CHARACTERISATION OF PI16+ T HELPER CELLS

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Abstract

CD4 T cells, a major component of the immune system, are a heterogeneous population comprising cells with known and unknown function in both health and disease. Biomarkers that identify pathogenic T cell subsets enable early diagnosis and may also provide leads for therapeutic intervention. Peptidase inhibitor 16 (PI16), a recently discovered biomarker by the Barry lab, for a functionally distinct subset of regulatory T cells, is also expressed in T helper cells. However, the characteristics of PI16 expressing T helper cells have not been previously studied. This thesis investigates the molecular and functional characteristics of PI16+ T helper (Th) cells in health and disease.

A high proportion of PI16+ Th cells express the chemokine receptors CCR4 and CCR6 in comparison with PI16- Th cells, indicating ability to respond to chemotactic stimuli, which was confirmed by in-vitro migration assays. Upon in-vitro stimulation, PI16+ Th cells express high levels of the Th17 transcription factor, ROR-γt, and produce more pro-inflammatory cytokines, including IL-17A, TNF, but less IFN-γ in comparison with PI16- Th cells. In the steady state, PI16+ Th cells express high levels of FAS receptor and low levels of CD38 and CXCR5, indicating a mature phenotype. Microarray analysis showed 649 genes were differentially expressed between PI16+ and PI16- Th cells and pathway analysis of the gene profile of PI16+ Th cells indicates a potential role in cell-mediated immune response, migration and inflammatory response. Intriguingly, nearly all PI16+ Th cells are memory (CD45RO+) cells, but not all memory cells are PI16+. Hence, PI16- cells are a mixture of CD45RA+ and CD45RO+.
In order to exclude the possibility that the distinct characteristics of PI16+ Th cells are simply due to their differing memory status, this study further compares the functional and phenotypical difference between purified PI16+ memory and PI16- memory Th cells. PI16+ cells may mimic the properties of long-term resting memory T cells by their high expression of Integrin β1, Hepatic leukemia factor and Clusterin and low expression of Tyrosine kinase and CCL5. In addition, high expression of histamine H4 receptor, Angiotensin converting enzyme 1 and Galectin-1 in PI16+ cells in comparison with PI16- memory Th cells may indicate their potential role in inflammation. Intracellular phosphoprotein analysis showed altered kinetics of STAT signalling by PI16+ cells in response to cytokine stimuli, compared with PI16- cells. Upon stimulation, PI16+ cells secrete more IL-2 than PI16- cells, indicating a potential autocrine driven proliferation status, which was confirmed by proliferation assay. However, PI16+ Th are more apoptotic on the one hand but resistant to suppression by Treg compared with PI16- mem Th cells on the other hand. A chemokine receptor profiling based method to segregate Th1, Th2, Th17 and Th22 subsets indicates, all the subsets express PI16 at varying proportions, but the Th22 subset almost uniformly expresses PI16.

In order to further investigate the role of PI16+ Th cells in disease, a suite of pilot clinical studies were performed including rheumatoid arthritis (RA), juvenile idiopathic arthritis, scleroderma, asthma, chronic sinusitis and type I diabetes. The preliminary data indicate that in the peripheral blood of sinusitis and RA patients, the proportion of PI16+ Th cells were higher compared with healthy
controls. In the peripheral blood of RA and scleroderma patients, higher numbers of CD45RA+PI16+ Th cells were present while these are almost undetectable in healthy control, which may indicate the peripheral expansion of this subset.

In conclusion, PI16 may be a biomarker for long-term memory Th cells with potent effector functions. Preliminary clinical studies on the role of PI16+ Th cells in autoimmune / inflammatory diseases provide promising first data warranting further investigation on the functional role of the PI16+ CD4+ CD25- cells to determine whether or not they represent a novel lineage.
Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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I also give permission for the digital version of my thesis to be made available on the web, via the University’s digital research repository, the Library Search and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

Arunesh Pullaniparambil Mohandas

Date:
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>7AAD</td>
<td>7-amino-actinomycin</td>
</tr>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CBA</td>
<td>Cytometric bead array</td>
</tr>
<tr>
<td>CC</td>
<td>α chemokine</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary Deoxyribonucleic acid</td>
</tr>
<tr>
<td>CFSE</td>
<td>Carboxyfluorescein succinimidyl ester</td>
</tr>
<tr>
<td>Ct</td>
<td>Cycle threshold</td>
</tr>
<tr>
<td>CXC</td>
<td>β chemokine</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Dinucleotide triphosphate</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine teta-acetic acid</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorter</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>FOXP3</td>
<td>Forkhead box Protein 3</td>
</tr>
<tr>
<td>g</td>
<td>Gravitational force</td>
</tr>
<tr>
<td>gDNA</td>
<td>Genomic DNA</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory bowel disease</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>mem</td>
<td>Memory</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean fluorescence intensity</td>
</tr>
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<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility</td>
</tr>
<tr>
<td>ml</td>
<td>Milliliter</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic acid</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>n</td>
<td>Sample size</td>
</tr>
<tr>
<td>nM</td>
<td>Nanomolar</td>
</tr>
<tr>
<td>°C</td>
<td>degree Celsius</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PI16</td>
<td>Peptidase inhibitor 16</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate-13-acetate</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROR</td>
<td>Retinoic acid receptor-related orphan receptor</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute medium</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription real time polymerase chain reaction</td>
</tr>
<tr>
<td>SA</td>
<td>Streptavidin</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>STAT</td>
<td>Signalling Transducer and Activator of Transcription</td>
</tr>
<tr>
<td>T cell</td>
<td>Thymus derived cells</td>
</tr>
<tr>
<td>T1D</td>
<td>Type 1 diabetes</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>Th</td>
<td>T helper cells</td>
</tr>
<tr>
<td>Treg</td>
<td>regulatory T cells</td>
</tr>
<tr>
<td>μg</td>
<td>microgram</td>
</tr>
<tr>
<td>μl</td>
<td>microliter</td>
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<td>μM</td>
<td>micro molar</td>
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CHAPTER 1: INTRODUCTION

1.1 Overview of the thesis

The aim of this thesis is to characterise a new subset of T helper cells that express peptidase inhibitor 16 (PI16) on their cell surface. The classification of a group of T cells as a “subset” is the subject of much debate. What qualifies a group of cells to become a subset? Does the subset require a unique function by secreting signature cytokine(s) or does it have to have a unique surface phenotype by expressing signature surface antigen(s) or both? For this thesis, the helper T cell compartment has been dissected into two separate populations based on their PI16 expression. The resulting PI16+ and PI16- T helper cells are referred to as “subsets” in a logical sense, and not necessarily in a biological sense. However, one of the implied questions that this thesis aims to answer is, whether PI16-positive cells comprise a new T helper cell subset.

The following section of Chapter 1 is a comprehensive review of the current literature on the phenotype of T helper subsets and their role in immune responses and autoimmunity, written as a journal manuscript. The aim of this review is to demonstrate the importance of T helper subset characterisation. The last section of this chapter briefly describes the rationale for the characterisation of PI16+ T helper cells and states the general hypothesis and aims of this thesis.
Chapter 2 describes in detail the general methods used in the experiments presented in this thesis. Specific additional materials and methods used for individual experiments are explained briefly in Chapters 3, 4 and 5.

Chapters 3, 4 and 5 describe and discuss the results of the three main pieces of research undertaken for this thesis. These chapters are written as individual journal manuscripts and therefore have minimal cross referencing. Furthermore, the fundamental rationale of this study is briefly stated in each of the manuscripts. Chapter 3 comprises the initial characterisation of PI16+ T helper cells and PI16- T helper cells. This includes surface phenotype, cytokine profile, transcription factor profile, gene profile using microarray and migratory capacity of PI16+ T helper cells. The results indicate differential characteristics between PI16+ and PI16- T helper cells. One of the key findings of these experiments is that PI16+ T helper cells have a memory phenotype while PI16- T helper cells are a mixture of naïve and memory cells. To exclude the possibility that the differential characteristics between PI16+ and PI16- T helper cells are merely due to biased naïve / memory distribution in these subsets, experiments presented in Chapter 4 have been conducted comparing purified PI16+ cells with purified PI16- memory T helper cells. This includes the validation of microarray data by flow cytometry and RT-qPCR, phosphoprotein analysis, proliferation assays, suppression and apoptosis assays and additional surface marker analysis and cytokine profiling.
Chapter 1

Overview

The aim of Chapter 5 is to identify if PI16+ T helper cells have a role in autoimmune and/or inflammatory diseases. The chapter combines unpublished data from multiple clinical pilot studies. Where appropriate, sample collection and some experimental work for these clinical studies performed by other members of the research group are acknowledged. Data analysis, data interpretation, statistical analysis and full manuscript preparation are credited towards this PhD thesis.

Chapter 6 discusses and speculates the general outcomes, their significance and limitations and future work of this research project.
1.2 Literature review: Human T helper cell subsetting: A brief journey from T cell origin and characterisation to targeted immunotherapy

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Wrote manuscript

Heddy Zola
Evaluated manuscript

Simon Barry
Evaluated manuscript

Doreen Krumbiegel
Evaluated manuscript

Signature...

Date...

10/11/14

10/11/14

11/11/14

11/11/14
Abstract

T helper (Th) cells, where “T” refers to their thymic origin, have been dissected into various subsets. T helper type 1 (Th1), T helper type 2 (Th2) and Th17 subsets are well characterised and widely regarded as major subsets. In the recent past, new subsets have been evaluated which include Th22, follicular B helper cells (Tfh) and Th9. However, as deeper analysis of the gene expression profiles of human T cell populations reveals increasing complexity, it is difficult to predict the number of subsets present in the T helper cell compartment. T helper cells form part of a diverse repertoire of cells responsible for protecting the human body from millions of pathogens present in the environment. Each T cell clone has been selected with unique T cell receptor arrangement to recognize specific pathogen-antigens that the body encounters every day and which are presented by antigen presenting cells (APC). These T helper subsets are broadly classified based on signature cytokine expression, signature transcription factors, cell surface markers, and function. Detailed characterisation of Th cell biomarkers enables classification of their role in immunity and autoimmunity. Furthermore, biomarkers are useful in diagnosis, monitoring disease activity, monitoring the impact of treatment and in some cases biomarkers could serve as target molecules for therapeutic intervention. This review discusses the origin and characterisation of human Th cells and how the signature markers of Th subsets have been a useful tool in understanding immune and autoimmune responses and in immunomodulatory treatments.
1.2.1 Introduction

Autoimmune diseases and inflammatory diseases have been a major focus of research for the past five decades. Although it is clear that an imbalance in T cell populations can lead to a wide range of autoimmune diseases, inflammatory diseases and allergies, it is still not clear what triggers this imbalance. Linking a single effector population with a specific disease is an oversimplification, as several effector populations may contribute to the pathology. This review aims to describe human T helper subset phenotypes in detail and their role in diseases.

1.2.2 T cell origin

The human body is protected from pathogens by the complex interaction of innate and adaptive immune responses. The current classification defines T cells as part of the adaptive immune system. T cells originate from hematopoietic stem cells, and unlike cells of the innate immune system, precursors of T cells migrate to the thymus, where they convert into thymocytes before maturing into either CD4+ T helper (Th) or CD8+ cytotoxic T cells. In early stages of development, progenitor thymocytes express a random repertoire of T cell receptors (TCR) and the surface molecule CD3. Initially thymocytes co-express CD4 and CD8 surface molecules. These cells are referred to as double positive (CD4+/CD8+) cells [1]. In the thymus epithelial region, thymocytes undergo processes called "positive and negative selection", where the cells are introduced to self-antigens through major histocompatibility
complex (MHC) molecules. Cells which do not recognize MHC (negatively selected) or cells binding strongly to self-antigen presented by the MHC (positively selected) undergo apoptosis. Intermediate affinity interactions result in cells which “pass” the selection process and leave the thymus as naive T cells. This selection process is one of the most important stages of T cell maturation, and is critical in maintaining immune tolerance to all organs and tissues of the body, as well as to harmless commensals and antigens. Any breakdown in this process could potentially cause autoimmune or auto-inflammatory diseases or allergy. The selection process also determines the initial lineage commitment. If double positive cells interact with MHC class II during the selection process, CD8 is down-regulated, and the cells mature into CD4+ single positive T cells. A significant proportion of CD4+ T cells differentiate into T helper cells and a subset of them into regulatory T cells (Treg). Treg are a critical subset of the CD4 compartment which emerge from the thymus with specificity for self-antigens in order to prevent inappropriate activation of T helper clones which may have escaped selection in the thymus. However, if the double positive cells interact with MHC class I, CD4 is down-regulated and the cells will mature into CD8+ cytotoxic T cells [2, 3].

After naive T cells leave the thymus, they migrate from one peripheral lymphoid tissue to another sampling the periphery for pathogen-associated antigens. T cells recognize antigens when presented on MHC by professional antigen presenting cells (APC). For example, naive CD4+ T cells will recognize antigen peptides with their TCR only if presented by MHC class II on antigen presenting
cells. After a multi receptor TCR complex interaction, which requires both signal 1; TCR stimulation, and signal 2; CD28 co-stimulation, naive T cells become activated and this shapes the type of response they illicit [4]. The activated T cells proliferate and differentiate into specific subsets of effector T cells / T helper cells depending on the cytokines in their microenvironment, the tissue context of the antigen, and other poorly understood triggers and cell-cell interactions [5]. These cells must not only facilitate an effective immune responses in an event of pathogenic invasion but then return to a resting state to down-regulate the immune responses once the pathogen has been cleared [6]. Signals from surrounding innate cells have a significant influence on the type of immune response generated [7].

1.2.3 Innate – adaptive link

The innate immune system consists of cells including, dendritic cells, macrophages, and neutrophils and innate lymphoid cells, which are among the first responders against pathogen invasion [8]. Originally, the innate immune system was described as non-specific, providing a generic immune response against any pathogens. However, more recent research suggests that the complexity of pattern recognition processes lead to a degree of sensing the type of pathogen to be identified and targeted more specifically [9]. Innate immune cells are equipped with pathogen recognition receptors (PRRs) which recognise molecular patterns present on and in a wide range of microbial organisms. These patterns are known as pathogen-associated molecular
pattern (PAMPs). When innate immune cells recognize PAMPs, an immune response cascade is activated to eliminate the pathogen. Innate immune cells secrete chemokines and cytokines to recruit more immune cells, activate the complement system, phagocytose pathogens or pathogen-infected cells and most importantly, they activate the adaptive immune system for more specialized immune responses. T cells of the adaptive immune system recognize antigens presented by antigen-presenting cells through their T-cell receptors (TCR). The antigen binding site of TCR comprises of α and β chains, which are formed by random gene arrangements during thymic maturation and selection. This process generates multiple clones of CD4 T cells which are able to recognize a great number of antigens. When a CD4 T cell is activated by an antigen, clonal expansion allows T cell clones specific to that particular antigen proliferate in order to combat pathogen invasion. However, in the absence of antigen and activation signals, antigen-specific T cell clones are present in very limited numbers in peripheral blood, which has made isolation and characterisation of antigen-specific T cells in healthy normal samples difficult.

To date, several types of CD4 positive T cells have been described; Th1, Th2, Th17, Tfh (Follicular T helper cells), Th22, Th9 and regulatory T cell (Treg) and each is classified loosely based on their cytokine production, expression of transcription factors, surface molecule expression pattern and function (Figure 1-1). However, more recent research suggests that these are not the only CD4-positive T cell subsets present in the human body. It is still unclear whether the different T helper subsets circulating in the periphery differentiate from
committed precursor cells or common naïve T cells. The cytokines present in the extracellular microenvironment play the primary role in T cell differentiation. These cytokines can induce transcription factors in cells and the enhanced transcription factor expression fine-tuned by micro RNA induces secretion of other cytokines. This feed forward loop commits and maintains the Th subset lineage. Alterations in these cytokines and transcription factors either result in de novo generation of a Th subset or the conversion of one subset to a new phenotype. There is growing evidence that some of the Th subsets might not be terminally differentiated but they could be plastic in nature [10].

1.2.4 T helper subsets and their role in protective immunity

1.2.4.1 T helper 1 cells (Th1)

T helper (Th) 1 cells, the first classified Th cell type, provide protection from intracellular pathogens such as Listeria monocytogenes and Leishmania major through cell-mediated immunity [11]. These cells are characterized by secretion of interferon gamma (IFN-γ) and interleukin 2 (IL-2) [12], and expression of the signature transcription factor, T-bet, which is required for Th1 differentiation [13]. T helper 1 differentiation is dependent on exposure to IFN-γ and IL-12 [14]. Briefly, when IFN-γ binds to IFN-γ receptor on the surface of CD4+ Th cells, it triggers phosphorylation of STAT1 (pSTAT1) [15]. Phosphorylated STAT1 dimerises and then translocates to the nucleus and promotes the transcription of other genes including T-bet. The transcription factors STAT1 and T-bet induce expression of the IL-12 receptor on the surface of the T cell.
When IL-12 binds to the IL-12 receptor, phosphorylation and translocation of STAT4 occurs [17]. The transcription factors STAT1, STAT4 and *T-bet* activate the IFN-γ gene, which results in the production of IFN-γ [18]. This positive feedback loop helps to maintain the Th1 phenotype. In addition to these key signature markers, Th1 highly express the chemokine receptors CXCR3 and CCR5 which together allows the cells to follow chemokine gradients towards CXCL9, CXCL10 CXCL-11, CCL3, CCL4 and CCL5 [19, 20].

Immediately after pathogens are sensed by the tissue, activated APC secrete IL-12 [21], whilst activated CD4 T cells express IL-12R [22]. By binding to its receptor on CD4 T cells, IL-12 induces high production of IFN-γ which itself reinforces the Th1 response. Secreted IFN-γ at the site of infection recruits more phagocytic cells to destroy intracellular pathogens [23]. Therefore, patients with mutations or deficiency in IL-12, IFN-γ or their receptors fail to initiate a Th1 immune response and are prone to severe infections by intracellular pathogens [24, 25]. For example, patients with severe chronic mycobacterial and salmonella infections were found to be deficient in IL-12R expression due to genetic mutation and their PBMC lacked the production of IFN-γ [26]. In addition, Ghalib *et al* showed that increased secretion of IL-12 plays an important role in Th1 mediated immune regulation of visceral leishmaniasis [27]. Analysis of T cell subsets isolated from patients who recovered from *Leishmania donovani* parasite infection showed activation of IFN-γ producing Th1 cells after antigen stimulation [28]. Moreover, elevated levels of IL-12 were observed in pleural fluids of patients with tuberculous
pleuritis but not in the serum of the same patients, suggesting that Th1 mediated immune response are initiated at the site of infection, rather than systemically [29]. In comparison with healthy individuals with latent tuberculosis (TB) infections and uninfected controls from TB endemic communities, TB infected individuals showed decreased levels IFN-γ suggesting a lack of protective immunity [30]. A Th1-mediated response was also observed in tuberculoid leprosy patients, where T cells showed increased Th1 cytokine secretion in response to *Mycobacterium leprae* antigen [31]. In summary, Th1 cells have been shown to play a protective role in many diseases and their signature biomarkers have been used for identification and monitoring.

1.2.4.2 *T helper 2 cells (Th2)*

In contrast to Th1 cells, which are primed to eradicate intracellular pathogens through cell mediated immune responses, Th2 cells fight pathogens in the extracellular space by inducing humoral or antibody-mediated responses. One major function of Th2-mediated host immunity is fighting parasitic helminthes infections. T helper 2 cells are characterized by their secretion of IL-4, IL-5 and IL-13 [32-34]. The transcription factor GATA-3, along with STAT6, induces Th2 differentiation [35, 36]. Briefly, when IL-4 binds to the IL-4 receptor, phosphorylation of STAT6 occurs, followed by its translocation to the nucleus [35]. This triggers the transcription of GATA-3 which activates the expression of Th2 cytokine genes including IL-4, IL-5 and IL-13 [36]. IL-4 secretion sets up a positive loop and maintains proliferation of Th2 cells [37]. In addition, the
chemokine receptors CCR4, CRTh2, CCR8 and CCR3 are highly expressed on this cell type [38-40].

During an infection, IL-4 and IL-13 secreted by Th2 cells activate B cells and increase the production of antibody against the pathogen-antigen [41-45], while IL-5 secretion at the site of infection, recruits eosinophils, resulting in local eosinophilia. These eosinophils are then triggered to degranulate and to destroy the pathogens [46, 47]. Therefore, T helper 2 cells initiate several responses including eosinophilia, intestinal mastocytosis and B cell activation to generate antigen-specific antibodies in order to fight the pathogens [48, 49]. For example, a strong correlation has been reported between increased IgE levels in serum and disease activity of malaria patients as an indication of a Th2-dominant role in parasite infections [50, 51]. In addition, patients with active cutaneous leishmaniasis have elevated levels of serum IL-4 compared with previously infected patients with healed lesions [52]. Likewise, aqueous humor samples of patients infected with Type I/III strain of ocular toxoplasmosis showed Th2-mediated responses as suggested by the presence of IL-5 [53]. In addition to a clear role in parasite clearance, T helper 2-mediated immune responses have also been reported in various types of bacterial and viral infection. For example, in patients with mycoplasma pneumonia infection, levels of IL-4 and IL-5 correlated with the severity of the infection [54]. In herpes zoster patients, an infectious disease caused by Varicella zoster, blister fluid contained increased amounts of Th2 cytokines including IL-4 [55]. A lower respiratory tract infection study in children showed increased levels of IL-4 and
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IL-5 in nasopharyngeal aspirate samples of patients with respiratory syncytial virus (RSV) and with adenovirus infection [56]. Collectively, Th2 not only plays a protective role against parasitic infections but also in bacterial and viral infections.

1.2.4.3 Th17 cells

More than a decade after the identification of the Th1 and Th2 cell lineage, a third subset, Th17, was classified based on their secretion of the newly discovered cytokines, IL-17A and IL-17F [57]. One of the main functions of Th17 cells is to provide immunity against extracellular pathogens in the mucosal regions of the body. The key transcription factors RORγt along with RORα induce Th17 differentiation [58, 59]. The cytokines IL-1β, transforming growth factor beta (TGF-β), IL-6, IL-21 and IL-23, each play a role in the stages of the differentiation of naive T helper cells into Th17 cells [59]. The initial step of Th17 differentiation begins with the activation of STAT3 by TGF-β and IL-6. STAT3 binds to the IL-21 promoter and regulates IL-21 production. Interleukin-21 and STAT3 trigger the transcription of RORγt/α and resulting in secretion of IL-17 and IL-21. Interleukin 21 acts in an autocrine fashion to maintain the differentiation and amplification of Th17 cells [59, 60]. In the later stage, IL-23 present in the microenvironment binds to the highly expressed IL-23 receptor (IL23R), which activates STAT3. This positive loop maintains the Th17 cell pool, which is also characterized by the expression of the chemokine receptors, CCR6 and CCR4 [61-64].
In response to an infection, activated Th17 cells secrete IL-17. The receptor for IL-17 is expressed on a wide range of cells including, but not limited to epithelial cells, endothelial cells and fibroblasts. When IL-17 binds to IL-17R on these cells at the site of infection, they in turn secrete IL-8, G-CSF and IL-6, which enable the recruitment of neutrophils to the site of infection [65]. Interleukin-17 further stimulates granulopoiesis to meet the high demand for the short lived neutrophils at the site of infection. It has been reported that patients with helicobacter pylori–associated gastritis show increased expression of IL-17 at RNA and protein level in gastric mucosal and lamina propria mononuclear cells [66]. T helper cells isolated from the synovium of patients infected with Lyme disease showed increased production of IL-17 after in-vitro polyclonal stimulation [67]. T helper 17 cells also respond to fungal and viral infections. For example, severely burned patients have elevated levels of IL-10 in their serum which inhibit Th17 differentiation as part of an anti-inflammatory mechanism. However, due to lack of Th17 cells, which otherwise provide host protection from Candida albicans infections in healthy adults, the burns patients are prone to these fungal infections [68]. Patients with corneal ulcers, an infection caused by Filamentous fungi, Aspergillus and Fusarium, have increased IL-17 RNA expression in their corneal ulcer samples [69]. Furthermore, patients infected with Epstein-barr virus have a significant number of IL-17 producing Th cells in their peripheral blood [70]. In conclusion, although Th17 cells have a pro-inflammatory phenotype, they are essential for protecting against certain bacterial, fungal and viral infections.
1.2.4.4 T follicular helper cell (Tfh)

Another more recently identified functionally distinct group of T helper cells is the T follicular helper cell subset (Tfh). One of the main functions of these cells is to promote the formation of germinal centers (GC) in B cell follicles [71]. T-cell dependent B cell responses require interactions between activated T helper cells and B cells to provide long-term humoral immunity. In the germinal centers, Tfh play an important role in the differentiation of B cells into plasma cells and memory B cells [72]. CXCR5, IL-6R, CD84 and the transcription factor BCL-6, which were traditionally associated with B cells, are also present in Tfh, suggesting a common genotype and phenotype between these cells [73]. The high expression of CXCR5 enables both Tfh and B cells to migrate to B cell follicles in response to CXCL13 [74]. The surface molecule CD40L, which is highly expressed on Tfh cells, enables the activation, proliferation and differentiation of B cells [45]. In contrast to earlier studies where CXCR5 expression was believed to be restricted to the Tfh subset, recent in-vitro studies show CXCR5-expressing Th2 and Th17 cells can assist co-cultured B cells in antibody secretion [75]. This raises the question whether Tfh are a unique subset or a transient phase of Th1/Th2/Th17 subsets that have localized to the germinal centers to promote an effective humoral response. Regardless of their origin or classification as a defined subset, current studies have shown a key role for Tfh in providing signals to induce B-cells to produce antibodies during host defense. While mouse studies indicate the dependency of Tfh on GC, a recent study showed that human Tfh can persist in the absence of
germinal centers, suggesting either that GC are not the only environment for Tfh or that human Tfh are more promiscuous than mouse Tfh [76]. However, excess Tfh differentiation could lead to autoimmune diseases as indicated by a strong correlation between Tfh numbers and several autoimmune diseases including Sjögren’s syndrome [77], rheumatoid arthritis, autoimmune thyroid disease [78], and systemic lupus erythematosus [79].

