THE ROLE OF HUMAN FORKHEAD BOX P3
IN CD4\(^+\) T CELL SUBSETS

by

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BSc, The University of Victoria, 2007

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES
(Experimental Medicine)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)

August 2012

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Abstract

The immune system eliminates threats to the body, but it must also prevent immune-mediated damage caused by inflammation and autoimmune disease. One way that immune responses are limited is by specialized T cells known as T regulatory (Treg) cells. The transcription factor forkhead box P3 (FOXP3) is highly expressed in Treg cells and is critical for their suppressive function. The importance of FOXP3 is demonstrated in humans with a severe autoimmune disease called immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) caused by mutations in FOXP3. Furthermore, conventional T (Tconv) cells can be re-programmed into suppressive cells upon stable over-expression of FOXP3. Therefore, there is tremendous interest in manipulating FOXP3 function and/or using Treg cells as a cellular therapy to modify immune responses of cancer patients, patients suffering from autoimmune disease, and transplantation patients. To better understand the function of FOXP3, the first goal was to investigate how mutant forms of FOXP3 found in IPEX patients were defective at programming Treg characteristics. Surprisingly, mutant forms of FOXP3 were not completely deficient at converting Tconv cells into Treg cells, suggesting that factors besides a defect in Treg cells may contribute to IPEX pathogenesis. FOXP3 is transiently up-regulated in human Tconv cells upon activation, but its role in these cells is unknown. Hence, the second goal was to examine the function of FOXP3 in Tconv cells by comparing FOXP3-deficient with wild type Tconv cells. FOXP3-deficient Tconv cells proliferated more and produced more cytokines than wild type Tconv cells. This finding suggests that FOXP3 has a role in the regulation of Tconv cell activation, especially in Th17 cells which were found to highly express activation-induced FOXP3. Lastly, the possibility of using FOXP3+ cells as a cellular therapy was investigated. A method to expand large,
pure populations of human and cynomolgus Treg cells was developed, and ex vivo expanded Treg cells were able to promote mixed chimerism and tolerance to a kidney transplant in cynomolgus macaques. Together, this work sheds light on the role of FOXP3 in CD4+ T cell subsets and helps pave the way for use of Treg cell therapy in the clinic.
Preface

Chapter 1 is composed of excerpts from two previously published articles. The first was reprinted from the Springer eBook Advances in Experimental Medicine and Biology, Volume 665, Section 1, McMurchy AN, Di Nunzio S, Roncarolo MG, Bacchetta R, and Levings MK, Molecular Regulation of Cellular Immunity by FOXP3, pages 30-45, Copyright (2010), with permission from Springer. I wrote the manuscript with the exception of the section entitled “Molecular and Cellular Biology of IPEX” which was written by Sara Di Nunzio. I edited the final manuscript together with the other authors.

Excerpts from the second article were reprinted from Seminars in Immunology, Jan;42(1), McMurchy AN, Bushell A, Levings MK, and Wood KJ, Moving to tolerance: clinical application of T regulatory cells, pages 27-34, Copyright (2011), with permission from Elsevier. The article was co-written with Andrew Bushell who was the primary author of the sections entitled “Early indications that Treg cells could prevent graft rejection,” “Induced Treg cells as a cellular therapy,” “The current clinical experience,” and “Impact of concurrent immunosuppression.” I was the primary author of the remaining sections, and I edited the final manuscript together with the other authors.

A version of chapter 2 has been published and was reprinted from The Journal of Allergy and Clinical Immunology, Dec;126(6), McMurchy AN, Gillies J, Allan SE, Passerini L, Gambineri E, Roncarolo MG, Bacchetta R, and Levings MK, Point mutants of forkhead box P3 that cause immune dysregulation, polyendocrinopathy, enteropathy, X-linked have diverse abilities to reprogram T cells into regulatory T cells, pages 1242-51, Copyright (2010), with permission from Elsevier. I wrote the manuscript and performed all
experiments except DNA methylation analysis, X-chromosome inactivation analysis, and generation of cDNA. Virus was produced by Jana Gillies and Rupi Dhesi.

I conducted all experiments in chapter 3, some with the help of co-authors: I cloned CD4⁺CD25⁻ T cells together with Sara Di Nunzio, and Jana Gillies conducted experiments with my supervision.

Research in chapter 4 was conducted in collaboration with Dr. Bruce Verchere at the Child & Family Research Institute, Vancouver, BC, and with Dr. Megan Sykes and Dr. Yong-Guang Yang at the Transplantation Biology Research Center, Harvard Medical School, Boston, MA, USA. I performed all procedures involving human cells including the optimization of Treg cell expansion procedures and generation of T cell lines for injection into NSG mice. Transplant of human islets, care of NSG mice, injection of human cells, and sacrifice of mice was performed by Derek Dai, a technician in Dr. Verchere’s laboratory. I performed all analysis of blood and spleen samples after sacrifice, except immunohistochemistry which was performed by Derek Dai.

I performed initial experiments on cynomolgus macaque T cells together with Dr. Leo Buhler during a foreign study term for 3 months at the Transplantation Biology Research Center. I performed all lentiviral transduction experiments and advised researchers how to expand Treg cells ex vivo and evaluate their function in vitro. Dr. Leo Buhler expanded the ex vivo cynomolgus Treg cells that were eventually injected into the cynomolgus macaque. Subsequent bone marrow transplant, kidney transplant, and monitoring of chimerism kidney function were performed by Dr. Leo Buhler and Dr. Hugo Sondermeijer.
All research involving human tissues was approved by the Clinical Research Ethics Board (H03-70062) and research involving animals was approved by the Animal Care Committee (A07-0438).
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Acknowledgements

I am extremely grateful to my supervisor Dr. Megan Levings who gave me exceptional guidance and support over the past five years. I am truly fortunate to have had such a rich graduate experience and to have such an inspiring mentor.

I thank Dr. Yong-Guang Yang and Dr. Megan Sykes for my time spent collaborating on a project at the Transplantation Biology Research Center in Boston, and I thank Dr. Bruce Verchere for guidance on our collaborative project. I am also grateful to my supervisory committee members Dr. Sandra Dunn, Dr. Tobias Kollmann, and Dr. Bruce Verchere for feedback on my research.

I thank Jana Gillies and Derek Dai for working with me on my projects and also Zheng Hu and Dr. Leo Buhler who I worked with closely in Boston. I am grateful for the support for cell sorting and flow cytometry provided by Lisa Xu at the Flow Cytometry Core Lab at the Child and Family Research Institute. Special thanks go to everyone in the Levings’ laboratory past and present for providing a supportive and collaborative atmosphere. I am particularly grateful to Sarah Allan for training me as a new graduate student and for being an inspiring role model.

My research was supported by the Canadian Institutes for Health Research (Canada Vanier Scholarship, Canadian Graduate Scholarship Master’s Award, and Transplantation Training Program Award) and the Michael Smith Foundation for Health Research (Junior Graduate Scholarship). My foreign study term in Boston was supported by the CIHR Foreign Study Supplement. Thanks are also owed to the blood donors who made this research possible.
For my parents Daryl and Ellen Alstad, whose support has allowed me to pursue my goals, and for my patient and encouraging husband Sean who inspires me every day to learn and grow professionally and personally.
Chapter 1: Introduction

The immune system provides protection from dangerous entities such as pathogenic microbes and cancerous cells. An important feature of the immune system is its ability to distinguish between harmful substances and the cells and structures that make up one’s own body. The lack of an immune response to self is called self-tolerance. When self-tolerance breaks down, disease can result. These diseases are called autoimmune diseases, and some examples include type 1 diabetes, multiple sclerosis, and rheumatoid arthritis.

Beyond autoimmune diseases, the concept of tolerance is also important for individuals who have undergone organ transplantation to replace a damaged organ. This is because the immune system recognizes cells from the donor organ as dangerous and responds by attacking and destroying those cells in a process called graft rejection. To prevent graft rejection, these patients must take drugs that suppress the function of their immune system for the rest of their lives, leaving them vulnerable to infections and cancer. Thus, it would be highly beneficial to better understand how tolerance works to develop better therapies to treat or prevent autoimmune diseases and also to find better ways to prevent graft rejection after organ transplantation.

