Expression of Trafficking Receptors by Regulatory T Cells in Human Health and Autoimmune Disease

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PREFACE

This thesis describes original research that I have conducted within the T Cell Biology Group at the Centenary Institute, under the supervision of Prof. Barbara Fazekas de St. Groth.

This is to certify that to the best of my knowledge, the content of this thesis is my own work and any contributions by others have been recognised accordingly. This work has not been previously submitted for the purpose of attaining another degree.

Suzanne Asad
June 2016
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ABSTRACT

Abnormalities in CD4+CD25+CD127loFoxP3+ regulatory T cells (Tregs) have been implicated in susceptibility to autoimmune disease. To define migratory Treg subsets that may be implicated in the pathogenesis of diseases manifested in different tissues, we designed 11 and 13 colour flow-cytometry panels to measure the expression of a range of chemokine receptors and integrins on Tregs in the peripheral blood (PB) of healthy individuals (n=44) and patients with rheumatoid arthritis (RA) (n=34) or psoriasis (n=44).

We showed that CLA, CCR4, CCR5, CCR6, CCR10 and CD62L were expressed on a higher proportion of CD4+CD25+CD127lo Tregs than conventional CD4+ T cells (Tconvs) in healthy adults. A significantly lower proportion of Tregs expressed the gut homing α4 (CD49d) and β7 when compared to Tregs that expressed the skin homing CLA, CCR4 and CCR10. Furthermore, the skin homing receptors, CLA, CCR4 and CCR10, were expressed on a much higher proportion of Tregs than Tconvs.

We used flow cytometry to analyse the levels of CXCR3+, CCR4+, CCR5+ and CCR6+ Tregs in the PB of RA patients. RA patients had significantly reduced levels of effector/memory Tregs in their PB. We also found a significantly reduced frequency of effector/memory Tregs expressing each of the chemokine receptors examined in the PB of RA patients. This could indicate a trafficking deficiency, resulting in less Tregs entering the joints and a consequent disruption of immune homeostasis at the site.

We examined the levels of β7+, CD49d+, CLA+, CCR4+, CCR10+, CCR5+, CD62L+, CXCR3+ and CCR6+ Tregs in the PB of psoriasis patients. We found Treg frequency to be increased in the PB of untreated psoriasis patients. However, the frequency of effector/memory Tregs expressing CXCR3 and CCR6 was significantly reduced in the PB of these patients. CXCR3+ and CCR6+ Tregs are known to be crucial to regulating Th1 and Th17 type immune responses, which are both involved in the pathogenesis of psoriasis. Therefore a reduction in
CXCR3 and CCR6 expression on Tregs could indicate a trafficking deficiency resulting in less Treg entry into psoriatic lesions.

We used bioinformatic tools to analyse all flow cytometry data obtained from patients and controls. We hope these methods of analysis will facilitate the uptake of flow cytometry in detecting preclinical disease or selecting patients for optimal treatment.
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<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>AF488</td>
<td>Alexa Flour 488</td>
</tr>
<tr>
<td>AF647</td>
<td>Alexa Fluor 647</td>
</tr>
<tr>
<td>Ag</td>
<td>Antigen</td>
</tr>
<tr>
<td>AIRE</td>
<td>Autoimmune regulator protein</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>APC</td>
<td>Allophycocyanin</td>
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<tr>
<td>CLA</td>
<td>Cutaneous lymphocyte antigen</td>
</tr>
<tr>
<td>CR</td>
<td>Chemokine Receptor</td>
</tr>
<tr>
<td>CST</td>
<td>Clonal Selection Theory</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic T lymphocyte Antigen-4</td>
</tr>
<tr>
<td>DAS28</td>
<td>Disease activity score in 28 joints</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic Cells</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activating cell sort</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>FMO</td>
<td>Fluorescence minus one</td>
</tr>
<tr>
<td>FOXP3</td>
<td>Forkhead box P3</td>
</tr>
<tr>
<td>FSC</td>
<td>Forward scatter</td>
</tr>
<tr>
<td>FW</td>
<td>FACS wash</td>
</tr>
<tr>
<td>HCL</td>
<td>Hierarchical clustering</td>
</tr>
<tr>
<td>HEV</td>
<td>High endothelial venules</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MeV</td>
<td>MultiExperiment Viewer</td>
</tr>
<tr>
<td>MHC</td>
<td>Peptide-Major Histocompatibility Complex</td>
</tr>
<tr>
<td>mTEC</td>
<td>Medullary Thymic Epithelial Cells</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MTX</td>
<td>Methotrexate</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen Associated Molecular Pattern</td>
</tr>
<tr>
<td>PASI</td>
<td>Psoriasis area severity index</td>
</tr>
<tr>
<td>PB</td>
<td>Peripheral blood</td>
</tr>
<tr>
<td>PB</td>
<td>Pacific Blue</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PD-1</td>
<td>Programmed death-1</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerytherin</td>
</tr>
<tr>
<td>PE/Cy7</td>
<td>Phycoerythrin-Cyanine 7</td>
</tr>
<tr>
<td>PerCp/Cy5.5</td>
<td>Peridinin chlorophyll protein/cyanine 5.5</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern Recognition Receptors</td>
</tr>
<tr>
<td>PSO</td>
<td>Psoriasis</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid Arthritis</td>
</tr>
<tr>
<td>RORγt</td>
<td>RAR-related orphan receptor gamma</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SSC</td>
<td>Side scatter</td>
</tr>
<tr>
<td>T-bet</td>
<td>T-box transcription factor</td>
</tr>
<tr>
<td>TCM</td>
<td>Tissue culture medium</td>
</tr>
<tr>
<td>Tconv</td>
<td>Conventional T cell</td>
</tr>
<tr>
<td>TCR</td>
<td>T Cell Receptor</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor-β</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-α</td>
</tr>
<tr>
<td>TNFi</td>
<td>Tumour necrosis factor-α inhibitor (therapy)</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T cell</td>
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CHAPTER 1: LITERATURE REVIEW

1.1 Introduction

In the early 1970s immunologists speculated that a subset of thymically derived T cells, then called ‘suppressor T cells,’ were involved in the peripheral regulation of immune responses. The widespread acceptance of these T cells, later termed regulatory T cells (Treg), as a significant subpopulation came after the identification in 1995 of CD25 as a marker of a subset of CD4+ T cells with suppressive activity in vivo [1]. CD4+CD25+ cells were later shown to express high levels of the transcription factor FoxP3 [2]. CD4+CD25+FoxP3+ Tregs are now widely regarded as primary mediators of peripheral tolerance and immune regulation. Due to their demonstrated importance in preventing allergy and autoimmunity, in contributing to immune evasion by tumours and in preventing transplant rejection, the study of mechanisms of Treg function and development is currently at the forefront of research in immunology.

An understanding of how Tregs function to prevent autoimmunity could help us develop targeted treatment for autoimmune disease. Although the mechanisms whereby Tregs suppress immune responses are still controversial, it has become clear that Tregs are involved in regulation at multiple points of an immune response. Their presence in both lymphoid and non-lymphoid tissues suggests they are involved in regulating both the priming and effector phases of conventional CD4 T cell responses. This led us to hypothesise that the trafficking and localisation of Tregs is crucial to their function and that deficiencies in Treg subsets expressing particular trafficking receptors may play a role in immune disease pathogenesis.
1.2 The Immune Response

The mammalian immune system consists of many cellular and molecular mechanisms that work together to protect the individual from invading microbes and abnormal host tissue. Immune defence can be viewed as two interrelated systems, termed innate and adaptive. Innate immunity is the first line of protection and consists of biochemical and cellular defences that are in place before a foreign substance is encountered, allowing a rapid response. The primary components of the innate immune system include selectively permeable barriers, commensal flora, acidic and mucous secretions and an array of cells and proteins that can eliminate microbes by recognising pathogen associated molecular patterns (PAMPs) characteristic of pathogenic organisms. Such recognition is mediated via pattern recognition receptors (PRRs), which are encoded by germline genes.

In contrast to the innate immune response, the defining features of the adaptive response are its ability to respond specifically to distinct molecules and generate ‘memory’ so that it can respond more rapidly to repeated exposure. The key mediators of the adaptive response are the T and B-lymphocytes derived from pluripotent haemopoietic stem cells. Lymphocytes recognise antigen by means of cell surface receptors that are formed by genetic recombination within the lymphocyte. This process of gene recombination generates a diverse range of receptors capable of recognising and responding to almost any molecular shape, including those that are made by the individual.

Both innate and adaptive immune responses can cause significant damage to host tissues and serious chronic disease states can ensue if they become deregulated. Hence the immune system has evolved multiple mechanisms designed to regulate its response and in particular to distinguish between self and non-self antigen (Ag). The maintenance of a balance between self-antigen-driven tolerance and pathogen-driven immunity is essential to human health. Despite constant exposure to self antigen, the immune system can maintain this balance whilst responding to microbes and remaining unresponsive to self.
The immune system represents a hierarchy of information transfer between different cell types. Information determining the type of immune response is passed from antigen presenting cells to T cells which finally activate B cells [3]. If T cell tolerance is incomplete, the self reactive T cells can provide help to self-reactive B cells and so the tissue damage becomes more severe and prolonged. When T lymphocyte processes are deregulated, inflammatory responses can develop against self-tissues. Such responses are termed autoimmune. As a group, autoimmune diseases have a significant health impact in terms of overall prevalence and mortality and are among the leading causes of death among young and middle aged women (reviewed in [4]).

Since most autoimmune diseases are believed to be T cell dependent, whether the major pathogenic mediators are cells or T-dependent antibodies, the processes involved in developing and maintaining self tolerance within the T cell compartment will be reviewed here.

1.3 T Cell Regulation

1.3.1 T Cell Receptor Development and Clonal Selection

The mammalian immune system has the potential to produce a vast array of receptors that can sense and react to virtually any chemical structure possible, hence giving it the capability to fight off any possible microorganism. The production of lymphocytes expressing such a large repertoire of different receptors raises the question of how certain cells are selected to survive and proliferate while others are not. This issue was first addressed by MacFarlane Burnet in 1959 in the ‘Clonal Selection Theory’ (CST), which hypothesised that once the immunological repertoire of receptors develops spontaneously in the host, selective interaction between antigen and receptor signals cell survival and proliferation (reviewed in [5]).
In respect to T lymphocytes, the diversity of T cell receptors (TCR) is generated in the thymus by a process of somatic genome modification that occurs selectively within lymphocytes (reviewed in [6]). This process is known as V(D)J recombination and involves the random splicing and recombination of the three separate segments of the TCR genes; the variable (V), the diversity (D) and the joining (J) genes [7]. A large diversity of receptors is formed in this process with a significant fraction, estimated to represent approximately 20-50%, having the capacity to bind self antigen with a potentially dangerous affinity [8]. This random receptor generating process is deliberate and is believed to have evolved in vertebrates in order to increase the efficiency of detection of foreign antigen. However, with it emerged the issue of self-reactivity [3].

1.3.2 Self Tolerance

Immunological tolerance is defined as antigen specific unresponsiveness induced by prior exposure to the antigen (reviewed in [9]). It is not fully understood what regulatory mechanisms control whether an immune response will lead to tolerance or immunity [5]. It is however known that failure of tolerance mechanisms results in ‘forbidden’ clones of cells that can find opportunity, upon antigen encounter, to proliferate and inflict damage on the body in an autoimmune response. The regulation of self-tolerance within the T cell repertoire is maintained at two levels: centrally and peripherally.

1.3.2.1 Central Tolerance

Once developing T cells within the thymus have undergone V(D)J recombination of their TCR genes, they express and display the rearranged TCR on their cell surface. The process of V(D)J recombination in the thymus may be accompanied by receptor editing and cellular deletion in a process known as thymic selection [10]. It is estimated that approximately 5x10⁷ T cells are produced daily, but only 1- 2x10⁶ of these exit the thymus to enter the
circulation (reviewed in [11]). This loss of ~95% of thymocytes reflects a strict selection process that determines the T cell repertoire in the periphery.

Central tolerance refers to events in the thymus (usually in the thymic medulla) that trigger clonal deletion of thymocytes expressing TCRs with high affinity for self-Ag [12]. The molecule that determines whether a T cell will be deleted is the TCR itself. It seems that the receptor and its signalling pathway are able to discriminate between low affinity and high affinity binding to Ag [13]. The processes that eliminate high affinity self-reactive cells are referred to as mechanisms of negative selection [13].

The microenvironment in the thymic medulla contains a significant measure of self-Ags. The large diversity of self-Ags available to T cells in the medulla determines the extent of central tolerance. These include Ags intrinsic to various cell types found within the thymus, such as epithelial cells, dendritic cells (DC), macrophages and thymic B cells. Self-Ags can also enter the thymus through the circulation or on immigrating cells [11]. In addition, analysis of thymic stromal cells by Derbinski and others in 2001 revealed the expression by medullary thymic epithelial cells (mTEC) of over 30 Ags previously believed to be synthesised only in non-thymic tissues [14].

The autoimmune regulator (AIRE) protein regulates the expression of these “tissue specific” Ags by thymic epithelial cells [15]. It has been shown that mutations in the gene encoding AIRE can result in a severe human autoimmune disease syndrome termed autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy or APECED [16]. The AIRE protein promotes the expression of tissue specific Ags and has several potential DNA-binding domains in order to regulate transcription of a wide range of genes [17]. The expression of tissue specific Ag in the thymus is of crucial importance; studies in mice in which medullary major histocompatibility complex (MHC) molecules are absent or blocked have shown that T cells with self-reactive TCRs can reach the periphery and cause systemic inflammatory conditions (reviewed in [6]).
Although the autoimmunity that develops in AIRE deficiency demonstrates the importance of central tolerance, central tolerance alone is not sufficient to prevent autoimmunity. T cells that recognise self-Ag at an affinity below the threshold required for deletion during central tolerance are released into the periphery. If not regulated, these T cells can generate an autoimmune response.

1.3.2.2 T Cell Maintenance, Growth and Differentiation

After development in the thymus, the T cells that escaped negative selection enter the peripheral blood circulation as naive T cells. These T cells migrate to and enter secondary lymphoid tissues such as draining lymph nodes, spleen and Peyer’s patches, where they are ready to encounter their antigen and become activated. Functionally naive T cells may survive for long periods of time in the absence of exposure to specific foreign antigen [18].

Antigen driven activation of functionally naive T lymphocytes changes them phenotypically into effector/ memory T cells. The formation of a complex between a TCR and a peptide-major histocompatibility complex (pMHC) ligand represents the molecular solution to the recognition of Ag and the activation of the T cell [19]. The TCR is ‘MHC restricted’ and can only bind Ag that is itself bound to MHC on the surface of the antigen-presenting cell (APC) [20]. T cells can be divided into two classes: CD4+ and CD8+. CD8+ T cells are restricted to the MHC class 1 molecules expressed on the surface of most nucleated cells and are generally called cytotoxic T lymphocytes (CTL). CD4+ T cells are termed T helper cells and recognise MHC class II molecules on a more restricted set of APCs such as macrophages and DCs (reviewed in [20]). The differentiation of naive CD8+ T cells transforms them into CTLs, which are activated by recognition of pMHC on infected cells allowing them to subsequently directly kill the cell. When activated CD4+ T cells encounter pMHC they release cytokines capable of activating macrophages or stimulating antibody (Ab) production by B cells.
The engagement of the TCR with pMHC is not sufficient to generate an activation signal within the T cell. For complete activation a second signal is required. Second signals include costimulators and cytokines that promote clonal expansion of specific T cells as well as their differentiation into effector/ memory cells. Two members of the B7 family, B7-1 (CD80) and B7-2 (CD86) are well-defined costimulators. Expression of these molecules on APCs is increased once they are activated by microbes or cytokines produced during the initial immune response [21].

Upon activation, T cells produce interleukin (IL)-2. IL-2 is the prototypical T cell growth factor and functions in an autocrine and paracrine way to cause clonal expansion of the T cells stimulated by Ag [22].

1.3.2.3 Peripheral Tolerance

Despite undergoing negative selection during central tolerance, a significant number of low-affinity self-specific TCRs fail to be edited or to trigger deletion, and hence self-reactive specificities enter the periphery expressed by naive T cells. It has been shown that T cells can become tolerant to peripherally administered Ag, even in the absence of the thymus [9]. Injection of protein into adult mice that had been thymectomised produced specific unresponsiveness to the Ag and hence demonstrated that processes in the periphery could achieve Ag specific tolerance [9]. Below we will discuss these processes.

Passive Tolerance

Lymphocyte responses can be prevented or terminated in the periphery by deprivation of necessary growth and survival factors, leading to a failure to initiate responses and hence functional inactivation or death of the lymphocytes involved (reviewed in [22]). Furthermore, peripheral Ags may not provoke an immune response if they elude T cells. This may be because they are sequestered in an immunologically privileged site or are expressed by cells
that do not express MHC molecules and are hence unable to present Ags (reviewed in [9]). This ‘ignorance’ of extrathymic autoantigen by the immune system may be important in avoiding autoimmune responses. However, this mechanism is potentially dangerous, as molecules may be released from dying cells, particularly following tissue damage.

Level of Costimulation

Bretscher and Cohen were the first to propose that the distinction between immunity and tolerance depended on whether the lymphocyte received a sufficient costimulatory signal at the time of Ag recognition (reviewed in [9]). The activation state of APCs is an important factor in determining whether this required level of costimulatory signal is present. Activated APCs provide high levels of costimulatory molecules and favour T cell proliferation. On the other hand, APCs in steady state express low levels of costimulatory molecules and favour ignorance in the form of either apoptosis of the T cell or the induction of functional anergy [23].

Because high affinity self-reactive T cells are removed in the thymus during central tolerance, the self-reactive T cells in the periphery have a comparatively weak affinity for self-Ag [12]. For this reason, these self-reactive T cells are more dependent on higher levels of costimulation for their activation. Steady-state APCs presenting self-Ag and expressing low levels of costimulatory molecules do not provide sufficient signals to these self-reactive T cells, therefore preventing them from undergoing activation, proliferation and differentiation to generate an autoimmune response [24].

Activation Induced Cell Death

Activation induced cell death occurs as a result of antigenic stimulation of T cells which triggers mechanisms that prevents their proliferation and survival. Experiments done by Thompson and Allison have demonstrated that a second receptor for the B7 molecules,
termed the cytotoxic T lymphocyte Ag-4 (CTLA-4), functions to shut off T cell activation [25]. The expression of CTLA-4 on the surface of T cells is known to increase upon activation, but its mechanism of action remains controversial. Two widely accepted theories are that it competes with CD28 for binding to B7 ligands and hence reduces CD28-mediated signalling, or that its binding to B7 generates alternative signals that interfere with signals from the TCR/CD28 pathways (reviewed in [26]).

Activation of T cells also induces the expression of the death receptor Fas and its ligand, FasL. Fas-FasL interactions trigger T cell death by inducing the caspase 8 proteolytic cascade and causing apoptosis; this pathway has been shown to be induced by repeated activation (reviewed in [6]). Expression of Fas and FasL is important for maintaining tolerance to self, as demonstrated by Nagata and Suda who showed that fatal autoimmune disease develops in mice with mutations in Fas or FasL [27]. Defects in Fas expression or function have also been shown to have similar effects in humans [28].

Tolerogenic APCs

Another model of peripheral tolerance has been proposed by Barbara Fazekas. This model suggests that lymphocytes are not capable of distinguishing between foreign and self-Ag and must rely on discrimination made by two subsets of APCs with distinct ontogenic origins, the lymphoid- and myeloid-derived DCs, which induce tolerance and immunity, respectively [3]. This model suggests that the induction of tolerance rather than immunity is determined not only by the level of costimulation but also by the subset of DCs presenting the Ag. This is determined by several factors such as the structure of the Ag and the location of the APC. Fazekas argues that this model can thus explain how peripheral tolerance and immunity can be induced in naïve T cells by different protocols of Ag administration [3]. A study by Kearney et al. confirmed these influences of Ag administration on the T cell response. They showed that subcutaneous injection of Ag produced immunity. On the other hand, when Ag was administered into the blood, cells at first proliferated for a short time but then
disappeared, leaving behind a small population that were hypo-responsive to Ag stimulation [29].

Regulatory T Cells

The existence of a subpopulation of T cells that specialise in the suppression of the immune response is now well established. These regulatory T cells (Treg) are widely regarded as the primary mediators of tolerance in peripheral tissues, where they have been shown to prevent autoimmune disease and limit chronic inflammation. Tregs are the major focus of this study and will be reviewed in more detail in section 1.5.

1.4 Autoimmune Disease

The existence of autoimmune disease suggests that central and peripheral tolerance are far from absolute. Many factors make it difficult to reach precise conclusions regarding the mechanisms of autoimmunity. The modelling of self tolerance in in vitro culture has proven to be difficult and has required that studies are performed in vivo. However the in vivo experimental systems are complex, often with multiple changes occurring in response to a single stimulus, making effects difficult to dissect [26]. Although the target Ag has been identified in a very few number of autoimmune diseases, such as celiac disease [30] and some forms of glomerulonephritis [31], the initiating stimulus and target Ag of most human autoimmune diseases have not yet been identified, therefore making it impossible to choose appropriate self-Ags to study in mice.

Although autoimmune diseases cause immense damage to self tissues, studies in transgenic mouse models suggest that this may be due more to the chronicity of the response rather than its initial magnitude. It is the persistence of self-Ag in the body that drives the chronicity of autoimmune responses. Often, auto-immune responses are initiated but never followed by any clinical manifestations unless additional events favour disease progression [32].
Progression of an autoimmune disease from initial activation to a chronic state often involves an increase in the number of auto-antigens targeted by T cells and antibodies (reviewed in [33]). This process is known as “epitope spreading” and both B cells and T cells contribute. Activated, autoreactive B cells can act as APCs generating and presenting peptides from self-Ags that have not yet been presented to naive T cells and thus are not tolerised against (reviewed in [33]). This cascade continues with T cells activating additional autoreactive B cells, leading to chronic inflammation.

Several strategies have been proposed to account for how a breakdown in central tolerance can allow auto-specific T cells to escape negative selection in the thymus. Cibotti and others have demonstrated that presentation of self-Ag at too low a concentration within the thymus can allow peripheral auto-reactivity to develop for some self proteins [34]. Furthermore, Mamula and others have shown that difficulty in processing and presenting particular self-Ags in the thymus can allow self reactive T cells specific for those Ags to escape central tolerance [35].

It can be predicted that gross defects in the induction of self-tolerance will lead to overwhelming autoimmunity to the entire range of self-Ags. This situation is not seen in human autoimmune disease. Thus human autoimmune disease is more likely to result from a partial dysregulation of peripheral tolerance rather than a major defect in central tolerance [3]. A common belief is that self-Ags can induce tolerance in T cells due to the absence of second signals whereby lack of costimulation induces deletion or anergy [22]. It can therefore be said that the initiation of autoimmunity can be caused by a stimulus that activates APCs to upregulate expression of costimulatory molecules. The frequently noted association between autoimmunity and infection has been attributed to this upregulation of costimulatory molecules on APCs in response to the infection [22]. It has also been suggested that the role of infection in predisposing to autoimmunity is mediated not by upregulation of constimulatory molecules on APCs but rather by the release of self-Ag, due to inflammation,
which is then taken up by immunogenic APCs which relocate to the secondary lymphoid organs to activate T cells [3].

In contrast to T cell-intrinsic tolerance, the role of regulatory T cells in preventing autoimmunity has been shown to be highly significant. According to Sakaguchi, any genetic abnormality or environmental insult can cause or predispose to autoimmune disease if it would tip the balance between Tregs and self reactive T cells towards the dominance of the latter [36]. Sakaguchi was the first to define the importance of Tregs in autoimmunity by demonstrating that mice depleted of Tregs developed histologically and serologically evident autoimmune disease [1].

Autoimmune diseases are heterogeneous, and hence it is possible that multiple areas of tolerance breakdown can exist, with different initiating triggers, genetic susceptibility loci and activity modifiers contributing to clinically recognised disease in different groups of patients [26]. One of the earliest classifications of autoimmune disease was to divide them into systemic versus organ-specific. The prototypical systemic disease is systemic lupus erythematosus (SLE). This disease is generalised and may involve multiple tissues including skin, muscle, kidney and virtually every other organ [26]. It is characterised by the presence of antibodies that can bind to double stranded DNA and other nuclear targets [37]. Organ-specific autoimmune disease is characterised by auto-reactivity to self-Ags that are specific to an individual tissue. They are usually mediated by autoreactive T cells which are initially activated in lymphoid tissue by an APC and then migrate to peripheral non-lymphoid tissue to cause damage. Type 1 diabetes is an example of an organ specific disease. It is caused by an autoimmune attack on self-Ags expressed by the pancreatic islet beta cells that make insulin, leading to cell loss and a deficit in insulin production [26].

1.4.1 Epidemiology of Autoimmune Disease
Autoimmune diseases are individually rare but together affect over 5% of the population in Western countries [33]. The incidence and prevalence of autoimmune disease is often dependent on genetic and environmental factors that also influence clinical manifestations and disease progression. Factors that initiate disease may not be identical to factors that influence severity or progression of the disease [38].

The incidence of human autoimmune diseases varies over a wide range: from less than one newly diagnosed systemic sclerosis to more than 20 cases of adult onset rheumatoid arthritis per 100,000 per year [4]. Autoimmune disorders can cause significant chronic morbidity and disability [39] and have become one of the leading causes of death among young and middle-aged women in the US [4]. There is strong epidemiologic evidence of a rise in the incidence of autoimmune diseases in developed countries over past three decades [40]. The diseases that have shown the most profound increases are multiple sclerosis (MS), type I diabetes (T1D) and Crohn’s disease [40].

Most autoimmune diseases more frequently affect women than men, with at least 65% of patients being female [4]. On the basis of population-based estimates of autoimmune disease incidence, Jacobson et al calculated that 2.7 times more women than men will develop an autoimmune disease during their lifetime [39]. Furthermore, there are notable differences in the age distribution among autoimmune diseases. Although disease can occur at almost any age, most autoimmune diseases have a characteristic age of onset. Juvenile rheumatoid arthritis and T1D have a mean age onset of 8-10 years [4]. MS and Graves’ disease generally occur in patients between ages 30 and 50 years whereas Hashimoto’s thyroiditis, and rheumatoid arthritis are diagnosed in patients with an older age group of 40-70 years [4].

The lifetime risk of specific autoimmune diseases varies considerably in different countries, with the highest prevalence of autoimmune disease evident in more developed countries [40]. One of the most profound geographical influences on prevalence is seen in MS with a
gradient of increasing prevalence with increasing latitude. This gradient is however currently seen only in Europe, the USA and Australasia [41].

1.4.2 Genetic Susceptibility

Epidemiological studies have demonstrated that genetic factors are important in determining susceptibility to autoimmune disease. The search for these genetic familial links was initiated by the observation of the evident clustering of autoimmune disease within families and the higher rate of concordance for autoimmune disease in monozygotic twins when compared to dizygotic twins [33].

Only a tiny fraction of autoimmune diseases arise from a mutation or defect in a single gene. As mentioned above, the rare APECED syndrome is caused by a mutation in the AIRE gene, which is essential for thymic negative selection [6]. More commonly, minor genetic defects in multiple genes responsible for central tolerance pathway may predispose an individual to autoimmunity rather than directly cause it.

The defective development or organization of thymic medullary epithelial cells can predispose to disease. This is evident in gene-targeted mice in which autoimmunity correlates with perturbed integrity of the medullary microenvironment [11]. In a study conducted by Boehm and colleagues, it was shown that a reduced number of mTECs and disrupted medullary architecture can cause inefficient central tolerance induction [42].

Central tolerance can also be disrupted due to mutations that alter the medullary expression of tissue-specific Ags and hence predispose to autoimmunity. Variations in the expression of insulin, ocular Ags or myelin can correlate with susceptibility to diabetes, experimental autoimmune uveitis or experimental autoimmune encephalomyelitis, respectively, in mouse models (reviewed in [11]).
Mutations in genes that down-regulate or maintain immune responses in the periphery may also contribute to autoimmunity. Most autoimmune diseases are multigenic, with multiple susceptibility genes working together to cause the abnormality. The susceptibility polymorphisms occur in normal, healthy people where they are compatible with normal immune function [33]. However, it is the individual combination of these susceptibility genes that is believed to predispose an individual to developing an autoimmune disease. MHC molecules make by far the biggest contribution to disease susceptibility, with the majority of autoimmune diseases being linked to a particular class I or class II MHC gene [43]. However it is believed that in some cases this MHC association to autoimmune disease may require a link to a mutation in another gene within the MHC gene locus, such as tumour necrosis factor (TNF)-α or complement (reviewed in [33]).

Mutations that can cause autoimmunity include those in genes encoding cytokines, co-receptors, co-stimulatory molecules, and signalling pathways that induce apoptosis. This has been demonstrated in mouse models where at least 25 genes were found to contribute to autoimmune disease when they are deleted or over expressed (reviewed in [33]). In humans, allelic variations of the gene encoding CTLA-4 causes a decrease in the inhibition of T cells and are associated with Graves’ disease [33]. However, whether a mutation in a gene causes disease depends on the host’s genetic background, which may either promote disease or be protective. Often complex genetic loci rather than a single gene have been linked to disease predisposition, and many loci are becoming an important focus of research in many autoimmune diseases.

1.4.3 Environmental Triggers

Autoimmunity is generally assumed to involve complex interactions between genetic traits and environmental factors [32]. That is to say, even in a genetically predisposed person, an environmental trigger is believed to be required to cause disease [33]. The importance of environmental triggers in promoting disease is supported by evidence that shows altered
disease development in similar populations living in different conditions. This is evident in MS where the number of MS cases in a genetically similar population changes as they migrate to other regions [44]. These observations, along with the lower than expected rate of disease concordance in identical twins (almost always less than 50%), suggest that an environmental factor promotes disease development [33]. For most autoimmune diseases, however, this trigger is not known.

It is generally believed that an infection can trigger autoimmune disease via two mechanisms. A popular theory is that infectious agents trigger autoimmunity in an Ag-specific way due to molecular mimicry. This is when antigenic epitopes of microbes have a similar structure to antigenic epitopes of the host proteins and thus the host immune system inadvertently responds to their own host Ags as well as those of the microbe [32, 33, 40]. Zhao and others described an example of molecular mimicry in mice following infection with herpes simplex virus Type 2. They showed that T cells that react to viral protein UL6 can also cross react with a peptide derived from corneal Ag [45]. Another example of molecular mimicry comes from an experimental model of MS in which hepatitis B virus was shown to cause signs of encephalomyelitis in rabbits (reviewed in [32]). However, in the case of autoimmune disease in humans, there is still no convincing evidence that these pathogenic cross-reactivities identified in animal studies are of significance to the human pathology. In a very few number of human autoimmune diseases, such as Guillain-Barre syndrome [46] and reactive arthritis [47], links to an infection have been established.

Another mechanism whereby microbes can induce autoimmunity is termed ‘bystander activation’. This Ag non-specific mechanism involves the release of previously sequestered self-Ags or stimulation of the innate response by microbial infection, resulting in activation of APCs that present self-Ag [32]. Activation of APCs in turn causes them to express costimulatory molecules and migrate to secondary lymphoid organs where they can activate naïve self-specific T cells. In a study by Miller and others, it was demonstrated that infection of the central nervous system by Theiler’s virus results in autoimmunity to myelin Ags in the
mouse [48]. The study concluded that the autoimmune attack resulted from the release and processing of myelin Ag by CNS APCs that had been activated by the viral infection. The bystander activation theory argues that infection stimulates the innate response to induce activation of APC, hence providing naïve self-reactive T cells with the second signal required for their activation.

Some of the mechanisms by which microbes can initiate autoimmune disease in animal models are also evident in humans. The challenge therefore is to ascertain why all infections do not induce autoimmunity. The general lack of autoimmune disease following infection demonstrates the importance of the fine-tuned regulatory mechanisms in the immune system, which are presumably the result of evolutionary pressure to protect the host from infection yet prevent extensive tissue damage.