1.2.4.5 Th22 cells

T helper 22 cells (Th22) are characterized by the secretion of IL-22. These cells are reportedly present in the epidermal layer in human skin for repair and protection [80]. Interleukin 6 and TNF-α promote the differentiation of Th22 cells. Aryl hydrocarbon receptor (AHR) is the key transcription factor for this subset [81]. High secretion of tumor necrosis factor alpha (TNF-α) along with IL-22 and high expression of CCR10, CCR4, CCR6 are the characteristic features of this subset [82, 83]. Even though IL-22 is a characteristic cytokine of Th22, it can also be produced by Th17 and NK cells. However Th22 do not produce IFN-γ, IL-4 or IL-17, cytokines produced by Th1, Th2 and Th17, respectively [80]. The main function of IL-22 is to defend against bacteria in epithelial cell-rich tissues such as the skin and mucosa [84]. And the receptor for IL-22 is restricted to non-hematopoietic cells which includes skin, lung and intestine cells [85]. Expression of skin homing receptors, CCR10 and CCR4, and secretion of the skin homeostatic cytokine IL-22, indicate the dominant role of Th22 in skin pathophysiology [86]. Moreover, Th22 cells have been associated
with pathogenesis of certain autoimmune diseases which have an involvement of epithelial cell surfaces, including psoriasis [87], rheumatoid arthritis [88], multiple sclerosis [89], and systemic lupus erythematosus [90].

1.2.4.6 Th9 cells

T helper 9 cells (Th9) were classified based on the excess production of IL-9, which has pleiotropic roles in immunity and inflammation [91]. The differentiation of naive CD4 T cells into Th9 cells is induced by the presence of IL-4 and TGF-β in the microenvironment [91]. Transcription factor PU.1 and interferon regulatory factor 4 (IRF4) play a role in the differentiation of CD4+ naive T cells into Th9 cells [92, 93]. Many cells both from the host tissues and from innate and adaptive immune compartments can produce IL-9. Therefore, the correlation of Th9 and disease conditions is still not clear. However, it has been suggested that Th9 plays a pathogenic role in allergic asthma [93], psoriasis [94] and in experimental autoimmune encephalomyelitis [95, 96].

1.2.5 The role of T helper subsets in disease

A tight balance in immune cell subsets is essential for a normal functioning of an immune system. Systemic elevation of specific cell subsets or enrichment of cells in certain tissue locations could potentially result in disease. Building a detailed understanding of this concept requires monitoring of subsets in both health and disease. Multiple T helper subsets are involved in many diseases. However the recruitment, activation or the relative contribution of these subsets
to the overall pathology may vary depending on the tissue affected in the
disease. This section highlights the role of T helper cell subsets, specifically
Th1, Th2 and Th17, in rheumatoid arthritis, type 1 diabetes, asthma,
scleroderma, inflammatory bowel disease and multiple sclerosis, as examples
of autoimmune diseases and chronic inflammatory diseases that affect different
tissues and organs.

1.2.5.1 Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease
classified as inflamed synovial tissue which can lead to joint disability. A
Th1/Th2 imbalance has been known to be an important factor in the
pathogenesis of RA. Part of this requires recruitment of T cells to the affected
tissues and there is enrichment of Th1 cells and to a lesser extent of Th2 in the
synovium of RA patients. For example, T cells isolated from synovial fluids of
inflamed joints of rheumatoid arthritis patients produced more IFN-γ than IL-4,
when activated \([97, 98]\). Expression of the chemokine receptors CXCR3 and
CCR5 on Th1 cells plays a key role in the migration of Th1 cells to inflamed RA
synovium, following the gradient of their respective ligands (CXCL10, CXCL19
and CCL3, CCL4) \([99, 100]\). However the Th1/Th2 ratio is not altered in
peripheral blood suggesting an active recruitment of Th1 to the site of
inflammation \([101]\). Supporting this, Kawashima et al. showed that the
increased number of Th1 cells in RA synovium did not correlate with the
expression of the Th1 transcription factor, \(T\text{-}bet\), in PBMC, which was low in
high disease activity RA patients, suggesting an active migration of these cells to synovium [102].

Although in the past RA was widely accepted as a Th1-associated autoimmune disease, Th17 are now also reported to play a role in inflammation of synovial tissues [103]. Higher levels of IL-21 and IL-23, key cytokines in Th17 differentiation, were also detected in the plasma of patients in the early stages of RA disease and are strongly correlated with disease activity [104]. Furthermore, higher concentrations of IL-23 in synovial fluid were observed in RA patients with bone erosion, and there was a strong correlation between the level of IL-23 and IL-17 in RA patients [105]. In the synovial membrane of RA patients, mRNA expression of IL-17 is strongly correlated with joint damage [106]. Th17 migrate to synovium by following chemokine gradient towards CCL20, which was found in higher concentrations in synovial fluid than in serum of RA patients [107] and higher frequencies of Th17-type CCR4 and CCR6 expressing cells were reported in the joints of RA patients. In addition, expression of Th17-lineage transcription factor ROR-γt was also highly expressed in CD4 T cells of these patients [108]. Taken together, both Th1 and Th17 are involved in the pathology of RA.

1.2.5.2 Type I Diabetes

The destruction of insulin-producing pancreatic β cells by auto-reactive T cells leads to type I diabetes (T1D). Although the specific genetic and environmental triggers are still poorly understood, several studies have confirmed a role for T
cell subsets in this disease. PBMC from first degree relatives of T1D patients produced high amounts of Th1 cytokine, IFN-γ, in the pre-diabetic phase (patients positive for Islet cell antibodies) [109]. However at the initial stages of the disease, during diagnosis, reduced numbers of CXCR3 and CCR5-expressing and IFN-γ-producing Th1 like-cells in peripheral blood, may indicate a possible extravasation of peripheral Th1 towards pancreatic lymph nodes [110]. Furthermore, increased concentrations of CXCR3-ligand, CXCL10 were found in the serum of T1D patients [111, 112]. In a Japanese cohort, polymorphisms in the Th1 transcription factor T-bet correlated with the risk of onset of T1D and the polymorphism resulted in increased IFN-γ gene transcription [113].

In contrast, a protective role for Th2 cells has been proposed in T1D. When stimulated, PBMC from T1D patients showed decreased secretion of Th2 cytokine, IL-4, if collected in the early stages, followed by a high secretion of IFN-γ when collected in the later stages of disease [114]. Lower secretion of IL-13, another Th2 cytokine, in T1D high risk first degree relative groups compared with low risk groups, further suggests a protective role of Th2 in this disease [115]. Furthermore, in immunotherapy experiments PBMC from patients treated with alum formulated glutamic acid decarboxylase 65 (GAD65), an auto-antigen targeted by auto-reactive T cells in T1D, showed steady increase in secretion of Th2 cytokines including IL-5 and IL-13 from the first month onwards beginning treatment [116].
Recent studies have shown that Th17 cells are also involved in T1D [117]. Peripheral blood CD4 T cells of T1D patients showed auto-reactivity to β cell antigens by secreting IL-17, suggesting a role of Th17 in β cell destruction [118]. Mechanistically, this may be linked to monocyte activation as monocytes isolated from T1D patients showed an active status and were primed to degranulate / release proinflammatory cytokines, IL-1β and IL-6, which in turn induces production of IL-17 [119]. In addition, IL-6, one of the driving factors for Th17 differentiation, was elevated in serum of these patients and there was a positive correlation between IL-6 cytokine levels and numbers of IL-17 positive CD4 T cells [120]. In common with other tissue specific pathologies, Type 1 diabetes patients showed an increased number of Th17 cells in pancreatic-draining lymph node but not in peripheral blood [121]. Furthermore, there is also a strong correlation between Th17 cells and C-reactive protein levels (CRP) in T1D patients suggesting an inflammatory role of Th17 in T1D [122]. In conclusion, while both Th1 and Th17 have pathological role in T1D, Th2 may have a protective role.

1.2.5.3 Asthma

T helper 2 cells are known to play an important role in asthma, a chronic inflammatory condition causing airway obstruction. Interleukin- 4, 5 and 13, key signature cytokines of the Th2 immune responses, are confirmed mediators of allergic asthma. Increased mRNA expressions of these cytokines in bronchoalveolar-lavage (BAL) were reported in asthmatic patients [123, 124].
During allergen challenge in asthmatic patients, airway epithelial cells highly express CCL17 and CCL22, ligands for CCR4, to recruit Th2 cells into the lungs. Endobronchial biopsies reveal that the majority of recruited T cells are IL-4+ and CCR4+ and also express elevated CCR8, consistent with a Th2 phenotype [125]. Th2 cells are also recruited to the site of inflammation by prostaglandin D2 (PGD₂), which is highly secreted by mast cells during asthmatic attack [126] and attracts cells expressing CRTH2, a key chemokine receptor expressed by Th2 cells [38]. Increased levels of PGD₂ and CRTH2 expression in BAL fluid were reported in patients with severe asthma [127]. Furthermore, bronchial biopsy specimens from asthma patients indicate increased expression of GATA-3 and higher numbers of STAT-6-immunoreactive cells which further implicate a Th2-mediated role in this disease [128].

While hyper-reactive Th2 are known to play a role in asthma, Th17 cells have also been shown to amplify inflammatory response in this disease. PBMC from asthmatic patients showed increased IL-17 secreting Th cells and also higher expression of the Th17 associated chemokine receptor CCR6 [129]. CCR6-ligand, CCL20, in BAL is up-regulated during asthmatic response enabling the recruitment of CCR6+ CD4 T cells [130]. By secreting IL-17, Th17 cells induce IL-8 secretion by many cells including macrophages, epithelial cells and other lymphocytes, which in turn recruits neutrophils. Sputum samples from asthmatic patients have high levels of IL-17A, and this correlated with IL-8 mRNA and
neutrophil counts [131]. In conclusion, both Th2 and Th17 aggravate the inflammation during asthmatic reactions.

1.2.5.4 Scleroderma

Scleroderma (SSc) is a connective tissue-related autoimmune disease with chronic inflammation and fibrosis. Although Th1 and Th17 subsets are responsible for the inflammation, Th2 is believed to play a role in scleroderma-induced fibrosis [132]. Sakkas et al. showed that Th2 signature cytokine, IL-4, in serum and IL-4 transcripts in peripheral blood were significantly higher in patients with scleroderma in comparison with controls [133]. Interleukin 13, another Th2 cytokine, was found in higher levels in serum of patients with localized scleroderma, causing fibrosis [134, 135]. Moreover, circulating T cells in patients with this disease showed higher proportion of cells expressing CRTH2, a Th2 surface marker [136]. And patients with idiopathic pulmonary fibrosis, a condition related to scleroderma, have higher expression of CCR4+ CD4 T cells than CXCR3+ CD4 T cells in BAL, further confirming the role of Th2 in fibrosis [137].

While Th2 has been shown to play a dominant role in the pathogenesis of scleroderma, Th1 and Th17 also play a role in the initial stages of the disease [132]. The Th1 differentiation-driving cytokine, IL-12, was elevated in the serum of scleroderma patients. Compared with controls, peripheral blood from scleroderma patients showed higher production of Th1 cytokines, IL-12, IFN-γ and IL-2 along with IL-4 [138, 139]. In addition, elevated levels of Th1
chemoattractants CXCL10 and CXCL9, and Th2 chemoattractants CCL17 and CCL22 were found in the serum of scleroderma patients in comparison with control groups [140]. However, CXCL10 serum levels dropped in later stages of the disease compared with initial stages, further supporting the dominant role of Th1 in the initial inflammatory stages [141].

Peripheral blood and fibrotic lesions of skin and lungs of scleroderma patients showed higher concentration of IL-17, suggesting a role of Th17 in the disease [142]. Interleukin-17+ CD4 T cells were found in higher proportion in severe diffuse scleroderma than in mild limited scleroderma [143]. In active stages, the number of circulating Th17 cells in the peripheral blood were elevated and correlated with disease activity. It is hence suggested that IL-17 promotes the proliferation of fibroblasts and excess production of collagen, a pathogenic feature of this disease [144].

1.2.5.5 Inflammatory bowel disease

Inflammatory bowel disease (IBD) is a chronic inflammatory condition in the gastrointestinal tract including ulcerative colitis (UC) and Crohn’s disease (CD). The Th17 subset is one of the major subsets responsible for the pathogenesis of UC and CD. Interleukin-6, an essential cytokine for the differentiation of Th17 cells was found to be secreted in higher levels in PBMC of patients with active inflammatory bowel disease [145]. In addition, expression of IL-17 was high in mucosa and serum of patients with active UC and CD [146]. A genome–wide association study revealed that there is a strong correlation between
polymorphism of IL-23 receptor, a key Th17 marker, and inflammatory bowel disease [147]. Mononuclear cells isolated from colonic specimens of IBD patients showed increased STAT-3 and phospho-STAT3 levels, a characteristic STAT molecule of Th17 [148]. Increased expression of CCR6 and its ligand CCL20 in colonic mononuclear cells further confirms the possible role of Th17 in IBD pathology [149, 150].

Th1 have also been shown to be involved in IBD. Patients with active UC and CD showed increased mRNA expression of Th1 signature cytokines, IFN-γ, in their intestinal mucosa [151]. IFN-γ producing cell numbers were elevated in lamina propria of CD patients compared with controls, however, there was no change in this cell number in UC [152]. High expression of IL-12 mRNA, a Th1 driving cytokine, in gastric mucosa of UC pediatric patients [153] and high proportion of Th1-like CXCR3+ CD4 T cells in submucosa of CD patients [154] indicate Th1 mediated inflammation in these diseases. CCR5, another Th1 chemokine receptor, was also highly expressed in T cells in inflamed colonic mucosa of UC patients [155].

However, Th2 only seems to have a major role in UC. Rectal biopsy from UC patients showed increased expression of Th2 cytokines, IL-4 and IL-13, in active UC compared with inactive UC [156]. Excess IL-13 production in UC patients is responsible for epithelial apoptosis leading to epithelial barrier disruption in the intestine, a major symptom in this disease [157]. In addition, intestinal biopsies of UC patients had higher IL-5 mRNA expression than in CD
Lymphocytes in rectal and gastric mucosa of pediatric UC patients also show increased expression of Th2 chemokine receptor CCR3 [159]. In short, there is a differential role of Th subsets in the pathophysiology of UC and CD.

1.2.5.6 Multiple sclerosis

Multiple sclerosis (MS) is a relapsing remitting autoimmune disease resulting in the demyelination of nerves, and this causes disruption to neurotransmission in the central nervous system. Th17 based inflammation is one of the major cause for this disease. IL-23, a key cytokine for Th17 lineage commitment, was found in elevated levels in the lesions of active MS patients [160]. In MS patients, IL-17 mRNA expression is high in both mononuclear cells in the peripheral blood and in cerebrospinal fluid (CSF), with higher expression in the periphery than in the CSF [161]. Under healthy conditions the spine and brain are immune privileged, and few migratory immune cells can cross the blood brain barrier. However, in MS, IL-17 plays a significant role in the disruption of the blood-brain-barrier (BBB), and disruption of this BBB is permissive for the transmigration of Th17 cells, which causes inflammation in the central nervous system [162]. T cells from MS patients that are reactive to myelin oligodendrocyte glycoprotein are almost entirely CCR6+, a key signature chemokine of Th17, suggesting their potential migration towards its ligand, CCL20 present in choroid plexus [163].

Before the discovery and characterization of the Th17 subset, a number of studies had shown the role of Th1 and Th2 in MS. Increased secretion of the
Th1-driving cytokine IL-12, by mononuclear cells was shown to correlate with MS disease activity [164]. PBMC from MS patients also showed elevated levels of IFN-γ and IL-4 mRNA expression [165]. IFN-γ and IL-4 were also higher in the serum of patients with acute stage MS, indicating the role of both Th1 and Th2 in this disease [166]. A recent study also showed that these cytokines were also higher in relapsing-remitting MS patients [167]. Higher numbers of IFN-γ producing auto-reactive T cells specific for myelin cell proteins, including myelin basic protein (MBP), proteolipid protein (PLP), and myelin-associated glycoprotein (MAG) were found in CSF of MS patients suggesting an active role of Th1 cells at the site of inflammation [168]. Furthermore, numbers of CXCR3+ Th1 cells were higher in MS than in controls [169]. At remission, decreased expression of CCR5, a Th1-type receptor, has been shown to be a maker for disease activity [170]. Patients with relapsing-remitting MS showed decreased Th2 transcription factor, GATA3, expression in blood compared with healthy controls [171]. In summary, Th1, Th2 and Th17 contribute to the pathology of MS.

### 1.2.6 Therapies targeting T helper cytokines and receptors

Phenotypical characterisation of Th subsets has not only helped in monitoring disease activity, but also in providing targets for therapeutic intervention. Various biologic drugs that inhibit inflammatory cytokine and/or signalling pathways or target surface molecules directly are currently in clinical use or
being tested for efficacy and safety in the treatment of various autoimmune and chronic inflammatory diseases (Table 1-1).

Anti-TNF therapy is one of the commonly used therapies to treat multiple diseases including rheumatoid arthritis, inflammatory bowel disease, and psoriasis, by targeting pro-inflammatory TNF. There are currently a number of approved anti-TNF / TNF inhibitor drugs available in the market including Etanercept, Adalimumab, Infliximab, Golimumab and Certolizumab-pegol [172]. More recently, an antibody against IL-6, one of the key cytokines that drive Th17 differentiation, Tocilizumab, has been approved for the treatment of RA. Rheumatoid arthritis patients treated with Tocilizumab have shown a decrease in the number of Th17 cells and a decrease in disease activity [80]. More interestingly, biologic drugs directly targeting IL-17A production by Th17 cells (Secukinumab and Ixekizumab) or targeting IL-17 receptor (Brodalumab) are currently in clinical trials. Secukinumab is currently in phase III trials and has shown significant clinical improvement in RA patients [173].

While multiple targeting strategies are required to treat these complex diseases, not all modalities are efficacious. For example, a clinical trial using Apilimod mesylate, which inhibits the Th1 cytokines IL-12/IL-23, showed no positive results in treating RA patients [174]. A therapy to treat asthma targeting the Th2 cytokine IL-13 (lebrikizumab) showed that patients with mild asthma had reduced late asthmatic response after they were challenged with allergen [175]. However, another randomized clinical trial with severe asthmatic patients
showed that anti-IL-13 (GSK679586) did not improve the condition [176]. These differences may in part be due to types of therapeutics, pharmacokinetics and delivery, and in part due to the heterogeneity of the disease.

As an alternative to targeting Th cytokines, antigen-based therapy has been tested in treating autoimmune disease. For example GAD, a normal antigen present in β-cells is a well-defined auto-antigen for islet-reactive T cells in T1D. Immunization with GAD showed positive results in treating Type 1 diabetes in mice, however this has not been tested in human clinical trials yet [177]. Adhesion receptors have also been targeted in T cell based therapy. Vedolizumab is a monoclonal antibody against α4β7, a key integrin for the migration of T cells to the gut, has been trialed to modulate inflammatory lymphocyte trafficking towards the gut in ulcerative colitis patients, and phase III clinical trials showed increased remission for patients taking this drug, however, there were also severe adverse effects [178].

In summary, T helper subsets have been targeted to treat T cell associated disease for many years. As new subsets or roles in new disease contexts are identified, new biomarkers expressed on and in T cells will be the basis of novel therapeutics in this exciting emerging field of medicine.
1.2.7 Conclusions

Formation and differentiation of T helper cell subsets involve a complex network of interactions. A considerable amount of research has been carried out on subsets of T helper cells and their associated biomarkers and cytokines, but there are still many unsolved questions including, the plastic nature of T helper cells and their ability to convert from one functional phenotype to another, versus the formation of separate functional subsets that perform only one task and have a fixed phenotype.

Characterization of new Th subsets is an essential part of understanding the human immune system. The subsets described in this review only represent the well-studied T helper subsets. Some of the more recently reported T cell subsets include: Rotovirus specific CD4 T cells, characterized by their intestine homing receptor [179]; GM-CSF producing Th cells, which have high apoptotic capabilities [180]; TCR-αβ+ CD4- and CD8- T cells which are highly immunosuppressive [181]; Th cells producing both IL-4 and IL-17, which are elevated in CD4 T compartment of asthmatic patients [182]. In addition, our group has characterised a new memory regulatory T cell subset based on the expression of peptidase inhibitor 16 (PI16) [183].

Although identification of different subsets of T cells by various research groups across the world adds complexity to the already complex network of T cells, defining function-specific T cells based on biomarkers will enable accurate
diagnosis and improve target-based therapies. Treating autoimmune / inflammatory diseases by targeting specific disease-causing auto-reactive T cells is predicted to yield much better results, with fewer side effects than non-specific immunosuppressive drugs which impact the entire immune system.
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**Figure 1-1: T helper subsets**

Figure shows currently known T helper subsets and their signature characteristics including, differentiation-inducing cytokines, secreted cytokines, chemokine receptors and transcription factors. Figure adapted from Dong, C and Martinez, G (2010), Nature Reviews Immunology.
Table 1-1: Monoclonal antibodies in therapeutics

<table>
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<tr>
<th>Target molecule</th>
<th>Disease</th>
<th>Biologic drug</th>
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<tr>
<td>TNF</td>
<td>RA, IBD, Psoriasis</td>
<td>Etanercept, Adalimumab, Infliximab, Golimumab and Certolizumab-pegol</td>
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<td>RA</td>
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1.3 Research rationale and hypothesis

Peptidase inhibitor 16 (PI16) was initially identified in a screen for potential biomarkers on regulatory T cells [183]. In order to evaluate this biomarker, monoclonal antibodies against PI16 were developed, and the expression and function of PI16-positive immune cells was investigated. Our laboratory focus on PI16 expressing regulatory T cells (Treg) led to the observation that PI16 identifies a memory Treg population with increased and stable FOXP3 expression [183]. However, as part of the analysis, we observed that PI16 was also expressed on T helper cells. This interesting finding was the basis for this study, and our interest was enhanced by publications suggesting a potential role of soluble PI16 in several diseases not directly linked to the immune system (cardiac hypertrophy and prostate cancer [184, 185]). However currently there is no information available regarding the structure, function or knock-out experiments for PI16. Nevertheless, as PI16 had not been previously reported in the immune system, this prompted two major questions:

1) Do PI16+ T helper cells have a distinct physiological or pathological role in the human immune system?

2) Is PI16 a biomarker of an existing T helper subset or does it represent a unique subset on its own?

This thesis aims to initiate a comprehensive characterisation of human PI16+ T helper cells and perform a pilot clinical study to determine a potential role in pathology.
Chapter 1

Hypothesis

The general hypothesis of this thesis is that “PI16+ T helper cells are phenotypically and functionally different from PI16- T helper cells.”

The aims of this PhD are:

1. To determine the surface phenotype, gene signature, chemokine profile, signaling molecule profile and cytokine profile of PI16+ T helper cells
2. To determine the proliferation capacity, ability to respond to suppression by regulatory T cells, apoptotic response and migratory properties of PI16+ T helper cells
3. To determine if there is a change in PI16 expression by T helper cells and/or change in naïve/memory phenotype of PI16+ T helper cells in autoimmune and/or inflammatory disease patients.
CHAPTER 2: GENERAL MATERIALS AND METHODS

2.1 Cell isolation methods and analysis of cell surface antigens

2.1.1 Isolation of peripheral blood mononuclear cells

Whole adult blood (10-20 ml) from healthy donors was collected in Lithium-Heparin anticoagulant tubes after informed consent. The project was approved by the Research Ethics Committee of Children, Youth and Women’s Health Service (REC 2007/11/2013). Buffy coats were obtained from the Australian Red Cross with approval for research use. Buffy coats were used in experiments where larger numbers of cells were required and cell sorting was used to isolate cells of interest. In all other experiments fresh whole blood was used. Peripheral blood mononuclear cells (PBMC) were isolated from whole blood or buffy coats using density gradient centrifugation. Blood was diluted 1:2 in sterile HyClone™ Dulbecco’s Phosphate-Buffered Saline / PBS (Thermo Fischer Scientific Inc., USA). 35ml of diluted blood was overlayed on 15ml of Lymphoprep™, a density gradient medium (Stemcell Technologies, Vancouver, Canada), in a sterile 50ml falcon tube. The tubes were centrifuged at 800g for 20 minutes at room temperature (RT), and the centrifuge was set to decelerate with breaks off to preserve the integrity of the mononuclear cell interface. The
PBMC layer was recovered with a pasteur pipette and transferred to a new 50ml falcon tube. The cells were washed in sterile PBS and pelleted by centrifugation twice; first at 800g for 5 min at RT and then at 400g for 5 minutes at RT. The cell pellet was resuspended in 1ml of PBS. Cell count and viability was checked using a Countess® automated cell counter (Life Technologies, Grand Island, NY, USA) by trypan blue staining (Life Technologies). Only cell preparations with viability of 90% or more were used for the subsequent experiments. Cells were resuspended at an appropriate concentration in the appropriate medium for the analyses described below.

2.1.2 Enrichment of CD4+ T cells

CD4+ T cells were isolated from buffy coats or whole blood by a negative selection rosetting strategy using RosetteSep™ Human CD4+ T cells enrichment cocktail (StemCell Technologies). The cell isolation procedure was followed according to manufacturer’s instructions. Briefly, the antibody cocktail which recognizes non-CD4 lineage antigens, including CD8 (non-CD4 T cells), CD16 (natural killer cells), CD19 (B cells), CD36 (erythrocytes, platelets, monocytes), CD56 (natural killer cells), CD66b (granulocytes), TCRγ/δ (non-CD4 T cells) and glycophorin A (red blood cells), was added at 1ml per 25ml of buffy or 50µl per 1ml of whole blood and incubated at RT on a rocker for 20 minutes. After incubation, CD4+ T cells were isolated using density gradient centrifugation as described in sec 2.1.1. The purity of the isolated CD4+ T cells was verified by staining with fluorochrome conjugated anti-CD4 monoclonal
antibody (Figure 2-1) as described in sec 2.1.4, and a stringent requirement that cell preparations should be greater than 90% pure before proceeding was imposed.

![Figure 2-1](image)

**Figure 2-1: Purity of isolated CD4 T cells**
Example histogram plot showing >90% purity of CD4 T cells post isolation using the RosetteSep™ Human CD4+ T cells enrichment method. Population broadcast from lymphocyte gate.

