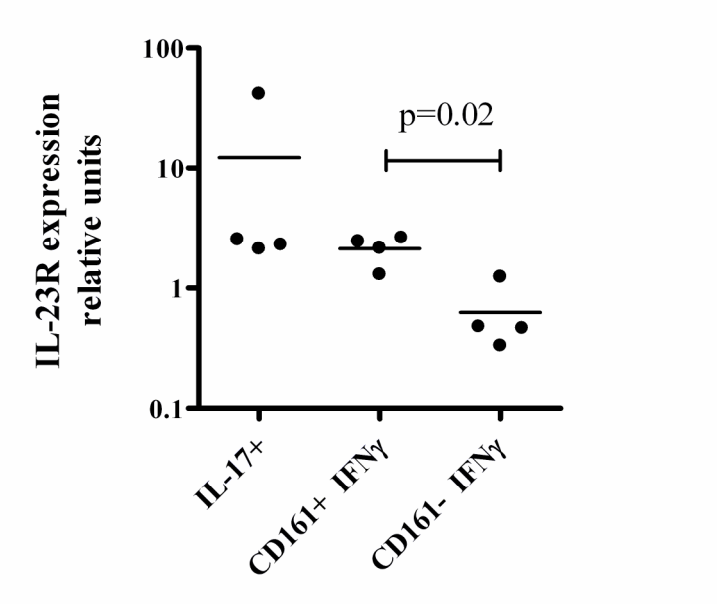


Figure 6.12: CD161+ Th1 cells have higher expression of IL-23R mRNA than CD161- Th1 cells.
Cytokine expressing synovial CD4+ T cells were detected using the cytokine capture assay and sorted into IL-17+, CD161+IFNγ+ and CD161-IFNγ+ populations. IL-23R mRNA expression measured by qPCR within sorted synovial populations, n=4. Lines represent mean levels.

To examine IL-23R expression at the protein level, on the different CD4+ T cell sub-populations SFMC were stimulated with PMA and ionomycin in the presence of brefeldin A, and intracellular cytokine expression was compared with IL-23R using flow cytometry. Figure 6.13 (left panel) shows SFMC CD4+ T cells stained for IL-17 production against the isotype control (biotinylated Goat IgG) which had no background staining. The polyclonal goat anti-human-IL-23R antibody demonstrated a discrete IL-23R+ population which made up 40% of the IL-17+ fraction (Figure 6.13, right panel). When analysed in a larger set of samples, CD161+ Th1 cells had significantly higher IL-23R expression (mean 55%±4% SEM) than CD161- Th1 cells (43%±3%). Surprisingly IL-17+ cells had intermediate IL-23R expression (49%±4%), though this did not differ significantly from the Th1 populations.

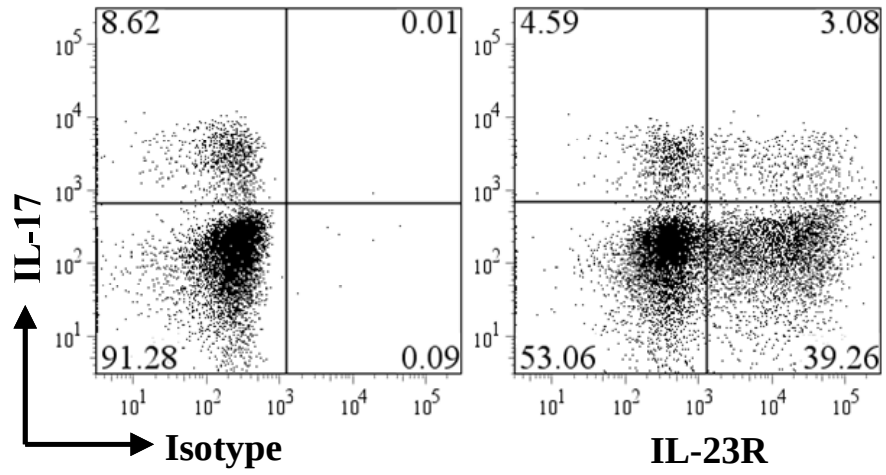


Figure 6.13: IL-23R expression on SFMC analysed by flow cytometry.

Representative flow cytometric analysis of SFMC analysing IL-17 expression against (left) goat biotinylated IgG isotype control and (right) IL-23R, plots gated on CD4+ T cell. Numbers indicated percentage of CD4+ T cells, representative of n=6.

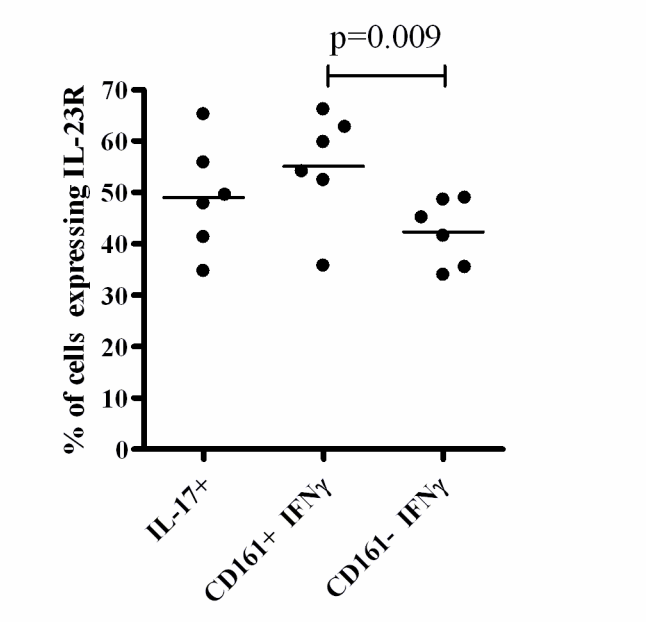


Figure 6.14: IL-23R expression is enhanced on CD161+ Th1 cells when compared to CD161- Th1 cells.

Percentage of IL-17+, CD161+IFNγ+ and CD161-IFNγ+ SFMC populations expressing IL-23R protein as detected by flow cytometry, (*n*=6). Lines represent mean levels.

6.3.7 SFMC enriched for CD161+ Th1 cells show a functional response to IL-23

To test if CD161+ Th1 cells from the joint showed a greater response to IL-23 in keeping with their increased IL-23R expression, SFMC were sorted into three populations, CD161+CCR6+, CD161+CCR6-, CD161-CCR6-. This method was chosen to avoid stimulating cells with PMA and ionomycin during cell purification, which is an integral part of the cytokine capture assay and risks re-opening multiple gene loci that were previously repressed (Lebkowski *et al,* 1987). As IL-17+ cells are almost universally CCR6+, gating out CCR6+CD4+ T cells provided a means of enriching Th1 cells without contaminating Th17 cells. This strategy however risks excluding those Th1 cells that express CCR6. Cells were first gated on CD4+CD25- cells to exclude regulatory T cells which could modulate cytokine secretion from Th17 or Th1 cells (Kleinschek *et al,* 2009). Figure 6.15 shows intracellular cytokine staining of the sorted populations after stimulation with PMA and ionomycin. This confirms that IL-17+ cells are retained with the CCR6+ fraction. Both CCR6- populations (CD161+ and CD161-) had a similar proportion of IFNγ expressing cells.

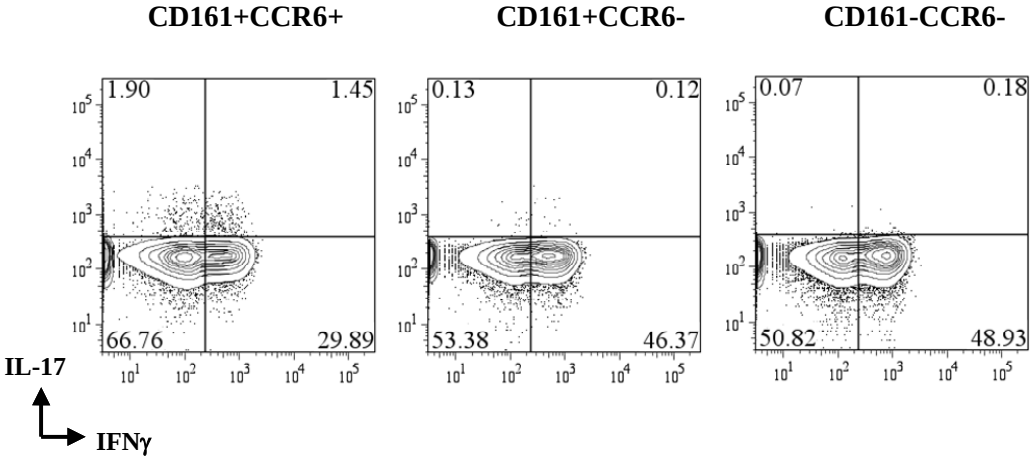


Figure 6.15: SFMC were enriched for CD161+ and CD161- Th1 populations by flow cytometry.
SFMC were sorted into (left) CD161+CCR6+, (middle) CD161+CCR6- and (right) CD161-CCR6- populations after gating on CD4+CD25- T cells. Enriched cells were then stimulated with PMA and ionomycin in the presence of brefeldin A before staining for intracellular cytokines as detected by flow cytometry. Representative dot plot of n=4.