1.1 Self-tolerance: a historical perspective

Research into self-tolerance has been closely linked with organ transplantation research. Peter Medawar is often referred to as the father of transplantation research and indeed was the first to observe that rejection of donor skin grafts was an immune-mediated process. Following Ray Owen’s observation in 1945 that fraternal twin cattle that share placental vasculature, called freemartin cattle, shared red blood cells (red blood cell
chimeras), Medawar and colleagues showed that these cattle could accept skin grafts from one another. This observation supported a previous hypothesis by Frank Macfarlane Burnet that the age of the animal when antigens are encountered determines its tolerance to those antigens. Hence, freemartin cattle, which share antigens from blood in utero, are tolerant to skin grafts from the same donor as adults. This hypothesis was further supported by seminal research by Billingham, Brent, and Medawar in 1953 in which they injected fetal or newborn mice with splenocytes from a mouse of another strain. These mice were subsequently able to accept skin grafts from the same donor, but not from a 3rd party strain. The hypothesis that the age of the animal determined tolerance was subsequently challenged with research that showed that adult mice that had undergone bone marrow transplants could also accept skin grafts from the same donor. Accordingly, Joshua Lederberg proposed in 1959 that it was not the age of the animal, but rather the age of the lymphocyte that determined tolerance. The idea that the age of the lymphocyte is the important determinant in tolerance is now known as central tolerance and is now understood to be a process whereby developing, self-reactive lymphocytes are deleted in the thymus (T cells) or bone marrow (B cells) before being released into the periphery.

Central tolerance is not the only mechanism, however, that prevents autoimmunity and promotes tolerance to self. Other peripheral mechanisms also operate including ignorance, anergy, and exhaustion. Ignorance is the physical partitioning of the immune system from immune privileged regions of the body; for example, immune cells have restricted access to the eye, brain, and testes. T cells can become anergic (resistant to subsequent activation and proliferation) when they encounter their antigen in the absence of any danger signals which provide co-stimulation. Furthermore, T cells become exhausted
after repeated encounters with antigen and danger signals; in this way, the immune response can be shut off to prevent an excessive response that could cause damage to healthy tissues. These peripheral mechanisms as well as central tolerance are considered to be recessive forms of tolerance because a tolerant state brought about by these mechanisms in one animal cannot be transferred to a naive animal. However, a dominant form of tolerance also operates in the form of regulatory cells that can actively suppress immune responses.

The first indication that cells with regulatory characteristics might exist and be important for self-tolerance came from work by Nishizuka and Sakakara in 1969 in which they observed that neonatal thymectomy of mice 2-4 days after birth resulted in autoimmunity. This autoimmunity could be transferred to immunodeficient mice by adoptive transfer of T cells, and the autoimmunity could be reversed in thymectomized mice by transfer of T cells from wild type mice, suggesting the presence of a T cell that develops in the first 4 days after birth that suppresses autoimmunity. Gershon and Kondo formally proposed the existence of “T suppressor” cells in 1970 after observing that T cells could suppress antibody responses. It wasn’t until after 1995, however, that intense research into T cells with regulatory properties began, following the discovery by Shimon Sakaguchi that T cells with regulatory capacity highly express the IL-2 receptor alpha chain (CD25), providing a means by which to enrich for and study these cells. Sakaguchi observed that transfer of CD4⁺CD25⁻ T cells into immunodeficient mice resulted in autoimmunity whereas co-transfer with CD4⁺CD25⁺ T cells protected mice from disease. The same year, the dominance of suppression by T regulatory (Treg) cells was demonstrated. These experiments showed that T cells could develop in a nude mouse (which does not have its own thymus) after transplant with a thymus from a mouse of another
strain. These mice could accept tissues from the same donor strain as the thymus graft. Importantly, if T cells were transferred from these mice into nude mice without thymus grafts, the mice could accept tissues from the thymus donor strain when a large number of T cells were injected, but rejected the tissues if a small number of T cells were transferred. These experiments showed that self-reactive T cells develop in the thymus and can escape into the periphery as indicated by their ability to cause rejection, but that when a sufficient number of Treg cells are present, the rejection can be inhibited.

1.2 **FOXP3+ Treg cells**

1.2.1 **Phenotype and function of FOXP3+ Treg cells**

Many different types of T cells with regulatory activity have been described including CD8+ T cells, \(^{12,18-21}\) CD4-CD8- double negative T cells, \(^{22,23}\) NK T cells, \(^{24}\) and \(\gamma\delta\) T cells, \(^{25}\) but these are all less well studied than their CD4+ T cell counterparts. In 2003, it was discovered that the suppressive function of CD4+CD25+ T cells relies on high level expression of the transcription factor forkhead box P3 (FOXP3). \(^{26-28}\) Since FOXP3 is a nuclear protein, live FOXP3+ cells cannot be isolated except from reporter mice; \(^{29-31}\) thus, the majority of studies rely on expression of CD25 to isolate Treg cells and investigate their biological properties, although CD25 is also expressed on activated effector T cells. Beyond expression of CD25, some other notable characteristics of FOXP3+ Treg cells include their inability to produce typically T-cell-derived cytokines such as IL-2, IFN-\(\gamma\), or TNF-\(\alpha\) and their in vitro hyporesponsiveness to T cell receptor (TCR) activation. \(^{32-34}\) They do, however, retain the ability to produce immunosuppressive cytokines such as IL-10, TGF-\(\beta\), and the recently described IL-35. \(^{35-41}\) The cell-surface proteins expressed by Treg cells are generally
characteristic of activated T cells, and in addition to CD25, include cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and glucocorticoid induced tumour-necrosis factor receptor family-related gene (GITR)\(^{42-44}\) but not CD127 (IL-7 receptor, alpha chain).\(^{45,46}\) Other potentially more Treg-specific proteins include folate receptor 4,\(^{47}\) a glycoprotein A repetitions predominant (GARP),\(^{48}\) and neuropilin-1,\(^{49}\) but further research is required to clarify the specificity and utility of these molecules.

Although much research has been done on FOXP3\(^+\) Treg cells, it is still unclear how these cells suppress immune responses to maintain self-tolerance and limit immune responses. What is clear is that they can suppress a number of different immune cells including the activation of antigen-presenting cells but can also directly suppress the activation, proliferation, and effector functions of CD4\(^+\) and CD8\(^+\) T cells.\(^{50}\) Furthermore, they can suppress B cells, mast cells, and NK cells.\(^{50}\) Cell contact is required for suppression, but these cells can also secrete immunosuppressive cytokines as discussed above.\(^{50}\) Importantly, TCR recognition of the cognate antigen is required to activate the suppressive function of Treg cells, but once activated, they can mediate indiscriminate bystander suppression. This bystander suppression is important for the process of linked-suppression in which tolerance to 3\(^{rd}\) party graft antigens is established if they are expressed together with antigens that are already tolerated.\(^{51-53}\)

There is extensive evidence from animal models that insufficient Treg cell numbers and/or function can lead directly to autoimmunity and allergy, whereas an over-abundance of these cells can suppress anti-tumour and anti-pathogen immunity. Evidence that adoptive transfer of Treg cells can not only prevent autoimmunity in FOXP3-deficient mice\(^{28}\) but can also prevent and/or reverse other pathologies such as type 1 diabetes,\(^{54}\) inflammatory bowel
disease,\(^5\) graft versus host disease,\(^5,6,7\) and rejection of transplanted organs\(^5,8,9\) has led to widespread interest in developing similar cell therapy-based approaches in humans. Indeed, in humans, a reduction in the number or function of Treg cells is often, but not always, associated with various autoimmune diseases including myasthenia gravis, autoimmune polyglandular syndrome type II, ulcerative colitis, and multiple sclerosis,\(^4,3,60-65\) graft-versus host disease and allograft rejection.\(^66,67\)

1.2.2 Role of FOXP3 in the development of Treg cells

1.2.2.1 Naturally occurring versus induced Treg cells

Treg cells may either arise directly in the thymus or be induced in the periphery when naive CD4\(^+\) T cells encounter their antigen in a tolerogenic environment. Currently, there are no known phenotypic markers that distinguish thymically-derived or naturally-occurring (n) Treg from peripherally-induced (i) Treg cells. There is evidence, however, that they likely differ in antigen-specificity since nTreg cells tend to recognize self-antigens\(^68,69\) and are selected when the strength of TCR signaling is above that of classical positive selection but below that of negative selection.\(^70\) In contrast, iTreg cells can be specific for any antigen presented in the context of tolerogenic dendritic cells\(^71\) or immunosuppressive cytokines such as TGF-\(\beta\) as discussed in more detail below.