**1.4.4 The Hygiene Hypothesis**

The incidence of autoimmune disease is on the rise in developed countries. Parallel with this is a decrease in the incidence of many infectious diseases due to improved hygiene and better socio-economic conditions [40]. Intestinal infections are the most notable of these, with their prevalence in developed countries significantly lower than that of less developed countries, particularly among children (reviewed in [40]). It is believed by many that the main factor contributing to increased levels of autoimmune disease in developed countries is the reduction in the incidence of infectious diseases and exposure to microbial agents due to higher levels of hygiene. As early as 1966, Leibowitz et al suggested that the risk of MS is increased amongst people who spent their childhood in a highly sanitized home [49].

There is also experimental evidence suggesting that exposure to infection is associated with a decreased incidence of autoimmune disease. It has been consistently observed that autoimmune diseases in a susceptible strain of mice develop earlier and are more severe in mice bred in a pathogen free environment [40]. Furthermore, Martins et al. demonstrated that
diabetes in non-obese diabetic (NOD) mice can be prevented by infecting the young mice with mycobacteria [50]. How infection protects against autoimmune disease is unknown. It was be suggested that infection stimulates the production of Tregs whose effects extend beyond the invading microbe.

1.5 Regulatory T Cells

With multiple mechanisms acting at different levels and in complementary fashions to maintain self-tolerance, it is important to determine at what level defects lead to autoimmune disease. Among the mechanisms of immunologic self-tolerance, there is increasing emphasis on the role of regulatory T cells (Tregs) as a key mechanism maintaining self-tolerance and immune regulation. The existence of a subpopulation of T cells that specialise in the suppression of immune responses was first proposed in the early 1970s [51]. The widespread acceptance of Tregs as a significant subpopulation came with the identification of CD25 as a reliable marker of CD4+ T cells with suppressive activity in vivo [1].

Tregs are now widely regarded as the most important primary mediators of peripheral tolerance. There exists a spectrum of T cell populations to which regulatory functions have been attributed [52]. Several subtypes of Tregs have been defined, each with a distinct phenotype, cytokine production profile and mechanism of action. These Tregs are found within both the CD8+ and CD4+ T cell compartments. Within CD4+ T cells, three subsets of Tregs have been defined. The T1 cells emerge during induction of anergy or after repetitive stimulation, and are characterised by their ability to secrete high amounts of IL-10 [53]. T1 cells have been shown to suppress the immune response of other T cells both in vivo and in vitro (reviewed in [52]). Another CD4+ Treg subset is termed Tn3. Tn3 cells are induced by oral Ag administration, produce high concentrations of transforming growth factor (TGF)-β and can inhibit development of immune pathology in mouse models [52].
'Natural’ or “thymic” Tregs develop in the thymus after recognition of self-Ag. These Tregs are distinct from conventional T cells in that they do not require TCR-mediated signals to constitutively express CD25, the α chain of the IL-2 receptor [54].

The natural Tregs that develop in the thymus are generated in a burst of activity during the early stages of foetal and neonatal T cell development [55]. Jordan et al. first demonstrated that expression of a self peptide in thymic stromal cells caused CD4+ T cells expressing TCRs specific for this peptide to differentiate into CD4+CD25+ cells [56]. Accumulating evidence indicates that thymic development of Tregs requires unique interactions between TCRs and self-pMHC complexes on thymic cells. These interactions must not be of such high affinity that they lead to deletion of the T cells, yet relatively high enough to promote Treg development. However, because the depletion of autoreactive T cells also involves high affinity interactions with self pMHC, other mechanisms, independent of TCR avidity, are suggested to be involved in Treg development [57]. These mechanisms may involve Ag-independent interactions via accessory molecules such as CD28 or programmed death (PD)-1, as well as cytokines including IL-2 and TNF-α [57]. The importance of the thymus in the development of Tregs has been demonstrated in mouse models in which athymic mice were shown to have impaired generation of Tregs leading to multi-organ autoimmunity [58].

1.5.1 Tregs in Autoimmune Disease

Early studies in a number of experimental animal models of autoimmune and immunoinflammatory disease provided substantial evidence that Tregs are an essential part of the T cell repertoire and are required to prevent autoimmunity in normal individuals. In 1969 Nishizuka and Sakakura showed that neonatal thymectomy of mice led to autoimmune destruction of the ovaries [59]. Later, in the 1980s, it was shown that the inoculation of NOD mice with CD4+ T cells from healthy animals prevented the spontaneous development of diabetes [36]. In 1995 Sakaguchi et al. demonstrated that the transfer of CD4+ T cell suspensions depleted of CD25-expressing cells into immunodeficient mice caused the
development of a number of organ-specific autoimmune diseases such as thyroiditis, gastritis and glomerulonephritis. Sakaguchi et al also showed that the co-transfer of CD4+CD25+ cells in this model prevented autoimmune disease in a dose-dependent fashion [1]. These results suggested that although self-reactive T cells exist in the periphery, their activation and expansion was regulated by CD4+CD25+ Tregs.

Previously, the lack of a marker that could be used to identify Tregs in humans made it difficult to elucidate the role of Tregs in autoimmune disease. In mice, Tregs make up a phenotypically distinct CD25+ population, whereas in humans ~20-40% of peripheral blood CD4+ T cells express CD25 to some extent [54]. When human CD4+ cells are subfractionated according to CD25 expression, only the top 1-2% that express the highest levels of CD25 were shown to have suppressive activity [60]. It was later shown that the lack of suppression by CD25+ cells as a whole resulted from interference in the assay by conventional (non Treg) CD25+ cells, rather than from the failure of Treg cells expressing lower levels of CD25 to suppress.

Since the report of FoxP3 being expressed exclusively by Tregs and the demonstration that gene transfer of FoxP3 confers a regulatory phenotype on murine naive CD4+CD25- T cells [2], FoxP3 was accepted as the first truly specific marker for CD4+CD25+ Treg cells in mice and humans. However, FoxP3 is an intracellular molecule and hence its detection requires fixation and permeabilisation of the cells, making it impossible to isolate viable Treg populations for functional studies [61]. This, along with the difficulty in reproducibly defining a CD25 ‘high’ subset on the basis of flow cytometric analysis, prompted the search for another marker that could be used to reliably identify the entire population of human Tregs.

The identification of one such marker was made in our lab, where it was demonstrated that low surface expression of CD127 (the α chain the IL-7 receptor) in combination with CD25 can distinguish CD127loCD25+ Tregs from conventional T cells [61]. This study confirmed
that the surface CD127<sup>lo</sup>CD25<sup>+</sup> phenotype was highly correlated with intracellular FoxP3 expression and hence provided a viable alternative for identifying human Tregs by surface markers alone.

Evidence that Tregs are crucial in maintaining immune tolerance in humans is the syndrome of multi-organ autoimmunity, dermatitis and allergy that results from a dysfunctional FoxP3 gene (immunodysregulation, polyendocrinopathy, enteropathy X-linked (IPEX) syndrome) [62]. However, most human autoimmune and immuno-inflammatory diseases are not caused by such an absolute dysfunction of Tregs, and it is thought that minor deficiencies in Treg frequency, function and development may predispose individuals for disease.

CD25<sup>hi</sup> ‘Tregs’ isolated from patients with autoimmune diseases including MS, myasthenia gravis and rheumatoid arthritis have been shown to have reduced suppressive capacity when compared to ‘Tregs’ from healthy controls (reviewed in [57]). However, these in vitro assays could be influenced by contamination with activated non-regulatory T-cells, as has recently been shown in a study of MS patients [63]. In vitro assays are also influenced by the susceptibility of the responder CD4<sup>+</sup>CD25<sup>-</sup> cells to suppression, and there is evidence that responder cells from autoimmune disease patients may be resistant to Treg-mediated suppression. For example, T cells from the synovial fluid of rheumatoid arthritis patients have been shown to be more difficult to suppress than T cells from peripheral blood (reviewed in [57]). This may be due to factors such as cytokines at the inflamed site, which may enhance the resistance of CD4<sup>+</sup>CD25<sup>-</sup> T cells to suppression.

The reported levels of circulating CD4<sup>+</sup>CD25<sup>hi</sup> T cells (the previous working definition of human Tregs) in healthy humans ranges from 0.6 to 7.9%. This indicates a lack of reproducible Treg phenotypic definitions between laboratories. Reduced levels of circulating CD4<sup>+</sup>CD25<sup>hi</sup> T cells have been described in individuals with juvenile arthritis [64], psoriatic arthritis [65], systemic lupus erythematosus [66] and Kawasaki disease [67]. In contrast to these findings, studies on other types of autoimmune disease such as spondyloarthritis and
multiple sclerosis have detected no significant difference in the number of CD4\(^+\)CD25\(^{hi}\) between healthy and diseased patients [65, 68]. Therefore it can be seen that a reduction in Treg numbers is not a general finding in patients with autoimmune disease.

Furthermore, studies in patients with juvenile idiopathic arthritis, rheumatic disease, inflammatory bowel disease (IBD), and rheumatoid arthritis have demonstrated increased numbers of CD4\(^+\)CD25\(^{hi}\) T cells within inflamed tissues in comparison to peripheral blood [64, 65, 69, 70]. Tregs at sites of inflammation have generally been shown to have an activated phenotype, with high levels of CTLA-4 and increased suppressive potency [57].

1.5.2 Mechanisms of Treg mediated suppression

Although it is clear that Tregs prevent autoimmune disease, the mechanisms by which they do this \textit{in vivo} are still unclear. One of the difficulties in elucidating Treg mechanism of action is the current opinion that Treg cells depend on cell-to-cell contact in order to mediate their function. Experiments done \textit{in vitro} support this claim; Treg cells have not been shown to suppress effector T cell proliferation when the two populations were separated by a permeable membrane [71]. There is however no evidence that contact between Treg cells and effector cells is required \textit{in vivo}. In fact, experiments using intravital microscopy to study the interactions of T cells, Tregs and antigen-bearing DCs in lymph nodes have demonstrated that there is no direct contact between Tregs and T cells \textit{in vivo} [72, 73].

Although Treg-mediated suppression is consistently cytokine independent \textit{in vitro}, there is evidence that Tregs utilise immunosuppressive cytokines \textit{in vivo}. Inhibitory cytokines such as IL-10 and TGF-β have been the focus of attention as mediators of Treg cell induced suppression. In an animal model of inflammatory bowel disease (IBD), protection from colitis is dependent on production of TGF- β and IL-10 by a population of T cells enriched for Tregs [74]. Interestingly, IL-10 knockout mice do not develop spontaneous autoimmune disease but do show enhanced pathology in the colon, suggesting that IL-10 is important in
preventing or reducing inflammation at that site [75]. Mice deficient in TGF-β have a severe multi-organ autoimmune disease, similar to that seen in FoxP3-deficient mice [76]. These TGF-β-deficient mice have significantly reduced numbers of Tregs and reduced expression of FoxP3 on their peripheral Tregs.

Tregs are also believed to be able to inhibit T cell proliferation in vivo. In Treg-sufficient non-obese diabetic (NOD) mice, fewer CFSE-labelled T cells proliferated; with 20-40% entering division compared 60-80% in Treg-deficient hosts [72]. In this study, Tregs were also shown to reduce the rate of T cell proliferation, thus preventing both activation and reducing the initial T cell proliferative burst. Similarly, the rapid proliferative response that takes place in RAG\(^{-}\) mice following T cell adoptive transfer has been shown to be inhibited by Tregs [77].

Tregs are able to mediate inhibition of T cell responses indirectly via dendritic cells. This provides insight into how a comparatively small Treg population can mediate control over a much larger conventional T cell population. In studies looking at the in vivo interactions of T cells, Tregs and antigen-bearing DCs in lymph nodes using microscopy, Tregs were shown to prevent the stable contact between T cells and DCs, therefore impairing T cell activation [72, 73]. Neither of these studies demonstrated direct contact between Tregs and T cells, with only Treg-DC and T cell-DC contact observed.

Several in vitro studies have shown that the co-culture of DCs with CD4\(^{+}\)CD25\(^{+}\) Tregs can downregulate the amount of costimulatory molecules (CD80 and CD86) expressed by the DCs [78]. CTLA-4 is constitutively expressed on Treg cells and is upregulated by TCR ligation. In these in vitro assays, the addition of CTLA-4 blocking Abs has been shown to inhibit the Treg-mediated modulation of CD80/CD86 expression [79]. Furthermore, Tregs isolated from CTLA-4-deficient mice are unable to modulate the expression of CD80/CD86. Although these studies provide evidence for the in vitro influence of Tregs on the expression of CD80/CD86 by DCs, evidence for the in vivo relevance of these findings was only
recently established in our lab. In this study, Treg-reconstitution of RAG\textsuperscript{−−} mice led to a Treg-specific, CTLA-4 dependent downregulation of CD80/CD86 on host DCs [80].

It is clear that Tregs are functionally heterogeneous and the mechanisms by which they mediate suppression may be tissue or context dependent. Tregs may have multiple mechanisms to limit autoimmunity and these may vary among Treg subsets that localise to distinct tissue environments [81]. The importance of Treg trafficking and entry into various tissues is central to this study and will be reviewed in detail below.

1.6 Regulatory T Cell Trafficking

1.6.1 Introduction on T cell trafficking

T cells constantly recirculate between blood and tissue in order to carry out their immune function [82]. This trafficking of lymphocytes directs subsets to specialised microenvironments depending on their state of differentiation and activation. Naïve T cells leave the thymus and specifically ‘home’ to secondary lymphoid organs in order to encounter antigen presented by dendritic cells. Activated effector T cells express a range of molecules that direct them to non-lymphoid tissues and sites of inflammation. Specialised endothelial surfaces are required to control the migration of T cells from blood into tissues; these are either the high endothelial venules in secondary lymphoid tissue or the post-capillary venules in non-lymphoid tissue. A combination of molecules on the endothelium and the surface of T cells are involved in the interaction that results in the extravasation of T cells from the blood into the tissue.

In order to leave the circulation, T cells must engage several adhesion pathways [83]. The first step involves the formation of ‘tethers’ or weak bonds between T cells and the endothelium. These tethers are mediated by the selectin family and their ligands that engage and disengage rapidly, allowing the cells to slow down and ‘roll’ along the endothelial lining
in order to sample the site for factors that can trigger firm adhesion [84]. The activation of firm adhesion is mediated by chemokines on the endothelium binding to chemokine receptors on T cells [85]. This interaction results in the activation of integrins on the T cell surface which then form high affinity bonds to their ligands on the endothelium, resulting in T cell arrest and transmigration through the endothelium into the tissue [86]. Chemokines then work as chemotactic factors directing the migration of T cells within the tissue.

T cells, including Tregs, express various combinations of selectins, integrins and chemokine receptors required for entry into a range of peripheral tissues. This differential expression determines their migration to distinct tissues and sites of inflammation. The expression of these homing receptors by Tregs is discussed below.

1.6.2 Tregs in lymphoid and non-lymphoid tissues

Where Tregs travel *in vivo* and where they act has recently been intensely studied. Studies have shown that under steady state conditions there exists a distribution of FoxP3*CD25*CD4* T cells in secondary lymphoid tissues [87]. In a mouse model of colitis it was demonstrated that CD25* Tregs transferred into mice migrated into both the mesenteric lymph nodes (LN) and the colon, suggesting Tregs are active at both these sites [88]. Additionally, the presence of Tregs at sites of ongoing inflammation demonstrates their ability to migrate into peripheral tissues. Tregs have been shown to increase in inflamed peripheral tissues in both mouse and human immuno-inflammatory disease [89]. Furthermore, Tregs have been shown to express a vast array of integrins and chemokine receptors that would facilitate their entry into peripheral tissue. These include CCR4, CCR8, CCR6, CLA and CCR5 (summarised in Table 1.1) [90-92].

Treg homing to secondary lymphoid organs such as LNs is important in several *in vivo* models. In a mouse model of graft versus host disease (GvHD), it has been shown that Tregs expressing higher levels of the lymph node homing molecule CD62L were superior *in vivo*
suppressors compared with Tregs expressing low levels of CD62L [93]. This study also showed that the CD62Llo and the CD62Lhi Tregs were similar in their suppressive capacity \textit{in vitro}, indicating a crucial role for Treg localisation to LNs in the control of inflammation \textit{in vivo}. Tregs also express the LN homing chemokine receptor CCR7. It has been shown that Tregs lacking expression of CCR7 are unable to prevent colitis due to their inability to home to lymph nodes and suppress primary T cell activation [94].

The importance of Treg homing to non-lymphoid tissues has also been demonstrated in numerous studies. In a study by Sather \textit{et al.}, it was shown that Tregs were present in all peripheral tissues tested (skin, lung, liver, lamina propria) and were particularly prevalent in the skin [81]. Furthermore, they demonstrated that mice with a complete loss of the chemokine receptor CCR4 on their Tregs develop severe inflammatory disease in the skin and lungs. Tregs have also been shown to recirculate from peripheral tissue to draining lymph nodes in the absence of ongoing inflammation. Using transgenic mice that express Kaede protein, which allows cell labelling in response to UV light exposure, Tomura \textit{et al.}, were able to show that Tregs recirculate from the skin to draining lymph nodes in the steady state [95]. They also showed that in order to downregulate cutaneous immune responses, Tregs within the circulation infiltrate the skin, migrate to draining lymph nodes and then recirculate back to the skin. This further indicates that Treg migration into peripheral sites is crucial for Treg-mediated suppression.

\subsection*{1.6.3 Treg Trafficking phenotype switch: from naïve to effector Tregs}

Upon activation, T cells in humans switch from expressing CD45RA, a naïve T cell marker, to expressing CD45RO, characteristic of effector/memory T cells. Most Tregs in the peripheral blood of adult humans express CD45RO, suggesting they have been previously activated [54]. On the other hand, CD45RA (naïve) Tregs are more prevalent in early life, with a high proportion of cord blood Tregs expressing this phenotype [96]. Naïve and effector memory Tregs have been shown to display phenotypic differences. Importantly, it
has been demonstrated that CD45RA (naïve) and CD45RO (effector/memory) Tregs preferentially localise to different tissues [97]. This is due to a switch in the expression of tissue specific homing receptors and integrins from lymphoid to non-lymphoid homing.

Naïve Tregs are CD62L⁺CCR7⁺ and migrate to secondary lymphoid tissues where, following antigen priming in these tissues, they downregulate CD62L and CCR7 and upregulate homing receptors including CCR2, CCR4, CCR6 and CCR8 that allow entry into peripheral tissue [92]. It has been shown that FoxP3⁺ Tregs undergo this activation-dependent switch in homing receptors at a faster rate than FoxP3⁻ T cells [92]. Expression of the integrin αₑ in mice has been shown to subdivide the CD25⁺ Treg compartment into two subpopulations that differ in their in vivo suppressive role and migratory pattern [98]. αₑ⁺ CD25⁺ Tregs were shown to express CD62L and CCR7 and recirculate through secondary lymphoid tissues. These cells lacked suppressive capacity under acute inflammatory conditions but were potent at suppressing naïve T cell expansion in lymph nodes. The αₑ⁺ CD25⁺ Tregs on the other hand migrated to inflamed tissues and had high suppressive potential in these tissues.

In humans, naïve FoxP3⁺ cells have also been shown to express lymphoid tissue homing receptors such as CCR7 and CD62L whereas effector/memory FoxP3⁺ cells express inflammatory chemokine receptors such as CCR5, CCR6 and CCR4 [99].

1.6.4 Tissue specific homing of conventional T cells and Tregs: Gut versus Skin

The migration of conventional T cells to different peripheral tissues results, at least in part, from expression of particular combinations of integrins and chemokine receptors. Of these tissue homing phenotypes, the gut specific α₄β₇⁺ [100, 101] and CCR9⁺ [102] and the skin specific CLA⁺, CCR4⁺ and CCR10⁺ [103, 104] are the best defined. Such tissue-specific trafficking plays an important role in the inflammatory response within that tissue. In a mouse model of inflammatory skin disease, only CCR4 expressing donor T cells
accumulated in the skin whereas CCR4 knock out (KO) T cells failed to cause inflammation [105].

Imprinting of tissue specificity on conventional CD4+ T cells is influenced by the tissue microenvironment and the presence of organ specific dendritic cells (DCs) during priming in secondary lymphoid tissue. Activated effector/memory T cells have often been shown to migrate preferentially to tissues within the lymphoid drainage site of their original primary lymphoid organ. For example, T cells activated by dendritic cells from mesenteric lymph nodes and Peyer’s Patches are induced to express high levels of α4β7 and CCR9 which then aid their future entry into gut tissue [106, 107].

Recently, two studies have demonstrated mechanisms by which skin and gut resident dendritic cells can induce expression of tissue specific integrins and chemokine receptors by conventional T cells. Iwata et al. reported that DCs from the gut associated lymphoid organs could produce retinoic acid from vitamin A (retinol), which in turn induced T cell expression of α4β7 and CCR9 [107]. Similarly, Sigmundsdottir et al. showed that the active form of vitamin D3 induced the expression of CCR10 on T cells which then enabled them to migrate in response to the skin specific chemokine CCL27 [103]. In this study, it was also demonstrated that the upregulation of the skin homing phenotype was accompanied by a downregulation of gut homing receptors.

Mechanisms responsible for a degree of tissue specificity in Ag-primed conventional T cells have also been shown to function during Treg differentiation. The priming of Tregs within skin draining LNs leads to the expression of P and E selectin ligands, which aid their migration into inflamed skin, whereas priming in mesenteric LNs results in the upregulation of integrin α4β7 and entry into the gut [108]. Furthermore, Sather et al. demonstrated that Treg expression of the skin homing receptor CCR4 is induced by activation in subcutaneous LNs [81].
Many studies of trafficking phenotypes of Tregs in adult mice and humans have demonstrated a skewed skin versus gut phenotype, with the majority of Tregs shown to express higher levels of skin homing CLA and CCR4 and less gut homing $\alpha_4\beta_7$ [81, 91, 109-111]. Interestingly, the reverse is true in early life, when the majority of Tregs express $\alpha_4\beta_7$ [110]. This indicates a possible role for the gut in Treg cell exposure to exogenous Ags in early life and could possibly shape the Treg compartment and influence homing receptor expression patterns and phenotype in later life.

1.6.5 Treg regulation of different immune responses

During their initial activation, conventional CD4$^+$ T cells can assume one of several functional fates (Th1, Th2 or Th17). These fates are largely determined by the cytokines that are present during activation and the transcription factors that are induced. Each of these defined immune responses can, if they are deregulated, cause harm to host tissue. In fact, known autoimmune, allergic and other immuno-inflammatory conditions are believed to be the result of uncontrolled Th1, Th2 and Th17 immune responses to harmless antigen. Defects in Treg function have been shown to result in Th1-, Th2- or Th17-mediated inflammatory disease and recently there has been increasing interest in mechanisms by which Tregs can regulate these specific types of inflammation.

Several studies suggesting that different Treg subsets are specialized in regulating polarized Th1, Th2 or Th17 immune responses have recently been published. The functional specialisation of Th1, Th2 and Th17 effector CD4$^+$ T cells is mainly dependent on differential expression of transcription factors that turn on programs of gene expression determining function and, importantly for this review, migration of the CD4$^+$ T cell (reviewed in [89]). It has recently been demonstrated that subsets of Tregs also express these transcription factors, which aid them in their suppressive function. A study by Koch et al. demonstrated that in response to IFN-$\gamma$, FoxP3$^+$ Tregs upregulated the Th1 associated transcription factor T-bet which subsequently induced the expression of CXCR3, a
chemokine receptor commonly associated with Th1 responses [112]. This upregulation of CXCR3 resulted in CXCR3+ T-bet+ Tregs accumulating at sites of Th1 mediated inflammation. Furthermore, it was shown that T-bet deficient Tregs failed to localise to inflamed tissue and could not control Th1 type inflammation [112].

It has also been shown that Treg expression of IRF4, a transcription factor involved in Th2 differentiation, is required for control of Th2 type inflammation. Mice with an IRF4 deletion in their FoxP3+ Tregs develop lymphoproliferative disease characterized by IL-4 producing CD4 T cells [113]. Additionally, Tregs with the IRF4 deletion displayed reduced levels of CCR8, a chemokine receptor that has been implicated in T cell homing to sites of Th2 type inflammation [114-116].

It has been shown that CCR6 mediates entry into sites of Th17 inflammation [117] and is required for Treg suppression of Th17 responses. The deletion of a transcription factor associated with Th17 inflammation, STAT3, in Tregs resulted in the development of intestinal inflammation characterised by IL-17 production and deregulated Th17 responses [118]. In this study, CCR6 expression on STAT3 KO Tregs was impaired. Furthermore, in a study of experimental autoimmune encephalomyelitis (EAE), a Th17 mediated disease model, a lack of CCR6 expression on Tregs resulted in a failure of Treg entry into the inflamed tissue and hence failure of disease control [119]. These studies all provide evidence for the importance of the co-localisation of Tregs with effector T cells at sites of inflammation.
1.7 Project Aims

The appropriate localisation of Tregs is crucial to their function. Despite this, very few studies have examined Treg trafficking phenotypes in patients with autoimmune disease. We hypothesise that deficiencies in Treg subsets expressing particular trafficking receptors may play a role in human autoimmunity.

Since Tregs have been shown to recirculate via the blood and are generally increased at sites of inflammation, analysis of their expression of chemokine receptors and integrins in peripheral blood should provide insights into their regulatory role in different tissues. The majority of studies that have examined Treg trafficking in humans defined Tregs as CD4⁺CD25hi cells. This definition makes it difficult to distinguish Tregs from conventional CD25⁺ T cells and has been shown to exclude a large percentage of the total Treg compartment.

We aimed to use the CD127loCD25⁺ phenotype, in association with a range of integrins and chemokine receptors, to define Treg subsets in healthy humans and in patients with rheumatoid arthritis and psoriasis, and to assess how these subsets may be implicated in disease severity and response to therapy in these diseases.

It is believed that Tregs can be subdivided into populations that express migratory receptors that target them to certain tissues, such as skin and gut, and specific sites, such as Th1, Th2 and Th17 mediated inflammation. Whether these phenotypes are specific and mutually exclusive has not been well defined. We aimed to take a novel approach by designing antibody panels that will allow for the analysis of up to 8 different chemokine receptors and integrins on Tregs, therefore allowing us to examine gut versus skin trafficking phenotypes and transcription factors and their associated homing phenotypes together in the same cell sample.
Flow cytometry involves the rapid quantification of multi-dimensional characteristics for millions of cells. The manual analysis of cell subset data derived from such high-throughput multidimensional experiments is extremely time consuming, particularly when determining correlations between multiple different cell subsets in multiple subject groups. For this reason, there is a need for more efficient data viewing and analysis methods. The final aim of this study was to develop bioinformatic methods to analyse multiparameter flow cytometric data and to perform automated comparisons between multiple conventional CD4$^+$ T cell and Treg subsets in patients and controls.

Future therapeutic strategies based on Tregs will need to take into consideration that appropriate trafficking properties are crucial to their success. A comprehensive study of the trafficking phenotypes of Tregs in humans has not been carried out. Here we hope to provide insight on the role of Tregs in peripheral tissue immune surveillance so that strategies can be developed to support Treg activity as a treatment option in autoimmune disease.
Table 1.1: Summary of chemokine receptors and integrins and their homing association.

<table>
<thead>
<tr>
<th>Receptor/Integrin</th>
<th>Ligand</th>
<th>Association</th>
<th>Key reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCR2</td>
<td>CCL2</td>
<td>Migration to inflamed tissue</td>
<td>• Upregulated on Tregs upon activation, allows entry into peripheral tissue [92]</td>
</tr>
<tr>
<td></td>
<td>CCL7</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CCL18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CXCR3</td>
<td>CXCL9</td>
<td>Migration to Th1-mediated inflammation</td>
<td>• Expression dependent on T-bet and allows entry to sites of Th1 inflammation [112]</td>
</tr>
<tr>
<td></td>
<td>CXCL10</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CXCL11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CX3CR1</td>
<td>CX3CL1</td>
<td>Migration to inflamed gut</td>
<td>Increased expression of CX3CR1 ligand on gut mucosal epithelium and increased expression of CX3CR1 on T cells in patients with IBD [120].</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCR4</td>
<td>CCL17</td>
<td>Migration to skin and sites of Th2-mediated inflammation</td>
<td>• Expressed by high proportion of Tregs [91]. CCR4 expression increased on T cells taken from skin inflammatory site AND deletion of CCR4 on Tregs causes skin inflammatory disease [81]. Deletion of CCR4 on Tregs causes lung inflammatory disease [81]. Directs homing and entry into sites of Th2 inflammation [114].</td>
</tr>
<tr>
<td>CCR5</td>
<td>CCL5</td>
<td>Migration to inflamed tissue</td>
<td>• Important for Treg homing to sites of infection [121]</td>
</tr>
<tr>
<td></td>
<td>CCL3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CCL4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCR6</td>
<td>CCL20</td>
<td>Migration to sites of Th17-mediated inflammation</td>
<td>• Deletion of STAT3 in Tregs results in inflammation characterised by IL-17 production and deregulated Th17 responses [118]. Mediates entry into sites of Th17 inflammation and is required for Treg suppression of Th17 responses [117].</td>
</tr>
<tr>
<td>CCR7</td>
<td>CCL19</td>
<td>Migration to Lymph nodes</td>
<td>• Required for prevention of T cell activation in a mouse model of colitis [94].</td>
</tr>
<tr>
<td></td>
<td>CCL21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chemokine</td>
<td>Chemokine Ligand</td>
<td>Function</td>
<td></td>
</tr>
<tr>
<td>------------</td>
<td>-----------------</td>
<td>--------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>CCR8</td>
<td>CCL8</td>
<td>Expression by Th2 cells and Tregs. Key regulator of Th2 cell recruitment into sites of allergic inflammation.</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Migration to lungs and sites of Th2-mediated inflammation.</td>
<td></td>
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<tr>
<td>CCR9</td>
<td>CCL25</td>
<td>Mediates T cell entry into small intestine. Increased expression of CCR9 by T cells taken from gut mucosa.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Migration to small intestine.</td>
<td></td>
</tr>
<tr>
<td>CCR10</td>
<td>CCL27</td>
<td>Allows migration to skin specific chemokine CCL27.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CCL28</td>
<td>Migration to skin.</td>
<td></td>
</tr>
<tr>
<td>CD62L</td>
<td>L-selectin</td>
<td>Required for Treg migration to LN.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Migration to lymph nodes.</td>
<td></td>
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<tr>
<td>α4β7</td>
<td>MadCAM-1</td>
<td>DCs from Peyer’s Patches induce expression of α4β7 which aid entry into gut tissue.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Migration to gut.</td>
<td></td>
</tr>
<tr>
<td>CLA</td>
<td>E-selectin</td>
<td>Highly expressed by Tregs. Homes Tregs to skin.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Migration to skin.</td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER 2: MATERIALS & METHODS

In order to study the frequency of Tregs and Treg subsets in patients and healthy donors, peripheral blood was collected from individuals and peripheral blood mononuclear cells (PBMCs) were isolated using density gradient centrifugation and stored. PBMCs were then stained with combinations of monoclonal antibodies and data was acquired on a custom built LSRII 10-laser flow cytometer (BD) and analysed using FlowJo software (Tree Star). Descriptions of patient recruitment and materials and methods are outlined below.

2.1 Patient and Control Cohorts

Informed written consent was obtained from all patients and controls prior to the study. The studies were approved by the Sydney Local Health District ethics review committees at Royal Prince Alfred Hospital, Camperdown and Concord Repatriation General Hospital, Concord. All personal and medical information collected from the patients, including sex, date of birth, diagnosis, treatment, disease duration and past clinical details, was stored confidentially.