IL-23R mRNA expression was assessed in these sorted sub-populations, to test if CD161+CCR6- cells were enriched for IL-23R compared to CD161- cells. IL-23R mRNA levels were highest in CD161+CCR6+ then CD161+CCR6- populations, with CD161-CCR6- cells having the lowest levels (Figure 6.16A). To limit the effects of large inter-individual variation the data were re-analysed after logarithmic conversion. This showed a significant elevation in IL-23R in both CD161+ populations compared to CD161- cells (Figure 6.16B). This result corresponds well with the data obtained by flow cytometry and PCR for IL-23R expression on CD161+ Th1 and CD161-Th1 cells (Figure 6.12 and Figure 6.14).

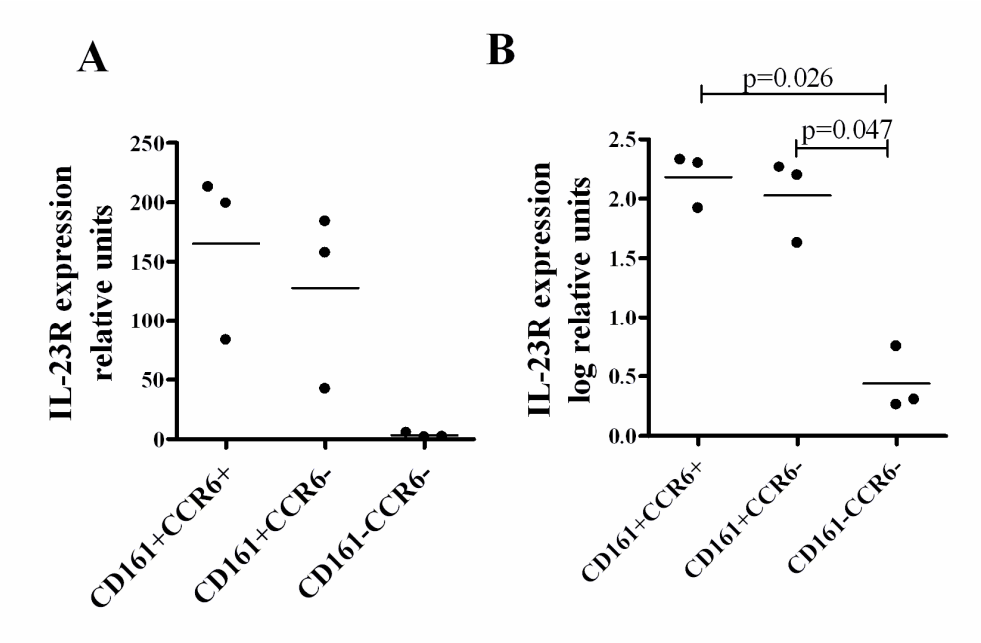


Figure 6.16: IL-23R expression is enriched on CD161+ SFMC.

A) IL-23R mRNA expression was measured by qPCR within sorted CD4+ T cell SFMC populations as shown in Figure 6.15, and B) after logarithmic transformation, n=3. Lines represent mean levels.

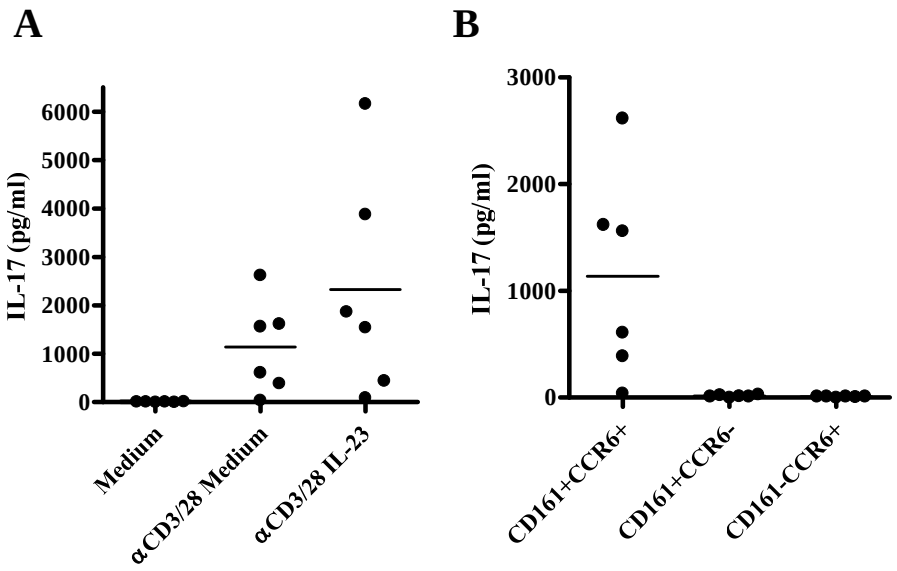


Figure 6.17: IL-17 secretion is limited to the CD161+CCR6+ population.

IL-17 protein levels analysed by multiplex immunoassay from SFMC sorted as in Figure 6.15. A) CD161+CCR6+ cells were left unstimulated, or stimulated with plate bound anti-CD3 and soluble anti-28 in medium alone or additionally in the presence of 10ng/ml IL-23. B) Sorted cells (as indicated) were stimulated with plate bound αCD3 and soluble αCD28. All cell supernatants were harvested on day 4, n=6. Lines represent mean levels.

In view of the enrichment of IL-23R expression on SFMC enriched for CD161+ Th1 it was hypothesised that this population would show a greater response to IL-23 than CD161- Th1 cells. To test this hypothesis, the sorted populations were stimulated in medium alone or in the presence of IL-23, or left unstimulated. Figure 6.17A shows that there was no detectable IL-17 secretion from CD161+CCR6+ cells when left unstimulated. However after stimulation with anti-TR signals, there was a trend for increased IL-17 secretion in response to IL-23 (mean 2330, ± SEM 940 pg/ml) when compared to medium alone (1140±340 pg/ml, p=0.2, paired t-test). IL-17 levels following stimulation of both CCR6- populations was less than 20pg/ml, demonstrating exclusion of Th17 cells from these populations.

CD161+CCR6- cells showed a greater response to IL-23 in terms of IFNγ secretion (mean IFNγ 881 vs 1315pg/ml, paired t-test p=0.06) than CD161-CCR6- cells (mean IFNγ 707 vs 789pg/ml, p=0.23, Figure 6.18A). There were similar results for TNFα, CD161+CCR6- cells showing a greater increase in cytokine secretion following the addition of IL-23 (mean TNFα 936 vs 1822pg/ml, p=0.07) than CD161-CCR6- cells

(mean TNFα 1000 vs 1005pg/ml, p=0.9, Figure 6.18B). TNFα secretion differed widely between SFMC sub-populations, being greatest in CD161+CCR6+ and least in CD161-CCR6- cells. Unexpectedly, only 2 of 6 CCR6+CD161+ samples demonstrating a clear response to IL-23, in terms of increase IFNγ or TNFα secretion.