**2.1.3 Isolation of CD4+CD25- and CD4+CD25+ cells using magnetic beads**

Human CD25 Microbeads II (Miltenyi Biotec GmbH, Germany) were used for the isolation of T helper cells (CD4+CD25-) and T regulatory cells (CD4+CD25+) from the purified CD4+ T cells (sec 2.1.2). Briefly, 20µl of CD25 MicroBeads were added per $10^7$ cells in 80µl of MACS® separation buffer
(Miltenyi Biotec) and incubated for 15 minutes at 4°C. The MicroBead-conjugated cells were washed in MACS® separation buffer and pelleted by centrifugation at 300g for 10 minutes and resuspended in 500µl of buffer. In order to magnetically trap the targeted cells, either MS columns, for <10^7 cells or LS columns for >10^8 cells, (Miltenyi Biotec) were placed in a MACS magnetic separator and were rinsed with 1.5-3ml respectively of MACS® separation buffer before applying the cell suspension. This strategy allows for simultaneous isolation of MicroBead-conjugated CD25+ (retained) cells and unconjugated CD25- cells (flow through). The columns were washed 3 times with the MACS buffer to remove the non-bead bound cells from the column matrix. The CD25- cells that flow through the column were collected and washed and pelleted by centrifugation at 300g for 10 minute at RT. Labelled CD25+ cells magnetically bound to the column were harvested by removal of the column from the magnetic separator and flushed out using the plunger provided in the kit, and then washed and pelleted by centrifugation at 300g for 10 minute at RT. The cell pellets were resuspended in 1ml of MACS buffer. Viability check and cell count was performed as described above (sec 2.1.1). Cells were resuspended at an appropriate concentration in the appropriate medium for the analyses described below.

2.1.4 Cell surface antigen staining for flow cytometry

Monoclonal antibodies (Table 2-1) were titrated to determine the optimal staining concentration. If an unconjugated antibody was present in the staining
panel, the unconjugated antibody was detected with a 2 layer anti-Ig biotin reagent strategy first, before staining the cells with streptavidin-fluorophore and other directly fluorochrome conjugated antibodies. Briefly, 0.5-1x10^6 cells in 50µl of PBS were stained per tube. For unconjugated antibodies, 100µl of primary unconjugated antibody (either hybridoma supernatant or diluted purified antibody) was added to each tube. Cells were incubated for 30 minutes on ice, then washed in cold PBS/Azide buffer (0.05% Sodium azide in PBS) (Sodium azide, Sigma) and pelleted by centrifugation at 360g for 5 minutes at RT. Secondary antibody binding was performed by adding 100µl of 1 in 50 diluted Biotinylated Horse Anti-Mouse IgG (H+L) (Vector Laboratories, Inc., Burlingame, CA, USA) in PBS/Azide buffer to each tube. Cells were washed in cold PBS/Azide and pelleted by centrifugation at 360g for 5 minutes at RT. In order to block non-specific immunoglobulin binding sites, cells were incubated in 5µl of 1 in 2 diluted Normal mouse serum (DakoCytomation, Denmark) in PBS/Azide buffer, for 10 minutes on ice. Without washing away the blocking agent, the cells were then stained with a fluorochrome-conjugated streptavidin antibody along with other directly fluorochrome-conjugated primary monoclonal antibodies for 30 minutes on ice in the dark. Cells were washed in PBS/Azide buffer and pelleted by centrifugation at 360g for 5 minutes at RT. Cells were resuspended in 0.5ml of the same buffer and analysed using a flow cytometer immediately.
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2.1.5 Flow cytometry analysis

Stained cells (sec 2.1.4) were analysed using a FACS Canto (BD Biosciences, San Jose, USA). To correct for fluorochrome generated spectral overlap, fluorescence compensation was performed using unstained cells and single-fluorochrome stained anti-mouse Ig k CompBeads (BD Biosciences) for every analysis. Further data analysis was performed using FlowJo software (FlowJo, LLC, USA).

A FACS Aria (BD Biosciences) was used to sterile sort PI16+ and PI16- cells. The gating strategy for sorting is shown in Figure 2-2. In order to maximise sort purity, a larger fluorescence intensity threshold was left between PI16+ and PI16- gates during sorting as shown in Figure 2-2b. Sorted cells were collected in X-Vivo™ 15 Serum-free medium (Lonza Walkersville, Inc., USA). Cells were washed, pelleted and resuspended in the appropriate medium and concentration for further analysis. Viability check and cell count were performed as described above (sec 2.1.1). If the cells were sorted for RNA-based gene expression analysis, they were resuspended in 300μl of RNAlater® solution (Life Technologies) per 10⁶ cells to stabilize and protect cellular RNA, and stored at -20°C until further analysis.
Figure 2-2: Gating strategy used for sorting PI16+ and PI16- cells
(A) Representative dot plot of unsorted cells gated on CD4 lymphocytes showing the gating strategy used for PI16+ and PI16- T helper (Th) cell sorting. (B) Representative QC dot plots showing sort purity. Dot plots show post-sort analysis of sorted PI16- (left) and PI16+ (right) Th cells gated on CD4 lymphocytes indicating the sort purity.

2.2 Functional immunoassay methods

2.2.1 Activation of cells under polarising and non-polarising conditions

Sorted PI16+ and PI16- T helper cells were activated under Th17 polarising and non-polarising conditions (Chapter 3). This protocol was adapted from J. Hiller
and C Traidl-Hoffmann’s method of “Effective and reliable in-vitro generation of human Th17 cells”. Sorted PI16+ and PI16- T helper cells resuspended in X-Vivo™ 15 Serum-free medium were seeded in 96-U-bottomed well plates (1x10^5 cells in a final volume of 200µl per well). Cells were activated using antibodies against CD3 and CD28 (Dynabeads® Human T-Activator CD3/CD28; Life Technologies), at a 1:2 bead-to-cell ratio. Cells were cultured without changing the medium for 7 days at 37 °C in the presence of the Th17-polarising cytokines; IL-1β (20 ng/ml), IL-6 (30 ng/ml), IL-23 (30 ng/ml), and TGF-β1 (2.25 ng/ml) from R&D Systems, anti-IFN-γ (1 µg/ml) and anti-IL-4 (2.5 µg/ml) from BD Biosciences. Control cells, cultured under non-polarising conditions, were activated with CD3/CD8 beads alone as described above. At day 7, culture supernatants for cytokine assay were collected and stored at -80°C until further analysis. For RNA based gene expression analysis, cells were released from the CD3/CD28 beads using the magnetic device provided with the kit and were stored in 300µl RNAlater® solution at -20°C until further analysis.

### 2.2.2 Measurement of secreted cytokines

To analyse secreted cytokine expression levels, the following BD Cytometry Bead Array (CBA) kits and/or BD Flex sets were used: Human Th1/Th2 cytokine kit; Human IL-17 Flex set; Human Th1/Th2/Th17 kit from BD Biosciences. Assay procedures were followed according to the manufacturer’s instructions. Briefly, 50µl of cell culture supernatants or cytokine standard
dilutions were mixed with 50µl of mixed capture beads and 50 µl of PE detection reagent, and incubated for 3 hours at RT in the dark. The capture beads bound with cytokines were washed with the wash buffer provided and pelleted by centrifugation at 200g for 5 minutes at RT. Beads were resuspended in 300µl of the same buffer. Data were analysed using FACS Canto and linear regression was performed using MS Excel. Cytokine concentrations were determined using linear regression analysis of standards.

![Image](image.png)

**Figure 2-3: Example standard curve plot for cytokine standards**
Figure shows an example standard curve plot. Mean fluorescence intensity (MFI) values determined using flow cytometry were plotted against the known concentration of cytokine standards. Unknown cytokine concentration (y) in samples was calculated using the linear regression analysis equation obtained from respective cytokine standards (inset in figure), where ‘x’ represents the MFI of the cytokine bound beads.
2.2.3 Analysis of intracellular cytokines

Intracellular cytokines were analysed using flow cytometry (Chapter 3). Cells were activated under polarising and non-polarising conditions as described in sec 2.2.1. Before intracellular cytokine measurement, cells were re-activated using Phorbo 12-Myristate 13-Aacetate (PMA; Sigma-Aldrich) and Ionomycin (Sigma-Aldrich) at a final concentration of 20ng/ml and 500ng/ml of cell suspension, respectively, for 4hrs at 37°C. In addition, cells were cultured with GolgiPlug™ (BD Biosciences) at 1µl/ml of cell suspension to block the intracellular protein transport process. After 4hrs, cells were washed and stained for surface antigens as described in sec 2.1.4. Intracellular cytokine staining was performed using a Cytofix/Cytoperm™ fixation permeabilization kit (BD Biosciences). Briefly, after cell surface antigen staining as described in sec 2.1.4, cells were resuspended in 250µl of Fixation/Permeabilization solution (BD Biosciences) and incubated for 20 minutes at 4°C. The fixed and permeabilized cells were washed and pelleted using the Perm/Wash™ buffer (BD Biosciences) by centrifugation at 360g for 10 minutes. The cells were then stained with the optimum concentration of fluorochrome-conjugated intracellular cytokine antibodies, determined by titration and incubated for 30 minutes at 4°C in the dark. The stained cells were washed and pelleted twice by centrifugation at 360g for 5 minutes and resuspended in the Perm/Wash™ buffer provided with the kit, followed by immediate flow cytometric analysis using the FACS Canto (BD). Unstained and non-activated cells were used as controls.
2.2.4 Analysis of intracellular phosphoproteins

Phosphorylation status of selected intracellular proteins was analysed using the BD Phosflow T cell Activation Kit (BD Biosciences) according to the manufacturer's instructions (Chapter 4). Buffers and antibodies required for staining phosphoprotein were provided with the kit. Purified PI16+ and PI16-memory Th cells obtained as described in sec 2.1.5 were treated with the following stimuli to induce phosphorylation. 200µl aliquots of 5x10⁵ cells/ml in X-Vivo™ 15 Serum-free medium were treated separately with PMA (400 nM, Sigma), hIFN-α (40,000 units/ml, PBL InterferonSource), hIL-2 (100ng/ml, BD Biosciences), hIL-4 (100 ng/ml, BD Biosciences) and hIL-6 (100 ng/ml, BD Biosciences) for 15 minutes at 37°C, to initiate ERK/MAPK, STAT1, STAT5, STAT6 and STAT3 signaling, respectively. After this phosphorylation induction, the cells were fixed using pre-warmed lyse/fix buffer and incubated at 37°C for 10-12 minutes. Cells were harvested by centrifugation at 600g for 6 minutes and the lyse/fix buffer was discarded after centrifugation. The cells were washed in 1X PBS and pelleted by centrifugation at 600g for 6 minutes. Then the cells were permeabilized using 1ml of cold Perm Buffer III on ice for 30 minutes. The cells were washed 3 times using the stain buffer and pelleted by centrifugation at 600g for 6 minutes, then resuspended in 100µl of stain buffer. The cells were stained with the appropriate Alexa Fluor® 488-conjugated anti-phosphoprotein antibodies (anti-pERK1/2 (20A), anti-pp38 MAPK (36/p38), anti-pSTAT1 (4a), anti-pSTAT3 (4/P-STAT3) anti-pSTAT5 (47) and anti-pSTAT6 (18/P-Stat6)) by incubating for 1hr at RT in the dark. The cells were
washed, pelleted by centrifugation and resuspended in 300µl of stain buffer for flow cytometric analysis. Unstained treated and untreated cells were used as controls. To measure the induction of signalling, the mean florescence intensity obtained for each phosphoprotein for both PI16+ and PI16- memory T helper cells were calculated using Cytobank software (www.cytobank.org) to determine the relative change in phosphorylation of signalling molecules between the two subsets with and without stimulation.

2.2.5 CFSE based suppression and proliferation assays

Suppression assays were performed to determine the ability of PI16+ and PI16- memory (responder) cells to respond to suppression by regulatory T cells (Treg). In this assay, PBMC act as antigen presenting cells which, together with anti-CD3 antibody, activate the T cell receptor (TCR) of PI16+/− T helper cells, and the degree of suppression of these cells is tested by addition of a limiting dilution of Treg. The assay is read out specifically on the responder cells by dye labelling them with CFSE (Carboxyfluorescein succinimidyl ester) prior to co-culture, as described below. Labelled responder cells were co-cultured with soluble anti-human CD3 antibodies (eBioscience, Inc., San Diego, USA) and irradiated PBMC and Treg, at varying Th:Treg ratios (1:1, 1:0.5 and 1:0). PI16+/− memory Th cells, Treg, and PBMC were obtained from three different donors as described in sec 2.1.5, 0 and 2.1.1.
Purified PI16+ and PI16- memory T helper (responder) cells were labelled with CFSE using the CellTrace™ CFSE Cell Proliferation Kit (Life Technologies) according to manufacturer’s instructions. Briefly, the cells were resuspended in pre-warmed sterile PBS /0.1% BSA (Bovine serum albumin, Sigma) at 1x10^6 cells/ml. CFSE stock solution (18µl of provided DMSO added to 1 vial of CFSE) was added at 2µl per ml of cell suspension and incubated for 10 minutes at 37°C. Then the cells were quenched with 5ml of ice-cold X-Vivo™ 15 Serum-free medium and incubated on ice for 5 minutes. Cells were washed and pelleted by centrifugation at 360g for 5 minutes at RT. The wash step was repeated 2 additional times and the pelleted cells were resuspended in the above media at appropriate concentration. Peripheral blood mononuclear cells (responder cell activators) were obtained from a second unmatched donor by density centrifugation as described in sec 2.1.1 were resuspended in X-Vivo™ 15 Serum-free medium and irradiated (30 gray) with a Blood Irradiator to prevent their proliferation. Regulatory T cells (suppressor) were isolated from a third unrelated donor as described in sec 2.1.3 and resuspended in X-Vivo™ 15 Serum-free medium.

Suppressor assays were set-up in U-bottom 96-well plates to facilitate maximum cell-cell contact. All wells were seeded with a constant number of irradiated-PBMC (1x10^5 cells/well) and responder cells (purified PI16+ and PI16- memory T helper) at 2x10^4 cells/well with varying numbers of Treg (2x10^4 cells for 1:1 Th:Treg ratio, 1x10^4 cells for 1:0.5 Th:Treg ratio and no cells for 1:0 Th:Treg ratio). The cells were cultured in 200µl of X-Vivo™ 15 Serum-free
medium with 100ng/ml of anti-CD3 (eBiosciences) for 4-5 days at 37 degrees and 5% CO2.

The samples were analysed using a BD FACS Canto (BD Biosciences) and FlowJo software. Based on the CFSE fluorescence intensity, populations of dividing and undivided cells were gated (Figure 2-4). As dye label per cell is halved with each cell division, discrete gates were set based on CFSE intensity as shown in Figure 2-4. The division index was calculated manually using the following formula as described in the FlowJo software manual:

\[ \text{Division Index} = \frac{\text{Gn}}{\text{G0}} \]

Figure 2-4: An example histogram monitoring lymphocyte proliferation
Histogram plot showing gating strategy used to determine the division index in suppression and proliferation assays.

The number of cells /events in each generation was denoted as G0, G1, G2, etc. G0 denotes number of cells in the undivided population and G1 denotes
number of cells that have divided once, G2 represent number of cells that have divided twice and so on.

Number of CFSE labelled cells at start= G0 + (G1/2) + (G2/4) + (G3/8)…

Total number of divisions= (G1/2)*1 + (G2/4)*2 + (G3/8)*3+…

Division Index= (Total number of divisions)/ (Number of CFSE labelled cells at start).

For proliferation assays, sorted PI16+ and PI16- memory T helper cells were labelled with CFSE as described above and then activated using Dynabeads® Human T-Activator CD3/CD28 (Life Technologies), at 1:2 bead-to-cell ratio, in X-Vivo™ 15 Serum-free medium for 3 days at 37°C and 5% CO₂. On day 3, cells were analysed on a BD FACS Canto and the division index was calculated as described above.

2.3 Molecular Biology

2.3.1 Isolation of RNA

For gene expression analysis, RNA was isolated from PI16+/− sorted cells (sec 2.1.5) in both the steady-state and post activation. In this thesis two methods and kits were used for mRNA isolation. For transcription factor analyses experiments in Chapter 3, mRNA was isolated using the QuickPrep™ Micro mRNA Purification Kit (GE Healthcare, UK), and for microarray experiments in Chapter 3 and the related gene validation experiments in Chapter 4, the
RNeasy® Plus Micro Kit (Qiagen, Hilden, Germany) was used. The usage of two different methods was due to the availability of the kit at the time of experiment. All buffers and reagents used in the RNA extraction processes were provided in the kit. Both the RNA isolation processes were performed according to their manufacturer’s instructions.

For mRNA isolation using QuickPrep™ Micro mRNA Purification Kit, cells stored in RNAlater® solution were pelleted and resuspended in 0.4ml of extraction buffer. 0.8ml of the elution buffer was added to lyse the cells and centrifuged at ~16,000g for 1 minute at RT, to remove the insoluble components and to obtain a clear homogenate. Oligo(dT)-cellulose resin pellet was prepared by centrifuging 1ml of Oligo(dT)-cellulose at ~16,000g for 1 minute at RT. The buffer was discarded and the clear cell lysate was mixed with the resin pellet to allow poly A tailed RNA binding. The tube was centrifuged at ~16,000g for 10 seconds and the supernatant was discarded. The pelleted resin was washed in the high salt buffer 5 times and recovered by centrifugation at ~16,000g for 10 seconds. Two more washes were performed with the low salt buffer and the resin recovered by centrifugation at 350g for 2 minutes and the final resin pellet was resuspended in 0.3ml of the same buffer. This suspension was transferred to a microspin column. The microspin column with resin was washed three times by centrifugation at ~16,000g for 5 seconds at RT and discarding the resulting supernatant after each spin and by adding 0.5ml of the low salt buffer. After the third spin, the microspin column was transferred to a sterile tube, and 0.2ml of the elution buffer at 65°C was added.
to the top of the resin in the microspin column. The column was centrifuged at ~16,000g for 5 seconds to collect the mRNA. The eluted mRNA was incubated at -20°C for 30 minutes after adding 20µl of potassium acetate solution per 200µl of eluted mRNA along with 10µl of glycogen solution and 1ml of chilled 95% ethanol. After incubation, mRNA was precipitated by centrifugation at ~16,000g for 5 minutes at 4°C. The resulting precipitate was resuspended in 20µl of RNase-free water.

For RNA extraction using the RNeasy® Plus Micro Kit, cells stored in RNAlater solution were pelleted and resuspended in 350µl of Buffer RLT Plus to lyse the cells. The resulting homogenized lysate was centrifuged through a gDNA eliminator spin column at 8000g for 30 seconds to eliminate the genomic DNA. 350µl of 70% ethanol was added to this flow-through and then transferred to RNeasy MinElute spin column. The column was centrifuged at 8000g for 15 seconds and the flow-through was discarded. 700µl of Buffer RW1 was added to the RNeasy MinElute spin column and centrifuged at 8000g for 15 seconds. The flow-through was discarded. 500µl of Buffer RPE was added to the spin column and centrifuged at 8000g for 15 seconds. The flow-through was again discarded. 500µl of 80% ethanol was added to the spin column and centrifuged at 8000g for 2 minutes. The flow-through was discarded with the collection tube. Finally, to elute the RNA, the spin column was placed in a new sterile collection tube and 14µl of RNase-free water was added to the spin column and centrifuged for 1 minute at maximum speed. The eluted RNA was stored at -80°C.
The quantity and the purity of the RNA were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). Purity of the RNA samples was verified by its $A_{260}/A_{280}$ ratio to be ~2.0 (Figure 2-5). The purity and the integrity of the RNA samples isolated for microarray experiments in Chapter 3 was further verified by determining the RNA integrity number (Figure 2-6) to be >7 using Agilent Bioanalyser RNA Picochip (Adelaide Microarray Centre, The University of Adelaide).

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>User ID</th>
<th>Date</th>
<th>Time</th>
<th>ng/ul</th>
<th>A260</th>
<th>A280</th>
<th>260/280</th>
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<td>-0.012</td>
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<td>0.499</td>
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<td>1.384</td>
<td>0.698</td>
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</table>

**Figure 2-5: Quantity and quality assessment of RNA using spectrophotometry**

Example RNA QC data generated by the NanoDrop spectrophotometer demonstrating the quantity and quality of RNA extracted from P16+ and P16-memory T helper cells. Sample IDs A, B, C and D represent different donors. The A260/A280 ratio of approximately 2.0 indicates high purity and integrity of RNA.
Figure 2-6: Electropherogram of an RNA sample used for microarray
An example electropherogram to determine RNA integrity number (RIN) of
samples using the Agilent Bioanalyser RNA Picochip performed by staff at
Adelaide Microarray Centre.

2.3.2 Synthesis of cDNA

For reverse transcription quantitative polymerase chain reaction (RT-qPCR),
complementary DNA (cDNA) synthesis was performed using RNA isolated as
described above. Similar to RNA extraction, two different methods were used to
synthesize cDNA. The usage of two different methods was due to the
availability of the kit at the time of experiment. For transcription factor analysis
experiments in Chapter 3, the SuperScript™ III First-Strand Synthesis System
(Life Technologies) was used and for gene expression analysis experiments in
Chapter 4, the QuantiTect Reverse Transcription Kit (Cat# 205311, Qiagen,
Germany) was used. All the buffers and reagents used in these experiments
were provided with the kits. The cDNA synthesis using both the methods was performed according to the manufacturer’s instructions.

For the SuperScript™ III First-Strand Synthesis System, 100ng of RNA was used per reaction. Briefly, 100ng of RNA was mixed with 1µl of 50µM oligo(dT)
primers and 1µl of 10mM dNTP, and DEPC-treated water to bring up volume to 10µl. RNA was denatured at 65°C for 5 minutes and placed on ice for 1 minute. Ten µl of cDNA synthesis mix (2µl of 10X RT buffer, 4µl of 25mM MgCl2, 2µl of 0.1M DTT, 1µl RNaseOUT™ (40 U/µl) and 1µl SuperScript™ III RT (200 U/µl)) was added to the RNA mix, and incubated at 50°C for 50 minutes for cDNA synthesis reaction. The reaction was terminated by incubating at 85°C for 5 minutes. Finally, 1µl of RNase H was incubated with the synthesised cDNA at 37°C for 30 minutes to remove any RNA present in the final product. The cDNA samples were stored at -20°C until further analysis.

For the QuantiTect Reverse Transcription method, 100ng of RNA was used per reaction. To eliminate contaminating genomic DNA, 2µl of gDNA wipeout buffer was added to the RNA sample along with RNase-free water to bring up the volume to 14µl. This mix was incubated at 42°C for 2 minutes and then placed on ice. Six µl of the reverse-transcription master mix was added to 14µl of the template RNA on ice and then incubated for 15 minutes at 42°C for cDNA synthesis followed by incubation at 95°C for 3 minutes to terminate the reaction. The cDNA samples were stored at -20°C until further analysis.
2.3.3 Relative quantification of genes using real-time PCR

Table 2-2: List of primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5’-3’)</th>
<th>Reverse (5’-3’)</th>
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<td>ACE</td>
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<tr>
<td>CCL5</td>
<td>CCAGCAGTCGTCTTTGCAC</td>
<td>TCTGGITTGGCACACACTT</td>
</tr>
<tr>
<td>CCR9</td>
<td>ATGTCAGGCGATTGCGAG</td>
<td>TGCAGTACCAGTAGACAAGGAT</td>
</tr>
<tr>
<td>CFH</td>
<td>GTGAAGTGTTTACCAGTGACAGC</td>
<td>AACCCTACTGCTTGTCCAAAA</td>
</tr>
<tr>
<td>CLU</td>
<td>CCAATCAGGGAAGTAGTAACGTC</td>
<td>CTTCGGCTCTTGCCTTTGTTTT</td>
</tr>
<tr>
<td>EOMES</td>
<td>GCCATGCTTTAGTGACACCCA</td>
<td>GGACTGGAGGTAGTACCAGC</td>
</tr>
<tr>
<td>FOXP3</td>
<td>GTGGCCCGGATGTGAAGGAG</td>
<td>GGAGCCCTTGTCGGATGATG</td>
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<tr>
<td>GATA3</td>
<td>GCAGGCTCTATACACAAAAATGA</td>
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</tr>
<tr>
<td>HLF</td>
<td>CCACCTTTATCCGCCTCC</td>
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<tr>
<td>HRH4</td>
<td>ATGCTAGGAAATGCTTTGGTCA</td>
<td>AGGAATGGAGATCACAACCAC</td>
</tr>
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<td>ITGA4</td>
<td>CACAACACGCTTCCGTTCGCTA</td>
<td>CGATCTGCATCGTGAATCAGC</td>
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<tr>
<td>LGALS1</td>
<td>TCGCCAGCAACCTGAATCCTC</td>
<td>GCACGAAGCTCTTACGCCTCA</td>
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<tr>
<td>PI16</td>
<td>GCAGTGGCCAGCTAAACCTCAG</td>
<td>GCACACCAGTATTCGATGTTG</td>
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<tr>
<td>ROR-γt</td>
<td>GTAACCCGGCCTACTCCTG</td>
<td>GTCTTGAGCCTAGTGCCTG</td>
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<tr>
<td>RPL13a</td>
<td>CGAGGTITGGCTGGAAGTACC</td>
<td>TTCTCGGAGCCTTGTCCGTAAG</td>
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<tr>
<td>T-bet</td>
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<td>TCAGCTGAGTAAATCTCAGGCATTC</td>
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<tr>
<td>TXK</td>
<td>CATCCAGTCCGTCTTTCTGCTG</td>
<td>TGCGACGCTGGGTGATTTTT</td>
</tr>
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</table>

For all RT-qPCR experiments, primer sequences (Table 2-2) were obtained from PrimerBank (http://pga.mgh.harvard.edu/primerbank/) and checked for their specificity using Primer-Blast (http://www.ncbi.nlm.nih.gov/tools/primer-
The primers were synthesized by Geneworks Australia. Real time PCR was performed using KAPA SYBR® FAST qPCR Master Mix (Kappa Biosystems, Boston, MA, USA). Every 10µl of PCR reaction mix contained 5µl of KAPA SYBR® FAST qPCR Master Mix, 0.4µl of cDNA, 0.2µl of 10nM forward primer, 0.2µl of 10nM reverse primer and 4.2µl of RNase-free water. PCR reaction and quantification was performed using a Corbett Rotor-gene 6000 (Qiagen). Ribosomal protein L13a (RPL13A) was used as the housekeeping gene for all experiments due to its stable expression in CD4 T cells (Wang, T 2012). Mean normalized gene expression was determined by using Q-gene software as reported by Perikles, S (2003). The Q-gene software determines the amplification efficiency of each gene (Figure 2-7 and Figure 2-8) before determining the normalised gene expression in samples. In Chapter 3, where the expressions of transcription factor genes in samples were detectable at lower levels, relative gene quantification was performed using comparative Ct method, as accuracy of quantitation at the low end of the standard curve is low.
Figure 2-7: Validation of primer efficiency
Example RT-qPCR quantitation raw data for validation of LGLS1 primer efficiency. cDNA from CD4 T cells was serially diluted and real-time PCR was performed with primers to the target gene (LGLS1) to quantitate expression levels relative to the housekeeping gene (RPL13a).

