GENETIC VARIANTS IN THE IL-2 PATHWAY DISRUPT THE IMMUNE BALANCE BETWEEN REGULATORY T CELLS AND TH17 CELLS IN HUMAN TYPE 1 DIABETES

by

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY in THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES (Pathology and Laboratory Medicine)

THE UNIVERSITY OF BRITISH COLUMBIA (Vancouver)

September 2014

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Abstract

Type 1 diabetes (T1D) is an autoimmune disease resulting from the destruction of insulin-producing β cells by autoreactive lymphocytes. CD4+FOXP3+ T regulatory cells (Tregs) are essential for immune tolerance, and murine studies suggest that their dysfunction can lead to T1D. Tregs require the cytokine interleukin-2 (IL-2) for maintenance of their suppressive function, and polymorphic variants in IL-2/IL-2R pathway genes are associated with T1D. Tregs can display plasticity by converting into Th17 cells, and intermediate FOXP3+IL-17+ cells have been identified. We hypothesized that pancreatic β cell destruction in T1D is driven by conversion of autoreactive Treg cells into a Th17 phenotype due to defective Treg IL-2 signaling in T1D subjects, who have polymorphic variants in the IL2RA gene.

We assessed by flow cytometry the proportion of Treg and Th17 subsets in peripheral blood mononuclear cells from T1D subjects. The subjects were genotyped to determine whether they had the T1D-associated IL2RArs3118470 CC risk haplotype. Samples from T1D subjects were also obtained before the onset of diagnosis.

We found that Tregs are potentially transitioning towards a Th17 phenotype in recent-onset T1D subjects as they have an elevated proportion of FOXP3+IL-17+ cells and Th17 cells in their peripheral blood. We went on to show that T1D subjects with the T1D-associated IL2RArs3118470 CC risk haplotype have Treg cells with IL-2 signaling deficits and an increase in the proportion of IL-17+FOXP3+ cells in their peripheral blood at diagnosis. We did not find changes in the overall proportions of Tregs and Th17 cells in T1D subjects sampled before the onset of diagnosis. However, we observed a subset of CD39-expressing Treg cells were reduced in proportion before disease onset and could act as a biomarker of T1D.
In conclusion, we show that defective IL-17-secreting Tregs are involved with T1D pathogenesis in a genetically identifiable subset of subjects, and provide a rationale for the treatment of T1D with therapeutics that target the IL-17 pathway.
Preface

All collection of human samples used in the study was approved by the Clinical Research Ethics Board of the University of British Columbia (H11-01834; H05-70019; H03-70046 and H07-01707).

A.K. Marwaha conceived all the research questions addressed in these studies and designed the experiments with guidance from R. Tan. A.K. Marwaha conducted all the experimental work and data analysis described herein, with technical assistance as follows (1). Peripheral blood mononuclear cells were isolated from all peripheral blood human samples by H. Qin (2). In Chapter 2, CD127loCD25+ staining was carried out by Q. Ouyang (2). In Chapter 2, Treg suppression assays were partially carried out in collaboration with K Berg (3). In Chapter 3, SNP genotyping was conducted by S. Staiger (an undergraduate summer student under the supervision of A.K. Marwaha), K Del Bel and A.Hirschfield (4). In Chapter 4, phosphoflow was carried out by S. Staiger under the direct supervision of A.K. Marwaha.

Some material in this thesis has been previously published. Data from Chapter 2 has been published in Marwaha et al (2010) J Immunol 185: 3814-3818. The manuscript was written by A.K. Marwaha, with editing from R. Tan and M.K. Levings. Parts of the introduction (Chapter 1) have been published in two reviews. Marwaha et al (2012) Front Immunol 3:129 was written and researched by A.K. Marwaha with editing from the other co-authors N. Leung and M.K. Levings. Marwaha et al (2014) Clinical Immunology 154 (1): 84-89 was written and researched by A.K. Marwaha with editing from the other co-authors S. Tan and J. Dutz.
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<th>Description</th>
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<tbody>
<tr>
<td>α</td>
<td>alpha</td>
</tr>
<tr>
<td>β</td>
<td>Beta</td>
</tr>
<tr>
<td>HbA1c</td>
<td>glycated hemoglobin</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine 5’- Monophosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine 5’- Triphosphate</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CDA</td>
<td>Canadian Diabetes Association</td>
</tr>
<tr>
<td>CpG</td>
<td>Cytosine – phosphate - Guanine</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic Lymphocyte</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>cytotoxic T-lymphocyte associated antigen 4</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EAE</td>
<td>Experimental autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FOXP3</td>
<td>Forkhead box P3</td>
</tr>
<tr>
<td>γ</td>
<td>gamma</td>
</tr>
<tr>
<td>GADA</td>
<td>Glutamic Acid Decarboxylase Antibody</td>
</tr>
<tr>
<td>GITR</td>
<td>Glucocorticoid-induced TNFR family related gene</td>
</tr>
<tr>
<td>GWAS</td>
<td>Genome wide association study</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency Virus</td>
</tr>
<tr>
<td>HLA</td>
<td>Human Leukocyte Antigen</td>
</tr>
<tr>
<td>IAA</td>
<td>insulin autoantibodies</td>
</tr>
<tr>
<td>IA2A</td>
<td>insulinoma-associated autoantigen 2</td>
</tr>
<tr>
<td>IDO</td>
<td>indoleamine 2,3-dioxygenase</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>iNKT</td>
<td>invariant natural killer cell</td>
</tr>
<tr>
<td>IPEX</td>
<td>Immune dysregulation, polyendocrinopathy, enteropathy, X-linked</td>
</tr>
<tr>
<td>iTreg</td>
<td>induced T regulatory cell</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean fluorescence intensity</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>MS</td>
<td>Multiple Sclerosis</td>
</tr>
<tr>
<td>NIH</td>
<td>National Institutes of Health</td>
</tr>
<tr>
<td>NK cell</td>
<td>Natural Killer cell</td>
</tr>
<tr>
<td>NOD</td>
<td>Non-obese diabetic</td>
</tr>
<tr>
<td>nPOD</td>
<td>Network for Pancreatic Organ Donors with Diabetes</td>
</tr>
<tr>
<td>nTreg</td>
<td>natural T regulatory cell</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral Blood mononuclear cells</td>
</tr>
<tr>
<td>PDL1</td>
<td>programmed cell death ligand</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol myristate acetate</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid Arthritis</td>
</tr>
<tr>
<td>ROR</td>
<td>retinoid orphan receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal Transducer and Activator of Transcription</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>T1D</td>
<td>Type 1 diabetes</td>
</tr>
<tr>
<td>Tbet</td>
<td>T-box transcription factor 21</td>
</tr>
<tr>
<td>Tc17</td>
<td>IL-17 secreting Cytotoxic T cell</td>
</tr>
<tr>
<td>Tconv</td>
<td>T conventional cell</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>Teff</td>
<td>T effector cell</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor-beta</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>Th1</td>
<td>IFN-γ secreting helper T cell</td>
</tr>
<tr>
<td>Th17</td>
<td>IL-17 secreting helper T cell</td>
</tr>
<tr>
<td>Treg</td>
<td>regulatory T cell</td>
</tr>
<tr>
<td>TSDR</td>
<td>Treg-specific demethylated region</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative Polymerase Chain Reaction</td>
</tr>
<tr>
<td>ZnT8A</td>
<td>Zinc transporter 8 autoantibody</td>
</tr>
</tbody>
</table>
Acknowledgements

I would like to thank all the agencies that have provided funding for this work. The Juvenile Diabetes Research Foundation (JDRF) provided operating grants for the majority of this work. I am grateful for scholarship funding provided by the Child and Family Research Institute and the Michael Smith Foundation for Health Research. I am particularly indebted to University College at the University of Oxford, who appointed me a Radcliffe Traveling Fellow for two years during the course of my studies.

I would like to thank my excellent mentor and supervisor, Rusung Tan, for providing continuous support and guidance during my time as a doctoral student. You have taught me how to stand on my own two feet as an independent researcher, who I hope, emulate your scientific acumen and prolific success in the future. I have truly appreciated your advice over the years, both professionally and personally.

I would also like to thank my supervisory committee: Jaki Chantler, Marc Horwitz, Megan Levings and Bruce Verchere. They have been extremely supportive over the course of my degree and have provided many excellent suggestions that have enabled me to complete my research.

We have been fortunate to develop several productive collaborations with other laboratories. I would like to acknowledge Stuart Turvey, Kate del Bel, Aaron Hirschfield, Sarah Chrome, Peter van den Elzen and Jan Dutz at the Child & Family Research Institute, The University of British Columbia. I would also like to thank Jane Buckner, our collaborator at the Benaroya Institute.
I have been grateful for the opportunity to mentor many intelligent and successful students, who have contributed to this work. I would like to thank Sara Tan, Spencer Staiger, Sze-Wing Wong, Catherine Biggs and Nicole Leung for all their efforts over the years. Research can’t happen without a collective laboratory effort, and so I would also like to thank other members of Rusung Tan’s laboratory for all their help and support over the years: John Priatel, Xiaoxia Wang, Huilian Qin, Lisa Xu, Rosemary Delnavine, Yu-Hsuan Huang and Brian Chung.

I would like to thank all my family in the UK and my chosen family in Vancouver for helping to provide me with the support needed to undertake this work. Finally, and in particular, I offer my utmost gratitude to my parents, Shakti Marwaha and Aruna Marwaha, for enabling me to have the very best opportunities in life with constant love and support. I hope I have made you proud.
Chapter 1: Introduction

The immune system has evolved to be one of the most complex and sophisticated processes within the human body. It displays a sentient-like ability to detect a pathogenic invader and mount an efficient targeted immune response, which can be moderated and remembered once the danger has passed. However, occasionally the immune system can mistakenly respond to stimulation from a self-antigen despite the series of fail-safes that exist to prevent such an occurrence. Autoimmunity now represents a considerable life-time burden of human disease. Unraveling the mechanisms underlying autoimmunity will continue to yield value by providing new therapeutic targets in autoimmune diseases such as T1D.

1.1 The balance between T regulatory cells and Th17 cells in the immune system

1.1.1 T helper subsets in the context of the immune system

The immune system is most easily thought of as a series of major divisions such as the innate and adaptive response arms. The innate immune system consists of many different cell types such as macrophages, natural killer (NK) cells and NKT cells. They work together to provide a quick first response to pathogenic invasion and are activated by local inflammatory signals and foreign pattern recognition. The adaptive immune system response requires the detection of a specific antigen presented by antigen presenting cells (dendritic cells and macrophages). There are two major groups of adaptive immune system cells. B cells produce an antibody-mediated humoral immune response activated by whole antigens. T cells invoke a cell-mediated immune response and are restricted to stimulation by peptide antigens presented in conjunction with a major histocompatibility complex protein (MHC). A further sub-division
specifies two major arms of the T cell response. Cytotoxic T cells (CTL) are restricted to peptide antigen recognition with MHCI and function to kill target cells. T helper cells are restricted to peptide antigen recognition with MHCII and function in multiple ways to help guide the immune response.

The naïve T helper cells, after activation via stimulation of their T cell receptor (TCR) and co-stimulatory molecules, can differentiate into multiple lineages, which each have their own cytokine secretion profile and function. The diversity of T helper function is continuously being expanded as new T helper cell subsets are identified. Their differentiation is controlled by different cytokines, which signal through distinct Signal Transducer and Activator of Transcription proteins (STATs) to switch on various master regulator transcription factors of the different T helper cell lineages. The first T helper cell lineages to be identified were named Th1 and Th2. Th1 cells are differentiated by the actions of interleukin(IL)-12 and interferon(IFN)-γ and signal predominately via STAT1 to switch on Tbet activity. They secrete IFN-γ and LT-α and promote a cell-mediated immune response. Th2 cells are differentiated by the actions of IL-4 and signal predominantly by STAT6 to switch on GATA3. They secrete IL-4, IL-13, IL-5 and IL-9 and have a role in allergy. Subsequently, many more T helper cell lineages have been identified.

Originally, it was thought that T helper lineages were terminally differentiated. However, it has now become well established that there is a significant amount of plasticity between the T helper cell subsets [1]. This has led to a change in thinking about how T helper cells might guide the immune response. Once recruited to a tissue, T helper cells can modulate their function and phenotype depending on the local inflammatory milieu guided by ‘crosstalk’ signaling from the innate immune system. A delicate balance between pro-inflammatory and suppressive cell T
helper subsets leads to the correct immune response to any antigen that is presented. This thesis focuses on the disturbance of the delicate balance between two types of more recently identified T helper subsets, regulatory T cells and Th17 cells, in the context of autoimmunity. A large amount of plasticity is known to exist between these two subsets, even though their function lies at opposite ends of the inflammatory response spectrum [2]. The function, balance and plasticity of regulatory T cells (Tregs) and Th17 cells is described in more detail below.

1.1.2 Regulatory T cells and their subsets

Regulatory T cells form 5-10% of circulating T helper cells in humans [3]. They have suppressive abilities, which act to balance the T effector cell pro-inflammatory function, in both acute and chronic inflammation [4-7]. They are able to suppress activity in either the innate or adaptive arms of the immune system [4]. Depletion of Tregs in adult mice results in autoimmunity [5, 8], and Tregs can also halt allograft rejection, inflammation and autoimmunity in vivo if they are adoptively transferred into mice [9-11] [12, 13]. Therefore, they also act to provide peripheral tolerance toward self antigen recognition[4].

The exact mechanism of Treg suppression is still being studied, but it is likely that they influence other cells in a contact-dependent manner in multiple ways [7]. Once activated, they do not require restimulation of their TCR to mediate suppression [7]. They can also spread their regulatory influence to other cells in a concept called ‘infectious tolerance’[14]. Many mechanisms of suppression have been shown in mice, but not all translate to humans. These include cytokine secretion (e.g. IL-10, IL-35 and TGF-β)[4, 15]; inhibition of cytokine secretion by other cells (e.g. IFN-γ secretion by CD8+ T cells) [16]; IL-2 consumption and inhibition of its
transcription in T effector cells [17, 18]; cytolysis via granzyme and perforin [19]; and inhibition of dendritic cell maturation and function [20-24].

Developing good markers to identify Tregs has been problematic. CD25/IL-2Rα is constitutively expressed in high levels on the surface of Tregs. This was the original marker used to identify Tregs when they were first discovered [5]. However, CD25 is upregulated on multiple cell types that respond to IL-2, and the identification of Tregs with this marker requires an arbitrary cutoff to determine a CD25hi state [25]. In the scurvy mouse, mutations in Forkhead box protein P3 (FOXP3) result in a fatal lymphoproliferative disorder due to a lack of Tregs [8]. In humans a similar immunodeficiency, IPEX syndrome (immune dysregulation, polyendocrinopathy, enteropathy, X-linked), also results from mutations in the FOXP3 gene, and the resulting Treg dysfunction results in overwhelming autoimmunity [26]. Therefore, FOXP3 was identified as a lineage defining transcription factor for Tregs. However, FOXP3 is also transiently expressed by activated Teff cells and not all FOXP3+ cells in humans are suppressive Tregs [27]. In fact, many of the proposed Treg ‘markers’ (CTLA-4, GITR, CD25 and FOXP3) are all expressed on conventional T cells upon their activation [27]. Bluestone et al identified a lack of surface expression of the IL-7R (CD127) on Tregs [28, 29]. CD127 in combination with CD25 easily identifies a CD127loCD25hi population without the need for intracellular staining and this population correlates with FOXP3+ status [28, 29]. This has been the main method of identifying Tregs in therapeutic applications [30]. However, the number of FOXP3+ cells and CD127loCD25hi cells are not equivalent in peripheral blood and the latter would exclude some Tregs if used in enumeration studies. The lack of validated human markers to identify suppressive Tregs was partially solved by the identification of CpG residues in a region in the FOXP3 promoter, called the TSDR (Treg cell-specific demethylated region), that seemed to be
demethylated in true suppressive Tregs, Epigenetic analysis based on qPCR methods can be used to measure the ratio of the TSDR versus CD3 cells, determining the ratio of natural Tregs in a sorted T cell population [31].

A number of subsets of Tregs have been identified, and it is clear that FOXP3+ suppressive cells do not form one large homogenous population. For example, there are natural (n)Tregs that originate from the thymus and induced (i)Tregs that develop from naïve T cells in the periphery[32, 33]. In the thymus, upon strong self-Ag/MHCII presentation to the T cell receptor (TCR) and CD28 co-stimulation, normal T effector cells are deleted to preserve tolerance [34]. However, this strong self-antigen recognition in the thymus can also give rise to natural Treg (nTregs) [35]. Comparisons on iTreg suppressive capability and TSDR demethylation status are hard to perform, because of the lack of specific markers for iTregs. Helios has been proposed as a potential marker of natural versus induced Tregs [36] but it has subsequently been shown to be present upon the activation and proliferation of conventional non-suppressive T cells[37], which brought into question its reliability.

In addition to the iTregs and nTregs, other FOXP3+ subsets have different functions that have been identified. Miyara et al showed that the memory marker CD45RA could be used in conjunction with FOXP3 or CD25 to delineate three subsets of cells [38]. The CD45RA+FOXP3+CD25+ naïve Tregs were suppressive in vitro and were analogous to the nTreg population previously described [38]. The CD45RA+FOXP3+CD25+ subset, after antigen stimulation, loses expression of CD45RA and upregulates expression levels of FOXP3 and CD25 to convert to a more highly suppressive CD45RA-FOXP3hiCD25hi subset [38]. Miyara et al also identified a third subset of CD45RA-FOXP3loCD25lo cells that lacked suppressive ability and secreted pro-inflammatory cytokines such as IL-17, IFN-γ and IL-2 [38].
The expression of CD39 (ENTPD1) defines another subset of Tregs that has increased suppressive capabilities and has been implicated in HIV, cancer and autoimmunity [39]. Tregs from CD39-deficient mice also show diminished suppressive capability in vivo and in vitro[40] and purified CD39+ Tregs have an anergic state and immunoregulatory function in vitro [40-42]. CD39 is an enzyme that degrades ATP to ADP and then AMP and is often co-expressed with CD73, whose role is to degrade AMP to adenosine. ATP causes activation of monocytes, lymphocytes and endothelial cells; helps promote the secretion of IL-1; and induces the migration/differentiation of dendritic cells [43-46]. CD39+ Tregs exert their highly suppressive effect by degrading extracellular ATP, which is released during cell damage [39]. Tregs are also highly sensitive to ATP induced cell death, which can be overcome by their surface expression of CD39 [41].