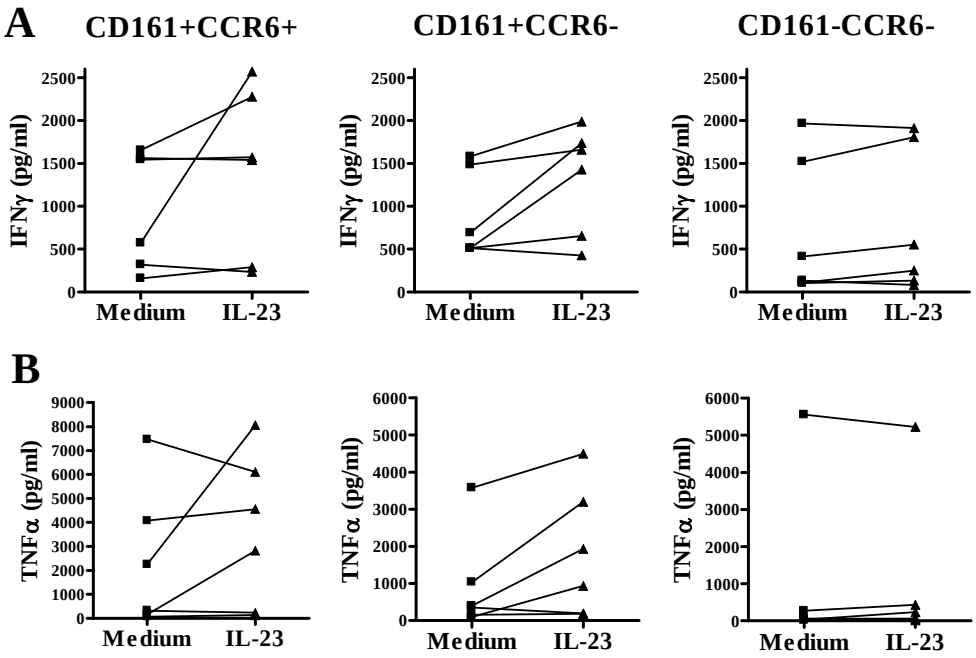


Figure 6.18: Cytokine secretion by CD161+ and CD161- SFMC in response to IL-23.
SFMC sorted as in Figure 6.15 were cultured in the presence of plate bound anti-CD3 and soluble anti-CD28, in the absence (square symbols) or presence of 10ng/ml IL-23 (triangles). A) IFNγ and B) TNFα protein levels (*n*=6) were analysed by multiplex immunoassay in cell supernatants harvested on day 4.

6.3.8 Clonal comparison of IL-17+ cells with Th1 cells within the inflamed joint

The results presented above indicated that synovial CD161+ Th1 cells shared with Th17 cells, their expression of "Th17 signature molecules" RORC2, IL-23R and CCR6. These data supported the hypothesis that CD161+ Th1 cells found within the joint originated from a Th17 pool. To test this, the TRBV clonality of the CD161+ Th1 population was compared with CD161-Th1 and IL-17+ T cells from the joints of patients with JIA (Figure 6.19). Some sub-family spectratypes were found to be similar between IL-17+ and CD161+Th1 populations (patient JAS16 BV6B,12, patient PA277 BV6B) but not in others (patient JAS16 BV3A, patient PA277 BV3A). The majority of spectra for CD161-Th1 were distinct from CD161+ Th1 and IL-17+ populations (patient JAS16 BV6B, 12, 18, patient PA277 BV3A, 23).

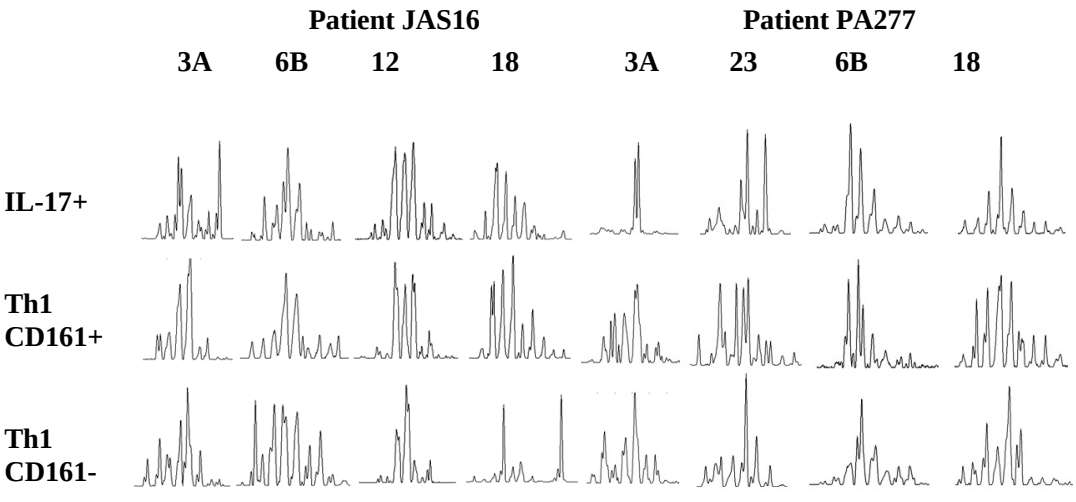


Figure 6.19: CD161+ Th1 cells show distinct clonality compared to CD161- CD4+ T cells from the joint.
Three populations, (top) IL-17+, (middle) CD161+ Th1 and (bottom) CD161- Th1 cells were purified from SFMC using a cytokine capture assay. TRBV spectratyping was performed on sorted populations, *n*=4. 4 representative TRBV families are shown, from 2 separate SFMC samples.

Analysis of oligoclonality or repertoire diversity by TRBV spectratype is dependent on the detection of non-Guassian distribution of CDR3 lengths. To confirm the putative links between the T cell sub-populations seen by spectratype, TRBV clones were analysed at the sequence level which allowed the identification of specific T cell clones as defined by the unique sequence of their re-arranged CDR3. PCR products from one BV sub-family (patient JAS16, BV18, Figure 6.19) were cloned and sequenced. Figure 6.20 summarises analysis of sequence homology between 383 individual clones. Interestingly, specific TRBV CDR3 sequences were identified which were common between IL-17+ cells and the CD161+IFNγ population (coloured segments). Importantly, these clones were not detected in the CD161- cells. The unique TR sequences of CD161+ and CD161- cells within the IFNγ+ population were distinct. The detailed TR sequences are listed in Table 6.5. These data suggested that at least a proportion of T cells within the CD161+ IFNγ+ population share a common ancestral clonality with Th17 cells.

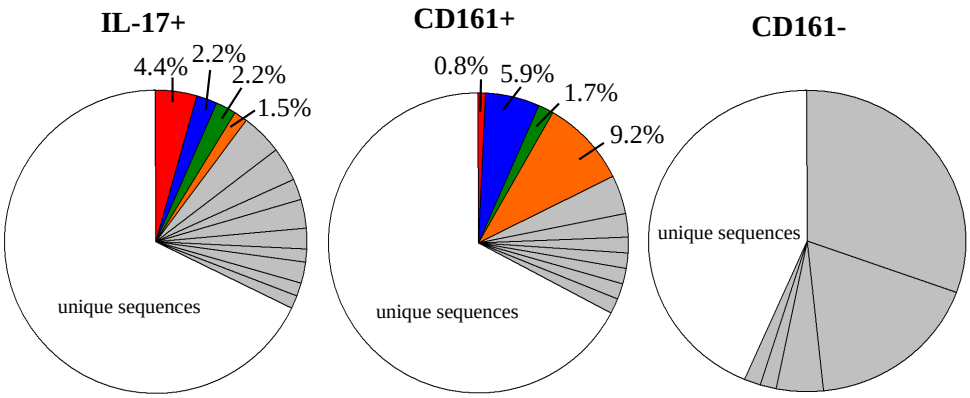


Figure 6.20: Shared origin between IL-17+CD4+ T cells and CD161+ Th1 but not CD161- Th1 cells within the joint.
PCR products for TRBV18 from patient JAS16 (Figure 6.19) were cloned and sequenced. Sequence results are illustrated as pie charts with coloured segments to indicate clones that overlap between cell populations (limited to IL-17+ and CD161+Th1 populations). Numbers indicate clone size as a percentage of total colonies sequenced for that cell population. Non-overlapping clones are indicated in grey, and unique sequences in white for all 3 populations.