2.1.1 Rheumatoid Arthritis Patients

Peripheral blood specimens were collected from 34 patients with rheumatoid arthritis (RA) (65% female, average age 56.4 years) and 34 sex and age matched controls (65% female, average age 55.6 years). Patients with RA, diagnosed according to the European League Against Rheumatism (EULAR) 2010 criteria [125], were enrolled in the study over a period of one year by Dr Diana Chessman at Concord Repatriation General Hospital (Concord, NSW, Australia). Clinical information including disease duration, disease activity, as assessed by the disease activity score in 28 joints (DAS28), treatment, smoking status and seropositivity was recorded at the time the blood was collected. Patient characteristics are summarised in Table 4.1 in Chapter 4.
2.1.2 Psoriasis Patients

Peripheral blood samples were obtained from patients treated by Professor Wolfgang Weninger and Dr Patricia Lowe at the Dermatology Centre of Royal Prince Alfred Hospital in Gloucester House (Camperdown, NSW, Australia). Specimens were collected from 44 patients with chronic psoriasis (77% male, average age 47.6 years) and 44 sex and age matched controls (77% male, average age 49.1 years). Clinical information including disease duration, disease activity, as assessed by the psoriasis area severity index (PASI) and treatment was recorded at the time the blood was collected. Patient characteristics are summarised in Table 5.1 in Chapter 5. The 44 control subjects were also analysed as a separate cohort in Chapter 3.

2.2 Reagents

2.2.1 Phosphate Buffered Saline (PBS)

Milli-Q filtered H₂O containing 8g/L NaCl, 0.2g/L KCl, 1.15g/L Na₂HPO₄ and at pH 7.2 was prepared at the Centenary Institute, kept sterile and stored at room temperature.

2.2.2 Ficoll-paque Plus

Each 100ml of Ficoll-Paque plus (GE Healthcare, UK) contains 5.7g Ficoll 400, 9.0g Diatrizoate Sodium with Edetate Calcium Disodium in water. This reagent was stored at room temperature and protected from direct light.

2.2.3 Tissue Culture Medium
RPMI Medium 1640 with L-glutamine and HEPES buffer (Invitrogen) supplemented with 10% heat activated foetal calf serum (FCS) (Trace MultiSer Biosciences, Australia) and 2mM L-Glutamine. 25mM 2-Mercapto-ethanol, and 1000 units/L of Penicillin (Invitrogen) or 1000 µg/L Streptomycin (Invitrogen) was added to prevent organism contamination. TCM was kept sterile and stored at 4°C.

2.2.4 Freezing Medium

2 x Freezing Medium consisted of RPMI 1640 and 20% Dimethyl sulfoxide (Sigma) filtered through a 0.22um sterile filter (Millipore) and then supplemented with 40% FCS. Reagent was kept sterile and stored at 4°C.

2.2.5 Fluorescence Activating Cell Sort (FACS) Wash

This reagent was used in wash and resuspension steps during antibody labelling for flow cytometry. FACS wash (F/W) contained 5% Foetal Calf Serum (FCS) and 0.05M sodium azide in PBS.

2.2.6 Fluorescence Activating Cell Sort (FACS) Fix

All samples analysed by flow cytometry were fixed with FACS Fix after staining. This reagent consists of 1% paraformaldehyde (BDH Laboratory Supplies) in PBS.

2.2.7 BD Pharmingen Transcription Factor Buffer Set

This set contains concentrated stock solutions of Fix/Perm buffer, Diluent buffer and Perm/Wash buffer (BD Pharmingen, 562574). Working solutions of the fix/perm buffer were made by mixing 1 part Fix/Perm concentrate with 3 parts Diluent buffer. Working solutions
of the perm/wash buffer were made by mixing 1 part Perm/Wash buffer concentrate with 4 parts triple distilled water. Working solutions were prepared fresh and stored at 4°C.

2.3 Isolation of mononuclear cells from peripheral blood

This protocol is for a single venous blood sample collected in a 9 ml Lithium Heparin tube (Greiner bio-one). All blood samples were handled in a Class 2 biosafety hood and under sterile conditions.

An automated cell count was first performed using a Sysmex KX-21 Haematology Analyser, to determine the concentration of leucocytes and lymphocytes in the venous blood. The blood was then diluted 1:2 with PBS in sterile 50ml tubes (BD Falcon). 12mls of Ficoll-Paque Plus solution (GE Healthcare) was next layered underneath the blood and the tube was centrifuged for 30 minutes at 600RCF at room temperature with the brake disengaged. The PBMCs were then collected from the interface between the Ficoll-Paque and the diluted plasma and transferred into sterile 15 ml tubes (BD Falcon) and washed twice with PBS (10 minutes, 230RCF, 22°C). Cells were resuspended in PBS, an aliquot taken for counting, and then centrifuged and resuspended in equal parts cold TCM and freezing medium at a concentration of 2-4 x 10⁶ cells per ml. 1ml aliquots were frozen at -1°C/minute using a Cryo-1°C/minute Freezing Container (Nalgene) and stored at -180°C in LNO₂.

2.4 Flow Cytometric Analysis

2.4.1 Monoclonal antibodies

Isolated mononuclear cells were stained for expression of a range of surface and intracellular markers using combinations of monoclonal antibodies (mAbs). The mAbs used in this study are listed in Table 2.1.
<table>
<thead>
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<th>Clone</th>
<th>Source</th>
<th>Cat#</th>
</tr>
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<td>BD V500</td>
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**Abbreviations:** APC- Allophycocyanin; PE- Phycoerytherin; PE/Cy7-Phycoerythrin-Cyanine 7; AF488- Alexa Fluor 488; AF647- Alexa Fluor 647; AF594- Alexa Fluor 594; PerCp/Cy5.5- Peridinin chlorophyll protein/ cyanine 5.5; BV- Brilliant Violet; BUV- Brilliant Ultraviolet;
2.4.2 Immuno-staining for cell surface markers

Frozen mononuclear cells were thawed in a 37°C water bath and transferred to tubes containing 9mls of pre-warmed TCM (37°C). The tubes were inverted to mix and then centrifuged (1000rpm, 10 mins, 22°C), resuspended in 2mls of cold FACS wash (FW), and an aliquot removed for counting. 1x10^6 viable cells from each sample were next transferred to a 96-well U-bottomed plate and washed twice with 150ul of FW (1500rpm, 3 mins, 4°C).

Following these wash steps, samples were incubated with a pre-diluted antibody and viability dye mix (containing antibodies and viability dye at optimal concentrations in a final volume of 100ul) for 45 mins at 4°C. If a biotinylated mAb was used and an avidin-fluorochrome conjugate was required, cells were washed three times with 150ul FW (1500rpm, 3 mins, 4°C) and the second layer of pre-diluted mix (50ul) was added for a further 45 mins at 4°C. Cells were then washed twice with 150ul FW (1500rpm, 3 mins 4°C), fixed in 150ul FACS fix for 15 mins at 4°C and washed again with 150ul FW (1500rpm, 3 mins, 4°C). Cells were then either stained for intracellular markers or filtered into 2mL FACS tubes immediately before acquisition on a flow cytometer.

2.4.3 Immuno-staining for intracellular markers

After the completion of cell surface staining, cells were transferred to FACS tubes and fixed in 1 ml Fix/Perm buffer (1x) (BD Pharmingen, 562574) (50min, 4°C, in the dark). 1 ml Perm/Wash buffer (1x) was next added and cells were centrifuged (350g, 6mins, 4°C), supernatant was removed and cells then washed again with 2 ml Perm/Wash buffer (1x) (350g, 6mins, 4°C). Cells were then resuspended in 100ul of pre-diluted antibody mix (contains antibodies at optimal concentrations in Perm/Wash buffer) and incubated for 50 minutes at 4°C in the dark. Cells were then washed twice in 2 ml Perm/Wash buffer (1x) (350g, 6mins, 4°C) and resuspended in 150ul FW for acquisition on a flow cytometer.
2.4.4 Compensation Controls

Compensation controls were prepared using antibody capture beads (CompBeads, BD). Beads were incubated with a single fluorochrome-conjugated antibody for 10 minutes prior to data acquisition on a flow cytometer. Unconjugated beads were used as negative controls.

2.4.5 Flow cytometric acquisition and analysis

Data were acquired on a LSRII 10-laser Flow Cytometer (Becton Dickinson, San Jose, CA, USA). Instrument performance was verified prior to acquisition using standard fluorescent microparticles (Spherotech) to check laser delays. Uncompensated data in FCS3 file format was collected using the FACSDiva digital software (Becton Dickinson). Data was then compensated manually using compensation controls to generate a compensation matrix in FlowJo software (Tree Star Inc., San Carlos, CA, USA). FlowJo software was subsequently used to analyse cell populations.

2.4.6 Gating strategy for the analysis of conventional T cells and Tregs

A general gating strategy was employed for the analysis of all PBMC flow cytometric data (Figure 2.1 A-E). A time gate was first used to exclude artefacts due to fluidics instability at the start and end of samples. A fixable dead cell exclusion dye was next used to remove dead cells from analysis. Doublets were next excluded by first gating on Forward Scatter-Area (FSC-A) versus Forward Scatter-Height (FSC-H) followed by a Side Scatter-Area (SSC-A) versus Side Scatter-Height (SSC-H) gate. CD4⁺ cells were then selected for analysis.

Tregs were identified within the CD4⁺ gate on the basis of expression of CD25, CD127 and FOXP3 (Figure 2.1 F&G). Tregs were defined as CD25⁺CD127lo and/or FOXP3⁺CD127lo cells. Conventional T cells (Tconvs) were defined as everything outside the indicated CD127loCD25⁺ Treg gate (Figure 2.1 G). CD45RO was used as a marker of post-thymic
Figure 2.1: General gating strategy for the analysis of Treg and Tconv subpopulations. Peripheral blood mononuclear cells (PBMC) were isolated from human blood and examined for the expression of markers that allowed for the identification of Tregs (CD4, CD25, CD127 and FoxP3) and then the further examination of subsets (CD45RO and CR/integrins). This is an example of a stain with CXCR3 as the trafficking receptor examined. A FSC-H vs time gate was used to exclude artefacts due to fluidics instability at the start and end of the samples (A). A fixable dead cell exclusion dye was next used to remove dead cells from analysis (B). Doublets were excluded using sequential FSC-A vs FSC-H (C) and SSC-A vs SSC-H (D) gates. CD4+ cells were then gated (E) and CD4+ Tregs were identified as CD127loCD25+ (G) or CD127loFOXP3+ (F). Conventional T cells (Tconvs) were defined as everything outside the indicated CD127loCD25+ Treg gate (G). Tregs and Tconvs were next subdivided on the basis of CD45RO expression in order to differentiate between CD45RO+ (effector/memory) and CD45RO− (naïve) subsets, and analysed for the various CRs and integrins examined. CXCR3 is used as an example here (H&I). CXCR3+CD45RO+ cells in the upper right gate and CXCR3−CD45RO− cells in the upper left gate were identified on both Tregs (H) and Tconvs (I). Numbers indicate cells within the gate as a percentage of the parent population.
activation and differentiation. Tregs and Tconvs were next subdivided on the basis of CD45RO expression in order to differentiate between CD45RO+ (effector/memory) and CD45RO- (naïve) subsets, and analysed for the various CRs and integrins examined. CXCR3 is used as an example in Figure 2.1 (H&I). CR+CD45RO+ cells in the upper right gate and CR+CD45RO- cells in the upper left gate were identified on both Tregs (Figure 2.1 H) and Tconvs (Figure 2.1 I).

2.5 Statistical Analysis

Statistical analysis was performed using Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA). The Mann-Whitney t-test was used to compare differences between two groups. The Kruskal–Wallis test followed by Dunn’s multiple comparison was used to analyse differences between three or more groups. The non-parametric Spearman method was used to calculate correlation coefficients. P-values <0.05 were considered significant.

Statistical analysis was also performed using MultiExperiment Viewer (MeV) (TM4–Microarray Software Suite). Data was loaded into MeV in .txt format and heatmaps were generated. Differences between groups were evaluated using an automated Mann-Whitney t-test. A Kruskal-Wallis test was used for identification of differences between three or more groups. P values less than 0.05 were considered significant. Unsupervised cluster analysis was performed using the hierarchical clustering (HCL) algorithm.

2.6 Flow Cytometric Panel Design for Accurate Measurement of T Cell Subsets

Polychromatic flow cytometry allows for the identification of specialised cell subsets within populations that were once considered to be homogeneous, allowing us to monitor changes in these subsets during autoimmune disease.
It is believed that Tregs can be subdivided into distinct populations that express migratory receptors that target them to certain tissues, such as skin and gut [81, 108], and specific sites, such as Th1, Th2 and Th17 mediated inflammation [112-116, 118, 119]. Despite this, no one has looked at these markers on conventional T cells (Tconvs) and Tregs in conjunction with each other using flow cytometry. This is probably due to the fact that developing a multi-colour flow cytometric panel is time consuming and requires a number of validation trials. We took a novel approach and designed two 10-13 colour antibody panels that will allow for the analysis of up to 8 different chemokine receptors (CR) and integrins on Tregs at a time, therefore allowing us to examine gut versus skin trafficking phenotypes and transcription factors and their associated homing phenotypes together in the one stain.

2.6.1 The LSRII-10-Laser Flow Cytometer

An LSRII-10-laser (BD) flow cytometer was used in this study. The standard bandpass and dichroic filter configuration of this instrument is summarised in Figure 2.2. An important step in developing a flow cytometric panel is to understand the optical configuration of the flow cytometer and to choose filters based on maximum emission spectra of the fluorochromes, while simultaneously avoiding unwanted spectral overlaps. This was particularly important for our study because we aimed to use a large number of fluorochromes to measure co-ordinately expressed cell markers. In order to provide ourselves with a guide in predicting optimal detection while minimising spillover, we calculated the %efficiency of fluorochromes on each of the filters available on the LSRII-10-laser flow cytometer (Figure 2.3).

However, despite the fact that we can predict relative dye brightness and spectral overlap, these factors are also influenced by the level of expression of the markers of interest, thus making it crucial to test antibody-fluorochrome combinations and perform trials on panels with the relevant controls.
Figure 2.2: Standard optical configuration of the 10-Laser BD FACS LSRII. The instrument is equipped with 10 lasers: UV (355nm), Infrared (786nm), Violet (405nm), Blue-Violet (445nm), Blue (488nm), Green (514nm), Green_2 (532nm), Yellow-Green (561nm), Orange (592nm) and Red (628nm). The optics are set up in octagon - (violet, blue-violet, green_2 and yellow/green lasers) and trigon – (UV, infrared, blue, green, orange and red lasers) shaped arrays, with a series of photomultiplier tubes (PMT) and filters (dichroic and band pass) that enable for the analysis of up to 30 parameters. The set of laser and detector combinations in the bottom panel represent the basic standard configuration of the machine for which cytometer setup and tracking (CST) is carried out daily by the flow cytometry facility. Any variations from this standard configuration will require the user to test and optimise settings.
Figure 2.3: %Efficiency of fluorochromes when excited by the lasers on the LSRII-10 Laser. The % Efficiency for each fluorochrome was calculated by multiplying the %Excitation by each given laser with the % total emission detected by the standard machine filters.
2.6.2 Fluorochromes and Optimal Detectors

In the current study, PBMCs are stained for the basic human Treg identification panel (CD4, CD25, CD127, FoxP3, CD45RO) plus combinations of CR/integrins and transcription factors. Due to the commercial unavailability of a wide range of fluorochrome conjugated antibodies directed at human chemokine receptors and integrins, we decided to move our standard Treg markers off the more popular lasers (blue, yellow-green and red), giving us room to make various combinations of CRs and integrins.

PE-CF594 on the Orange Laser

One of the main advantages of the LSRII-10-laser cytometer is the availability of an orange 592nm laser. However despite this, human antibodies directly conjugated to fluorochromes designed for this laser, such as the Alexa Fluor 594 (AF594), are not available commercially. This is probably due to the fact that flow cytometers installed with an orange laser are extremely rare, resulting in little demand for antibodies designed for this laser.

The PE-CF594 fluorochrome is designed for the yellow-green 561nm laser. However the orange 592nm laser can also significantly excite this fluorochrome. Because spectral spillover makes PE and PE-CF594 incompatible together when both are detected off the yellow-green laser, we decided to trial PE-CF594 detection in the 610/20 filter off the orange laser (Figure 2.4). In order to do this, PBMCs were either stained with biotinylated CD127 followed by streptavidin-AF594 (the fluorochrome designed for the 610/20 channel on the orange laser) (Figure 2.4, A) or CD127 conjugated to PE-CF594 (Figure 2.4, B&C).

Although PE-CF594 is designed for the 615/10 channel on the yellow-green laser, it is evident that it is comparable on the 610/20 channel on the orange laser (Figure 2.4, B&C), hence allowing us to utilise the PE-CF594 fluorochrome while eliminating the issue of emission spillover from other fluorochromes excited by the yellow/green laser, particularly
Figure 2.4: Detecting PE-CF594 on the 610/20 filter off the 592nm orange laser. PBMCs were examined for the expression of markers that allowed for the identification of Tregs (CD4, CD25, CD127, FoxP3). CD4+ cells were gated and examined for the expression of CD127 and CD25 or FoxP3 and Tregs were gated as CD127loCD25+ or CD127loFoxP3+. (A) Treg staining using CD25 BV421 and CD127 conjugated to biotin followed by streptavidin-AF594. The AF594 fluorochrome is designed for the O_610 channel on the orange laser. (B&C) Treg staining using CD25 BV421 and CD127 PE-CF594. PE-CF594 is designed for the YG_615 channel on the yellow/green laser (C) however it is comparable on the O_610 channel on the orange laser (B). Detecting PE-CF594 on the orange laser minimises emission spillover from fluorochromes excited by the yellow/green laser, particularly PE. (D) Treg staining using CD127 BUV737 and FoxP3 PE-CF594 detected on the O_610 channel on the orange laser. Numbers indicate cells within the gate as a percentage of the parent population.
PE. PE-CF594 detected on the orange laser also proved to work well with intracellular FoxP3 (Figure 2.4, D). Because we were later presented with an alternative fluorochrome for CD127 (Figure 2.8, B), we decided to make FoxP3 PE-CF594 a standard in our intracellular panel.

**Brilliant Violet**

Because CD25 discrimination is relatively poor, it is necessary for it to be conjugated to a dye that is both bright and has minimal spillover from other channels. In our initial Treg panel, prior to our attempts to create a 10-13-colour panel, CD25 was conjugated to APC. In these initial stains, because the CD25 signal in APC was both crucial and low, no other antibody conjugates were detected on the red laser. In order to have more flexibility with antibody combinations, we decided to trial CD25 on a range of dyes. Preferably, we wanted to move CD25 off APC, making the three channels on the red laser available for other commercially available antibodies.

The recent introduction of the brilliant violet-421 (BV421) fluorochrome presented us with an alternative dye that was both bright and in a channel where not many of the markers we are interested in are available. Figure 2.5 looks at Treg differentiation, using the CD127^loCD25^+ gate, with CD25 conjugated to a range of fluorochromes (APC, FITC, PE, Brilliant Violet 421 and Brilliant Violet 605). We tested CD25 on both BV421 and BV605 (Figure 2.5, bottom panel). Although CD25-BV605 was found to be as bright and discriminating as CD25-PE and CD25-APC, CD25-BV421 offered a much brighter, more distinguished Treg population. For this reason, it was decided to use CD25-BV421 as our standard CD25 conjugate in all our panels.

When testing BV421 conjugated antibodies, we noticed a remarkable difference in Treg differentiation and signal when comparing the Canto II and the LSRII-10-laser flow cytometers (Figure 2.6, A). The Canto II provides a more suitable filter for the detection of
Figure 2.5: Comparing fluorochrome conjugates of CD25 on the CANTO II. PBMCs were examined for the expression of markers that allowed for the identification of Tregs (CD4, CD25, CD127) with a different CD25 fluorochrome conjugate tested in each stain. CD4+ cells were gated and examined for the expression of CD127 and CD25 and Tregs were gated as CD127loCD25+. Numbers indicate cells within the gate as a percentage of the parent population.
Figure 2.6: Comparing Brilliant Violet 421 conjugated CD25 and CD127 on the Canto II (filter 450/40) and LSRII-10 Laser (filter 470/20). (A) PBMCs were examined for the expression of markers that allowed for the identification of Tregs (CD4, CD25, CD127). CD4\(^+\) cells were gated and examined for the expression of CD127 and CD25 and Tregs were gated as CD127\(^{lo}\)/CD25\(^+\). Top panel shows Treg discrimination with CD25 conjugated to brilliant violet 421 on the Canto II (left) and LSRII-10 (right). Bottom panel shows Treg discrimination with CD127 conjugated to brilliant violet 421 on the Canto II (left) and LSRII-10 (right). Numbers indicate cells within the gate as a percentage of the parent population. (B) The brighter BV421 signal seen on the Canto II was due to a more suitable filter for detection of BV421 emission (450/40 compared to 470/20). A more suitable filter (447/60) was available to use on the LSRII-10 however this filter was found to be incompatible with the two violet lasers on the machine. The 447/60 filter permits the 445nm violet laser through to the detector, resulting in a strong positive signal in the BV421 channel.
Figure 2.7: Optimal lasers and filters for detection of Brilliant Violet 421 (BV421) and Brilliant Violet 711 (BV711). (A) A more suitable filter (447/60) was added in place of the standard 470/20 filter for optimal detection of BV421 emission. The 445nm violet laser was also switched off to eliminate interference with signal in the 447/60 filter. The standard 780/60 filter and 755LP band-pass in the A position were also removed and replaced with a 710/50 filter and 685LP band-pass for optimal detection of BV711 emission. These filters were then used for detection of CD25 BV421 (B) and T-bet BV711 (C).
Chapter 2: Materials and Methods

BV421 emission (450/40 on the Canto II vs 470/20 on the LSRII-10-laser), resulting in a brighter BV421 signal. A more suitable filter (447/60) was available to use on the LSRII-10-laser, however this filter was found to be incompatible with the two violet lasers on the machine (Figure 2.6, B). The 447/60 filter permits the 445nm violet laser through to the detector, causing everything to be positive in the BV421 channel.

In order to fix this, it was necessary to switch off the 445nm laser and use only the 405nm violet laser in combination with the 447/60 filter for optimal BV421 detection (Figure 2.7 A&B). The standard 780/60 filter and 755LP band-pass in the A position on the violet laser were also removed and replaced with a 710/50 filter and 685LP band-pass for optimal detection of BV711. This channel was used for detection of T-bet conjugated to BV711 in our transcription factor panel (Figure 2.7 A&C).

**Brilliant Ultraviolet**

Brilliant ultraviolet (BUV) dyes are UV-excitable fluorochromes that provide great population resolution and hence expanded the capabilities of the 355nm UV laser on flow cytometers. We used BUV dyes for two of our standard Treg panel markers, CD127 on BUV737 and CD45RO on BUV395 (Figure 2.8). A more suitable filter (740/35) and long-pass (690LP) was added in place of the standard 825/40 filter for optimal detection of BUV737 emission (Figure 2.8, A). The standard 450/50 filter in the C position on the UV laser was also removed and replaced with a 379/28 filter for optimal detection of BUV395 emission. These filters were then used for detection of CD127 BUV737 (Figure 2.8, B) and CD45RO BUV395 (Figure 2.8, C).

**Detecting Th1, Th2 and Th17 associated transcription factors**

Our aim was to take a novel approach and design an antibody panel that will allow for the analysis of transcription factors and their associated homing phenotypes together in the one
Figure 2.8: Optimal filters and band-passes for detection of Brilliant Ultraviolet 737 (BUV737) and Brilliant Ultraviolet 395 (BUV395). (A) A more suitable filter (740/35) and band-pass (690LP) was added in place of the standard 825/40 filter for optimal detection of BUV737 emission. The standard 450/50 filter in the C position was also removed and replaced with a 379/28 filter for optimal detection of BUV395 emission. These filters were then used for detection of CD127 BUV737 (B) and CD45RO BUV395 (C).
stain. Due to the lack of flow cytometric data on the expression of these transcription factors by CD4 T cells in peripheral blood, we tested each antibody using fluorescence minus one (FMO) controls (Figure 2.9). PBMCs were surface stained for CD4 followed by intracellular staining for RORγt, T-bet and GATA-3. Lymphocytes were examined for the expression of CD4 and RORγt (Figure 2.9, top panel), CD4 and T-bet (Figure 2.9, middle panel) and CD4 and GATA-3 (Figure 2.9, bottom panel). Small populations of CD4+ cells are seen to express RORγt and T-bet while almost the entire CD4+ population (~99%) expresses GATA-3. For this reason, it was decided to eliminate GATA-3 from the stain and replace it with FoxP3.

2.6.3 Two optimised 11 and 13 – colour panels

The two final 11 and 13 colour panels are summarised in Table 2.2. Figures 2.10 & 2.11 represent a basic overview of the gating strategy and subsets of Tconvs and Tregs examined. A general gating strategy was first employed for the analysis of all PBMC flow cytometric data (Figure 2.1 A-E). Tregs were then identified within the CD4+ gate on the basis of expression of CD25, CD127 and FOXP3 (Figure 2.10 and 2.11, B). Tregs were defined as CD25+CD127lo and/or FOXP3+CD127lo cells. Conventional T cells (Tconvs) were defined as everything outside the indicated CD127loCD25+Treg gate. CD45RO was used as a marker of post-thymic activation and differentiation. Tregs and Tconvs were next subdivided on the basis of CD45RO expression in order to differentiate between CD45RO+ (effector/memory) and CD45RO− (naïve) subsets, and analysed for the various CRs, integrins, and transcription factors examined (Figures 2.10 & 2.11, C&D).

Further analysis of the subset data derived from these two flow cytometric panels will be presented and discussed in Chapters 3 and 5. The experiments in Chapter 4 were performed before these two panels were finalised, however, an optimised 9-colour panel, that formed the basis for the two final panels, was used.
Figure 2.9: Flow cytometric analysis of the transcription factors RORγt, T-bet and GATA-3. PBMCs were surface stained for CD4 followed by intracellular staining for RORγt, T-bet and GATA-3. Lymphocytes were gated based on FSC and SSC and examined for the expression of CD4 and RORγt (top panel), CD4 and T-bet (middle panel) and CD4 and GATA-3 (bottom panel). A fluorescence minus one (FMO) control was included for each transcription factor to verify populations. Numbers indicate cells within the gate as a percentage of the parent population.
**Table 2.2:** Two flow cytometric panels for the analysis of conventional T cell and Treg subsets

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13-colour: CD45RO CD127 CD25 Dead cell exclusion - - CD4 CLA CD49d CCR10 CD45RA β7 CCR4 CCR5 CD62L

11-colour: CD45RO CD127 CD25 - CD4 T-bet - CXCR3 - CCR6 CCR4 FoxP3 RORγt Dead cell exclusion
Figure 2.10: 11-colour flow cytometric panel for the analysis of Treg and Tconv subpopulations. Peripheral blood mononuclear cells (PBMC) were isolated from human blood and examined for the expression of markers that allowed for the identification of Tregs (CD4, CD25, CD127 and FoxP3) and then the further examination of subsets (CD45RO, CR/integrins and transcription factors T-bet and RORγt). A FSC-H vs time gate was used to exclude artefacts due to fluidics instability at the start and end of the samples and a fixable dead cell exclusion dye was used to remove dead cells from analysis. Doublets were excluded using sequential FSC-A vs FSC-H and SSC-A vs SSC-H gates (refer to figure 2.1). CD4+ cells were then gated (A) and CD4+ Tregs were identified as CD127loCD25+ or CD127loFOXp3+ (B). Conventional T cells (Tconvs) were defined as everything outside the indicated CD127loCD25+ Treg gate (B). Tregs and Tconvs were next subdivided on the basis of CD45RO expression in order to differentiate between CD45RO+ (effector/memory) and CD45RO− (naïve) subsets, and analysed for the various CRs and integrins and transcription factors examined (C&D). Numbers indicate cells within the gate as a percentage of the parent population.
Figure 2.11: 13-colour flow cytometric panel for the analysis of Treg and Tconv subpopulations. Peripheral blood mononuclear cells (PBMC) were isolated from human blood and examined for the expression of markers that allowed for the identification of Tregs (CD4, CD25, CD127) and then the further examination of subsets (CD45RO and CR/integrins). A FSC-H vs time gate was used to exclude artefacts due to fluidics instability at the start and end of the samples and a fixable dead cell exclusion dye was used to remove dead cells from analysis. Doublets were excluded using sequential FSC-A vs FSC-H and SSC-A vs SSC-H gates (refer to figure 2.1). CD4\(^+\) cells were then gated (A) and CD4\(^+\) Tregs were identified as CD127\(^lo\)CD25\(^+\) (B). Conventional T cells (Tconvs) were defined as everything outside the indicated CD127\(^lo\)CD25\(^+\) Treg gate (B). Tregs and Tconvs were next subdivided on the basis of CD45RO expression in order to differentiate between CD45RO\(^+\) (effector/memory) and CD45RO\(^-\) (naïve) subsets, and analysed for the various CRs and integrins examined (C&D). Numbers indicate cells within the gate as a percentage of the parent population.
CHAPTER 3: EXPRESSION OF TRAFFICKING RECEPTORS BY REGULATORY T CELLS IN HEALTHY HUMANS

3.1 Introduction

Naturally occurring CD4^+CD25^hiCD127^loFoxP3^+ regulatory T cells (Tregs) are crucial for the maintenance of immune tolerance. For this reason, Treg based therapeutic strategies may offer therapeutic benefit in many immune disorders and early clinical trials in humans appear promising [126-128]. However, an understanding of Treg trafficking could optimise Treg selection and contribute to the development of more targeted Treg-therapy.

The appropriate localisation of Tregs is crucial to their function. Despite this, very few studies have examined the migratory potential of Tregs in humans. In mice, Treg homing to lymphoid organs has been shown to be crucial to their role in suppressing T cell responses and limiting inflammation [88, 93, 94, 98]. Furthermore, naïve Tregs have been shown to undergo priming in murine secondary lymphoid tissues, where they upregulate homing receptors required for entry into peripheral non-lymphoid tissues [92]. The expression of these homing receptors by Tregs has been demonstrated to be critical to their suppressive ability, allowing them to co-localise with effector T cells at sites of inflammation [81, 112-116, 118, 129] (reviewed in Chapter 1, section 1.6).

The majority of studies that have examined the expression of Treg trafficking molecules in humans defined Tregs as CD4^+CD25^hi cells. This definition makes it difficult to distinguish Tregs from conventional CD25^+ T cells and has been shown to exclude a large percentage of the total Treg compartment [61]. Here, we used the CD127^loCD25^+ phenotype, in combination with a range of integrins and chemokine receptors, to define Treg migratory subsets and compare them to conventional T cells (Tconvs) in the peripheral blood of 44 healthy humans. We hypothesised that the migratory behaviour of Tregs in humans
influences their suppressive capacity in vivo and postulate that an understanding of these trafficking phenotypes will provide insights into their regulatory role in different tissues.

3.2 Summary of methods

The study was approved by the Sydney Local Health District ethics review committee at Royal Prince Alfred Hospital, Camperdown. Informed written consent was obtained from all individuals prior to the study. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinised venous blood by density gradient centrifugation and stored in 1 ml aliquots at -180°C in LNO₂ (outlined in Chapter 2, section 2.3).

Cells were later thawed in batches and immuno-stained with two optimised 11 and 13-colour flow cytometric panels (Table 2.2, Figures 2.10 & 2.11). Details of the monoclonal antibodies used are listed in Table 2.1 and staining procedure is described in Chapter 2, section 2.4. Data was acquired on a LSRII 10-laser flow cytometer (BD) and analysed using FlowJo software (Tree Star). A general gating strategy was employed for the analysis of all flow data (Figure 2.1, described in Chapter 2, section 2.4.6).