There is little doubt that FOXP3 is required for the normal function of Treg cells since the lack of this gene results in autoimmunity, but there is debate on the precise role of FOXP3 in the thymic differentiation of nTreg cells. Several years ago, mixed bone marrow chimera experiments revealed that stem cells from mice genetically deficient for FOXP3 were unable to give rise to CD4\(^+\)CD25\(^+\) T cells,\(^28\) leading to the conclusion that FOXP3 was
necessary for this process. In more recent experiments, however, it was found that although expression of FOXP3 is required for suppressive activity, cells expressing a Treg-associated gene signature and cell surface molecules still developed in the absence of FOXP3. These findings suggest that the role of FOXP3 may be to amplify and stabilize the pre-determined Treg lineage rather than to define the lineage itself. In support of this idea, it has been reported that many aspects of the Treg cell signature are not directly controlled by FOXP3 since it contains gene clusters that are co-activated with, rather than trans-activated by, FOXP3.

When naive peripheral T cells are activated in the presence of TGF-β and IL-2, expression of FOXP3 is induced, and the resulting iTreg cells are suppressive in vitro and in vivo. Interestingly, in vitro addition of all-trans retinoic acid, a vitamin A metabolite, can enhance this conversion, and it has recently been shown that the source of a similar vitamin A signal could be from gut derived CD103+ dendritic cells. TGF-β may also be involved in the development of nTreg cells since it has recently been shown that conditional deletion of TGF-βRI blocks the appearance of Foxp3+ thymocytes. An outstanding question is whether these TGF-β-induced Treg cells are stable and persistent FOXP3+ cells. Floess and colleagues observed that when TGF-β was removed, FOXP3 expression is rapidly reduced, suggesting that exposure to TGF-β alone is not sufficient to generate stable iTreg cells. Indeed, beyond TGF-β there is also clearly a requirement for IL-2 in this conversion. Evidence that expression of FOXP3 in TGF-β-induced Tregs is stabilized in inflamed environments in vivo suggests that cytokine signals in addition to IL-2 may be required for the stability, survival and/or expansion of iTreg cells.
1.2.2.2  Re-programming CD4+ T cells into Treg cells by ectopic expression of FOXP3

The finding that retrovirus-mediated over-expression of FOXP3 in naive CD4+ T cells from mice is sufficient to recapitulate all of the known features of Treg cells, including suppression of autoimmunity and rejection of transplanted grafts in vivo,27,28,87 led to the idea that similar gene-and-cell therapy based approaches should be possible in humans. This idea was first investigated in 2003, following the original reports from the groups of Sakaguchi, Rudensky and Ramsdell.26-28 It was initially found, however, that retrovirus-mediated over-expression of FOXP3 in human CD4+ T cells was not sufficient to generate a population of cells with potent in vitro suppressive capacity.88 Further investigation into why there was an apparent discrepancy between the ability of FOXP3 to re-program mouse and human cells into Treg cells led to the finding that in order for human CD4+ T cells to be efficiently converted into Tregs, they must express not only high, but also stable levels of FOXP3.89

A lentivirus-based method was developed to ectopically express FOXP3 under control of the elongation factor one alpha promoter, the activity of which does not depend on the activation state of the cell. When FOXP3 was over-expressed in naive or memory CD4+ T cells from adult peripheral blood using this method, a population of T cells was generated that was phenotypically and functionally identical to ex vivo Treg cells based on in vitro assays of proliferation, cytokine production, cell surface marker expression and suppressive capacity. These data indicate that the ability of FOXP3 to convert conventional CD4+ T cells into Treg cells is more tightly regulated in humans than in mice, possibly due to the fact that FOXP3 can also be expressed transiently in activated Tconv cells90 as discussed below. The ability to generate and efficiently expand stable populations of suppressive Treg cells in vitro
is an important advance in the development of therapeutic applications to translate the immunosuppressive powers of these cells to the clinic.

The lentivirus-based system was also used to investigate the dose- and time-dependent requirements for expression of FOXP3 to mediate efficient conversion into Treg cells. Using a version of FOXP3 fused to the hormone binding domain of the estrogen receptor (ER), an inducible form of the transcription factor was created, the activity of which can be pharmacologically controlled by the estrogen analog 4-hydroxytamoxifen (4HT).91 The relative suppressive capacity of transduced T cells was correlated with higher concentrations of 4HT corresponding to higher levels of FOXP3 activity. Moreover, the suppressive capacity was maximal when cells were kept in 4HT for 7-12 days. Since 4HT-induced nuclear translocation of FOXP3 happens with a few hours, these data suggest that significant changes in gene expression are necessary before the functional effects are manifested. Notably, the suppressive capacity was rapidly reversed when 4HT was withdrawn from the FOXP3-ER-expressing cells.

1.3 Molecular and cellular biology of IPEX

FOXP3 is an X-linked gene (located in Xp11.23) whose fundamental importance in immune regulation was first recognized in 2001 when three parallel studies identified it as the gene mutated in the Scurfy mouse92 and in humans with IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked).93,94 Scurfy mice arose as a spontaneous mutation at the Oak Ridge National Laboratory in 1949 and suffer from an X-linked, rapidly fatal autoimmune disease mediated by CD4⁺ T cells.95
Patients with IPEX suffer a similar autoimmune disease, the main features being enteropathy, characterized by refractory diarrhoea, multiple endocrine organ autoimmunity, such as type 1 (insulin-dependent) diabetes and thyroiditis, hyper IgE and eczema (www.ipexconsortium.org).93,94,96-98 The onset of the disease is often in early infancy and the course can be rapidly lethal. The disease is rare, but retrospective data on clinical cases of early autoimmune enteritis associated with type 1 diabetes or of neonatal diabetes of unknown origin suggests that the actual frequency of the disease may be underestimated.98 In addition to supportive care (including parenteral nutrition, blood transfusions, and treatment of diabetes) immunosuppressive drugs, such as high-dose steroids, cyclosporin A, tacrolimus, methotrexate, infliximab, and rituximab, are commonly used to treat IPEX patients.97 Unfortunately, immunosuppression is usually only partially effective and the dose is limited by infectious complications and toxicity. More recently, sirolimus (rapamycin) has been used to treat IPEX patients99,100 since it can specifically suppress Tconv cells and spare, or even promote, Treg cell expansion.101,102

Currently, the only curative treatment for IPEX patients is bone marrow transplantation,98,103 but this approach is limited by toxicity and availability of HLA-compatible donors. Thus, a gene-therapy based approach to correct genetic defects in FOXP3 in T cells would be a novel treatment option. In healthy females who are heterozygous for different types of FOXP3 mutations, it was found that their peripheral blood mononuclear cells and CD4+ naive, effector and memory T cells have a random pattern of X-chromosome inactivation.104 In contrast, circulating Treg cells in these women exclusively express the wild type allele of FOXP3, suggesting either that wild type FOXP3 is necessary for the development of Treg cells, or that in vivo it gives a selective advantage for the normal
homeostasis of Treg cells. The latter possibility would be in line with the notion that FOXP3 is required for the "competitive fitness" of Treg cells.\textsuperscript{72} The fact that Treg cells expressing wild type FOXP3 can maintain peripheral tolerance in subjects whose other haematopoietic cells are mosaics of wild type and mutant FOXP3, supports the rational for gene or cellular-based therapeutic approaches to restore Treg cell function in IPEX patients.