TRBV Sequence across CDR3 region **TRBV18** N region **TRBJ** TRBC2 unless otherwise indicated	**Population**		
	IL-17+ cells	CD161+ Th1 cells	CD161- Th1 cells
TRBJ2.5 **TGT GCC AGC TCA CCA** CAA CAG AGA **GAG ACC CAG TAC TTC GGG CCA GGC ACG CGG CTC CTG GTG CTC** GAG GAC CTG AAA AAC **C A S S P** Q Q R **E T Q Y F G P G T R L L V L** E D L K N	**6/136 (4.4)**	**1/119 (0.8%)**	**0**
TRBJ2.7 **TGT GCC AGC TCA** GTA AGG GAA ATC CAC **GAG CAG TAC TTC GGG CCG GGC ACC AGG CTC ACG GTC ACA** GAG GAC CTG AAA AAC **C A S S** V R E I H **E Q Y F G P G T R L T V T** E D L K N	**3/136 (2.2%)**	**7/119 (5.9%)**	**0**
TRBJ2.5 **TGT GCC AGC TCA CCG** ACA AGA CAA **GAG ACC CAG TAC TTC GGG CCA GGC ACG CGG CTC CTG GTG CTC** GAG GAC CTG AAA AAC **C A S S P** T R Q **E T Q Y F G P G T R L L V L** E D L K N	**3/136 (2.2%)**	**2/119 (1.7%)**	**0**
TRBJ1.1 **TRBC1** **TGT GCC AGC TCA** CTG GCC TCG GAC **ACT GAA GCT TTC TTT GGA CAA GGA ACC AGA CTC ACA GTT GT** GAG GAC CTG AAC AAA **C A S S** L A S D **T E A F F G Q G T R L T V V** E D L N K	**2/136 (1.5%)**	**11/119 (9.24%)**	**0**
TRBJ2.7 **TGT GCC AGC TCT** CTT AGC **TCC TAC GAG CAG TAC TTC GGG CCG GGC ACC AGG CTC ACG GTC ACA** GAG GAC CTG AAA AAC **C A S S** L S **S Y E Q Y F G P G T R L T V T** E D L K N	**6/136 (4.4%)**	**0**	**0**
TRBJ2.1 **TGT GCC AGC TCC CCC** GGG TCT CAA GAC **AAT GAG CAG TTC TTC GGG CCA GGG ACA CGG CTC ACC GTG CTA** GAG GAC CTG AAA AAC **C A S S P** G S Q D **N E Q F F G P G T R L T V L** E D L K N	**5/136 (3.7%)**	**0**	**0**
TRBJ2.3 **TGT GCC AGC TCA CCG** GGA CAG GGA CCT **AGC ACA GAT ACG CAG TAT TTT GGC CCA GGC ACC CGG CTG ACA GTG CTC** GAG GAC CTG AAA AAC **C A S S P** G Q G P **S T D T Q Y F G P G T R L T V L** E D L K N	**4/136 (2.9%)**	**0**	**0**
TRBJ2.2 TGT GCC AGC TCA **CGT AAG ACG GGG ACC GCC** ACC GGG GAG CTG TTT TTT GGA GAA GGC TCT AGG CTG ACC GTA CTG **GAG GAC CTG AAA AAC** **C A S S** R K T G T A **T G E L F F G E G S R L T V L** E D L K N	**3/136 (2.2%)**	**0**	**0**
TRBJ2.7 **TGT GCC AGC TCA CCA** CTG GGA CTC **TAC GAG CAG TAC TTC GGG CCG GGC ACC AGG CTC ACG GTC ACA** GAG GAC CTG AAA AAC **C A S S P** L G L **Y E Q Y F G P G T R L T V T** E D L K N	**3/136 (2.2%)**	**0**	**0**
TRBJ2.3 **TGT GCC AGC TCG TCC** TTT AGC **ACA GAT ACC CAG TAT TTT GGC CCA GGC ACC CGG CTG ACG GTG CTC** GAG GAC CTG AAA AAC **C A S S S** F **S T D T Q Y F G P G T R L T V L** E D L K N	**3/136 (2.2%)**	**0**	**0**
TRBJ2.7 **TGT GCC AGC TCA CCG** GGA CAG CGC AGC **TCC TAC GAG CAG TAC TTC GGG CCG GGC ACC AGG CTC ACG GTC ACA** GAG GAC CTG AAA AAC C A S S P **G Q R S** S Y E Q Y F G P G T R L T V T **E D L K N**	**2/136 (1.5%)**	**0**	**0**
TRBJ2.7 **TGT GCC AGC TCA CCG** GGA GGG **GAG CAG TAC TTC GGG CCG GGC ACC AGG CTC ACG GTC ACA** GAG GAC CTG AAA AAC **C A S S P** G G **E Q Y F G P G T R L T V T** E D L K N	**2/136 (1.5%)**	**0**	**0**
TRBJ2.6 **TGT GCC AGC TCA CCA** CCG GTT GGG GGT **TCT GGG GCC AAC GTC CTG ACT TTC GGG GCC GGC AGC AGG CTG ACC GTG CTG** GAG GAC CTG AAA AAC **C A S S P** P V G G **S G A N V L T F G A G S R L T V L** E D L K N	**2/136 (1.5%)**	**0**	**0**

TRBJ2.2 **TGT GCC AGC TCA CCA** CAA CCC CCG GGA CAC ATC **ACC GGG GAG CTG TTT TTT GGA GAA GGC TCT AGG CTG ACC GTA CTG** GAG GAC CTG AAA AAC **C A S S P** Q P P G H I **T G E L F F G E G S R L T V L** E D L K N	**0**	**5/119 (4.2%)**	**0**
TRBJ2.2 **TGT GCC AGC TCG** GCC GGG GAG **AAC ACC GGG GAG CTG TTT TTT GGA GAA GGC TCT AGG CTG ACC GTA CTG** GAG GAC CTG AAA AAC **C A S S** A G E **N T G E L F F G E G S R L T V L** E D L K N	**0**	**3/119 (2.5%)**	**0**
TRBJ2.1 **TGT GCC AGC TCG TCG** GGT CTC TTA **GAG CAG TTC TTC GGG CCA GGG ACG CGG CTC ACC GTG CTA** GAG GAC CTG AAA AAC **C A S S S** G L L **E Q F F G P G T R L T V L** E D L K N	**0**	**2/119 (1.7%)**	**0**
TRBJ2.3 **TGT GCC AGC TCA CCG** CCG TGG AGT GGG GGG ACC **ACA GAT ACG CAG TAT TTT GGC CCA GGC ACC CGG CTG ACA GTG CTC** GAG GAC CTG AAA AAC **C A S S P** P W S G G T **T D T Q Y F G P G T R L T V L** E D L K N	**0**	**2/119 (1.7%)**	**0**
TRBJ2.1 **TGT GCC AGC TCA CCA** CCG GGG TGG GGC GAT **GAG CAG TTC TTC GGG CCA GGG ACA CGG CTC ACC GTG CTA** GAG GAC CTG AAA AAC **C A S S P** P G W G D **E Q F F G P G T R L T V L** E D L K N	**0**	**2/119 (1.7%)**	**0**
TRBJ1.1 TRBC1 **TGT GCC AGC TCA** CTG GCC TCG GAC **ACT GAA GCT TTC TTT GGA CAA GGC ACC AGA CTC ACA GTT GTA** GAG GAC CTG AAC AAG **C A S S** L A S D **T E A F F G Q G T R L T V V** E D L N K	**0**	**2/119 (1.7%)**	**0**
TRBJ1.1 TRBC1 **TGT GCC AGC TCC** CAG GGA CAG GTC **AAC ACT GAA GCT TTC TTT GGA CAA GGC ACC AGA CTC ACA GTT GTA** GAG GAC CTG AAC AAG C A S S **Q G Q V** N T E A F F G Q G T R L T V V **E D L N K**	**0**	**2/119 (1.7%)**	**0**
TRBJ2.3 **TGT GCC AGC TCA CCA** CTT CCT AGC GGG GAA GGG GCG TTA **AGC ACA GAT ACG CAG TAT TTT GGC CCA GGC ACC CGG CTG ACA GTG CTC** GAG GAC **C A S S P** L P S G E G A L **S T D T Q Y F G P G T R L T V L** E D CTG AAA AAC L K N	**0**	**0**	**39/128 30.5%**
TRBJ2.5 **TGT GCC AGC TCA CCA** CTG CCT AGC GAA **GAG ACC CAG TAC TTC GGG CCA GGC ACG CGG CTC CTG GTG CTC** GAG GAC CTG AAA AAC **C A S S P** L P S E **E T Q Y F G P G T R L L V L** E D L K N	**0**	**0**	**23/128 18.0%**
TRBJ1.1 TRBC1 **TGT GCC AGC TCA CCT** CTC CCA TGG GAC AGG GAT GCT **GAA GCT TTC TTT GGA CAA GGC ACC AGA CTC ACA GTT GTA** GAG GAC CTG AAC AAG **C A S S P** L P W D R D A **E A F F G Q G T R L T V V** E D L N K	**0**	**0**	**6/128 (4.7%)**
TRBJ2.1 TGT GCC AGC TCA CCA **AGC GGG GGT TTT CCC GGG GGT** GAG CAG TTC TTC GGG CCA GGG ACA CGG CTC ACC GTG CTA **GAG GAC CTG AAA AAC** **C A S S P** S G G F P G G **E Q F F G P G T R L T V L** E D L K N	**0**	**0**	**2/128**
TRBJ1.3 TRBC1 **TGT GCC AGC TCA CCA** CCG ACA AAC **TCT GGA AAC ACC ATA TAT TTT GGA GAG GGA AGT TGG CTC ACT GTT GTA** GAG GAC CTG AAC AAG **C A S S P** P T N **S G N T I Y F G E G S W L T V V** E D L N K	**0**	**0**	**2/128**
Unique sequences	**92/136 66%**	**80/119 65%**	**56/128 44%**

Table 6.5: Unique TRBV18 sequences for clones from patient JAS16 (Figure 6.20).