1.1.3 Th17 cells and their role in autoimmunity

Th17 cells were identified in 2005 [47-49], and, as for other CD4+ T cell lineages, their development is controlled by a combination of cytokines that initiates a program of transcription factor expression and epigenetic re-modeling [50]. In humans, the cytokines that instruct Th17 cell lineage development likely include IL-6, IL-21, IL-23, and IL-1β [51-55], with a potential synergistic role for TGF-β [56-58] via its ability to suppress Th1 cell lineage commitment [59]. Cytokine-driven activation of the signal transducer and activator of transcription (STAT) 3 pathway is an essential step in Th17 cell differentiation [60-62], ultimately leading to expression of their lineage-defining transcription factor: retinoid orphan receptor (ROR)C2 in humans or RORγt in mice [51, 55, 57, 63, 64]. Although the IL-17 cytokine family includes six members
Th17 cells are thought to only produce IL-17A and IL-17F, which are 55% identical [65]. IL-17A can combine with IL-17F to form a heterodimer and both can form homodimers [66].

Th17 cells have many phenotypic characteristics that distinguish them from other Th cell lineages. In addition to IL-17A and IL-17F, Th17 cells secrete other signature cytokines including IL-21 and IL-22 [67]. They have also been reported to produce IFN-γ [68], IL-4 [69], IL-10 [70], IL-9 [71], IL-26, CXCL8 and CCL20 [72]. They are poor producers of IL-2, which may result in their poor proliferative potential in vitro [73]. They constitutively co-express CCR4 and CCR6, but not CXCR3 [74], and are derived from CD161+ precursors [75]. Th17 cells are not the sole producers of IL-17A. Other possible sources of this cytokine include γδ T cells [76], mast cells [77], neutrophils [77] and Tregs [78]) and so it is possible that in certain disease processed Th17 cells are not the major source of IL-17A.

Th17 cells have diverse effects on multiple cell types in different diseases. However, the precise mechanism of their action is still being elucidated [79-85]. IL-17 induces inflammatory cytokines, chemokines, growth factors and matrix metalloproteinases in various cell types in target tissues [86]. This results in neutrophil recruitment and promotes the survival and activation of B cells [86]. IL-17 also acts on epithelial cells of peripheral tissues to promote release of defensins, which have antimicrobial activities and protect the host against infection [86].

The first recognition of the importance of Th17 cells in autoimmunity came from studies of EAE (experimental autoimmune encephalomyelitis). The notion of EAE as a Th1 mediated disease was challenged when it was found that only mice deficient in the p40 subunit of IL-12 were resistant to EAE, whereas mice deficient in the p35 subunit were actually more susceptible to disease [87]. Cua et al solved this paradox by using genetically-deficient mice to show that IL-23p19 and IL-12p40, but not IL-12p35, were essential for EAE development [88]. IL-23, which
shares the IL-12p40 subunit with IL-12, was subsequently found to stabilize Th17 cells and these
cells were found to be the main contributing factor in EAE [49]. Subsequently, a further
correlation between Th17 cells and human autoimmunity was sought.

A large body of evidence now shows a pro-inflammatory role of Th17 in autoimmunity,
and this axis has been successfully targeted therapeutically in psoriasis. Initially, increased levels
of IFN-γ, TNF-α, and IL-12 in the serum and lesions of psoriasis subjects labeled this as a Th1-
mediated disease [89]. However, RORC, IL-1β, IL-6 and IL-23 are also increased in psoriatic
skin lesions [89] leading to the possibility that Th17 and Th1 act in synergy to produce psoriatic
inflammation. Several monoclonal antibodies targeting the p40 subunit shared by IL-12 and IL-
23 (Ustekinumab) have been approved for clinical use in psoriasis. Targeting IL-17 alone with
Secukinumab (AIN457) or Ixekizumab, both fully human neutralizing antibodies to IL-17A, is
also effective in psoriasis [90, 91], confirming that this is likely a major pathogenic cytokine in
this skin disease. Another autoimmune disease with strong links to Th17 cells is RA, a chronic
disease that leads to joint destruction. Direct clinical evidence for the role of IL-17 in RA comes
from recent clinical trials which found that Secukinumab and another anti-IL-17A therapeutic
known as LY2439821 significantly benefit these subjects [92, 93]

Th17 cells therefore initially developed a reputation as a destructive element in several
diseases, including multiple sclerosis, rheumatoid arthritis (RA), psoriasis and inflammatory
bowel disease (IBD). However, these original conclusions were over-simplified and in some
diseases Th17 cells clearly have a protective role. Th17-derived cytokines exert a protective
functional role in the intestine by two mechanisms. First, IL-17A improves barrier function by
strengthening tight junctions after inducing claudin and mucin expression [94, 95]. Second, IL-
22 improves barrier function by inducing epithelial cell proliferation [96] and enhancing goblet
cell restoration and mucus production [97]. Also, a novel suppressive Th17 subset dubbed regulatory Th17 (rTh17) cells has been described. When Esplugeues et al used a CD3-antibody strategy to induce mucosal tolerance, Th17 cells were recruited to the gut but then re-programmed into suppressive, FOXP3-negative, rTh17 cells [98]. The function of rTh17 cells depends on IL-10, TGF-β and CTLA-4, and does not occur in CCR6-deficient mice where Th17 are not recruited to the gut. The latter data indicate that the mucosal immunity microenvironment is critical for the development of rTh17 cells. Therefore, Th17 cells seem to have a mostly pathogenic role in the context of autoimmunity but there are definitely subsets of Th17 cells that are more protective in specific autoimmune diseases such as IBD.

1.1.4 Th17 plasticity

T helper cell subsets were original thought to consist of a small number of easily defined permanently differentiated T helper lineages. As more new lineages were identified, it became apparent that the fate of a naïve T helper cell could vary immensely according to the local cytokine inflammatory milieu guiding its differentiation. It was hypothesized that the local immune response depended on a finely tuned balance between multiple permanently differentiated regulatory and pro-inflammatory T helper cell subsets.

However, this model of balance between terminally differentiated cells was complicated by the fact that intermediate Th subsets sharing characteristics of different Th lineages were being described. Th17 can co-secrete IFN-γ [68, 99-101] or co-express FOXP3 [38, 102-106], indicating the existence of multiple intermediate subsets of Th17 cells with functional specialization. Master regulators of T helper cell differentiation (Tbet for Th1, RORγT for Th17 and FOXP3 for Treg cells) were co-expressed in these intermediate cell phenotypes [105, 107-
The precise nature and function of these intermediate cells is still being studied. However, the function of the intermediate FOXP3+IL-17+ cell phenotype was of particular interest, since they seemed to represent a merger between the highly pro-inflammatory and suppressive T helper lineages [38, 102-104, 110, 111].

A number of hypotheses have been suggested to account for the origins of FOXP3+IL-17+ intermediate cells. They could simply be a separate distinct lineage derived from naïve T helper cells that share Th17 and Treg characteristics. Alternatively, they could represent activated Th17 effector cells that have transiently upregulated the expression of FOXP3 but do not maintain a regulatory T cell suppressive function. Finally, they could be Tregs that have been transitioning to a more pro-inflammatory Th17 cell type at the site of inflammation. This transition process may be an effort to express transcription regulators to mimic the phenotype of the T effector cells that Tregs are trying to regulate. This would endow Tregs with finely tuned homing, survival or functional properties. For example, Tregs exhibiting plasticity could express chemokine receptors that would allow them to be guided toward sites of tissue inflammation with Teff cells. However, in some circumstances plasticity could allow secretion of pro-inflammatory effector cytokines by Tregs, which would be a method of inhibiting peripheral tolerance when faced with a genuine pathogenic threat. In autoimmunity this transition from a Treg phenotype toward a T effector phenotype could be driving disease. This hypothesis is supported by the fact that it is well established, in vivo and in vitro, that Tregs can transition to Th17 in mice and human autoimmunity [2].

Plasticity has also been described between the Th17 and Th1 lineages. Cells have been identified that co-express Tbet and RORγt and therefore secrete both IL-17 and IFN-γ. These IFN-γ +IL-17+ cells have a role in the pathogenesis of autoimmune disease [107, 108]. There is
now evidence that Th17 cells up-regulate IFN-γ and also downregulate IL-17 in response to IL-12 or IL-23 in the absence of TGF-β in vitro[68, 100]. Therefore, Th17 cells actively transition towards a Th1 phenotype and these ex-Th17 IFN-γ+ T helper cells (identifiable by CD161 surface expression) [109] are pathogenic in certain autoimmune diseases. For example IFN-γ +IL-17+ cells can be isolated from the inflamed joint in children with arthritis [109]. In T1D, on adoptive transfer of Th17 from the BDC2.5 non-obese diabetic (NOD) mice into NOD/SCID recipients, there is a transition to a Th1 phenotype[112, 113]. Th1 and Th17 cells often work in synergy with each other; transition between these two pro-inflammatory lineages would allow secretion of either IFN-γ or IL-17 and contribute actively to the autoimmune response [2].

The high degree of plasticity of Th17 cells is another reflection of their dual pathogenic and protective role in autoimmune disease. This is summarized in the Figure 1.1 below.
Figure 1.1 Plasticity leading to protective and pathogenic functions of Th17 cells

Depending on the local cytokine environment, different subsets of Th17 cells arise and mediate distinct effector function. In the presence of IL-23, Th17 cells seem to be pro-inflammatory and can either make IFN-γ themselves or work in concert with Th1 cells to drive a positive feedback pathway of tissue damage such as that seen in psoriasis or RA. In Crohn’s disease, Th17 cells can differentiate in the gut into protective or pathogenic Th17 cells. In the presence of TGF-β and IL-10, Th17 are re-programmed into “regulatory” Th17 cells, which seem to protect from intestinal inflammation.

1.1.5 The effect of IL-2 on Treg and Th17 function

Interleukin-2 (IL-2) is a 15.5kDa α-helical bundle cytokine [114], which was first identified 35 years ago as a suspected ‘T cell growth factor’, after being isolated from
supernatants of activated human T cells [115]. It is primarily produced by T helper cells following antigen stimulation but can also be produced to a lesser extent by CTL, NKT cells [116], dendritic cells [117] and mast cells [118].

IL-2 binds to a receptor that is made up of various combinations of three subunits that guide the affinity of the interaction. These three subunits are the IL-2Rα (CD25), IL-2Rβ(CD122) and the common cytokine receptor subunit IL-2Rγ (CD132) [119-121]. The IL-2Rβ (CD122) subunit is shared with the IL-15R [122]. The common γc subunit forms a part of five other cytokine receptors, namely, IL-3, IL-7, IL-9, IL-15 and IL-21 [123, 124]. IL-2Rα (CD25) is constitutively expressed on Treg cells but needs to be upregulated upon activation on other cell types and can also exist in a soluble form [125]. All three subunits together form the IL-2 high affinity receptor; the IL-2Rβ and IL-2Rγ together form an intermediate affinity receptor; and the IL-2Rα subunit alone forms a low affinity receptor [126]. IL-2 signals predominately in Tregs via the JAK 1/3-STAT 5 pathways to target genes encoding FOXP3, FasL and CD25 [125]. Signaling can also occur, mainly in Teff cells, via the P13-K-AKT pathways, which target genes encoding IL-2, CTLA-4, Bel-2 and Cyclins [125]. A third signaling pathway, SHC-RAS-MAPK, is also activated upon IL-2R stimulation [125].

IL-2 can have multiple effects on different cell types. IL-2 stimulates the expansion of conventional T cells and NK cells and upregulates their CD25 surface expression [125, 127]. IL-2 has different effects on T conventional cells depending on the concentration of the cytokine [128]. At very high concentrations IL-2 can induce activation-induced cell death (AICD); in moderate concentrations it induces the T effector phenotype, and in low concentrations it induces a memory phenotype [128].
IL-2 is critical for Treg differentiation in the periphery and maintenance of stable expression of FOXP3 and CD25[129]. If mice are lacking expression of IL-2, IL-2Rα or IL-2Rβ, a lethal autoimmunity develops [123], which can be rescued by the adoptive transfer of regulatory T cells [121, 130, 131]. IL-2 is known to inhibit differentiation of the Th17 lineage. Mice receiving adoptively transferred IL-2<sup>−/−</sup> CD4<sup>+</sup> T cells generate more Th17 cells than mice receiving wild-type cells [132]. IL-17A transcription is inhibited because IL-2-activated STAT5 competes with STAT3 for binding sites in the IL-17A locus [133]. Another proposed mechanism for Th17 inhibition includes the action of IL-2 to induce the Th17 inhibitor Tbx21 and inhibit the IL-6R [134].

The alternate signaling abilities of IL-2 via different affinity receptors on different cell types mean that the local concentration of IL-2 available can produce drastically different effects in the immune system. This makes clinical use of IL-2 as a therapeutic agent highly dose dependent. IL-2 (proleukin) has been used in high doses to treat melanoma but in lower doses has shown efficacy in Graft Versus Host Disease (GVHD) [135]. In general, IL-2 promotes Treg differentiation and stability and inhibits Th17 differentiation and so if given at an appropriately low dose can alter the finely tuned balance between these T helper lineages to help prevent autoimmunity [136].

1.2 Type 1 Diabetes

1.2.1 Overview

T1D arises from an autoimmune mediated self-destruction of pancreatic β cells, whose function is to secrete insulin. The lack of insulin results in hyperglycaemia, which must be treated with exogenous insulin to prevent severe ketoacidosis and death. T1D subjects can
present in this severe ketoacidotic state or with symptoms of polydipsia, polyuria and weight loss. T1D is diagnosed according to Canadian Diabetes Association criteria, which sets minimum blood levels for glucose (11.1mmol/dl) or glycosylation of haemoglobin (6.5%)[137]. Poor control of these glycaemic levels will result in long-term macrovascular and microvascular complications such as cardiovascular disease, retinopathy and renal failure. Treatment currently relies on daily injections of insulin [137] or insulin pumps [138]. Pancreatic β cell islet transplant therapy is reserved for subjects with very severe complications [139], but a recent analysis of its efficacy showed up to 70% of subjects will require insulin due to transplant failure within 2 years [139].

1.2.2 Etiology

The worldwide incidence of T1D is rapidly increasing and current rates would see global incidence double over the next decade if continued [140, 141]. According to the International Diabetes Federation (IDF), 79,100 children under 15 years worldwide currently develop T1D annually, and a total of 497,100 children live with this disease. That the incidence of T1D can be altered according to changes of season [142] has led to the hypothesis that a number of environmental factors may be contributing to T1D disease pathogenesis. These include enteroviral infection [143, 144]; vitamin D levels [145-147]; and breastfeeding duration [148]. However, substantive mechanistic evidence is lacking for these environmental influences in T1D [149]. It is possible that multiple environmental factors correlate to the onset of disease.

The prevalence of T1D also varies depending on geographical location and race [150]. Although the lifetime risk of developing T1D is 1 in 300, this will increase to 1 in 20 if individuals have a first-degree relative with T1D [151]. A significant difference is also observed
in the lifetime risk between monozygotic twins (50%) and dizygotic twins (10%) [152]. This implies that genetic susceptibility plays a major role in developing T1D. Over 40 genetic loci have now been linked to T1D [153], and the most common (accounting for up to 50% of the genetic susceptibility) is the major histocompatibility complex (MHC) class II region on chromosome 6p21 (IDDM1 locus) [154] [155]. The second most common linkage occurs between all the combined single nucleotide polymorphisms (SNPs) in genes involved in the IL-2/IL-2R pathway and T1D [156-158]. In particular, the interleukin-2 receptor α chain (IL2RA) locus on chromosome 10p15 has been frequently confirmed to associate with risk of T1D and autoimmunity [159-162]. Other loci that have been repeatedly associated with disease include the insulin locus (INS) on chromosome 11p15 [163-165]; protein phosphatase-22 (PTPN22) gene on chromosome 1p13 [166-168]; cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) on chromosome 2q31 [169, 170]; and CLEC16A, a C-lectin of undefined function that resides on chromosome 16p13 [159-161]. However, apart from the MHC class II locus, only the insulin VNTR, PTPN22, CTLA4 and IL2RA genetic loci are associated with T1D with odds ratios of greater than 1.1[171].

1.2.3 Pathology

The dogma that there are no functioning β cells and insulin/C-peptide secretion in the pancreata of subjects with T1D after diagnosis has recently been challenged by the work of investigators in Belgium, Finland, and the USA (Network for Pancreatic Organ Donors with Diabetes [nPOD]), who have collected samples from cadaveric donors with T1D-associated autoantibodies [172]. These samples have shown that, although 70% of islets are insulin depleted [173, 174], there are definitely insulin-secreting functional β cells in the pancreas[175, 176].
There is also a great deal of variability between subjects [149, 177], which implies that there are multiple subsets of T1D disease pathogenesis. The spread of the insulitic lesions is in a lobular pattern [178], which has led to the hypothesis that there is a relapsing remitting pattern of disease perhaps initiated by multiple viral infections in early childhood [149].

1.2.4 Immunopathogenesis

There is a growing body of evidence to suggest that the composition of gut microbiota can prime the immune system’s response to β cell self-antigen. Several studies have found differences in the gut microbiota (e.g. higher abundance of *Bacteroides*) of children with T1D and healthy controls but these studies were all performed with fairly small cohorts [179]. In terms of other immune mechanisms underlying T1D, there has always been a measurable autoantibody response that has been predictive of disease, with up to 90% of T1D subjects having at least one autoantibody[180]. The autoantibodies are reactive to insulin (IAA), glutamic acid decarboxylase (GADA), insulinoma-associated autoantigen 2 (IA2A) and zinc transporter 8 (ZnT8A) [181]. Recently it has been shown that subjects with over two autoantibodies have a 70% risk of developing T1D within 10 years [182], which has justified their inclusion in preventative clinical trials to test therapeutics before the onset of T1D. Although the autoantibodies are indicative of an immune response, they are not causative, since there is a predominant innate and adaptive cell-mediated immune response that eventually leads to β cell destruction [149]. The composition of the immune cells that have been reported in the insulitic lesions from the pancreatic islets during an autoimmune T1D response also supports a cell-mediated pathology. CD8+ cytotoxic T cells are the most predominant population within the insulitis lesion, followed by (in declining order): macrophages (CD68+); CD4+ T cells; B
lymphocytes (CD20+); and plasma cells (CD138+)[183]. FOXP3+ Treg cells are rare in this lesion, perhaps reflecting their potential dysfunction in T1D [183].