Statistical analysis was performed using Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA). The Mann-Whitney t-test was used to compare differences between two groups. The non-parametric Spearman method was used to calculate correlation coefficients. P-values <0.05 were considered significant.

Statistical analysis was also performed using MultiExperiment Viewer (MeV) (TM4–Microarray Software Suite). Data was loaded into MeV in .txt format and heatmaps were generated. Unsupervised cluster analysis was performed using the hierarchical clustering (HCL) algorithm.
3.3 Results and Discussion

3.3.1 Identifying regulatory T cells in human peripheral blood

Using the surface expression of CD4 and CD25 to isolate human Treg cells, it was originally demonstrated that only CD4$^+$ cells that express the highest levels of CD25 had suppressive activity in vitro [60]. This CD25$^{hi}$ population constitutes 1-2% of human peripheral blood CD4$^+$ T cells. This percentage is considerably smaller than that found in mice (6-7% of circulating CD4$^+$ T cells), suggesting that the CD25$^{hi}$ gate may have excluded some human Tregs from analysis.

The production of monoclonal antibodies reactive with human FoxP3 in 2005 improved the specificity of Treg cell detection over that provided by the combination of anti-CD4 and anti-CD25 antibodies. However, FoxP3 is an intracellular molecule and its detection requires fixation and permeabilisation to disrupt the cell surface membrane. It has previously been shown in our lab that the fixation and permeabilisation steps involved in FoxP3 detection affect many of the fluorochromes used in multiparameter flow cytometry, especially APC and tandem dyes such as PerCp-Cy5.5 and PE-Cy7 (Figure 3.1 A, taken from a methods paper published in our lab, [130]). In addition to this, on days where experiments are prolonged and the cells are subjected to many steps, FoxP3 staining may have unanticipated consequences, including a remarkable increase in autofluorescence, seen particularly in the detectors on the violet laser (Figure 3.1 B). This degree of autofluorescence makes differentiation between signal and background impossible.

We previously showed in our laboratory that the surface expression of CD127, the α-chain of the interleukin-7 receptor, allows distinction between CD127$^{lo}$ Treg cells and CD127$^{hi}$ conventional T cells in human peripheral blood and lymph node [61]. With the demonstrated problems with FoxP3 staining, we tested whether FoxP3 was absolutely required to identify Treg cells, by comparing Treg numbers calculated as CD127$^{lo}$CD25$^+$ with those calculated as
CD127<sup>lo</sup>FoxP3<sup>+</sup>. PBMCs were stained with CD127, CD25, CD4 and FoxP3 and gated as indicated in Figure 2.1, F&G. Figure 3.1 C shows the correlation between the percentage of cells within CD127<sup>lo</sup>CD25<sup>+</sup> and CD127<sup>lo</sup>FoxP3<sup>+</sup> gates in the 44 healthy individuals analysed in this study. The percentages of Tregs within the two gates in each individual were very highly correlated. It was therefore decided that it was not necessary to include FoxP3 in all subsequent stains, allowing a more sensitive and stable detection of multiple surface molecules.

In instances where it was necessary to stain for intracellular transcription factors, as was the case in one of our panels (Table 2.2), cells were split pre the fixation and permeabilisation steps. This ensured that only half of the PBMCs from each sample underwent fixation/permeabilisation therefore allowing us to monitor effects on tandem dyes and autofluorescence.

### 3.3.1.1 Frequency of CD4<sup>+</sup>CD127<sup>lo</sup>CD25<sup>+</sup> Tregs in the peripheral blood of healthy volunteers

Peripheral blood samples from our cohort of 44 healthy volunteers were examined for the number of total, effector/memory (CD45RO<sup>+</sup>) and naïve (CD45RO<sup>-</sup>) CD127<sup>lo</sup>CD25<sup>+</sup> Tregs (Figure 3.2). While conventional CD4<sup>+</sup> T cells are almost equally divided into CD45RO<sup>+</sup> and CD45RO<sup>-</sup> cells, the majority of Tregs fall within the CD45RO<sup>+</sup> compartment. The mean number (± SEM) of effector/memory Tregs (CD4<sup>+</sup>CD127<sup>lo</sup>CD25<sup>+</sup>CD45RO<sup>+</sup>) as a percentage of total CD4<sup>+</sup> T cells was 6.2 ± 0.24, while the percentage of naïve Tregs (CD4<sup>+</sup>CD127<sup>lo</sup>CD25<sup>+</sup>CD45RO<sup>-</sup>) was 2.1 ± 0.19, giving a total of 8.3 ± 0.25% of CD4<sup>+</sup> T cells (Figure 3.2 B). It has previously been shown that the frequency of naïve Tregs in peripheral blood declines with age, possibly due to thymic involution [54, 61, 131]. We have shown this to be true in our cohort (Figure 3.2 C). In contrast, the frequency of effector/memory Tregs is stable.
Figure 3.1: Effect of FoxP3 fix/perm treatment on detection of Tregs. (A) Effect of FoxP3 fix/perm treatment on fluorescence of conjugated antibodies. (i) Expression of CD25 and CD127 on CD4⁺ cells and (ii) the same sample, with additional staining for FoxP3 according to the manufacturer’s instructions. (iii) Effect on fluorescence of various dyes. Samples were stained with a single colour and half of each sample was then treated with the FoxP3 fix/perm buffers as per the manufacturer’s instructions. The treatment was found to reduce the signal and increase the background/noise for each fluorochrome tested. Signal:noise ratios were calculated for all fluorochromes and the reduction in signal:noise after FoxP3 treatment was expressed as a percentage of signal:noise without FoxP3 treatment. (B) Prolonged staining procedures and mistreatment of cells sometimes results in significantly increased background autofluorescence, particularly on the violet laser. This makes it impossible to differentiate signal from background (B, left panels – normal autofluorescence/background, right panels – increased autofluorescence/background. Gated on CD4⁺ cells). (C) Correlation between the frequencies of CD4⁺ Tregs identified by gating for CD127loCD25⁺ versus CD127loFOX3⁺ cells. PBMCs from healthy individuals (n=44) were stained and Tregs gated as in Figure 2.1 F&G. The correlation was statistically evaluated using a non-parametric Spearman test and was found to be significant (p<0.0001 ****).

Figure 3.2: Levels of CD4⁺CD127loCD25⁺ cells in the peripheral blood of 44 healthy donors. (A) CD4⁺ Tregs were identified as CD127loCD25⁺ and then subdivided into CD45RO⁺ and CD45RO⁻ subpopulations. Numbers indicate cells within the gate as a percentage of total CD4⁺ cells. (B) CD45RO⁺ (effector/memory), CD45RO⁻ (naïve) and total Tregs expressed as a percentage of total CD4⁺ T cells. Each point represents an individual subject (n=44) and horizontal bars represent the group means. (C) Relationship between CD45RO⁺ and CD45RO⁻ Treg subpopulations and age. The correlation was evaluated using a non-parametric Spearman test.
3.3.2 Expression of trafficking receptors by Tconvs and Tregs

It has long been known that lymphocytes not only leave the blood to enter peripheral tissue, but also continuously recirculate back to the blood, therefore maintaining immunological surveillance \[82\]. Difficulty in acquiring tissue from healthy individuals makes the peripheral blood the most accessible tissue for the analysis of lymphocytes that recirculate. Using transgenic mice that express Kaede protein, which allows for T cell labeling in the periphery under physiological conditions, it has been shown that Tregs and Tconvs within the circulation infiltrate the skin, migrate to draining lymph nodes and then recirculate back to the skin in both the steady state and in response to inflammation \[95\]. This establishes that Tregs recirculate between the blood and peripheral tissues to both maintain homeostasis and control ongoing immune responses. Furthermore, it has been demonstrated that at steady state in mice, the vast majority of skin CD4\(^+\) T cells equilibrate with the circulation rather than remaining fixed in the tissue \[132\].

We hypothesised that the analysis of surface molecules required for migration to peripheral tissues would provide an indication of Treg activity within those tissues. The trafficking receptor profile of conventional CD4\(^+\) T cells (Tconvs) has been well characterised. We defined this phenotype in Tregs and compared the expression of these molecules on Tregs and Tconvs. To do this, mononuclear cells from the peripheral blood of 44 healthy individuals were stained using a range of monoclonal antibodies to various chemokine receptors (CRs) and integrins (Table 2.1 & 2.2) and then subjected to 11 or 13 colour flow cytometry. The integrins chosen for comparison were \(\beta7, \alpha4\) (CD49d), CLA and CD62L. The CRs examined were CCR4, CCR5, CCR6, CCR10 and CXCR3.

Studies in mice have shown that, upon activation, Tregs undergo a trafficking receptor switch from molecules that enable recirculation through lymphoid tissues to molecules that enable migration and entry to non-lymphoid peripheral tissues \[92, 98\]. In order to study the trafficking phenotypes of effector and naïve Tregs in humans, we subfractionated cells on the
basis of CD45RO expression. In humans, T cells exit the thymus expressing CD45RA and switch to CD45RO upon antigen recognition [133]. Hence, CD45RO is a marker of post thymic differentiation and effector/memory cells. By including CD45RO in our stains, we were able to examine the trafficking receptor phenotype on both CD45RO$^+$ effector/memory and CD45RO naïve human Tregs.

### 3.3.2.1 Expression of trafficking receptors on CD45RO$^+$ Tconvs and Tregs

Representative dot plots showing the expression of β7, α4 (CD49d), CLA, CCR4, CCR10, CCR5, CD62L, CXCR3 and CCR6 on CD45RO$^+$ Tconvs and Tregs can be seen in Figure 3.3 with numbers for the entire cohort in Figure 3.4.

Among CD45RO$^+$ Tconvs, integrin α4 (CD49d), CCR4, CD62L, CXCR3 and CCR6 are the most highly expressed trafficking receptors (Figure 3.4A). Within CD45RO$^+$ Tregs, CLA, CCR4, CD62L and CCR6 are expressed on the highest proportion of cells (Figure 3.4B). CLA is expressed on a higher proportion of CD45RO$^+$ Tregs than Tconvs (mean 36% vs 9% respectively), as is CCR4 (70% vs 32%), CCR10 (30% vs 6%), CCR5 (21% vs 8%), CD62L (45% vs 21%) and CCR6 (49% vs 23%). No differences were found between Tconvs and Tregs in terms of α4 (CD49d), β7 and CXCR3 expression.

While no differences were found between effector/memory Tconvs and Tregs in terms of expression of the gut-associated β7, a significantly smaller proportion of Tregs express β7 (mean 8%) when compared to Tregs that express CLA (mean 36%), CCR4 (mean 70%) and CCR10 (mean 30%). Furthermore, these skin homing receptors, CLA, CCR4 and CCR10, are expressed on a much higher proportion of Tregs than Tconvs (36% vs 9%, 70% vs 32% and 30% vs 6% respectively). For this reason, adult human Tregs can be considered to showing a bias towards migration to the skin, indicating an important role for Treg immune regulation at this site.
Figure 3.3: Expression of chemokine receptors and integrins by CD45RO⁺ Tconvs and Tregs. (A) CD4⁺ Tregs were identified as CD127loCD25⁺ and conventional T cells (Tconvs) were defined as everything outside the indicated CD127loCD25⁺ Treg gate. (B) Tregs and Tconvs were next subdivided on the basis of CD45RO expression in order to identify CD45RO⁺ (effector/memory) subsets, and analysed for the various chemokine receptors and integrins examined (β7, CD49d, CLA, CCR4, CCR10, CCR5, CD62L, CXCR3 and CCR6). Numbers indicate cells within the gate as a percentage of the parent population.
Figure 3.4: Expression of chemokine receptors and integrins by Tconvs and Tregs in healthy individuals (n=44). Expression of chemokine receptors and integrins (CR/I) by CD45RO^+ Tconvs (A) and Tregs (B), gated as in Figure 3.3. Each point represents an individual donor and horizontal bars represent the group means. The Mann-Whitney t-test was used to compare differences between Tconvs and Tregs, **** = p<0.0001, ns= not significant.
L-selectin (CD62L) has been shown to be required for the homing of naïve and central memory cells to secondary lymphoid tissue [134]. Based on CD62L expression, CD45RO+ cells can be subdivided into CD62L− memory cells that migrate to non-lymphoid peripheral tissues and CD62L+ central memory cells that migrate to lymphoid tissues. We have shown that Tregs contain a significantly higher proportion of these CD62L+ central memory cells when compared to Tconvs (45% vs 21%), suggesting an enhanced role for Tregs in regulating systemic immunity within lymphoid tissues.

All the trafficking receptors examined, except for α4 (CD49d), β7 and CXCR3, are expressed on a higher proportion of effector/memory Tregs than Tconvs. For this reason, it appears that Tregs in healthy humans are a highly migratory subset with roles in both lymphoid and non-lymphoid tissues. Tregs are diverse in terms of trafficking receptor expression and this heterogeneity is different to that seen in Tconvs. Figure 3.5 is a heat map representing the level of each CR and integrin examined (rows) on CD45RO+ Tconvs (left) and Tregs (right) in our cohort of 44 healthy individuals. Unsupervised hierarchical clustering resulted in Tconvs and Tregs clustering in two separate groups, highlighting that effector/memory Tregs have a unique trafficking receptor signature that differentiates them from effector/memory Tconvs.

**Trafficking phenotypes on Tregs gated as CD25hi versus CD127loCD25+**

A study using FoxP3 to identify Tregs showed that the proportion of Tregs expressing CLA, CCR4, CCR6, and α4β7 was similar to the proportions we determined using CD127loCD25+ to identify Tregs [99]. Although studies that used CD25hi to define Tregs also demonstrated that the highest proportions of Tregs expressed CLA, CCR4, CCR6 and CD62L and the lowest expressed α4β7 [90, 91, 109, 110, 135], the size of these proportions differed significantly to ours. For example, one of these studies reported that ~80% of Tregs expressed CLA and ~73% expressed CCR6 [91] whereas we found ~36% of CD127loCD25+
**Figure 3.5. Tregs and Tconvs have distinct trafficking phenotypes.** Data obtained from flow cytometric experiments is visualised colorimetrically in a heat map. Each row represents the subsets of chemokine receptors and integrins examined and each column represents the levels on either Tconvs (left) or Tregs (right) in each individual (n=44). Frequencies that are increased relative to the row’s mean are indicated in yellow, decreased in blue and frequencies that show no difference are indicated in black. Unsupervised hierarchical clustering resulted in Tconvs (green) and Tregs (red) clustering in two separate groups.
cells expressed CLA and ~49% expressed CCR6. Furthermore, published studies have shown that 80-95% of CD25hi Tregs in adults express CCR4 [91, 109, 110, 135] while ~7% express α4β7 [91, 110], whereas we found ~70% of Tregs to express CCR4, ~25% to express α4 (CD49d) and ~8% to express β7. Additionally, studies that measured the levels of CD62L+ cells on CD25hi Tregs reported that ~80-90% of Tregs express CD62L [91, 135] while we found that only ~45% of Tregs express CD62L.

These data suggested that the expression of trafficking receptors may correlate with that of CD25; with cells that express the highest levels of CD25 also expressing the highest levels of CLA, CCR4, CCR6 and CD62L. In order to test this, we examined the trafficking receptor phenotype of Tregs gated as CD4+CD25hi (top 3% of CD25 expression) and compared them to Tregs gated as CD4+CD127loCD25+ (Figure 3.6). In doing this, we demonstrated that the proportions of cells expressing almost all of the trafficking receptors examined were significantly increased within the CD25hi “Treg” gate. The three receptors whose expression was unchanged between CD25hi and CD127loCD25+ Tregs were α4 (CD49d), β7 and CXCR3, the three receptors we found to be equally expressed by Tregs and Tconvs (Figure 3.4 & 3.5).

We wanted to test whether this correlation between expression of trafficking receptors and CD25 is also evident in conventional CD4 T cell populations. In order to do this, we selected the CD45RO+ trafficking receptor-expressing Tconvs (Figure 3.7, A) and calculated the MFI of CD25 expression within this population (Figure 3.7, B). Interestingly, CD25 expression was highest on CLA, CCR4, CCR10, CD62L and CCR6 expressing cells and lowest on α4 (CD49d) and β7 expressing cells. This indicates that the trafficking phenotypes commonly associated with Tregs may in fact be a function of CD25 expression rather than the Treg phenotype itself.

3.3.2.2 Expression of trafficking receptors on CD45RO+ Tconvs and Tregs
Figure 3.6: Expression of chemokine receptors and integrins by Tregs gated as either CD127^{lo}CD25^{+} or CD25^{hi}. Expression of chemokine receptors and integrins (CR/I) by CD45RO^{+} Tregs gated as either CD127^{lo}CD25^{+} (black) or CD25^{hi} (red). Flow plots of the gates are displayed on the right hand side. Each point represents an individual donor (n=44) and horizontal bars represent the group means.
Figure 3.7: Differing levels of CD25 expression on the various CR/Integrin$^+$ Tconv subsets. (A) Tconvs were gated as cells that fall outside the CD127$^{lo}$CD25$^+$ Treg gate and then examined for CR/Integrin$^+$ effector/memory cells (red gate). (B) CR/Integrin$^+$ CD45RO$^+$ Tconvs were selected and examined for their mean fluorescence intensity (MFI) of CD25. Each point represents an individual donor (n=44) and horizontal bars represent the group means.
Two of the trafficking receptors examined, β7 and CD62L, were found to be expressed on a significant proportion of CD45RO naïve Tconvs and Tregs. Representative dot plots showing the expression of β7 and CD62L on CD45RO naïve Tconvs and Tregs can be seen in Figure 3.8 B with numbers for each individual within the entire cohort of subjects in Figure 3.8 C.

Among Tconvs, CD45RO β7+ and CD45RO CD62L+ cells make up equal proportions of total Tconvs (mean 48%). Among Tregs, CD45RO CD62L+ cells are found at slightly higher levels than CD45RO β7+ cells (21% vs 14%). Both β7 and CD62L are expressed on a higher proportion of CD45RO Tconvs than Tregs. Interestingly, CD62L is expressed on a higher proportion of CD45RO+ Tregs than CD45RO Tregs, whereas the opposite is true for Tconvs, suggesting a possible important role of circulating central memory Tregs in lymphoid tissue.

It has been described that a lower proportion of circulating Tregs in adults express the gut homing integrin β7 when compared to Tconvs [91, 110]. However these studies did not divide Tregs into effector/memory and naïve subsets. By dividing Tregs this way, we have shown that the decrease in expression of gut homing β7 commonly associated with adult Tregs is due to less β7+ cells in the CD45RO naïve Treg compartment.

### 3.3.2.3 Concluding remarks on section 3.3.2

In this part of the study, we set out to analyse the expression of migratory receptors on Tregs in the peripheral blood of healthy individuals. In doing so, we have shown that Tregs express receptors that enable entry into lymphoid and non-lymphoid tissues and that this migratory phenotype is distinct to that seen in Tconvs. This demonstrates that Tregs exert their regulatory function at various sites and are involved in both regulating the priming of cells in lymphoid tissue and limiting inflammation in non-lymphoid tissue, even in the absence of any ongoing inflammation. While it is crucial for Tregs to be able to co-localise with Tconvs in order to suppress responses at sites of ongoing inflammation, it is important to emphasise
Figure 3.8: Expression of chemokine receptors and integrins by CD45RO− Tconvs and Tregs. (A) CD4+ Tregs were identified as CD127loCD25+ and conventional T cells (Tconvs) were defined as everything outside the indicated CD127loCD25+ Treg gate. (B) Tregs and Tconvs were next subdivided on the basis of CD45RO expression in order to identify CD45RO− (naïve) subsets, and analysed for the expression of β7 and CD62L, two markers found to be significantly expressed by naïve T cells. (C) Levels of expression of β7 and CD62L on naïve Tconvs (black) and Tregs (red). Each point represents an individual donor (n=44) and horizontal bars represent the group means. The Mann-Whitney t-test was used to compare differences between Tconvs and Tregs, **** = p<0.0001
their role in immunosurveillance and maintaining homeostasis in the absence of inflammation. Tregs migrate in a coordinated fashion in order to carry out optimal immunosurveillance and function to prevent both the initiation of aberrant immune responses and limit the progression of these responses. We also demonstrated that the use of CD25 alone to study Tregs results in an inaccurate interpretation of their migratory phenotype.

The use of drugs targeting T cell trafficking has recently been trialled as a strategy to combat immune-mediated disease. However these drugs haven’t been as effective as anticipated [136-138] or have had unpredicted adverse events [139]. It is likely that this is partly due to the overlooked effects these drugs have on the trafficking of Tregs. A study that examined the binding specificity of Vedolizumab, an anti-α4β7 therapeutic antibody, on various cell subsets, examined total CD4 and CD8 T cells, B cells, natural killer cells, basophils, monocytes, eosinophils and neutrophils but not Tregs [140]. We have shown that Tregs are highly migratory and express trafficking receptors that target them to peripheral tissues, therefore emphasising that future therapeutic interventions targeting cell migration need to take into account the effects on Tregs.

### 3.3.3 Tissue specific homing on Tconvs and Tregs

It has long been known that a proportion of circulating CD4⁺ T cells display some degree of tropism for certain tissues, particularly the gut and skin. This selective entry of lymphocytes is due to their expression of specific chemokine receptors and adhesion molecules that allow them to traffic through and engage in immunosurveillance of various tissues. Of these tissue-associated homing phenotypes, the gut specific α4β7 [100, 101] and the skin specific CLA, CCR4 and CCR10 [103, 104, 141] are the most well known (reviewed in Chapter 1, section 1.6.3).

The integrin α4β7 allows lymphocytes to bind to the mucosal vascular addressin cell adhesion molecule-1 (MAdCAM-1) [142] resulting in the efficient trafficking of
lymphocytes into gut tissue [100, 101]. On the other hand, CD4+ T cells that express the cutaneous lymphocyte antigen (CLA), an adhesion molecule that binds E-selectin, have a tropism for cutaneous tissue [141]. CCR4 is expressed by most CLA+ skin homing T cells [143, 144] and is found at high levels on skin infiltrating lymphocytes [104]. Furthermore, the CCR4 ligand CCL17 (TARC) is associated with cutaneous endothelial cells [145]. More recently, another cutaneous T cell attracting chemokine, CCL27, was found to be expressed by cutaneous tissue and to bind the receptor CCR10 on T cells [146, 147].

While a few studies have examined the co-expression of these adhesion molecules and receptors on total CD4+ T cells in humans [143, 144, 147, 148], none have focused on Tregs. We designed a flow cytometric panel that allowed us to analyse combinations of these chemokine receptors and integrins on both Tconvs and Tregs (Table 2.2, Figure 2.11). Because CLA, CCR4 and CCR10 are mainly expressed on CD45RO+ CD4 T cells, for this next section we will focus only on this subset.

3.3.3.1 Gut and skin–associated trafficking receptors on Tconvs and Tregs

In order to analyse α4β7+ cells, we co-stained PBMCs with antibodies to α4 (CD49d) and β7 integrins and analysed them in combination (Figure 3.9). A higher proportion of CD45RO+ Tconvs expressed α4β7 when compared to Tregs (mean 18.1 vs 8.9%) (Figure 3.9 B). In Figure 3.9A it is clear that within peripheral blood CD45RO+ T cells, all β7+ cells fall within the α4β7+ gate whereas a large proportion of α4+ (CD49d) cells do not. This means that expression of β7 alone is sufficient for identifying α4β7+ CD4 T cells in blood circulation. In order to confirm this, we gated total β7+ cells on CD45RO+ Tconvs and Tregs and overlayed them onto the α4 versus β7 cytometry plot, verifying that these β7+ cells made up all the cells in the α4β7+ gate (Figure 3.10).

In order to explore the differences between the expression of CCR4 and CCR10 on CLA+ skin homing Tconvs and Tregs, we co-stained samples for CLA, CCR4 and CCR10 and
Figure 3.9: Expression of gut homing α4β7 by CD45RO⁺ Tconvs and Tregs. (A) Representative cytometry plots from three individual donors. CD45RO⁺ (effector/memory) Tregs and Tconvs were gated and examined for the expression of α4 (CD49d) and β7. Co-expressing α4β7⁺ cells are in the indicated gates on both Tconvs (left panel) and Tregs (right panel). (B) Levels of co-expressing α4β7⁺ cells as a percent of total CD45RO⁺ Tconvs and Tregs. Each point represents an individual donor (n=44) and horizontal bars represent the group means. The Mann-Whitney t-test was used to compare differences between Tconvs and Tregs. **** = p<0.0001
Figure 3.10: β7 alone is enough to gate α4β7+ cells on CD45RO+ Tconvs and Tregs.

(A) CD45RO+ (effector/memory) Tconvs and Tregs were selected and examined for the expression of β7. β7+ cells are within the indicated gate (red). (B) β7+ cells, as gated in A, were selected and overlayed onto the α4 vs β7 cytometry profile (red). β7+ cells fall within the α4β7+ gate on both Tconvs and Tregs. Representative cytometry plots from three individual donors.
examined co-expression amongst these molecules (Figure 3.11). In doing so, we were able to show that CCR4 is expressed on ~90% of CLA⁺ Tconvs but also on a significant proportion of CLA⁻ Tconvs (only ~30% of CCR4⁺ Tconvs express CLA). Furthermore, almost all (99%) CLA⁺ Tregs expressed CCR4 where as only 58% of CCR4⁺ Tregs express CLA (Figure 3.11, C i & ii). This demonstrates that CLA⁺CCR4⁺ cells exist in both the Tconv and Treg compartments, indicating a role for CCR4 in homing to other non-cutaneous peripheral tissues.

In contrast, CCR10 is expressed on significantly fewer CLA⁺ skin homing T cells. Only 50% of CLA⁺ skin-homing Tconvs and 73% of CLA⁺ skin-homing Tregs express CCR10 (Figure 3.11, C, i). Interestingly, 73% of CCR10⁺ Tconvs express CLA, therefore demonstrating that CLA⁻CCR10⁺ Tconvs also exist and suggesting that not all CCR10⁺ cells can be assumed to be skin-homing. The minor population of CCR10⁺ CD4 T cells that do not express CLA has been previously described [148]. Here we show that a larger proportion of CCR10⁺ Tregs express CLA when compared to Tconvs (mean 90% vs 73%) (Figure 3.11, C, iii).

Interestingly, we found that virtually all CCR10⁺ Tconvs and Tregs expressed CCR4 (Figure 3.11, C, iii), indicating that almost all of the CCR10⁺ Tconvs and Tregs are CLA⁺CCR4⁺CCR10⁺. Since both CCL17 and CCL27 (the ligands for CCR4 and CCR10) are upregulated on inflamed cutaneous endothelium, CCR4 and CCR10 may offer redundant mechanisms to allow CLA⁺CCR4⁺CCR10⁺ cells to enter inflamed skin. However, under non-inflammatory homeostatic conditions, entry to the skin may be controlled differently. For example, CCR10 has been shown to be required for the entry of T cells into murine skin under homeostatic conditions but was dispensable for the infiltration of T cells into skin under inflammatory conditions [149]. In addition, CCR10 knockout mice displayed over-reactive immune responses in the skin, likely due to the significantly reduced frequency of Tregs in the skin of these mice [149].
Figure 3.11: Co-expression of skin associated receptors by CD45RO\(^+\) Tconvs and Tregs. (A) CD45RO\(^+\) (effector/memory) Tconvs and Tregs are examined for the expression of CLA, CCR4 and CCR10. Co-expressing cells are in the indicated gates on both Tconvs (left panel) and Tregs (right panel). (B) Levels of co-expression of CLA, CCR4 and CCR10 as a percent of total CD45RO\(^+\) Tconvs and Tregs. (C) Percent CLA\(^+\) (i), CCR4\(^+\) (ii) and CCR10\(^+\) (iii) cells co-expressing other skin associated receptors on both Tconvs (black) and Tregs (red). Each point represents an individual donor (n=44) and horizontal bars represent the group means. The Mann-Whitney t-test was used to compare differences between Tconvs and Tregs, **** = p<0.0001
In this study, we showed that CCR10 is expressed more highly on Tregs than Tconvs (Figure 3.4) and on a higher proportion of both CLA+ and CCR4+ Tregs than Tconvs (Figure 3.11). This suggests a potential role for Tregs to maintain homeostasis in the skin under steady-state non-inflammatory conditions. Therefore, developing strategies that can increase the number of CCR10+ Tregs might help restore immune homeostasis in skin related immune disease.

In conclusion, we have shown that the skin homing compartment of CD4+ Tconvs and Tregs can be divided into subpopulations based on trafficking receptor expression, and that the proportions of these subpopulations differ amongst Tconvs and Tregs. Although both CCR4 and CCR10 are associated with skin-homing CLA+ T cells, the association is incomplete in both cases. CCR4 is expressed on almost all skin-homing CLA+ T cells, but also on a subset of other cells. In contrast, CCR10 is almost exclusively found within a subset of CLA+ skin homing cells.

3.3.3.2 Co-expression of gut and skin–associated trafficking receptors on Tconvs and Tregs

Since we showed that β7 alone is sufficient for identifying α4β7+ cells (Figure 3.10), we used only β7 when examining the co-expression of gut and skin associated trafficking receptors. Expression of CLA and β7 is generally believed to be mutually exclusive [150], but we have shown here that a small proportion of cells co-express these markers (Figure 3.12). On total CD45RO+ Tconvs, ~1.3% of cells are CLA+β7+ (Figure 3.12, B). On the other hand, ~3.8% of total CD45RO+ Tregs are CLA+β7+. Interestingly, 37% of β7+ effector/memory Tregs express CLA while only 8% of β7+ effector/memory Tconvs express CLA (Figure 3.12, C).

CCR4+β7+ cells make up equal proportions of total CD45RO+ Tconvs and Tregs (~11-13%). Additionally, 67% of β7+ effector/memory Tconvs also express CCR4 while almost all (93%) β7+ effector/memory Tregs also express CCR4. This indicates that the expression of CCR4 is
**Figure 3.12: Co-expression of skin and gut associated receptors by CD45RO^+ Tconvs and Tregs.**

(A) CD45RO^+ (effector/memory) Tconvs and Tregs are examined for the co-expression of gut associated \( \beta 7 \) with skin associated CLA, CCR4 and CCR10. Co-expressing cells are in the indicated gates on both Tconvs (left panel) and Tregs (right panel). (B) Levels of co-expression of \( \beta 7 \) with CLA, CCR4 and CCR10 as a percent of total CD45RO^+ Tconvs and Tregs. (C) Percent \( \beta 7^+ \) cells co-expressing skin associated CLA, CCR4 and CCR10 on both Tconvs (black) and Tregs (red). Each point represents an individual donor (n=44) and horizontal bars represent the group means. The Mann-Whitney t-test was used to compare differences between Tconvs and Tregs, **** = p<0.0001
distributed within both gut and skin homing cells. ~90% of CLA+ Tconvs and 67% of β7+ Tconvs express CCR4 while almost all CLA+ and β7+ Tregs express CCR4. This again indicates a role for CCR4 in trafficking cells to non-cutaneous peripheral tissues, the gut included. On the other hand, CCR10 and β7 are generally mutually exclusive on Tconvs, with only 4% of β7+ Tconvs expressing CCR10. In a study that examined the co-expression of CCR10 and β7 on memory CD4+ T cells, it was concluded that “practically no” CCR10+ cells express detectable β7 [148]. However this study did not examine the expression of these molecules on Tregs. We have shown that 30% of β7+ Tregs express CCR10 (Figure 3.12, C).

3.3.3.3 Concluding remarks on section 3.3.3

The co-expression of adhesion molecules and CRs on a subset level suggests a wide variety of combinations that can target cells into diverse microenvironmental niches. The in vivo significance of the proportions of cells expressing these various combinations is currently unknown. Nevertheless, studying these subsets on both Tconvs and Tregs can provide clues for the design of future therapy.