IL-17+, CD161+ Th1 and CD161- Th1 cells were sorted from SFMC using cytokine capture assay. A total of 383 PCR products for TRBV18 were cloned and sequenced (136 from Th17 cells, 119 from Th1 CD161+ cells and 128 from Th1 CD161- cells). TRBV sequence across the CDR3 region is displayed and frequency of clones in each of the three populations (percentage of total sequences in parentheses). TRBV18 and J region sequence are displayed in bold. TRBJ and TRBC segments are indicated. TRBC1 and TRBC2 refer to the two TR constant genes Nomenclature as defined by Arden and colleagues (Arden *et al,* 1995).

6.4 Discussion

6.4.1 Links between Th17 and Th17/1 cells found at the inflammatory site

Data from Chapter 5 supported the hypothesis that IL-12 drives Th17 plasticity towards a Th17/1 and Th1 phenotype *in vitro*. To test if the elevated IL-12 levels found at the inflammatory site, promoted Th17 plasticity in a similar fashion *in vivo,* SFMC were sorted into Th17, Th17/1 and Th1 populations and evidence for shared clonal origins was assessed by TRBV sequence analysis. The greatest overlap in TRBV sequence was between Th17/1 (upto 30%) and Th17 cells supporting the hypothesis that Th17 cells may show plasticity towards a Th17/1 phenotype within the inflamed joint. Less than 10% of Th1 clones overlapped with either Th17 or Th17/1 clones. This relatively low frequency of overlap may reflect the rarity of conversion of IL-17+ cells to a Th1 cell phenotype in the joint or may indicate that the majority of Th1 cells within the joint are independent of a Th17 origin. At first sight these data would appear to contradict murine studies which showed that adoptive transfer of Th17 cells polarised *in vitro,* rapidly transitioned to a Th1 phenotype (Bending *et al,* 2009; Lee *et al,* 2009). However these two studies were in the context of an immunodeficient host. Th17 cells transferred into an immunocompetent recipient successfully maintained a IL-17+ phenotype, and only converted to a Th17/1 and Th1 phenotype in small numbers following an inflammatory signal (Nurieva *et al,* 2009). Unexpectedly the Th17 program was also more stable in murine Th17 cells harvested directly *ex vivo,* when compared to Th17 generated *in vitro* (Lexberg *et al,* 2008). Taken together these data suggest that the imprinting and stability of the IL-17 locus differs depending on the *in vivo* cues received during and after differentiation. A recent study of the epigenetic control of IL-17 and IFNγ expression in murine Th17 cells, showed that the IFNγ locus has histone modifications that are permissive to IFNγ secretion, in effect making Th17 “poised” for plasticity towards a Th1 phenotype (Mukasa *et al,* 2010). Upregulation of IFNγ after exposure of Th17 cells to IL-12, is dependent on STAT-4, as is the downregulation of RORC2, which is important in silencing the IL-17 locus. Yet Mukasa and colleagues did not explain the

apparent lack of plasticity in ex-vivo Th17 cells from mice when compared to the results from human cells presented in Chapter 5. .

6.4.2 CD161 as a marker of Th17 ancestry

If a proportion of Th17 cells do convert to a Th1 phenotype in humans, then it was predicted that CD161 expression could be used to identify these "converted" cells. To test this hypothesis *in vitro,* CD161+ Th17 cells were purified using the capture assay and cultured in the presence of IL-12 to induce a Th1 phenotype. Consistent with the stated hypothesis, Th17 cells maintained CD161 expression in culture as did Th17/1 and Th1 cells induced by IL-12. Importantly CD161- CD4+ T cells did not upregulate CD161 expression in the presence of IL-12, as previous reports had indicated that NK cells may upregulate CD161 under these conditions (Azzoni *et al,* 1998).

Before concluding that CD161 expression is maintained after Th17 plasticity, one confounder needs to be considered. Contaminating Th17/1 and Th1 cells make up a small proportion of the sorted Th17 population (Chapter 4). Although, Th17 cells, Th17/1 and Th1 express similar levels of the IL-12 receptor β2chain (Annunziato *et al,* 2007), it is still possible the Th17/1 and Th1 "contaminants" may have a selective growth advantage over Th17 cells in short term culture with IL-12. As a result CD161+ Th1 cells detected on day 6 of culture could represent outgrown Th1 contaminants, rather than Th17 cells converting to a CD161+ Th1 phenotype *in vitro*. To address this concern, further evidence was sought to test if Th17 convert to CD161+Th1 cells. SFMC were examined directly ex-vivo to analyse similarities in the expression of Th17 signature molecules between the two populations. It was hypothesised that CD161+ Th1 cells would express higher levels of Th17-related molecules than CD161- Th1 cells, as a sign of their Th17 origins.

6.4.3 A Th17 molecular signature in Synovial CD161+ Th1 cells

Th1 cells from the joint fall into 2 distinct populations according to their expression of CD161 (Figure 6.4). When analysed for the dominant Th17 related transcription factor, RORC2, synovial CD161+ Th1 cells expressed significantly higher levels than CD161- cells. Similarly expression of CCR6 and the IL-23 receptor, both linked to a Th17

phenotype, was enhanced in the CD161+ Th1 population. Both genes, CCR6 and IL-23R have been shown to be upregulated following the forced over-expression of RORC2 (Manel *et al,* 2008). Interestingly, CD161 is also expressed in a subset of cells following lenti-viral expression of RORC2 (Crome *et al,* 2009; Maggi *et al,* 2010). As CD161, CCR6 and IL-23R expression is downstream of RORC2, the enrichment of these genes on synovial CD161+Th1 could be explained if synovial Th17 transition to a Th1 phenotype, whilst maintaining residual expression of RORC2 and its downstream targets, but not IL-17. It is possible that the epigenetic control of RORC2 targets such as CD161, IL-23R and IL-17 is differentially regulated, such that CD161 but not IL-17 expression is maintained following exposure to IL-12.

Is CD161 expression solely dependent on RORC2? Over-expression of RORC2 only led to upregulation of CD161 in less than 10% of cells (Crome *et al,* 2009). As shown in this study and elsewhere (Azzoni *et al,* 1998), CD161 expression is modulated by several factors, some of which are likely to be independent of RORC2. For example, IL-12 has been shown to downregulate RORC2 expression (Annunziato *et al,* 2007) but has the opposite effect of upregulating CD161 expression, at least in NK cells (Azzoni *et al,* 1998).

CD161 expression on synovial Th1 cells may be under complex regulation but it was still important to ask if the association between CD161 and RORC2 observed, resulted from a Th17 ancestry. To help answer this, clonal links between Th17 cells, CD161+ and CD161- Th1 cells from the joint were assessed by analysis of TRBV. If CD161+ Th1 cells originated from a Th17 population, there should be clear clonal links between the two sets of cells. If however, CD161 expression on Th1 cells is independent of a Th17 ancestry, then clonal links between Th17 cells and Th1 cells should be stochastic and not skewed to the CD161+ Th1 population.

6.4.4 Analysis of clonal links between IL-17+ cells and CD161 Th1 cells

Analysis of TRBV CDR3 sequences in IL-17+, CD161+ and CD161- Th1 cells from the inflamed joints of JIA patients showed a remarkable pattern of results. Data from 383 individual clones showed shared sequences only between IL-17+ and CD161+ cells and

not CD161- cells. This result is in line with the previous data demonstrating the maintenance of CD161 expression following Th17 plasticity *in vitro*, and a Th17 molecular signature in synovial Th1 cells expressing CD161. Taken together these data would suggest that Th17 cells undergo plasticity towards a Th1 phenotype in children with arthritis, and that these cells maintain expression of CD161 and to less extent RORC2.

It was notable there were no overlapping sequences between CD161+ and CD161- Th1 cells. This would suggest that CD161- have a distinct clonal origin to both IL-17+ and CD161+ Th1 cells and that CD161 expression, or the lack of it, is in some way maintained with successive cell division. It is interesting to speculate if this study's findings of shared Th17 and CD161 Th1 clonality within the joint would be the same for other disease sites, or indeed healthy control PBMC. Inflammatory sites, such as affected skin in psoriasis, are likely to share a similar biology to the joint, with increased expression of IL-12p70 promoting Th17 plasticity (Yawalkar *et al,* 2009). From murine data it would appear that Th17 are relatively stable in vivo without inflammatory signals (Lexberg *et al,* 2008), and so overlapping Th17 and Th1 clones may be less evident in peripheral blood.