Studies in animal models, especially the NOD mouse, have identified roles for these and other immune cells in β cell destruction [184]. The interaction of the innate and adaptive immune response in allowing the pathogenesis of T1D to occur has also been partially confirmed in human samples [184]. The initiation of the immune response is likely β cell damage by an unknown environmental trigger such as a viral infection [185, 186]. The dendritic cells engulf self-antigen, transfer it to the pancreatic lymph nodes and prime pathogenic antigen-specific CD4+ and CD8+ T cells [187]. B cells can also act to present β cell antigen to diabetogenic T cells and secrete non-pathogenic autoantibodies [184]. The primed CTL return to the pancreas and recognize self-antigen presented on β cell surface in conjunction with MHCI, enabling the latter to be killed by perforin and granzyme release or the direct actions of cytokines such as IFN-γ [184]. Other innate immune cells may release factors that also contribute toward β cell death. Macrophages may also be involved in promoting activation of the immune response through the secretion of IL-12, which sustains the effector functions of activated diabetogenic T cells and NK cells [188, 189]. NK cells have cytotoxic function via the release of IFN-γ, granzymes and perforin [190, 191]. However, NK cells are rarely found in human insulitic lesions [183] and so this mechanism is likely not the predominant mechanism of β cell death. Macrophages can also contribute directly to β-cell death through the release of TNF, IL-1β and nitric oxide [192-194].

Despite similar exposure to environmental triggers, this autoimmune process does not occur in healthy subjects who do not develop T1D. This is because a number of immunoregulatory mechanisms exist to help prevent the pathogenic process described above.
Tregs can inhibit diabetogenic T cells and innate immune cells through contact dependent mechanisms and the release of inhibitory cytokines such as IL-10 and TGFβ [195-197]. The release of indoleamine 2,3-dioxygenase (IDO) by some dendritic cells would help expand Treg cells and suppress diabetogenic T cells [198, 199]. iNKT cells could also promote the recruitment of tolerogenic subsets of dendritic cells to the site and would inhibit the differentiation of T effector cells during their priming in the pancreatic lymph node [200, 201]. Finally, programmed cell death ligand (PDL1) expression by β-cells and engagement by dendritic cells would further inhibit the action of diabetogenic T cells [184].

A complex mechanism underlies autoimmune pancreatic β cell destruction, which involves many different cells from the innate and adaptive immune system working together. It is possible that multiple underlying pathogenic mechanisms can result in the clinical manifestation of T1D. However, the autoimmune response is likely the result of a disruption in the finely tuned balance of Treg and effector T cells, which both respond to the cytokine IL-

1.2.4.1 IL-2 and T1D

Studies in the NOD mouse model of diabetes and in humans with T1D have identified several IL-2/IL-2R pathway genes that are associated with disease susceptibility. These include genes encoding IL-2, IL-2Rβ, PTPN2, CTLA-4 and CD25 (IL-2Rα) [202]. CD25 (IL-2Rα) is the only protein in this list that not shared with other cytokine signaling pathways and the IL2RA locus on chromosome 10p15 has been frequently confirmed to associate with risk of T1D [159, 160, 162, 203].

The IL-2 locus in the NOD mouse Idd3 and in humans (4q27) confer susceptibility to disease [204]. In the NOD mouse a protective Idd3.B6 allele will ameliorate the reduced IL-2
production that is observed in this model [205]. The reduced IL-2 secretion in the pancreas of the NOD mouse is linked to decline in Treg function during progression towards diabetes [205-207][208]. The Tregs starved of IL-2 predictably exhibit poor maintenance of FOXP3 protein expression, increased apoptosis and reduced suppressive capabilities [207]. These findings of reduced IL-2 production and Treg dysfunction have been confirmed in humans [209]. Tregs isolated from T1D subjects also have reduced STAT5 phosphorylation in response to IL-2[210]. Exogenous IL-2 treatment can protect NOD mice from developing diabetes; however, this effect is highly dependent on the amount given, the timing of the intervention and whether any co-treatments were administered [207, 211-214]. These murine data are recapitulated in human clinical trials involving IL-2. An initial trial of IL-2 in subjects with T1D involved the use of rapamycin, which has an antiproliferative effect that promotes growth of CD4+CD25+FOXP3+ Tregs and selectively depletes CD4+CD25- T effector cells [215]. Unfortunately, this trial showed a transient decrease in C-peptide levels, perhaps due to an increase in NK cells despite an increase in the number of Tregs and a long-term rescue of their IL-2 signaling dysfunction [135]. However, Klatzman et al have recently completed a trial that used low doses of IL-2 without rapamycin. They concluded that this treatment promotes the expansion of Tregs with few adverse effects and no decline in clinical condition [136]. Therefore, the dose and timing are critical in IL-2 administration in the context of T1D in order to promote the Treg response, while not inducing other effector arms of the immune system that would cause pathogenic side effects in the context of an autoimmune disease.
1.2.4.2 Role of Th17 cells in T1D

The literature shows some controversy over the role of IL-17 in murine models of T1D. In particular, one study suggested that IL-17 mRNA silencing has no effect in the NOD mouse model [216]. However, mRNA silencing does not knock-down cytokine function absolutely; IL-17 knock-out NOD mice demonstrate delayed onset of diabetes and diminished severity of insulitis [217]. In fact, most rodent studies implicate the IL-17 pathway in the pathogenesis of autoimmune diabetes. In diabetes-prone BioBreeding (DP-BB) rats, the Th17 cell population rises in the first months of age, but the proportion and function of T regulatory cells does not change [218]. Furthermore, in a mouse model of low-dose streptozotocin-induced diabetes, administration of IL-23 induced both IL-17 production and development of autoimmune diabetes [219]. In the same model, the frequencies of both Th17 (CD4⁺) and Tc17 (CD8⁺) cells were increased in the pancreatic lymph nodes, and those mice lacking expression of the IL-17 receptor had slower progression of disease [220]. In non-obese diabetic (NOD) mice, spontaneous disease onset is delayed by inhibition of IL-17 through the use of blocking antibodies [221-224]. However, Mathis, Cooke and colleagues showed that, when β cell specific CD4⁺ T cells from TCR-transgenic BDC2.5 NOD mice were polarized to a Th17 phenotype and then transferred to non-diabetic NOD/SCID recipients, the cells accelerated diabetes, but only after differentiating into a Th1-like phenotype [112, 113]. Antigen-specific Tc17 cells that target hemagglutinin on pancreatic β cells induced diabetes only when co-transferred with diabetogenic CD4⁺ T cells that secrete IL-12 (possibly allowing Tc17 conversion to an IFN-γ secreting phenotype) [225]. More recently, a study has confirmed the presence of IL-17⁺ T cell populations in the pre-diabetic stage of diabetes development in the NOD mouse. This study also suggested that antigen-specific
Th17 cells could confer disease when adoptively transferred, without the need for conversion to a Th1 phenotype, if TNF-α were present [226].

IL-17 is strongly implicated in the pathogenesis of T1D in humans. Honkanen et al have shown an up-regulation of Th17 immunity in stimulated peripheral blood mononuclear cells (PBMCs) and in memory T helper cells in children with T1D [227]. Other groups have shown that T1D subjects have a higher proportion of memory CD4+ T cells with the capacity to transition into Th17 cells that also secrete IL-9 [71]. Reports also show that, in addition to peripheral T cells, T1D subjects have an increased proportion of monocytes that secrete Th17 polarizing cytokines [228] and islet antigen-specific Th17 cells [229]. Pancreatic lymph nodes from T1D subjects have an expansion in Th17 cells [230], and islets from recent-onset T1D subjects express IL-17A, RORC (the human, lineage-defining IL-17 transcription factor) and IL-22 [229]. The role of IL-17 in the pathogenesis of T1D could also be mediated by direct cytokine effects on the pancreatic islets. IL-17 is able to, in conjunction with IL-1-β and IFN-γ, upregulate the expression of stress response genes and proinflammatory chemokines in β cells, leading to their apoptosis [227, 229, 231]. The human and animal data together provide a strong case for a potential role of IL-17 immunity in the pathogenesis of human T1D.

1.2.4.3 Role of regulatory T cells in T1D

After the discovery that suppressive Tregs perform critical roles in preventing autoimmunity in animal models and human disease [232], they became a prime candidate for dysfunction in the pathogenesis of T1D. A mutation in Treg-defining transcription factor FOXP3 results in IPEX syndrome, which gives an insight into the effects of absolute Treg dysfunction in humans [233]. Greater than 60% of IPEX subjects present with T1D at or near birth [233], and
so Tregs are essential for preventing the development of autoimmune T1D in a large proportion of individuals.

Treg dysfunction has been shown to be an essential part of the immunopathogenesis in the NOD mouse [207, 234], and Treg transplantation delays disease onset in this model of T1D [235, 236]. Several studies have tested Treg numbers in the peripheral blood of subjects with T1D, but have produced contradicting results. Some show T1D subjects or their relatives have a decrease in the proportion of Tregs [237]; others have shown no change [238-240]; and one study has even shown an increase in the proportion of Tregs in subjects with T1D and autoimmune gastritis [241]. Buckner et al suggested that the dysfunction lies in T1D CD4+ effector cells because they are more resistant to suppression by Tregs in T1D [242].

These contradictory results might be explained by the different extracellular and intracellular markers that have been used to identify Tregs in peripheral blood. Most previous studies rely on identifying Treg cells by the expression of a winged helix transcription factor FOXP3 [232]. FOXP3 was thought to be essential for genetic programming and therefore a master regulator of Tregs in humans and mice [232]. However, several studies have now established that FOXP3 is transiently expressed by cells that do not have a regulatory function. Furthermore, FOXP3 expression in Tregs has been shown to be unstable and loss of expression in so-called ‘ex-FOXP3’ cells can result in murine Treg cells taking on the phenotype of the pro-inflammatory cells they once suppressed. Inconsistencies in previous studies enumerating Tregs in T1D may have arisen, therefore, because they inadvertently included cells that express FOXP3 but do not exhibit the classical Treg phenotype and secrete pro-inflammatory cytokines.

A completed Phase I trial, conducted in Poland, has demonstrated that infusion of autologous Tregs in children with recently diagnosed T1D was safe and tolerable [243]. The
study concluded that this treatment held considerable potential, since some improvement in the β cell function was observed; however, the study was not powered to assess definitively the efficacy of autologous Treg therapy in recent-onset T1D subjects. A recently-published follow-up of the 12 subjects showed no adverse effects, with two of the children in the treatment group remaining insulin-independent after 1 year [30]. Bluestone et al have also initiated a large-scale trial involving infusion of CD4⁺CD127lo/-CD25⁺ polyclonal Tregs (#NCT10210664) into recently diagnosed T1D subjects that has so far proved safe.

1.3 Thesis hypothesis and objectives

1.3.1 Summary of rationale

The autoimmune nature of T1D has long suggested a defect in T cell immune tolerance to β cell antigens. In particular, investigators have searched for evidence in humans that a subset of CD4⁺ (Treg) cells characterized both by expression of the forkhead transcription factor, FOXP3 and by suppressor function are reduced or defective in T1D. In support of a role in T1D pathogenesis, dysfunction of CD4⁺FOXP3⁺ T cells is important for progression of autoimmunity in non-obese diabetic (NOD) mice [207, 234] and the enhancement of Treg function in NOD mice prevents hyperglycemia [235, 236]. As yet, functional studies of Treg cells in humans with T1D have been difficult to achieve and attempts by many groups to enumerate and phenotype human Treg cells have produced inconsistent results [237-239, 244-246]. Therapy using endogenously supplied Tregs from recent-onset T1D subjects, which have been expanded in vitro and returned to the host have provided some efficacy in a small cohort of subjects with T1D [30, 243].
Since their recent discovery in 2005, Th17 cells have been linked to multiple autoimmune diseases, and multiple murine studies indicate IL-17 may be involved in T1D pathogenesis. For example, there is evidence from experiments in the NOD mouse model of human T1D, where administration of IL-17 blocking antibodies prevents development of diabetes [221] and IL-17 knock-out NOD mice demonstrate a delayed onset of diabetes [217].

The well-documented plasticity that exists between Tregs and Th17 cells implies that the appearance of intermediate FOXP3+IL-17+ cells and/or the conversion of dysfunctional Tregs to an ‘exTreg’ IL-17 producing phenotype may be a possible cause of autoimmunity. These improperly differentiated ‘deviant’ Treg cells that have “gone rogue” in secreting pro-inflammatory cytokines such as IL-17 may play a critical role in the pathogenesis of diabetes.

An overwhelming body of GWAS (Genome Wide Association Study) evidence now shows that polymorphic variants in the IL-2/IL-2R pathway are associated with T1D [202]. These findings are supported by the NOD mouse model of diabetes, in which the IL-2 locus Idd3 confers susceptibility to disease [204]. IL-2 seems to be essential for the maintenance of Treg function and Tregs from subjects with T1D have reduced STAT5 signaling in response to exogenous IL-2 stimulation [210]. This Treg signaling deficit can be reversed with the exogenous administration of IL-2 as a therapeutic treatment in T1D [136]. IL-2 has also been found to inhibit the differentiation of Th17 [133].

Although they have all been separately implicated, the exact relationships between T1D-associated polymorphic variants in the IL-2/IL-2R pathway; Treg dysfunction; and Th17 cells, in the pathogenesis of human autoimmune T1D, are still to be determined.
1.3.2 Hypothesis and objectives

We hypothesize that pancreatic β cell destruction in T1D is driven by the conversion of autoreactive Treg cells into a Th17 phenotype due to defective Treg IL-2 signaling in T1D subjects who have polymorphic variants in the *IL2RA* gene.

**Aim 1** (Chapter 2): To determine if subjects with recent-onset T1D have changes in the proportions of Treg and Th17 cells in their peripheral blood.

**Aim 2** (Chapter 3): To determine whether development of Th17 cells from Treg cells is influenced by T1D-associated polymorphic variants in the *IL2RA* gene.

**Aim 3** (Chapter 4): To determine whether changes in Treg or Th17 populations occur before the onset of T1D and so can be used as biomarkers of disease.
Chapter 2: Increased IL-17 secreting T-Cells in children with recent-onset T1D

2.1 Introduction

The autoimmune destruction of pancreatic β cells by self-reactive T cells leads to T1D. Evidence that regulatory CD4+ T cells (Tregs) suppress the activation of autoreactive T cells and maintain self-tolerance [232] has led to the hypothesis that Treg dysfunction is a major factor underlying the development of T1D. The best-characterized Tregs are those that express the FOXP3 transcription factor, but although FOXP3 appears to be an accurate marker of Tregs in mice, it is also expressed by activated non-suppressive T cells in humans [247].

Interleukin (IL)-17 is a pro-inflammatory cytokine secreted by a distinct lineage of CD4+ T helper (Th17) cells. Th17 cells have an established pathogenic role in several autoimmune diseases [248]. In animal studies, the function for Th17 in T1D is supported by the observation that IL-17 is expressed in the pancreas of non-obese diabetic (NOD) mice [224] and that inhibition of IL-17 in this model leads to delayed onset of T1D during the effector phase of the disease [221, 222]. In addition, adoptive transfer of islet-specific Th17 cells into NOD/SCID mice induces diabetes but only after conversion into a Th1-like phenotype [112, 113]. In humans, the frequency of IL-17-secreting CD4+ T cells in lymphocytes from established T1D subjects is increased compared with healthy controls [228] but their relevance in recent-onset T1D has not been shown. Recently, several groups have reported that human IL-17+FOXP3+ cells can be isolated ex vivo and that IL-17–Tregs can transform into IL-17 producing cells [38, 102-104, 110, 111]. Of particular interest, human FOXP3+CD4+ T cells may be divided into
three phenotypically and functionally distinct subpopulations, depending on the expression of FOXP3, CD25 and the human naive cell marker, CD45RA [38]. Whereas naïve Tregs (CD45RA+CD25intFOXP3lo) and memory Tregs (CD45RA-CD25hiFOXP3hi) possess suppressor function, a third group of FOXP3+ memory T cells (CD45RA-CD25intFOXP3lo) are non-suppressive and secrete IL-17 [38].

Earlier studies have reported that T1D subjects have decreased [237] increased [241] or equivalent [238-240] proportions of Tregs compared with controls. Given the recently recognized complexity of FOXP3+ T cell subsets, we hypothesized that inconsistencies in the enumeration of Tregs in T1D may have been the result of inadvertent inclusion of cells that express FOXP3 but do not exhibit the classical Treg phenotype.

Here, we show that, although there is an increase in the overall proportion of FOXP3-expressing CD4+ T cells in new onset T1D subjects compared with controls, the increase is restricted to the CD45RA-CD25intFOXP3lo subset that secretes significantly more IL-17 and is not suppressive compared to other FOXP3+ subsets. In addition, we report that T1D subjects have a globally increased proportion of both CD4+ and CD8+ T cells that secrete IL-17.

2.2 Methods
2.2.1 Subject recruitment

Peripheral blood was obtained from 64 subjects with recent-onset (<6 months from diagnosis) T1D and 53 age-matched controls without T1D attending BC Children's Hospital (Table 2.1). The study protocol was approved by the Clinical Research Ethics Board of the University of British Columbia (Certificate: H07-01707) and all parents or subjects provided informed written consent or assent.
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<tr>
<td>Mean A1C</td>
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**Table 2.1 Characteristics of T1D subject and healthy control groups from the study**

Data are shown as the means ± standard deviation. All T1D subjects were being treated with insulin and did not show evidence of other autoimmune diseases. Controls were age-matched children with no autoimmune, metabolic or other significant endocrine diseases.

### 2.2.2 Flow cytometry

PBMC were isolated on a Ficoll-Hypaque gradient, cryopreserved and later stained with different combinations of antibodies to CD4 (RPA-T4), CD14 (MφP9.1), CD8 (RPA-T8), and CD127 (hIL-7R-M21) (BD Biosciences); to CD45RA (HI100) and CD25 (BC96) (eBioscience). For intracellular staining, cells were treated with fix/perm buffer (eBioscience) and stained with anti-FOXP3 (259/C7, BD) or anti-IL17A (eBio64DEC17). Data were acquired by FACSaria (BD). The unpaired Student’s t-test or 1 way ANOVA test was used for statistical comparisons.
2.2.3 Detection of IL-17 by flow cytometry or ELISA

PBMC (5 x10^5 cells/ml) or sorted CD25+ subsets (2 x10^5 cells/ml) were incubated with human anti-CD3/CD28 Dynabeads (Invitrogen) at a 1:1 ratio for 72 hours, without polarizing cytokines, to optimize the detection of IL-17. PBMC samples were suspended in R10 media with PMA (100 ng/ml), ionomycin (1mg/ml) and Brefeldin A (10 mg/ml) (Sigma) for 5 hours before intracellular staining. CD25+ sorted cell-free supernatants were assessed for IL-17 concentration by ELISA (eBioscience).

2.2.4 Microsuppression assays

To assess suppressive capacity, ex vivo allogeneic CD4+ responder T cells were stimulated at 8,000 cells/well in the presence of anti-CD3 and anti-CD28 beads. Different subsets of CD4+CD25+FOXP3+ T cells (Fractions I, II and III) were added to the responder cells and suppressive capacity was assessed by measuring the amount of [^3H]-thymidine incorporation in the final 16 hours of a 6-day culture.