Figure 2-8: Determining PCR amplification efficiency of genes
An example standard curve plot of LGLS1 gene primers using Q-gene software. The software calculates the amplification efficiency of the gene from the slope of the curve, to determine the correction factor for normalised gene expression in samples. Amplification efficiency of the gene = $10^{1-1/SLOPE}$.
2.3.4 Microarray data analysis

In order to evaluate differences in gene expression between PI16+ and PI16- T helper cells a microarray analysis was performed (Chapter 3). Sorted PI16+/cells (sec 2.1.5) from PBMC of four different healthy adult donors were used for this experiment. RNA was isolated as described above. In order to determine the purity of the PI16+ and PI16- Th cells sorted for microarray analysis $FOXP3$ gene expression in these samples were verified using RT-qPCR, with donor matched purified Treg samples as controls. Purity of the sorted cell samples was also determined as shown in Figure 2-2.

Figure 2-9: QC to determine $FOXP3$ expression in PI16+ Th cells
A representative RT-qPCR quantitation experiment showing the $FOXP3$ expression in PI16+ Th cells and in donor matched PI16+ Treg (3 technical replicates per gene).
Gene expression analysis was performed by the Adelaide Microarray Centre, University of Adelaide using the Affymetrix Human Gene 2.0 ST array. After hybridisation and quantitation, background correction and normalisation, with the help of a bioinformatician (Steve Pederson, The University of Adelaide), relative log-fold changes in gene expression levels were obtained for 1654 genes in PI16+ Th cells in comparison with PI16- Th cells.

The obtained relative gene expression values of PI16+ Th cells were further analysed using Qiagen’s Ingenuity pathway analysis (IPA) software to predict the biological significance and pathways impacted by these genes. The following filters were applied to the data set when analysing in IPA software; species (Rat OR Mouse OR Human), confidence (experimentally observed), tissues (T lymphocytes) and cut-off (Log Ratio = \( \pm 0.5 \)).
CHAPTER 3 : PEPTIDASE INHIBITOR 16
IDENTIFIES MEMORY T HELPER CELLS WITH PRO-INFLAMMATORY CHARACTERISTICS

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Statement of Authorship

Title of paper:
Peptidase inhibitor 16 identifies memory T helper cells with proinflammatory characteristics

Publication status:
Written in manuscript style

Author Contributions:
By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that the permission is granted for the manuscript to be included in candidate's thesis

Arunesh Pullaniparambil Mohandas (Candidate)
Performed all experiments, analysed and interpreted data, wrote manuscript

Signature: .......................... Date: 1.0.11/1.4

Stephen Pederson
Performed statistical analysis for microarray data

Signature: .......................... Date: 5.11.1.4

Heddy Zola
Involved in experimental design and helped to evaluate manuscript

Signature: .......................... Date: 6.11.1.4

Simon Barry
Supervised development of work, involved in experimental design, assisted with data interpretation, evaluated manuscript

Signature: .......................... Date: 11.11.1.4

Doreen Krumbiegel
Designed experimental framework, supervised development of work, assisted with data interpretation and evaluated manuscript

Signature: .......................... Date: 11.11.1.4
**Abstract**

T helper cells (Th) play a major role in an immune reaction. Unique signature biomarkers of T helper subsets not only enable the identification of cells with distinct functions, but also serve as a tool for therapeutic applications. As part of a gene and biomarker discovery program in our group, peptidase inhibitor 16 (PI16) was recently identified on a functionally important subset of regulatory T cells (Treg). We also observed expression of PI16 on a subset of CD25-CD4+ cells, and we have now compared the phenotype of PI16-positive T helper cells with PI16-negative T helper cells in healthy humans. We found that nearly all PI16+ Th cells express CD45RO and CD95. In addition, PI16+ Th subset have higher percentages of CCR4+ and CCR6+ cells but lower percentages of CD38+ and CXCR5+ cells in comparison with PI16- Th subset. When stimulated with anti-CD3/CD28 beads, PI16+ Th cells produce more IL-17A, IL-10, TNF, but less IFN-γ and have a higher expression of ROR-γt in comparison with PI16- Th cells. Transwell migration assays revealed that PI16+ Th cells migrate better than PI16- Th cells in response to CCL17 and / or CCL20. Microarray data showed that 649 genes were differentially expressed between PI16+ and PI16- Th cells. In addition, pathways analysis of the gene profile of PI16+ Th cells indicated that they may have an important role in cell-mediated immune response, migration patterns and inflammatory response. In summary, we have identified a biomarker of a proinflammatory Th subset with the potential of migrating to inflammatory sites in response to chemokine gradients, which might play an important role in infection or inflammation.
3.1 Introduction

With the advent of genomics based gene discovery, the number of T helper (Th) cell subsets has grown in the past three decades. Since the description of Th1/Th2 by Mosmann and Coffman in 1986, several additional subsets have been described (Th17, Th9, Tfh and Th22), mostly identified by a signature surface marker profile and expression of specific transcription factors, signalling molecules and cytokines [1-5]. More recently, new subsets have been reported based on their unique function; including GM-CSF producing Th cells, which modulate the immune response of other T cells [6]; CXCL13 producing CD4+ T cells which recruit CXCR5+ cells to the site of inflammation in RA patients leading to chronic inflammation [7]; IL-17 producing Th2 cells which increase the severity of allergic asthma [8]; IL-4 / IL-17 producing CD4+ T cells which are present in increased numbers in patients with bronchial asthma in comparison to healthy adults [9].

There is much debate regarding the overlapping function of the multiple Th subsets in health and disease, their interactions and their differentiation potential. Given that these cells function in a complex tissue microenvironment and are exposed to multiple signalling networks involving surface receptors and cytokines, the theory of T cell plasticity has been proposed. This had led to the need for new biomarkers to segregate functionally distinct T cell subsets. Intrigued by our study on peptidase inhibitor 16 (PI16)+ regulatory T cells and in order to gain further insight into Th biology, we propose another Th subset,
which is identified by surface expression of PI16. We have previously shown that PI16 is expressed on a significant proportion of $\text{FOXP3}^{\text{high}}$ regulatory T cells as well as CD25- T helper cells [10]. In healthy humans, PI16+ Treg express the chemokine receptors CCR4 and CCR6 similar to Th17, suggesting that they can follow the same tissue cues as Th17 cells to regulate inflammation [11]. Our studies in juvenile idiopathic arthritis patients (JIA) supported our hypothesis showing an enrichment of Treg in inflamed joints of JIA patients whilst the number of PI16 expressing Treg was low [12]. Although the full biological role of this peptidase inhibitor is still not known, it was originally identified as a key biomarker of prostate cancer prognosis and heart disease [11-19]. Although not in immune cells, other studies report a strong correlation of PI16 with a number of disease conditions. A recent study shows PI16 was elevated in cardiac-selective fibrosis mice model, suggesting an anti-hypertrophic role [19]. PI16 was proposed as a prognostic marker for prostate cancer recurrence [13]. Patients with acute renal allograft rejection showed an increased level of PI16 in their plasma [20]. In heart failure, PI16 inhibits the growth of cardiomyocytes in-vivo and in-vitro [16]. Another study conducted with mammary tumour cell lines indicated that PI16 suppressed cell growth [17]. Together these studies suggest that PI16 could have an anti-proliferative role.

However, little data have been published regarding the role of PI16 within the immune system. Therefore, we have characterised the phenotype and functional characteristics of PI16+ Th cells and compared them with PI16- Th cells. In the steady state, PI16 expressing Th cells represent a pool of CCR4+,
CCR6+ and CXCR3+ cells with memory phenotype and do not exclusively represent Th1, Th2 or Th17 cells. However, upon activation these cells produce higher levels of the pro-inflammatory cytokines, IL-17A and TNF, while also secreting IL-10, and high expression of the transcription factor, ROR-γt. In addition, PI16+ Th cells migrate towards inflammatory chemokines and have a higher proliferation rate compared with P16- Th cells, suggesting a greater role during inflammation.

3.2 Materials and methods

3.2.1 Isolation of peripheral blood mononuclear cells (PBMC)

Peripheral blood was collected in Lithium-Heparin anticoagulant tubes from healthy adults after informed consent. The project was approved by the Research Ethics Committee of Children, Youth and Women’s Health Service (REC 2007/11/2013). Buffy coats were obtained from the Australian Red Cross. PBMC were isolated from whole blood or buffy coat using standard density gradient centrifugation (Lymphoprep, Axis-Shield, Oslo, Norway).

3.2.2 Analysis of cell surface protein expression

Monoclonal antibodies to CD4, CD25, CD45RO, CD45RA, CD27, CD38, CD62L, CD95, CCR4, CCR5, CCR6 and CXCR3 were obtained from BD Biosciences (San Jose, CA) and CXCR5 from R&D Systems (Minneapolis, MN). These markers were selected based on the information from Zola, H et al.
Chapter 3

(2010), Leukocyte and Stromal Cell Molecules: The CD Markers. Anti-human PI16 antibody, which was generated in-house as previously described, is an IgG antibody and does not have any agonistic effect [11]. Surface molecules were stained using standard flow cytometry protocols and analysed using a FACS Canto (BD Biosciences).

3.2.3 Th17 polarising conditions

PBMC stained with CD4, CD25 and PI16 were sorted into CD4+CD25-PI16+ (PI16+ Th) and CD4+CD25-PI16- (PI16- Th) populations using a FACS Aria (BD Biosciences). Sorted cells were cultured in normal and Th17 polarising conditions based on the Miltenyi Biotec protocol [22]. Briefly, for Th17 polarised condition, cells were cultured in X-Vivo 15 serum-free medium (Lonza, Basel, Switzerland) with IL-1β (20 ng/ml), IL-6 (30 ng/ml), IL-23 (30 ng/ml), and TGF-β1 (2.25 ng/ml) from R&D Systems and anti-IFN-γ (1 µg/ml) and anti-IL-4 (2.5 µg/ml) from BD Biosciences. Cells were stimulated with anti-CD3/CD28 beads (Dynal T cell expander kit; Invitrogen) for 7 days at 37°C without changing the media. For comparison, cells were stimulated with beads alone without any polarising cytokines.

3.2.4 Intracellular and secreted cytokine profiling

Cells cultured in Th17 polarising conditions as described in sec 3.2.3 were restimulated using Phorbol 12-myristate-13-acetate (10 ng/ml, Sigma-Aldrich, Steinheim, Germany) and Ionomycin (1 µg/ml, Sigma-Aldrich) along with
GolgiPlug (BD Biosciences) for 4 hrs on day 7. Cells were co-stained for intracellular IL-17A and IFN-γ according to the manufacturer’s instructions (BD Biosciences). Comparison with cells cultured under non-polarising conditions was also performed.

Cell culture supernatants were collected and stored at -80°C for later measurement of the amounts of secreted cytokines. Th1/Th2 cytokine Cytometric Bead Array kit (BD Biosciences) was used to measure, IL-4, IL-5, IL-10, TNF, IFN-γ and a Flex set (BD Biosciences) was used to measure IL-17A. Cytometric bead array analysis was performed using the manufacturer’s protocol and the data was analysed using a FACS Canto (BD Biosciences).

### 3.2.5 Transcription factor profiling

To evaluate the relative expression of key transcription factors in these cells, mRNA samples were isolated from cultured cells from the experiment described in sec 3.2.3 using an *illustra* QuickPrep mRNA Purification Kit (GE Life sciences). cDNA templates were synthesised using SuperScript® III First-Strand Synthesis System (Life technologies). Real-time quantitative PCR was performed using KAPA SYBR FAST qPCR 2X kit (Kapa Biosystems, Wilmington, MA). Relative gene expression for *T-bet* (forward 5’-GATGTTTGTGGACGTGGTCTTG, reverse 5’-TCAGCTGAGTAATCTCGGCATTC); *GATA3* (forward 5’-GCGGGCTCTATCACAAAATGA, reverse 5’-GGCTCAGGGAGGACATGTGT),
ROR-γt (forward 5’-GTAACGGGCCTACTCCTG, reverse 5’-GTCTTGACCACCTGGTTCCTGT), FOXP3 (forward 5’-GTGGCCCGATGTGAGAAG, reverse 5’-GGAGCCCTTGTCCGATGATG) and PI16 (forward 5’-GCGTGAGCACTACAACCTCAG, reverse 5’-GCACACCAGTAATTGTGTTGG) were determined using Rotor-gene 6000 (Qiagen). Gene expression was normalised to house-keeping gene, RPL13a (forward 5’-CGAGGTTGGCTGGAAGTACC, reverse 5’-CTTCTCGGCCTGTCTCCGTA). Primers were obtained from GeneWorks, Australia. The method is described in detail in Chapter 2 Sec 2.3.3.

### 3.2.6 *In-vitro* leukocyte migration

To determine the differential migration capacity of PI16- and PI16+ T helper cells, *in-vitro* transwell migration was analysed using transwell plates (5um pore size, Corning). Migration assays were performed as previously reported [11]. Briefly, 1X10^6 PBMC were seeded in the upper chamber. CCL17 (100 ng/ml) and/or CCL20 (100ng/ml) chemokine were added to the lower chamber. After 2 hours incubation, non-migrated cells in the top chamber and migrated cells in the bottom chamber were stained with CD4, CD25 and PI16 antibodies. Differential migration was calculated by comparing the percentage of PI16+Th cells and PI16- Th cells in the top chamber and bottom chamber.
### 3.2.7 Microarray data analysis

To determine the gene signature of PI16 expressing Th cells the Affymetrix Human Gene 2.0 ST array was used. RNA was isolated from FACS purified PI16+ and donor matched PI16– Th cells from 4 independent donors. Quality of RNA was assessed using Agilent bioanalyzer before performing the microarrays. Samples were amplified, hybridised and scanned at Adelaide Microarray Centre according to the standard Affymetrix protocols.

All subsequent analysis was performed in R (R Development Core Team), using v16.0.0 of an Ensembl centric gene-level chip description file (CDF) (http://brainarray.mbni.med.umich.edu/) which targeted 37217 unique Ensembl gene (ENSG) identifiers. The aroma.affymetrix framework [23] was used in conjunction with the package limma [24]. For detection above background (DABG), parameters for a modified MAT (Model-based Analysis of Tiling-arrays) model [25] were estimated using the set of background probes from data which was optical-corrected & quantile normalised. Probes were assigned to one of 10 equally sized bins based on the fitted values obtained, and empirical distributions of the residuals were obtained for each bin. Fitted MAT values were subsequently obtained for the remainder of the signal-targeting probes across the array, probes were assigned to one of the bins and p-values obtained based on the empirical distributions. A single gene-level p-value for $H_0: S=0$, was obtained using Fisher’s method across all arrays & genes with a p-value $\geq 0.05$ were declared as undetectable. For the remaining genes, probe-
level p-values were calculated using the same method and those for which \( p \geq 0.05 \) were excluded. Identifiers with less than four probes remaining after this process were also removed, leaving a total of 19874 unique Ensembl identifiers for downstream analysis. Background correction on the complete dataset was performed using Robust Multi-array Average (RMA) [26] & array quality was assessed using RLE (Run-length encoding) & NUSE (Normalised Unscaled Standard Error) plots [27] with no arrays being flagged as low quality arrays. Gene-level signal estimates were obtained for each array using probe-level modelling after DABG filtering as described above.

Differences in gene expression levels were obtained for each donor. Donor-based weights were estimated for each comparison & applied during model fitting, with FDR (false discovery rate)-adjusted p-values being obtained for each identifier. Based on visual inspection of volcano plots a two-step gene selection method was applied. An initial set of genes was selected based on unadjusted p-value, with a second set of genes being selected based on unadjusted p-value, but with a threshold applied to the absolute value of log fold-change. FDR estimates were also obtained for each group based on the p-values alone.

To further understand the potential functional role of PI16+ Th cells based on gene expression, Qiagen’s Ingenuity pathway analysis software was used. The following filters were used for analysis; species (Rat OR Mouse OR Human),
confidence (experimentally observed), tissues (T lymphocytes) and cut-off (Log Ratio = ±0.5).

### 3.2.8 Statistical analysis

GraphPad Prism (San Diego, CA) was used to perform statistical tests. For surface marker analysis, secreted cytokine profiling and transcription factor profiling, the Holm-Sidak method was used to determine statistical significance. For migration assay and intracellular cytokine profiling, ratio-paired T test was used. Alpha value was set to 0.05 to determine statistical significance in all tests and to ensure only less than 5% chance of error is possible in accepting / rejecting the hypothesis.

### 3.3 Results

#### 3.3.1 Surface characteristics of PI16 expressing T helper cells

We confirmed that PI16 is expressed on a subset of Treg and T helper cells as previously reported [10, 11]. We first analysed the proportion of PI16 expression on Th cells in peripheral blood of healthy adults. The expression of PI16 on T helper cells was analysed in 22 healthy adult donors. A gating strategy was followed as shown in Figure 3-1a. Expression of PI16 on T helper cells ranged from 6.5% to 26.7% with a median of 11.75% (n=22) (Figure 3-1b).
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To further determine the surface phenotype of PI16+ Th cells, PBMC stained with CD4, CD25 and PI16 were co-stained with antibodies to CD27, CD38, CD62L, CD95, CCR5, CXCR3, CXCR5, CD45RO, CCR4 and CCR6. The differential expression of these markers on PI16+ Th cells were compared with PI16- Th cells (Figure 3-1c & d). In comparison with PI16- Th cells, a significantly lower percentage of PI16+ Th cells express CD27 and CD62L in comparison with PI16- Th cells (84.7% vs 95.0% and 79.2% vs 90.5%; p<0.05). Almost all of the PI16+ Th cells express CD45RO and CD95 (mean, 93.2% and 92.1% respectively). However, these cells have very low percentages of CD45RA and CD38 (mean 4.5% and 4.7% respectively). Further investigation of chemokine receptor expression showed that, PI16+ Th cells have high proportion of CCR4+ (mean; 60.8% vs 10.3%) and CCR6+ (mean; 61.6% vs 22.2%) cells, but barely express CXCR5 (mean; 2.2% vs 17.5%), in comparison with PI16- Th cells.

3.3.2 Intracellular and secreted cytokine profile

Results from the investigation of surface characteristics suggest that PI16+ Th cells have more similarities with Th17 cells than Th1 or Th2 cells. To investigate this further, we analysed cytokine expression capability under Th17 polarising conditions. PI16+ and PI16- sorted T helper cells were stimulated with anti-CD3 / CD28 beads, under Th17 polarising conditions for 7 days. On day 7, after restimulation with PMA / Ionomycin, intracellular production of IL-17 and IFN-γ in PI16+ Th cells was compared with PI16- Th cells. Under non-polarising
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PI16+ T helper cells

conditions PI16+ Th cells produced significantly more IL-17 (mean; 8.5% vs 1.6%, p<0.05) but less IFN-γ (mean; 19.5% vs 39.3%, p<0.05) than PI16- Th cells (Figure 3-2a). Under Th17 polarising conditions (Figure 3-2b) the number of PI16+ Th cells producing IL-17 is higher compared with the PI16- cells (11.9% vs 3.7%, p<0.05), and the induction of IL-17 production by polarised PI16- cells was less than that of unpolarised PI16+ cells (3.7% vs 8.5%).

Cytokine levels in culture supernatant were measured and compared between PI16+ Th cells and PI16- Th cells. As shown in Figure 3-2c & d, under non-polarising conditions, high levels of IL-10 (mean; 11.2 ng/ml vs 6.0 ng/ml), TNF (mean; 12.4 ng/ml vs 8.2 ng/ml), and IL-17 (mean; 4.6 ng/ml vs 1.1 ng/ml) were detected in the supernatant of PI16+ Th cells in comparison with PI16- Th cells (p<0.05). Whereas, under Th17 polarising conditions decreased levels (p<0.05) of IL-2 (mean; 4.2 ng/ml vs 9.2 ng/ml) were detected in PI16+ Th cell supernatant in comparison with PI16- Th cell supernatant (p<0.05). However, the IL-17 level was higher (mean; 3.8 ng/ml vs 1.5 ng/ml; p<0.05) in PI16+ Th cells supernatant compared with PI16- Th cells under Th17 polarising conditions.

3.3.3 Transcription factor profile

Gene expression levels of the transcription factors FOXP3, T-bet, GATA3 and ROR-γt, were examined under non-polarising and Th17 polarising conditions. Relative gene expression was calculated for PI16+ Th cells with PI16- T helper
as the comparator control. As shown in Figure 3-3a, under non-polarising conditions, PI16+ Th cells express significantly higher levels \((p<0.05, n=4)\) of \(ROR-\gamma t\) in comparison with PI16- Th cells. \((p<0.05, n=4)\). However, there is no relative difference in the expression of \(T-bet\) and \(GATA3\). There is a marginal increase in \(FOXP3\) expression in PI16+ Th cells than in PI16- Th cells. However it is not statistically significant and the \(FOXP3\) expression is several orders of magnitude below the levels detected in regulatory T cells (data not shown). Although there was a modest relative increase in the expression of \(ROR-\gamma t\) and \(GATA3\) in PI16+ Th cells under Th17 polarising conditions, \((n=4)\), it did not achieve statistical significance (Figure 3-3b). Similarly, the apparent decrease in expression of \(T-bet\) and \(FOXP3\) \((n=3)\) is also not statistically significant. Expression of PI16 gene is significantly higher in PI16+ Th cells than in PI16- Th cells \((n=3, P<0.05)\) after activation in non-polarised conditions. However its expression was under detectable levels after treating with Th17 polarising mediators.

### 3.3.4 Chemotaxis assay

To examine the \textit{in-vitro} migration capacity of PI16+Th cells in response to the CCR4-ligand CCL17 and the CCR6-ligand CCL20, a transwell migration assay was performed. The percentage of PI16+ Th cells to PI16- Th cells were calculated in each of the top and bottom chambers. As can be seen in Figure 3-4, the percentage of PI16+ Th cells was significantly higher \((p<0.05)\) in the
bottom chamber, when ligands CCL17 and/or CCL20 were present, indicating that PI16+ cells migrate towards inflammatory cytokines CCL17 and CCL20.

3.3.5 Gene profile

In order to determine if PI16 expressing Th cells express a unique gene profile, donor matched RNA from PI16+ and PI16- Th cells were analysed using Affymetrix microarray. 649 genes were differentially expressed between PI16+ and PI16- Th subset (Figure 3-5a). Table 3-1 and Table 3-2 show the top under-expressed and over-expressed genes respectively. Furthermore, in order to understand the functional difference between PI16+ and PI16- Th subsets, microarray data was analysed using Ingenuity Pathway Analysis software (Qiagen). Figure 3-5b shows the top diseases and functions related to gene expression of PI16+ Th cells which include cellular movement, immune cell trafficking, cell mediated immune response, inflammatory response, hereditary disorder, neurological disease, cell death and survival, cellular growth and proliferation and haematopoiesis.

3.4 Discussion

This study describes for the first time a unique subset of T helper cells identified by the expression of Peptidase Inhibitor 16 (PI16) on the cell surface which have resting memory phenotype with pro-inflammatory characteristics.
Whilst PI16- Th cells can be further divided based on expression of CD45RA or CD45RO, essentially all PI16+ Th cells express CD45RO indicating a memory phenotype. In addition, nearly all PI16+ Th cells express CD95 but only a few express CD38. CD95 is traditionally associated with apoptotic signalling, however, recent studies suggest that it has a multifunctional role including anti-apoptosis, proliferation, migration and activation [31-34]. Furthermore, CD38 has been associated with activation of naive T cells and is lost as T cells mature [28]. Studies have shown that CD4+CD38- are more hyper-proliferative in comparison with CD4+CD38+ T cells [29, 30]. Collectively, expression of CD45RO, CD95 and absence CD38 on PI16+ Th cells suggest a mature, hyper-proliferative memory phenotype. Further analyses of the surface characteristics revealed that PI16+ Th subset have a very high proportion of cells expressing CCR4 and CCR6, key chemokine receptors in the pathogenesis of autoimmune / inflammatory diseases [35] and associated with Th17 cells [36]. Interestingly, CCR4 is also a chemokine receptor expressed on the Th2 subset. In addition, PI16+ Th cells also express CXCR3, a Th1 marker [37]. However, these cells do not express CXCR5. Although CXCR5 expressing T cells are known to help B cells in germinal centers [38], studies have shown aged memory cells lack CXCR5 [39] which may explain why there is no CXCR5 expression on PI16+ Th cells. In short, based on the chemokine receptor profile, PI16 expressing T helper cells do not fit neatly into the existing Th1, Th2 and Th17 paradigm.
In order to evaluate whether PI16+ Th status may identify a plastic Th subset capable of switching lineages, PI16+ Th cells and PI16- Th cells were stimulated under non-polarising and Th17 polarising conditions. Our results indicate that PI16+Th cells produce more pro-inflammatory cytokines including IL-17 and TNF and less IFN-γ. Under non-polarising conditions, more IL-10 was present in the culture supernatant of PI16+Th cells than in PI16- Th cells, which might be an inherent mechanism of these cells to self-regulate their excess proliferation or to regulate other T cell subsets. Under Th17 polarising conditions less IL-2 was present in PI16+ Th cell culture supernatant than in PI16- Th culture supernatant. Although the reason is still not clearly known, an inverse correlation between IL-2 expression and Th17 differentiation has previously been reported [21, 40], suggesting that PI16+ Th may be permissive to Th17-like conversion. Furthermore, relative gene expression analysis between PI16+ Th cells and PI16- Th cells indicates the presence of the Th17 promoting transcription factor RORγt in PI16+ Th helper subset, supporting the pro-inflammatory phenotype. However, under Th17 polarising condition there is no relative difference in levels of RORγt between PI16+ and PI16–subsets indicating that PI16+ T helper cells are not selectively predisposed to differentiate into Th17 cells. Although not statistically significant, increased expression of FOXP3 in PI16+ Th cells along with significant increase in IL-10 secretion is consistent with a transient immunoregulatory mechanism in a pro-inflammatory subset [41-43]. Even though PI16+Th cells express CCR4, CCR6, IL-17A and ROR-γt, similar to a pro-inflammatory Th17 subset, they also express CXCR3 and produce some IFN-γ, which are markers identifying Th1
cells. Nevertheless, further investigation was performed to identify their potential role in inflammation. The results from the chemokine migration assay suggest that PI16+ Th cells migrate better in response to CCR4 and CCR6 ligands, indicating an active role of the PI16+ Th subset in response to inflammation.