The optimal subpopulation of Tregs for immuno-therapy may be defined by trafficking receptors that favour certain tissues. Certainly, studies in an animal model of graft versus host disease (GvHD) have shown that CLA expressing Tregs are more efficient at preventing skin destruction [151]. More importantly, increased frequencies of CLA+ Tregs at the time of neutrophil engraftment in allogeneic stem cell transplant (ASCT) patients has been shown to be associated with the prevention of acute GvHD involving the skin [152]. It has also been shown that increased frequencies of CLA+ or β7+ Tregs are associated with reduced incidence [153] or severity [152] of skin or gut aGvHD, respectively. This confers that Tregs are not a single uniform subset, but that various migratory subsets within Tregs can represent a target that is potentially modifiable by cellular therapy.
3.3.4 Trafficking of CD4⁺ T cells to sites of Th1, Th2 and Th17 type inflammation

During their initial activation, conventional CD4⁺ T cells can assume one of several functional fates (Th1, Th2 or Th17). Each of these subsets is believed to be defined by master regulator transcription factors and distinct patterns of cytokine production. Th1 cells express the transcription factor T-bet and secrete IFN-γ [154], Th2 cells express the transcription factor GATA-3 and secrete IL-4, IL-5 and IL-13 [155] and Th17 cells express the transcription factor RORγt and secrete IL-17 [156, 157].

Once naïve CD4⁺ T cells are primed and develop into Th1, Th2, and Th17 type cells, they migrate from lymphoid organs to target peripheral tissues. It is thought that different CRs are preferentially expressed on Th1, Th2 and Th17 type cells, resulting in the selective migration of these subsets to particular tissues [158]. Generally, Th1 cells are believed to be CXCR3⁺ [159-162] while Th2 cells are CCR4⁺ [159-164] and Th17 cells CCR6⁺ [165, 166]. However, more recently these definitions have been modified, with Th1 cells being described as consisting of both CXCR3⁺CCR6⁻ and CXCR3⁺CCR6⁺ subsets, while Th2 cells are CXCR3⁻CCR6⁺CCR4⁺ and Th17 cells CXCR3⁺CCR6⁻CCR4⁺ [167, 168].

It is important to note that these studies examined the CR expression of Th1, Th2 and Th17 type cells on cells artificially polarised in vitro [159, 160, 163] or by isolating circulating CR expressing CD4⁺ T cells from human blood and examining their cytokine profiles following in vitro stimulation [161, 162, 167, 168]. For example, in two of these studies, a small proportion of isolated CD4⁺ memory T cells expressing CCR4 were shown to produce IL-4 following in vitro stimulation whereas a proportion of CXCR3 cells produce IFN-γ [161, 162]. It is worth mentioning that even following artificial stimulation, only a minority of the CR expressing subsets express the relevant cytokines, thus casting doubt on the use of CR expression patterns to study and measure all Th1, Th2 and Th17 type cells.
3.3.4.1 Chemokine receptor expression on circulating $T$-bet$^+$ and RORγt$^+$ cells

In order to study the CR expression patterns of Th1 and Th17 type T cells, we took a novel approach and designed an 11 colour antibody panel that allowed us to combine detection of the transcription factors T-bet and RORγt with their commonly associated CRs (Table 2.2, Figure 2.10). In doing so, we were able to identify circulating CD4$^+$ T cells that express T-bet and RORγt without the need for prior in vitro stimulation (Figure 2.9).

When T-bet$^+$CD4$^+$ cells were examined for the expression of CXCR3, CCR4 and CCR6, they were found to express only CXCR3, the CR commonly associated with Th1 T-bet$^+$ cells (Figure 3.13). Numerous studies have described circulating CXCR3$^+$ CD4 T cells as Th1 type cells [159-162, 167-169]. However, it is evident in our study that in human PB not all CXCR3$^+$ cells express T-bet. In fact, in the 44 healthy donors examined, 89% (mean) of CXCR3$^+$CD4$^+$ T cells did not express T-bet (Figure 3.15, B, subset 3). It should be noted, however, that T-bet expression is also seen in a proportion of cells that fall within the CXCR3 negative gate (Figure 3.13).

When RORγt$^+$CD4$^+$ cells were examined for the expression of CXCR3, CCR4 and CCR6, they were all found to express CCR6, the CR commonly associated with Th17 RORγt$^+$ cells (Figure 3.14). In fact, the combination of RORγt and high CCR6 identifies a distinct population of RORγt$^+$CCR6$^+$ CD4 T cells in adult human blood. It is important to note however, that although all RORγt$^+$ cells express CCR6, not all CCR6$^+$ cells express RORγt. Interestingly, we also show that subsets of RORγt$^+$ cells express the CRs CCR4 and CXCR3. This trafficking receptor profile is consistent with their inflammatory role in an array of peripheral tissues [170-172].

In a few highly regarded studies, Th1, Th2 and Th17 cells in human PB were identified as CXCR3$^+$, CXCR3CCR6CCR4$^+$ and CXCR3CCR6$^+$CCR4$^+$ memory CD4 T cells, respectively [167-169]. Although these studies found that “Th1” and “Th17” cells isolated
Figure 3.13: Not all CXCR3⁺ T cells express T-bet. Peripheral blood mononuclear cells (PBMC) were isolated from human blood and surface stained for CD4, CD25, CD127, CD45RO and the CRs CXCR3, CCR4 and CCR6 followed by intracellular staining for T-bet, RORγt and FoxP3. CD4⁺ T cells here are examined for the co-expression of the transcription factor T-bet with Th1, Th2 and Th17 associated chemokine receptors (CXCR3, CCR4 and CCR6 respectively). Representative cytometry plots from four individual donors.
Table: All ROR-γt+ cells express CCR6 but not all CCR6+ T cells express ROR-γt. Peripheral blood mononuclear cells (PBMC) were isolated from human blood and surface stained for CD4, CD25, CD127, CD45RO and the CRs CXCR3, CCR4 and CCR6 followed by intracellular staining for T-bet, ROR-γt and FoxP3. CD4+ T cells here are examined for the co-expression of the transcription factor ROR-γt with Th1, Th2 and Th17 associated chemokine receptors (CXCR3, CCR4 and CCR6 respectively). Representative cytometry plots from four individual donors.
this way were enriched for IFN-γ and IL-17 producing cells respectively, they did not find them all to produce these cytokines. In order to examine “Th1” and “Th17” cells, identified as described in the literature [167-169], for the expression of T-bet and RORγt, we gated CXCR3+ (“Th1”) and CXCR3CCR6−CCR4+ (“Th17”) cells within CD45RO+CD4+ cells (Figure 3.15, A, subsets 1&4). We next examined these subsets for T-bet and RORγt expression (Figure 3.15, A, subsets 3&6) and compared them to the percentages of total T-bet+ and RORγt+ CD4 T cells (Figure 3.15, A, subsets 2&5).

Figure 3.15B shows the quantification of these subsets in the PB of the 44 healthy individuals examined. “Th1” cells, defined as CXCR3+ effector/memory T cells, make up a mean of 23% of PB CD4+ T cells whereas T-bet+ cells makeup only a mean of 5.3%. Importantly, 89% of CXCR3+ effector/memory T cells do not express T-bet in human PB. “Th17” cells, defined as CXCR3CCR6−CCR4+ effector/memory T cells, make up a mean of 10.8% of PB CD4+ T cells whereas RORγt+ cells makeup only 7% of PB CD4+ T cells. Significantly, 71.5% of CXCR3CCR6−CCR4+ effector/memory T cells do not express RORγt in human PB.

Based on our findings, CRs alone cannot be used to isolate pure Th1 and Th17 type cells, defined as expressing T-bet and RORγt respectively. The use of these surface markers to isolate Th1 and Th17 cells markedly overestimates their frequency in comparison to defining Th1 and Th17 cells based on intracellular staining for T-bet and RORγt.

3.3.4.2 Th1 and Th17 associated Tregs

If Th1, Th2 and Th17 type cells are deregulated, they can cause harm to host tissue. Recently, there has been increasing interest in mechanisms by which Tregs can regulate these specific types of inflammation, with several studies suggesting that Treg subsets are specialized in regulating polarized Th1, Th2 or Th17 immune responses [112-116, 118, 119] (reviewed in Chapter 1, section 1.6.4).
Figure 3.15: The surface expression of chemokine receptors cannot be used to identify Th1 (T-bet+) and Th17 (RORγt+) CD4 cells in peripheral blood. (A) “Th1”(CXCR3+CD45RO+) and “Th17” (CXCR3CCR6+CCR4+CD45RO+) cells were gated as described in the literature (1&4) and then examined for the expression of T-bet and RORγt, respectively (3&6). Total T-bet+ and RORγt+ CD4 cells were also gated (2&5). (B) Percentages of all the cell subpopulations identified in A in the peripheral blood of 44 healthy individuals. Each point represents an individual donor and horizontal bars represent the group means.
In mice, it has recently been demonstrated that subsets of Tregs co-express the transcription factors T-bet or ROR\(\gamma\)t together with FoxP3 [112, 173, 174]. These studies demonstrated that the expression of T-bet and ROR\(\gamma\)t by Tregs in turn induces the expression of CXCR3 and CCR6 respectively, thus allowing Tregs to co-localise with inflammatory effector cells at sites of Th1 and Th17 mediated inflammation.

The co-expression of T-bet and ROR\(\gamma\)t with FoxP3 on unstimulated CD4 T cells in human PB has not been examined. We could not identify any CD4\(^+\) T cells that co-expressed T-bet and FoxP3 within human PB (Figure 3.16A). However interestingly, a study in ovarian cancer patients has shown that FoxP3\(^+\) Tregs that co-express T-bet and CXCR3 can be found in tumour tissue [175]. Despite the fact that FoxP3\(^+\) Tregs in our study do not express T-bet, they do express CXCR3 at levels equal to Tconvs (Figure 3.16B).

When we examined CD4\(^+\) T cells for the co-expression of ROR\(\gamma\)t and FoxP3, we identified a clear population of ROR\(\gamma\)t\(^+\)FoxP3\(^+\) cells (Figure 3.17A). Despite the fact that only a small proportion of FoxP3\(^+\) cells express ROR\(\gamma\)t, Tregs express CCR6 at levels higher than Tconvs (Figure 3.17B).

In order to study the ROR\(\gamma\)t\(^+\)FoxP3\(^+\) population more closely and compare it to ROR\(\gamma\)t\(^+\) and FoxP3\(^+\) cells, we divided CD4 T cells into 3 populations based on the expression of FoxP3 and ROR\(\gamma\)t (Figure 3.18A). ROR\(\gamma\)t\(^+\)FoxP3\(^+\), ROR\(\gamma\)t\(^+\)FoxP3\(^-\) and ROR\(\gamma\)t\(^+\)FoxP3\(^+\) cells were identified and their levels in the PB of the 44 healthy individuals were quantified (Figure 3.18B). ROR\(\gamma\)t\(^+\)FoxP3\(^+\) cells made up a mean of 7.4% of total CD4\(^+\) T cells, ROR\(\gamma\)t\(^+\)FoxP3\(^-\) cells made up a mean of 6.1% and ROR\(\gamma\)t\(^+\)FoxP3\(^+\) co-expressing cells made up only a mean of 1.1% of circulating CD4 T cells. Interestingly, when we examined whether the circulating levels of these subsets correlate with each other, we found that the ROR\(\gamma\)t\(^+\)FoxP3\(^+\) co-expressing cells in each individual highly correlated with total ROR\(\gamma\)t\(^+\) cells (Figure 3.18C).
Figure 3.16: Peripheral blood FoxP3⁺ CD4⁺ T cells do not co-express T-bet. (A) CD4⁺ T cells are examined for the co-expression of the transcription factors T-bet and FoxP3. Representative cytometry plots from four individual donors. (B) Percentage of cells that express the T-bet associated chemokine receptor CXCR3 on both Tconvs and Tregs. Each point represents an individual donor (n=44) and horizontal bars represent the group means.
Figure 3.17: A small percentage of CD4\(^+\) T cells in peripheral blood co-express the transcription factors ROR\(\gamma\)t and FoxP3. (A) CD4\(^+\) T cells are examined for the co-expression of the transcription factors ROR\(\gamma\)t and FoxP3. Representative cytometry plots from four individual donors. (B) Percentage of cells that express the ROR\(\gamma\)t associated chemokine receptor CCR6 on both Tconvs and Tregs. Each point represents an individual donor (n=44) and horizontal bars represent the group means.
Figure 3.18: Percentage of RORγt⁺FoxP3⁺ CD4 T cells in peripheral blood of 44 healthy subjects. (A) CD4⁺ T cells are examined for the expression of the transcription factors RORγt and FoxP3. Three cell populations were identified; RORγt⁺FoxP3⁺ (1), RORγt⁺FoxP3⁻ (2) and RORγt⁺FoxP3⁺ (3). (B) Percentages of RORγt⁺FoxP3⁺, RORγt⁺FoxP3⁻ and RORγt⁺FoxP3⁺ within CD4⁺ cells. Each point represents an individual donor (n=44) and horizontal bars represent the group means. (C) Correlations between the three identified subpopulations. The correlation was evaluated using a non-parametric Spearman test.
We also examined the expression of CD45RO and CRs CXCR3, CCR4 and CCR6 on the RORγt FoxP3+, RORγt FoxP3- and RORγt FoxP3+ cells identified above (Figure 3.19, B&C). We found that almost all of the cells in the RORγt FoxP3- and RORγt FoxP3+ subsets were of the effector/memory phenotype (CD45RO+) (mean 94%). When examined for the expression of CXCR3, the two FoxP3 expressing subsets (RORγt FoxP3+ and RORγt FoxP3-) expressed significantly less CXCR3 than the FoxP3- subset. However when CCR6 was examined, it was the two RORγt expressing subsets (RORγt FoxP3- and RORγt FoxP3+) that displayed the highest levels of CCR6 expression. It is important to note however that the RORγt FoxP3+ cells expressed significantly less CCR6 than the RORγt FoxP3- cells. When examined for expression of CCR4, the FoxP3 expressing subsets (RORγt FoxP3- and RORγt FoxP3+) displayed the highest percentage of CCR4+ cells. Interestingly, the RORγt FoxP3+ co-expressing cells contained the highest percentage of CCR4+ cells.

It has previously been reported that a small proportion (3-4%) of human memory Tregs, purified as CD4+CD25hi, secrete IL-17 following in vitro stimulation [176, 177]. In one of these studies, a large proportion (34%) of “Tregs”, isolated as CD25hi, were found to express RORγt following stimulation [176]. This indicates that in vitro stimulation leads to a change in RORγt expression, leading to an overestimation of the frequency of IL-17 producing RORγt Tregs in human PB, and highlighting the importance of our work in identifying RORγt+ cells within PB CD4 T cells.

When we compared the CR profile of FoxP3+ Tregs and RORγt+ cells, we found a high level of similarity. Both subsets contained higher proportions of CCR4+ and CCR6+ cells when compared to Tconvs (% for Tconvs in Figure 3.4). Interestingly, RORγt+ cells contained the highest proportion of CCR6+ cells while FoxP3+ cells contained the highest proportion of CCR4+ cells with RORγt FoxP3+ co-expressing cells taking on both these features, expressing CCR6 at levels similar to RORγt+ cells and CCR4 at levels similar to FoxP3+ cells. The CR expression profile of IL-17 producing stimulated CD4 T cells and FoxP3+ Tregs has been
Figure 3.19: Expression of CD45RO, CXCR3, CCR4 and CCR6 by RORγt*FoxP3⁺, RORγt*FoxP3⁻ and RORγt*FoxP3⁺ CD4 T cells in peripheral blood. (A) CD4⁺ T cells were examined for the expression of the transcription factors RORγt and FoxP3 and three cell populations were identified; RORγt*FoxP3⁺ (1), RORγt*FoxP3⁻ (2) and RORγt*FoxP3⁺ (3). (B&C) Representative cytometry plots (B) and percentages (C) of total CD45RO⁺, CD45RO⁺CXCR3⁺, CD45RO⁺CCR4⁺ and CD45RO⁺CCR6⁺ cells on RORγt*FoxP3⁺, RORγt*FoxP3⁻ and RORγt*FoxP3⁺ CD4 T cells in the peripheral blood of 44 healthy individuals. Each point represents an individual donor and horizontal bars represent the group means. Differences were analysed using a Kruskal Wallis test, p values <0.05 were considered significant.
previously compared. This study, like us, found Tregs to be higher for CCR4 while IL-17 producing cells were higher for CCR6 and CXCR3 [117].

3.3.4.3 Using combinations of CRs to define Th1, Th2 and Th17 –like Tregs

With the discovery in mice of Treg subsets that co-express the transcription factors T-bet and RORγt with FoxP3 [112, 173, 174], there has been increasing interest in methods for isolating these Tregs for functional studies. In order to do this, one study has claimed to identify, using CRs, functionally distinct subsets of Tregs that resemble polarised Th1, Th2 and Th17 type cells [169].

These Th1, Th2 and Th17 – “LIKE” Tregs were defined as CXCR3+, CXCR3CCR6CCR4+ and CXCR3CCR6CCR4+ cells respectively. In doing this, the study claimed that Th17 cells were present at a substantially higher frequency within the Treg compartment when compared to Tconvs [169]. Only ~8% of the cells in the Th17 – “LIKE” Treg compartment were found to produce IL-17. Interestingly, Th17 – “LIKE” Tregs in this study were defined as CXCR3CCR6CCR4+, that is positive for two CRs known to be highly expressed by Tregs. This prompted us to examine the relationship between the co-expression of the CRs CXCR3, CCR4 and CCR6 in order to determine whether the increased expression of CCR4 and CCR6 within the Treg compartment resulted in the increased co-expression of these CRs.

In order to do this, we gated CXCR3CCR4+, CXCR3CCR6+ and CCR4CCR6+ co-expressing cells on Tconvs and Tregs and graphed their percentages against predicted frequencies of co-expressing cells (calculated by multiplying the percent of cells that express CR1 with the percent of cells that express CR2) (Figure 3.20A). In doing this, we showed that the co-expression of any two CRs examined was random and can be predicted (with high accuracy, r = 0.9+) based on the frequencies of cells that express each CR individually (Figure 3.20B). We also found this relationship to be true for Th2 and Th17 – “LIKE” Tregs identified as CXCR3CCR6CCR4+ and CXCR3CCR6CCR4+ (Figure 3.20C).
Figure 3.20: Examining the co-expression of the chemokine receptors CXCR3, CCR4 and CCR6 on Tconvs and Tregs in the peripheral blood of 44 healthy individuals. (A) CD45RO⁺ Tconvs and Tregs were examined for the co-expression of CCR4 and CXCR3 (i), CCR6 and CXCR3 (ii) and CCR6 and CCR4 (iii). Co-expressing cells are in the indicated gates. Numbers indicate cells within the gate as a percentage of the parent population. (B) Examining the relationship between the frequency of cells that express each chemokine receptor individually ((i), CR1⁺ (green box) and CR2⁺ (blue box)) and co-expressing cells ((ii), CR1⁺CR2⁺ (red box)). In order to do this, we calculated predicted frequencies of co-expressing cells (CR1⁺CR2⁺) by multiplying the percent of cells that express CR1 with the percent of cells that express CR2 and graphing them against the co-expressing cells gated by flow cytometry (ii). This was done on both Tconvs (blue) and Tregs (red). (C) “Th2” (CXCR3⁺CCR6⁻CCR4⁺) and “Th17” (CXCR3⁺CCR6⁺CCR4⁺) cells were gated as described in the literature on both Tconvs (blue) and Tregs (red) and graphed against predicted frequencies of CXCR3⁺CCR6⁻CCR4⁺ and CXCR3⁻CCR6⁺CCR4⁺ cells. Correlations were evaluated using a non-parametric Spearman test.
3.3.4.4 Concluding remarks on section 3.3.4

Many studies on inflammatory disease in humans have used various combinations of CRs to measure Th1, Th2 and Th17 type cells in human PB and/or organs [178-180]. For example, one study that used CCR6 to measure Th17 cells in children with asthma concluded that Th17 cells were increased in the PB of patients [178]. In the current study, we were able to identify circulating CD4+ T cells that express T-bet and RORγt without the need for in vitro stimulation. In doing so, we were able to study the CR expression profile of these T-bet+ and RORγt+ cells. None of the CRs examined was found to be exclusively expressed by T-bet+ Th1 cells or RORγt+ Th17 cells. In fact, RORγt+ Th17 cells were found to express all the CRs examined, not just CCR6 or CCR6 and CCR4 in combination. We also showed that most of the CXCR3, CCR4 and CCR6 expressing CD4 T cells express neither T-bet or RORγt in human PB.

IL-17 has been shown to be necessary for the induction of maximal autoimmune inflammation in models of experimental autoimmune encephalomyelitis (EAE) [181], collagen induced arthritis [182, 183] and colitis [184]. Th17 type inflammation has also recently been implicated in a number of immune-mediated diseases in humans [185]. Indeed, the levels of IL-17 producing cells have been shown to be increased in inflamed tissues of patients with inflammatory bowel disease [170], multiple sclerosis [171] and rheumatoid arthritis [172]. These studies highlight the importance of being able to detect Th17 type cells in the PB and tissue of patients. Therefore, our method for identifying RORγt+ cells by flow cytometry is of great importance. An important challenge in this field of research is to find ways to isolate viable Th17 cells with high purity so that they can be used in functional studies. Now that we have shown that RORγt expression can be detected in unstimulated PB, this should aid the search for markers that correlate better with RORγt expression than the CRs currently used for that purpose.
CHAPTER 4: EXPRESSION OF TRAFFICKING RECEPTORS 
BY REGULATORY T CELLS IN RHEUMATOID ARTHRITIS

4.1 Introduction

CD4+ regulatory T cells (Tregs) expressing the IL-2Rα chain (CD25) and the master regulator Foxp3 transcription factor play an important role in controlling autoimmune responses [36]. The strongest evidence for the importance of Tregs in maintaining immune tolerance in humans is the syndrome of multi-organ autoimmunity, dermatitis and allergy that results from a dysfunctional FoxP3 gene (immunodysregulation, polyendocrinopathy, enteropathy X-linked (IPEX) syndrome) [62]. However, most human autoimmune diseases are not caused by such a complete dysfunction of Tregs, and it is believed that more subtle imbalances in Treg frequency, function and development may serve as predisposing factors for disease.

Rheumatoid arthritis (RA) is an inflammatory autoimmune disease characterised by the accumulation of inflammatory cells in the joints, resulting in tissue destruction and severe disability [186, 187]. The first evidence of self-reactivity in RA was the identification of rheumatoid factor, an autoantibody, in the blood of affected patients [188]. It became apparent that T cells played a crucial role in the pathogenesis of RA in the early 1980s, when the synovial tissue and fluid from patients was shown to be enriched with T cells, predominantly CD4+ T cells [189, 190]. The processes that initiate and cause the progression of a dysregulated T-cell dependent response in RA are still unknown.

Tregs have been shown to play a critical role in the control of autoimmune arthritis in experimental models [191-193]. However, the involvement of Tregs in human RA is still not well understood. Some studies have shown that Treg numbers in the peripheral blood (PB) of RA patients are reduced compared with controls [65, 194] whereas others have found them to
be increased [195, 196] or unchanged [197-199]. Reduced suppressive function of Tregs derived from the PB of RA patients has been reported in some studies [197, 198, 200], whereas others have reported normal suppressive capacity of Tregs from RA patients [194, 195, 201].

It is clear there is conflicting evidence on the frequency and function of Tregs in RA. This is partly due to the fact that human Tregs were originally identified as CD25\textsuperscript{high} [60] making it hard to distinguish Tregs from CD25\textsuperscript{+} conventional T cells, particularly in human blood where up to 20-40\% of PB CD4\textsuperscript{+} T cells express CD25 to some extent [54]. There is a lack of consensus on the precise demarcation between CD25\textsuperscript{high} and CD25\textsuperscript{+} expression. For this reason, studies in RA patients have defined Tregs as CD4\textsuperscript{+}CD25\textsuperscript{+} [195, 199], CD4\textsuperscript{+}CD25\textsuperscript{high} [194, 196, 197, 200] and CD4\textsuperscript{+}CD25\textsuperscript{bright} [65, 201]. This has made it difficult to compare results from individual studies and to reach conclusions on the role of Tregs in RA.

Nevertheless, Tregs have consistently been shown to be enriched in the synovial fluid of RA patients when compared to PB [65, 194, 195, 199, 201, 202]. To facilitate their entry into peripheral tissues, Tregs express a vast array of integrins and chemokine receptors (CR) that have been shown to be crucial to their role in regulating immune responses [81, 90-92]. Emerging evidence also suggests that distinct CR expression patterns may contribute to selective trafficking and compartmentalization of Tregs at sites where regulation is required [112, 113, 118]. Despite this, chemokine receptor expression by Tregs in the PB or joints of RA patients has not been examined.

We have previously shown that low surface expression of CD127, the \(\alpha\) chain of the IL-7 receptor, in combination with CD25 can distinguish CD25\textsuperscript{+}CD127\textsuperscript{lo} Tregs from conventional CD4\textsuperscript{+} T cells (Tconvs) in human PB [61]. In the current study, we used nine-colour flow cytometry to analyse the levels of CXCR3\textsuperscript{+}, CCR4\textsuperscript{+}, CCR5\textsuperscript{+} and CCR6\textsuperscript{+} conventional T cells and CD25\textsuperscript{+}CD127\textsuperscript{lo} Tregs in the PB of RA patients. We hypothesised that the correct localisation of Tregs is vital to their role in preventing and regulating joint inflammation and
that deficiencies in Treg migratory subsets may play a role in disease pathogenesis. We also applied a novel method of using bioinformatic analysis software to analyse flow cytometric data, allowing us to simultaneously draw unsupervised, automated comparisons between Tconv and Treg subsets in RA patients and controls.

4.2 Summary of methods

4.2.1 Patients

Peripheral blood specimens were collected from 34 patients with rheumatoid arthritis (RA) (65% female, average age 56.4 years) and 34 sex and age matched controls (65% female, average age 55.6 years). Patients with RA, diagnosed according to the European League Against Rheumatism (EULAR) 2010 criteria, were enrolled in the study over a period of one year by Dr Diana Chessman at Concord Repatriation General Hospital (Concord, NSW, Australia). All patients and controls gave written, informed consent and the study was approved by the Sydney Local Health District ethics review committee at Concord Repatriation General Hospital.

Clinical information including disease duration, disease activity, as assessed by the disease activity score in 28 joints (DAS28), treatment, smoking status and seropositivity was recorded at the time the blood was collected from RA patients.

4.2.2 Cell isolation

Peripheral blood mononuclear cells (PBMC) were isolated from heparinised venous blood by density gradient centrifugation using Ficoll-Paque Plus (GE Healthcare). The isolated mononuclear cells were stored in 1 ml aliquots at -180°C in LNO2 (details in Chapter 2, section 2.3)
4.2.3 Flow cytometric analysis

Cells were thawed in batches and surface stained at 4°C for 45 minutes (details in Chapter 2, section 2.4) using the following antibodies: V500-conjugated CD4 (RPA-T4, BD Horizon), Brilliant violet 421-conjugated CD25 (M-A251, BD Horizon), phycoerythrin (PE)-CF594-conjugated CD127 (HIL-7R-M21, BD Horizon), biotin-conjugated CD45RO (UCHL1, BioLegend), streptavidin-DyLight-800 (Thermo Scientific), Alexa Fluor 488-conjugated CXCR3 (1C6, BD Pharmingen), PE-conjugated CCR6 (11A9, BD Pharmingen), allophycocyanin (APC)-conjugated CCR4 (L291H4, BioLegend) and Alexa Fluor 700-conjugated CCR5 (HEK/1/85a, BioLegend). A LIVE/DEAD Fixable Blue stain (Invitrogen) was used to exclude dead cells from analysis (antibodies listed in Table 2.1).

Data was acquired on a LSRII 10-laser flow cytometer (BD) and analysed using FlowJo software (Tree Star). A general gating strategy was employed for the analysis of all flow data (Figure 2.1, description in Chapter 2, section 2.4.6).

A batch of samples from RA patients and their matched controls were thawed on each day to avoid variability in comparing patients and controls due to flow cytometer instability. PBMCs from a single control not included in the study were included in each run to allow us to monitor flow cytometer stability and standardize performance.

4.2.4 Statistical analysis

Statistical analysis was performed using MultiExperiment Viewer (MeV) (TM4–Microarray Software Suite). Data was loaded into MeV in .txt format and heatmaps were generated. Differences between RA patients and controls were evaluated using an automated Mann-Whitney t-test. A Kruskal-Wallis test was used for identification of differences between RA patients treated with TNF inhibitors, RA patients not treated with TNF inhibitors and controls. P values less than 0.05 were considered significant. Unsupervised cluster analysis was performed using the hierarchical clustering (HCL) algorithm.
We also used Graph Pad Prism 6 for analysis. Group means were compared using the Mann-Whitney t-test or the Kruskal-Wallis test. Correlations were determined using the Spearman correlation test. P values less than 0.05 were considered significant.

4.3 Results

4.3.1 Chemokine receptor expression by conventional and regulatory T cells in the peripheral blood of patients with RA and healthy controls.

We analysed the chemokine receptor (CR) expression by conventional T cells (Tconvs) and regulatory T cells (Treg) in the peripheral blood (PB) of 34 RA patients and 34 age and sex matched controls. Demographic and clinical information for the RA patients are presented in Table 4.1.

We have previously shown that the expression of CD127, the α-chain of the interleukin-7 receptor, along with CD25 allows for the accurate identification of CD25+CD127lo Tregs in human PB [61]. In this study, we developed a nine-colour flow cytometry panel that allowed us to use this definition of Tregs to investigate the expression of the chemokine receptors CXCR3, CCR4, CCR6 and CCR5 on Tregs and Tconvs in hope that an analysis of circulating Treg/Tconv subsets could provide a more accurate indication of their activity within tissues.

Representative histograms showing expression of these chemokine receptors on Tregs and Tconvs can be seen in Figure 4.1. Tregs were defined as CD25+CD127lo (Figure 4.1A) and Tconvs as everything outside this gate. Figure 4.1C shows the correlation between the percentage of Tregs identified as CD25+CD127lo and FOXP3+CD127lo in 15 of the RA patients. The frequency of Tregs within the two gates in each individual was very highly correlated.
Table 4.1 Clinical characteristics of RA patients included in study

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Patients (n=34)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, median (range), years</td>
<td>59 (29-82)</td>
</tr>
<tr>
<td>% Female</td>
<td>65</td>
</tr>
<tr>
<td>Disease duration, median (range), years</td>
<td>8 (0.5-30)</td>
</tr>
<tr>
<td>% Ever smoked</td>
<td>47</td>
</tr>
<tr>
<td>% Seropositive (RF and/or CCP) (n=29)</td>
<td>69</td>
</tr>
<tr>
<td>Disease activity (DAS28), mean ± SD</td>
<td>3.07 ± 1.5</td>
</tr>
<tr>
<td>% Methotrexate users</td>
<td>71</td>
</tr>
<tr>
<td>% Corticosteroid users</td>
<td>62</td>
</tr>
<tr>
<td>% TNFi users</td>
<td>29</td>
</tr>
</tbody>
</table>

RA = rheumatoid arthritis; RF = rheumatoid factor; CCP = Cyclic citrullinated peptide; TNFi = Tumour necrosis factor inhibitor
Figure 4.1. Representative histograms of the expression of chemokine receptors (CRs) CXCR3, CCR4, CCR6 and CCR5 by conventional T cells (Tconvs) and regulatory T cells (Tregs) in the peripheral blood of patients and controls. Mononuclear cells were isolated as described and examined by flow cytometry for the expression of markers that allowed for the identification of Tregs, defined as CD4⁺CD127loCD25+ (A), and Tconvs, defined as everything outside the indicated Treg gate. Tregs and Tconvs were subdivided on the basis of CD45RO expression in order to differentiate between CD45RO⁺ (effector/memory) and CD45RO⁻ (naïve) subsets, and analysed for the indicated CRs. Expression of the CRs by Tconvs and Tregs was analysed using two gates (B). CR⁺CD45RO⁺ cells in the upper right gate and CR⁺CD45RO⁻ cells in the upper left gate. Numbers indicate cells within the gate as a percentage of total CD45RO⁺ or CD45RO⁻ Tconvs and Tregs. (C) Correlation between the frequencies of CD4⁺ Tregs identified by gating for CD127loCD25⁺ versus CD127loFOXP3⁺ in 15 RA patients. The correlation was statistically evaluated using a non-parametric Spearman test (****).
Because the ability to enter non-lymphoid tissues is closely correlated with post-thymic differentiation in response to antigen, expression of chemokine receptors was examined along with CD45RO, an effector/memory marker, on both Tconvs (Figure 4.1B, upper panel) and Tregs (Figure 4.1B, lower panel). Effector/memory (CD45RO+) Tconvs and Tregs expressing the chemokine receptor of interest, calculated as a percent total CD45RO+ Tconvs/Tregs, appear in the upper right gate and naïve (CD45RO-) Tconvs and Tregs expressing the chemokine receptor of interest, calculated as a percent total CD45RO- Tconvs/Tregs, in the upper left gate. These subsets were quantified for all 68 patients and controls and used for further analysis.