2.3 Results

2.3.1 TID subjects have an increased proportion of CD4+ T cells that express markers characteristic of Tregs

We determined the proportion of classically defined Tregs in a cohort of recent-onset T1D subjects and age-matched controls without T1D. Two different flow cytometric strategies were used to identify Tregs: one based on the intracellular expression of FOXP3 (Figure 2.1A), and the other based on high surface levels of the IL-2 receptor (CD25) and low IL-7 receptor (CD127) α chain (Figure 2.1B) [29]. T1D subjects exhibited an increased frequency of Treg-like
cells relative to controls when determined either by FOXP3 expression or CD25/CD127 levels respectively (FOXP3+: T1D = 6.2 ± 0.4% vs. Control = 4.7 ± 0.3%, p = 0.0041; CD25+CD127lo/-: T1D = 4.8 ± 0.3% vs. Control = 3.5 ± 0.3%, p = 0.0045).

Figure 2.1 T1D subjects have an increased proportion of CD4+ T cells that express markers characteristic of Treg cells

(A) The proportion of CD4+ T cells that express FOXP3 is increased in T1D subjects compared to healthy controls (T1D: n = 23, Control: n = 23, p = 0.004). (B) The fraction of CD4+ T cells that are CD25+CD127lo is elevated in T1D subjects relative to healthy controls (T1D: n = 14, Control: n =13, p = 0.005). Representative density plots are displayed for T1D and control subjects. Percentage of CD4+CD14- T cells within each gate is shown in the top right corner of density plots.
2.3.2 T1D subjects display an elevated frequency of CD45RA-CD4+ T cells that express low levels of FOXP3

We observed that T1D subjects exhibited an increased frequency of Treg-like cells relative to controls when determined either by FOXP3 expression or CD25/CD127 levels respectively (Figure 2.1A, 2.1B). Human FOXP3+CD4+ T cells may be divided into three functional subsets based on the expression of FOXP3 and CD45RA [38]. Therefore, we assessed the frequency of all FOXP3+ subsets in T1D subjects. (Figure. 2.2A, 2.2B). In T1D subjects, the proportion of CD4+ T cells that was CD45RA-FOXP3lo (Fraction [Fr.] III) was increased 1.5-fold relative to healthy controls (T1D = 3.9 ± 0.3 % vs. Control = 2.6 ± 0.2 %, p = 0.003). In contrast, there was no significant difference in the proportion of CD4+ T cells that were CD45RA+FOXP3lo (Fr. I: T1D = 1.2 ± 0.2 % vs. Control = 1.2 ± %, p = 0.93) or CD45RA-FOXP3hi (Fr. II: T1D = 1.1 ± 0.2 % vs. Control = 0.9 ± 0.1 %, p = 0.37). Thus, in T1D subjects, the increase in CD4+ T cells that express FOXP3 is due solely to an increase in the CD45RA-FOXP3lo subset.
Figure 2.2 T1D subjects display an elevated frequency of CD45RA-CD4+ T cells that express low levels of FOXP3

CD4+ T cells expressing FOXP3 can be subdivided into three distinct subsets based on expression levels of CD45RA and FOXP3: Fr. I, CD45RA+FOXP3lo cells; Fr. II, CD45RA-FOXP3hi cells; Fr. III, CD45RA-FOXP3lo cells. (A) Representative density plots are displayed for T1D and control subjects. Background staining in the FOXP3-detecting channel is shown through presentation of a fluorescence-minus-one (FMO) control, lacking treatment with fluorescently labeled anti-FOXP3 antibody. Percentage of CD4+CD14- T cells within each Fraction is shown in the top right corner of density plots. (B) T1D subjects possess an increased proportion of CD4+ T cells bearing a CD45RA-FOXP3lo (Fr. III) phenotype. (T1D: n = 23, Control: n = 23, p = 0.003).
2.3.3 CD45RA-CD25intFOXP3lo cells from T1D subjects produce more IL-17 than other Treg subsets and are not suppressive

CD45RA-FOXP3lo cells are known to produce substantially more IL-17 than other Treg subsets and are not suppressive [38]. Therefore, we sought to determine whether CD45RA-FOXP3lo cells present in T1D subjects also have enhanced capability to secrete IL-17 and a reduction in suppressive capability. Since cells stained for FOXP3 are not viable, we isolated subsets of FOXP3+ cells on the basis of CD25 and CD45RA expression [38]. This was possible because CD25 levels on T1D CD4+ T cells correlate strongly with FOXP3 expression, allowing the isolation of the three FOXP3+ (Fr. I-III) subsets by sorting for CD45RA+CD25int (Fr. I), CD45RA-CD25hi (Fr. II) and CD45RA-CD25int (Fr. III) cells, respectively (Figure 2.3A).

Sorted CD4+ T cells bearing a CD45RA+CD25int (Fr. I), CD45RA-CD25int (Fr. III), or CD25- (Fr. IV) phenotype, were stimulated with anti-CD3/CD28 beads, and IL-17 secretion was quantified by ELISA (Figure 2.3B). Consistent with previous observations [38], T1D CD45RA-CD25int cells secreted considerably more IL-17 than CD45RA+CD25int (137-fold increase; 548 ± 132 pg/ml vs. 4 ± 0.01 pg/ml; \( p = 0.001 \)) or CD25- cell subsets (6.6-fold increase; 548 ± 132 pg/ml vs. 82 ± 5.5 pg/ml; \( p = 0.0193 \)). IL-17 secretion could not be determined on CD45RA-CD25hi (Fr. II) cells because of insufficient cell numbers after sorting.
Figure 2.3 CD45RA-CD25intFOXP3lo cells from T1D subjects produce more IL-17 than other Treg subsets

(A) The level of FOXP3 expressed by CD4+ T cells is proportional to surface CD25 levels. CD45RA+FOXP3lo, CD45RA-FOXP3hi and CD45RA-FOXP3lo CD4+ T cells can be isolated as live cells by cell sorting CD45RA+CD25int (Fr. I), CD45RA-CD25hi (Fr. II) and CD45RA-CD25int (Fr. III) CD4+ T cells. (B) CD45RA-CD25int (Fr. III) CD4+ T cells produce more IL-17 than CD45RA-CD25int (Fr. I) and CD25- (Fr. IV) CD4+ T cells (Fr. III vs. Fr. I, n = 8, p = 0.001; Fr. III vs. Fr. IV, n = 8, p = 0.02). Purified CD45RA+CD25int (Fr. I), CD45RA-CD25int (Fr. III) and CD25- (Fr. IV) CD4+ T cells were activated with human anti-CD3/CD28 Dynabeads for 72 hrs in vitro, cell-free supernatants were collected and concentrations of IL-17 in pg/ml assessed by ELISA.
To test the suppressive capacity of subsets of CD4+CD25+ T cells, cells were sorted into Fractions I, II and III and tested for their ability to suppress the proliferation of CD4+ T cells. At a 1:8 ratio (putative suppressor:responder), CD45RA+CD25int (Fr. I) cells and CD45RA-CD25hi (Fr. II) cells suppressed CD4+ responder cell proliferation significantly more than CD45RA-CD25int (Fr. III). (67 ± 11% and 51 ± 10% vs 18 ± 9%, n= 5, p = 0.02 and 0.003, Figure 2.4). Thus, CD4+ T cells from T1D subjects contain larger fractions of CD45RA-CD25intFOXP3lo cells that secrete IL-17 and have lost suppressive capability.

Figure 2.4 CD45RA-CD25intFOXP3lo cells from T1D subjects do not have suppressive capability

CD45RA+CD25int (Fr. I) cells and CD45RA-CD25hi (Fr. II) cells suppressed CD4+ responder cell proliferation significantly more than CD45RA-CD25int (Fr. III). Purified CD45RA+CD25int (Fr. I), CD45A-CD25hi (Fr.II) and CD45RA-CD25int (Fr. III) cells were added to CD4+ responder T cells, stimulated at 8,000 cells/well in the presence of anti-CD3 and anti-CD28 beads and suppressive capacity was assessed by measuring the amount of[^3H]-thymidine incorporation in the final 16 hours of a 6 day culture. The data shown are the mean +/- standard error from 5 T1D subjects.
2.3.4 CD4+ and CD8+ T cells from recent-onset T1D subjects are also skewed towards IL-17 secretion

The observation that T1D subjects exhibited an increase in IL-17-secreting FOXP3+CD4+ T cells led us to investigate whether CD4+ and CD8+ T cells were also biased towards IL-17 secretion. PBMC from control and T1D subjects were stimulated with anti-CD3/CD28 beads for three days and re-stimulated with PMA and ionomycin before IL-17 detection by intracellular staining (Figure 2.5A,B). The respective proportions of CD4+ and CD8+ T cells that secrete IL-17 in T1D subjects were 2.6-fold and 3.1-fold greater than controls (CD4: 0.47 ± 0.11 % vs. 0.18 ± 0.03 %, \( p = 0.02 \); CD8: 0.22 ± 0.02 % vs. 0.07 ± 0.03 %, \( p = 0.004 \)). Thus, in recent-onset T1D subjects, both CD4+ and CD8+ T cell populations are biased toward IL-17 secretion.
Figure 2.5 CD4+ and CD8+ T cells from recent-onset T1D subjects are also skewed towards IL-17 secretion (A & B)

The proportion of CD4+ and CD8+ T cells that secrete IL-17 is increased in T1D subjects compared to healthy controls (CD4+: T1D: n = 17, Control: n = 17, p = 0.015; CD8+: T1D: n = 17, Control: n = 17, p = 0.0004). Representative density plots are displayed for T1D and control subjects. Percentages of CD4+ or CD8+ T cells that secrete IL-17 are shown in the top right corner of density plots. PBMC were activated with anti-CD3/CD28 and after three days the capacity of T cells to secrete IL-17 was determined by intracellular staining.
2.4 Discussion

This is the first study of children with T1D to assess whether alterations exist in the proportion of FOXP3+CD4+ cells that have a regulatory versus inflammatory (IL-17) phenotype. We observed that, although T1D subjects have an increased proportion of cells that express FOXP3, the increase is limited to a subset that co-expresses IL-17 and is not suppressive. These results suggest that earlier studies enumerating FOXP3-expressing cells in the context of T1D should be re-evaluated, and our data support the hypothesis that an increase in IL-17-producing T cells underlies the pathogenesis of T1D.

In previous studies, where the data has been inconsistent regarding the proportion of Tregs in T1D subjects [237-241], the differences seen may have been the result of using CD25 alone as a marker of Tregs [238]; the use of a control population older than that of the T1D group [244]; and the use of a less specific FOXP3 antibody clone (PCH101) that precluded greater discrimination of FOXP3hi versus FOXP3lo cells [249]. Given these differences, preceding studies that assessed FOXP3+ T cells in T1D subjects would not easily have identified the CD45RA-FOXP3lo population [38]. Our study is the first to compare Treg cells in a large cohort of recent-onset T1D using a highly specific FOXP3 antibody clone and age-matched controls.

The data observed here suggest that the increased proportion of CD4+ T cells expressing FOXP3 does not represent a true increase in naïve (CD45RA+CD25intFOXP3lo) or memory (CD45RA-CD25hiFOXP3hi) Tregs that are suppressive, but rather, is confined to IL-17-secreting CD45RA-CD25intFOXP3lo cells. These findings recapitulate those of Miyara et al [38], who found that CD45RA-CD25intFOXP3lo cells are non-suppressive and also increased in
systemic lupus erythematosus subjects. Further investigation will be required to determine if this observation extends to other autoimmune diseases.

Several groups have identified T helper cells that, like CD45RA-CD25intFOXP3lo cells, are IL17+FOXP3+ [38, 102, 103, 110, 111]. However, their origin remains unknown. They may be activated CD4+ effector T cells that transiently express FOXP3 [247], or they may derive from Tregs that failed to maintain sufficient levels of FOXP3 expression (‘ex-FOXP3’ cells) and subsequently have converted to a pro-inflammatory IL-17 secreting phenotype [250]. The well-described plasticity of the Treg and Th17 lineages [38, 102-104, 110, 111] suggests that IL17+FOXP3+ T cells may be an intermediate lineage, with a suppressive versus pro-inflammatory role that depends on the local cytokine milieu. A recent study in NOD mice immunized with the tolerogen IgG-GAD1 found that FOXP3+ T cells which express the Th17 lineage transcription factor, RORγt, arise before islet inflammation, and may differentiate in vitro to either Th17 or Tregs [251]. The elevated population of IL-17-secreting CD45RA-FOXP3lo cells described here in humans may be an analogous intermediate cell population that lies between “true” Tregs and Th17 and exhibits plasticity.

The observation that CD4+ and CD8+ T cells from T1D subjects secrete increased levels of IL-17 may be ascribed to the presence of a pro-inflammatory polarizing cytokine milieu. In support of this possibility, Bradshaw et al showed that monocytes from T1D subjects spontaneously secrete pro-inflammatory cytokines, necessary for Th17 cell differentiation and expansion [228]. The skewing toward IL-17 secretion would likely disrupt the delicate balance of islet T cells in favor of autoimmune inflammatory destruction.

This report highlights importance of discriminating different FOXP3-expressing subsets, and the findings demonstrate that multiple T cell subsets are biased toward IL-17 secretion in
T1D. Together, these data imply a potentially important role for IL-17 secretion in the pathogenesis of human autoimmune diabetes and suggest the possibility of therapeutic approaches that target the IL-17 axis in T1D.
Chapter 3: T1D subjects with *IL2RA* risk haplotypes have Tregs with reduced IL-2 signaling capabilities and an increase in FOXP3+IL-17+ cells

3.1 Introduction

The immune response in T1D is guided by the T helper cell population. In particular, a balance occurs between the regulatory T cells (Tregs) and T effector cells such as the IFN-γ-secreting Th1 cells or the IL-17-secreting Th17 cells [252]. Regulatory T cells (Tregs) are crucial in regulating and suppressing the function of T effector cells to maintain cellular homeostasis [253]. This delicate balance is disrupted in the autoimmune pathogenesis of T1D by a defective Treg population and the expansion of a T effector cell population that recognizes pancreatic antigens. Recently, it has been shown that a large amount of plasticity exists in the T helper cell population [254], and so transition of Tregs to the Th17 lineage and vice versa can occur. In Chapter 2, we observed a population of FOXP3+IL-17+ (CD45RA-FOXP3lo) cells which are elevated in proportion in the blood of recent-onset subjects with T1D.

Interleukin-2 was first identified as a generic T cell growth factor, but subsequently was found to be most important in maintaining the function of Tregs, which constitutively express the IL-2Rα subunit CD25 on their surface [208, 255]. IL-2 signals predominantly via a high-affinity heterotrimeric receptor made up of α (CD25), β (CD122) and common γ (CD132) subunits. IL-2 induced proliferation of Tregs is thought to be mediated in part by the intracellular signaling molecule STAT5 [256], whose phosphorylation induces the transcription of FOXP3, CD25 and CD122 [256].
The risk for T1D is clearly influenced by multiple genetic loci, with first-degree relatives of subjects with T1D showing a 15-fold higher risk for developing the disease than the general population. In large-scale Genome Wide Association Studies, several single nucleotide polymorphisms (SNPs) have been linked to both T1D and autoimmunity in general. Several genes that encode proteins in the IL-2/IL-2R signaling cascade are associated with T1D [156-158]. Autoimmune variants have been identified in genes encoding CD25, IL-2 itself and the protein tyrosine phosphatase N2 (PTPN2). CD25 is the only one of those proteins that is specific to the IL-2/IL-2R pathway and not shared with other cytokine signaling cascades. CD25 is encoded by the \textit{IL2RA} locus on chromosome 10p15, which has been frequently associated with risk of T1D and autoimmunity in cohorts of European, Japanese and Chinese subjects [159-162]. Multiple risk or protective \textit{IL2RA} haplotypes have been identified, and all of the associated variants are non-coding, falling upstream of the \textit{IL2RA} gene or intron 1. T1D-associated protective haplotypes that are tagged by single nucleotide polymorphisms (SNPs) in the \textit{IL2RA} locus include rs12722495 (previously rs41295061) (OR =0.62), rs2104286 (OR = 0.80) and rs11594656 (OR = 0.87) [257-259]. Healthy controls with the T1D-associated IL2RArs2104286 or IL2RArs12722495 risk haplotypes have diminished IL-2 responsiveness in Treg cells, as measured by phosphorylation of STAT5 [156, 210]. This difference in Treg signaling upon IL-2 stimulation has not been confirmed in subjects with T1D.

The IL-2RArs3118470 SNP is also a good candidate for potential functional relevance. A haplotype containing this SNP was significantly protective (P=3.2 x 10(-5)) in T1D affected sib-pair families [260] and this observation was replicated in a Japanese [261] and Polish [262] population. Also, this SNP has been associated with differential allele expression of IL2RA.
mRNA [263]. We therefore chose to study T1D-associated risk and protective variants in IL-2RArs3118470 in the context of T1D.

Given the crucial nature of IL-2 for the generation and maintenance of FOXP3+ Treg development and function [121]; that GWAS studies have identified T1D-associated polymorphic variation in IL-2/Il-2R pathway genes; and that Tregs have been shown to be defective in IL-2 signaling in T1D and capable of secreting IL-17, we hypothesized that T1D subjects homozygous for a T1D-associated IL-2RArs3118470 CC risk haplotype, have Tregs that signal defectively in response to IL-2 and therefore an increase in Tregs that secrete IL-17.

3.2 Methods

3.2.1 Subject recruitment and blood collection

For Figure 3.1, peripheral blood was obtained from 25 subjects, with recent-onset (<6 months from diagnosis) T1D, attending British Columbia Children's Hospital. PBMC were isolated using a Ficoll-Hypaque gradient and frozen at a concentration of between 6 - 9 x 10^6 cells/ml. Parents of all participants provided informed written consent and subjects provided written assent where applicable. The study protocol was approved by the Clinical Research Ethics Board of the University of British Columbia (Certificate Number: H07-01707).

For Figures 3.2-3.4, frozen PBMC was obtained from the TrialNet Consortium Pathway to Prevention (Natural History) Study, which has been previously described [264]. This included 52 subjects who developed T1D within 3 months and 42 control subjects, who were autoantibody-negative relatives of subjects with T1D.
3.2.2 Genotyping

The *IL2RA* haplotypes defined by single nucleotide polymorphisms (SNPs) rs706778 and rs3118470 and the PTPN2 haplotypes defined by SNPs rs478582 and rs45450798 were genotyped on all subjects described in section 3.2.1 using commercially available or custom designed TaqMan assays (4331349, C___1882662_10, C___1623598_10; Life Technologies). SNPs were deemed acceptable for analysis if they had call rates > 95%, frequencies that did not deviate from Hardy–Weinberg equilibrium (*p* > 0.05), and if no Mendelian errors were observed in the available complete trios.