To date, more than thirty different types of FOXP3 mutations have been described, including mis-sense mutations, splice site alterations, and deletions.\textsuperscript{97} Importantly, the type of mutation does not necessarily correlate with clinical manifestations. Fourteen unrelated affected males were recently studied to investigate whether FOXP3 mutations and changes in protein expression correlated with molecular and clinical data. Notably, the only known mutations that completely prevent transcription and expression of FOXP3 protein are those that occur in the ATG start codon, and these IPEX patients have very severe disease.\textsuperscript{98} All patients, and particularly those carrying mutations in known functional domains of FOXP3 or mutations that alter protein expression, have enteropathy, generally associated with endocrinopathy and eczema. Similar genotypes, however, did not always result in similar disease presentation and severity. These data indicate that beyond FOXP3, other genetic and environmental factors contribute to the development of IPEX.\textsuperscript{98}

In order to understand how FOXP3 regulates autoimmunity in humans, whether IPEX patients have numerical or functional defects in Treg cells has been investigated. Depending on the type of mutation, surprisingly many IPEX patients have normal numbers of circulating FOXP3\(^+\) T cells.\textsuperscript{98,105} Moreover, only Treg cells from patients with mutations in the ATG start codon, which completely abrogates expression of FOXP3, completely lack suppressive activity \textit{in vitro}.\textsuperscript{105} In contrast, patients who have mutations in the FKH domain of FOXP3
have more subtle changes in Treg cell function that depend on the strength of TCR activation and whether the targets of suppression are allogeneic or autologous. Thus, normal DNA-binding activity of FOXP3 is not essential for Treg development and moreover, IPEX is not simply a result of a global defect in Treg cells.

IPEX patients have an increased frequency of Th17 cells, and Treg cells in these patients may differentiate into Th17 cells in the absence of functional FOXP3. Another finding from studies on IPEX patients is that, in some cases, their conventional CD4⁺CD25⁻ T cells display a defect in production of IL-2 and IFN-γ, suggesting that IPEX could be due not only to impaired Treg cell function but also to a parallel defect in Tconv cell function. These data are in direct contrast to the finding that in FOXP3-deficient mice Tconv cells are hyper-activated and produce increased levels of pro-inflammatory cytokines. They do, however, correlate with the fact that in humans FOXP3 is also expressed transiently in activated Tconv cells, suggesting that there is a functional role for FOXP3 outside of Treg cells that has yet to be defined.

1.4 Role of FOXP3 in conventional T cells

A unique property of conventional CD4⁺ T cells in humans is that they also express FOXP3 transiently when they are activated. It has been suggested that this is exclusively a TGF-β-mediated process, but this has not been confirmed. Activated Tconv cells express FOXP3 maximally three days after TCR-stimulation and, under conditions of strong activation, almost 100% of Tconv cells become FOXP3⁺. However, a key difference between activated Tconv cells and Treg cells remains evident: although the intensity of FOXP3 expression of activated Tconv cells reaches that of resting Treg cells, it

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remains significantly lower than that in similarly-activated Tregs cells.\textsuperscript{108} These data correlate with the finding that only stable and high expression of FOXP3 can convert human CD4\textsuperscript{+} T cells into Treg cells,\textsuperscript{89,91} and further support the conclusion that Treg cell development and function does not simply rely on the presence or absence of FOXP3, but rather on the magnitude and stability of its expression.

A consistent finding is that the transient expression of FOXP3 in activated Tconv cells is not sufficient to suppress cell division or cytokine production.\textsuperscript{108-110} These data indicate that the molecular activity of FOXP3 in Treg and Tconv cells is fundamentally different, likely as a consequence of other yet-to-be-defined interactions with other Treg-specific proteins. One point of controversy that has arisen from these studies is whether FOXP3\textsuperscript{+} Tconv cells transiently acquire suppressive activity. Some reports indicate that FOXP3\textsuperscript{+} Tconv cells temporarily become suppressive\textsuperscript{111,114,115} whereas others could not confirm this finding.\textsuperscript{108-110,112,113,116} It should be noted that it is very difficult to make meaningful conclusions from \textit{in vitro} suppression assays performed with activated Tconv cells as they are not only susceptible to activation-induced-cell death but also have fundamentally different kinetics of proliferation. Thus, it is possible that "suppression" of thymidine incorporation could be due to either induction of cell death or that the peak of proliferation was missed, as was previously shown in comparisons of T cells activated by immature or mature dendritic cells,\textsuperscript{117} rather than true suppression. Nevertheless, further investigation into the role of FOXP3 in Tconv cells is clearly warranted.
1.5 Molecular characteristics of FOXP3

1.5.1 Key structural features of FOXP3

Sequence analysis of *FOXP3* revealed a classical forkhead (FKH) domain and ultimately recognition of a novel member of the FKH family of transcriptional factors.\(^{92}\) Early structure-function studies confirmed that FOXP3 was able to bind the consensus FKH binding sites in the transthyretin and immunoglobulin variable regions V1 (V1P) promoters.\(^{107}\) Although FOXP3 was originally thought to exclusively repress transcription since it inhibited luciferase activity driven by an SV40 promoter appended to three V1P forkhead consensus sites,\(^{107}\) more recent studies have revealed that it can also act as an activator of transcription.\(^{118-120}\)

In humans, FOXP3 is a 431 amino acid (aa) protein consisting of an amino terminal proline-rich region (aa 1-193), a C2H2 zinc finger domain (aa 200-223), a leucine zipper motif (aa 240-261), and a carboxyl-terminal forkhead domain (aa 338-421).\(^{121}\) Its FKH and zinc finger/leucine zipper sequences are 73-80% and 44-51% identical to FOXP1, FOXP2, and FOXP4, respectively, while the remainder of the sequence is divergent.\(^{122}\) Specifically, the proline-rich amino terminal region of FOXP3 contrasts significantly with the glutamine-rich amino termini of the other FOXP members.\(^{123}\) Furthermore, FOXP3 lacks the C terminal binding protein 1 (CtBP1) transcriptional repressor domain present between the leucine zipper and FKH domains in FOXP1 and FOXP2, suggesting that the mechanisms by which it represses transcription differ from other FOXP family members.\(^{123}\) The amino-terminal 106 to 190 aas have recently been identified as necessary, but not sufficient for the repressive activity of FOXP3.\(^{124,125}\) As discussed in more detail below, this region is also responsible for binding to the histone acetyltransferase TIP60 and histone deacetylase HDAC7.\(^{125}\) The
leucine zipper region is essential for homo- and hetero-dimerization\textsuperscript{124,126,127} while the zinc finger does not seem to be essential for either homodimerization or repressive activity.\textsuperscript{124} As for all FKH proteins, the FKH domain of FOXP3 contains a DNA-binding sequence, as well as a nuclear localization sequence.\textsuperscript{124}

**Figure 1.1 Schematic diagram of FOXP3.**

The domains of FOXP3 are shown. Some proteins that associate with FOXP3 are depicted above the domain of FOXP3 with which they interact.

**1.5.2 Isoforms of FOXP3 in humans**

In humans, T cells co-express two isoforms of FOXP3: FOXP3a is the full-length transcript that is the only form expressed in mice, whereas FOXP3b is a splice variant that lacks exon 2.\textsuperscript{88,128} Whether FOXP3a and b differ in their ability to drive Treg development was investigated, and it was found that although FOXP3b suppresses transcription of a luciferase reporter under control of the IL-2 promoter, ectopic expression of FOXP3b in T cells is less effective than FOXP3a at suppressing IL-2.\textsuperscript{88} In contrast, Smith et al. reported that FOXP3b was equally effective as FOXP3a at suppressing IL-2 production.\textsuperscript{129} Functional differences between FOXP3a and b have recently been attributed to differential interactions between another transcription factor involved in T cell development: retinoic acid-related orphan receptor (ROR)-\textalpha. Specifically, FOXP3 and ROR-\textalpha interact via an LxxLL motif in exon 2 of FOXP3, resulting in inhibition of ROR-\textalpha-mediated transcriptional activation.\textsuperscript{130} In addition, it has been reported that while FOXP3a is distributed both in the nucleus and
cytoplasm, FOXP3b is primarily located in the nucleus, possibly due to lack of a lysine-rich region encoding a typical nuclear export signal present in exon 2.¹²²

Another splice form of FOXP3 that lacks both exon 2 and exon 7, which contains part of the leucine zipper domain, has also been identified and referred to as FOXP3 Δ2Δ7.¹²⁹ Recently, preferential expression of FOXP3b and Δ2Δ7 was observed in a subgroup of patients with Sezary syndrome (SS), a form of cutaneous T-cell lymphoma (CTCL) characterized by lymphocytes with atypical cerebriform nuclei (Sezary cells) in the skin, lymph nodes, and blood.¹³¹ Interestingly, both the FOXP3b and Δ2Δ7 isoforms are defective at repressing an NFkB-driven luciferase reporter.¹³¹ Although CTCL cell lines from SS patients were suppressive, this function was mediated by IL-10 and independent of FOXP3. These data suggest that the alternative splice forms of FOXP3 may mediate malignant Treg cell differentiation but not their suppressive action.