**4.3.2 Decreased frequency of CXCR3+, CCR4+, CCR5+ and CCR6+ effector/memory Tregs in the PB of RA patients when compared with healthy controls.**

We used a bioinformatic analysis software, MeV, to draw simultaneous, automated comparisons between multiple Tconv and Treg subset data obtained from flow cytometry. Data was uploaded into the software and a heat map was generated (Figure 4.2A), providing a simple way to visualize all the data simultaneously. Each column represents an RA patient (left) or control (right) and each row the percent of cells within each Tconv or Treg subset, gated as indicated in Figure 4.1.

Once data is loaded into MeV and a heatmap is generated, various statistical and clustering tools can be applied to allow for the identification of significant similarities and differences between subject groups. We next performed an automated t-test to identify the significant differences in Tconv and Treg subset frequencies between RA patients and controls. This identified nine subsets that were shown to be significantly different between patients and controls, seven of which were decreased in RA and two of which were increased, shown in a heat map in Figure 4.2B. Three of these subsets were in the Tconv compartment and six were within Tregs.
**Figure 4.2.** Decreased expression of CXCR3, CCR4, CCR5 and CCR6 on effector/memory Tregs in the peripheral blood of rheumatoid arthritis (RA) patients (n=34) when compared with healthy controls (n=34). Peripheral blood mononuclear cells from patients and controls were examined by flow cytometry for the expression of CXCR3, CCR4, CCR5 and CCR6 within their Tconv and Treg (CD4+CD127loCD25+) compartment. (A) Data obtained from flow cytometric experiments is visualised colorimetrically in a heat map. Each row represents a Tconv or Treg subset; each column a patient or control. Frequencies that are increased relative to the row’s mean are indicated in yellow, decreased in blue and frequencies that show no difference are indicated in black. (B) An automated Mann-Whitney t-test indicated that nine subsets displayed significantly different frequencies between patients and controls (p<0.05), shown here in a heat map. Seven of these subsets were found to be significantly decreased in RA patients. (C) The nine Treg and Tconv subsets selected by statistical analysis to be significantly different between patients and controls were next used for an unsupervised hierarchical clustering where subjects are organized based on the overall similarity in expression patterns. Subjects clustered in two groups; one consisting mainly of RA patients (red) and the other controls (green). The nine Tconv (D) and Treg (E, F) subsets displayed as bar graphs. Each point represents an individual subject and horizontal lines represent means. The Mann-Whitney t-test was used to compare differences between patients and controls. * = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001.
Having identified nine subsets that were significantly different between RA patients and controls, we next asked whether these subsets in combination could be used to differentiate between patients and controls. We performed an unsupervised hierarchical clustering of the 68 patients and controls based on the frequencies of the nine subsets that differed significantly between patients and controls (Figure 4.2C). 30 out of the 34 RA patients clustered together, with 4 patients clustering in the control group. Furthermore, 30 out of the 34 controls clustered together with 4 controls clustering with patients.

Figure 4.2 D-F illustrates the nine subsets identified by MeV to be significantly between patients and controls in traditional graphs. CXCR3+ and CCR5+ effector/memory Tconvvs were found to be decreased in the PB of RA patients when compared to controls (mean 29.7% vs 49%, and 13.2% vs 20.8%) whereas CCR4+ effector/memory Tconvvs were increased in RA (mean 70.4% vs 66.4%) (Figure 4.2D). Total effector/memory (CD45RO+) Tregs were found to be significantly decreased in RA patients (mean 54.4% vs 63.9%), subsequently, naïve (CD45RO−) Tregs were increased in RA patients (mean 45.6% vs 36.2%) (Figure 4.2E). Within the effector/memory Treg compartment, frequencies of all the chemokine receptors examined were shown to be decreased in patients with RA (Figure 4.2F) (CXCR3+; mean 16.2% vs 37.2%, CCR4+; mean 90.6% vs 94.2%, CCR5+; mean 19.5% vs 31.1%, CCR6+; mean 48.6% vs 60.4%).

Having found significant differences between patients and controls in the proportions of Tconv and Treg subsets, we next examined whether the percent of cells within subsets correlate with disease activity as measured by DAS28 (Figure 4.3). The frequency of both total effector/memory (CD45RO+) Tconvvs and CXCR3+ effector/memory (CD45RO+) Tconvvs correlated negatively with DAS28 (p < 0.01). Thus we showed that CXCR3+ effector/memory Tconvvs were greatly reduced in RA (p < 0.0001) (Figure 4.2D) and that this decrease was more pronounced in patients with more active disease. No significant correlations were found with seropositivity and smoking status.
Figure 4.3. Total effector/memory conventional T cells (Tconvs) and CXCR3+ effector/memory Tconvs negatively correlate with DAS28 in RA patients (n=32). Peripheral blood mononuclear cells from RA patients were examined by flow cytometry for the expression of the indicated chemokine receptors by their Tcon and Treg (CD4+CD127loCD25+) compartment. The disease activity score based on 28 joints (DAS28) was calculated for each patient at the time the peripheral blood specimen was collected. The Spearman correlation test was used to test for significance. ** = p < 0.01. Dashed lines represent the mean in the control group.
4.3.3 TNF inhibitor therapy may alter the frequency of total effector/memory Tregs, CCR4+ effector/memory Tregs and CCR6+ effector/memory Tregs.

We next wished to determine whether incorporating clinical parameters into the analysis of data from RA patients would give us insights into whether any of these clinical parameters significantly affect the frequency of Tconv and Treg subsets (Figure 4.4). We performed an unsupervised hierarchical clustering of the 34 RA patients based on the frequencies of their Tconv and Treg subsets and four clinical parameters; DAS28, seropositivity, smoking status and TNF inhibitor (TNFi) use. This analysis clustered patients into three groups, one of which consisted 7 out of the 10 RA patients on TNFi therapy (boxed in red).

To explore the effect of TNFi use on the frequencies of the Tconv and Treg subsets examined, we categorized patients into two groups, TNFi treated (n=10) and non-TNFi treated (n=24). There were no significant differences between TNFi treated and non-TNFi treated in age (mean 58 vs 57), DAS28 (mean 3.1 vs 3.0) and methotrexate (MTX) use (70% vs 70.8% MTX users). The TNFi treated patients had slightly longer disease duration (mean 13.3 vs 7.5 years). We performed an automated Kruskal-Wallis test in order to identify the significant differences in Tconv and Treg subset frequencies between RA TNFi treated, RA non-TNFi treated and controls. This identified eleven subsets that were significantly different between these three groups. An unsupervised hierarchical clustering was generated on these eleven subsets, again resulting in 7 out of the 10 TNFi treated patients falling into one cluster (Figure 4.5A).

Graphs of the eleven subsets identified by the automated Kruskal-Wallis test are presented in Figure 4.5, B&C. Effector/memory Tconvs were elevated in the PB of RA patients on TNFi therapy when compared to RA patients not on TNFi therapy (mean 45.0% vs 28.6%). In Figure 4.2 we showed that the frequency of effector/memory Tregs were decreased in the PB of RA patients. This decrease is not evident when comparing TNFi treated with controls (mean 62.1% vs 63.9%), suggesting TNFi use normalises effector/memory Treg frequency.
Unsupervised hierarchical cluster analysis of RA patients based on frequencies of Tconv/Treg subsets and clinical parameters results in patients on TNF inhibitor therapy to fall closely together. Peripheral blood mononuclear cells from RA patients were examined by flow cytometry for the expression of CXCR3, CCR4, CCR5 and CCR6 within their Tconv and Treg (CD4+CD127loCD25+) compartment. Data obtained from flow cytometric experiments and clinical information recorded at the time the peripheral blood specimen was taken is visualised colorimetrically in a heat map. Each row represents a Tconv or Treg subset or a clinical parameter; each column represents a patient (n=34). Frequencies that are increased relative to the row’s mean are indicated in yellow, decreased in blue and frequencies that show no difference are indicated in black. Unsupervised hierarchical clustering caused patients to fall into three groups; one consisting of 7 out of the 10 RA patients on TNF inhibitor therapy (boxed in red).
Figure 4.5. Frequencies of eleven Tconv and Treg subsets were significantly different between RA patients on TNF inhibitors (RA+TNFi), RA patients not on TNF inhibitors (RA) and controls. Peripheral blood mononuclear cells from patients and controls were examined by flow cytometry for the expression of CXCR3, CCR4, CCR5 and CCR6 within their Tconv and Treg (CD4+CD127loCD25+) compartment. (A) An automated Kruskal-Wallis test indicated that eleven subsets displayed significantly different frequencies between RA+TNFi (n=10), RA (n=24) and controls (n=34) (p<0.05). These eleven subsets were used for an unsupervised hierarchical clustering where subjects are organized based on the overall similarity in expression patterns. One cluster consists of 7 out of the 10 RA patients on TNF inhibitor therapy (boxed in red). Data is visualised here in a heat map where each row represents a Tconv or Treg subset; each column a patient or control. Frequencies that are increased relative to the row’s mean are indicated in yellow, decreased in blue and frequencies that show no difference are indicated in black. The eleven Tconv (B) and Treg (C) subsets selected by statistical analysis to be significantly different between the three groups displayed as bar graphs. Each point represents an individual subject and horizontal lines represent means. The Kruskal-Wallis test was used to compare differences between RA+TNFi, RA and controls. * = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001.
This trend was also true for CCR4\(^+\) and CCR6\(^+\) effector/memory Tregs, where no significant differences between TNFi treated patients and controls were evident, whereas non-TNFi treated patients had significantly reduced frequency of these subsets when compared to controls (Figure 4.5C). This trend was not evident for CXCR3 and CCR5 expressing effector/memory Tregs, where both TNFi treated and non-TNFi treated patients showed significantly decreased frequency when compared to controls. Interestingly, we found a significantly reduced frequency of CXCR3\(^+\) naïve Tregs in the PB of RA patients on TNFi therapy when compared to controls (mean 4.8\% vs 8.6\%). This trend was not seen in RA patients not on TNFi therapy.

4.4 Discussion

There has been a degree of confusion regarding the level of Tregs in the PB of RA patients [65, 194-199]. This is in part due to the fact that Tregs were originally identified as CD25\(^{\text{high}}\) [60], making it difficult to determine the precise demarcation between CD25\(^{\text{high}}\) and CD25\(^{+/\text{intermediate}}\). We have previously shown that human Tregs can be accurately identified as CD4\(^+\)CD25\(^{\text{CD127lo}}\) [61]. We set out to use this definition to analyse the total frequency of Tregs, as well as that of various subsets of effector/memory and naïve Tregs that express the chemokine receptors CXCR3, CCR4, CCR5 and CCR6, in the peripheral blood of patients with RA (Figure 4.1).

We report that the total frequency of CD4\(^+\)CD25\(^{\text{CD127lo}}\) Tregs in RA PB is unchanged when compared to controls. However RA patients display significantly reduced frequency of effector/memory Tregs in their PB (Figure 4.2). Two other studies have used the low expression of CD127 in combination with CD25 to examine Treg levels in RA [203, 204]. One of these studies also reported no difference in the CD25\(^{\text{CD127lo}}\) Treg frequency when comparing patients and controls, but demonstrated that RA Tregs displayed impaired suppressive function [203]. The other study reported that CD25\(^{\text{hi}}\)CD127\(^{\text{low}}\). Tregs were decreased in RA PB, particularly in active RA [204]. However on close examination of the
flow cytometric gating strategy used in that study, it is clear that CD25<sup>hi</sup> cells were first selected and then examined for low CD127 expression, therefore excluding a significant proportion of CD25<sup>-</sup>CD127<sup>lo</sup> Tregs from analysis. Interestingly, the CD25<sup>hi</sup>CD127<sup>low/</sup> Tregs this study described may be representative of effector/memory Tregs, as it is known that activated Tregs display higher expression of CD25 [54, 131]. Then this study is consistent with our finding that effector/memory Tregs are reduced in RA PB.

A number of studies have used FoxP3 expression to identify Tregs in RA. Our results are consistent with one group that found CD45RA FoxP3<sup>hi</sup> activated/effector Tregs to be reduced in the PB of RA [205]. Total CD25<sup>high</sup>FoxP3<sup>+</sup> cells have also been reported to be reduced in RA PB, but in this report CD25<sup>high</sup> cells were first selected and then examined for Foxp3 expression, again eliminating a large proportion of Tregs [206]. Levels of CD4<sup>+</sup>FoxP3<sup>+</sup> cells have been reported by one group to be unchanged in the PB of RA, although the average frequency of these CD4<sup>+</sup>FoxP3<sup>+</sup> cells was 1.7% in controls and 1.9% in RA, indicating that the majority of Tregs were again missing from analysis [207]. It is clear that there is a critical need to standardize methods for Treg identification in the study of RA, allowing for comparisons across studies and for broader conclusions to be made.

CD4<sup>+</sup> conventional T cells (Tconvs) make up a large proportion of the inflammatory cells within the RA joint and contribute significantly to the inflammation [188]. CD25<sup>+</sup>CD127<sup>lo</sup> Tregs have also been shown to be enriched in the synovial fluid (SF) of RA patients [202], although difficulty in accessing control SF makes it hard to draw conclusions on whether the Tconv to Treg ratio is altered in RA. PB, on the other hand, is accessible to analysis and the detection of CR expression by blood Tconv and Treg subsets in RA patients may provide insights into the number of Tconvs and Tregs that migrate into joints. The co-localisation of Tregs with Tconvs in tissues has been shown to be crucial to their role in regulating immune responses [112, 113, 118]. It has been demonstrated that CXCR3<sup>+</sup> Tregs migrate to sites of Th1 mediated inflammation whereas CXCR3 deficient Tregs fail to localise and control this
type of inflammation [112]. Furthermore, CCR6 deficient Tregs fail to enter sites of Th17 mediated inflammation resulting in dysregulated Th17 responses [118].

Despite the known importance of Treg homing, the chemokine receptor expression by Tregs in the PB or joints of RA patients has not been examined. We hypothesised that a reduced frequency of Tregs expressing certain chemokine receptors may result in less Treg entry into the joint prior to ongoing inflammation, consequently leading to the initiation and progression of a chronically dysregulated immune response. We found a significantly reduced frequency of effector/memory Tregs expressing all the chemokine receptors examined (CXCR3, CCR4, CCR5 and CCR6) in the PB of RA patients. We also found that a smaller proportion of effector/memory Tconvs in RA PB expressed CXCR3 and CCR5 (Figure 4.2). This could indicate an initial, primary trafficking deficiency resulting in fewer total CD4⁺ cells entering the joints, with the consequence of disrupted homeostasis. It has been shown that co-culturing synovial tissue cultures from RA patients with CD4⁺ cells (containing Tregs) results in reduced IFN-γ production [208]. This regulatory effect was more pronounced when CD4⁺ cells were enriched for CD4⁺CD25⁺ Tregs were used. It is therefore possible that, under normal conditions, mixtures of Tconvs and Tregs enter the joint to prevent uncontrolled inflammation and that a homing deficiency on these cells could result in disrupted homeostasis and inflammation.

T cells infiltrating the joint have been shown to predominantly express the chemokine receptors CXCR3 and CCR5 whereas CCR4 and CCR6 are largely absent [209-214]. Interestingly, CXCR3 and CCR5 are the receptors that showed the most marked decrease in the PB of RA patients. Thus it could be argued that a reduction in CD4⁺ cells expressing these receptors in the blood could be due to the accumulation of these cells in the joint. This hypothesis would indicate a strong negative correlation between PB CXCR3⁺ and CCR5⁺ Tregs and disease activity. However, in our analysis, none of the frequencies of effector/memory Treg subsets that express CXCR3, CCR4, CCR5 and CCR6 correlated with disease activity scores calculated based on 28 joints (Figure 4.3). If the reduction of these
Tregs in the blood was due to an accumulation in the joint, higher joint inflammation scores should correspond to fewer effector/memory cells within these subsets in the blood.

Furthermore, studies in mice have shown that during the later stage of an immune response, disease specific CD4+ T cells equilibrate with the circulation [215, 216], thus suggesting that the measurement of CD4+ T cell subsets in the blood can provide a relevant correlate of chronic immune responses in the joint. In a murine melanoma model developed in our lab to study the effects of tumour-specific CD4+ T cells on tumour control, it was demonstrated that CD4+ T cells in the blood, lymph nodes, spleen and within the tumours were in equilibrium by day 40 of the response [215]. Additionally, another study in mice investigating the dynamics associated with T-cell localisation following infection of the skin by herpes simplex virus has shown that as the response enters the memory phase, virus specific memory CD4+ T cells within the dermis are largely in equilibrium with the circulation [216].

The importance of chemokine receptor driven cell recruitment in inflammatory disease has led to the development of drugs targeting these receptors. However clinical trials have not yielded promising results [217, 218]. This could be due to a large gap in our understanding of the importance of Treg homing to these sites and the finely tuned Tconv to Treg balance required to maintain immune homeostasis.

TNF-α is a key cytokine involved in RA joint inflammation and its blockade has proven to be an effective treatment [219]. Although TNF-α-inhibitors have a moderate to high (60%) rate of drug continuance due to sustained low disease activity and improved quality of life, inefficacy remains the main cause of discontinuation [220, 221]. The mechanism underlying the effectiveness of TNF-α blockade is not completely understood, consequently hindering our ability to select patients who will effectively respond to treatment.

TNF-α-inhibitors have been shown to reduce inflammatory cell migration into the RA joint [222]. It has been suggested that an increase in CD4 T cells expressing CXCR3 in the PB of
Chapter 4: Tregs in Rheumatoid Arthritis

RA patients following TNF-α-inhibitor therapy is a result of reduced infiltration of CXCR3+ cells into the joint [223, 224]. We showed a significant increase of total effector/memory T cells in the PB of patients on TNF-α-inhibitors when compared to patients not on TNF-α-inhibitors (Figure 4.5). We also saw a trend towards increased CXCR3+ CD4 T cells, but this did not reach statistical significance. It is possible that this is due to altered lymphocyte trafficking as a result of therapy, causing cells to accumulate in the blood.

Recent evidence suggests that TNF-α-inhibitors are able to normalise Treg cell function and induce an increase in distinct populations of Tregs in patients with RA [197, 206, 225-227]. The first of these studies demonstrated that CD4+CD25+ “Treg” cells isolated from patients with RA were unable to suppress pro-inflammatory cytokine secretion and that this effect could be reversed with anti-TNF-α treatment [197]. It has also been reported that Tregs from patients responding to adalimumab, a TNF-α-inhibitor, were able to inhibit IL-17 production in vitro whereas Tregs from non-TNF-α-inhibitor treated patients could not [226]. One study attributed impaired Treg cell function in RA to TNF-α in the inflamed joint inducing FoxP3 dephosphorylation. In this study, Treg function was again shown to be restored by treatment of patients with TNF-α-inhibitors [227].

The levels of total CD4+FoxP3+ Tregs in the PB of patients have been shown to be unaffected by TNF-α-inhibitor therapy [228, 229]. However, subsets of CD4+FoxP3+CD62L- Tregs have been shown to be increased in the PB of patients on anti-TNF-α therapy [225]. We also showed the levels of total Tregs to be unaffected, but we showed that treatment with TNF-α-inhibitors was associated with normalisation of the numbers of effector/memory Tregs and effector/memory Tregs expressing the chemokine receptors CCR4 and CCR6 (Figure 4.5). Ours is the first study to look at the effect of TNF-α-inhibitor therapy on the chemokine receptor expression of Tregs in RA.

A high degree of heterogeneity has been recognised in RA, making it a suitable condition for the application of personalised medicine. The identification of biomarkers that can guide
diagnosis, prognosis and treatment selection will have many benefits at the individual and societal level. High throughput genomic and transcriptomic technologies are increasingly being applied to clinical samples in RA in hope of identifying signatures that can predict and improve outcome. However, no one has yet applied bioinformatic analysis tools to flow cytometric data. Flow cytometry involves the rapid quantification of multiple parameters for millions of cells. The manual analysis of cell subset data derived from such high-throughput experiments is extremely time consuming, particularly when determining correlations between multiple cell subsets in multiple subject groups. For this reason, there is a need for more efficient data viewing and analysis methods. We used bioinformatic tools to analyse flow cytometry data obtained in our study. Automated clustering allowed us to identify nine cell subsets that in combination were able to differentially cluster RA patients and controls (Figure 4.2).

In order to find a signature that can predict clinical outcome, it is important to be able to combine experimental data with clinical measures, again highlighting the significant role bioinformatic analysis can play in the identification of relevant clinical correlations. When we combined clinical measures into our analysis, we found that patients on anti-TNF-α therapy clustered together (Figure 4.4).

Although anti-TNF-α therapies are among the most effective, they have serious potential adverse effects and not all patients respond [230]. Despite this, we still do not have ways to select patients for anti-TNF-α treatment. It is important to ask whether the patients on anti-TNF-α therapy in our study clustered together due to the effects of treatment or because they had different subset frequencies to begin with, highlighting the possibility of identifying patients suited to anti-TNF-α therapy before they are trialled on other non-effective treatment. This highlights the power of bioinformatic tools for identifying predictive signatures in data obtained from flow cytometry. This type of information will be obtained in future experiments with longitudinal studies in which pre and post anti-TNF-α treatment samples are compared.
In RA, selecting the most effective treatment is currently a case of trial and error. Therefore, finding factors that can identify preclinical RA or select patients for treatment are crucial. We hope our methods of analysis will help the many groups that use flow cytometry to monitor cell subsets in RA patients, allowing them to retrieve the most out of their data. It is also crucial to study the effects other therapies, such as methotrexate and corticosteroids, have on Treg subsets in RA patients. Future studies need to take this into account and recruit larger well-defined patient cohorts including patients that are treatment naïve.
CHAPTER 5: EXPRESSION OF TRAFFICKING RECEPTORS BY REGULATORY T CELLS IN PSORIASIS

5.1 Introduction

CD4⁺CD25⁺FoxP3⁺ regulatory T cells (Tregs) are crucial in preventing autoimmune responses. This role was first demonstrated in mice when it was shown that the transfer of CD4⁺ T cells depleted of CD25-expressing cells into immuno-deficient mice caused the development of a number of organ-specific autoimmune diseases [1]. In this model, the co-transfer of CD4⁺CD25⁺ cells prevented autoimmune disease in a dose-dependent fashion. In humans, a dysfunctional FoxP3 gene causes multi-organ autoimmunity (immunodysregulation, polyendocrinopathy, enteropathy X-linked (IPEX) syndrome) [62], signalling that Tregs are essential for suppressing autoimmune responses. However, a complete dysfunction of Tregs is not evident in most human autoimmune diseases and it is thought that minor deficiencies in Treg frequency, function and development may predispose individuals for disease.

Psoriasis is a chronic autoimmune skin disease of unknown etiology characterised by the formation of well demarcated raised skin lesions containing hyperproliferative keratinocytes and a dense lymphocytic infiltrate [231]. The disease affects ~2% of the world’s population [232] and leads to a significant impairment in the quality of life in patients due to social stigmatisation, pain, physical disability and psychological distress [233]. In the past, psoriasis was believed to be caused by an accelerated turnover of keratinocytes, but various studies have since established an immunological basis for the disease. As early as 1979, histological studies showed that the earliest events in the pathogenesis involved the dilation of blood vessels in the dermis followed by the appearance of a mononuclear cell infiltrate [234]. This mononuclear cell infiltrate was later shown to consist mainly of T cells [235, 236], predominantly CD4⁺ T cells [237].
Given the presence of a dense T cell infiltrate, it is plausible to hypothesise that impaired development and function of Tregs might be involved in the pathogenesis of the disease, resulting in deregulated T-cell mediated immune responses and disrupted skin homeostasis. We have shown that a large proportion of Tregs in healthy humans express CLA (Figure 3.4), an adhesion molecule that allows migration into the skin. This suggests that Tregs may be involved in maintaining skin homeostasis, even in the absence of ongoing inflammation.

There is conflicting evidence on the frequency and function of Tregs in psoriasis. Because human Tregs were originally identified as CD25\textsuperscript{high} [60], it was difficult to distinguish Tregs from CD25\textsuperscript{+} conventional T cells. For this reason, some studies have shown that Tregs comprise similar proportions of CD4\textsuperscript{+} T cells in psoriatic and normal blood [238-240], while others have reported Tregs to be reduced [65, 241] and others increased [242]. In one study, it was found that psoriatic patients had dysfunctional blood and target tissue CD4\textsuperscript{+}CD25\textsuperscript{hi} cell activity [238]. CD4\textsuperscript{+}CD25\textsuperscript{hi} cells taken from these patients were functionally deficient in suppressing effector T cell responses \textit{in vitro}, although this deficiency was not found to be absolute, with higher numbers of these CD4\textsuperscript{+}CD25\textsuperscript{hi} “Treg” cells providing similar suppression to normal cells.

Nevertheless, numerous studies have consistently shown Tregs to be enriched in psoriatic skin lesions [242-250]. In order to enter peripheral tissues, Tregs express a range of integrins and chemokine receptors (CR) that have been shown to be essential to their role in regulating inflammation [81, 90-92]. Recent evidence also suggests that distinct CR expression patterns may contribute to selective trafficking and compartmentalisation of Tregs at sites where regulation is required [112, 113, 118]. Despite these findings, the expression of adhesion molecules and CRs by Tregs in the PB or skin of psoriasis patients has not been examined.

We have previously shown that low surface expression of CD127, the \(\alpha\) chain of the IL-7 receptor, in combination with CD25 can distinguish CD25\textsuperscript{+}CD127\textsuperscript{lo} Tregs from conventional
CD4+ T cells (Tconvs) in human PB [61]. In the current study, we used 11 and 13-colour flow cytometry to analyse the levels of nine chemokine receptors and integrins (β7, CD49d, CLA, CCR4, CCR10, CCR5, CD62L, CXCR3 and CCR6) on conventional T cells and CD25+CD127lo Tregs in the PB of psoriasis patients. Since psoriasis has been shown to involve both Th1 and Th17 type immune processes [251, 252], we also examined the levels of T-bet+ Th1 and RORγt+ Th17 type cells in the PB of patients and controls.

We hypothesised that the trafficking and localisation of Tregs is crucial to their function in regulating inflammation in human skin and postulate that an understanding of Treg trafficking in psoriasis could contribute to the characterisation of disease subsets and the development of more targeted Treg-therapy.

### 5.2 Summary of methods

**5.2.1 Patients**

Peripheral blood samples were obtained from patients treated by Professor Wolfgang Weninger and Dr Patricia Lowe at the Dermatology Centre of Royal Prince Alfred Hospital in Gloucester House (Camperdown, NSW, Australia). Psoriasis was diagnosed based on clinical presentation. Psoriatic arthritis was diagnosed using the Classification Criteria for Psoriatic Arthritis (CASPAR) [253]. Specimens were collected from 44 patients with chronic psoriasis (77% male, average age 47.6 years) and 44 sex and age matched controls (77% male, average age 49.1 years). Clinical information including disease duration, disease activity as assessed by the psoriasis area severity index (PASI), and treatment were recorded at the time of blood collection.

All patients and controls gave written, informed consent and the study was approved by the Sydney Local Health District ethics review committee at Royal Prince Alfred Hospital, Camperdown.
5.2.2 Cell isolation

Peripheral blood mononuclear cells (PBMC) were isolated from heparinised venous blood by density gradient centrifugation using Ficoll-Paque Plus (GE Healthcare). The isolated mononuclear cells were stored in 1 ml aliquots at -180°C in LNO₂ (detailed in Chapter 2, section 2.3).

5.2.3 Flow cytometric analysis

Cells were thawed in batches and immuno-stained with two optimised 11 and 13- colour flow cytometric panels (Table 2.2, Figures 2.10 & 2.11). Details of the monoclonal antibodies used are listed in Table 2.1 and staining procedure is described in Chapter 2, section 2.4. Data were acquired on a LSRII 10-laser flow cytometer (BD) and analysed using FlowJo software (Tree Star). A general gating strategy was employed for the analysis of all flow data (Figure 2.1, described in Chapter 2, section 2.4.6).

A batch of blood samples from psoriasis patients and their matched controls were thawed on each day to avoid any possible discrepancy in comparing patients and controls due to flow cytometer instability. PBMCs from a single control, not included in the study, were included in each run to allow monitoring of flow cytometer stability and to standardise cytometer performance.

5.2.4 Statistical analysis

Statistical analysis was performed using MultiExperiment Viewer (MeV) (TM4–Microarray Software Suite). Data was loaded into MeV in .txt format and heatmaps were generated. Differences between patients and controls were evaluated using an automated Mann-Whitney t-test. An automated Kruskal-Wallis test was used to identify significant differences between
three or more subject groups. P values less than 0.05 were considered significant. Unsupervised cluster analysis was performed using the hierarchical clustering (HCL) algorithm.

We also used Graph Pad Prism 6 for analysis. Group means were compared using the Mann-Whitney t-test or the Kruskal-Wallis test. Correlations were determined using the Spearman correlation test. P values less than 0.05 were considered significant.

5.3 **Results**

5.3.1 **Trafficking receptor expression by conventional and regulatory T cells in the peripheral blood of patients with psoriasis and healthy controls.**

We analysed integrin and chemokine receptor (CR) expression by conventional T cells (Tconvs) and regulatory T cells (Tregs) in the peripheral blood (PB) of 44 psoriasis patients and 44 age and sex matched controls. Demographic and clinical information for the patients are provided in Table 5.1.

We have previously shown that the expression of CD127, the α-chain of the interleukin-7 receptor, along with CD25 allows for the identification of CD25⁺CD127lo Tregs within CD4⁺ T cells in human PB [61]. In this study, we developed 11 and 13-colour flow cytometry panels to investigate the proportion of cells expressing nine chemokine receptors and integrins (β7, CD49d, CLA, CCR4, CCR10, CCR5, CD62L, CXCR3 and CCR6) within circulating Tconvs and Tregs with the aim of providing an indication of their likelihood of entering tissues.