Furthermore, our microarray data reveal that PI16+ Th cells have a unique gene signature with 649 genes differentially expressed between PI16+ and PI16- Th cells. Comprehensive analysis of these genes of interests using Ingenuity software suggest that PI16+ Th cells have a significant role in the immune system, especially in immune cell trafficking, cell mediated immune response and inflammatory response.

In conclusion, we describe for the first time a novel subset of T helper cells identified by PI16. Our results suggest that these cells might represent long term memory Th cells with an ability to migrate towards inflammatory sites. Furthermore, although these PI16+ Th cells show Th17-like chemokine receptors and transcription factors, and produce significantly more IL-17 and less IFN-γ compared with PI16- T cells, polarisation experiments do not confirm plasticity capabilities. Therefore, PI16 does not exclusively detect the already described Th1, Th2 or Th17 subsets, but identifies a potentially new subset that might control the immune response at the site of inflammation. Further investigations are necessary to understand the functional properties of this subset and its role in disease.
Acknowledgements

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References


23. Bengtsson, H. and K. Simpson..., aroma. affymetrix: A generic framework in R for analyzing small to very large Affymetrix data sets in bounded memory. *Department of ..., 2008*.


Figure 3-1: Surface characteristics of PI16+ Th cells.

a) Representative dot plot of PI16 staining against CD25 is shown. b) Percentage of PI16 expressing Th cells in healthy adult peripheral blood (n=22). Bar represents median. c) Mean percentage expression of surface markers and chemokine receptors on PI16+ Th cells and PI16- Th cells. Bar graph shows mean + SEM (n=5 except for CCR4, CCR5 and CXCR5; n=4, **p<0.05). d) Representative plot showing staining pattern of PI16 against other surface markers (n=5 except for CCR4 and CXCR5; n=4).
PI16+ and PI16- Th cells were stimulated for 7 days with anti-CD3/CD28 beads alone (left) and with Th17 polarising conditions (right). a) & b) Intracellular IFN-γ and IL-17A were measured after 4hr re-stimulation with PMA / Ionomycin. Representative plot is shown (n=6 for anti-CD3/CD28; n=5 for Th17 polarising conditions). Statistics in the plots represent mean ± (SD). c) & d) Secreted cytokine were measured in supernatant using BD CBA. Bar graph shows mean ± SEM (n=4, *p<0.05).
Figure 3-3: Transcription factor profile of PI16+ and PI16- Th cells
Relative change in T-bet, ROR-γt, GATA3, FOXP3 and PI16 were measured between PI16+ and PI16- Th cells using RT-qPCR. a) Cells were stimulated with anti-CD3/CD28 beads alone (n=4; p<0.05). b) Cells were stimulated under Th17 polarising conditions (n=4 for ROR-γt and GATA3, n=3 for T-bet, FOXP3 and PI16). Bar graphs show mean±SEM.
Figure 3-4: *in-vitro* migration of PI16+ Th cells
Chemotaxis assay was performed using Transwell migration plates. PBMC seeded in the upper chamber. Bottom chamber contained CCL17, CCL20, CCL17+CCL20 or media (control). Percentage of PI16- and PI16+ Th cells was compared between upper and lower chamber. Lines in the graph connect matched donors (*p<0.05).
Figure 3-5: Microarray data comparing PI16+ and PI16- Th cells
Log fold-change values of gene expression in PI16+ Th cells were calculated relative to PI16- Th cells. a) Volcano delta plot showing the spectrum of differential expression of under expressed and over expressed genes on a log scale when comparing PI16+ Th vs PI16- Th cells. b). Relative log fold-change values were also analysed using IPA software. List shows top functions and diseases that are potentially linked to PI16+ Th cells based on gene expression.
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**PI16+ T helper cells**

## Table 3-1: Under expressed genes in PI16+ Th cells

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CHAPTER 4: PEPTIDASE INHIBITOR IDENTIFIES OLD RESTING MEMORY T HELPER CELLS WITH POTENT EFFECTOR FUNCTION

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Chapter 4

PI16+ memory Th cells

Signature.

Date.

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Designed experimental framework, supervised development of work, assisted with data interpretation and evaluated manuscript

Signature:

Date.

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Abstract

Our initial comprehensive screening experiments revealed that PI16+ Th cells uniformly express CD45RO, have better migratory properties, have a pro-inflammatory phenotype and have a distinct gene signature. Here, we further investigate CD45RO (memory) matched T helper subsets by comparing PI16+ and PI16- memory T helper cells. Our results indicate PI16+ cells have a high expression of Integrin β1 (CD29), Hepatic leukaemia factor and Clusterin and low expression of Tyrosine kinase, CCL5 and CXCR5 which resemble the properties of old resting memory T cells. In addition, high expression of CCR4, CCR6, histamine H4 receptor, Angiotensin converting enzyme 1 and Galectin-1 in PI16+ cells suggest an active role in inflammation. When cultured with cytokines, these cells show more STAT signalling activity suggesting a faster immune response. When stimulated with anti-CD3/CD28 beads, PI16+ cells secrete both anti-inflammatory IL-10, and pro-inflammatory TNF and IL-17A along with IL-2. We also observed that PI16+ cells proliferate faster and are more apoptotic, whilst suppression assays showed that they are more resistant to suppression by Treg compared with PI16- mem Th cells. Most interestingly, although Th1, Th2, Th17, Th1/Th17 subsets express PI16 at varying proportions, nearly all Th22 cells express PI16. In conclusion, in healthy adults PI16 identifies old resting memory T helper cells which when activated have a rapid effector function.
Chapter 4

4.1 Introduction

The body is challenged with different types of foreign pathogens throughout life. In the event of pathogen invasion, the body is equipped with an innate and adaptive immune system to first, destroy the pathogens and second, to remember the challenge in order to provide long term protection from future infection. This ‘memory’ ability of the immune system explains why secondary immune responses are quicker and more effective than primary immune responses. Antigen-specific lymphocyte responses include T cell help (CD4), T cell mediated cytotoxicity (CD8) and antibody production (B cells).

When naïve CD4 T cells are presented with pathogen antigen by antigen-presenting cells (APC), they undergo clonal expansion and differentiate into specific effector subsets (T helper (Th) 1, Th2, Th17, follicular T helper cells (Tfh) or Th22) depending on the type of requirement for pathogen clearance. Signature chemokine profiles have proven to be one of the key factors for distinguishing these subsets. Th1 cells express CXCR3; Th2 cells express CCR4 [1]; Th17 express CCR4 and CCR6 [2]; Th22 express CCR4, CCR6 and CCR10[3]; and Tfh express CXCR5 [4]. After destroying the pathogens, activated T cells either become apoptotic or attain ‘memory’ status.

Memory CD4 T cells form a heterogeneous population. They are broadly classified into central memory T cells (T_{CM}), that are CCR7+ and CD62L+ and migrate to secondary lymphoid organs and effector memory T cells (T_{EM}) that
are CCR7- and migrate to peripheral tissues. Originally it was reported that \( T_{CM} \)
lack the ability to produce effector cytokines in comparison to \( T_{EM} \), however
more recent data disproved this idea \([5, 6]\), suggesting other phenotypic
characteristics distinguish these subsets.

Other studies have shown that memory CD4 T cells can be classified based on
tissue tropism. Memory T cells have a migratory pattern that is dependent on
the expression of chemokine receptors, integrins and selectins on their cell
surface. The expression of these migration mediators is greatly dependent on
the location of the primary T cell activation \([7, 8]\). For example, gut, lung and
skin are a major port of entry for pathogens, and memory cells generated in
these tissues/ organs are known to be imprinted with their respective migratory
receptors, such that they can enter the peripheral circulation, but be recalled to
the tissue upon antigen exposure. For example, naïve T cells that were
originally activated in the skin-draining lymph node turn into skin-tropic memory
T cells. These cells express CLA, CCR4 and CCR10 \([10-12]\). Gut-tropic T cells
which are thought to originate in gut-associated lymphoid tissues express
CCR9 and Integrin \( \alpha 4\beta7 \) \([13, 14]\). However, tissue specific T cells are known to
have restricted entry or offer less immune response in other tissue regions \([8, 9]\).
For example, T cells activated by lung dendritic cell, express CCR4 and
provide more effective protective immunity to the lungs than gut or skin tropic T
cells \([9]\).
Although there are these different classifications of T cells based on receptor expression, cytokine production, memory status and tissue tropism, it is unlikely that each class represent a mutually exclusive group of cells. However, collectively research studies have demonstrated that based on the surface phenotype of a T cell, its functional and migratory pattern can be predicted. Identification and isolation of functionally distinct subsets of T cells is a key aspect of disease-specific immunotherapy.

Our research group has identified a functionally distinct subset of CD4 T cells that express Peptidase Inhibitor 16 (PI16) on their surface, both on CD25+ regulatory T cells (Treg) and CD25- T helper cells. Although the function of PI16 is poorly understood, our initial characterisation has shown that PI16 is expressed on a distinct subset of T helper cells with different phenotype and function compared with PI16- T helper cells [Chapter 3]. Our microarray data revealed that there are 649 genes that were differentially expressed between PI16+ and PI16- Th cells. Furthermore, in healthy adults PI16+ Th cells are all CD45RO+. They have a high expression of CCR4, CCR6, ROR-γt and IL17, suggesting they are able to produce effector cytokines and capable of migrating to inflammatory sites [Chapter 3]. We propose that PI16 identifies a novel T helper subset, and in order to exclude the possibility that PI16 is a coincidental surface protein on memory cells, we compare the functional and phenotypical difference between PI16+ memory Th cells and PI16- memory Th cell. We also validate a molecular signature of genes that were significantly differentially expressed in our microarray experiment.
4.2 Methods

4.2.1 Isolation of peripheral blood mononuclear cells (PBMC)

Peripheral blood was collected in Lithium-Heparin anticoagulant tubes from healthy adults after informed consent. The project was approved by the Research Ethics Committee of Children, Youth and Women’s Health Service (REC 2007/11/2013). Buffy coats were obtained from the Australian Red Cross. PBMC were isolated from whole blood or buffy coat using standard gradient centrifugation (Lymphoprep, Axis-Shield, Oslo, Norway).

4.2.2 Analysis of cell surface protein expression

Monoclonal antibodies to CD4, CD25, CD45RO, CD45RA, CD27, CD29, CD35, CD38, CD62L, CD95, CCR4, CCR5, CCR6, CCR7, CXCR3 and CXCR5 were obtained from BD Biosciences (San Jose, CA) and CXCR5 (R&D Systems, Minneapolis, MN). Anti-human PI16 antibody was generated in-house as previously described [15]. Surface molecules where stained using standard flow cytometry protocols and analysed using a BD FACS Canto.

Expression of PI16 on T helper subsets (Th1, Th2, Th17, Th1/Th17 and Th22) was determined by segregating T helper subsets by chemokine receptor profile as reported by Duhen et al [17]. Using this method, percentages of Th1, Th2, Th17, Th1/Th17 and Th22 subsets present in PI16+ and PI16- mem Th cells were also compared. CD4+CD25- cells were isolated from whole blood using
RosetteSep™ Human CD4+ T Cell Enrichment Cocktail (STEMCELL technologies, Vancouver) and CD25 MicroBeads II (Miltenyi Biotec, Germany). Isolated CD4+ CD25- cells were stained for CD45RA, PI16, CXCR3, CCR4, CCR6 and CCR10.

4.2.3 Gene expression analysis using RT-qPCR

As a follow up of our microarray analysis, functionally important genes of interest were selected and validated using RT-qPCR. CD4+ CD25- CD45RA- PI16- (PI16- memory Th) and CD4+ CD25- CD45RA- PI16+ (PI16+ memory Th) cells were sorted to >90% purity from buffy coats with a BD FACS Aria (BD Biosciences, CA). RNA samples were isolated from sorted PI16+ and PI16- mem Th cells using RNeasy Plus Micro Kit (Qiagen). cDNA synthesis from isolated RNA was performed using QuantiTect Reverse Transcription Kit (Qiagen). Real-time quantitative PCR was performed using KAPA SYBR FAST qPCR 2X kit (Kapa Biosystems, Wilmington, MA). Gene expression of Angiotensin converting enzyme I (ACE), Hepatic leukemia factor (HLF), Complement factor H (CFH), Clusterin (CLU), Histamine receptor H4 (HRH4), Galectin-1 (LGLS1), Tyrosine kinase (TXK), CCL5, CCR9, EOMES, Integrin α4 (ITGA4) were calculated using Rotor-gene 6000 (Qiagen). Gene expression was normalised to house-keeping gene, RPL13a. Mean normalised expression for these genes were calculated for PI16- and PI16+ mem Th cells as reported by Perikles [16]. Primers were obtained from GeneWorks, Adelaide, Australia (Table 4-1).
4.2.4 Phosphorylated STAT protein analysis

To study PI16+ Th subset-specific signalling events, the BD Biosciences Phospflow Kit (San Jose, CA) was used to analyse intracellular phosphorylated proteins. Activation and staining was performed according to the manufacturer’s protocol. Briefly, PI16+ and PI16- mem Th sorted cells were activated using biological modifiers for 15min at 37°C to detect STAT signalling. Phorbol 12-myristate-13-acetate (400 nM, Sigma) was used to initiate ERK and MAPK signalling; hIFN-α (40,000 units/ml, PBL InterferonSource) for STAT1, hIL-2 (100ng/ml, BD Biosciences) for STAT5; hIL-4 (100 ng/ml, BD Biosciences) for STAT6; hIL-6 (100 ng/ml, BD Biosciences) for STAT3. Control cells were incubated in X-Vivo 15 serum-free media (Lonza, Basel, Switzerland) only. Treated and control cells were then permeabilised and stained with STAT antibodies and analysed in a BD FACS Canto. Relative change in phosphorylation of signalling molecules was analysed using Cytobank software (www.cytobank.com).

4.2.5 Analysis of secreted effector cytokines

To evaluate cytokines secreted by PI16+ mem Th cells, the BD Cytometric Bead Array Human Th1/Th2/Th17 cytokine kit was used. PI16+ and PI16-mem Th sorted cells were activated with anti-CD3/CD28 beads in X-Vivo 15 serum- free media for 5 days at 37°C. Culture supernatants were collected and stored at -80°C before measuring secreted IL-2, IL-4, IL-10, IL-17F, TNF and
IFN-γ. Staining and analyses were performed according to the manufacturer's protocol.

### 4.2.6 Proliferation and suppression assay

To determine whether PI16 expressing Th cells proliferate faster and to determine their response to suppression by Treg, *in-vitro* proliferation and suppression assays were performed. Proliferation and suppression of PI16+ mem Th cells were compared with PI16- mem Th cells. Sorted PI16+ and PI16- mem Th cells were labelled with CFSE using CellTrace™ CFSE Cell Proliferation Kit (Invitrogen). CFSE staining was performed according to the manufacturer’s instructions. Proliferation assay was performed by stimulating the cells with anti-CD3/CD28 beads for 3-4 days in X-Vivo 15 serum-free medium. For suppression assays, the sorted cells were co-cultured with Treg (CD4+CD25+) from another unmatched donor at different Th:Treg ratios (1:1, 1:0.5, 1:0). The cells were stimulated for 3-4 days using 100 ng/ml anti-CD3 (eBioscience) and PBMC from a third donor. Division index (DI, average number of cell divisions, including divided and undivided cells) was calculated as described in the FlowJo software manual (FlowJo LLC, Ashland, OR USA) to compare the proliferation and suppression of PI16+ mem Th and PI16- mem Th cells.
4.2.7 Apoptosis assay

To compare the susceptibility to apoptosis of PI16+ and PI16- mem cells, an Activation Induced Cell Death (AICD) assay was performed. PBMC isolated from whole blood were treated with high dose PMA (40 ng/ml, Sigma-Aldrich, Steinheim, Germany) / Ionomycin (4 µg/ml, Sigma- Aldrich) at 37°C for 1hr and 2hr time points. Control cells were incubated with X-Vivo 15 SFM only. After 1 hr and 2hr respectively, cells were stained with monoclonal antibodies against CD4, CD45RA, PI16 (sec 4.2.2). Apoptotic and dead cells were distinguished by Annexin V (BD Biosciences) and 7-aad (Sigma) staining, respectively. Samples and data were analysed using a FACS Canto (BD Biosciences).

4.2.8 Statistical analysis

GraphPad Prism (San Diego, CA) was used to perform statistical tests. For surface marker analysis and Th subset distribution analysis student’s- T test was used to determine statistical significance. For gene expression, signalling molecules, proliferation assay and suppression assays a ratio-paired T test was used. For cytometry bead array assay and apoptosis assay Sidak multiple comparison method was used to determine statistical significance. Alpha value was set to 0.05 to determine statistical significance in all tests and to ensure only less than 5% chance of error is possible in accepting / rejecting the hypothesis.
Chapter 4

PI16+ memory Th cells

4.3 Results

4.3.1 PI16+ memory T helper cells have a distinct surface phenotype in comparison with PI16- memory T helper cells

Our initial flow cytometry and microarray data revealed that PI16+ Th cells show a unique genotype [Chapter 3]. Most significantly, in normal healthy adults, all PI16+ Th cells are CD45RO+ but not all CD45RO+ Th cells are PI16+. PI16- Th cells are a mixture of CD45RO+ and CD45RA+. Therefore further characterisation of CD45RO matched subsets was warranted. To do this we compared PI16+ mem Th cells with PI16- mem Th cells, and then further validated the microarray data to determine if the unique phenotype of PI16+ Th compared with the PI16- Th populations was not simply because of its homogeneous CD45RO expression. To investigate functional capability of the cells, the surface phenotype was compared before and after activation using PMA / Ionomycin for 4 hrs.

PBMC (n=4) stained with CD4, CD25, CD45RA, PI16 were co-stained with antibodies to CD27, CD29, CD35, CD38 CD62L, CD95, CCR4, CCR5, CCR6, CCR7, CXCR3 and CXCR5. As shown in Figure 4-1a, nearly all PI16+ mem Th cells express CD29 and CD95 and significantly more in comparison with PI16- mem Th cells (mean; 95.35% vs 60.37% and 96.07% vs 86.75% respectively). PI16+ mem T helper cells express little or no CD38 in comparison with PI16- mem Th cells (Mean; 2.40% vs 23.85). Expression of CD35 is significantly
higher in PI16+ mem Th cells (mean; 22.0% vs 11.67%) in comparison with PI16- mem Th cells but similar to naïve (CD45RA+) Th cells (21.5%).

As shown in Figure 4-1b, PI16+ mem Th cells have a higher proportion of cells expressing the chemokine receptors CCR4 and CCR6 expressing cells in comparison with PI16- mem Th cells (mean; 58.90% vs 25.15% and 61.93% vs 48.13%). Similar to naïve Th cells but in contrast to PI16- mem Th cells, PI16+ mem Th do not express CXCR5 (mean; 4.00% vs 28.47%). Although these cells express CCR5, CCR7 and CXCR3, the percentage of expression is not significantly different from PI16- mem Th cells.

4.3.2 Validation of microarray data using RT-qPCR

Microarray data comparing PI16+ Th with PI16- Th cells revealed that there were 649 significantly differentially expressed genes. In order to validate the microarray data RT-qPCR was performed for the top up-regulated and down-regulated genes. In the microarray data PI16- Th cells contained a mixture of CD45RA+ and CD45RO+ cells while PI16+ Th cells contained only CD45RO+ cells (Chapter 3). Here, RT-qPCR was performed on CD45RO matched samples by comparing PI16- mem Th cell with PI16+ mem Th cells (n=4). Confirming the microarray data, RT-qPCR data show (Figure 4-2a & b) PI16+ mem Th cells have a higher expression of ACE, HLF, CFH, CLU, HRH4, LGLS1 and a lower expression of TXK, CCL5, CCR9, EOMES and ITGA4 compared with PI16- mem Th cells. As it is not possible to determine the
relative abundance of individual genes on cell subsets from RNA analysis, as cells are pooled, key genes are also validated at the protein level (Surface staining for CCR4, CD29, CD35 and CXCR5 in sec 4.3.1).

### 4.3.3 PI16+ memory T cells display a higher activation status

In order to study the signalling pathways in PI16+ mem Th subset, phosphorylation of intracellular proteins was analysed (n=3). Figure 4-3a shows a representative plot of three donors. Phorbol 12-myristate-13-acetate, hIL-2 and hIL-4 induce significantly more phosphorylation of ERK, STAT5 and STAT6, respectively, in PI16+ mem Th cells compared with PI16- mem Th cells (p<0.05). Although activation of STAT1 and STAT3 induced by hIFN-α and hIL-6, respectively, are not statistically different between the two subsets, preliminary data suggest that their expression is trending higher in the PI16+ subset in comparison with the PI16- subset, for all three donors (Figure 4-3b).

In order to compare the secreted cytokine profile of PI16+/+ mem Th cells, sorted cells were stimulated and cytokine concentration in culture supernatants was analysed (n=3, Figure 4-3c). The PI16+ Th subset produce significantly more IL-2 (mean; 2192 pg/ml vs 818 pg/ml), IL-10 (mean; 496 pg/ml vs 132 pg/ml) and IL-17A (mean; 555 pg/ml vs 177 pg/ml) in comparison with the PI16-Th subset. We could not detect a significant amount of IL-4 and IL-6 (data not shown) in the supernatant after 5 days of stimulation, whilst TNF was detected
in the PI16+ cell subset supernatant but not in the PI16- Th cell subset supernatant (142 pg/ml vs 24 pg/ml).

4.3.4 **PI16+ memory Th cells are hyper-proliferative, less susceptible to suppression by regulatory T cells but more susceptible to apoptosis**

CFSE-based assays were performed to determine the response of PI16 expressing Th cells to activation and Treg mediated suppression. After 3-4 days of culture, the division index (DI) of PI16+ mem Th cells was compared with PI16- mem Th cells. In the proliferation assay (Figure 4-4a), DI is significantly higher (mean fold difference; 1.65, p<0.05) in PI16+ mem Th cells in comparison to PI16- mem Th cells. In the suppression assay (Figure 4-4b & c), regardless of the Th:Treg ratio (1:1, 1:0.5 and 1:0), DI is significantly higher (mean fold difference; 2.13, 1.76 and 1.71 respectively; p<0.05) in PI16+ mem Th cells in comparison with PI16- mem Th cells.

Activation induced cell death assays were performed to determine the apoptotic response of PI16+ and PI16- memory subsets as well as naïve CD4 T cells. As shown in Figure 4-4d, PI16+ memory cells are more prone to apoptosis in response to stimulation than PI16- memory T cells and naïve T cells (mean; 83.97% vs 64.17% and 33.87% respectively).
4.3.5 All Th22 cells express PI16

We next investigated whether PI16 is present in all subset of T helper cells. To do this, Th1, Th2, Th1/Th17, Th17 and Th22 subsets were segregated utilizing the chemokine profile as reported by Duhen et al [17]. As shown in Figure 4-5a, T helper subsets were gated based on surface expression of PI16 and their chemokine receptor profile; CXCR3+ CCR6- = Th1, CXCR3-CCR6-CCR4+ = Th2, CXCR3+CCR6+ = Th1/Th17, CXCR3-,CCR4+,CCR6+CCR10- = Th17 and CXCR3-CCR4+CCR6+CCR10+ = Th22. All Th cell subsets except Th22 express PI16 in less than 50% of cells (n=3; mean; Th1 - 14.3%, Th2 - 34.83%, Th1/Th17 - 21.57%, Th17 - 41.63%), however Th22 cells homogeneously express PI16 (mean; 94.57%).

Using the same chemokine profile method, Th subset proportion was also compared between PI16- and PI16+ mem Th cells (Figure 4-5b). Presence of Th1, Th17 and Th22 subsets are significantly different (p<0.05) between PI16- and PI16+ Th cells. There are less Th1 cells (mean; 23.8% vs 34.2%) and more Th17 cells (mean; 15.1% vs 5.42%) in PI16+ subset compared with PI16- cells. Th22 cells are present only in PI16+ mem Th subset (mean; 3.46% vs 0.15%).

4.4 Discussion

Over the past decade, many intracellular and cell surface molecules have been discovered with single or multiple physiological functions in healthy and diseased individuals. Understanding the expression and function of these
molecules/ biomarkers at the gene and protein level in a cell or pool of cells will help better understand the physiological role of these cell subsets, and may provide new tools to treat diseases which are associated for example with defects in CD4 T cell-mediated immunological processes.

We have previously shown that PI16 is expressed on a sub population of CD4 regulatory and Th cells. However, because PI16+ Th cells homogeneously express CD45RO, whilst PI16- Th cells are comprised of a mixture of CD45RA+ and CD45RO+ cells [Chapter 3], further investigation of PI16 expressing Th cells was conducted by comparing PI16+ mem Th cells with PI16- mem Th cells. This study shows that PI16 dissects the CD4 memory Th cells into two populations, and that PI16 identifies a functionally distinct subset of memory CD4 T cells.