6.4.5 Pathological implications of differences between CD161+Th1 and CD161-Th1 cells?

These data indicated major differences in the transcriptional program and clonal origins of CD161+Th1 and CD161-Th1 within the joint. Given the links to Th17 cells, are CD161+Th1 more "pathogenic" than CD161-Th1 cells *in vivo*? Synovial CD161+ Th1 cells expressed greater levels of the chemokine receptor CCR6 than CD161- Th1 (Figure 6.11). CCR6 expression has been shown to be critical for the entry of pathogenic auto-immune T cells to the site of inflammation in disease models (Hirota *et al,* 2007; Reboldi *et al,* 2009) and may afford entry to CD161+ Th1 cells generated outside of the joint. Related to this, the ligand for CCR6, CCL20 was found to be highly expressed in synovial fluid (Chapter 4). CD161+ Th1 expressed high levels of the IL-23 receptor and responded to IL-23 with increased secretion of IFNγ and TNFα than CD161- Th1 cells. There is strong evidence supporting a genetic link between the IL-23R and human

autoimmune diseases including psoriasis (Filer *et al,* 2008b), ankylosing spondylitis (Rahman *et al*, 2008) and IBD (Schmechel *et al,* 2008). The exact mechanism by which the IL-23R confers disease susceptibility in humans remains unclear, but data from murine studies suggests that the IL-23R is essential for the stabilisation and terminal differentiation of Th17 cells *in vivo* (McGeachy *et al,* 2009). Recent data from a mouse model of HLA-B27 associated ankylosing spondylitis have suggested a link between HLA Class I protein mis-folding and increased IL-23 production which drives a Th17 response, resulting in gut and joint inflammation (DeLay *et al,* 2009). The genetic association of human autoimmunity with IL-23 may have been finally explained by a brilliant study coordinated between many centres focused on T cell research (Ghoreschi *et al,* 2010). Here, the authors clearly show that the Th17 cells can be differentiated without TGFβ signalling and that IL-1β, IL-6 and IL-23 together were sufficient to induce a Th17 phenotype, realigning previously contradictory murine and human data. Importantly, IL-23 induced Th17 cells were more pathogenic in inducing EAE than TGFβ generated cells. Th17 cells induced by IL-23 expressed RORγt but additionally T-bet, again tying together disparate strands of research highlighting the importance of T-bet in the pathogenicity of autoimmune disease (Bettelli *et al,* 2004; Yang *et al,* 2009). This study may also explain the previously noted discrepancy in the pathogenicity of Th17 generated *in vitro* using TGFβ and IL-6 and those harvested *ex-vivo,* which presumably would have been exposed to IL-23 (McGeachy *et al,* 2009).

CD161 may also provide a pathogenic role independent of IL-17. As discussed in Chapter 1, ligands for CD161 are expressed in the joint and following receptor-ligand binding on synovial T cells there is increased secretion of IFNγ (Huarte *et al,* 2008). As IFNγ and IL-17 may synergise within the joint, the ligation of CD61 may have an indirect pro-inflammatory effect (cytokine synergy with be discussed further in Chapter 7).

6.4.6 Study Limitations

The experiments discussed above exploring TRBV sequence do have potential limitations than merit consideration. In the case of experiments comparing the clonality of Th17 with Th17/1 cells (Figure 6.3), the separation of synovial IFNγ+ and IFNγ- cells

by cytokine capture was relatively poor when compared to PBMC (Figure 4.18). This is probably due to a much higher concentration of IFNγ secreting T cells within the joint than in PBMC. A high concentration of IFNγ secreted by SFMC into the media during the "capture phase" of the assay could lead to false (bystander) capture onto non-IFNγ-secreting cells, blurring the distinction between IFNγ+ and IFNγ- cells. Ultimately this would lead to contamination between "cytokine captured" Th17 and Th17/1 populations after FACS sorting and TRBV analysis would over-estimate the degree of shared sequences. Contamination between IL-17+ and IL-17- captured populations is much less likely given the relative rarity of IL-17+ cells in both SFMC and PBMC, which would limit the false capture of IL-17. As a result, comparisons of TRBV clonality between IL-17+ and Th1 populations are more robust. The next concern is related to the sequence of TR re-arrangement during T cell maturation within the thymus. The TRBV locus undergoes re-arrangement first followed by TR alpha variable region (TRAV), so it is possible for two T cells in the periphery to share TRBV sequence, but be distinct clones as defined by their unique TRAV sequence. In addition the "leaky" nature of allelic exclusion at the TRAV locus has been showed to result in cells expressing two different TR (shared β chain but distinct α chain), and early but distinct TR signals could, in theory, lead to two clones, which share TRBV sequence but express different TR (von Boehmer 1992). Although clonal analysis using TRAV sequence would preclude these rare occurrences, the TRAV locus is poorly defined in humans, which has led to the use of the TRBV locus for most studies. Another explanation for shared TR sequence between distinct T cell subsets would be for Th17 and Th1 cells to differentiate from a common naïve pre-cursor in the popliteal lymph node and migrate together to the joint. As naïve T cells are rarely found within the joint (Wedderburn *et al*, 2000) it is unlikely that Th17 and Th1 cells could differentiate directly in situ. Finally the presence of a common "arthritogenic" antigen could lead to Th17 and Th1 cells with a shared TRBV sequence, independent of clonal origin. This possibility remains relatively unlikely as research over the last three decades has yet to discover an arthritogenic peptide (Ravelli & Martini 2007).

6.4.7 Summary

This Chapter examined the links between Th17 and Th1 cells within the inflamed joints of children with arthritis. The data presented showed Th1 cells harvested from the inflamed joint and selected according to the marker CD161, shared a molecular pattern and clonal ancestry with synovial Th17 cells, supporting the evidence for Th17 plasticity, presented in Chapter 5. The functional consequences of this T cell plasticity within the joint on disease severity, and the potential therapeutic benefits of blocking this conversion will be addressed in the final Chapter.

7 : Final Discussion

7.1 Role of Th17 cells in childhood and adult arthritis

Since experiments in murine models discovered a link between Th17 cells and autoimmune arthritis (Murphy *et al,* 2003), there has been an expectation that Th17 cells would also be important, if not central, to the pathology of human arthritis. However, to date, this question remains unanswered. There are several pieces of data that support a role for Th17 cells in human arthritis. As previously noted, the secreted cytokine, IL-17 is elevated in the inflamed joints of patients with rheumatoid arthritis (RA) (Chabaud *et al,* 1999), the most common form of human autoimmune arthritis. CD4+ T cells from RA patients but not healthy controls show proliferative responses to cirtullinated proteins, putative autoantigens in RA, and these responses are associated with secretion of IL-17 in samples from RA patients but not controls. (von Delwig *et al,* 2010). Synovial fibroblasts maybe a target for IL-17 secreted within the rheumatoid joint, as they respond to co-culture with Th17 but not Th1 cells by secreting IL-6, IL-8 and tissue-destructive enzymes (MMP-1 and MMP-3) (van Hamburg *et al,* 2010). Interestingly, the frequency of Th17 but not Th1 cells correlates with serum markers of inflammation and synovitis identified by power doppler ultrasound (Gullick *et al,* 2010).

However, there is also data that undermines the hypothesis that RA is a "Th17 disease". Th17 cells are not enriched within the joints of patients with RA (Yamada *et al,* 2008) and there is little evidence to support a genetic predisposition for Th17 related genes in the disease. In contrast, Th17 cells have been found to be enriched in the peripheral blood and synovial fluid of patients with ankylosing spondylitis (AS) and psoriatic arthritis (Jandus *et al,* 2008; Pene *et al,* 2008; Shen *et al,* 2009) and the IL-23R is clearly linked with both these diseases (Chapter 6 discussion).

This report was the first to demonstrate the enrichment of Th17 cells within the joints of children with arthritis and show a clear link between the frequency of synovial Th17 cells and the severity of disease course. This would suggest that Th17 cells play a pathogenic role in childhood arthritis, which may reflect the actions of IL-17, in promoting a neutrophil influx into the joint, and synergising with TNFα and IL-1β to drive cartilage and bony destruction. Th17 cells may be resistant to regulatory T cell suppression. Several studies have shown than FOXP3+ Treg can suppress TNFα and IFNγ response,

but fail to inhibit IL-17 secretion from human effector T cells, (Annunziato *et al,* 2007; Flores-Borja *et al,* 2008), which may explain the association between Th17 cells and disease course.

Chronic autoimmune uveitis is another disease linked with Th17 cells (Amadi-Obi *et al,* 2007). Interestingly in JIA, two subtypes, persistent and extended oligoarthritis are strongly associated with chronic uveitis, but only one, extended oligoarthritis, was linked with increased frequency of Th17 cells within the joint. The mechanism(s) that explain why Th17 cells traffic to the eyes in some patients, and to the joints in others, are still unclear.