1.5.3 Notable FOXP3 protein interactions

1.5.3.1 Interactions with Runx1

FOXP3 binds to the runt-related transcription factors Runx1, Runx2, and Runx3. Most investigations are focused on Runx1 since it is expressed in Treg and Tconv cells and regulates expression from the IL-2 and IFN-γ promoters.¹³² Indeed FOXP3 suppresses Runx1-stimulated IL-2 production in Tconv and Treg cells.¹³² The Runx1-binding domain of FOXP3 is located within aa 278-336, a region between the leucine zipper and FKH domain (Figure 1.2). A mutant form of FOXP3 unable to bind to Runx1 fails to suppress IL-2 production, demonstrating that interaction is critical for this function.¹³² The importance of Runx1 to Treg cell function is further illustrated by the fact that mouse CD4⁺ T cells
transduced with a mutant FOXP3 that cannot bind to Runx1 are less suppressive than cells transduced with wild type FOXP3, and moreover that knockdown of Runx1 in human Treg cells attenuates their suppressive capacity.\textsuperscript{132} Recent data indicate that interactions between Runx1 and Foxp3 are also necessary for Foxp3-mediated inhibition of IL-17-producing T cells.\textsuperscript{133} Of note, one of the known point mutations of FOXP3 that causes IPEX occurs in the Runx1-binding region of FOXP3 and causes a late-onset, mild, and spontaneously remitting disease.\textsuperscript{105} Further investigation will be required to define if altered interactions between FOXP3 and Runx1 may underlie autoimmunity in this patient.

Figure 1.2  FOXP3 interacts with Runx1.

Interaction of FOXP3 with Runx1 is important for FOXP3-mediated gene repression and maintenance of a suppressive phenotype in Treg cells. This interaction is also required for the ability of FOXP3 to inhibit Th17 cell differentiation.

1.5.3.2  Interaction with ROR-\(\alpha\)

ROR-\(\alpha\) was first shown to interact with the amino terminal region of FOXP3 in a yeast-two hybrid screen, and the interaction was confirmed in co-immunoprecipitation studies.\textsuperscript{130} FOXP3 suppresses ROR-\(\alpha\)-mediated transcription, but unlike repression of NFAT-mediated transcription, this function does not depend on the FKH domain, but rather on a region in exon 2 (Figure 1.3), as discussed above. Recently the association between Foxp3 and ROR-\(\alpha\) was also found to occur in mouse cells.\textsuperscript{134} Since ROR-\(\alpha\) has a newly
recognized role in the development of Th17 cells, it will be of interest to investigate how FOXP3 may affect this process.

Figure 1.3 FOXP3 interacts with RORα.

FOXP3 interacts with RORα via a region in exon 2. More research is needed to determine how interaction of FOXP3 with RORα may affect Th17 versus iTreg development.

1.5.3.3 Interaction with ROR-γt

After finding that TGF-β-induced Foxp3 expression represses expression of ROR-γt-driven IL-17 production, it was of interest to investigate whether ROR-γt and FOXP3 interact. Indeed, the two proteins co-immunoprecipitate in transfected 293T cells via a mechanism that is DNA-independent. Notably, co-transduction of CD4+ T cells with ROR-γt and FOXP3 suppresses IL-17 production, but there are conflicting data on the domains of FOXP3 required for this effect. Zhou et al. found that a FKH deletion mutant of FOXP3, and a form containing a point mutation (R397W) in the FKH domain from an IPEX patient, lost their ability to suppress IL-17. On the other hand, Yang et al. found that mutant forms of FOXP3 that either lacked the FKH domain or had a mutant leucine zipper, retained their ability suppress IL-17 and Th17 cell differentiation. Notably, the interaction also involves a region encoded by exon 2 (Figure 1.4). Further studies will be required to fully elucidate the molecular interactions between these two proteins. Interestingly, the inhibitory effects of FOXP3 on ROR-γt-induced IL-17 production can be reversed by IL-6 or
IL-21, suggesting that these cytokines may cause inhibitory or stimulatory post-translational modifications of FOXP3 or ROR-γt, respectively.¹³⁵

**Figure 1.4 FOXP3 interacts with ROR-γt.**

FOXP3 interaction with ROR-γt via a region in exon 2 suppresses IL-17 production and Th17 cell development.

### 1.5.3.4 The FOXP3-TIP60-HDAC7 complex

FOXP3 interacts with the histone acetyltransferase (HAT) protein HIV-1 TAT-interactive protein, 60kDa (TIP60) via an amino terminal domain (106-190) of FOXP3.¹²⁵ Since this region of FOXP3 is also required for transcription repression,¹²⁴ Li et al. investigated whether TIP60 was required for this function. Indeed, when expression of TIP60 was knocked down, FOXP3-mediated repression of reporter activity was reduced.¹²⁵ Moreover, a HAT-deficient form of TIP60 reduces the ability of FOXP3 to repress transcription, suggesting that the HAT activity of TIP60 is important for the repressive activity of FOXP3.¹²⁵ One hypothesis is that the HAT activity of TIP60 may be directly involved in acetylation of FOXP3. Since acetylated FOXP3 binds to chromatin preferentially,¹³⁶ TIP60 may function to enhance the ability of FOXP3 to bind to promoters. Since TIP60 is known to recruit Class II histone deacetylase 7 (HDAC7) in other transcriptional repressor complexes, the possibility that FOXP3 also interacts with this protein was investigated. Indeed, co-immunoprecipitation studies demonstrated that FOXP3 associates with HDAC7 in human Treg cells. As for TIP60, the HDAC7-association domain
is in the amino terminal 1-190 aa. Thus, the amino terminal 106-190 aas are key for the ability of FOXP3 to repress transcription via a mechanism that depends on a tri-molecular complex of FOXP3, TIP60 and HDAC7 (Figure 1.5).

Figure 1.5 The FOXP3-TIP60-HDAC7 complex.

Interaction of FOXP3 with TIP60 and HDAC7 via amino terminal amino acids 106-190 is required for FOXP3-mediated suppression of genes. TIP60 may acetylate FOXP3, enhancing its stability and function. TIP60 and HDAC7 may also modify chromatin at FOXP3 target genes.

1.5.3.5 Interaction with HDAC9

Beyond associations with HDAC7, depending on the state of activation, FOXP3 also interacts with HDAC9 in Treg cells. In resting Treg cells, HDAC9 is primarily located in the nucleus, but upon stimulation, it is transported out of the nucleus, suggesting that it only associates with FOXP3 in the resting state and that release of this interaction is required for suppression (Figure 1.6). Further evidence for the role of HDAC9 as a negative regulator of FOXP3 function comes from analysis of Hdac9-/- mice. These mice not only have a 50% increase in Treg cells in lymphoid tissues, but their Treg cells are also three to fourfold more suppressive than control cells. Inhibiting the catalytic activity of HDAC9 has a similar effect on Treg cells as the absence of the protein: treatment of mice with the HDAC-inhibitor trichostatin A (TSA) increases Foxp3 expression in Treg cells, and Treg cells from TSA treated mice were more suppressive in vitro and in vivo than the cells from control-treated mice. HDAC9 may inhibit Treg cell activity by deacetylating FOXP3, thereby decreasing
its ability to bind DNA. Consistent with this notion, treatment with TSA increased the amount of acetylated FOXP3 in Treg cells and binding to the IL-2 promoter.\textsuperscript{137}

**Figure 1.6 HDAC9 inhibits FOXP3 function in resting Treg cells.**

In resting Treg cells, HDAC9 interacts with and deacetylates FOXP3, reducing its stability and decreasing its ability to bind target genes. When Treg cells are activated, HDAC9 is exported from the nucleus, allowing acetylation of FOXP3, which enhances its function.