Traditionally memory T cells have been categorised into central (T\textsubscript{CM}) and effector (T\textsubscript{EM}) memory T cells according to the expression of CCR7, CD62L and CD27 [18, 19]. T\textsubscript{CM} are known to circulate but can migrate towards secondary lymphoid organs and T\textsubscript{EM} are known to be present in the peripheral tissue. However, as we have now shown, none of these markers are differentially expressed between PI16+ and PI16- memory Th cells. Hence PI16 does not uniquely represent T\textsubscript{CM} or T\textsubscript{EM} memory cells. However, distinguishing memory T cells merely into simple subsets as T\textsubscript{CM} and T\textsubscript{EM} has been controversial [6, 20, 21].
As part of establishing the signature gene expression profiles of the PI16+ T helper cells, we performed validation of differentially expressed genes from the expression array dataset. The candidates for validation were selected on log fold change and relevance to immunological function, and differential expression confirmed by RT-qPCR. PI16+ memory Th cells have a high expression of Histamine H4 receptor (HRH4/ H₄R) at the mRNA level, whilst PI16- memory Th cells express little or none of this gene. Histamine plays an important role in allergy mediated inflammation. H₄R has been reported to be present on CD4 T cells with an enhanced function in Th2 and Th17 subsets [22, 23]. Although known for its role in inflammation, histamine can also act as an anti-inflammatory mediator by recruiting regulatory T cells [24].

In recent years, the role of T cells in hypertension and cardiovascular inflammation and disease has been demonstrated. Renin-angiotensin system (RAS), which plays a key role in regulating blood pressure, is also found in T cells [25-28]. Our results indicate that in healthy adults, the PI16+ T helper subset has a high expression of Angiotensin converting enzyme 1 (ACE) gene, a key component of the RAS system. The functional implications of this finding are currently unknown.

Hepatic leukemia factor (HLF) plays a key role in cellular circadian rhythms and up-regulates anti-apoptotic genes [29]. HLF is also listed as one of the key signature markers for stem cells [30]. Although its role in T cells is not clearly
understood, the PI16+ subset has a high expression of this gene in comparison to PI16- memory Th cells.

Complement plays a key role in the innate immune system in clearing pathogens. Complement regulator, factor H (CFH), is known to be secreted or expressed on the cell surface by host tissues to prevent self-damage during pathogen clearance [31-34]. However, expression of factor H by T cells has not yet been reported. PI16+ cells highly express factor H gene in comparison with the PI16- subset, which may suggest resistance to complement mediated lysis.

Clusterin, known to be an immunosenescence marker [35], is highly expressed in the PI16+ subset in comparison to PI16- subset. This suggests that PI16+ memory Th cells are much older than PI16- subset, and are perhaps the long term memory cells that are predicted to provide life-long recall responses to harmful pathogens.

PI16+ Th cells have a high expression of Galectin-1 in comparison with the PI16- subset. Galectin-1 plays a key role in homeostasis. At the site of inflammation or infection, the effector T cells proliferate rapidly to destroy the pathogens. After pathogen clearance, activated T cells increase expression of galectin-1 to undergo apoptosis and/or secreted galectin-1 present in the microenvironment will trigger a regulatory effect in Th cells by inducing secretion of IL-10 [36-38]. This function of Galectin-1 correlates with the fact that the PI16+ subset is hyper proliferative and secretes IL-10 when activated.
PI16+ cells express less Tyrosine kinase (TXK) gene in comparison to PI16-memory Th cells. TXK, a member of TEC family, plays a key role in INF-γ production in Th1 cells. However, it is normally expressed at low levels in steady state resting CD4 T cells, but is up-regulated only in activated T cells [39-41]. This suggests that PI16-memory T cells are recently activated T cells.

PI16+ cells do not express CCL5 / RANTES. CCL5 is a late activation gene and it appears in T cells 3-5 days after activation [42]. It was reported to be one of the highly expressed genes in memory subsets of both CD4 and CD8 T cells and is virtually absent in naïve T cells. Even though the gene is present in memory T cells, they do not secrete CCL5 until stimulated [43]. To our knowledge, absence of this gene in a subset of memory cells has not yet been reported. Hence, the physiological implication of under-expression of this gene by PI16+ memory Th cells, similar to naïve T cells, is intriguing.

CCR9 and α4β7 integrin expressing CD4 T cells play a key role in intestinal mucosal immunity [44-46]. However, PI16+ cells express less CCR9 and α4 integrin genes in comparison with PI16- cells. This suggests that PI16+ cells do not preferentially traffic to the gut.

EOMES belongs to the same T-box factor family as T-bet. Recent studies have shown that it plays a key role in regulating IFN-γ expression and driving Th1 development [47, 48]. However, PI16+ memory Th cells have a low expression of EOMES which correlates with their low IFNg production.
Surface expression of immunologically relevant functional markers was analysed and compared between PI16+ and PI16− mem Th cells. PI16+ Th cells homogeneously express CD29 / Integrin beta-1. Integrins play an important role in T cell migration, adhesion, retention and survival. Expression of integrins along with chemokine receptors suggest unique migration capacity of different T cell subsets [49]. One study has shown that CD29 is essential for long-term and steady maintenance of memory CD4 in bone marrow [50]. This is in conjunction with the high expression of CD45RO supporting our hypothesis that PI16 identifies the oldest subset of memory Th cells.

Interestingly, CD38, which is present in naïve CD4 T cells and recently activated cells [51] is not expressed on the PI16+ subset. Studies have shown that CD45RA+ naïve CD4 T cell lose their high expression of CD38 once they mature and become antigen-experienced. CD38− CD4 T cells are hyper-proliferative in comparison to CD38+ CD4 T cells [52]. Furthermore, memory T cells with stem cell like properties have been shown to lack CD38 [53], and it is possible that long term memory cells will require stem like properties to persist for the lifetime of the adult.

Differential expression of chemokine receptors among T cells subsets is one of the key discriminators that determine migratory pattern and localisation. Therefore T helper subsets can be dissected based on their chemokine receptor expression patterns. CXCR3+ and CCR4+ T cells are known to differentiate into Th1 and Th2 subsets, respectively [54]. CCR6+CCR4+ and
CCR6+CXCR3+ memory Th cells have been identified as Th17 and Th1/Th17 cells [2]. Th22 cells express CCR10 along with CCR6 and CCR4 [55]. Memory Th cells expressing CXCR5 are denoted as Tfh [56]. In accordance with our findings in mixed populations of Th cells, expression of CCR4 and CCR6 is higher on PI16+ memory Th cells indicating that PI16+ T cells are capable of migrating to inflammatory sites along CCL20 and CCL17 gradients. Our results show that PI16 is present on a sub-population of all subsets of memory Th cells (Th1, Th2, Th1/Th17 and Th22) except for Tfh. Interestingly, nearly all the Th22 express PI16 whilst the other subsets only have intermediate proportions of PI16+ cells. Moreover, the PI16+ Th cells express CCR4, CCR6 and CCR10, which have been associated with skin-homing functions [10, 12, 57]. In contrast, CXCR5 is expressed only in a small subset of memory T cells and not on PI16+ Th cells [56]. CXCR5+ T cells / Tfh are directed to B-cell follicle to help B cell in producing antibodies. These cells are known to represent recently activated T cells. However, long-lived memory T cells lack CXCR5 [4]. When antigen specific-T cells are activated, they acquire CXCR5 and migrate to germinal centres to initiate T-cell mediated antibody response [58]. This further suggests that PI16+ cells might represent a pool of long-lived antigen-experienced T cells.

To investigate the effector potential of the PI16 Th cells we analysed cytokine expression after stimulation. PI16+ memory Th cells produced increased amounts of IL-2, IL-10, TNF and IL-17A compared with PI16- memory Th cells. This is consistent with previous observation when comparing PI16+ Th and
mixed PI16- Th cells (Chapter 3). Taken together with the high expression of “Th17-like” CCR4 and CCR6, this further suggests that PI16+ cells have pro-inflammatory characteristics.

IL-2 production was higher in the PI16+ subset in comparison with PI16-memory subsets. IL-2 secretion directly correlates with increased proliferation and survival. This is supported by our proliferation assay data, which shows that, despite the cells were derived from the same donor, PI16+ memory Th cells are more proliferative in comparison to PI16- memory Th cells. Intriguingly, when co-cultured with regulatory T cells (Treg) from a different donor, PI16-memory Th cells were more susceptible to Treg suppression than PI16+ Th cells. These results confirm that PI16 expressing T helper cells are hyper-proliferative, and more resistant to Treg suppression. Rapid proliferation of effector T cells and apoptosis are the key aspects of a healthy and balanced immune response. Dysfunction in any of these would lead to a weak immune response or an excessive inflammation resulting in tissue damage. PI16+ cells have a high expression of CD95 / Fas receptor and are more susceptible to apoptosis than PI16- memory Th cells when activated with high dose PMA / Ionomycin. We speculate that in order to control inflammation, PI16+ T helper cells have an inherent mechanism to create a negative feedback loop mediated by IL-10 [59], and this may trigger apoptosis.

As intracellular signalling molecules have been associated with specific T helper differentiation, we next analysed the expression and activation of key
signalling molecules in the PI16+ Th cells. STAT1 is an inducer of T-bet, a master gene for Th1 differentiation [60]; STAT6 up regulates GATA3 expression in IL-4 dependent Th2 differentiation [61]; STAT5 regulates expression of FOXP3 in Treg, and is also known to play a role in FOXP3 expression in activated CD4+CD25- Th cells [62]; STAT3 plays a key role in Th17 generation which is mediated by IL-6 and IL-23 [63]. Upon short term stimulation, PI16+ subsets expressed more phosphorylated STAT signalling molecules in comparison with PI16- subsets. However, the data do not suggest that the PI16+ cells are pre-disposed to a specific Th lineage, they rather seem to be heterogeneous and overall, their response to all stimuli is significantly higher in comparison with PI16- memory Th cells. In addition, the bimodal peaks for STAT5 may indicate there is a mixture of responsive and unresponsive cells to IL-2 in both the subsets. Furthermore, increased expression of phosphorylated ERK 1/2 in PI16+ subsets compared with PI16- subset revealed that PI16+ Th cells quickly proliferate and differentiate in response to activation signals [64].

In summary, we have shown that PI16 identifies an immunologically distinct component of the CD4 memory T cell compartment. We suggest that PI16+ Th cells comprise long lived resting memory Th cells with proinflammatory capacity and with the capacity to migrate to inflammatory sites potentially preferentially to skin. When activated, these cells proliferate rapidly and are resistant to regulatory T cell suppression. However, in healthy adults they seem to have an inherent immunoregulatory function to control hyper proliferation. Further
studies in disease cohorts is performed to better understand the pathophysiological relevance of the PI16+ T helper memory subset, which may potentially translate into new diagnostic biomarkers or a tool for cell therapy.

Acknowledgements

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Chapter 4

PI16+ memory Th cells

References


55. Trifari, S., et al., Identification of a human helper T cell population that has abundant production of interleukin 22 and is distinct from T(H)-17, T(H)1 and T(H)2 cells. *Nat Immunol*, 2009. 10(8): 864-871.


Figure 4.1: Surface phenotype of PI16+ memory T helper cells. 
Differential expression of surface markers on PI16+ mem Th cells, PI16- mem Th cells and naïve Th cells before and after activation for 4hrs using PMA and Ionomycin. a) Expression of CD27, CD29, CD35, CD38, CD62L and CD95. n=4, mean±SEM. b) Expression of chemokine receptors CCR4, CCR5, CCR6, CCR7, CXCR3 and CXCR5. n=4, mean±SEM.
Figure 4-2: Gene profile of PI16+ memory T helper cells

Gene expression in PI16+ and PI16- memory Th cells in steady state was determined using RT-qPCR. Mean normalized expression of genes were calculated and RPL13a was used to normalise gene expression. Lines in graphs connect matched donors. Over-expressed (a) and under-expressed (b) functionally important genes in PI16+ cells were validated using RT-qPCR. All gene expression are significantly (p<0.05) different between PI16+ and PI16-mem Th cells (n=4; 3 technical replicates per donor).
Figure 4-3: STAT and secreted cytokine profile of PI16+ memory T helper cells
PI16+ and PI16- mem Th cells were treated with hIFN-α, hIL-2; hIL-4; or hIL-6 to initiate phosphorylation of STAT1, STAT5, STAT6 and STAT3 respectively. PMA was used to initiate phosphorylation of ERK and MAPK. a) Representative histogram for 3 donors. b) Normalised MFI of ERK, MAPK, STAT1, STAT3, STAT5 and STAT6 for 3 donors (*p<0.05). Lines connect matched donors. c) PI16+ and PI16- mem Th cells were stimulated for 5 days with anti-CD3 / CD28 beads. Secreted cytokines were measured using BD CBA. Lines connect matched donors (*<0.05).
Figure 4-4: Proliferation, suppression and apoptosis assays.
Cells stained with CFSE were stimulated with anti-CD3/CD28 beads for 3-4 days. Division Index (DI) was compared between PI16+ and PI16- mem Th cells. a) Representative histogram of 3 donors is shown (p<0.05). b) & c) Cells were stimulated with PBMC and CD3 antibody and co-cultured with Treg at different Th: Treg ratios (1:1, 1:0.5, 1:0). DI was compared in 3 donors. Lines in graph connects matched donors (*p<0.05). c) Representative histogram for 3 donors. d) Cells treated with PMA/ I for 1-2hrs were measured for apoptosis by Annexin V staining. Bars represent mean±SEM (n=3, *p<0.05).
Figure 4-5: PI16 on the memory T helper subsets.
T helper subset type was determined by the expression of CXCR3, CCR4, CCR6 and CCR10. a) Representative dot plot of 3 donors in determining Th1, Th2, Th17, Th1/Th17 and Th22 subsets is shown. Percentage expression of PI16 on Th subsets in all three donors is plotted. b) Based on the same chemokine profile method, distribution of Th subsets in PI16+ and PI16- mem Th cells were determined. Stacked bars represent mean±SEM of 3 donors (*p<0.05).
## Tables

### Table 4-1: List of primers

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CHAPTER 5: PILOT CLINICAL STUDY: DO PI16+ T HELPER CELLS HAVE A ROLE IN DISEASE?

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Chapter 5

Clinical study

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Signature... Date

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Signature... Date 7/11/2014

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..................Date..................
Abstract

There is an ongoing debate regarding the mechanism of pathogenesis of autoimmune and inflammatory diseases. The current knowledge of involvement of heterogeneous CD4 T cells suggests that one or more specific subsets of these cells play a dominant role in disease initiation or progression. T cell subset biomarkers might provide an effective tool for early predication, diagnosis, monitoring and therapeutic intervention. One potential biomarker, peptidase inhibitor 16 (PI16), is expressed on a functionally distinct subset of T helper (Th) cells. This study is a compilation of multiple pilot clinical studies aimed to survey the role of PI16+ Th cells in a group of diseases with different tissue and organ involvement; rheumatoid arthritis, juvenile idiopathic arthritis, scleroderma, asthma, chronic sinusitis and type I diabetes. The expression of PI16 on Th cells in the peripheral blood and the affected organs or tissues (example, sinus tissue or synovial fluid), wherever applicable, was compared between disease and healthy controls. In addition, in rheumatoid arthritis, scleroderma and asthma studies, the naïve (CD45RO-) / memory (CD45RO+) ratio in PI16+/− Th cells and total Th cells was also compared between disease group and healthy controls. Our preliminary data indicate that only in sinusitis and RA, the proportion of PI16 expressing Th cells in peripheral blood is higher in the disease group in comparison with healthy controls. In RA and scleroderma the naïve/ memory ratio is higher in comparison with healthy controls. In conclusion, these pilot study results indicate that PI16+Th cells may have a role in autoimmune / inflammatory diseases. Further studies are
warranted to confirm the pathophysiological role of PI16+ Th cells in these diseases.
Chapter 5  
Clinical study

5.1 Introduction

The primary role of the immune system is to protect us from pathogens through a controlled immune reaction against foreign antigens [1]. However in certain individuals, the immune system reacts against self-antigens or becomes hypersensitive to harmless antigens including food [2]. Autoimmune diseases, which are classified into organ-specific and systemic diseases, are often chronic, debilitating, and can potentially lead to death. The American Autoimmune Related Diseases Association Inc. (AARDA) lists a total of 159 autoimmune and autoimmune-related diseases [3]. While the reasons for autoimmunity are not completely understood, genetic and environmental factors are thought to predispose individuals to these diseases [4]. In addition, research has shown a strong correlation between alterations in numbers, location or function of T cell subsets and autoimmune diseases. T helper (Th) subsets, including T helper type 1 (Th1), T helper type 2 (Th2), T helper 17 (Th17), Th22, Th9 and follicular T helper (Tfh) each have a specific role in providing host defence against pathogens in healthy individuals. However, Th subsets have also been strongly linked to the pathogenesis of autoimmune diseases [2]. Discovery of novel biomarkers of T cell subsets is essential for early diagnosis and for developing target specific therapeutic interventions.

Previously, our group described a new subset of CD4 T cells characterised by the surface expression of peptidase inhibitor 16 (PI16). A comprehensive genome wide array analysis revealed that PI16 is highly expressed in
CD25+FOXP3+ regulatory T cells (Treg), and in conjunction with CD25 it could potentially be used as a biomarker for human Treg. Furthermore \textit{in-vitro} studies showed an enhanced potential of PI16+ Treg to migrate to inflammatory sites in response to CCL17 and CCL20 to regulate pro-inflammatory responses [5, 6]. A potential role of PI16 expressing CD25+ CD4 T cells in autoimmune diseases was first described in juvenile idiopathic arthritis (JIA). Our group has shown that the inflamed joints of JIA patients have an increased number of pro-inflammatory Th17 cells. Whilst regulatory T cells were enriched in the inflamed joints of JIA patients, the number of PI16+ Treg were found to be low compared with peripheral blood. This suggests that the more abundant PI16- Treg have not been able to control the inflammation at this site and we hypothesised that PI16+ Treg are more potent than PI16- Treg [7]. However the role / characteristics of PI16 expressing T helper cells in these patients have not been previously studied.

This thesis demonstrates that PI16 expressing T helper (Th) cells isolated from healthy adults have potent effector function. PI16+ Th cells are a heterogeneous population with regards to Th subsets but homogenous with regards to memory phenotype. These cells are hyper-proliferative and secrete more pro-inflammatory cytokines like IL-17 and TNF in comparison with PI16-memory Th cells, suggesting a potential role in inflammatory diseases [Chapter 3 & 4].
Here we further investigate the role of PI16 expressing T helper cells (CD4+ CD25- PI16+) in JIA along with other autoimmune and inflammatory diseases including rheumatoid arthritis (RA), scleroderma, type 1 diabetes (T1D), sinusitis, and asthma during pregnancy. The objective in the selection of these diverse disease cohorts was to investigate whether PI16+ Th cells have a tissue / organ specific role. Because the pathogenesis and progression of disease activity of these diseases are different in each cohort, by analysing multiple disease cohorts, we may be able to infer specific roles for PI16+ Th cells in skin, mucosa, internal organs, and joints/bones. However, these are pilot studies and are not intended to be powered for a specific endpoint, but to generate a preliminary survey of the involvement of PI16+ cells in clinically relevant conditions before conducting major clinical trials.

*Rheumatoid Arthritis (RA)* is characterised by chronic inflammation in synovium leading to cartilage degradagation and bone erosion [8]. The advanced stage of this disease results in limited or lost mobility and chronic immune activation can also further lead to systemic disorders including cardiovascular disease [9]. Cells of both the innate and adaptive immune system play a role in the pathogenesis of this disease, including neutrophils and T helper subsets (Th1 and Th17), which are mainly responsible for the chronic inflammation leading to tissue damage [10-12]. B cells aggravate the disease by secreting autoantibodies to autoantigens including rheumatoid factor and citrullinated proteins [13].
**Juvenile idiopathic arthritis (JIA)** is the most common rheumatologic disease occurring in children under the age of 16 [14]. Unlike in adults, JIA is heterogeneous and is classified into four major types, depending on the disease symptoms; oligoarthritis, polyarthritis, systemic arthritis and enthesitis-related arthritis [15]. T cells, especially cells with pro-inflammatory functions like Th17 have been described to play a key role in the pathogenesis of JIA by promoting active inflammation and tissue damage in the joints [7].

**Scleroderma** is a chronic autoimmune disease characterised by thickening or sclerosis in the skin and other organs. The localised form of the disease, known as "morpha", can be disabling but may not be fatal [16]. The systemic form, known as systemic sclerosis, can be fatal as a result of heart, kidney, lung or intestinal damage. Systemic sclerosis can be further divided into Limited and Diffuse variants depending upon the extent of cutaneous thickening. Primarily, scleroderma begins with vascular endothelial damage, lymphocyte activation and excessive collagen deposition in the extracellular matrix which results in an abnormally hard tissue architecture [17]. Th1, Th2 and Th17 play key roles at different stages of pathogenesis of the disease; Th1 and Th17 during the early inflammatory stages while Th2 are involved in fibrosis during the later stages of the disease [18].

**Asthma** is characterised by chronic inflammation of the airways. While the non-allergic asthma triggered by external factors including cold air, hyperventilation, exercise, etc which do not involve an immune response in the first instance
[19], the allergen induced asthmatic response begins with Th2 activation and IgE secretion by activated B cells followed by the recruitment of eosinophils, mast cells and basophils, resulting in airways inflammation [20-22]. Asthma exacerbation in pregnant women is a common problem which can complicate pregnancy, leading to preterm delivery, intrauterine growth restriction (IUGR) or fetal death [23]. Although there are several effective clinical interventions, including glucocorticoid therapy, to control asthma, in at least 50% of pregnant women with pre-existing asthma, pregnancy worsens their symptoms, suggesting a change in leukocyte phenotype, activation status and function during pregnancy [24].

*Chronic rhinosinusitis or sinusitis* is a Th2 mediated inflammatory disorder causing inflammation in nasal and paranasal cavities. Chronic sinusitis is classified into two types; chronic rhinosinusitis with polyps (CRSwNP) and without polyps (CRSsNP). Th1 and Th17 cells, along with Th2 cells play a role in the nasal inflammation in both CRSwNP and CRSsNP patients [25, 26].

*Type 1 diabetes (T1D)* is an autoimmune disease characterised by the lack of insulin production due to self-destruction of insulin-producing beta cells in the pancreas [27]. Both innate and adaptive immune cells including monocytes, Th1 and Th17 are responsible for the autoimmune reaction against β-cell auto-antigens [28, 29].
Collectively, the pathophysiology of an autoimmune disease might depend on a dominant role of one or more specific subsets of immune cells. This study was designed to gain insights into the characteristics of PI16+ Th cells by investigating whether PI16+ T helper cells have an association with any autoimmune and inflammatory diseases. However, this study does not include any functional analysis of PI16+ Th cells from these pilot disease cohorts, as it is beyond the scope of the project. The percentage of Th cells expressing PI16 was compared between healthy and disease groups. The ratio of naïve and memory cells in PI16+ Th subset (N/M_{PI16+}), PI16- Th subset (N/M_{PI16-}) and in total Th cells (N/M_{Th}) were also compared between healthy subjects and disease cohorts.

5.2 Methods

5.2.1 Specimen collection

5.2.1.1 Normal donors

Peripheral blood from healthy adults (n=22) and children (n=8) was collected after informed consent. The study was approved by the Women’s and Children’s Hospital Research Ethics Committee (REC 2007/11/2013). Samples from healthy children were used as a control in JIA and T1D study, while normal random donor adult blood was used as a control for the adult disease cohort studies.
5.2.1.2 Type 1 diabetes

Peripheral blood from children with T1D and children at pre-clinical stage was collected after informed consent. This study was approved by the Women’s and Children’s Hospital Research Ethics Committee (REC2264/3/14). Blood samples from patients in the pre-clinical stage, defined as 2 or more autoantibody positive but not insulin dependent, were collected only once at the time of diagnosis. A total of 12 patient samples were collected in this group. Blood samples from insulin dependent T1D patients were collected at 3, 6 and 9 months after the onset of disease, wherever possible. In the T1D group, samples were collected from 35 patients at 3 month time-point, out of which 8 were lost to follow up at 6th month and 7 more lost to follow up at 9th month. Clinical notes and diagnostic reports for these patients, indicating the level of Islet-cell antibodies (ICA), glutamate decarboxylase antibodies (GAD) and Islet antigen 2 antibodies (IA2) in their peripheral blood, were also obtained for the 3 different time points. Peripheral blood mononuclear cells (PBMC) isolated from peripheral blood were stored in liquid nitrogen as described in sec 5.2.2 for further analysis.

5.2.1.3 Chronic sinusitis

Peripheral blood and nasal tissue were obtained from chronic sinusitis patients after informed consent. This study was approved (#2009192) by the South Australian Health, Human Research Ethics Committee for the participating hospitals (The Queen Elizabeth Hospital, Lyell McEwin Hospital and Modbury
Hospital). Inflamed sinus tissues from ethmoid sinuses were obtained from CRSwNP (n=5) and CRSsNP (n=2) patients during routine surgery. In CRSwNP patients, polypoid ethmoid sinus mucosa protruding into the nasal cavities was also harvested. Tissue samples were collected and transported in RPMI 1640 (HyClone Laboratories, South Logan, UT, USA). Samples were processed immediately, as described in sec 5.2.2.

5.2.1.4 Juvenile idiopathic arthritis

Peripheral blood and synovial fluid were drawn from children with idiopathic juvenile arthritis after informed consent. This study was approved by the Women’s and Children’s Hospital Research Ethics Committee (REC2101/9/11). Samples were collected during their routine visit to the hospital for therapeutic steroid joint injection. Juvenile Arthritis Disease Activity Scores 100 (JADAS) of the patients were determined at the time of sample collection. Out of the 15 patients recruited for this study, 10 were categorised oligoarthritis, 3 polyarthritis and 2 enthesitis-related arthritis. Donor-matched peripheral blood and synovial fluid samples were obtained from 11 donors. Collected samples were processed immediately for further analysis, as described in sec 5.2.2.