7.2 The fate of Th17 cells within the joint

Why do patients with rheumatoid arthritis have high levels of IL-17 protein within their joints but a low frequency of Th17 cells? It is possible that local secretion of IL-17 is predominately of a non T cell origin. IL-17 can be secreted by NK cells, CD8+ T cells and gamma delta T cells (Kolls & Linden 2004). Synovial membrane from patients with rheumatoid arthritis was examined using immunohistochemistry by Hueber and colleagues (Hueber *et al,* 2010). They found that most IL-17+ cells are mast cells and not Th17 cells, which are present at a much lower frequency. An alternative explanation for the apparent absence of Th17 cells in the rheumatoid joint, is that synovial Th17 cells appear very early in the pathology of these patients, but then undergo plasticity and shift towards a Th1 phenotype, leading to a very high ratio of Th1 to Th17 cells (Yamada *et al,* 2008). In this report the fate of Th17 cells in the inflammatory environment was examined using an *in vitro* assay. IL-12 was shown to drive Th17 plasticity towards a Th7/1 and Th1 phenotype and this was predicted to occur within the JIA joint. This hypothesis was tested by comparing clonal links between synovial Th17 and Th1 cells. Analysis of unique TRBV CDR3 sequence showed strong links between synovial Th17 cells and a subset of Th1 cells that expressed the Th17 related surface marker, CD161. This Th1 sub-population, had significantly higher expression of the Th17 transcription factor, RORC2 and genes downstream of RORC2, CCR6 and IL-23R, than CD161-Th1 cells. In addition, CD161-Th1 cells were clonally distinct from CD161+Th1 cells. This would suggest that Th1 cells within the joint have distinct clonal origins, and that a

proportion of Th1 cells within the CD161+ compartment, arise from a Th17 population that has subsequently extinguished IL-17 secretion. These conclusions are dependent on the T cell populations tested being sufficiently pure to prevent cross contamination which could result in overlapping clonal ancestry and gene expression profiles between Th17 and CD161+Th1 cells. Annunziato and colleagues have since isolated CD161+Th1 and Th17 cells from JIA SFMC by single cell cloning, which limits concerns of contamination (personal communication, Prof Annunziato, University of Florence, Italy). Analysis of TRBV spectratypes from these samples showed a skewed clonal distribution within the joint, but with very similar patterns between CD161+Th1 and Th17 cells, supporting the conclusions drawn in this report that these two populations are related.

7.3 The implications of Th17 conversion to Th17/1 or Th1 cells

The functional implications of the conversion of Th17 cells to Th17/1 or Th1 cells and the consequent co-localisation of IL-17 and IFNγ in the inflamed joint are intriguing. The overall outcome, in terms of the capacity to drive disease in JIA, is dependent on the relative pathogenicity of IFNγ and IL-17 within the joint and the potential for synergy. The role played by IFNγ in autoimmune arthritis is a complex one, with variable results depending on the specific model studied (Hu & Ivashkiv 2009). It has long been appreciated that IFNγ can promote innate immunity by "priming" responsiveness to other pathogen associated molecules (Chapter 5), activating STAT-1 (Hu *et al*, 2002) which upregulates multiple genes encoding phagocytic receptors, chemokines and cytokines and abrogates inhibitory feedback loops (Stark 2007). IFNγ priming disrupts induction of IL-10 in response to TLR agonists, by increasing activity of glycogen synthase kinase 3β (GSK3β) which itself inhibits AP-1 and CREB, two transcription factors critical for IL-10 expression (Hu *et al*, 2006).

As well as priming the immune response, several studies have shown that IFNγ can play a homeostatic role in the immune system. IFNγ is important in the contraction phase of the acute immune response as well as directly limiting inflammation associated tissue damage (Haring & Harty 2006). IFNγ suppresses several matrix metalloproteinases (MMPs) which contribute to the damage of cartilage with the joints of arthritis patients

(Sanceau *et al,* 2002). Tissue infiltration by neutrophils and monocytes and bone resorption are attenuated by IFNγ signalling (Takahashi *et al,* 1986; Vermeire *et al,* 1997). A further immuno-regulatory role for IFNγ has come to light following the re-appraisal of models of autoimmune disease, in the light of the discovery of Th17 cells. As discussed in Chapter 1, in the CIA model, ablation of IFNγ or the receptor for IFNγ leads to exacerbation of disease (Ferber *et al,* 1996; Kageyama *et al,* 1998). IFNγ cross-regulates Th17 differentiation and also Th17 effector functions. So it has been suggested that removal of IFNγ exaggerates Th17 mediated pathology, at least in the murine system (Park *et al,* 2005).

Although these data support a pathogenic role for Th17 cells and a homeostatic or regulatory one for Th1 cells, further studies complicate this simple picture. In proteoglycan induced arthritis (PGIA), IFNγ is the dominant mediator of inflammation, and in EAE, either Th17 or Th1 cells can mediate disease, pathogenicity being linked to expression of T-bet, rather a specific cytokine profile (Yang *et al,* 2009). Is there any evidence from patient studies to help resolve the relative contribution of Th1 or Th17 cells to the disease process in arthritis? Trials of recombinant IFNγ and anti-IFNγ have been limited to pilot studies, with insufficient numbers to definitively answer questions of efficacy. In one of the largest studies, involving 54 patients with RA, recombinant human IFNγ did not confer a benefit over placebo (Cannon *et al,* 1989), questioning a regulatory role for IFNγ in human disease.

There are more recent data from *in vitro* studies of human samples, examining the interaction between IFNγ and IL-17. In skin samples from psoriatic patients, IFNγ from Th1 cells acts on resident APC to promote IL-1β and IL-23 secretion which leads to the induction of Th17 cells. IFNγ primed APC also secrete CCL20, driving the recruitment of Th17 cells to the target site (Kryczek *et al,* 2008). Taken in the context of this thesis, Kryczek's results would predict a cycle of positive feedback whereby Th17 are recruited to the joint, convert to Th17/1 or Th1 in response to local IL-12 and the resulting production of IFNγ, promotes further recruitment of Th17 by virtue of the secreted CCL20. These data lead to the proposal that both Th17 and Th1 cells have the potential to contribute to inflammation in arthritis.

7.4 Targeting Th17 and Th1 cells in human autoimmune arthritis

A new therapeutic agent, simultaneously targeting Th17 and Th1 cells, has therefore generated considerable interest in the treatment of human autoimmune disease. Ustekinumab, a monoclonal antibody against the shared IL-12p40 subunit is under investigation in a range of autoimmune diseases. The blockade of IL-12p40 is likely to simultaneously attenuate the induction and stabilisation of Th17 cells by blocking IL-23, and Th1 cells by blocking IL-12. If the predictions discussed in 7.2 are correct, the attenuation of IL-12 alone would stabilise Th17 cells and possibly exacerbate arthritis, but in the case of Ustekinumab this effect may be counter-acted by the additional actions of IL-23 blockade. Ustekinumab may also have important actions on non-T cells. Remarkably two lymphoid populations of the innate immune system have also been shown to express RORγt, and IL-17 (Buonocore *et al,* 2010; Takatori *et al,* 2009). Lymphoid tissue inducer cells (LTi), previously recognised to play a role in the development of lymph nodes, express IL-23R and secrete IL-17 and IL-22, although it is unclear if this has a function in host defence. Another LTi like innate cell is the predominant source of IL-17 secretion within the gut in a model of infection induced colitis (Buonocore *et al,* 2010). This novel population, secretes IL-17 in response to IL-23, and is critical for the development of colitis in a Rag knockout animal. Both of these innate sources of IL-17 may be amenable to targeting with treatment with anti-IL-12p40.

Ustekinumab has been compared to the anti-TNFα agent, Etanercept, in a large randomised controlled trial of moderate to severe psoriasis. 73.8% of patients who received high dose Ustekinumab had a 75% improvement in psoriasis severity, as compared with only 56.8% with Etanercept (Filer *et al,* 2008b). Although infection risks remain a concern, this relatively small trial did not detect a significant infection hazard. Another trial of the same agent in Crohn's disease, found less impressive results, with 53% of patients showing a clinical response compared 30% of patients on placebo (Sandborn *et al,* 2008). Similarly results from the treatment of psoriatic arthritis were disappointing, with only 42% of patients reaching the very modest American College of Rheumatology (ACR) response rate of 20% improvement (Gottlieb *et al,* 2009).

Anti-IL-17 monoclonal antibodies are also under investigation in psoriasis and RA, but published studies are limited to a phase 1 trial in RA (Genovese *et al,* 2010), which suggests a modest superiority of the drug against placebo. Ultimately clinical trials in patients will confirm the prediction derived from murine models, that Th17 cells are critical to disease in autoimmune arthritis. From the trial results discussed, it would appear that IL-17 blockade may be more efficacious in diseases with a clear Th17 signal, such as Psoriasis, rather than RA. These results offer hope for the success of anti-IL-17 therapy in JIA, especially subtypes with severe arthritis linked to uveitis, such as extended oligoarthritis.