3.2.3 Phosphoflow

Phosphoflow was performed by adapting a standard protocol, which has been previously published [265]. Briefly, PBMC were activated with or without IL-2 200U/ml for 10 minutes, fixed with BD Biosciences Phosflow Buffer I, and permeabilized using BD Phosflow Buffer III prior to staining with anti-pSTAT5(Y694), anti-FOXP3 (150D/E4), anti-CD4(RPA-T4), and anti-CD25(BC96). All pSTAT5 data shown are from previously frozen PBMCs. Data were acquired using a FACS-LSRII (BD Biosciences, San Diego, CA) and analyzed using FlowJo (TreeStar, Ashland, OR). Change in pSTAT5(Y694) mean fluorescence intensity (MFI) was calculated as the difference in the geometric MFI of the IL-2 stimulated sample and non-stimulated paired sample.

3.2.4 Flow cytometry

PBMC were stained with a combination of antibodies comprising anti-CD4 (RPA-T4), anti-CD14 (MYP9.1), anti-CD8 (RPA-T8), and anti-CD127 (hIL-7R-M21) (all from BD
Biosciences); anti-CD45RA (HI100) anti-CD25 (BC96), and anti-CD39 (eBioA1) (all from eBioscience). PBMC samples were suspended in R10 media with PMA (100 ng/ml), ionomycin (1mg/ml) and Brefeldin A (10 mg/ml) (Sigma) for 4 hours before intracellular staining. For intracellular staining, cells were treated with perm/fix buffer (eBioscience) and stained with anti-FOXP3 (259/C7, BD) or anti-IL17A (eBio64DEC17) and anti-IFN-γ (4S.B3, BD). Data were acquired by FACS- LSRII (BD). The unpaired Student’s t-test was used for statistical comparisons.

3.3 Results

3.3.1 Tregs from T1D subjects homozygous for the IL-2RArs3118470 CC risk haplotype, have reduced pSTAT5 signaling in response to IL-2

Polychronakos et al showed that two IL2RA SNPs (rs706778 and rs3118470) had statistically significant T1D association ($P = 6.96 \times 10^{-4}$ and $8.63 \times 10^{-4}$, respectively). We performed genotyping for these SNPs and phosphoflow on a small cohort of recent-onset T1D subjects, recruited at the BC Children’s Hospital, and found that T1D subjects who carry the IL2RArs3118470(C) risk variant, have reduced IL-2 signaling in CD25+ FOXP3+ Tregs, as measured by phosphorylation levels of the downstream signaling molecule STAT5 following IL-2 stimulation ($\Delta$MFI pSTAT5: CC = $184.6 \pm 41.80$ vs TC = $334.5 \pm 46.83$, $p = 0.03$; CC = $184.6 \pm 41.80$ vs TT = $438 \pm 91.97$, $p = 0.013$; CC vs TC vs TT, 1 way ANOVA, $p = 0.018$, Figure 3.1). Altered IL-2 induced STAT5 phosphorylation was not observed those carrying the IL2RArs706778 (A) risk variant ($\Delta$MFI pSTAT5: AA = $368.2 \pm 99$ vs AG = $326.6 \pm 46$ vs GG = $254.1 \pm 111$, 1 way ANOVA, $p = 0.07$, Figure 3.1B) suggesting that this effect is specific to the IL-2RArs3118470 CC risk haplotype.
Figure 3.1 Tregs from T1D subjects homozygous for the IL-2RArs3118470 CC risk haplotype, have reduced pSTAT5 signaling in response to IL-2

STAT5 phosphorylation in FOXP3+CD25+ T cells following IL-2 stimulation from (A) T1D subjects with the CC, T/C and TT genotype at IL2RAsrs3118470 (B) T1D subjects with the AA, A/G and GG genotypes at IL2RAsrs706778. PBMCs were stimulated with or without the presence of 200IU/ml of IL-2 for 10 minutes. Cells were fixed/permeabilized and phosphoflow staining performed. Analysis of change in MFI of pSTAT5 upon IL-2 stimulation was calculated by gating on CD4+FOXP3+CD25+ Treg cells and measuring the change in pSTAT5 MFI between cells stimulated with and without IL-2. FOXP3+CD25+ Tregs from T1D subjects homozygous for the
IL-2RArs3118470 CC risk haplotype, had reduced STAT5 phosphorylation upon IL-2 stimulation, compared to subjects with the T/C (CC = 184.6 ± 41.80 vs TC = 334.5 ± 46.83, p = 0.03) or TT (CC = 184.6 ± 41.80 vs TT = 438 ± 91.97, p = 0.013) IL2RArs3118470 genotypes (CC vs TC vs TT, 1 way ANOVA; p = 0.018) (A). No significant difference in FOXP3+CD25+ levels of pSTAT5 upon IL-2 stimulation was found between different genotypes at IL2RArs707778 (B). T1D-associated risk haplotypes (IL2RArs3118470 CC and IL2RArs707778 AA) are shown in red. (C) A representative plot of pSTAT5 levels after 30 minutes of IL-2 stimulation of T1D PBMC gated on CD4+FOXP3+CD25+ cells is shown. Red = IL2RArs3118470 CC genotype, Green = IL2RArs3118470 TC genotype, Blue = IL2RArs3118470 TT genotype and Grey = background levels with no IL-2 staining.

3.3.2 The CD45RA-FOXP3lo subset of CD4+ T cells is increased in proportion in T1D subjects homozygous for the IL2RArs3118470 CC risk haplotype

In Chapter 2, we have previously shown that recent-onset T1D subjects have an elevated proportion of CD45RA-FOXP3lo cells. This subset of FOXP3lo Treg cells are known to secrete pro-inflammatory cytokines such as IL-17 and have a reduced suppressive function in vitro. However, we observed that, while most subjects had an increase in the proportion of the CD45RA-FOXP3lo subset, not every subject had an elevated fraction of these cells. Some T1D subjects clearly carried an increased proportion of CD45RA-FOXP3lo cells, while others had proportions similar to the control group. We therefore hypothesized that the presence of unique genetic polymorphisms in each subject may underlie these phenotypic and functional differences. Since T1D subjects homozygous for the IL-2RArs3118470 CC risk haplotype have reduced IL-2 signaling in CD25+ FOXP3+ Tregs and IL-2 signaling is essential for the maintenance of FOXP3 expression in Tregs [266], we hypothesized that T1D subjects with the IL-2RArs3118470 CC risk haplotype may also have an increased proportion of CD45RA-FOXP3lo cells.
We obtained a new cohort of T1D subjects that were recruited from the TrialNet Consortium Pathway to Prevention (Natural History) Study. With the larger pool of genetic diversity from which this cohort is drawn, we anticipated it to be more likely to include T1D subjects with the TT genotype at IL2RAs3118470. However, this TT population was too rare to include in the analysis (n=1). We therefore used flow cytometry analysis to identify the CD45RAFOXP3lo subset, as shown previously (Figure 2.2), and compared T1D subjects homozygous for the IL2RAs3118470 CC risk haplotype and T1D subjects carrying the T/C genotype at rs3118470. We found that the T1D subjects, who are homozygous for the IL-2RAs3118470 CC risk haplotype, have an increased proportion of CD45RAFOXP3lo cells compared with T1D subjects carrying the T/C genotype at IL2RAs3118470 (T1D subjects CD45RAFOXP3lo: CC = 2.02 ± 0.12% vs TC = 1.59 ± 0.12%, \( p = 0.014 \), Figure 3.2A). The same elevation was not seen in control subjects (AB- controls CD45RAFOXP3lo: CC 2.7 ± 0.2% vs TC 2.4 ± 0.16%, \( p = 0.24 \), Figure 3.2B).

PTPN2 is a phosphatase with functional significance in the IL-2/IL-2R signaling pathway [157], and Tree et al have found that reduced Treg IL-2 signaling was associated with subjects carrying T1D risk alleles at PTPN2 SNPs, rs45450798 (C) and rs478582 (T) [156, 267]. We therefore examined whether CD45RA-FOXP3lo subset proportions varied in T1D subjects with different genotypes at PTPN2rs45450798 and PTPN2rs478582. However, we found no significant difference in these genotypes. (T1D subjects CD45RA-FOXP3lo: [PTPN2rs478582]: TT = 1.76 ± 0.13% vs CT = 1.72± 0.12% vs CC = 2.39± 0.28%, 1 way ANOVA \( p=0.07 \); [PTPN2rs45450798]: CC = 1.85± 0.13% vs CG = 2.19± 0.21% vs CC = 1.5± 0.23%, 1 way ANOVA \( p=0.19 \)). This indicates that alterations in the proportion of CD45RA-FOXP3lo cells in the peripheral blood is likely specific to T1D subjects homozygous for the IL2RAs3118470 CC
risk haplotype, and does not extend to T1D-associated haplotypes marked by SNPs in the *PTPN2* gene.

Figure 3.2 The Fr. III, CD45RA-FOXP3lo cell subset of CD4+ T cells is increased in proportion in T1D subjects homozygous for the IL-2RArs3118470 CC risk haplotype

Percentage of CD4+ cells that are Fr. III, CD45RA-FOXP3lo in PBMC of (A) TID or (B) control subjects with the CC and T/C genotypes at IL2RArs3118470 (C) T1D subjects with TT, C/T and CC genotypes at PTPN2rs478582. (D) T1D subjects with CC, C/G and GG genotypes at PTPN2rs45450798. PBMC was stained using flow cytometry techniques to identify the CD45RA-FOXP3lo subset. CD45RA-FOXP3lo CD4+ T cells in the PBMC of T1D subjects homozygous for the IL-2RArs3118470 CC risk haplotype were elevated in proportion (CC = 2.02 ± 0.12 vs
TC 1.59 ± 0.12, \( p = 0.014 \) (A). This difference was not observed in the control subjects (B) or at different genotypes in PTPN2rs478582 or PTPN2rs4545098 (C & D). T1D-associated risk haplotypes (IL2RArs3118470 CC and PTPN2rs478582 TT, PTPN2rs4545098 CC) are shown in red.

3.3.3 T1D subjects homozygous for the IL-2RArs3118470 CC risk haplotype do not have a difference in CD25 expression on the surface of Tregs or T effector cells

In addition to the phenotypical difference in FOXP3+ subsets observed, we wanted to discover if there were any functional consequences of the IL-2RArs3118470 CC risk haplotype in T1D. Since, the IL-2 receptor subunit CD25 is usually constitutively expressed on the surface of FOXP3hi Treg cells and IL-2 signaling provides a positive feedback loop to upregulate and maintain CD25 expression on T cells [157], we hypothesized that the polymorphic variation in \textit{IL2RA} might be altering the expression of CD25 on Tregs or T effector cells.

However, when we analyzed PBMC from T1D subjects for expression of CD25 on CD4+CD127loFOXP3hi Treg cells or CD4+FOXP3- T effector cells we saw no difference between the T1D subjects homozygous for the IL2RArs3118470 CC risk haplotype and T1D subjects carrying the T/C genotype at rs3118470 (T1D subjects CD25 MFI on CD127loFOXP3+: CC = 309± 26 vs TC = 267 ± 23, \( p=0.23 \); CD25 MFI on CD4+ FOXP3-: CC = 39± 2.2 vs TC = 33 ± 2.1, \( p = 0.08 \), Figure 3.3). This would indicate that the functional effects of the IL2RArs3118470 CC risk haplotype do not involve altering CD25 expression on the surface of the cells.
Figure 3.3 T1D subjects homozygous for the IL-2RArs3118470 CC risk haplotype do not have a difference in CD25 expression on the surface of Tregs or T effector cells

MFI of CD25 on CD4+CD127loFOXP3hi Treg cells (A) or CD4+FOXP3- T effector cells (B) in PBMC of TID subjects with the CC and T/C genotypes at IL2RArs3118470. PBMC was stained using flow-cytometry techniques, median fluorescence intensity of CD25 was assessed on gated CD4+CD127loFOXP3hi Treg cells or CD4+FOXP3- T effector cells. The IL-2RArs3118470 CC risk haplotype does not effect CD25 expression on CD4+CD127loFOXP3hi Treg cells (A) or CD4+FOXP3- T effector cells (B). T1D-associated risk haplotype IL2RArs3118470 CC is shown in red. (CC: n = 27 , TC: n = 25)

3.3.4 T1D subjects homozygous for the IL-2RArs3118470 CC risk haplotype have a higher proportion of IL-17 secreting FOXP3+ cells

Since the CD45RA-FOXP3lo subset has been shown by ourselves and others to secrete increased levels of pro-inflammatory cytokines such as IL-17 and IFN-γ, we hypothesized that another functional consequence of the IL-2RArs3118470 CC risk haplotype is to alter cytokine secretion in FOXP3+ Treg cells or the proportion of T helper cell subsets (Th17 and Th1). We stimulated PBMCs with PMA and ionomycin in the presence of Brefeldin A to detect cytokine
secretion by intracellular flow cytometry. The most striking observation is that there is a strong association between T1D subjects homozygous for the IL-2RAr3118470 CC risk haplotype and an elevated proportion of FOXP3+IL-17+ cells compared with T1D subjects with the T/C genotype at rs3118470. (T1D subjects FOXP3+IL-17+: CC = 0.15 ± 0.02% vs TC = 0.06 ± 0.007%, \( p=0.002 \), Figure 3.4A). Again, a significant difference was not observed in the control population (AB- controls FOXP3+IL-17+: CC 0.19± 0.03% vs TC = 0.14± 0.03%, \( p =0.18 \), Figure 3.4B). Also, this difference was not accounted for by changes in the frequency of Th17 or CD4+FOXP3+ Treg cells. (T1D subjects CD4+IL-17+: CC = 1.19 ± 0.13% vs TC = 0.9± 0.1%, \( p=0.08 \); CD4+FOXP3+: CC = 5 ± 0.3% vs TC = 4.3 ± 0.4%, \( p=0.24 \), Figure 3.4C,D). Since other groups have also indicated an importance for FOXP3+ IFN-\( \gamma \) + secreting subsets in T1D [252], we wanted to know if T1D subjects homozygous for the IL-2RAr3118470 CC risk haplotype would have an increase in IFN-\( \gamma \) secreting subsets. However, no difference in these subsets was found in T1D subjects with different genotypes at IL2RAr3118470. (T1D subjects CD4+ IFN-\( \gamma \)+: CC = 10.72± 1.2% vs TC = 7.9 ± 1.4%, \( p =0.14 \); [IFN-\( \gamma \)+FOXP3+]: CC = 14.8± 1.5% vs TC 11.9± 1.3%, \( p =0.15 \), Figure 3.4E,F)

This suggests that functional immune changes in individuals homozygous for the IL-2RAr3118470 CC risk haplotype are specific to an increase in the capacity of Tregs to secrete IL-17 in subjects with T1D.
A. **% IL-17+FOXP3+ CD4 T cells**

B. % IL-17+ FOXP3+CD4 T cells

C. % CD4 Th17 cells

D. % CD4 Treg cells

E. % IFNγ+FOXP3+ CD4 T cells

F. % CD4 Th1 cells
Figure 3.4 T1D subjects homozygous for the IL-2RAs3118470 CC risk haplotype have a higher proportion of IL-17 secreting FOXP3+ cells

Percentage of CD4+ cells that are IL-17+FOXP3+ in PBMC of (A) T1D or (B) control subjects with the CC and T/C genotypes at IL2RAs3118470. Percentage CD4+ cells that are Th17 (C), Treg (D), IFN-γ +FOXP3+ (E) or Th1 (F) in TID subjects with the CC and T/C genotypes at IL2RAs3118470. PBMC was stimulated with PMA (100 ng/ml), ionomycin (1mg/ml) in the presence of Brefeldin A (10 mg/ml) for 4 hrs before flow-cytometry analysis. T1D subjects homozygous for the IL-2RAs3118470 CC risk haplotype have an increased proportion of CD4+ cells that are IL-17+FOXP3+ (CC = 0.15 ± 0.02% vs TC = 0.06 ± 0.007%, p =0.002). This difference is not observed in control subjects (B) or in any other T helper cell subset (C-F). T1D-associated risk haplotype IL2RAs3118470 CC is shown in red. (Th17 = CD4+CD14-IL-17+ cells, Tregs = CD4+CD14-FOXP3+ cells, Th1 = CD4+CD14-IFNg+ cells)

3.4 Discussion

We demonstrate a genetically identifiable subset of T1D subjects, who are homozygous for the IL-2RAs3118470 CC risk haplotype and have different functional and phenotypical Treg differences compared to subjects carrying the T/C genotype at rs3118470. T1D subjects homozygous for the IL-2RAs3118470 CC risk haplotype have Tregs with reduced phosphorylation of the STAT5 signaling molecule upon IL-2 stimulation (Figure 3.1). They also have an increased proportion of the CD45RA-FOXP3lo subset that Miyara et al and we have previously identified as a cytokine-secreting Treg population with reduced suppressive capacity[38] (Figure 3.2). The functional consequence of the homozygous IL-2RAs3118470 CC risk haplotype did not seem to be associated with CD25 expression on the surface of Treg or T effector cells (Figure 3.3). This could be because this variant is non-coding, falling upstream of the IL2RA gene or intron 1. Long et al found that subjects homozygous for the IL2RAs2104286 risk haplotype with reduced Treg IL-2 responsiveness also do not display decreased CD25
expression on T cells. However, we found that T1D subjects homozygous for the IL-2RArs3118470 CC risk haplotype did have elevated proportions of IL-17 secreting FOXP3+ cells (Figure 3.4). Combined, these data suggest that the homozygous IL-2RArs3118470 CC risk haplotype can identify T1D subjects who have dysfunctional Treg cells, which are less responsive to IL-2 and therefore more prone to secreting the pro-inflammatory cytokine IL-17.

Subjects with T1D are arbitrarily grouped together because of the fulfillment of a set of clinical criteria that would result from any immune mechanism that could destroy pancreatic β cell function. However, it is more likely that a number of alternate immunopathogenic processes might occur to give result to different subsets of T1D. Different genetic susceptibility loci could guide the immune response to be biased toward a specific autoimmune mechanism. Other groups have identified T1D-associated variants in the IL2RA gene, IL-2 itself and the protein tyrosine phosphatase N2 (PTPN2). They have shown that these variants decrease the IL-2 responsiveness of Tregs in healthy controls [156, 157]. To this mounting body of evidence we add a newly identified IL-2RArs3118470 CC risk haplotype that seems to impact the ability of Tregs in T1D subjects to signal in response to IL-2 and select for an increase in circulating Tregs that secrete IL-17.