5.2.1.5 Rheumatoid arthritis

Peripheral blood was collected from rheumatoid arthritis patients (n=45) after informed consent. This study was approved by the Royal Adelaide Hospital Research Ethics Committee (RAH 090414). Patient’s Disease Activity Score
(DAS), Disease Activity Score 28 (DAS28) and CRP levels were also obtained at the time of sample collection. PBMC isolated from peripheral blood were stored in liquid nitrogen as described in sec 2.2 for further analysis.

5.2.1.6 Scleroderma

Peripheral blood was collected from patients with scleroderma after informed consent. The study was approved by the Royal Adelaide Hospital Research Ethics Committee (RAH 090414). Samples were collected from 42 patients and clinical information about their disease classification (limited scleroderma, diffuse scleroderma or other) and treatment methods (steroid, non-steroid) were also obtained. Two patients were neither in the limited or diffuse category. PBMC isolated from peripheral blood were stored in liquid nitrogen, as described in sec 2.2 for later analysis.

5.2.1.7 Asthma in pregnancy

Blood samples were collected from pregnant women who were between 18 and 37 weeks gestation. Samples were collected from pregnant women with (n=18) and without (n=18) asthma. PBMC were isolated from peripheral blood and stored in liquid nitrogen as described in sec 5.2.2, for later analysis. This study was approved by the Queen Elizabeth Hospital Human Research Ethics Committee (ERN 2009045).
5.2.2 Cell processing

Peripheral blood was collected in Lithium-Heparin anticoagulant tubes. Peripheral blood mononuclear cells (PBMC) and synovial fluid mononuclear cells (SFMC; only in JIA study) were isolated using the standard density gradient centrifugation method (Lymphoprep Axis-Shield, Oslo, Norway) to separate RBC, platelets and plasma from the mononuclear fraction. Sinus tissues collected in RPMI 1640 (HyClone Laboratories, South Logan, UT, USA) were minced using a scalpel and gently filtered through 70µm cell strainer to obtain a single-cell suspension. For the JIA study and the sinusitis study, flow cytometry analysis was performed on freshly isolated cell samples. For all other studies, PBMC were stored in liquid nitrogen for later batch analysis. Healthy normal donor samples were also frozen as a control. Briefly, isolated cells were stored in 90% fetal calf serum (SAFCBiosciences, Lenexa, KS, USA) with 10% dimethyl sulfoxide (Sigma Aldrich, Steinheim, Germany) at -80°C freezer then transferred to liquid nitrogen. Samples were thawed in RPMI 1640 (HyClone Laboratories, South Logan, UT, USA) supplemented with 2 mM L-glutamine (SAFCBiosciences, Lenexa, KS, USA), penicillin/streptomycin (Sigma Aldrich, Steinheim, Germany), and 10% fetal calf serum (SAFCBiosciences). The viability of the cells was checked using trypan blue (Sigma Aldrich) exclusion before flow cytometry analysis.
5.2.3 Analysis of cell surface protein expression

For flow cytometry analysis, PBMC / SFMC / cells from sinus tissue (0.5-1 X 10^6 cells) were stained with monoclonal antibodies against CD4, CD25, (BD Biosciences, San Diego, USA) and PI16 (Co-operative Research Centre for Biomarker Translation, Australia). For RA, scleroderma and pregnancy-asthma studies, cells were stained with monoclonal antibodies against CD45RO (BD Biosciences) in addition to above mentioned antibodies. Staining was performed according to standard surface molecule staining protocols for flow cytometry. Data was acquired using FACSCanto (BD Biosciences) and analysed using FlowJo software (FlowJo LLC, Ashland, OR, USA).

5.2.4 Statistical analysis

For all studies, unless specified, the Holm-Sidak’s comparison test was used to compare the percentage of Th cells expressing PI16 between disease groups and control groups. The same method was used to compare the N/M_{PI16+}, N/M_{PI16-}, and N/M_{Th} ratios between disease groups and healthy controls. Linear regression analysis was performed to identify the relationship between PI16 expression in T1D patients at 3, 6 and 9 months, and their autoantibody levels (ICA, GAD and IA2). For the JIA study, ratio paired t-test was performed to compare the percentage of T helper cells expressing PI16 in the peripheral blood with donor-matched synovial fluid. Linear regression was performed to identify the relationship between JADAS and PI16 expression in T helper cells in peripheral blood and synovial fluid. For the sinusitis study, a ratio paired t-test
was performed to compare the percentage of T helper cells expressing PI16 in the peripheral blood with donor-matched sinus tissue. Alpha value was set to 0.05 for all the studies to determine the significance and to ensure only less than 5% chance of error is possible in accepting / rejecting the hypothesis.

5.3 Results

5.3.1 Type 1 diabetes

To determine the role of PI16 in type 1 diabetes, PI16 expression on T helper cells at different stages of the disease was determined. The percentage of PI16 expression on Th cells was compared between patients at pre-clinical stage (n=12), patients at 3, 6 and 9 months (n=35, 27 and 20 respectively) after the onset of the disease, and in healthy children (n=8). Figure 5-1a & b show that the mean percentage of T helper cells expressing PI16 is not significantly different between any of these groups. Moreover, there is no linear relationship between percentage of PI16 expression on Th cells and T1D related autoantibodies (ICA, GAD and IA2) in peripheral blood at 3 and 6 months after the onset of the disease. Interestingly, at nine months after the onset of the disease, there is a significant negative correlation between ICA in the peripheral blood and percent PI16 expression on Th cells, \( R^2=0.2, p=0.0475 \) (Figure 5-1c). However, there is no significant association between PI16 expression and the other two autoantibodies, IA2 and GAD, at nine months after the onset of disease.
5.3.2 Chronic sinusitis

To determine the role of PI16+ Th cells in chronic sinusitis, the percentage of T helper cells expressing PI16 in the peripheral blood was compared with donor-matched nasal tissue in the patient cohort. As shown in Figure 5-2 the percentage of Th cells expressing PI16 is significantly higher in peripheral blood compared with donor matched nasal tissue (p<0.05, n=7). The mean percentage of Th cells expressing PI16 in the peripheral blood is higher in patients than in healthy adults (24.27%; n=7 vs 13.42%; n= 22). Patients were categorised into chronic rhinosinusitis with polyps (CRSwNP; n=5) and without polyps (CRSsNP; n=2). However, the sample size is too low to draw comparison based on the disease type (data not shown). It is also not possible to analyse nasal tissue-resident cells from healthy donors as it is not ethical to harvest that tissue.

5.3.3 Juvenile idiopathic arthritis

To determine the role of PI16+ Th cells in the peripheral blood and at the site of inflammation in juvenile idiopathic arthritis (JIA), the percentage of Th cells expressing PI16 in the peripheral blood of JIA patients was determined and compared with donor-matched synovial fluid. The mean percentage of PI16 expression on Th cells of JIA peripheral blood and synovial fluid was also compared with that of peripheral blood of healthy juvenile controls. Comparison with synovial fluid from healthy controls is not possible as it is unethical to collect this sample. Figure 5-3a shows that there is no significant difference in
the percentage of PI16 expressing cells between peripheral blood and synovial T helper cells in JIA patients. In addition, there is no significant difference in the mean percentage of PI16 expressing peripheral blood Th cells between JIA patients and healthy controls. Linear regression was performed to estimate the relationship between Juvenile Arthritis Disease Activity Score (JADAS) and PI16 expression in synovial fluid and peripheral blood T helper cells. As shown in Figure 5-3b, there is no significant relationship between JADAS and percent PI16 on Th cells in synovial fluid and peripheral blood.

5.3.4 Rheumatoid arthritis

To determine the role of PI16+ Th cells in RA, the percentage expression of PI16 on Th cells was compared between healthy controls and patient groups with different disease activity levels. As shown in Figure 5-4, RA patients were grouped into their clinical classifications; remission (n=9), low disease activity (n=7), moderate disease activity (n=5) and high disease activity (n=23), based on their disease activity score (DAS). The same patients were also grouped based on a second disease activity score DAS28, which was: remission (n=16), low (n=5), moderate (n=17) and high (n=7) disease activity. The main difference between these two scores is that the DAS measures 44-swollen/ tender joints in addition to the Ritchie Articular Index, while DAS28 measures only 28 tender joints. In addition, both the scores include erythrocyte sedimentation rate, patient global assessment of disease activity or general health assessment on a visual analog scale [30]. Patient grouping was also performed based on low
(n=33) and high (n=12) C-reactive protein (CRP) levels in peripheral blood. Figure 5-4a shows that the mean percentage of T helper cells expressing PI16 is significantly higher only in the remission group (categorized based on DAS and DAS28 scores) and in low CRP level group, compared with healthy controls (23.67%, 21.88% and 19.78% vs 13.22 respectively; p<0.05).

To determine if there is a change in the proportion of naïve (CD45RO-) and memory (CD45RO+) cells in the PI16+ Th subset in rheumatoid arthritis (RA) patients, naïve/memory ratios (N/M) were determined. Our nomenclature for naïve/memory ratios in total T helper cells, PI16+ T helper cells and PI16- T helper cells are N/M_{Th}, N/M_{PI16+} and N/M_{PI16-}, respectively. Figure 5-4b shows the comparisons between N/M_{PI16+} and N/M_{Th} ratios in patient groups based on the DAS, DAS28 and CRP level, and healthy adults. The mean N/M_{PI16+} ratio of patients in remission is significantly higher than in healthy adults and in RA patients with moderate and high disease activity (0.75 vs 0.12, 0.31 and 0.33 respectively; p<0.05). In addition, the mean N/M_{PI16+} ratio in low disease activity is significantly higher than in healthy adults (0.54 vs 0.12; p<0.05). However, there is no significant change in the mean N/M_{PI16+} ratio between low, moderate and high disease activity patients. When comparing the N/M_{Th} ratios, there are no significant differences between any patient groups or between all patient groups and healthy adults. Similarly, there are no significant differences in N/M_{PI16-} ratios between any of the groups (data not shown). When the patients were grouped using DAS28 score (Figure 5-4b), the only mean N/M_{PI16+} ratio in the remission group is significantly higher than in the healthy group (0.47 vs 0.12,
p<0.05). However, mean N/M\textsubscript{Th} (Figure 5-4b) and N/M\textsubscript{PI16-} (data not shown) ratios are not significantly different between any of the patient groups and healthy controls. The third grouping of RA patients using CRP levels (Figure 5-4c), shows that mean N/M\textsubscript{PI16+} ratios in both the higher CRP level group and the lower CRP level group are significantly higher than in healthy controls (0.56 and 0.39 vs 0.12; p<0.05). However, the mean N/M\textsubscript{Th} (Figure 5-4c) and N/M\textsubscript{PI16-} (data not shown) ratios are not different in any of these groups.

### 5.3.5 Scleroderma

To determine the role of PI16+ Th cells in scleroderma, the percentage of PI16 expressing Th cells and mean N/M\textsubscript{Th}, N/M\textsubscript{PI16+} and N/M\textsubscript{PI16-} ratios were compared between patients and healthy controls. The comparisons were performed between patients with two types of scleroderma, limited (n= 6) and diffuse (n=33) and between treatment groups, steroid (n=7) and non-steroid (n=34). There is no significant difference in mean percentage of T helper cells expressing PI16 between limited scleroderma patients, diffuse scleroderma patients and healthy controls (Figure 5-5a). The mean N/M\textsubscript{PI16+}, N/M\textsubscript{Th} (Figure 5-5b) and N/M\textsubscript{PI16-} (data not shown) ratios of diffuse, but not limited scleroderma patients are significantly higher than the control group (2.65, 3.56 and 3.9 vs 0.12 respectively; p<0.05). The mean N/M\textsubscript{PI16+} ratio of the steroid group is significantly higher compared with non-steroid group and healthy control group (2.87 vs 1.00 and 0.12 respectively; p<0.05) (Figure 5-5c). However, there is no
difference in mean $N/M_{Th}$ (Figure 5-5c) and $N/M_{PI16-}$ (data not shown) ratios between steroid, non-steroid and healthy control groups.

### 5.3.6 Asthma in pregnancy

We have compared the mean percentage of Th cells expressing PI16 and mean $N/M_{Th}$, $N/M_{PI16+}$ and $N/M_{PI16-}$ ratios between asthmatic and non-asthmatic pregnant patients (Figure 5-6). For comparison, we also included normal healthy adult controls, which include both men and women. Previous experiments have established no difference in percent PI16 expression on Th cells between men and women (data not shown). The mean percentage of Th cells expressing PI16 is not significantly different between healthy controls and pregnant women; either asthmatic or non-asthmatic (Figure 5-6a). The mean $N/M_{Th}$, and $N/M_{PI16+}$ ratios were not significantly different between asthmatic and non-asthmatic pregnant women. Interestingly, $N/M_{Th}$ ratio is higher in pregnant women with asthma and without asthma in comparison with normal healthy adults (3.1 and 2.8 vs 1.2 respectively; $p<0.5$). Finally, the $N/M_{PI16+}$ ratio in pregnant women without asthma is significantly higher than in normal healthy adults (2.0 vs 0.12; $p<0.05$).

### 5.4 Discussion

Autoimmunity and hyper inflammation are a result of an unregulated immune response, as unwarranted effector function of immune cells at the site of disease activity results in inflammation. The tissue damage that is caused by
this chronic effector activity and the failure of the immune system to regulate it drives the pathology. The objective of curative immune therapy is to regain control over this process. T helper subsets have been the subject of many studies in various autoimmune disease cohorts. Therefore, it is essential to create novel tools to efficiently detect T helper subsets that might play protective or pathological roles in diseases. Peptidase Inhibitor 16 identifies a hyperproliferative memory T helper subset with potent effector functions [Chapter 3 & 4]. However, their function in autoimmune disease is unknown. In this pilot study we aimed to determine whether PI16 expressing T helper cells have a role in type I diabetes rheumatoid arthritis, juvenile idiopathic arthritis, scleroderma, sinusitis and asthmatic pregnancy.

Our analysis of PI16+ Th cells in type 1 diabetes (T1D) revealed no association between peripheral PI16+ Th cell numbers and T1D disease status. The mean proportion of Th cells expressing PI16 in patients at the pre-clinical phase and patients at 3, 6 and 9 months post diagnosis were not significantly different in comparison with healthy children. Although the level of autoantibody ICA at nine months after onset of the disease decreased with increasing PI16+ Th cell numbers, overall there was no significant correlation between PI16+ Th numbers in the peripheral blood and the levels of autoantibodies. Auto-reactive CD4 T cells specific for islet cell antigens are primarily present in the pancreatic lymph node, and are difficult to detect in the peripheral blood without in-vitro manipulation [31]. Therefore it can be speculated that even if PI16+ Th cells
have an active role in β-cell destruction, they might be present in increased numbers only in the pancreatic lymph nodes and not in the peripheral blood.

In our previously reported study in juvenile idiopathic arthritis (JIA), we observed a significant enrichment of PI16+ Treg and an increase in Th17 cells in synovial fluid of JIA patients [7]. Therefore, in this study we hypothesised that PI16+ Th cells which have a pro-inflammatory phenotype and produce increased amounts of IL-17 in comparison with PI16- Th cells [Chapter 3 & 4], might be increased in the inflamed synovium. Contrary to our hypothesis, our results indicate a trend that the number of PI16+ Th cells in the synovial fluid is lower when compared with peripheral blood. However, the sample size is too low for this to be confirmed statistically significant. Although the T cell subset proportions in synovial fluid of JIA patients compared with donor matched peripheral blood may indicate abnormal T cell activity in the synovium, it is difficult to interpret without a comparative synovium sample from healthy donors. However, the phenotype of Th cells in synovium of JIA cannot be compared with healthy controls as it is impossible and unethical to collect synovial fluid from healthy children. In addition, the linear regression analysis demonstrate that there is no significant relationship between the disease activity score (JADAS) and PI16 expression in peripheral blood or synovium Th cells of JIA patients. We speculate that JADAS may not reflect the true disease activity status, as the majority of the patients in this study were treated with non-steroidal anti-inflammatory drugs (NSAID). On the one hand, NSAIDs which inhibit prostaglandin synthesis, mainly act as analgesics by decreasing
pain and inflammation and do not decrease disease progression in rheumatism [32]. On the other hand, the JADAS scoring system includes the parent / patient assessment of well-being which is indirectly dependent on the pain and disease symptoms [33]. Therefore statistical analysis comparing a numerical measure with a subjective score is difficult to validate, and this may explain why our data fail to show any correlation between the disease activity (JADAS) and the percentage of PI16+ Th cells.

Our previously published data indicate that in chronic sinusitis, despite an increase in Treg in the mucosal tissue, inflammation is not controlled [34]. This may be due to lack of functional Treg in the tissue. Consistent with this result, an increase in number of PI16 expressing Th cells was found in the peripheral blood of sinusitis patients in comparison with healthy controls. However, in comparison with peripheral blood, the proportion of PI16 expressing Th cells is lower in the donor matched nasal tissue of sinusitis patients. Even though comparing T cell numbers and subset proportions in sinusitis nasal tissue with a control nasal tissue from healthy donors would be optimal, it is not possible for ethical reasons. Regardless, this preliminary data suggests a potential role of PI16+ Th cells in sinusitis.

Based on earlier phenotypical studies [Chapter 3], PI16+ Th cells have a memory phenotype (CD45RO+) in the peripheral blood of healthy controls. In this study we investigated whether there is a change in naïve / memory phenotype in PI16+ Th subset in RA, scleroderma and asthmatic pregnancy
patients. Naïve versus memory homeostasis is essential for an effective balance in the immune system, such that the immune system is poised to respond to challenge, but restrained to prevent chronic pathology. The proportion of naïve to memory T cells changes with age, and a prime example is cord blood which is almost all naïve (CD45RA) at birth, but the proportion of naïve T cells decrease and memory (CD45RO) T cells increase in the peripheral blood with age, suggesting a decrease in the naïve/ memory ratio with age [35]. It is uncommon for this ratio (CD45RA+ cells/CD45RO+ cells) to increase, but in some diseases re-induction of naïve status has been observed, suggesting a peripheral expansion. For example; in myelodysplastic syndrome and multiple myeloma patients and in HIV patients after IL-2 therapy, an increased number of naïve regulatory T cells (Treg) is reported. The increased number of naive Treg is thought to be due to extra-thymic expansion of peripheral Treg which are not derived from the thymus [36-38]. A related study showed that in HIV patients, where CD4 T cells are constantly destroyed, replenishment of these cells occurs through peripheral expansion of T cells therefore increasing the naïve/memory ratio [39]. The peripherally expanded naïve T cells in all these studies were distinguished from other naïve T cells by the loss of T cell receptor excision circles (TREC) because naïve T cells originating from the thymus have high expression of TREC and the TREC content decrease with each cell division [35, 40].

Consistent with this, our results show that PI16+ Th cells which mainly have a memory phenotype in healthy adults, express CD45RA in some patients with
rheumatoid arthritis. The RA patients were grouped based on their CRP levels, DAS and DAS28. Patients with both lower and higher CRP levels have increased proportion of naive cells in the PI16+ subset in comparison with healthy controls. Moreover based on DAS and DAS28 groupings, the proportion of naïve cells in the PI16+ Th subset has increased in patients under remission compared with healthy adults. In addition the proportion of PI16 expressing Th cells have increased in the peripheral blood of patients under remission and in patients with lower CRP levels. A closely related study indicates that patients with JIA have peripherally expanded naïve T cells, indicated by lower TREC expression in comparison with healthy donors [41]. The proportion of naïve T cell is dependent on the thymic output, but the function of the thymus is affected in autoimmune disease, leading to decreased thymic output which may alter T cell homeostasis. However, the decreased production of T cells by the thymus may be compensated by post-thymic expansion. This was shown by a decrease in TREC content in total peripheral CD4 T cells in RA patients [42]. Together, this may suggest that PI16+ Th cells proliferate more in the remission patients which may imply a protective effect against RA pathology. However, there was no significant change in the naive to memory ratio in total Th cells in comparison with healthy adults, suggesting no dramatic perturbations in the Th compartment as a whole.

Similar trends were observed in scleroderma patient cohorts. The naïve/memory ratio in PI16+ Th and in total Th cells in diffuse scleroderma patients is significantly higher in comparison with healthy controls. The statistics
also indicated the ratio was significantly different between steroid and non-steroid treatment groups. However, due to low sample size and high standard deviation in diffuse scleroderma and steroid treatment group, a biological conclusion cannot be reached from this cohort. However in limited scleroderma, although not statistically significant, the proportion of naïve cells is trending to be higher in PI16+ Th cells of scleroderma patients in comparison with healthy adults. The mean naïve / memory cell ratio value >1 in PI16+ Th cells in limited scleroderma patients indicate higher naïve cell numbers in comparison with mean ratio of about 0.1 in healthy adults, which indicates higher numbers of memory cells in the subset. However, there is no significant change in the proportion of naïve and memory cells in total Th cells. This may indicate that peripheral expansion of Th cells primarily occurs in the PI16+ subset in scleroderma patients which has been suggested in RA patients above. However, there is no change observed in total Th cells as a whole. Although our studies are limited by the lack of information about the TREC content of PI16+ naïve Th cells and their proliferative and / or activation status, the results from the RA and scleroderma studies suggest that PI16+ Th cells may have a potential role in these diseases.

In line with the RA and scleroderma studies, the naïve/memory ratio in PI16+/- and in total Th subset also analysed in asthmatic pregnancy. Our study revealed that irrespective of asthmatic status, the proportion of naïve cells in PI16+ Th cells and in total Th cells is higher in pregnancy than in normal healthy adults. Although the reason is not clearly known, this is consistent with
other studies showing elevated proportions of CD45RA T cells during normal pregnancy [43]. During normal pregnancy the immune system develops tolerance to the partially allogeneic foetus, which is controlled by the induction of Treg. An increase in CD45RA+ Treg was reported to be essential during normal pregnancy and a lower percentage of this subset leads to preeclampsia [44, 45]. These are consistent with our results of increased naïve Th population in the peripheral blood during pregnancy in comparison with normal healthy adults. However, during pregnancy, mother’s thymus undergoes involution, resulting in decreased thymic output of naïve T cells [46]. This raises the question about the origin of the increased naïve T cells in the peripheral blood during pregnancy. It can be speculated that these are post-thymic expanded cells and also it may be possible that the increased CD4+ CD25- Th cells provide a source for peripherally induced Treg. Regardless, based on the results from this study it is difficult to conclude if the change in naïve/memory ratio in PI16+ Th and in total Th subset is because of asthma or pregnancy.

In conclusion, these pilot studies show that PI16+ Th cells may play a role in autoimmune diseases, especially in sinusitis, RA and scleroderma. This role may be disease-specific, and as the pathophysiology of different autoimmune diseases is complex, it is difficult to make general conclusions. Although a change in naïve/memory phenotype of PI16+ Th cells may indicate an active function in diseases, it needs to be determined whether they have a protective or a pathogenic role. While the RA study may suggest a protective role of PI16+ Th cells as they are increased in patients under remission, a time course
experiment will be required to confirm the change in proportion PI16+ Th cells at different stages of the disease of a patient. Understanding the dynamics of PI16 expression on Th cells before, during and after the onset of a disease is essential to confirm their function. This will also enable us to understand the effect of therapy on PI16+ Th subset. In summary these pilot studies indicate a potential role of PI16+ Th subset in disease. The preliminary results build a foundation for further functional assays in order to better understand their role in health and disease.

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References


Activity, Disease Activity Score (DAS) and Disease Activity Score with 28-Joint Counts (DAS28), Simplified Disease Activity Index (SDAI), Clinical Disease Activity Index (CDAI), Patient Activity Score (PAS) and Patient Activity Score-II (PASII), Routine Assessment of Patient Index Data (RAPID), Rheumatoid Arthritis Disease Activity Index (RADAI) and Rheumatoid Arthritis Disease Activity Index-5 (RADAI-5), Chronic Arthritis Systemic Index (CASI), Patient-Based Disease Activity Score With ESR (PDAS1) and Patient-Based Disease Activity Score without ESR (PDAS2), and Mean Overall Index for Rheumatoid Arthritis (MOI-RA). Arthritis care & research, 2011. 63 Suppl 11: S14-36.


Figure 5-1: Type 1 diabetes
The percentage of T helper cells expressing Pi16 was determined in peripheral blood of T1D patients and compared with healthy controls. a) shows the percent Pi16+ Th cells in patients at 3 months (n=35), 6 months (n=28) and 9 months (n=21) after the onset of disease, patients at the pre-clinical stage (n=12) and juvenile control (n=8). b) shows donor matched comparison at 3, 6 and 9 months after the onset of disease. Lines connect matched donors. c)
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shows the association between the level of Islet-cell antibodies (ICA), glutamate decarboxylase antibodies (GAD) and Islet antigen 2 antibodies (IA2) in the peripheral blood and percent PI16 expression on Th cells, at 3, 6 and 9 months after the onset of disease. Linear regression analysis was performed to test degree of correlation ($\alpha=0.05$).
Figure 5-2: Chronic sinusitis
The percentage of T helper cells expressing PI16 was determined in peripheral blood and nasal tissue of sinusitis patients (n=8) and compared with peripheral blood of healthy controls (n=16). Lines connect matched donors (*p<0.05).
Figure 5-3: Juvenile idiopathic arthritis
a) The percentage of T helper cells expressing PI16 was determined in peripheral blood and synovial fluid of JIA patients (n=11) and compared with peripheral blood of juvenile healthy controls (n=8).
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[Graphs showing the relationship between DAS, DAS28, and CRP with different disease activity scores (Remission, Low, Moderate, High, Healthy).]