7.5 The evolution of regulatory and effector T cell balance

So far this discussion has focused on effector T cells in isolation. Data from this report suggest that it is the balance of Th17 cells with regulatory T cells in the joint that may influence the clinical phenotype of patients with JIA. This inverse relationship between Treg and Th17 has been seen in many different murine settings, and may represent a true signature of Th17 biology. Weaver and Hatton, examined this relationship and argue that the close biologic links between Th17 and Treg is a consequence of primitive vertebrates adapting to the interaction with microbes within the GI tract (Weaver & Hatton 2009). Most vertebrate species retain a vast array of microbes within the gut, providing access to bacterial enzymatic, metabolic pathways and vitamin derivates, potentially unavailable within host tissue itself. This commensal flora could afford the host an evolutionary advantage allowing a flexible diet when experiencing food shortages. However the complexity of immune response needed to regulate this symbiotic relationship would have been far greater than the simple host-pathogen system maintained by the innate immune system. To distinguish “good” or commensal flora from potentially pathogenic invasive bacteria, a more specific form of immunity would be required. Hence the pressure to evolve an adaptive immune system with memory. To prevent a host response to the resident commensal flora generating bystander tissue damage, the default state would be a regulatory one. Specialised dendritic cells within the gut express the enzyme retinal dehydrogenase, which converts a vitamin A precursor, retinal to retinoic acid, which in a TGFβ rich environment, such as the gut, can drive the generation of Treg

from naïve cells (Coombes *et al,* 2007; Mucida *et al*, 2007). Following danger signals from pathogenic microbes, IL-6 secreted by antigen processing cells in concert with TGFβ over-rides the regulatory program and instead leads to differentiation of Th17 cells. As well as steering T cell differentiation from Treg to Th17, inflammatory cytokines, such as IL-1β and IL-23 can induce mature Treg to upregulate IL-17 secretion, again promoting the switch from tolerance to immunity (Chapter 4 discussion). It is a possible that this reciprocal relationship between Treg and Th17 cells, which is finely controlled in the normal gut, becomes dysregulated in autoimmune disease. Genetic factors may play a role in tipping the Treg/Th17 balance against Treg and towards Th17 cells. A genetic association between the IL-2 receptor alpha chain (also known as CD25) and autoimmune arthritis may act on the Treg side of the balance. A group of single nucleotide polymorphisms (SNP), including rs12722495, in the non coding regions spanning 18kb directly 5′ of *IL2RA*, to 25 kb into intro 1 of the gene, have been associated with several autoimmune diseases (Lowe *et al,* 2007). A related SNP, rs2104286, is in linkage disequilibrium with the SNPs detected by Lowe and colleagues, and has been associated with JIA patients (Hinks *et al*, 2010). The G allele of the rs12722495 SNP is protective of diabetes and is found at a reduced frequency in diabetic patients when compared to health controls. Investigators from the Todd laboratory examined healthy controls with the "protective" GG haplotype and showed that CD4+ memory T cells from these individuals expressed higher levels of surface CD25 protein and also had greater IL-2 production than individuals with the AA haplotype (Dendrou *et al,* 2009). Given the critical dependence of peripheral Treg on exogenous IL-2 for their homeostatic expansion and function (Fontenot & Rudensky 2005), it is possible that the AA haplotype, with reduced IL-2 secretion leads to fewer, less suppressive Treg which may tip the balance away from Treg and towards Th17. This effect may be specific to the inflammatory environment, as Treg frequency defined as either CD25hi or FOXP3+ cells was equivalent in the peripheral blood of healthy volunteers with both haplotypes.

The genetic association between *IL-23R* and autoimmune arthritis (discussed in chapter 6), may also alter the Th17/Treg balance by acting on the Th17 side of the equation. Although a functional role for the *IL-23R* polymorphism is still to be confirmed, it is likely that the haplotype linked with disease susceptibility will show an increased

response to IL-23. Given the role for IL-23 in the differentiation of naïve T cells towards Th17 (Manel *et al*, 2008; Wilson *et al*, 2007) and also up-regulation of IL-17 expression in mature Treg, a susceptible *IL-23R* haplotype may tip the balance towards Th17 and away from Treg. Finally environmental cues may impact on this delicate balance. Several studies have identified viral and bacterial proteins and DNA within the joints of JIA patients (Wilkinson *et al*, 1999). It is possible that these molecules represent pathogen associated molecular patterns (PAMPs) which activate the innate immune system leading to a pro-inflammatory signal (e.g., IL-6 or IL-23 secretion) which in the context of individuals with a genetic predisposition, drives T cell plasticity towards a high Th17, low Treg phenotype and consequently autoimmunity.

7.6 T cell plasticity – the new paradigm

When work on this thesis first began in 2006, Th17 cells were an exciting new addition to the well established paradigm of Th1 and Th2 lineages. The subsequent four years have been a challenging time for this model of distinct T cell subsets, each supposedly expressing a unique transcription factor, and a terminally differentiated effector phenotype. The almost simultaneous discovery of Treg plasticity towards a Th17 phenotype and Th17 cells towards a Th1 phenotype has completely revolutionised our understanding of cell "lineages". Cells with an intermediate phenotype sharing master transcription factors from apparently distinct cell types (Th17/1 cells expressing T-bet and RORC2) are readily detectable within mice and humans. As demonstrated in this thesis, the plasticity of T cells is not just a feature of genetically manipulated murine models but an active process in human autoimmune disease which may have implications for future therapies. As understanding grows of the epigenetic control of transcription factor and cytokine gene expression, it is clear that T cell subsets can no longer be viewed as distinct, fixed populations. Rather, as some authors have suggested, the cell's phenotype is more akin to energy states of electrons in atoms (Murphy & Stockinger 2010). In some circumstance cells may be in a "high" energy state, and thus relatively unstable (such as Th17 cells) allowing transition towards a "low" energy state (Th1 cells) which no longer have the potential or "energy" to undergo further change.

This new paradigm has some important implications for the future immune therapies. One example relates to the field of regulatory T cells. Over the last decade there has been great interest in generating regulatory T cells in the laboratory, with the aim of re-infusing cells back into patients, to treat autoimmune disease. Now in the light of data demonstrating Treg plasticity towards a Th1 and Th17 phenotype *in vivo* (Zhou *et al*, 2009), it is clear this approach needs to be carefully reconsidered since the "converted" Th1 and Th17 may drive autoimmunity. More generally, if T cells can switch phenotype or the predominant cytokine secreted, it may be more effective to target the differentiation of effector T cells (e.g., Ustekinemab) than trying to target a single effector cytokine. A better understanding of how T cell plasticity impacts on the balance between regulatory and effector T cells will help shape future therapeutic strategies that target the immune system in JIA.

In conclusion this thesis of work has established a role for Th17 cells in juvenile arthritis, has examined the migratory phenotype of both Treg and Th17 cells in the context of this autoimmune disease and demonstrated a reciprocal relationship between Th17 and Treg cells in the joint. Furthermore, the analysis of the novel "intermediate" Th17/1 population and their relationships (phenotypic, functional and transcriptional) with the Th17 and Th1 populations has generated strong evidence for the plasticity of Th17 cells, perhaps via the intermediate phenotype, to Th1 cells. Finally the thesis has provided evidence that those Th1 cells in the inflamed site which express the marker CD161, may have arisen from Th17 cells and that the inflammatory cytokine milieu may enable such conversion from Th17 to Th1 cells.

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List of publications arising from this work or contributed to during this PhD programme

1. **Nistala, K**., Adams A., Cambrook H., Ursu S., Olivito B., de Jager W., Evans J., Cimaz R., Bajaj-Elliott M., Wedderburn LR. Th17 plasticity in human autoimmune arthritis is driven by the inflammatory environment, *Proc Natl Acad Sci*. 2010 Aug 17;107(33):14751-6.
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4. Hunter, P., **Nistala K**., Jina, N., Eddaoudi A., Thomson W., Hubank M., and Wedderburn, L.R. Biological Predictors of Extension of Oligoarticular Juvenile Idiopathic Arthritis from Synovial Fluid Cellular Composition and Gene Expression. *Arthritis Rheum*, 2010 Jan 7;62(3):896-907.
5. **Nistala, K**., Moncrieffe, H., Newton, K. R., Varsani, H., Hunter, P., & Wedderburn, L. R., "Interleukin-17-producing T cells are enriched in the joints of children with arthritis, but have a reciprocal relationship to regulatory T cell numbers", *Arthritis Rheum*, 2008 vol. 58, no. 3, pp. 875-887.