Although previous clinical trials with IL-2 and rapamycin have been unsuccessful because of the presumed activation of NK cells [135], Klatzman et al have recently found that lower doses of IL-2 are safe and could cause expansion of the Treg population without impacting NK or T effector cell populations [136]. Low-dose IL-2 will soon be used in larger randomized controlled trials to assess efficacy in recent-onset T1D. We would suggest that those T1D subjects homozygous for the IL2RArs3118470 CC risk haplotype could be more reactive to stimulation with exogenous IL-2. It would be important to genotype participants to see if there is
any correlation between response to IL-2 therapy and T1D-associated polymorphic variants in the IL-2/IL-2R pathway. Strong interest also exists in developing cellular autologous Treg therapy for T1D, and preliminary data shows that the \textit{ex vivo} culture of autologous Tregs can restore the IL-2 stimulation/pSTAT5 signaling deficits that are observed in T1D [135]. Our data indicate T1D subjects homozygous for the IL-2RArs3118470 CC risk haplotype might need to be excluded from undergoing autologous Treg therapy because they are likely to have a higher propensity of Tregs that secrete IL-17 during culture.

These data begin to uncover the complexity of multiple genetic susceptibility loci and how they may relate to immunological dysfunction in the autoimmune process of T1D. This emerging field of immunogenetics will help better direct new therapies, such as low dose IL-2 or cellular Treg therapy to T1D subjects with risk haplotypes. This pharmacogenetic approach will likely have a higher chance of achieving efficacy, even if the treatment can only be targeted at a smaller subset of genetically identifiable individuals. T1D is unlikely to have a single underlying pathogenic mechanism and uncovering of the immune mechanisms underlying different genetically identifiable subsets will help us direct therapy to those who are most likely to benefit.
Chapter 4: Determining the Treg and Th17 subsets that can be used as biomarkers of T1D

4.1 Introduction

Previous studies report that human T1D subjects have decreased [268, 269], increased [241] or equivalent [238, 239, 244, 245] proportions of Tregs in their peripheral blood compared to controls. These studies typically used non-specific Treg definitions that have since been refined. In Chapter 2, we have used two different sets of phenotypic definitions (FOXP3+ or CD127−), and found that Treg cells are paradoxically increased in subjects with T1D. Miyara et al first demonstrated that, when PBMC were stained with a combination of antibodies to CD4, FOXP3, CD25 and CD45RA, it was possible to identify a subset of CD45RA−CD25intFOXP3lo cells that secreted IL-17 and lacked suppressive capability [38]. Applying these techniques to T cells in diabetes, we observed in Chapter 2 that the same subset of CD45RA−CD25intFOXP3lo cells is significantly enlarged in a cohort of children with recent-onset T1D compared with age-matched healthy controls. We also reconfirmed, in T1D subjects, Miyara et al’s observation that the CD45RA−CD25intFOXP3lo subset of cells secrete elevated levels of IL-17 and are not suppressive.

In chapter 2 we also showed that, in contrast to age-matched healthy controls, peripheral lymphocytes from children with recent-onset T1D have an increased proportion of IL-17 secreting CD4+ (Th17) and CD8+ T cells (Tc17). These data are supported by a study from Honkanen et al, which found similar elevations in the Th17 population in a different cohort of recent-onset T1D subjects [227]. Reports also show that, in addition to peripheral T cells, T1D
subjects have an increased proportion of monocytes that secrete Th17 polarizing cytokines [228] and islet-antigen specific Th17 cells [229].

These observations suggest that there are potentially pathogenic alterations in a proportion of Treg, Th17 and FOXP3+IL-17+ intermediate subsets in recent-onset T1D. However, all previous studies, including ours, have been performed on blood obtained from T1D subjects several months after diagnosis. To decide on the significance of these findings, it would be important to assess how Treg and IL-17 secreting subsets change during the natural history of T1D. In particular, is there any increase in pro-inflammatory IL-17 secreting subsets or decrease in regulatory subsets before individuals develop T1D? This would allow us to ascertain if peripheral blood T cell proportion changes could be used as biomarkers of T1D akin to autoantibody status.

To answer this question, we collaborated with the National Institutes of Heath (NIH) T1D TrialNet Natural History Study. In this study, TrialNet has accumulated cryopreserved PBMC samples from relatives of T1D subjects, including those who have not yet developed diabetes and those who have been tracked from pre-diabetes to the onset of disease and beyond. Autoantibody status has been assessed in all these subjects, allowing identification of autoantibody negative and positive relatives of T1D subjects who have or have not progressed to T1D.

We hypothesized that subjects who eventually develop T1D will have decreased proportions of Treg subsets and increased proportions of IL-17 secreting subsets (including FOXP3+IL-17+ cells) before the diagnosis of disease.
4.2 Methods

4.2.1 Subject recruitment and blood collection

Frozen PBMC was obtained from the TrialNet Consortium Pathway to Prevention (Natural History) Study, which has been previously described [264]. All subjects were tested (Phase 1) for the presence of three autoantibodies (to GAD, insulin and ICA512). Individuals who are positive for >1 autoantibody enter Phase 2, in which they receive an oral glucose tolerance test (OGTT); those who test positive for ≤1 autoantibody are re-tested at a later date, progressing to Phase 2 only when they test positive for ≥2 autoantibodies. In Phase 2, risk stratification is based on the OGTT: among individuals with normal OGTT, the risk of developing diabetes is <25% for individuals with one autoantibody; ≥25% if two autoantibodies; and ≥50% if three autoantibodies [264]. All subjects enrolled are tested frequently to assess if they meet the clinical criteria for a diagnosis of T1D. We received blinded PBMC samples from: 50 autoantibody negative relatives of subjects with T1D (AB- Controls); 40 autoantibody positive relatives of T1D subjects, who did not progress to T1D (AB+ non-progressors); 61 T1D subjects whose blood was taken at mean 575± 43 days before diagnosis (T1D before diagnosis); 18 subjects whose blood was taken at mean day 0.9± 0.3 diagnosis (T1D at diagnosis); and 20 subjects whose blood was taken at 170± 6 days after diagnosis (T1D after diagnosis). We were unable to run staining on PBMC from an average of three subjects per group because of the samples’ poor viability upon defrosting.

4.2.2 Flow cytometry

PBMC were defrosted in randomized batches of 45-50 samples on four separate occasions and two panels of flow cytometry staining were performed on the same day.
For the first panel Treg immunophenotyping was assessed. One million PBMC were stained directly *ex-vivo* with a combination of antibodies comprising anti-CD4 (RPA-T4), anti-CD14 (MYP9.1), anti-CD8 (RPA-T8), and anti-CD127 (hIL-7R-M21) (all from BD Biosciences); anti-CD45RA (HI100) anti-CD25 (BC96), and anti-CD39 (ebioA1) (all from eBioscience). These cells were then treated with perm/fix buffer (eBioscience) and stained with the intracellular antibody anti-FOXP3 (259/C7, BD). Data were acquired by FACS-LSRII (BD). The unpaired Student’s *t*-test was used for statistical comparisons. An example of the flow cytometry gating strategy used for the Treg immunophenotyping (panel 1) is shown in Figure 4.1.

For the second panel, T cell cytokine secretion profiles were assessed. Two million PBMC were suspended in R10 media with PMA (100 ng/ml), ionomycin (1mg/ml) and Brefeldin A (10 mg/ml) (Sigma) for 4 hrs before staining. They were stained with a combination of antibodies comprising anti-CD4 (RPA-T4), anti-CD14 (MYP9.1), anti-CD8 (RPA-T8). For intracellular staining, cells were treated with perm/fix buffer (eBioscience) and stained with anti-FOXP3 (259/C7, BD), anti-IFN-γ (4S.B3, BD) anti-IL17A (eBio64DEC17). Data were acquired by FACS-LSRII (BD). The unpaired Student’s *t*-test was used for statistical comparisons. An example of the flow cytometry gating strategy used for T cell cytokine secretion profiles (panel 2) is shown in Figure 4.2.
Figure 4.1 Flow cytometry panel gating strategy for Treg immunophenotyping

All samples were initially gated on lymphocytes and CD4+CD14+ cells. Gating strategies for (A) CD127loFOXP3+, (B) CD127loCD25+, (C) CD45RA+FOXP3+, CD45RA-FOXP3lo and CD45RA-FOXP3hi and (D) CD39-FOXP3+ and CD39+FOXP3+ cell populations are shown.
Figure 4.2 Flow cytometry panel gating strategy for T cell cytokine secretion profiles

All samples were initially gated on lymphocytes and CD8+CD14+ (A) or CD4+CD14+ (B & C) cells. Gating strategies for (A) Tc17: CD8+IL17+; (B) Th1:CD4+ IFN-γ+; IL17+ IFN-γ+; (C) Th17: CD4+IL17+; FOXP3+IL-17+ cell populations are shown.

4.3 Results

4.3.1 The proportion of CD127loFOXP3+ cells is decreased after the onset of T1D

In view of conflicting reports about the levels of regulatory T cells in recent-onset subjects with T1D [237] [241] [238-240], we wanted to assess the proportion of Tregs before diagnosis using the samples obtained from the TrialNet Natural History Study. These samples contained PBMC isolated from autoantibody negative and positive T1D subjects and their relatives. These subjects were followed to ascertain who did or did not progress to develop T1D. This allowed us to study five cohorts: AB-controls; AB+ non-progressors; T1D before diagnosis (AB+ progressors); T1D at diagnosis; and T1D after diagnosis.

Enumerating Tregs from human blood is difficult because of the different markers that are available, none of which absolutely exclude activated T effector cells [27]. The use of
FOXP3 and CD127 in combination (CD127loFOXP3+) is the most validated method to identify a specific proportion of Tregs [270]. Therefore, we decided to define Tregs by flow cytometry as the CD127loFOXP3+ cell subset in the PBMC of our five cohorts. Since the CD127loCD25hi subset of cells is used to identify Tregs for therapeutic purposes in T1D [30], we also assessed by flow cytometry the proportion of CD127loCD25+ cells in the PBMC of our cohorts for comparison. Examples of the gating strategies are shown in Figure 4.1.

Our hypothesis was that proportions of Treg cells would be decreased in autoantibody positive relatives of T1D subjects (AB+ non progressors) or T1D subjects before they were diagnosed. However, we did not observe any significant changes in the Treg population (assessed as either CD127loFOXP3+ or CD127loCD25+) before the onset of disease. (CD127loFOXP3+: AB- controls 4.8±0.24% vs AB+ non progressors 5±0.37%; T1D before diagnosis 4.7±0.21%; T1D at diagnosis 4.9±2.4%; p>0.05; CD127loCD25+: AB- controls 2.3±0.18% vs AB+ non progressors 2.5±0.2%; T1D before diagnosis 2.4±0.12%; T1D at diagnosis 2.5±0.31%, p>0.05, Figure 4.3). We did observe that T1D subjects 170±6 days after diagnosis had elevated proportions of CD127loFOXP3+ cells compared to AB- controls (CD127loFOXP3+: AB- controls = 4.78±0.24% vs T1D after diagnosis = 3.81±0.4%, p = 0.048, Figure 4.3A). This observation was not replicated in the CD127loCD25+ population (CD127loCD25+: AB- controls = 2.27±0.17% vs T1D after diagnosis = 2.36±0.3%, p = 0.78, Figure 4.3B).
4.3.2 The proportion of CD39+FOXP3+ cells is decreased before the onset of T1D

Since there was no significant change in the overall levels of CD127loFOXP3+Treg cells before diagnosis of T1D, we wanted to see if there were differences in various subsets of Treg cells. Tregs that express CD39 on their surface are highly suppressive and reduced in proportion in other autoimmune diseases such as relapsing/remitting multiple sclerosis.[39] CD39 polymorphisms have also been associated with diabetes [39]
We therefore decided to assess by flow cytometry the proportion of CD39+ and CD39-FOXP3+ cells in PBMC obtained from our five cohorts (AB- controls; AB+ non-progressors; T1D before diagnosis; T1D at diagnosis; and T1D after diagnosis).

We found that autoantibody positive relatives of T1D subjects who did not progress to disease (AB+ non-progressors) and T1D subjects sampled before or after diagnosis had a reduced proportion of CD39+FOXP3+ cells compared to AB- controls (CD39+FOXP3+: AB- controls = 1.95 ± 0.14% vs AB+ non-progressors = 1.32 ± 0.15%, p=0.003; T1D before diagnosis = 1.37 ± 0.12%, p=0.002 and T1D after diagnosis = 1.38 ± 0.15%, p=0.02, Figure 4.4A). This reduction in proportion of CD39+FOXP3+ cells was maintained almost to a significant level in T1D subjects at diagnosis (CD39+FOXP3+: AB- controls = 1.95 ± 0.14% vs T1D at diagnosis = 1.41 ± 0.21%, p=0.05). Similar to the findings in multiple sclerosis, no statistically significant differences were observed between any of our five cohorts in the proportions of CD39-FOXP3+ cells (AB- controls 3.8 ± 0.21% vs AB+ non progressors 4.6 ± 0.42%; T1D before diagnosis 4.1 ± 0.18%; T1D at diagnosis 4.2 ± 0.51%; T1D after diagnosis 4.3 ± 0.65%, p>0.05, Figure 4.4B)
Figure 4.4 The proportion of CD39+FOXP3+ cells is decreased before the onset of T1D

Percentage of CD4+ T cells that are (A) CD39+FOXP3+ or (B) CD39-CD25+ in PBMC of AB- controls, AB+ non-progressors, T1D mean 575± 43 days before diagnosis, T1D at mean day 0.9± 0.3 of diagnosis and T1D mean 170± 6 days after diagnosis. PBMC was stained using flow-cytometry techniques to identify the CD39+FOXP3+ and CD39-FOXP3- subsets. Autoantibody positive non-progressors and T1D subjects before or after diagnosis had a reduced proportion of CD39+FOXP3+ cells compared to AB- controls (AB- controls = 1.95 ± 0.14% vs AB+ non-progressors = 1.32 ± 0.15%, t-test p=0.003; T1D before diagnosis = 1.37± 0.12%, t-test p=0.002; T1D after diagnosis = 1.38 ± 0.15%, p=0.02) (Anova and Bonferroni’s post test, p = 0.005) (A). No significant differences between any cohort were observed in the proportions of CD39-FOXP3+ cells (B). (Anova and Bonferroni’s post test, p = 0.56)

4.3.3 FOXP3+IL-17+ cells are increased in proportion after diagnosis of T1D

Miyara et al first demonstrated that human FOXP3+CD4+ T cells can be divided into three phenotypically and functionally distinct subpopulations, depending on the expression of FOXP3 and the human naive cell marker, CD45RA [38]. Whereas naïve Tregs (CD45RA+FOXP3lo) and memory Tregs (CD45RA-FOXP3hi) possess suppressor function, a
third group of FOXP3+ memory T cells (CD45RA-FOXP3lo) are non-suppressive and secrete IL-17 [38]. We went on to demonstrate in Chapter 2 that the CD45RA-FOXP3lo population is increased in children with recent-onset T1D (within 6 months) compared with age-matched healthy controls. Subsequent to this study, we optimized the simulation conditions with PMA and ionomycin in the presence of the golgi blocker Brefeldin A, that enabled direct detection of IL-17 secretion, while preserving direct ex-vivo levels of FOXP3 expression (IL-17+FOXP3+ cells).

Since we found, in Chapter 2, that the proportion of intermediate Th subsets, which express the Treg transcription factor FOXP3 but also secrete IL-17, is enlarged in a cohort of children with recent-onset T1D compared with age-matched healthy controls, we wanted to know if this change occurred before diagnosis. We therefore assessed by flow cytometry the proportions of all the three FOXP3+ subsets observed by Miyara et al (gating strategy in Figures 3.2 & 4.1) as well as FOXP3+IL-17+ cells directly (gating strategy in Figure 4.2) in our five sample cohorts previously described (AB-controls; AB+ non-progressors; T1D before diagnosis; T1D at diagnosis; and T1D after diagnosis). We hypothesized that both the CD45RA-FOXP3lo subset and FOXP3+IL-17+ subset would be reduced before diagnosis in the natural history of T1D.

Consistent with our previous results, we found no significant difference between any cohorts in the proportions of naïve CD45RA+FOXP3+ Tregs (CD45RA+ FOXP3+: AB-controls 1.97± 0.17% vs AB+ non progressors 2.3± 0.2%; T1D before diagnosis 2.3 ± 0.17%; T1D at diagnosis 2.1 ± 0.36%; T1D after diagnosis 1.4 ± 0.26%, p>0.05, Figure 4.5A) or memory CD45RA-FOXP3hi Tregs (CD45RA-FOXP3hi: AB- control 0.75± 0.07% vs AB+ non progressors 0.78± 0.1%; T1D before diagnosis 0.59± 0.05%; T1D at diagnosis 0.67± 0.21%;
T1D after diagnosis 0.92± 0.2%, \( p > 0.05 \), Figure 4.5B). We also found no significant difference in either the proportion of CD45RA-FOXP3lo cells or the IL-17+FOXP3+ cells before subjects developed T1D or at the time of diagnosis (CD45RA-FOXP3lo: AB- controls 2.4 ± 0.12% versus AB+ non-progressors 2.1± 0.185% ; T1D before diagnosis 1.9± 0.1% ; T1D at diagnosis 2.098 ± 0.3%, \( p > 0.05 \); IL-17+FOXP3+: AB- controls 0.15 ± 0.02% vs AB+ non-progressors 0.13± 0.08%; T1D before diagnosis 0.11± 0.01% ; T1D at diagnosis 0.10± 0.02%, \( p > 0.05 \), Figure 4.5C,D). However, consistent with previous findings, T1D subjects 170± 6 days after diagnosis had elevated proportions of CD45RA-FOXP3lo and IL-17+FOXP3+ cells compared to AB- controls (CD45RA-FOXP3lo: AB- controls = 2.4 ± 0.12%, T1D after diagnosis = 3.34± 0.35%, \( p = 0.002 \); IL-17+FOXP3+: AB- controls = 0.15 ± 0.02%, T1D after diagnosis = 0.24± 0.03%, \( p = 0.01 \), Figure 4.5C,D).
Figure 4.5 FOXP3+IL-17+ cells are only increased in proportion after diagnosis of T1D

Percentage of CD4+ T cells that are (A) FrI, CD45RA+FOXP3+, (B) Fr II, CD45RA-FOXP3hi, (C) FrIII, CD45RA-FOXP3lo and (D) IL-17+FOXP3+ in PBMC of AB- controls, AB+ non-progressors, T1D mean 575± 43 days before diagnosis, T1D at mean day 0.9± 0.3 of diagnosis and T1D mean 170± 6 days after diagnosis. PBMC was stained using flow-cytometry techniques either with (D) or without (A-C) stimulation with PMA/Ionomycin in the presence of Brefeldin A. T1D subjects 170± 6 days after diagnosis had elevated proportions of Fr III, CD45RA-FOXP3lo (C) and IL-17+FOXP3+ (D) cells compared to AB- controls (CD45RA-FOXP3lo: AB- controls = 2.4 ± 0.12% vs T1D after diagnosis = 3.34± 0.35%, t-test $p=0.002$ (Anova and Bonferroni’s post test, $p = 0.0001$) (C); IL-17+FOXP3+: AB- controls = 0.15 ± 0.02% vs T1D after diagnosis = 0.24± 0.03%, t-test $p=0.01$ (Anova and Bonferroni’s post test, $p = 0.0004$) (D)). No significant differences between any cohort were observed in the
proportions of Fr I, CD45RA+FOXP3+ (Anova and Bonferroni’s post test, \( p = 0.07 \)) (A) or Fr II, CD45RA-FOXP3hi (B) cells (Anova and Bonferroni’s post test, \( p = 0.24 \)).