### 1.5.4 Epigenetics of genes regulated by FOXP3

One way to promote or repress transcription is to modify chromatin structure at target genes. It is becoming clear that this is an important way in which FOXP3 regulates genes, through interaction with chromatin-modifying proteins such as TIP60, HDACs 7 and 9, Eos, males-absent on the first (MOF), and linker histone H1.5. An open chromatin structure conducive to gene transcription is associated with acetylation of histones 3 and 4 on lysine residues and trimethylation of histone 3 at lysine 4 (H3K4me3), while trimethylation of histone 3 at lysines 9, 27, and 20 (H3K9me3, H3K27me3, H3K20me3) marks closed chromatin and repressed gene transcription.\textsuperscript{138-144}

Early studies showed that binding of FOXP3 to promoters it represses (e.g. IL-2, IL-4 and IFN-\(\gamma\)) results in histone 3 deacetylation at these promoters.\textsuperscript{145,146} In contrast, for the promoters that it transactivates (e.g. GITR, CD25, and CTLA-4), Foxp3 binding is correlated with increased histone acetylation.\textsuperscript{146} More recent studies have uncovered a variety of
binding partners that co-operate with FOXP3 to bring about changes in chromatin structure at target genes. First, FOXP3 interacts with linker histone H1.5, a histone protein that has a role in the assembly of closed chromatin structures, so FOXP3 may differentially repress or activate genes by recruiting or excluding H1.5, respectively.\textsuperscript{147}

Second, FOXP3 employs the histone modifying protein CtBP1 via direct interaction with Eos.\textsuperscript{148} The Eos/CTBP1 complex appears to have a role specifically in the repression of FOXP3 target genes because when Eos was knocked down in Treg cells, 90\% of genes known to be suppressed by FOXP3 were no longer repressed while more than 95\% of genes known to be up-regulated by FOXP3 were unaffected by knock-down of Eos.

Lastly, FOXP3 can bind to the histone acetyltransferase MOF that specifically acetylates histone H4K16, a mark of active transcription. Furthermore, FOXP3-activated genes contain binding sites for the H3K4me3 demethylase PLU-1 that overlap with FOXP3 binding sites. Thus, FOXP3 gene activation may occur at sites of H4K16 acetylation combined with H3K4me3, accomplished by FOXP3 recruitment of MOF to acetylate H4K16 and displacement of PLU-1 to allow H3K4 methylation.\textsuperscript{149}

\subsection*{1.5.5 Post-translational modifications of FOXP3}

There are many ways that proteins can be post-translationally modified to affect their function, stability, or degradation. So far, it is known that FOXP3 can be acetylated, phosphorylated, ubiquitinated, and proteolytically processed. Most research has been performed on acetylation of FOXP3. Focusing on lysine residues conserved between mouse and humans in the FKH domain, the Hancock group found that mutation of K383 and K393 significantly reduced the capacity of FOXP3 to suppress targets including IL-2 and to confer
suppressive capacity to naive CD4\(^+\) T cells.\(^{137,150}\) However, whether these lysines are the targets of acetylation was not determined. Another group has identified 3 lysine residues, K31, K262, and K267, by mass spectrometry that are acetylated in both mouse and human FOXP3 and developed K31 and K262 acetylation-specific antibodies to confirm their results.\(^{151}\) FOXP3 is acetylated by p300 and deacetylated by SIRT1,\(^{152,153}\) and indeed, this was the case for K31 and K262. Prevention of FOXP3 deacetylation by inhibition of SIRT1 decreased Foxp3 poly-ubiquitination and proteosomal degradation,\(^{152}\) leading to the hypothesis that acetylation of FOXP3 on lysine residues increases its stability by preventing ubiquitination on those same residues.

Notably, the possibility of pharmacological inhibition of HDACs to enhance FOXP3 acetylation and improve Treg function has important clinical implications. Work in animal models has shown that TSA-treated mice have increased numbers of Foxp3\(^+\) Treg cells in lymphoid tissues and reduced disease severity in the mouse model of colitis induced by dextran sulphate sodium. Mice transplanted with MHC-mismatched cardiac and islet grafts and treated with TSA have a small survival advantage that is greatly enhanced by the combined treatment of TSA with rapamycin to inhibit the proliferation of alloreactive T cells.\(^{137}\) Furthermore, treatment with the HDAC inhibitor valproic acid increased the number and function of Treg cells \textit{in vivo} and decreased the severity of collagen-induced arthritis.\(^{154}\) Lastly, inhibition of SIRT1 can increase FOXP3 expression in human and mouse T cells\(^{153}\) and specific inhibition of SIRT1 \textit{in vivo} can increase the stability of adoptively transferred iTreg cells.\(^{151}\)

Another post-translational modification of FOXP3 is proteolytic processing by proprotein convertases. Mouse Foxp3 was shown to be cleaved at both the NH\(_2\)- and C-
terminal ends at proprotein convertase motifs, and these sequences are conserved in human FOXP3. The C-terminal end was found to be cleaved by PC1/3 and PC7, both found in Treg cells, but it is still unclear what cleaves the C-terminal end. Interestingly, NH2-cleaved, C-cleaved, or NH2 + C-cleaved FOXP3 had different abilities to confer Tconv cells with suppressive capacity \textit{in vitro} and \textit{in vivo}, with C-cleaved and NH2 + C-cleaved programming the most potent suppression.\textsuperscript{155}

FOXP3 can be post-translationally modified and targeted for degradation in the presence of hypoxia-induced factor 1 (HIF1α), though more work is required to fully elucidate the mechanism of this process.\textsuperscript{156} Likewise, more work is required to understand the significance of phosphorylation of FOXP3.

1.5.6 Epigenetic regulation of FOXP3 expression

In general, expression of FOXP3 is induced by short and weak TCR stimulation, and pharmacological treatment with inhibitors of the phosphatidylinositol 3' kinase (PI3K) pathway favour FOXP3 expression.\textsuperscript{101,157-159} Activation of STAT5 downstream of IL-2 is also key for induction and maintenance of FOXP3 in both nTreg and iTreg cells.\textsuperscript{160,161} Moreover, as discussed above, TGF-β can directly stimulate de novo expression of FOXP3 and contribute to the development of iTreg cells.\textsuperscript{86} Ultimately, these factors all contribute to the epigenetic changes that determine the stability and magnitude of FOXP3 expression. Since stable and high levels of expression are necessary for Treg lineage-commitment and function,\textsuperscript{89,91,162} understanding how epigenetic changes contribute to FOXP3 expression is fundamental for the development of cell-therapy based applications.
Currently, three main regions of \textit{FOXP3}, which are highly conserved in mice and humans, are known to be subject to epigenetic modifications that impact transcriptional activity of the locus: the promoter, the so-called TGF-\(\beta\)-sensor region, and the Treg-cell-specific demethylated region (TSDR).\textsuperscript{163} Bisulphite sequencing to analyze the methylation state of CpG motifs in these regions revealed that they were highly methylated (indicative of inactive chromatin) in Tconv cells but almost completely demethylated in Treg cells.\textsuperscript{85,164} Further examination of the histone modifications associated with these regions by chromatin immunoprecipitation demonstrated that in Treg cells these regions also have increased H3K4me3 and acetylation of histone 3 compared with Tconv cells, indicating an open chromatin structure for Treg cells and a condensed structure for Tconv cells.\textsuperscript{165}

There is a great deal of interest in determining how the epigenetic changes in these three regions act as on/off switches, and whether they also determine the stability and magnitude of FOXP3 expression. For example, evidence that the degree of demethylation in the TSDR is less in thymic than peripheral Tregs cells suggests that expression of FOXP3 may be stabilized in the periphery.\textsuperscript{85} Moreover, when the effects of TGF-\(\beta\) on epigenetic changes in these loci were investigated it was found that although there was a certain amount of demethylation of the locus, it was not to the same extent and transient in comparison to \textit{ex vivo} Treg cells.\textsuperscript{85} The fact that TGF-\(\beta\) alone may not be sufficient to irreversibly open the FOXP3 locus could underlie the variable reports on the capacity of TGF-\(\beta\) to induce suppressive Treg cells.\textsuperscript{86} Epigenetic analysis also provided further evidence that the expression of FOXP3 upon TCR activation of Tconv cells is transient.\textsuperscript{166} Importantly, the stability of FOXP3 expression can be manipulated pharmacologically using compounds that alter epigenetic changes. For example, blocking maintenance DNA methylation induces
stable activation-dependent FOXP3 expression in Tconv cells and also confers stability to TGF-β-mediated induction of Foxp3 expression.\textsuperscript{167} Thus, the development of successful cell therapies based on generating iTreg cells \textit{in vitro} may rely on the use of strategies to ensure the epigenetic changes in the FOXP3 locus are stable.