Representative histograms showing expression of these chemokine receptors on Tregs and Tconvs can be seen in Figure 5.1. Tregs were defined as CD25⁺CD127lo (Figure 5.1A) and Tconvs as CD4⁺ T cells outside this gate. Figure 5.1C shows the correlation between the
Table 5.1 Clinical characteristics of psoriasis patients included in study

<table>
<thead>
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<th>Characteristic</th>
<th>Patients (n=44)</th>
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<td>Age, median (range), years</td>
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<tr>
<td>% Male</td>
<td>77</td>
</tr>
<tr>
<td>Disease duration, median (range), years</td>
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<tr>
<td>% Psoriasis + psoriatic arthritis</td>
<td>27</td>
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<tr>
<td>Disease activity (PASI), mean ± SD</td>
<td>7.8 ± 7.8</td>
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<tr>
<td>% Methotrexate users</td>
<td>25</td>
</tr>
<tr>
<td>% TNFi (Infliximab) users</td>
<td>36</td>
</tr>
<tr>
<td>% Untreated (includes topical only)</td>
<td>45</td>
</tr>
</tbody>
</table>

PASI = Psoriasis Area Severity Index; TNFi = Tumour necrosis factor inhibitor
Figure 5.1: Expression of chemokine receptors and integrins by conventional T cells (Tconvs) and regulatory T cells (Tregs) in the peripheral blood of patients and controls. Mononuclear cells were isolated as described and examined by flow cytometry for the expression of markers that allowed for the identification of Tregs, defined as CD4⁺CD127⁻CD25⁺ (A), and Tconvs, defined as everything outside the indicated Treg gate. (B) Tregs and Tconvs were next subdivided on the basis of CD45RO expression in order to identify CD45RO⁺ (effector/memory) and CD45RO⁻ (naïve) subsets, and analysed for the various chemokine receptors and integrins examined (ß7, CD49d, CLA, CCR4, CCR10, CCR5, CD62L, CXCR3 and CCR6). Numbers indicate cells within the gate as a percentage of the parent population. (C) Correlation between the frequencies of CD4⁺ Tregs identified by gating for CD127⁻CD25⁺ versus CD127⁻FOXP3⁺ in 44 psoriasis patients. The correlation was statistically evaluated using a non-parametric Spearman test (****).
percentage of Tregs identified as CD25$^+$CD127$^{lo}$ and FOXP3$^+$CD127$^{lo}$ in the 44 psoriasis patients studied. The frequency of Tregs within the two gates in each individual was very highly correlated.

Because the ability to enter non-lymphoid tissues is closely correlated with post-thymic differentiation in response to antigen, expression of integrins and chemokine receptors was examined along with CD45RO, an effector/memory marker, on both Tconvs (Figure 5.1B, left panel) and Tregs (Figure 5.1B, right panel). Effector/memory (CD45RO$^+$) Tconvs and Tregs expressing the integrin or chemokine receptor of interest, calculated as a percent total CD45RO$^+$ Tconvs/Tregs, appear in the upper right gate of each panel and naïve (CD45RO$^-$) Tconvs and Tregs expressing the integrin or chemokine receptor of interest, calculated as a percent total CD45RO$^-$ Tconvs/Tregs, in the upper left gate. These subsets were quantified for all 88 patients and controls and used for further analysis.

5.3.2 Levels of conventional and regulatory T cell subsets in the peripheral blood of psoriasis patients and healthy controls.

We used a bioinformatic analysis software, MeV, to draw simultaneous, automated comparisons between multiple Tconv and Treg subset data obtained from flow cytometry. Data were uploaded into the software and a heat map was subsequently generated (Figure 5.2A), providing a simple way to simultaneously visualize the data. Each column represents a psoriasis patient (left) or control (right) and each row a Tconv or Treg subset, gated as indicated in Figure 5.1.

Once data is loaded into MeV and a heatmap is generated, various statistical and clustering tools can be applied to allow for the identification of significant similarities and differences between subject groups. We next performed an automated t-test to identify the significant differences in Tconv and Treg subset frequencies between psoriasis patients and controls. This identified ten subsets that were significantly different between patients and controls; all
Figure A: Heatmap showing PSO patients and controls with Tconv and Treg classification.

Figure B: Heatmap with PSO patients and controls comparing expression levels.

Figure C: Scatter plots showing CXCR3+, CLA+, CD62L+, Beta-7+ percentages in PSO patients and controls.

Figure D: Scatter plots showing CXCR3+, CCR6+, CCR5+, CCR4+ percentages in PSO patients and controls.

Legend:
- CXCR3+ effector/memory Tconv
- CLA+ effector/memory Tconv
- CD62L+ naive Tconv
- Beta-7+ naive Tconv
- CXCR3+ effector/memory Treg
- CXCR3+ naive Treg
- CCR4+ effector/memory Treg
- CCR6+ effector/memory Treg
- CCR5+ effector/memory Treg
- Beta-7+ naive Treg

Low to High color scale.
Figure 5.2. Decreased expression of trafficking receptors on Tregs in the peripheral blood of psoriasis (PSO) patients (n=44) when compared with healthy controls (n=44). Peripheral blood mononuclear cells from patients and controls were examined by flow cytometry for the expression of \( \beta \)7, CD49d, CLA, CD62L, CCR4, CCR5, CCR6, CCR10 and CXCR3 within their Tconv and Treg (CD4\(^+\)CD127\(^{lo}\)CD25\(^+\)) compartment. (A) Data obtained from flow cytometric experiments is visualised colorimetrically in a heat map. Each row represents a Tconv or Treg subset; each column a patient or control. Frequencies that are increased relative to the row’s mean are indicated in yellow, decreased in blue and frequencies that show no difference are indicated in black. (B) An automated Mann-Whitney t-test indicated that ten subsets displayed significantly different frequencies between patients and controls (p<0.05), shown here in a heat map. All ten of these subsets were found to be significantly decreased in PSO patients. The ten Tconv (C) and Treg (D) subsets displayed as bar graphs. Each point represents an individual subject and horizontal lines represent means. The Mann-Whitney t-test was used to compare differences between patients and controls. * = p < 0.05, ** = p < 0.01, **** = p < 0.0001.
were decreased in psoriasis, as shown in the heat map in Figure 5.2B. Four of these subsets were within the Tconv compartment and six were within Tregs.

**Figure 5.2C & D** illustrates the ten subsets identified by MeV in a more traditional format. CXCR3+ and CLA+ effector/memory Tconvs were decreased in the PB of psoriasis patients when compared to controls (means 37% vs 47%, and 18% vs 22%). Furthermore, CD62L+ and β7+ naïve Tconvs were decreased in the PB of psoriasis patients when compared to controls (means 67% vs 78%, and 78% vs 82%) (Figure 5.2C). Within the effector/memory Treg compartment, frequencies of CXCR3+, CCR6+, CCR5+ and CCR4+ Tregs were significantly reduced in patients with psoriasis (Figure 5.2D) (CXCR3+; mean 24% vs 34%, CCR6+; mean 56% vs 64%, CCR5+; mean 27% vs 31%, CCR4+; mean 90% vs 93%). Additionally, CXCR3+ and β7+ naïve Tregs were significantly decreased in psoriasis patients (mean 9% vs 11%, and 33% vs 39%) (Figure 5.2D).

None of the Tconv and Treg subset percentages analysed correlated with disease activity score (PASI) or disease duration for the psoriasis patients (data not shown).

### 5.3.3 Levels of conventional and regulatory T cell subsets in the peripheral blood of untreated psoriasis patients, treated psoriasis patients and healthy controls.

In order to determine whether treatment contributed to differences between psoriasis patients and controls, patients were divided into untreated (n=20, includes patients using topical therapy) and treated (n=24) groups. There were no significant differences between untreated and treated patients in age (mean 45 vs 50 years) and disease duration (18 vs 23 years). However the treated patients had a significantly lower disease activity score as measured by PASI (mean 4 vs 12). An automated Kruskal-Wallis test was subsequently performed in MeV in order to identify the significant differences in Tconv and Treg subset frequencies between untreated psoriasis patients, treated psoriasis patients and controls. This identified nine subsets that were significantly different between the three groups (Figure 5.3A).
A) Heatmap showing the expression levels of various T-cell subsets in PSO_Treated, PSO_Untreated, and Controls. The subsets include CXCR3+ effector/memory Tconvs, CLA+ effector/memory Tconvs, CD62L+ naive Tconvs, Beta-7+ naive Tconvs, Total CD127loCD25+ Tregs, Total CD127loFoxP3+ Tregs, CXCR3+ effector/memory Tregs, CCR6+ effector/memory Tregs, Beta-7+ naive Tregs, and CD49d+ naive Tregs.

B) Scatter plots showing the percentage of T-cell subsets in different conditions. The plots compare untreated (Unstimulated), treated (Treated), and control (Controls) groups for CXCR3+, CLA+, CD62L+, and Beta-7+ Tconvs, and for total Tregs, CXCR3+, CCR6+, Beta-7+, and CD49d+ Tregs.

C) Similar scatter plots as in B, but for total Tregs, with the percentage of CD4+ cells included.
Figure 5.3: Frequencies of nine Tconv and Treg subsets were significantly different between untreated PSO patients, treated PSO patients and controls. Peripheral blood mononuclear cells from patients and controls were examined by flow cytometry for the expression of β7, CD49d, CLA, CD62L, CCR4, CCR5, CCR6, CCR10 and CXCR3 within their Tconv and Treg (CD4$^{+}$CD127$^{lo}$CD25$^{+}$) compartment. (A) An automated Kruskal-Wallis test indicated that nine subsets displayed significantly different frequencies between untreated PSO patients (n=20), treated PSO patients (n=24) and controls (n=44) (p<0.05). Data is visualised here in a heat map where each row represents a Tconv or Treg subset; each column a patient or control. Frequencies that are increased relative to the row’s mean are indicated in yellow, decreased in blue and frequencies that show no difference are indicated in black. The nine Tconv (B) and Treg (C) subsets selected by statistical analysis to be significantly different between the three groups displayed as bar graphs. Each point represents an individual subject and horizontal lines represent means. The Kruskal-Wallis test was used to compare differences between groups. * = p < 0.05, ** = p < 0.01, *** = p < 0.001.
Data for the nine subsets identified by MeV are presented in Figure 5.3, B&C. In regards to the Tconv compartment, all four subsets that were decreased in the psoriasis group as a whole were found to be significantly different amongst the three groups (Figure 5.3B). Interestingly, the decrease in two of these subsets, the CXCR3$^+$ and CLA$^+$ effector/memory Tconvs, was entirely due to a decrease in the treated psoriasis patient group. CXCR3$^+$ effector/memory Tconvs were significantly reduced in the treated psoriasis group when compared to controls (means 34% vs 47%) while no difference was seen in the untreated group. CLA$^+$ effector/memory Tconvs were also significantly reduced only in the treated psoriasis group when compared to controls (mean 16% vs 22%). On the other hand, CD62L$^+$ naïve Tconvs were significantly decreased in the PB of both untreated and treated psoriasis patients. β7$^+$ naïve Tconvs were also slightly decreased in both untreated and treated patients.

Interestingly, we found that the number of total Tregs is significantly increased in the PB of untreated psoriasis patients when compared to treated psoriasis patients (mean 11.4% vs 7.7% of CD4$^+$ cells) (Figure 5.3C). In Figure 5.2, we showed that the levels of CXCR3$^+$ and CCR6$^+$ effector/memory Tregs were significantly reduced in the PB of patients. This decrease was evident in both treated and untreated patients. On the other hand, β7$^+$ and CD49d$^+$ naïve Tregs are significantly decreased only in untreated psoriasis patients (means 30% vs 39% and 14% vs 20%) (Figure 5.3C).

5.3.4 Unsupervised hierarchical cluster analysis of patients based on frequencies of Tconv and Treg subsets and clinical parameters.

We next wished to determine whether incorporating clinical parameters into analysis of flow-based data from psoriasis patients would give us further insights into whether any of these clinical parameters significantly affected the frequency of Tconv and Treg subsets (Figure 5.4). We performed an unsupervised hierarchical clustering of the 44 patients based on the frequencies of their Tconv and Treg subsets and five clinical parameters; PASI, presence of
Figure 5.4: Unsupervised hierarchical cluster analysis of PSO patients based on frequencies of Tconv and Treg subsets and clinical parameters results in patients on TNF inhibitor therapy to cluster together. Data obtained from flow cytometric experiments and clinical information recorded at the time the peripheral blood sample was taken is visualised colorimetrically in a heat map. Each row represents a Tconv/Treg subset or a clinical parameter; each column represents a patient (n=44). Frequencies that are increased relative to the row’s mean are indicated in yellow, decreased in blue and frequencies that show no difference are indicated in black. Unsupervised hierarchical clustering caused patients to fall into three groups; one consisting of 11 out of the 16 PSO patients on TNF inhibitor therapy (boxed in red).
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arthritic, disease duration, TNF inhibitor (TNFi) use (n=16) and methotrexate (MTX) use (n=11). This resulted in patients clustering into three groups; one consisting of 11 out of the 16 psoriasis patients on TNFi therapy (boxed in red).

5.3.5 Levels of conventional and regulatory T cell subsets in the peripheral blood of psoriasis patients on TNF inhibitor therapy.

To investigate the effect of TNFi use on the frequencies of the Tconv and Treg subsets examined, we categorized patients into two groups, TNFi users (n=16) and non-TNFi users (n=28). There were no significant differences between TNFi users and non-TNFi users in age (mean 47 vs 48 years), disease duration (24 vs 19 years) and methotrexate (MTX) use (25% MTX users in both groups). However the TNFi users had a significantly lower disease activity score as measured by PASI (mean 2 vs 11, p=0.0003). We performed an automated Kruskal-Wallis test in order to identify the significant differences in Tconv and Treg subset frequencies between psoriasis TNFi users, psoriasis non-TNFi users and controls. This identified ten subsets that were shown to be significantly different between these three groups, displayed in a heatmap in Figure 5.5A.

Traditional graphs of the ten subsets identified by the automated Kruskal-Wallis test are presented in Figure 5.5, B&C. Although we previously showed that CXCR3+ effector/memory Tconvs were significantly reduced only in treated psoriasis patients (Figure 5.3B), this decrease was not due to TNFi use (Figure 5.5B). Patients not on TNFi therapy displayed reduced levels of CXCR3+ effector/memory Tconvs when compared to controls (mean 35% vs 47%) while TNFi users were not significantly different from controls. Interestingly, TNFi therapy appears to have a significant effect on subsets in the naïve Tconv compartment. CCR6+ naïve Tconvs were significantly increased in TNFi users when compared to non-TNFi users (mean 2.2% vs 1.7%). Furthermore, CD62L+ and β7+ naïve Tconvs are significantly reduced only in non-TNFi users when compared to controls.
Figure 5.5: Frequencies of ten Tconv and Treg subsets were significantly different between PSO patients on TNF inhibitors (PSO+TNFi), PSO patients not on TNF inhibitors (PSO) and controls. Peripheral blood mononuclear cells from patients and controls were examined by flow cytometry for the expression of β7, CD49d, CLA, CD62L, CCR4, CCR5, CCR6, CCR10 and CXCR3 within their Tconv and Treg (CD4⁺CD127loCD25⁺) compartment. (A) An automated Kruskal-Wallis test indicated that ten subsets displayed significantly different frequencies between PSO+TNFi (n=16), PSO (n=28) and controls (n=44) (p<0.05). Data is visualised here in a heat map where each row represents a Tconv or Treg subset; each column a patient or control. Frequencies that are increased relative to the row’s mean are indicated in yellow, decreased in blue and frequencies that show no difference are indicated in black. The ten Tconv (B) and Treg (C) subsets selected by statistical analysis to be significantly different between the three groups displayed as bar graphs. Each point represents an individual subject and horizontal lines represent means. The Kruskal-Wallis test was used to compare differences between groups. * = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001.
In Figure 5.2 we showed that levels of CXCR3$^+$, CCR4$^+$ and CCR6$^+$ effector/memory Tregs and β7$^+$ naïve Tregs were decreased in the PB of psoriasis patients. Here we showed that this decrease was not evident in TNFi users, whereas non-TNFi users had significantly reduced levels of all these subsets when compared to controls (Figure 5.5C). In addition, patients on TNFi therapy had significantly increased levels of CCR6$^+$ effector/memory and naive Tregs when compared to patients not on TNFi therapy (mean 65% vs 51% and 11% vs 8%). Furthermore, patients on TNFi therapy had significantly increased levels of β7$^+$ naïve Tregs when compared to patients not on TNFi therapy (mean 39% vs 29%). This suggests that TNFi use normalises the frequency of Treg subsets expressing CXCR3, CCR4, CCR6 and β7 in the PB of psoriasis patients.

5.3.5.1 Longitudinal analysis of Tconv and Treg subsets in the peripheral blood of psoriasis patients pre and post TNF inhibitor therapy.

An analysis of TNFi treated patients provided only correlative data and cannot be used to conclude that the changes seen in the TNFi group are specifically due to treatment. TNF inhibitors are used to treat patients after they have failed other therapies, suggesting that this group of patients may be inherently different to other patients. In order to answer this question, it is important to monitor the effects of anti-TNF on Treg and Tconv subsets pre and post treatment. We were able to collect PB samples from seven patients pre and post therapy with Infliximab, a TNFi. An overview of the samples collected from these seven patients is outlined in Table 5.2.

When examining Tconv subsets, no significant differences were found pre and post therapy with a TNFi (Figure 5.6). However it is important to note that it is difficult to achieve significance with a small number of patients, of which only four had a complete set of samples for each of the 5 time points (Table 5.2). In Figure 5.5 we showed that there was a significant increase in CCR6$^+$ naïve Tconvs in TNFi users when compared to non-TNFi users. When patients were examined longitudinally, there was a slight increase in the
Table 5.2 Longitudinal samples from patients pre and post TNFi (Infliximab) therapy (n=7)

<table>
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<th></th>
<th>Week</th>
<th>0 (pre)</th>
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<td>√</td>
<td>√</td>
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<td>√</td>
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<tr>
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PASI = Psoriasis Area Severity Index; TNFi = Tumour necrosis factor inhibitor
Figure 5.6: Longitudinal analysis of Tconv subsets pre and post TNFi therapy in seven PSO patients. Peripheral blood samples were collected from seven patients pre (week 0) and post treatment with TNFi Infliximab (Table 5.2) and examined by flow cytometry for the expression of trafficking receptors CXCR3, CCR4, CCR6, CLA, CCR5, CD62L, Beta-7 and CCR10 within their effector/memory (CD45RO⁺) and naïve (CD45RO⁻) Tconv compartment. Points represent the group means at each of the time-points (weeks 0, 2, 6, 15 and 52+). The Kruskal Wallis test was used to identify differences between groups. No significant differences were found. Horizontal lines on the right hand side represent means (±SEM) from the three groups analysed in Figure 5.5; PSO (black), PSO+TNFi (red) and Controls (blue).
frequency of this subset (mean 1.4% to 1.9%), but this did not reach statistical significance. CD62L⁺ naïve Tconvs were also slightly increased in TNFi treated patients (Figure 5.5). Interestingly, when TNFi patients were examined longitudinally, we saw a gradual increase in the frequency of CD62L⁺ cells within the naïve (mean 55% to 80%) and effector/memory (mean 30% to 51%) Tconv compartments pre and 52 weeks post treatment (Figure 5.6). Once again this change did not reach statistical significance. On the other hand, although we found that β7⁺ naïve Tconvs were significantly reduced only in non-TNFi users when compared to controls, TNFi use itself did not increase the frequency of this subset when comparing pre and 52 weeks post treatment.

In terms of Tregs, in Figure 5.5 we showed that CXCR3⁺, CCR4⁺ and CCR6⁺ effector/memory Tregs and β7⁺ naïve Tregs were reduced only in patients not on TNFi therapy, suggesting that TNFi therapy may increase the frequency of these subsets. We also showed that patients on TNFi therapy had significantly increased levels of CCR6⁺ effector/memory and naïve Tregs when compared to patients not on TNFi therapy. When examined longitudinally, TNFi therapy did not increase the frequency of CXCR3, CCR6 or β7 expressing Tregs (Figure 5.7). However, there was a trend towards increased frequency of CCR4⁺ effector/memory Tregs (mean 86% to 91%) following TNFi use, which did not reach statistical significance. Interestingly, there was a gradual increase in the percentage of CD62L⁺ effector/memory (mean 46% to 70%) and naïve (mean 29% to 65%) Tregs, similar to what we saw in Tconvs. This difference reached statistical significance in naïve Tregs when comparing pre TNFi therapy to 52 weeks of therapy, indicating that treatment with a TNFi results in increased levels of CD62L⁺ cells in the PB of psoriasis patients.

5.3.6 Levels of conventional and regulatory T cell subsets in the peripheral blood of psoriasis patients treated with methotrexate.

To investigate the effect of methotrexate (MTX) on the frequencies of the Tconv and Treg subsets examined, we categorized patients into two groups, MTX users (n=11) and non-MTX
### Tregs

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**Key:**
- **CD45RO+** and **CD45RO-** indicate the status of the CD45 receptor.
- The graphs illustrate changes over time, with error bars indicating variability.
Figure 5.7: Longitudinal analysis of Treg subsets pre and post TNFi therapy in seven PSO patients. Peripheral blood samples were collected from seven patients pre (week 0) and post treatment with TNFi Infliximab (Table 5.2) and examined by flow cytometry for the expression of trafficking receptors CXCR3, CCR4, CCR6, CLA, CCR5, CD62L, Beta-7 and CCR10 within their effector/memory (CD45RO⁺) and naïve (CD45RO⁻) Treg compartment. Points represent the group means at each of the time-points (weeks 0, 2, 6, 15 and 52+). The Kruskal Wallis test was used to identify differences between groups. * = p < 0.05. Horizontal lines on the right hand side represent means (±SEM) from the three groups analysed in Figure 5.5; PSO (black), PSO+TNFi (red) and Controls (blue).
users (n=32). There were no major differences between MTX users and non-MTX users in age (mean 53 vs 46 years), disease duration (24 vs 19 years), PASI (mean 5.9 vs 8.4) and TNF inhibitor use (36% vs 38%). We performed an automated Kruskal-Wallis test in order to identify the significant differences in Tconv and Treg subset frequencies between psoriasis MTX users, psoriasis non-MTX users and controls. This identified 13 subsets that were significantly different between these three groups, displayed in a heatmap in Figure 5.8A.

The data from the 13 subsets identified by the automated Kruskal-Wallis test are presented in Figure 5.8, B&C. Psoriasis patients on methotrexate had reduced levels of CXCR3+ and CLA+ effector memory Tconvs in their PB when compared to controls (CXCR3; means 27% vs 47%, CLA; means 14% vs 22%) (Figure 5.8B). The frequency of total Tregs was also significantly reduced in psoriasis patients taking methotrexate when compared to patients not taking methotrexate (mean 6.7% vs 10.4%) (Figure 5.8C). In terms of trafficking receptors, both groups of patients on methotrexate and not on methotrexate display decreased levels of CXCR3+ effector/memory Tregs when compared to controls. However methotrexate use also appeared to have a significant effect on the levels of other CRs and adhesion molecules on Tregs, with patients on this therapy displaying reduced levels of CCR4+, CCR6+, CLA+ and CCR10+ effector/memory Tregs and increased levels of β7+ effector/memory Tregs in their PB.

5.3.7 Levels of skin-associated trafficking receptors correlate with total Tregs in the blood of psoriasis patients.

We examined the relationship between the levels of skin and gut-associated trafficking receptors and total effector/memory Tregs in the PB of 44 psoriasis patients and 44 age and sex matched controls (Figure 5.9). In doing so, we found that levels of Tregs expressing the skin-associated trafficking receptors CLA, CCR4 and CCR10 positively correlated with total effector/memory Tregs in the PB of psoriasis patients but not controls (Figure 5.9A). This indicates that while the number of Tregs expressing skin-homing-molecules CLA, CCR4 and
Figure 5.8: Frequencies of thirteen Tconv and Treg subsets were significantly different between PSO patients on methotrexate therapy (PSO+MTX), PSO patients not on methotrexate (PSO) and controls. Peripheral blood mononuclear cells from patients and controls were examined by flow cytometry for the expression of β7, CD49d, CLA, CD62L, CCR4, CCR5, CCR6, CCR10 and CXCR3 within their Tconv and Treg (CD4⁺CD127loCD25⁺) compartment. (A) An automated Kruskal-Wallis test indicated that 13 subsets displayed significantly different frequencies between PSO+MTX (n=11), PSO (n=32) and controls (n=44) (p<0.05). Data is visualised here in a heat map where each row represents a Tconv or Treg subset; each column a patient or control. Frequencies that are increased relative to the row’s mean are indicated in yellow, decreased in blue and frequencies that show no difference are indicated in black. The 13 Tconv (B) and Treg (C) subsets selected by statistical analysis to be significantly different between the three groups displayed as bar graphs. Each point represents an individual subject and horizontal lines represent means. The Kruskal-Wallis test was used to compare differences between groups. * = p < 0.05, ** = p < 0.01 *** = p < 0.001.
Figure 5.9: Levels of Tregs expressing the skin-associated trafficking receptors CLA, CCR4 and CCR10 correlate with total Tregs in psoriasis patients only. Peripheral blood mononuclear cells from patients and controls were examined by flow cytometry for the expression of skin-associated trafficking receptors CLA, CCR4 and CCR10 and gut-associated receptors β7 and CD49d on their Tregs. (A&B) Relationship between total CD45RO+ Tregs and Treg subsets expressing CLA, CCR4 and CCR10 (A) and β7 and CD49d (B). The correlation was evaluated using a non-parametric Spearman test.
CCR10 does not differ significantly between untreated psoriasis patients and controls (Figure 5.3), the proportion of Tregs expressing these molecules increases as total Treg frequency increases in patients, suggesting a selective increase in skin-homing and an effort to get Tregs into the skin. This relationship was not evident for gut-associated molecules β7 and CD49d (Figure 5.9B).

5.3.8 Levels of T-bet\(^+\) Th1 and ROR\(_{\gamma}t\)^+ Th17 type CD4 T cells in peripheral blood of psoriasis patients and controls.

Psoriasis has been shown to involve both Th1 and Th17 type immune processes [251, 252]. We wished to examine the levels of T-bet\(^+\) Th1 and ROR\(_{\gamma}t\)^+ Th17 type cells in the PB of patients and controls. In order to do this, we developed a flow cytometry panel that allowed us to identify circulating CD4\(^+\) T cells that express T-bet and ROR\(_{\gamma}t\) without the need for \textit{in vitro} stimulation (Figure 5.10A). Furthermore, we examined CD4\(^+\) T cells for the co-expression of ROR\(_{\gamma}t\) and FoxP3 and identified a population of ROR\(_{\gamma}t^t\)FoxP3\(^+\) cells.

The levels of T-bet\(^+\) Th1 type cells, ROR\(_{\gamma}t^t\) Th17 type cells and ROR\(_{\gamma}t^t\)FoxP3\(^+\) cells did not differ between patients and controls (Figure 5.10B). Interestingly however, these subsets displayed different relationships with age in psoriasis patients and controls (Figure 5.10C). T-bet\(^+\) Th1 type cells positively correlated with age in controls but not in patients while ROR\(_{\gamma}t^t\) Th17 type cells and ROR\(_{\gamma}t^t\)FoxP3\(^+\) cells negatively correlated with age in psoriasis patients only. Psoriasis patients had increased levels of ROR\(_{\gamma}t^t\) Th17 type cells at a younger age when compared to controls. No correlations were found between T-bet\(^+\) Th1 type cells, ROR\(_{\gamma}t^t\) Th17 type cells or ROR\(_{\gamma}t^t\)FoxP3\(^+\) cells and disease duration (data not shown).

We also studied the relationship between the number of T-bet\(^+\) Th1 type cells, ROR\(_{\gamma}t^t\) Th17 type cells and ROR\(_{\gamma}t^t\)FoxP3\(^+\) cells and total effector/memory Tregs in the PB of patients and controls (Figure 5.10D). No significant correlations were seen between levels of T-bet\(^+\) Th1 type cells and Tregs in patients or controls. However the level of ROR\(_{\gamma}t^t\) Th17 type cells
Figure 5.10: Levels of T-bet$^+$ Th1 and ROR$\gamma$t$^+$ Th17 type CD4 T cells in peripheral blood of psoriasis patients and controls. Peripheral blood mononuclear cells from patients and controls were examined by flow cytometry for the expression of T-bet, ROR$\gamma$t and FoxP3. (A) CD4$^+$ T cells are examined for the expression of the transcription factors T-bet, ROR$\gamma$t and FoxP3. Three cell populations were identified; T-bet$^+$ Th1 cells, ROR$\gamma$t$^+$ Th17 cells and ROR$\gamma$t$^+$FoxP3$^+$ cells. (B) Percentages of T-bet$^+$ Th1 cells, ROR$\gamma$t$^+$ Th17 cells and ROR$\gamma$t$^+$FoxP3$^+$ cells within CD4$^+$ cells in psoriasis patients (n=44) and controls (n=44). Each point represents an individual donor and horizontal bars represent the group means. The Mann-Whitney t-test was used to compare differences between patients and controls, no significant differences were found. (C) Relationship between T-bet$^+$ Th1 cells, ROR$\gamma$t$^+$ Th17 cells and ROR$\gamma$t$^+$FoxP3$^+$ cells and age. (D) Relationship between the frequency of T-bet$^+$ Th1 cells, ROR$\gamma$t$^+$ Th17 cells and ROR$\gamma$t$^+$FoxP3$^+$ cells and total effector/memory (CD45RO$^+$) Tregs. Correlations were evaluated using a non-parametric Spearman test.
very significantly correlated with total effector/memory Tregs in controls (p<0.0001) but not in patients, indicating a possibly deregulated Th17/Treg balance.

5.4 Discussion

Tregs in human PB were originally defined as CD4⁺CD25[^high] making it difficult to determine the exact delineation between CD25[^high] and CD25⁺/intermediate cells. This has partly contributed to the conflicting results regarding the level of Tregs in the PB of psoriasis patients [65, 238-242]. We have previously shown that human Tregs can be accurately identified as CD4⁺CD25⁺CD127[^lo] [61]. We set out to use this definition to analyse the total frequency of Tregs, as well as various subsets of effector/memory and naïve Tregs that express the trafficking receptors β7, CD49d, CLA, CCR4, CCR10, CCR5, CD62L, CXCR3 and CCR6 in the peripheral blood of patients with psoriasis (Figure 5.1).

We report that the frequency of total CD4⁺CD25⁺CD127[^lo] Tregs is unchanged when comparing the PB of our entire psoriasis patient cohort with controls (Figure 5.2). In two other studies that used FoxP3 to identify Tregs, it was also demonstrated that levels of Tregs were not different in the PB of psoriasis patients [240, 242]. However in one of these studies, Tregs were shown to be significantly increased in patients with high disease activity, defined as a PASI score > 25 [242]. When we examined Tregs in relation to disease activity, we did not find a correlation (data not shown). This may be because our cohort did not include patients with PASI scores > 25.

Although we found Treg frequency to be unchanged in the PB of our entire patient cohort, it was increased in the PB of untreated patients when compared to treated patients (Figure 5.3). While the statistical significance of this increase was reliant on a couple of outliers in the untreated patients, there is still a trend towards increased Treg frequency in untreated patients when these outliers are removed. This is consistent with a report from another group that demonstrated an increase in CD4⁺FoxP3⁺ Tregs in the PB of untreated psoriasis [244]. This
indicates that there may be a disease specific increase in Treg frequency in psoriasis patients. It could be argued that if Tregs are increased in psoriasis, then a Treg deficiency is unlikely to be implicated in disease pathogenesis. However it is likely that the increase in Treg numbers is a result of ongoing inflammation and may indicate an attempt by Tregs to control the aberrant immune response. For this reason, it is important to examine Tregs that express various trafficking receptors in order to identify potential deficiencies in Treg subsets with the potential to migrate to diseased tissue.

CD4+ T cells make up a large proportion of the inflammatory cells within psoriatic skin lesions [235-237]. The differential expression of trafficking receptors by CD4+ T cells determines their migration to specific tissue environments including psoriatic plaques. T cells infiltrating psoriatic skin lesions have been shown to express the adhesion molecules CLA [254-257], CCR4 [255, 256], CCR5 [258], CCR6 [255, 259], CCR10 [260] and CXCR3 [256, 261]. Although numerous studies have also shown CD4+FoxP3+ Tregs to be enriched in psoriatic skin lesions [242-250], it has been demonstrated that the ratio of Tregs to Tconvs is higher in distant symptomless skin than in psoriatic plaques, where the ratio is skewed towards inflammatory effector T cells [250]. It is therefore plausible that an imbalance of Tregs and Tconvs at psoriatic sites could contribute to disease.