4.3.4 IL-17 secreting T cells are increased in proportion after diagnosis of T1D

In Chapter 2, we have shown that the proportion of CD4+IL-17+ Th17 cells and CD8+IL-17+ Tc17 cells is increased in the peripheral blood of subjects with recent-onset T1D (within 6 months) compared with healthy controls. Therefore, we hypothesized, based on these observations, that Th17 and Tc17 cells may also be increased in proportion before the onset of T1D.

PBMC from our five sample cohorts (AB- controls; AB+ non-progressors; T1D before diagnosis; T1D at diagnosis; and T1D after diagnosis) were stimulated with PMA/ionomycin in the presence of Brefeldin A prior to flow cytometry analysis to detect CD4+IL-17+ Th17 cells and CD8+IL-17+ Tc17 cells. We found no significant difference in either the proportion of Th17 cells or the Tc17 cells before subjects developed T1D or at the time of diagnosis (CD4+IL-17+: AB- controls 1.3± 0.11% \textit{versus} AB+ non-progressors 1.2 ± 0.1%; T1D before diagnosis 1.1 ± 0.07%; T1D at diagnosis 0.95± 0.13%, \( p>0.05 \); CD8+IL-17+: AB- controls 0.21± 0.02% \textit{versus} AB+ non-progressors 0.22± 0.04%; T1D before diagnosis 0.22± 0.02%; T1D at diagnosis 0.21± 0.03%, \( p>0.05 \), Figure 4.6A,B).

However, consistent with our previous results, we found T1D subjects 170± 6 days after diagnosis had elevated proportions of Th17 and Tc17 compared to AB- controls (CD4+IL-17+: AB- controls = 1.38 ± 0.11% \textit{vs} T1D after diagnosis = 1.81 ± 0.21%, \( p =0.02 \); CD8+IL-17+: AB- controls = 0.21 ± 0.02% \textit{vs} T1D after diagnosis = 0.49± 0.1%, \( p=0.0003 \), Figure 4.6 A,B).
An intermediate Th subset that seems to co-secrete IL-17 and IFN-γ has been described. This subset of IL-17+IFN-γ+ cells is thought to be highly pathogenic in other autoimmune diseases, such as autoimmune juvenile arthritis [109]. We therefore hypothesized that IL-17+IFN-γ+ cells may also be increased in proportion in T1D subjects compared to AB-controls. We used flow cytometry to identify IL-17+IFN-γ+ cells in the PBMC of the five cohorts described above (gating strategy in Figure 4.2). We found that, while there was no significant difference before subjects developed T1D or at the time of diagnosis compared with AB-controls (IL-17+IFN-γ+: AB- controls 0.24± 0.03% vs AB+ non-progressors 0.24 ± 0.18%; T1D before diagnosis 0.2± 0.02%; T1D at diagnosis 0.18 ± 0.05%, p>0.05, Figure 4.6C), consistent with other IL-17 secreting T cell subsets there was an increase in the proportion of IL-17+IFN-γ+ cells 170± 6 days after diagnosis of T1D compared with AB- controls. (IL-17+IFN-γ+: AB-controls = 0.25 ± 0.03% versus T1D after diagnosis= 0.52 ± 0.1, p = 0.001, Figure 4.6C)

We wanted to ascertain if this difference in IL-17+ IFN-γ+ cells could be accounted for by a change in the proportion of CD4+ IFN-γ+ Th1 cells in our five sample cohorts. We therefore also gated on the CD4+ IFN-γ+ Th1 cells (gating strategy shown in Figure 4.2), but saw no statistical difference between the AB- controls and the T1D subjects 170± 6 days after diagnosis (CD4+ IFN-γ+: AB- controls = 14.6 ± 1.1% vs T1D after diagnosis = 12.2 ± 1%, p=0.27, Figure 4.6D) However, we did observe that T1D subjects 575± 43 days before diagnosis and T1D subjects at day 0.9± 0.3 of diagnosis had reduced proportions of CD4+ IFN-γ+ Th1 cells compared with AB- controls (CD4+IFN-γ+: AB- controls = 14.59 ± 1.1%, T1D before diagnosis = 9.19 ± 0.8%, p=0.0001 and T1D at diagnosis = 8.38 ± 1.35%, p=0.003, Figure 4.6D)
Figure 4.6 IL-17 secreting T cells are increased in proportion after diagnosis of T1D

Percentage of CD4+ T cells (A, C & D) or CD8+ T cells (B) that are (A) Th17: CD4+IL-17+ (B) Tc17: CD8+IL-17+ (C) IL-17+ IFN-γ+ and (D) Th1: CD4+ IFN-γ+ in PBMC of AB- controls, AB+ non-progressors, T1D mean 575± 43 days before diagnosis, T1D at mean day 0.9± 0.3 of diagnosis and T1D mean 170± 6 days after diagnosis. PBMC was stained using flow-cytometry techniques after stimulation with PMA/ionomycin in the presence of Brefeldin A. T1D subjects 170± 6 days after diagnosis had elevated proportions of (A) Th17: CD4+IL-17+ (B) Tc17: CD8+IL-17+ and (C) IL-17+ IFN-γ+ compared with AB- controls (CD4+IL-17+: AB- controls = 1.38 ± 0.11%, T1D after diagnosis = 1.81 ± 0.21%, t-test p = 0.02 (Anova and Bonferroni’s post test, p = 0.0004) (A); CD8+IL-17+: AB- controls = 0.21 ± 0.02%, T1D after diagnosis = 0.49± 0.1%, t-test p = 0.0003 (Anova and Bonferroni’s post test, p = 0.0001), (B); IL-17+ IFN-γ+: AB- controls = 0.25 ± 0.03%, T1D after diagnosis = 0.52± 0.1%, t-test p = 0.001 (Anova and Bonferroni’s post test, p = 0.0001), (C)). T1D subjects 575± 43 days before
diagnosis and T1D subjects at day 0.9 ± 0.3 of diagnosis had reduced proportions of CD4+ IFN-γ+ cells compared with AB- controls (AB- controls = 14.59 ± 1.1%, T1D before diagnosis = 9.19 ± 0.8%, \( p=0.0001 \) and T1D at diagnosis = 8.38 ± 1.35%, \( p=0.003 \) (Anova and Bonferroni’s post test, \( p = 0.0009 \)), (D)).

### 4.4 Discussion

All the IL-17 secreting subsets that we assessed (including intermediate phenotypes such as FOXP3+IL-17+ and IL-17+ IFN-γ+ T helper cells) are only elevated in proportion in the peripheral blood during the recent-onset period after and not before T1D diagnosis (Figures 4.5 & 4.6). We have also shown that any alterations in overall Treg proportions in recent-onset T1D subjects depend on the markers that are used to identify these cells (CD127loFOXP3+ versus CD127loCD25+) and do not occur before T1D diagnosis (Figure 4.3). However, the CD39+ subset of FOXP3+ Tregs is decreased in proportion both before diagnosis and in the peripheral blood of AB+ non-progressor relatives of those with T1D (Figure 4.4). This study therefore identifies a reduction in the proportion of CD39+FOXP3+ cells as a potential biomarker of disease that correlates with change in autoantibody status.

CD39 is an enzyme that is expressed on the surface of immune cells (including Tregs) and functions to degrade ATP to ADP and then AMP [271, 272]. Since ATP is released extracellularly by damaged tissue and has many pro-inflammatory functions, it is thought that CD39 on the surface of Tregs uses the breakdown of ATP to exert generic suppressive functions in the local inflammatory milieu [39]. Tregs have a higher sensitivity to ATP-induced cell death than T effector cells, and this can be prevented if Tregs express functional CD39 on their surface [41]. In the context of HIV, high numbers of CD39+ Tregs are associated with a poor prognosis and it has been shown that the CD39+ Tregs inhibit IL-2 transcription in T effector cells, limiting
their growth and expansion [273]. CD39+FOXP3+ cells have also been implicated in autoimmune diseases. CD39-/- mice develop autoimmunity and are more susceptible to the Th17-driven experimental auto-immune encephalomyelitis model of multiple sclerosis (MS) [39]. A role of CD39+ Tregs in MS is further implicated by the observation that subjects having the relapsing/remitting form of MS have significantly lower numbers of CD39+ Tregs in their peripheral blood [41]. In T1D the genetic expression of the ENTPD1 transcript, which encodes CD39, shows increased levels of variation in Tregs [274]. This may indicate that the activity of CD39-mediated suppression is variable in different subsets of subjects with T1D. T1D polymorphisms have also been associated with diabetes [39], and so a reduction in the proportion of CD39+ Tregs may contribute to T1D pathogenesis.

Overall, CD39 seems to be expressed on a subset of highly suppressive Treg cells [39] and this subset is the only one assessed that seems to be reduced in proportion prior to diagnosis. The proportion of CD39+FOXP3+ cells is also reduced in relatives of T1D subjects with autoantibody positive status who do not go on to develop disease. This suggests that CD39 expression could identify a Treg subset that is a potential biomarker of T1D and further work is needed to validate this initial observation. Further experiments to sort (using extracellular markers such as CD39+CD127loCD25hi) and determine the function of the human CD39+ Treg population in T1D subjects would help determine if CD39+ Tregs are contributing to the pathogenic process in the context of T1D.

For multiple reasons, IL-17 secreting subsets may not be increased in proportion in the peripheral blood of T1D subjects until months after onset of disease. This could reflect the commonly cited disadvantage of peripheral blood analysis, which is that it is not reflective of tissue specific changes in immune cell proportion. There is evidence that pancreatic lymph nodes
from T1D subjects have an expansion in Th17 cells [230] and that islets from recent-onset T1D subjects express IL-17A, RORC and IL-22 [229]. It could be possible that, before diagnosis, IL-17 secreting cells are actively being recruited and expanded in the pancreatic lymph nodes and tissue. Only after diagnosis, when the majority of β cells are destroyed, are chemokine levels and auto-antigen availability reduced, and so the IL-17 secreting cells start circulating in increased numbers in the blood.

Another hypothesis as to why IL-17 secreting cells are only elevated in proportion after diagnosis is that recent-onset T1D subjects have some characteristic, unrelated to pathogenesis, that is causing the increase in IL-17 secreting subsets in the blood. IL-17 is known to be elevated as a response to candida infections, which are prevalent in subjects with T1D [275]. There is also evidence of a skew toward Th17 subsets in the peripheral blood of subjects with type 2 diabetes, which may imply a role for hyperglycaemia directly promoting Th17 differentiation [276].

It is also possible that only a small subset of T1D subjects have an increase in the proportion of IL-17 secreting cells. As described in Chapter 3, these subjects could be genetically determined. Figure 4.6 shows a subset of T1D subjects who have an increase in the IL-17 secreting cell proportion before disease, and a subset of T1D subjects who do not show this increase. We include all these subjects in a single ‘before diagnosis’ cohort and this would not show an overall increase in the proportion of IL-17 secreting cell subsets before diagnosis if the effect is small or not universal.

It has been acknowledged that plasticity exists between the Treg and Th17 population, such that conversion from Treg to Th17 cells can occur \textit{in vitro} and \textit{in vivo} [277]. It is possible that this process is slowly occurring in subjects with T1D during the natural history of disease, and that it is not detectable in artificially grouped cohorts that have a different level of
pathogenesis on the day of diagnosis. Therefore, differences are only observed after diagnosis, when all T1D subjects in the cohort have undergone significant pathogenesis and Treg to Th17 conversion is mostly complete.

We also observed that IL-17+ IFN-γ+ T helper cells are increased after diagnosis in recent-onset T1D subjects. These dual cytokine secreting cells have been implicated in other autoimmune diseases as potentially pathogenic. In humans, the conversion of Th17 cells into Th17/Th1-type cells has been reported in the synovial fluid of children with juvenile arthritis (14); in subjects with Crohn’s disease IFN-γ-expressing Th17 cells have been demonstrated in the gut (15). We have shown a similar increase in IL-17+IFN-γ+ cell proportion in the recent-onset subjects with T1D. The significance of this, though, is marred because this change is not observed before the onset of T1D. However, the Th1 population is reduced before the onset of T1D. This might suggest that a slow transition is occurring from the Th1 to the Th17 phenotype in vivo, which would explain a reduction in the Th1 cells before diagnosis, followed by an increase in the IL-17+ IFN-γ+ and Th17 cells after diagnosis. This hypothesis is contradicted by evidence in the NOD mouse that the conversion of Th17 cells to a Th1 phenotype seems to be the prevailing pathogenic mechanism and not vice versa [112, 113]. The origin of these IL-17+IFN-γ+ cells that appear after diagnosis needs to be ascertained by further studies.

These observations show that all previous studies on recent-onset diabetes, where samples have been assessed many months after diagnosis, are not going to be reflective and consistent with disease pathogenesis before T1D diagnosis. Therefore, previous and future studies should try to use cohorts such as the NIH Natural History TrialNet Study to validate any changes in peripheral blood post-diagnosis and to see if they occur earlier in the natural history of disease. This adds further support to the theory that clinical trial failures in recent-onset
subjects within 100 days of diagnosis may not be reflective of the treatment’s efficacy in people at high risk of T1D development. A subject with two or more positive autoantibodies has a 70% chance of developing diabetes within 10 years [182]. It is possible therefore to identify a cohort of ‘at risk’ subjects for clinical trials of novel therapeutics in T1D. These data support the re-testing in this ‘at risk’ cohort of immunomodulatory therapies that may have not shown significant efficacy in recent-onset T1D subjects.
Chapter 5: Conclusion

5.1 Summary of data in relation to original hypothesis and rationale

Our original hypothesis was that pancreatic β cell destruction in T1D is driven by the conversion of autoreactive Treg cells into a Th17 phenotype due to defective Treg IL-2 signaling in T1D subjects who have polymorphic variants in the IL2RA gene. In Chapter 2, we show an elevation in the proportion of IL-17 secreting subsets (including a CD45-FOXP3loIL-17+ subset) in the peripheral blood of recent-onset T1D subjects compared with controls. These data support our original hypothesis that Tregs are potentially transitioning towards a Th17 phenotype in recent-onset T1D subjects. In Chapter 3 we show T1D-associated polymorphic variants in the IL2RA gene result in an IL-2 signaling deficit in Treg cells and an increase in proportion of IL-17+FOXP3+ cells in the peripheral blood. These data provide evidence for the hypothesis that the elevated FOXP3+IL-17+ proportion occurs in a genetically identifiable subset of children with T1D who have polymorphic variants in the IL2RA gene. In Chapter 3, we did not find evidence to support the hypothesis that changes in proportions of Tregs and Th17 cells occur before the onset of T1D diagnosis. However, we observed that CD39-expressing Treg cells were reduced in proportion before disease onset and could act as a biomarker of T1D.

5.2 Data in the context of current literature

In recent-onset T1D subjects we found an increase in a FOXP3+ subset of cells that secreted IL-17 and was not suppressive in vitro. We also found an increase in the proportion of Th17 and Tc17 cells in the peripheral blood of recent-onset T1D subjects. This study was one of the first to link IL-17 immunity with T1D in humans after some previously described murine
studies suggesting an association. These data are supported by a study from Honkanen et al., which found similar elevations in the Th17 population and demonstrated an increase in IL-17 mRNA transcription in cells from children with T1D [227]. Reports also show that, in addition to peripheral T cells, T1D subjects have an increased proportion of monocytes that secrete Th17 polarizing cytokines [228] and islet-antigen specific Th17 cells [229]. Pancreatic lymph nodes from T1D subjects have an expansion in Th17 cells [230], and islets from recent-onset T1D subjects express IL-17A, RORC (the human, lineage-defining IL-17 transcription factor) and IL-22 [229]. IL-17 has a direct effect on the pancreatic islets, in conjunction with IL-1-β and IFN-γ, to up-regulate the expression of stress response genes and proinflammatory chemokines in β cells, leading to their apoptosis [227, 229, 231]. Taken together, these human and animal data present compelling evidence that autoimmune responses driven by IL-17 have a pathogenic role in T1D.