1.5.7 Role of FOXP3 in Treg versus Th17 cell development

When naive CD4\textsuperscript{+} T cells encounter their antigen they differentiate into subsets defined by differences in cytokine production and effector function. For example, Th1 cells develop in the presence of IL-12 and secrete IFN-γ thereby promoting cellular immunity and elimination of intracellular pathogens. By contrast, IL-6, IL-1β, and TGF-β promote the development of Th17 cells which contribute to host defense against pathogens that require robust tissue inflammation to be cleared.\textsuperscript{168} Pathologically, both Th1 and Th17 cells can also mediate autoimmunity. The fact that TGF-β seems to be required, either directly or indirectly, for the development of pro-inflammatory Th17 cells is at odds with the parallel role for this cytokine in the development of anti-inflammatory Treg cells. This has led to a great deal of interest in defining the molecular basis for the developmental relationship between Th17 cells and Treg cells, since therapeutic strategies to reduce autoimmunity must not be confounded by parallel promotion of Th17 cells.

As discussed above, ROR-α, ROR-γt and Runx1 are critical transcription factors in Th17 development and function, and there is much interest in defining how their interactions with FOXP3 define the Treg versus Th17 cell lineage.\textsuperscript{133,168,169} It is thought that in the presence of TGF-β alone, expression of FOXP3 is induced and that it interacts with Runx1 and ROR-γt to inhibit expression of IL-17, either directly via a Foxp3/Runx1 complex or
indirectly by preventing Runx1 from enhancing ROR-γt-mediated IL-17 transcription. Conversely, in Th17 polarizing conditions, signals from TGF-β and IL-6 are thought to combine to decrease FOXP3, allowing Runx1 to preferentially bind to ROR-γt and enhance IL-17 expression.

In addition to direct interactions with ROR-γt and Runx1, interactions between Foxp3 and the TIP60/HDAC7 complex also contribute to repression of ROR-γt-mediated transcription and thus IL-17 production. Analysis of a mutant form of Foxp3 (∆105-190) that cannot bind to TIP60 or HDAC7 revealed that its ability to suppress ROR-α or ROR-γt mediated-transcription was attenuated. Mutation of both the ROR-α-interacting domain (the LxxLL motif) and the TIP60/HDAC7 binding domain completely abolished the ability of Foxp3 to suppress ROR-α and ROR-γt-mediated reporter activity. Thus, Foxp3 cooperates with both TIP60/HDAC7 and the ROR transcription factors to repress ROR-mediated transcription and Th17 cell differentiation.

Recent experiments have shown that HIF1α co-operates with ROR-γt to tip the balance toward Th17 rather than Treg. HIF1α deficient T cells failed to differentiate into Th17 cells in vitro, even though ROR-γt and ROR-α expression and STAT3 phosphorylation remained unchanged. IL-23R expression was decreased, however, and FOXP3 and Treg markers such as CTLA-4 were increased. The role of HIF1α in Th17 versus Treg differentiation was further revealed by another study which showed that HIF1α transcription can be induced via the JAK-STAT3 pathway involved in Th17 cell polarization, that HIF1α can promote ROR-γt gene transcription, and that HIF1α can bind to the ROR-γt complex and enhance transcription of target genes. Moreover, HIF1α can bind to FOXP3 to promote its degradation, thereby preventing its inhibition of ROR-γt function.
Because FOXP3 inhibits Th17 differentiation, it was of interest to determine if, in the absence of FOXP3, TGF-β was sufficient to induce Th17 cells. However, when CD4+ T cells from Scurfy mice were stimulated with TGF-β they did not produce IL-17, indicating that the role of IL-6 in Th17 cell differentiation is not simply to inhibit Foxp3. Moreover, when Foxp3- T cells were stimulated with TGF-β and IL-6, they had reduced IL-17 and enhanced IFN-γ production compared to wild type cells. Further investigation revealed that although FOXP3 is not directly required for Th17 cell development, it is indirectly required via its ability to suppress Th1 cell development. These data lend further support to the paradox that FOXP3 has a dual role in immune homeostasis: beyond defining the Treg lineage it can also potentially contribute to the development of Th17 cells. Since CD4+ T cells which co-express FOXP3 and RORC2 (the human equivalent of ROR-γt) are suppressive, Treg development may be dominant in this process.

A further complexity in the relationship between FOXP3, Treg and Th17 cells is that several groups have recently shown that Treg cells can be reprogrammed into Th17 cells. When Treg cells are activated in the presence of pro-inflammatory cytokines such as IL-6 or IL-1β, expression of FOXP3 is down-regulated, and there is a parallel increase in IL-17 expression and loss of suppressive capacity. This conversion is blocked by inhibition of histone deacetylases, suggesting that the differentiation of Treg cells into IL-17 producing cells depends on epigenetic modifications. Evidence that this conversion can also occur in vivo comes from studies in which CD45.2+ GFP+ Treg cells from Foxp3 reporter mice were mixed with congenic CD45.1+ Tconv cells and transferred into Rag1-/- recipients. When experimental autoimmune encephalomyelitis is induced in these mice, the CD45.2+ cells, which were originally Treg cells, down-regulate Foxp3 and start to secrete IL-17.
Thus, there appears to be a considerable amount of plasticity in the Treg cell lineage, and further research into how expression of FOXP3 contributes to this process will be key to understanding how this process may go awry and lead to autoimmunity.

1.6 Treg cells in transplantation tolerance

1.6.1 Early indications that Treg cells could prevent graft rejection

Almost 30 years ago in a rat heart transplant model, Hall et al. showed in the MHC-mismatched PVG-to-DA strain combination that a two-week course of cyclosporine (CyA) led to indefinite allograft survival without further therapy. Importantly, when harvested 100 days post-transplant and tested in adoptive transfer models, T cells from these animals had the capacity to prevent rejection mediated by normal effector cells. These data provided a clear indication that long-term allograft survival independent of long-term immunosuppression (operational tolerance) involved T cells with the ability to regulate naïve alloreactive T cells. Subsequently, Hall and colleagues demonstrated that regulation was associated with CD4+ T cells and were the first to suggest that CD25 is a useful Treg marker. Similar data were obtained in a rat renal allograft model where operational tolerance was induced by donor-specific blood transfusion.

To determine whether cells isolated on the basis of CD25 expression could be used therapeutically in the transplant setting, Hara et al. reconstituted immunodeficient CBA mice with naïve CBA effector T cells with or without CD4+CD25+ T cells isolated from CBA mice bearing fully allogeneic B10 heart allografts. The reconstituted mice were then transplanted with test B10 skin grafts. Mice reconstituted with effector T cells alone rejected their skin grafts acutely but, in stark contrast, co-transfer of CD4+CD25+ T cells from tolerant animals
led to indefinite skin graft survival in 80% of recipients. Strikingly, when used at equivalent cell doses, naïve CD4\(^+\)CD25\(^+\) T cells were unable to control rejection suggesting that exposure to alloantigen in a tolerogenic environment either enhances nTreg function and/or generates a population of induced Treg.

At present, there are three main strategies being pursued to develop Treg-cell based therapies for transplantation: 1) the use of nTreg cells, either freshly isolated or expanded \textit{in vitro}; 2) the generation of iTreg cells, generated by donor antigen stimulation \textit{in vitro}; and 3) the generation of Treg cells by ectopic gene expression.