The co-localisation of Tregs with Tconvs in tissues has been shown to be crucial for their capacity to in regulate immune responses [112, 113, 118]. Despite this, the expression of trafficking receptors by Tregs in the PB or skin of psoriasis patients has not previously been examined. We hypothesised that deficiencies in trafficking receptor expression by Tregs may result in less Treg entry into the skin, consequently leading to the initiation and progression of a chronically dysregulated immune response. We found a significantly reduced frequency of effector/memory Tregs, but not Tconvs, expressing CXCR3 and CCR6 in the PB of both treated and untreated psoriasis patients (Figure 5.2 & 5.3). CXCR3+ Tregs have been shown to accumulate at sites of Th1 mediated inflammation whereas CXCR3- deficient Tregs fail to localise and control this type of inflammation [112]. Furthermore, impaired CCR6 expression
on Tregs has been shown to result in dysregulated Th17 responses [118]. Psoriasis has been shown to involve both Th1 and Th17 type immune processes [251, 252] and therefore a reduction in CXCRI+ and CCR6+ Tregs could indicate a trafficking deficiency resulting in less Treg entry into psoriatic lesions and consequential disrupted control of inflammation.

No differences in the proportion of skin homing CLA-, CCR4- or CCR10- expressing Tregs were seen between untreated psoriasis patients and controls, either in the naïve or effector/memory compartments (Figure 5.3). However, while the number of Tregs expressing skin-homing-molecules did not differ between psoriasis patients and controls, the proportion of Tregs expressing these molecules increased with total effector/memory Treg frequency in patients only, suggesting a selective increase in skin homing and perhaps an attempt by Tregs to control tissue-specific inflammation (Figure 5.9). Interestingly, naïve Tregs expressing the gut homing phenotypes β7 and α4 (CD49d) were reduced in the blood of patients with psoriasis.

We also found smaller proportions of β7+ and CD62L+ naïve Tconvs in the PB of psoriasis patients. Expression levels of CD62L have previously been shown to be reduced on CD4+ T cells in the PB of psoriasis patients, and it has been suggested that this was due to shedding of the molecule as a result of increased activation [262]. However we have shown that the reduction in CD62L+ Tconvs in psoriasis patients was due to a reduction in the naïve, not activated, compartment of Tconvs.

TNF-α plays an important role in the inflammatory process of psoriasis, as indicated by increased levels of TNF-α in psoriatic lesions [263] as well as the effectiveness of treatment with TNF-α inhibitors [264]. Due to the high costs and risks associated with TNF-α inhibitors, such as increased risk of infection, sepsis and the reactivation of tuberculosis, guidelines suggest that TNF-α inhibitors should be reserved for patients unresponsive to topical therapy, phototherapy and disease-modifying anti-rheumatic drugs (DMARD) [265-267]. Despite this, the mechanism underlying the effectiveness of TNF-α blockade is not
completely understood, hence hindering our ability to select patients that will fail other therapy but respond effectively to TNF-α inhibitors.

It has been demonstrated that lesions from psoriasis patients treated with TNF-α inhibitors contain a higher Treg to Tconv ratio [268], suggesting that Tregs may have a role in the therapeutic effects of treatment. Very few studies have examined the effects of TNF-α blockade on Tregs in the PB of psoriasis patients. We found that the TNF-α inhibitor Infliximab had no significant effect on the frequency of Tregs in the PB of psoriasis patients (Figure 5.5 & 5.7). In support with our findings, CD4⁺CD25⁺FoxP3⁺ Treg frequency has been shown to increase from baseline to 24 weeks in patients following treatment with the TNF-α inhibitor Etanercept, but not Infliximab [269]. This suggests a drug class effect and outlines the importance of studying the immune effects of each individual TNF-α inhibitor.

TNF-α inhibitors have been shown to reduce T cell numbers in psoriatic lesions, suggesting a possible decrease in cell infiltration [246, 270, 271]. Despite this, we are the first to examine the effects of TNF-α inhibition on trafficking receptor expression in psoriasis. Without longitudinal studies on a group of patients undergoing TNF-α inhibitor therapy, it is difficult to conclude that the changes seen in the TNF-α inhibitor users are specifically due treatment. TNF-α inhibitors are used to treat patients after they have failed other therapies, suggesting that it is possible that this group of patients may be inherently different to other patients. Therefore it is important to monitor the effects of TNF-α inhibitors on Treg and Tconv subsets pre and post treatment. We analysed PB samples from seven patients pre and post therapy with Infliximab.

Although we saw an increase in the frequency of CCR6⁺, CD62L⁺ and β7⁺ naïve Tconvs in TNF-α inhibitor users (Figure 5.5), only the increase in CD62L appeared to be due to TNF-α inhibitor use. When TNF-α inhibitor users were examined longitudinally, we saw a gradual increase in the frequency of CD62L⁺ and effector/memory Tconvs pre and 52 weeks post treatment (Figure 5.6). In terms of Tregs, although the levels of CXCR3⁺, CCR4⁺ and CCR6⁺
effector/memory Tregs and CCR6+ and β7+ naïve Tregs were higher in TNF-α inhibitor users, suggesting that TNF-α inhibitor therapy may have increased the frequency of these subsets, when examined longitudinally TNF-α inhibitor therapy did not increase the frequency of CXCR3, CCR6 or β7 expressing Tregs (Figure 5.7). However, there was a trend towards increased frequency of CCR4+ effector/memory Tregs following TNF-α inhibitor use. Interestingly, there was a gradual increase in the percentage of CD62L expressing effector/memory and naïve Tregs, similar to what we saw in Tconvs. This difference reached statistical significance in naïve Tregs when comparing pre TNF-α inhibitor therapy to 52 weeks of therapy, indicating that treatment with a TNF-α inhibitor results in increased levels of CD62L+ cells in the PB of psoriasis patients.

CD62L and CCR7 are involved in the recruitment of T cells to lymphoid tissue. Interestingly, it has been demonstrated that in psoriatic lesions, lymphoid-like tissue exists in the dermis [272]. In a study that examined CCR7 and its ligand CCL19 in psoriatic lesions, it was demonstrated that disease resolution by anti-TNF-α agents is associated with the inhibition of the CCR7/CCL19 axis and the dissolution of lymphoid-aggregates [273]. Therefore, the increase in CD62L+ cells in the PB of psoriasis patients we observed following TNF-α inhibitor therapy could be due to reduced entry into the skin as a result of the dissolution of these lymphoid aggregates.

In psoriasis, disease-modifying anti-rheumatic drugs (DMARD), such as methotrexate (MTX), are often the first line of treatment [266]. The mechanism of the anti-inflammatory effect of methotrexate is not fully understood, although it has been suggested that MTX alters T cell activation and adhesion molecule expression [274]. PBMCs cultured with MTX contain a significantly lower frequency of T cells expressing CLA and CD25 [274]. Furthermore, a reduced frequency of CLA+ T cells has been observed in the PB and lesions of patients on MTX therapy [275]. In this study, the frequency of CLA+ T cells was shown to correlate inversely with the dose of MTX, with patients on the highest doses having the lowest frequency of CLA+ T cells [275]. Our work has extended on previous studies by
showing that MTX significantly reduces the levels of CXCR3+ and CLA+ effector/memory Tconvs and CCR4+, CCR6+, CLA+ and CCR10+ effector/memory Tregs in the PB of psoriasis patients (Figure 5.8), suggesting an effect on a more diverse range of adhesion molecules than previously documented. It is possible that the reduction in CD25 previously observed [274] in turn reduces activation-induced expression of adhesion molecules. Interestingly, we also found significantly reduced levels of total Tregs in psoriasis patients taking MTX. This may also be due to the reduction in CD25 previously described.

Psoriatic skin lesions have been shown to contain IFN-γ producing Th1 type cells and IL-17 producing Th17 type cells [252, 276-278], thus prompting the analysis of these cell types in the PB of patients. *In vitro* stimulated peripheral blood mononuclear cells from patients with severe psoriasis have consistently been shown to contain increased levels of CD4+ IL-17 producing effector/memory T cells [277, 279-281]. These IL-17 producing cells have been demonstrated within both the CLA+ (skin-homing) and CLA- CD4 T cell compartments [279]. In terms of IFN-γ producing cells in the PB of psoriasis patients, one study found no difference in the levels of these cells in the PB when compared to controls [279], while others have reported a slight increase [280].

It is uncertain whether these artificially stimulated IFN-γ and IL-17 producing cells correspond to true Th1 and Th17 phenotypes. We developed a flow cytometry panel that allowed us to identify circulating CD4+ T cells that express the Th1 transcription factor T-bet and the Th17 transcription factor RORγt without the need for *in vitro* stimulation (Figure 5.10).

We report that the levels of T-bet+ Th1 type cells and RORγt+ Th17 type cells did not differ between psoriasis patients and controls. Interestingly however, these subsets displayed different relationships with age in psoriasis patients and controls. T-bet+ Th1 type cells positively correlated with age in controls only while RORγt+ Th17 type cells negatively correlated with age in psoriasis patients only. Psoriasis patients have increased levels of
RORγt+ Th17 type cells at a younger age when compared to controls, perhaps indicating an early initial phase of the disease marked by increased levels of Th17 cells.

Interestingly, the levels of RORγt+ Th17 type cells very significantly correlated with total effector/memory Tregs in controls but not in patients. This indicates that there is a direct relationship between Tregs and RORγt+ Th17 cells in healthy humans and that this balance is deregulated in psoriasis. The balance between Tregs and RORγt+ Th17 cells may be an important determinant of whether skin inflammation is initiated and whether it progresses. Future experiments should aim to design a flow cytometry panel that will allow for the analysis of RORγt+ Th17 cells within CLA+ skin-homing T cells. This would allow us to delineate whether the abnormalities observed in the relationship between RORγt+ Th17 cells and age and RORγt+ Th17 cells and Treg frequency are apparent within, or indeed restricted to, the CLA+ skin-homing compartment.

Due to a high degree of heterogeneity in psoriasis, it is a suitable disease for the application of personalised medicine. The recognition of biomarkers that can guide diagnosis, prognosis and treatment selection will provide many benefits to disease management. Flow cytometry entails rapid quantification of multi-dimensional characteristics for millions of cells. For this reason, there is a need for more efficient data viewing and analysis methods. We used bioinformatic tools to analyse flow cytometry data obtained in our study.

In order to find a signature that can predict clinical outcome, it is important to be able to combine experimental data with clinical measures, again highlighting the significant role bioinformatic analysis can play in the identification of relevant clinical correlations. When we combined clinical measures into our analysis, we found that patients on anti-TNF-α therapy clustered together (Figure 5.4).

However, although these patients clustered together, our longitudinal analysis of patients on anti-TNF-α therapy indicated that although some changes in Tconv and Treg subsets were
due to treatment, most were not. For example, although levels of CCR6$^+$ effector/memory Tregs were significantly increased in patients on TNF-α inhibitors (Figure 5.5), we did not observe an increase in this subset in patients following TNF-α inhibitor therapy (Figure 5.7). In fact, we observed a slight, non-significant decrease in this subset following therapy (mean 65 to 59). Interestingly, the frequency of CCR6$^+$ effector/memory Tregs in patients pre TNF-α inhibitor therapy is equal to the frequency of this subset in patients on TNF-α inhibitor therapy and controls (mean 65.1 vs 64.6). This suggests that this group of patients has inherently increased levels of CCR6$^+$ Tregs, and this could be intrinsic to patients selected for TNF-α inhibitor therapy.

Therefore it is possible that the patients on anti-TNF-α therapy in our study clustered together because they had different subset frequencies to begin with, highlighting the possibility of identifying candidates for anti-TNF-α therapy before they are trialled on other non-effective treatment. This highlights the power of bioinformatic tools for identifying predictive signatures in data obtained from flow cytometry. These observations need to be confirmed in studies with larger well-defined patient cohorts including patients that are treatment naïve. Furthermore, longitudinal studies are needed for both MTX and anti-TNF-α therapy.

In psoriasis, biomarkers that can be used to identify preclinical disease or select patients for future treatment will aid in disease management and improve clinical outcome. We hope our methods will facilitate the uptake of flow cytometry and bioinformatic analysis in the study of psoriasis.
CHAPTER 6: GENERAL DISCUSSION

6.1 Regulatory T cell expression of trafficking receptors in healthy humans

Over recent years, our understanding of the importance of Treg trafficking in the control of inflammation has increased. Indeed, in the application of Treg-based cellular therapy, the ability of infused cells to migrate to the appropriate tissue may be a crucial factor in determining the success of therapy. Hence an understanding of the factors controlling Treg localisation could be used to optimise Treg-selection and to contribute to the development of such therapy.

Previous studies on Treg migration in humans used CD25$^{hi}$ to define Tregs [90, 91, 109, 110, 135]. In Chapter 3 (section 3.3.2.1) we demonstrated that using CD25$^{hi}$ to identify Tregs in humans leads to inaccurate findings regarding their expression of trafficking receptors. We are the first to use the CD127$^{lo}$CD25$^{+}$ phenotype to accurately identify all Tregs in order to study their trafficking phenotype. To do this, we examined the expression of β7, α4 (CD49d), CLA, CD62L, CCR4, CCR5, CCR6, CCR10 and CXCR3 on CD127$^{lo}$CD25$^{+}$ Tregs and compared them to conventional T cells (Tconvs) in the peripheral blood of 44 healthy humans.

We found that within effector/memory Tregs, CLA, CCR4, CD62L and CCR6 are expressed on the highest proportion of cells. In comparing effector/memory Tconvs and Tregs, we established that CLA, CCR4, CCR10, CCR5, CD62L and CCR6 are expressed on a higher proportion of Tregs than Tconvs. No differences were found between effector/memory Tconvs and Tregs in terms of α4 (CD49d), β7 and CXCR3 expression. These experiments described in Chapter 3 establish that Tregs are diverse in terms of trafficking receptor expression and that this heterogeneity is different to that seen in Tconvs. Indeed, in an unsupervised hierarchical cluster analysis, we found that Tconvs and Tregs clustered in two
separate groups, highlighting that Tregs have a unique trafficking receptor signature that differentiates them from Tconvs.

A proportion of circulating CD4⁺ T cells exhibit a degree of tropism for certain tissues, particularly the gut and skin. The expression of certain adhesion molecules influences the selective entry of lymphocytes into tissues. Of these tissue-associated homing phenotypes, the gut specific α4β7 [100, 101] and the skin specific CLA, CCR4 and CCR10 [103, 104, 141] are the most defined.

While no differences were found between effector/memory Tconvs and Tregs in terms of expression of the gut-associated β7, a significantly lower proportion of naïve Tregs express β7 when compared to naïve Tconvs. Furthermore, the skin homing receptors CLA, CCR4 and CCR10, are expressed on a much higher proportion of Tregs than Tconvs. For this reason, adult human Tregs can be concluded to show a bias towards migration to the skin, indicating an important role for Treg immune regulation at this site. Interestingly, the reverse has been shown to be true in early life, when the majority of Tregs express α4β7 [110]. This suggests a role for the gut in early immune imprinting, whereby Treg cell exposure to exogenous antigens in early life could shape the Treg compartment and influence homing receptor expression patterns and phenotype in later life.

We also found that effector/memory Tregs contain a significantly higher proportion of CD62L⁺ central memory cells when compared to Tconvs, suggesting a role for Tregs in regulating systemic immunity as central memory cells within lymphoid tissues. This supports the claim that Tregs are involved in both regulating the priming of cells in lymphoid tissue and limiting inflammation in non-lymphoid tissue.
6.1.1 Co-expression of tissue specific homing receptors on Tconvs and Tregs

While a few studies have examined the co-expression of skin and gut associated adhesion molecules and receptors on total CD4\(^+\) T cells in humans [143, 144, 147, 148], none have focused on Tregs. In Chapter 3 (section 3.3.3) we analysed these chemokine receptors and integrins in combination with each other on both Tconvs and Tregs.

Expression of CLA and \(\beta7\) is generally believed to be mutually exclusive [150]. However we have shown that a proportion of cells co-express these markers. Interestingly, we found that an average of 37\% of \(\beta7^+\) effector/memory Tregs express CLA while only 8\% of \(\beta7^+\) effector/memory Tconvs express CLA. Additionally, an average of 67\% of \(\beta7^+\) effector/memory Tconvs express CCR4 while almost all (93\%) of \(\beta7^+\) effector/memory Tregs express CCR4. This demonstrates that CCR4 is expressed by both gut and skin homing cells, indicating a role for CCR4 in trafficking cells to non-cutaneous peripheral tissues, the gut included. This is consistent with its association with homing to Th2 type inflammation (discussed below). On the other hand, we found that CCR10 and \(\beta7\) are generally mutually exclusive on Tconvs, with only 4\% of \(\beta7^+\) Tconvs expressing CCR10. In a study that examined the co-expression of CCR10 and \(\beta7\) on memory CD4\(^+\) T cells but not Tregs, it was concluded that “practically no” CCR10\(^+\) cells express detectable \(\beta7\) [148]. We showed that, in contrast to Tconvs, 30\% of \(\beta7^+\) Tregs express CCR10.

When the co-expression of trafficking receptors is used to define cell subsets, they reveal a broad variety of combinations that can target cells into diverse microenvironmental niches. The \textit{in vivo} significance of the proportions of cells expressing these various combinations is currently unknown. Nevertheless, studying these subsets on both Tconvs and Tregs can generate hypotheses for future study and potential application to therapy.

It can be postulated that the expression of trafficking receptors that favour certain tissues may define the best subpopulation of Tregs for therapy. Certainly, it has been demonstrated that
CLA expressing Tregs are more efficient at preventing skin destruction in an animal model of graft versus host disease (GvHD) [151]. In humans, increased frequencies of CLA⁺ Tregs at the time of neutrophil engraftment in allogeneic stem cell transplant (ASCT) patients have been shown to be associated with a reduction in acute GvHD involving the skin [152]. Furthermore, increased frequencies of CLA⁺ or β7⁺ Tregs are associated with reduced incidence [153] or severity [152] of skin or gut aGvHD, respectively.

6.1.2 Th1 and Th17 associated chemokine receptors and Tregs

It is thought that the preferential expression of CRs can be used to identify Th1, Th2 and Th17 type Tconvs and Th1, Th2 and Th17 – “like” Tregs. Generally, Th1 cells are believed to be CXCR3⁺ [159-162] while Th2 cells are CCR4⁺ [159-164] and Th17 cells CCR6⁺ [165, 166]. However, more recently these definitions have changed with Th1 cells being described as consisting of both CXCR3⁺CCR6⁻ and CXCR3⁺CCR6⁺ subsets, while Th2 cells are CXCR3 CCR6 CCR4⁺ and Th17 cells CXCR3 CCR6⁺CCR4⁺ [167, 168]. Studies that defined these CR expression patterns either examined cells artificially polarised in vitro [159, 160, 163] or isolated circulating CR expressing CD4⁺ T cells from human blood and examined their cytokine profiles following in vitro stimulation [161, 162, 167, 168].

In order to study the CR expression patterns of Th1 and Th17 type T cells, we took a novel approach and designed an 11 colour antibody panel that allowed us to combine the transcription factors T-bet and RORγt with their commonly associated CRs; CXCR3, CCR4 and CCR6. We identified circulating CD4⁺ T cells that express T-bet and RORγt without the need for in vitro stimulation (Chapter 3, section 3.3.4).

T-bet⁺ CD4⁺ cells were found to only express CXCR3, the CR commonly associated with Th1 T-bet⁺ cells. Numerous studies have described circulating CXCR3⁺ CD4 T cells as Th1 type cells [159-162, 167-169]. However, it is evident in our study that in human PB not all CXCR3⁺ cells express T-bet. In fact, in the 44 healthy donors examined, an average of 89%
of CXCR3+CD4+ T cells did not express T-bet. All RORγt+CD4+ cells expressed CCR6, the CR commonly associated with Th1 RORγt+ cells. However, CCR6+ cells did not all express RORγt. Interestingly, we also showed that subsets of RORγt+ cells express the CRs CCR4 and CXCR3. This pattern of expression of trafficking receptors is consistent with a pro-inflammatory role in an array of peripheral tissues [170-172].

In order to examine “Th1” and “Th17” cells, identified as described in the literature [167-169], for the expression of T-bet and RORγt, we gated CXCR3+ (“Th1”) and CXCR3-CCR6-CCR4+ (“Th17”) cells within CD45RO+CD4+ cells and examined them for expression of T-bet and RORγt (Chapter 3, section 3.3.4.1). We found that “Th1” cells, defined as CXCR3+ effector/memory T cells, make up, on average, 23% of PB CD4+ T cells whereas T-bet+ cells makeup only an average of 5.3%. Importantly, an average of 89% of CXCR3+ effector/memory T cells do not express T-bet in human PB. However, the flow cytometry data revealed that in many individuals, T-bet+ cells would fall within the CXCR3+ gate, because CXCR3 expression on T-bet+ cells is generally lower than on T-bet+ cells. “Th17” cells, defined as CXCR3-CCR6-CCR4+ effector/memory T cells, make up an average of 10.8% of PB CD4+ T cells whereas RORγt+ cells makeup an average of 7% of PB CD4+ T cells. Significantly, 71.5% of CXCR3-CCR6-CCR4+ effector/memory T cells do not express RORγt in human PB.

Based on our findings, it appears that CRs alone cannot be used to isolate pure Th1 and Th17 type cells. The use of these surface markers to isolate Th1 and Th17 cells overestimates their frequency in comparison to defining Th1 and Th17 cells based on intracellular staining for T-bet and RORγt.

6.1.2.1 RORγt+FoxP3+ cells

In mice, it has recently been demonstrated that subsets of Tregs co-express the transcription factors T-bet and RORγt with FoxP3 [112, 173, 174]. We were the first to examine the co-
expression of T-bet and RORγt with FoxP3 on unstimulated CD4 T cells in human PB (Chapter 3, section 3.3.4.2). No cells co-expressing T-bet and FoxP3 were identified in human PB. However we did identify a subset RORγtFOXp3+ cells. These cells made up 1.1% of circulating CD4+ T cells, were of the effector/memory phenotype (CD45RO+) and the numbers were highly correlated with the frequency of total RORγt+ cells in each individual.

We also examined the expression of CRs CXCR3, CCR4 and CCR6 on the identified RORγtFOXp3+ cells and compared them to FoxP3+ Tregs and RORγt+ Th17 cells. CR expression by FoxP3+ Tregs and RORγt+ Th17 cells was similar. Both these subsets expressed higher levels of CCR4 and CCR6 than Tconvs. RORγt+ cells expressed the highest levels of CCR6 while FoxP3+ cells expressed the highest levels of CCR4, with RORγtFOXp3+ co-expressing cells taking on both these features, expressing CCR6 at levels similar to RORγt+ cells and CCR4 at levels similar to FoxP3+ cells.

It has been reported that 3-4% of human memory CD4+CD25hi “Tregs” secrete IL-17 following in vitro stimulation [176, 177]. In one study, a large proportion (34%) of “Tregs”, isolated as CD25hi, were found to express RORγt following stimulation [176]. This indicates that in vitro stimulation in combination with the inaccurate identification of Tregs as CD25hi leads to the overestimation of the frequency of RORγt+ Tregs in human PB, highlighting the importance of our work in identifying RORγt expression within unstimulated FoxP3+CD4+ T cells. Our studies did not determine whether de novo expression of transcription factors and cytokines after in vitro stimulation reflects physiological processes that would occur in response to antigen recognition in vivo. In the absence of a method to detect transcription factor expression in viable cells before sorting, the answer to this question remains outside the limits of current technology.
Chapter 6: General discussion

6.2 Regulatory T cell expression of trafficking receptors in autoimmune disease

The syndrome of multi-organ autoimmunity (immunodysregulation, polyendocrinopathy, enteropathy X-linked (IPEX) syndrome) that results from a dysfunctional FoxP3 gene [62] highlights the importance of Tregs in maintaining tolerance to self-antigen in humans. The IPEX phenotype is also evidence that gross defects in Tregs will lead to overwhelming autoimmunity to a whole range of self-antigens. This is not the case in sporadic human autoimmune disease. Thus human autoimmune disease is more likely to result from a partial dysregulation of peripheral tolerance through subtle abnormalities in Treg subset frequencies, function and development.

Epidemiological studies have demonstrated that genetic factors are important in determining susceptibility to autoimmune disease. These observations include the evident clustering of autoimmune disease within families and the higher rate of concordance for autoimmune disease in monozygotic twins when compared to dizygotic twins [33]. The association of several autoimmune diseases within families suggests a level of shared genetic susceptibility amongst them. MHC molecules make the biggest contribution to disease susceptibility, with susceptibility to the majority of autoimmune diseases, including RA [282] and psoriasis [283], being linked to MHC alleles. Other risk loci shared amongst several autoimmune diseases are associated with pathways relevant to Tregs, such as IL2RA [284, 285] and CTLA-4 [286]. However, these susceptibility polymorphisms also occur in normal, healthy people where they are compatible with normal immune function. Furthermore, migration studies provide evidence for differences in the incidence of autoimmune diseases in genetically similar populations living in different conditions [44]. This suggests that an environmental factor predisposes individuals to autoimmune disease development. Interestingly, birthplace has the strongest influence [287], hence signifying an environmental role in early life [288].
The “hygiene hypothesis” was originally proposed to explain the increasing incidence of hay fever and eczema in the UK and the very significant link to birth order, with decreasing susceptibility as birth order increased [289]. The hypothesis suggests that allergic diseases are prevented by infection in early childhood, and that improvements in household amenities, declining family size and higher standards of personal hygiene have reduced the chances of childhood infection. This hypothesis has since been extended to apply to other immunoinflammatory diseases, and is believed to be the cause of the rising incidence of autoimmune disease in parallel with improved hygiene and socio-economic living conditions [40]. It has been argued by Barbara Fazekas de St Groth that there is increasing evidence [290, 291] that the gut microbiome is the environmental factor most influential on predisposition to immunoinflammatory disease [288]. It has been demonstrated that commensal gut microbes are established in early life [292] and this could provide a mechanism underpinning the hygiene hypothesis by explaining how exposure to microbes early in life can have life-long impact on the immune system [288].

How infection protects against autoimmune disease is unknown. It has been suggested that infection stimulates the development of Tregs whose effects extend beyond the invading microbe. In a recent study carried out to assess the influence of heritable vs non-heritable factors on immune cell population frequencies in 210 healthy twins, it was found that most subsets were dominantly influenced by non-heritable factors [293]. Importantly, this study demonstrated that with age, the frequency of Tregs in monozygotic twins diverged, suggesting an environmental influence on Tregs that increases with time [293].

It has been suggested that the control of DC stimulatory capacity is the first point at which Tregs could work to prevent autoimmune disease [80]. Tregs downregulate the steady-state DC expression of co-stimulatory molecules in vivo, therefore preventing autoimmunity by fine-tuning costimulation to control the peripheral threshold for T cell activation [80]. This provides an explanation of how Treg deficiencies could result in susceptibility to autoimmune disease. If costimulation increases due to a Treg defect, auto-reactive T cells
could receive a signal sufficient for driving proliferation and differentiation, steps crucial for a pathogenic autoimmune response [288]. An individual’s genotype affects the specificity of their peripheral auto-reactive cells, the expression of self-antigens and their affinity for self-MHC. Therefore, when an individual’s Treg network fails to perform at its full potential, they would become susceptible to the development of those diseases to which they had a genetic predisposition [288]. This explains how abnormalities in Tregs could result in a wide range of autoimmune responses.

As exposure to infection changes with improved living standards, defects in the Treg compartment may become more frequent, thus explaining the increasing incidence of autoimmune disease, the decreasing age of onset and why more disease predisposition genotypes become involved [288]. According to this model, the severe tissue damage that results from autoimmune disease is therefore not due an aggressive response, but rather the inability of the immune system to clear the self-antigen, hence leading to chronic inflammation [288]. Consequently, the Treg abnormalities that initiate such responses may in fact appear minor and insignificant.

We aimed to search for abnormalities in migratory Treg subsets that may be implicated in the pathogenesis of diseases localised to different tissues. To do this, we measured the proportions of Tregs expressing a range of adhesion molecules in the PB of patients with rheumatoid arthritis (RA) (Chapter 4) and psoriasis (Chapter 5). A summary of the results for Tregs is presented in Table 6.1.

We found a reduction in migratory subsets of Tregs in the PB of both RA and psoriasis patients. RA patients had a significantly reduced frequency of total effector/memory Tregs and effector/memory Tregs expressing CXCR3, CCR4, CCR5 and CCR6 in their PB. Psoriasis patients had a significantly reduced frequency of effector/memory Tregs expressing CXCR3 and CCR6 in their PB. These differences were independent of disease activity, suggesting that these findings could indicate a primary Treg deficiency resulting in less Tregs
Table 6.1: Summary of significant differences in frequencies of effector/memory and naïve Tregs expressing trafficking receptors in the peripheral blood of rheumatoid arthritis and psoriasis patients when compared to controls.

<table>
<thead>
<tr>
<th>Treg subset (%)</th>
<th>RA (n=34)</th>
<th>RA+TNFi (n=10)</th>
<th>Psoriasis (n=44)</th>
<th>Psoriasis (untreated)* (n=20)</th>
<th>Psoriasis+TNFi (n=16)</th>
</tr>
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<tbody>
<tr>
<td>CD45RO⁺ CXCR3⁺</td>
<td>↓</td>
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Significant differences are in comparison to matched controls.
TNFi = Tumour necrosis factor inhibitor
*Untreated psoriasis includes patients using topical therapy only
entering the joints and skin, with consequential disrupted homeostasis. Interestingly, in psoriasis it has been demonstrated that the ratio of Tregs to Tconvs is higher in distant symptomless skin than in psoriatic plaques, where the ratio is skewed towards inflammatory effector T cells [250]. This supports the hypothesis that an imbalance of Tregs and Tconvs at the site could contribute to disease.

When we compared TNF-α inhibitor users to non-users and controls in RA and psoriasis, we found significant differences in the TNF-α inhibitor users. However, it is difficult to conclude that these differences are specifically due to treatment without longitudinal studies on a group of patients undergoing TNF-α inhibitor therapy. Because TNF-α inhibitors are used to treat patients after they have failed other therapies, it is possible that this group of patients may be inherently different to other patients. Therefore it is important to monitor the effects of TNF-α inhibitors on Treg subsets pre and post treatment. In psoriasis, we analysed PB samples from seven patients pre and post therapy with Infliximab and found that although some changes in Tconv and Treg subsets could be attributed to treatment, most were not. For example, although levels of CCR6+ effector/memory Tregs were significantly increased in patients on TNF-α inhibitors, we did not observe an increase in this subset in patients following TNF-α inhibitor therapy. This suggests that this group of patients has inherently different levels of Treg subsets and this could be intrinsic to patients who receive TNF-α inhibitor therapy because of a failure to respond to other therapies. This further reinforces that differences in Treg subset frequencies may represent primary abnormalities in individuals that may not only predispose them to disease, but can also be indicators of disease severity and future response to treatment.

Autoimmunity is a multifaceted disease with variations in response to therapy, clinical manifestations and age of on-set evident within a single disease. The elucidation of all the possible Treg phenotypes and how they vary from health to disease brings us one step closer to understanding the pathogenesis of autoimmunity and to the development of effective therapeutic intervention.
Our observations need to be confirmed in future studies with larger well-defined patient cohorts at different stages of disease and therapy regime. Furthermore, future longitudinal analysis and follow-up of response in patients undergoing therapy is crucial in establishing a definite relationship between pre-treatment Treg subsets and therapeutic response.
REFERENCES

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References


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