Chapter 2 shows there is a subset of those with recent-onset T1D who have an elevated level of IL-17 secreting cells or intermediate FOXP3+IL-17+ cells compared with controls and a subset that do not. This could simply be because all the T1D subjects present at different stages of pathogenesis at diagnosis and that inter-individual biological variability is common within human peripheral blood cellular analyses. An alternative hypothesis is that additional factors will determine whether a T1D subject has a higher proportion of IL-17 secreting cells in their peripheral blood. These factors may be age, sex or HBA1c level. However, controls were age- and sex-matched to the T1D subjects, and HBA1c levels would not necessarily correlate to disease pathogenic state in T1D subjects treated with insulin. Another reason for variability within T1D subjects would be the likelihood that there is inter-individual variation in genetic susceptibility loci for T1D. It is intuitive to assume that T1D may arise by many alternate
pathogenic mechanisms, which lead to the same clinical endpoint of pancreatic dysfunction. These alternate pathogenic mechanisms could be guided by the possession of risk haplotypes in the many T1D-associated polymorphic variants that have been identified by GWAS analysis. As mentioned previously, secondary to HLA, the T1D-associated polymorphic variants in the IL-2/IL-2R pathway genes have the largest association with T1D [202]. In addition, IL-2 is a critical cytokine for iTreg differentiation and maintenance of suppressive functionality in the periphery [129]. Treg dysfunction in T1D is linked to lack of IL-2 availability [209] and there is an overall reduction in Treg STAT5 signaling upon IL-2 stimulation [210]. Exogenous IL-2 has been shown in a low therapeutic dose to boost Treg numbers and rescue the STAT5-mediated signaling defects observed [135]. This evidence led us to hypothesize that subjects with T1D-associated polymorphic variants in the IL-2/IL-2R pathway genes may have reduced IL-2 signaling in peripheral Tregs. CD25 is the only protein in the IL-2/IL-2R pathway that is not shared with other cytokine signaling cascades. Therefore, we decided to focus our initial assessment on T1D-associated haplotypes defined by SNPs in the *IL2RA* gene. In Chapter 3, we describe that T1D subjects homozygous for the IL-2RArs3118470 CC risk haplotype have reduced phosphorylation of STAT5 in response to IL-2 stimulation. This phenomenon did not extend to other T1D-associated polymorphic variants in the *IL2RA* gene that we assessed, but other groups have shown similar effects in polymorphic variants in the *IL2RA* and *PTPN2* genes. Tree et al have published that, in healthy controls, the disease-associated *IL2RA* rs12722495 risk haplotype is associated with reduced pSTAT5 signaling in Tregs; they have recently extended this finding to T1D subjects [156]. Long et al have also published a similar finding in healthy controls homozygous for the IL2RArs2104286 risk haplotype, who display reduced IL-2 induced STAT5 phosphorylation in their CD4+CD25+ cells [210]. Buckner et al have shown CD4+ T
cells of healthy individuals who carry the risk allele of PTPN2rs1893217 display a reduced response to IL-2 as measured by pSTAT5 and expression of FOXP3, a STAT5-dependent protein. Combined with our data, these studies provide a compelling rationale that T1D-associated polymorphic variations in the IL-2/IL-2R pathway genes result in a functional immune deficit in Tregs’ ability to signal adequately in response to IL-2. Each of these T1D-associated haplotypes defined by different genes in the IL-2/IL-2R pathway may individually have a very subtle effect in the immune response or specifically the IL-2 signaling capability of Tregs. These genetic variants also commonly occur in healthy controls who do not develop T1D. However, combined with other unidentified immune or environmental influences specific to T1D pathogenesis, they could have a large enough effect to disrupt Treg suppression of β cell destruction in T1D.

The plastic ability of Tregs to convert to the Th17 phenotype [38, 102-104, 110, 111] and the increased proportion of FOXP3+IL-17+ cells in T1D, identified in Chapter 2, led us to hypothesize that T1D subjects homozygous for the IL-2RArs3118470 CC risk haplotype, which decreased the signaling response of Tregs to IL-2, could in fact be leading to their conversion to a Th17 phenotype. In Chapter 3, we go on to show that T1D subjects homozygous for the IL-2RArs3118470 CC risk haplotype also have an increased proportion of FOXP3+IL-17+ cells in their peripheral blood. We were able to identify genetically a subset of recent-onset T1D subjects who have an increased proportion of intermediate CD45RA-FOXP3loIL-17+ cells in their peripheral blood. Interestingly, this novel finding does not extend to other T1D-associated polymorphic variants in the IL2RA or PTNP2 genes. Furthermore, the increase in FOXP3+IL-17+ cells was not observed in autoantibody negative controls homozygous for the IL-2RArs3118470 CC risk haplotype. This indicates that other factors, such as the precise
composition of the inflammatory milieu, might influence the increase in the proportion of FOXP3+IL-17+ cells seen in T1D subjects near diagnosis. Interestingly, we did not observe an increase in Th17 cells in the T1D subjects homozygous for the IL-2RAs3118470 CC risk haplotype. Th17 proportion increased, but it did not reach statistical significance. This could be because the sample size was too small and needs to be increased to reduce variability and demonstrate significance. Alternatively, Tregs may not totally downregulate FOXP3 expression and complete the conversion to Th17 at the time of diagnosis, but they do lose suppressive function and attain the ability to secrete IL-17.

We were able to identify genetically a subset of T1D subjects who have Treg IL-2 signaling pathway deficits and an increase in FOXP3+IL-17+ cells in their peripheral blood near diagnosis. However, these correlations do not prove a causative link between the two observations. Further work would be needed to isolate Tregs from T1D subjects homozygous for the IL-2RAs3118470 CC risk haplotype and assess their ability to convert to FOXP3+IL-17+ intermediate cells and Th17 cells. This work was limited by the availability of samples with the correct genotypes. In our cohort we were unable to identify enough T1D subjects with the TT genotype at IL-2RAs3118470. Comparisons between T1D subjects homozygous for the CC risk haplotype and TT genotype at IL-2RAs3118470 would have been optimal to detect differences in Treg dysfunction and conversion to FOXP3+IL-17+ cells.

However, these data described in Chapter 3, imply that genotyping T1D subjects for polymorphic variation in the IL-2/IL-2R pathway is likely to identify a subset of subjects that would respond differently to exogenous IL-2 therapy in clinical trials for T1D. In addition, autologous Treg therapy is being used in phase I/II clinical trials in T1D subjects [30, 243]. It will be important to assess the participants recruited to future Treg therapeutic trials for T1D-
associated risk haplotypes marked by SNPs in the *IL2RA* genes that we found to be associated with FOXP3+ cell conversion to an IL-17 secreting phenotype. This would ensure that FOXP3+IL-17+ cells are not being transplanted back into subjects genetically susceptible to Treg plasticity, with potential deleterious effects.

The results in Chapter 2 showed an increase in IL-17 subsets and FOXP3+IL-17+ cells in recent onset T1D subjects. In Chapter 4, we wanted to address the hypothesis that the changes we observed in Treg and Th17 populations occur before diagnosis of T1D, which would enable identification of biomarkers of T1D. Autoantibody status of subjects is a currently used biomarker and can be a useful predictor of disease onset [180]. The higher number of autoantibodies translates to an increased risk of T1D. However, not all autoantibody positive relatives of T1D subjects will progress to T1D. Therefore, more specific and sensitive biomarker assays that will predict disease onset need to be developed. Biomarkers are important because they enable the initiation of therapeutic intervention before the onset of T1D, when there is likely a higher β cell mass to rescue.

We therefore assessed a cohort of subjects obtained from TrialNet’s Natural History Study that followed relatives of T1D subjects to assess whether they became autoantibody positive and whether they eventually went on to develop disease [264]. These rare samples allowed us to assess the peripheral blood of T1D subjects before they progressed to disease. We hypothesized that subjects before the onset of disease may have a decrease in Treg proportion or an increase in the proportion of IL-17-secreting subsets (including the FOXP3+IL-17+ intermediate subset). However, in analyzing overall Treg proportions, defined as CD127loFOXP3+ or CD127loCD25+, we did not find any difference in the proportion of Tregs before diagnosis. We did find a decrease in the proportion of CD127loFOXP3hi cells in T1D
subjects after diagnosis, which seemed to contradict our findings in Chapter 2 that CD4+FOXP3+ were elevated in proportion in the peripheral blood of recent-onset subjects. However, the cohorts assessed were very different, and enumeration studies of Tregs in T1D have yielded vastly contradictory results depending on the markers used to identify a suppressive Treg cell (all of which are also expressed in activated human T conventional cells [27]) and the cohort of subjects being studied [237] [241] [238-240]. The only way to look effectively at bona fide suppressive Tregs would be to identify T cells that are fully demethylated at the TSDR region of the FOXP3 promoter [278]. Future work could include epigenetic analysis based on qPCR methods to measure the ratio of the TSDR versus CD3 cells and determine the ratio of natural Tregs in the T cell population [31].

We identified a CD39-expressing Treg subset that seemed to be reduced in proportion in T1D subjects before the onset of disease. This could potentially be a new biomarker of T1D, since decreased proportions of CD39+ Tregs are also observed in autoantibody negative relatives of T1D subjects who do not progress to disease. The CD39+ Tregs are a relatively newly identified subset that seem to have a highly suppressive function mediated by the conversion of ATP to AMP by CD39 on their surface [272]. They have also been identified as reduced in proportion compared with controls in subjects with other autoimmune diseases, such as relapsing/remitting multiple sclerosis [41]. This work leads to the hypothesis that the subset of CD39+ Treg cells is pathogenically involved in T1D onset or that Tregs that are dysfunctional in T1D actively downregulate CD39. A further hypothesis is that the CD39+FOXP3+ cells are actively converting towards an IL-17 secreting phenotype in disease pathogenesis. Further work is required to validate these preliminary findings and to determine whether CD39+ Tregs act as a biomarker for T1D and secrete IL-17.
We also found changes in proportions of the IL-17 secreting subsets only after T1D diagnosis and not before. This may be for a number of reasons including; a direct role for hyperglycaemia in promoting T cell IL-17 secretion; our sample size being too small to observe subtle effects; and large variability in islet cell immune pathogenesis at diagnosis within the cohorts. However, taken in context with the data from Chapter 3, it is strongly possible that a conversion of Tregs to an IL-17 secreting phenotype is occurring before the time of diagnosis, but that this only occurs in a subset of subjects who possess the T1D-associated risk haplotypes marked by SNPs in the \( IL2RA \) gene. Therefore, the grouping together of a large cohort of subjects in arbitrary ‘before diagnosis’ or ‘at diagnosis’ time points leads to subtle differences guided by genetic variation not being observed.

5.3 Model

These data combined suggest the following model of pathogenesis in a genetically identifiable subset of T1D subjects (illustrated in Figure 5.1 below).

An initial environmental pathogenic trigger causes the release of auto-antigen from pancreatic \( \beta \) cells. Innate immune system cells such as macrophages and dendritic cells arrive at the pancreatic islet causing an initial inflammation. These cells help recruit naïve Tregs to the site of inflammation and, upon recognition of self-antigen and local IL-2 stimulation, initially express high levels of FOXP3+ and act in a suppressive capacity to stop the autoimmune destruction of the islets by previously self-antigen primed diabetogenic CTL. However, a subset of T1D subjects, with T1D-associated risk haplotypes marked by SNPs in the IL-2/IL-2 signaling pathway, have Tregs with defects in their ability to phosphorylate STAT5 in response to IL-2, and so their Tregs can not maintain high levels of FOXP3+. Their Tregs slowly lose FOXP3
expression and transition to a IL-17 secreting phenotype that have reduced suppressive capabilities. We therefore observe a decrease in the CD39+FOXP3+ cells before disease onset and an increase in FOXP3+IL-17+ (CD45RA-FOXP3lo) cells at disease onset in T1D subjects homozygous for the IL-2RArs3118470 CC risk haplotype. The Treg to Th17 conversion process is only complete after disease onset of T1D. That is why we observed that recent-onset T1D subjects have an increased Th17 cell proportion in their peripheral blood compared with controls.

Figure 5.1: Model of T1D pathogenesis
In addition, a Treg to Th1 conversion could occur in the pathogenesis of T1D. Also Th17 cells could transition to a Th1 phenotype. The altered Treg/Th17 balance proposed would therefore promote the ability of conventional Th1 cells to contribute to disease pathogenesis.

This model suggests a number of further experiments that could be performed to confirm its validity. There is also a possibility that the FOXP3+IL-17+ cells described above have some immunosuppressive capacity and are not pathogenic in T1D. It would, therefore, be important to develop a sorting strategy for these populations to assess their suppressive capabilities in response to a number of stimuli. CD39-expressing Tregs from T1D subjects could be isolated and the factors that enable them to lose CD39 expression and transition toward an IL-17-secreting phenotype could be assessed in vitro. One limitation is the fact that CD39+FOXP3+ cells cannot be sorted from the peripheral blood of T1D subjects, because they require intracellular staining to be identified; validated extracellular markers for CD39+ Tregs would need to be developed. Further experiments could also be done to determine the TCR repertoire of CD39+Tregs and FOXP3+IL-17+ cells, and so identify which pancreatic antigens they recognize in the context of T1D. Finally, it would be important to isolate Tregs from T1D subjects with the IL2RARS3118470 CC risk haplotype and determine whether they are more easily able to transition in vitro into an IL-17 secreting phenotype. However, this work would be limited by the fact that the precise in vitro conditions required to convert human Tregs into FOXP3+IL-17+ cells are not completely known. Our data shows that the increase in FOXP3+IL-17+ only occurs in T1D subjects and not autoantibody negative controls, which implies that possession of the IL-2RARS3118470 CC risk haplotype is not likely enough by itself to lead to the conversion of Tregs
to FOXP3+IL-17+ cells. It is likely that in vitro work will not replicate the in vivo local pancreatic environment that would be needed to observe this plasticity.

5.4 General limitations

The limitations of this research and performing the above experiments are generic to most research involving human T1D subjects. The samples available for T1D research, for ease of analysis, are grouped into time points in relation to disease onset. However, within these cohorts of subjects, much inter-individual variation will occur in genetically associated T1D loci, sample processing and stage of disease pathogenesis at diagnosis.

In vivo data and tissue samples to validate findings observed in the peripheral blood of T1D subjects are also lacking. For example, detection of large numbers of antigen specific T cells is likely to occur only in the pancreatic lymph nodes at the height of disease pathogenesis. In addition, as mentioned previously, it is very hard to replicate in vitro the precise local inflammatory milieu in the pancreas that would lead to the ability of Tregs to transition toward an IL-17 secreting phenotype. This in vivo data can be obtained from genetically manipulated mouse models of T1D. However, not all the murine and human Treg and Th17 pathways are equivalent. For example, CD39 is constitutively expressed on all murine Tregs but not in humans [279]. The mouse models of disease are genetically identical clones, which implies they do not reflect the genetic diversity apparent in human T1D subjects and are only representative of a small genetic subset of human T1D subjects [280]. Almost all of the 100+ reagents that have been shown to be effective in delaying disease onset in the NOD mouse model of human T1D have not been translated into successful human clinical therapeutic trials [281]. Therefore, a
general limitation prevails in T1D research because of lack of \textit{in vivo} verification of human T1D peripheral blood findings in mouse models that do not necessarily reflect human disease.

5.5 \textbf{Therapeutic future directions}

The search for a cure for T1D has generated many experimental approaches, including islet or \( \beta \) cell transplantation coupled with sufficient protection from autoimmune destruction. The Edmonton Protocol introduced islet transplantation as an effective means of treating T1D [282]; however, its widespread use is hampered by a shortage of donor tissue, the lifelong need for immunosuppressive therapy, and uncertainty regarding the long-term function of islet grafts [283-287]. Moreover, at present, gross immune suppression of children with recent-onset T1D to block autoimmunity does not represent a viable treatment because of the unacceptable risks and adverse effects inherent to broad-spectrum immunosuppressive drugs. What is needed is the development of specific and well-tolerated therapies that will halt T cell and cytokine-mediated destruction of \( \beta \) cells.

Toward developing such a therapy, permanent or long-term interruption of autoimmune \( \beta \) cell destruction at the time of clinical presentation may preserve sufficient \( \beta \) cells to maintain physiological insulin secretion indefinitely. This approach is potentially possible because functional \( \beta \) cells remain present within islets at the time of disease presentation, as confirmed by histological analysis. Animal studies have also indicated that interrupting autoimmunity after the onset of overt disease induces long-term remission of disease [288, 289].

Two promising therapeutic modalities that have shown preventative effects in the NOD mouse model are exogenous IL-2 (though this is heavily dose- and timing-dependent) and autologous Treg therapy [30, 136, 243]. These therapies have also both shown some promise in
very early phase studies but are yet to be validated with large-scale randomized control trials. In a previous trial involving IL-2 and rapamycin, a transient deterioration occurred in pancreatic function despite a rescuing of the Treg IL-2 signaling defect observed in T1D subjects [135]. Klatzman et al found that lowering the dose of IL-2 avoids the transient dip in insulin/c-peptide secretion [136]. For future clinical trials, it would be important to identify the participants with T1D-associated risk haplotypes defined by SNPs in the IL2RA and PTPN2 genes. This may explain differences in response to IL-2 therapy and allow pharmacogenetic targeting of this therapy to particular subsets of the T1D heterogeneous population, who are more likely to have defects in the IL-2/IL-2R pathway leading to their particular pathogenesis of disease. Autologous Treg therapy has shown some anecdotal success in preserving β cell function in individual subjects [30]. However, we have identified genetically a subset of subjects whose Tregs might be more prone to IL-17 secretion and conversion to a pathogenic state. Our data suggests that Treg therapy is best targeted toward those genetically identifiable subsets of T1D subjects who do not have Treg IL-2 signaling defects that promote the generation of IL-17+FOXP3+ cells.

Taken in context with the other murine and human studies, these data provide a strong rationale for a role for IL-17, which promotes the apoptosis of β-cells, in the pathogenesis of T1D [227, 229, 231]. We have helped establish a justification for the use of agents that target the IL-17 pathway in T1D. One possibility is to use the specific IL-17 inhibitor secukinumab that has been shown to be effective in psoriasis [92, 93]. However, this targeting of a single pathway would suggest that IL-17 is the sole or primary mechanism by which T1D pathogenesis occurs. Our data suggests that the IL-17+ secreting Tregs would only be pathogenic in a small subset of T1D subjects who have T1D-associated polymorphic variation in the IL2RA gene. A better
approach, then, would be to target secukinumab use in T1D subjects who possess functionally relevant T1D-associated polymorphic variation in the IL2RA and PTPN2 genes.

Despite the heterogeneous T1D population, it would be possible to increase chances of efficacy by targeting multiple immune pathways simultaneously. We showed an increase in IL-17+ IFN-γ positive cells in recent-onset T1D subjects and this might suggest that there is a synergistic effect between these two pro-inflammatory pathways in T1D. The conversion of Th17 cells into intermediate Il-17+ IFN-γ type cells has been reported in the autoimmune conditions juvenile arthritis and Crohn’s disease [68, 109]. Mathis and colleagues showed that, when β cell specific CD4+ T cells from TCR-transgenic BDC2.5 NOD mice were polarized to a Th17 phenotype and then transferred to non-diabetic NOD/SCID recipients, the cells accelerated diabetes, but only after differentiating into a Th1-like phenotype [112, 113]. In addition, Tc17 cells that target hemagglutinin on pancreatic β cells induced diabetes only when co-transferred with diabetogenic CD4+IL-12+ T cells that would allow Tc17 conversion to an IFN-γ secreting phenotype[225]. Recently, it was shown that NOD mice doubly deficient in both IL-17 and IFN-γ receptors showed greater protection from diabetes in comparison to IL-17 single-knockout NOD mice [217].

These data provide a strong rationale to test agents that will suppress multiple pro-inflammatory axes simultaneously in the early phases of T1D. The anti-p40 monoclonal antibody ustekinumab is currently in use for psoriasis, where it has been shown to be both safe and effective. This antibody, inducing a blockade of both IFN-γ and IL-17 signaling pathways, is a logical candidate for evaluation in T1D clinical trials. We have therefore initiated a phase I/II trial with this compound in recent-onset T1D subjects. This clinical trial will definitively test the
hypothesis, supported by these data, that blockade of IL-17 pathways can halt the conversion of Tregs into a pathogenic IL-17 secreting phenotype in recent-onset T1D subjects.
References


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