DAS

DAS28

CRP

Disease activity score

Naive / Memory ratio in P116+ Th cells

Low, Moderate, High, Healthy

Naive / Memory ratio in P116+ Th cells

Low, Moderate, High, Healthy

Naive / Memory ratio in P116+ Th cells

Low, Moderate, High, Healthy

Naive / Memory ratio in P116+ Th cells

Low, Moderate, High, Healthy

Naive / Memory ratio in P116+ Th cells

Low, Moderate, High, Healthy

Naive / Memory ratio in P116+ Th cells

Low, Moderate, High, Healthy
Figure 5-4: Rheumatoid arthritis
The percentage of T helper cells expressing PI16 was determined in peripheral blood of rheumatoid arthritis patients and compared with healthy controls. a) Percent PI16 on Th was compared between patients grouped based on DAS (remission, n=9; low n=7; moderate n=5; high n= 23), DAS28 (remission n=16; low n=5; moderate n=17; high n= 7) and CRP level (low n=33; high n=12) and healthy controls (n=16). b) The ratio of naïve and memory cells in PI16+ Th cells and total Th cells was compared between patients grouped based on DAS, DAS28 and CRP level and healthy controls. Bar indicates mean (*p<0.05).
Figure 5-5: Scleroderma
The percentage of T helper cells expressing PI16 was determined in peripheral blood of scleroderma patients and compared with healthy controls. a) Percent PI16 on Th were compared between patients with diffuse (n=6) and limited (n=33) scleroderma. b) The ratio of naïve and memory cells in PI16+ Th cells and total Th cells was compared between patients grouped based on limited and diffuse scleroderma, and healthy controls. c) The ratio of naïve and memory cells in PI16+ Th cells and total Th cells was compared between patients grouped based on treatment groups; with (n=7) and without (n=34) steroids, and healthy controls. Bar indicates mean (*p<0.05).
Figure 5-6: Asthmatic pregnancy
The percentage of T helper cells expressing PI16 was determined in peripheral blood of scleroderma patients and compared with healthy controls. a) Percent PI16 on Th were compared between pregnant women with (n=18) and without (n=18) asthma and normal healthy adults including both men and women (n=16) b) The ratio of naïve and memory cells in PI16+ Th cells and total Th cells was compared between pregnant women with and without asthma and normal healthy adults. Bar indicates mean (*p<0.05).
6.1 Helper T cell paradigm

T helper cells (CD4+ CD25-), a key component of the adaptive immune system, provide pathogen specific immune response by activating other immune cells including phagocytic cells, cytotoxic T cells and B cells, primarily by secreting cytokines. T helper cells (Th) are a heterogeneous population, comprising subsets of cells with different phenotypical and functional characteristics. The heterogeneity of Th cells may signify the need for division of labour to combat the diverse range of pathogens we encounter every day. Currently several subsets of Th cells have been defined including Th1, Th2, Th17, Th22, Tfh and Th9, each with a distinct role in protective immunity and autoimmunity as described in Chapter 1. In order to segregate a functionally distinct subset in a heterogeneous population of Th cells, biomarkers are essential, not just for in-vitro studies but also for diagnosis and for the emerging field of target-specific therapies.

As part of a genome-wide array study to identify potential biomarkers for regulatory T cells, PI16 emerged as a potential biomarker for a functionally distinct subset of regulatory T cells (Treg). The intriguing characteristics of PI16+ Treg (CD4+CD25+) have led to the question whether the T helper cells (CD4+CD25-) expressing PI16 also have unique characteristics. This study, for the first time has investigated the characteristics of PI16+ CD25- CD4+ T cells.
(PI16+ Th). By an extensive survey of surface antigen expression, gene expression, intracellular signalling molecules and cytokine production of PI16+ Th cells, along with in-vitro functional assays, this study predicts the potential functional role of this Th cell subset in health and disease.

### 6.2 Th17 cell association?

One of the main questions this thesis aims to answer is whether PI16+ Th cells have a phenotype identical to a previously described Th subset in literature, which includes Th1, Th2, Th17, Th22, Tfh and Th9, or alternatively, whether PI16 identifies a new Th subset. The initial screening for surface markers [Chapter 3] co-expressed by PI16+ Th cells revealed that a high proportion of the PI16+ subset express CCR4 and CCR6, which is consistent with a Th17 phenotype [1]. However, when PI16+ cells were activated with Th17 polarising cytokines there was only a marginal, not significant increase in the Th17 signature transcription factor, ROR-γt, in comparison to PI16- Th cells. The apparent lack of difference in the induction of ROR-γt between PI16+ Th and PI16- Th cells in this culture system [Chapter 3] might be because PI16- Th cell pools contain a mixture of naïve (CD45RA+) and memory (CD45RO+) cells, while PI16+ Th cell pools are almost all CD45RO+ memory cells. The current literature suggests that the CD45RA+ naïve T cells are more plastic than CD45RO+ memory T cells and have the potential to differentiate more readily into any different type of T helper subset primarily depending on the cytokine cues [2]. Hence, in the above experiment where cells were activated with Th17
polarising cytokines, there might be a significant induction of ROR-γt in the naïve cells which are present only in the PI16- subset. This *in-vitro* experiment of activating cells with polarising cytokines may closely resemble *in-vivo* pathology induced condition. However, comparing the response of CD45RO homogeneous PI16+ Th cells against the heterogeneous (CD45RA+ and CD45RO+) PI16- Th cells may mask the distinct characteristics of the PI16+ subset. Therefore, a parallel experiment was performed activating cells without any polarising conditions, which may not resemble *in-vivo* conditions, but may reveal the pre-programmed effector characteristics of cells, especially of memory cells. This is because, naïve cells require polarising / inducing cytokines to commit to a given differentiation path, whereas, memory cells express pre-programmed genes that were induced by their primary response and are committed to a given differentiation path in the event of antigen re-exposure. Although the differentiation path of memory cell response can also depend on cytokine milieu, when memory cells are non-specifically re-stimulated in the absence of polarising cytokines, they will presumably deliver an effector recall response similar to their primary response. Therefore, it can be speculated that the higher expression of ROR-γt by PI16+ cells in comparison with PI16- cells, under non-polarized conditions, may indicate that the PI16+ Th subset are enriched for cells committed to Th17 lineage origin [Chapter 4].
6.3 Chemokine profile

To further investigate the heterogeneity of the PI16+ subset, the Duhen et al. [3] method of resolving Th subsets (Th1, Th2, Th17 and Th22) by chemokine profile [Chapter 4] was used to compare the distribution of Th subsets in PI16+ and PI16- memory cells. This experiment revealed that the PI16+ memory subset, while homogeneous for CD45RO expression, is a heterogeneous population containing Th1, Th2, Th17, Th1/Th17 and Th22 cells. In addition, a higher proportion of Th17-like cells in the PI16+ subset in comparison with the PI16- subset, may explain the higher ROR-γt expression and IL-17 secretion in the PI16+ subset. Interestingly, while there are both PI16+ and PI16- cells in the Th1, Th2, Th17 and Th1/Th17 subsets, almost all Th22-like cells express PI16. However, further investigation is required to identify if these Th22 cells secrete IL-22 and express the transcription factor AHR; both are key signature markers of Th22 [4]. Therefore it may be possible to isolate Th22 cells by using PI16 in combination with CCR10 instead of the conventional method of using three surface markers; CCR4, CCR6 and CCR10. In conclusion, PI16 may not be a sole biomarker for any of the already described subsets, but based on other experimental analyses as described below, it may segregate a functionally distinct subset.

In addition to the utility of the chemokine receptors as passive biomarkers of particular subsets, they are also functional receptors. Chemokine receptors define the migratory pattern of T cells. Circulating T cells expressing specific
chemokine receptors respond and migrate towards the tissue location where the chemokine receptor ligands are secreted [5]. The type of chemokine receptor expressed by memory CD4 T cells is dependent on the tissue from where the memory cells were generated. For example, memory cells generated during an infection in the lung express the chemokine receptor CCR4, presumably to traffic to and protect the lungs from future infections [6]. Combined data from surface expression analysis and gene expression analysis reveal that PI16+ Th cells have a higher expression of CCR4, CCR6, CCR8 and CCR10 which suggest a skin-homing fate [7, 8]. However, these analyses were performed on cells isolated from the peripheral blood and therefore only predict the migratory pattern. Further studies using skin samples are required to confirm this hypothesis.

6.4 Immune memory

A key finding of this research is that nearly all PI16+Th cells have a memory phenotype (CD45RO+) in healthy adult peripheral blood. In this study, memory cells are classified based on the conventional method of CD45RO+ / CD45RA-status.

Currently there are two main models of memory cell generation. The conventional model is that, in response to pathogen recognition by the immune system, naïve T cells are activated and become effector T cells which proliferate to resolve the infection, and once the pathogen is cleared, most of
the effector T cells undergo apoptosis, while some cells are retained to become memory cells / antigen-experienced cells [9]. However, recent studies suggest another model, in which memory cells are a precursor to effector cells. According to this model, upon antigen stimulation naïve T cells become stem memory T cells (T_{SCM}), which differentiate into central memory T cells (T_{CM}) and then effector memory T cells (T_{EM}) before becoming effector T cells (T_{EFF}) [10]. This suggests that formation of memory can be before or after resolution of infection. Nonetheless, T_{SCM}, a relatively newly classified memory subset, are primarily characterised based on the expression of CD45RA, and T_{CM} and T_{EM} are classified based on the presence or absence of CCR7 expression, respectively [9, 11]. On the one hand, PI16+ memory cells seem to be heterogeneous and do not fit the phenotype of any of these populations, as they do not express CD45RA and have a mixture of CCR7+ and CCR7-expression [Chapter 4]. On the other hand, based on other surface markers and gene expression analysis, PI16+ Th cells may represent old resting memory cells while PI16- memory cells may represent recently activated cells [Chapter 3 & 4]. For example, in comparison with PI16- memory Th cells, PI16+ memory Th cells have high expression of Clusterin, an immunosenescence marker [12] and β1 integrin, which is essential for the maintenance of memory cells in bone marrow, a site considered a niche for long term survival of memory cells [13]. PI16+ cells also have low expression of tyrosine kinase, CXCR5 and CD38, markers that are only present in recently activated cells [14-16]. Tissue resident memory T cells (T_{RM}), another recently described subset of memory T cells, are known to be long-term resting memory cells [10]. However, these cells are
absent in peripheral blood, and the mechanism that drives the migration of these cells from peripheral blood to tissues after antigen-exposure and the mechanism of recall responses are poorly understood. Can PI16+ memory Th cells be a precursor for these cells? With the data currently available it is difficult to definitively categorise the PI16+ subset within the $T_{RM}$, $T_{SCM}$, $T_{CM}$ or $T_{EM}$ classification system. Considering only a small proportion of memory T cells in the human body circulate in blood, while the majority of them reside in tissues [10], future studies of PI16 expression in tissue samples may provide clarification of the role of PI16+ Th cells \textit{in-vivo}.

### 6.5 Effector phenomenon

While the surface phenotype of PI16+ mem Th cells may not reveal a memory subset classification, some of the \textit{in-vitro} functional assays reveal potent effector capabilities. The current dogma suggests that an immune response by antigen-experienced / memory cells is significantly faster and of higher magnitude in comparison with a primary immune response by naïve cells [17]. Specifically, during a secondary encounter of a pathogen, memory cells with the particular pathogen-antigen specificity respond rapidly by secreting effector cytokines in order to eliminate the pathogen before it reaches a disease-causing threshold level. For example, when ovalbumin-specific naïve and memory CD4 T cells were stimulated with ovalbumin peptide and antigen presenting cells, naïve cells secreted peak amounts of IFN-$\gamma$ at 48-72 hrs, whereas, memory cells reached peak secretion at $\sim$6hrs post stimulation [18].
In addition, differential effector responses amongst memory cell subsets have also been reported [9]. Based on the in-vitro polyclonal activation experiment in [Chapter 4], PI16+ memory Th cells have a faster effector response than PI16-memory cells, as PI16+ memory Th cells show increased phosphorylation of ERK, STAT1, STAT3 and STAT5 and STAT6 and secrete higher amounts of IL-2, TNF, IL-17 and IL-10, in comparison with PI16- memory Th cells. In these experiments, polyclonal stimulation and assay of pooled supernatants masks the individual response of each cell, and there may be differences in the response depending on the antigen. At this stage the antigen-specificity of PI16+ cells is unknown. It is clear that a T cell pool purified from peripheral blood comprises of several/ many T cell clones and each clone is equipped to recognize a particular antigen among a vast variety of potential pathogen antigens. Accurate and sensitive identification of antigen-specificity of cells from PBMC remains technically difficult. However, recent advances in the cytometry and sequencing fields including single-cell analysis and high sensitivity sequencing are providing solutions to this problem. This includes single-cell sequencing of TCR genes to map the repertoire and the use of peptide loaded MHC tetramer binding to physically identify antigen specificity [19]. Using these newly advanced high-throughput methodologies, further investigation needs to be performed to determine the antigen-specificity of PI16+ cells. Based on the diversity of cytokines released by PI16+ Th cells as described in Chapter 3 & 4, it can be predicted that these cells are most likely a polyclonal population with multiple antigen specificities.
Controlled proliferation of effector populations is one of the key aspects of a healthy immune response to pathogen, which otherwise leads to unwarranted hyper-inflammation causing tissue damage. However, an inadequate proliferative response is indicative of a weak immune system causing susceptibility to infection. The proliferation assays [Chapter 4] suggest that PI16+ cells proliferate faster than PI16- memory Th cells under the same in-vitro conditions. In addition, presence and absence of certain markers which are indicative of hyper-proliferation characteristics of a cell were also examined in PI16+ memory Th cells in comparison to PI16- memory Th cells [Chapter 3 & 4]. These markers include low expression of CD38, and high secretion of IL-2 by PI16+ mem Th cells in comparison with PI16- mem Th cells. Following a proliferation / clonal expansion phase of T cells, which aims to eliminate the pathogen is essential, there has to be a contraction phase, where the cells undergo apoptosis to maintain immune cell homeostasis. Apoptotic assays [Chapter 4] indicated that PI16+ memory cells have a higher sensitivity to apoptosis than PI16- memory cells. This correlates with high expression of CD95, an apoptotic marker [Chapter 4]. However, CD95 also acts in anti-apoptotic signalling [20], which may help in the long-term survival of these cells as true tissue resident memory cells. Apart from CD95-mediated apoptosis, there are other molecular mechanisms that control the contraction phase. For example, Galectin-1, which triggers apoptosis as well as induce secretion of IL-10, a regulatory cytokine, is highly expressed in the PI16+ subset [21]. This is consistent with the cytokine profile data [Chapter 3 & 4] which revealed high secretion of IL-10 by PI16+ mem Th cells in comparison with PI16- memory Th
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cells. This may indicate an inherent autocrine proliferation control mechanism in these cells post antigen stimulation. Intriguingly, these cells were less susceptible to suppression by Treg \textit{in-vitro}, in comparison to PI16- memory Th cells [Chapter 4]. This may be due to higher proliferation rate of PI16+ memory Th cells in comparison with PI16- memory Th cells. In conclusion, when activated, PI16+ memory Th cells have a quicker effector response with greater magnitude in comparison with PI16- memory Th cells.

6.6 Clinical relevance

While the phenotypical characterisation of PI16+ cells was performed using random healthy controls, pilot data from six clinical studies including: rheumatoid arthritis (RA), scleroderma, type 1 diabetes (T1D), chronic sinusitis, juvenile idiopathic arthritis and asthmatic pregnancy, were examined to investigate whether PI16+ Th cells have a role in any of these autoimmune/inflammatory diseases [Chapter 5]. There is an increase in the proportion of PI16+ Th cells in the peripheral blood of RA and chronic sinusitis patients in comparison with healthy controls. In RA, only the patients under remission and patients with low CRP levels showed an increase in the percentage of PI16+ Th, in comparison with healthy adults, suggesting protective characteristics against this disease. In the other pilot studies there was no significant increase in PI16 expressing Th cells in comparison with healthy controls. However, in all the cohorts where naïve: memory ratios were measured, which includes RA, scleroderma and asthmatic pregnancy cohorts,
there was an increase in naïve cells in the PI16+ Th subset, whereas the ratio of naïve and memory cells in total Th cells was not significantly different between disease groups and healthy groups. While almost all PI16+ Th cells are CD45RO+ and CD45RA- in healthy adults, expression of CD45RA or loss of CD45RO in a significant proportion of PI16+ Th cells in disease groups is intriguing. Since PI16+ Th cells represent less than ~25% of Th cells in most individuals, changes in the naïve: memory proportion in PI16+ subset does not dramatically alter the overall naïve: memory ratio in total Th cells and there is no significant change in this ratio in PI16- Th cells. Further studies need to be performed to investigate if PI16+ Th cells are changing phenotype by losing CD45RO and gaining CD45RA or if naïve Th cells are beginning to express PI16 in disease cohorts. However, tracking the fate of a cell is difficult in humans unlike in mice where many research groups have used genetically marked mice to track cell fate. For example, Rubtsov et al have conducted experiments tracking the fate of fluorescent protein tagged regulatory T cells in-vivo in knock-in mice [22]. Nevertheless, this thesis has focused only on some autoimmune / inflammatory diseases so cannot conclude whether this change in naïve: memory ratio of PI16+ Th cells occurs in any other disease cohorts.

Taking into consideration the other published studies that examine naïve/memory status, two possible explanations can be speculated for the potentially abnormal behavior of PI16+Th cells expressing CD45RA. These CD45RA expressing PI16+ Th could either be effector memory cells re-expressing CD45RA or they could be post-thymic expanded cells.
The first possibility is based on the idea that terminally differentiated effector memory T cells re-express CD45RA (TEMRA). In CD8 populations, these cells are differentiated from central memory T cells re-expressing CD45RA in the absence of antigen. These cells have low proliferation potential, are highly resistant to apoptosis and have highest amounts of perforin. Unlike naive cells, these cells are CCR7- and CD27- [23, 24]. These cells are found in increased numbers in blood in the cases of persistent viral infections and ageing [25]. For example, an increase in TEMRA has been reported in Hepatitis C virus patients, HIV and cytomegalovirus patients [26, 27]. The percentages of CD4+ and CD8+ TEMRA have been shown to increase with age [28]. In addition, an increase in this population was also reported by Matteucci, et al. in T1D patients. Although the biological significance for this in T1D is not yet known, the authors of that study predict that repeated stimulation of cells in-vivo over a long-term could be a potential reason for the increase in TEMRA [29]. However, this thesis cannot confirm if CD45RA expressing PI16+ Th cells are TEMRA, as their CCR7 and CD27 expression was not investigated in the disease cohorts. Furthermore it might be possible that TEMRA exist in the PI16- Th population as well. If PI16+ Th cells expressing CD45RA are in fact TEMRA, and these are exclusively present only in the PI16+ subset, as there is no increase in CDR45RA expression in PI16- subset, then their expansion in autoimmune patients could be due to the chronic activation status of Th cells in response to auto-antigens.
Another possibility is based on the concept that post-thymic expanded CD4 T cells express CD45RA. Expression of CD45RO increases with age until adulthood. While the T cells in newborn are almost entirely naïve /CD45RA+, they begin to express CD45RO as they encounter antigen and become antigen-experienced or attain memory status [30]. However, the thymic output of naïve T cells decreases with age due to thymic involution. In order to maintain homeostasis, peripheral expansion of T cells occurs and these can express CD45RA [31]. Peripheral expansion is also possible in patients with chronic disease, where there is a constant demand for T cells due to prolonged infection or antigen exposure [32]. These peripherally expanded CD45RA+CD4 T cells can be distinguished from recent thymic emigrants by low CD31 (PECAM-1) expression and decrease in TCR excision circles (TREC). TREC, a DNA episome formed in the thymus, is highly expressed in naïve T cells that are recent thymic emigrants, and is diluted during cell divisions [31, 33, 34]. Based on these markers, increased peripheral expansion of naïve regulatory T cells (Treg) was observed in HIV-patients after IL-2 treatment [35]. A related study showed a similar peripheral expansion of Treg with CD45RA expression in patients with multiple myeloma [35, 36]. Therefore, it can be hypothesised that the CD45RA expressing PI16+ Th cells in the RA and scleroderma cohorts are peripherally expanded T cells, generated to meet the constant demand for T cells during chronic active inflammation. Future studies examining CD31 and TREC expression in PI16+ subset are required to confirm this hypothesis.
Although this clinical study is presented as the last results chapter of this thesis, the study was conducted in parallel with phenotypical characterisation of PI16+ Th subset in healthy controls. Therefore the key findings about the functional characteristics of PI16+ Th cells in healthy donor samples as described in Chapter 3 & 4, could not be investigated in disease cohorts. Hence the clinical studies only looked at the PI16 expression in the patient groups and naïve/memory phenotype of PI16+ Th cells only in the RA, scleroderma and asthma patient groups. Further investigation is necessary as suggested below to prove the functional capacity of PI16+ cells in disease.

### 6.7 Future directions

This thesis, for the first time, encapsulates a wide category of phenotypical and functional characteristics of PI16+ Th cells. Preliminary characterisation of PI16-expressing Th cells have produced intriguing results and further studies are warranted to fully evaluate their phenotype, their function and the potential of PI16 to be used as a diagnostic and / or therapeutic biomarker. Suggested experiments for further studies are summarised below.

In order to confirm the chemokine receptor profile data which indicated all Th22 express PI16, the Th22 status of PI16+ Th cells could be determined by their IL-22 cytokine secretion and AHR transcription factor gene expression which are signature biomarkers of Th22. To investigate this, sorted PI16+ Th cells from peripheral blood can be activated using CD3/CD28 beads with / without
Th22 polarising cytokines; recombinant human IL-6 and TNF-α. Secretion of IL-22 could be detected both in supernatant by ELISA and in cells by intracellular flow cytometry staining. Gene expression of AHR could be determined using RT-qPCR.

As PI16+ cells have a memory phenotype, their recall response can be compared with PI16- memory Th cells by in-vitro antigen re-call response assays. PBMC from individuals who had been previously vaccinated, for example, with tetanus or diphtheria toxoid could be re-stimulated in-vitro with the same antigen. Recall response could be determined and compared between PI16+ and PI16- memory Th cells by profiling their cytokine secretion and by activation markers like CD69 using flow cytometry.

The results from this thesis indicate PI16+ memory Th cells are less responsive to suppression by CD4+ CD25+ Treg. To determine the response of PI16+ Th cells to PI16+ Treg, a suppression assay determining the division index of PI16+ Th cells when co-cultured with PI16+ Treg cells could be performed similar to the assay described in Chapter 2.

Further clinical studies can be performed to determine the functional role of PI16+ Th cells in disease. Cytokine profile and activation status of these cells could be determined. Based on the results from this thesis, future studies could be focused on rheumatoid arthritis and scleroderma. To determine the activation status of PI16+ cells, fresh peripheral blood, synovial fluid and skin
biopsies obtained from patients can be stained with Ki-67 along with CD4, CD25, CD45RA and PI16. Cells positive for Ki-67 will indicate active proliferation status \textit{in-vivo} suggesting their dominant role in the disease. In addition cell can be stimulated \textit{in-vitro} using Phorbol 12-myristate-13-acetate and Ionomycin for 4-6 hours to profile their cytokine secretion in comparison with healthy controls.

To further determine if PI16+ Th cells play a pathogenic or protective role in rheumatoid arthritis and scleroderma, patients could be enrolled for the study at the time of diagnosis. Blood samples and if possible, synovial fluid or skin biopsies can be obtained at the time of diagnosis and during follow up visits which presumably will be post treatment. The change in proportion of PI16+ Th cells in blood and other tissue samples, if applicable, can be determined and correlated with the disease status at various time points.

In order to understand whether the increase in CD45RA-expressing PI16+ Th cells in disease are peripherally expanded cells, peripheral blood drawn from patients can be stained with CD31 along with CD4, CD25, CD45RA and PI16. Cells positive for CD31 and CD45RA will indicate recent thymic emigrants and cells that are CD45RA+ but CD31- will indicate peripheral expanded cells. This could be further confirmed by TREC expression. CD45RA+PI16+ Th cells can be sorted to determine TREC expression using RT-qPCR. Higher expression of TREC will indicate recent thymic emigrant and lower expression will indicate peripheral expansion. However, there might be technical difficulties in obtaining
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enough clinical samples for RT-qPCR analysis. PicoPure® RNA isolation kit (Life technologies) could be used for RNA isolation, which could isolate total RNA from fewer than 10 cells.

Although, this project has been focused only on human samples, mouse models may help in answering several important questions including origin and fate of PI16 expressing cells. Although the mouse models do not necessarily translate well in humans, PI16 knock out mice may reveal the significance of PI16 in T cells. However, expression of PI16 in mouse has not yet been studied although the gene homolog is detectable on mouse CD4 cells.

6.8 Conclusions

This PhD project aimed to perform an extensive analysis of the CD4+ CD25- PI16+ Th subset. One of the underlying questions this thesis aimed to answer is whether PI16+ Th cells are a functionally distinct subset or whether PI16 is a biomarker for any of the T helper subsets. Based on the results from this study (Figure 6-1), it can be concluded that the PI16+ Th subset might not uniquely resemble an already described Th subset but have distinct phenotypical and functional characteristics in comparison with PI16- Th cells. It can be argued that dissecting a heterogeneous T cell population, into two, based on any single surface marker, may in theory yield phenotypically if not functionally two different populations. The resulting populations may still be heterogeneous by
other criteria. The use of chemokine receptor profiling strategy clearly suggest this is the case for PI16+ and PI16- cells.

Figure 6-1: PI16+ Th in the existing T helper paradigm
Model suggesting the generation of PI16+ memory T helper cells from the currently known T helper subsets. Figure shows the signature biomarkers of Th1, Th2, Th17, Thf, Th22 and Th9 subsets and the characteristics of PI16+ Th cells and the diseases they are implicated in from this study.
One of the rationales behind this thesis, driving the need for new tools to subset T cells, is to identify the functionally aberrant T cell subsets that cause autoimmune and/or inflammatory diseases. Therefore it is essential to determine the function of T cell subsets not just in diseases but also in healthy controls. Biomarkers identifying these subsets act as vital targets for biologic drugs and/or could potentially be used for monitoring disease activity. The current study on PI16+ Th cells provides promising results in this direction. The in-vitro studies indicate PI16+ cells may represent long-term memory cells with capacity to exert potent effector functions and in contrast, PI16- Th cells represent a mixture of naïve and memory cells with decreased effector functions. Furthermore, preliminary clinical studies also indicate a potential role of PI16+ subset in disease. Therefore if PI16+ cells exhibit a confirmed pathogenic role in a particular disease, PI16 can be used as a target molecule for drug delivery systems. However if PI16+ cells exhibit a protective role in an infection/disease, PI16 can be used as a biomarker for isolation of cells for cell therapy. In conclusion, PI16 expressing T helper cells represent a distinct phenotype with functional implication in disease. Further functional and clinical studies may reveal the full characteristics of this subset and may provide future directions in using PI16 as a potential biomarker in therapeutics/targeted therapy.
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