### 1.6.2 Natural Treg cells as a cellular therapy

The small number of nTreg cells accessible in the peripheral circulation means that for cellular therapy, it will almost certainly be necessary to use a polyclonal stimulus to expand nTreg \textit{in vitro}. Thus, the first challenge is deciding the basis on which Treg cells should be isolated to maintain pure populations after \textit{in vitro} expansion. Purity is an issue because of the potential out-growth of non-Treg that could contribute to rejection or cause autoimmunity. In the absence of a Treg-specific cell surface marker, two different combinations of markers appear to be promising for Treg isolation. The first combination seeks to isolate CD4\(^+\)CD25\(^{hi}\) nTreg cells but with the addition of an antibody to select for CD45RA\(^+\) cells and so eliminate antigen experienced or memory T cells. The second combination also uses CD4 and CD25 but includes an antibody to CD127 (IL-7R\(\alpha\)) on the basis that in human nTreg, there is a reciprocal expression of CD127 and FOXP3 and thus CD127 provides a sortable surrogate maker for FOXP3\(^+\) nTreg cells.
Notably, isolation of CD25\(^{−}\)CD45RA\(^{+}\) (naive) T cells yields Treg cells with a greater suppressive capacity than total CD25\(^{hi}\) cells.\(^{184}\) The reason for this became clear when Miyara et al. examined subpopulations of human FOXP3\(^{+}\) T cells and discovered that the CD25\(^{+}\)CD45RA\(^{-}\)FOXP3\(^{lo}\) population is not suppressive \textit{in vitro}, contains IFN-\(\gamma\) and IL-2 producing cells, and also may contain Th17 precursors.\(^{186}\) Furthermore, after three weeks of \textit{in vitro} expansion, CD127\(^{−}\) Treg became methylated at the TSDR while CD45RA\(^{+}\) expanded Treg remained de-methylated,\(^{187}\) and the CD127\(^{−}\) Treg that lost FOXP3 expression were CD45RA\(^{−}\) demonstrating that naïve Treg may represent the most stable population for expansion.\(^{187,188}\) Naïve Treg have additional benefits of having the greatest expansion potential\(^{188}\) and of expressing the homing receptors CD62L and CCR7 even after \textit{in vitro} expansion which may be beneficial for cellular therapy to target the cells to lymphoid organs.\(^{184}\) One drawback, especially in an ageing population, is that numbers of naïve Treg decline in peripheral blood with age,\(^{46,189}\) hence, isolation based on CD127 expression may still be a practical approach.

Work continues to identify additional markers that may give purer or more potent Treg, or provide more straightforward isolation procedures. In addition to selection criteria based on CD45RA or CD127, there have been studies investigating the utility of CD121a/CD121b and TGF-\(\beta\)/LAP,\(^{190}\) CD39,\(^{191-193}\) and glycoprotein-A repetitions predominant (GARP)\(^{48,194,195}\) as new Treg markers. However, all of these proteins are only expressed on activated Treg so they would only be useful for re-purifying \textit{in vitro} expanded Treg if contamination with effector T cells was suspected. Markers have also been identified that separate Treg into different functional subsets. For example, human ICOS\(^{+}\)FOXP3\(^{+}\) cells produce IL-10 and TGF-\(\beta\) whilst ICOS\(^{−}\)FOXP3\(^{+}\) cells only produce TGF-\(\beta.\(^{196}\) Thus
depending on the type of immune response to be suppressed, it may be useful to isolate subsets of nTregs which have specific mechanisms of action.

In view of their low abundance, much work has gone into developing in vitro methods to expand nTreg. Methods employed to stimulate Treg include anti-CD3/anti-CD28 coated beads as well as cell-based artificial antigen presenting cells expressing co-stimulation molecules and/or Fc receptors. In addition to stimulus through the TCR, Treg cells require CD28 co-stimulation and exogenous IL-2. Adding rapamycin to the culture has been shown to preserve Treg purity and allow selective Treg expansion. Notably, as discussed above, rapamycin-expanded Treg retain suppressive capacity in vitro and, when tested in a GVHD model, were more effective than nTreg expanded under conventional conditions. However, rapamycin also significantly inhibits the proliferation of Treg; thus, addition of this compound for only a portion of the expansion period may be optimal. Importantly, expansion outcomes among individuals are heterogeneous, potentially affecting the number or purity of Treg that can be obtained from a given individual. Indeed, in a recently reported clinical study, target doses of expanded nTreg were not achieved for all patients. Improvements in expansion methods that remain GMP compliant should allow Treg therapy to be applicable for a wider range of patients.

While it has been essential to use in vitro suppression assays to determine whether expanded nTreg retain regulatory function, a much more critical matter is whether these cells can regulate alloreactivity in vivo. Fortunately, in the past few years, several humanised-mouse models have been described in which immunodeficient mice are reconstituted with components of the human immune system. In a unique study, Nadig et al. reconstituted immunodeficient BALB/c Rag-/- common γ chain-/- mice with human peripheral blood
mononuclear cells (PBMC) with or without expanded nTreg isolated from the same cell donor, then transplanted these mice with segments of human left internal mammary artery side branches as interposition grafts in the descending aorta.\textsuperscript{209} Grafts were harvested at day 30 and examined for intimal hyperplasia, one of the hallmarks of immune-mediated vascular damage. Mice transplanted without cellular reconstitution showed no signs of intimal proliferation, whereas mice reconstituted with allogeneic PBMC showed extensive vasculopathy which in some cases resulted in almost complete intimal occlusion. However, co-transfer of expanded nTreg isolated on the basis either of CD4 and CD25 or CD4, CD25 and low expression of CD127, had a striking impact on vasculopathy with some vessels being entirely free from occlusion. On the basis of their ability to prevent vasculopathy in this model, cells sorted on the basis of CD127 appear to be some five times more effective on per cell basis,\textsuperscript{209} an observation that could have important implications for the design of future clinical studies. This approach has recently been extended to a human skin graft model in BALB/c Rag\textsuperscript{+/-} common \(\gamma\) chain\textsuperscript{-/-} mice reconstituted with allogeneic human PBMC and, as in the vessel model, \textit{in vitro} expanded CD4\textsuperscript{+}CD25\textsuperscript{+}CD127\textsuperscript{lo} nTreg have been shown to be powerful regulators of allograft rejection emphasizing their clinical potential.\textsuperscript{210}

### 1.6.3 Induced Treg cells as a cellular therapy

Broadly speaking, the CD4\textsuperscript{+} T cell compartment of rodents and man contains approximately 5-10\% of FOXP3\textsuperscript{+} nTreg and, as discussed earlier, these clearly play an essential role in normal immune homeostasis.\textsuperscript{16,211-216} However, as discussed above, in humans it is difficult to isolate pure populations of nTreg and their low expansion potential continues to limit clinical application. Many groups have thus pursued the development of
strategies to induce iTreg \textit{in vitro}. GVHD models allowed the unequivocal demonstration that stimulation of donor CD4$^+$ T cells with recipient strain APC in the presence of TGF-β and IL-2 resulted in a population of Treg that could prevent lethal GVHD \textit{in vivo}.\textsuperscript{56}

Subsequently, two groups made the important observation that Foxp3$^+$ cells with suppressive function \textit{in vitro} and \textit{in vivo} could be generated from CD4$^+$CD25$^-$ precursors.\textsuperscript{217,218} The ability to generate Treg from non-Treg precursors could have important consequences for eventual Treg cell therapy because CD4$^+$ conventional T cells are 10-20-fold more abundant than pre-existing Treg and thus provide a larger pool of potential Treg precursors.

The potential of induced Tregs has been extended from GVHD models to models of organ and tissue transplantation, and several groups have shown that \textit{in vitro} generated iTreg can control allograft rejection. For example, Feng et al. developed a novel \textit{in vitro} protocol in which recipient strain CD4$^+$ T cells are stimulated for 14 days with donor APC in the presence of IFN-γ.\textsuperscript{219} Without further purification, the resulting cells prevent donor-specific skin and vessel allograft rejection mediated by CD25$^-$ effector T cells in a sensitive adoptive transfer model.\textsuperscript{220,221} Importantly, T cell stimulation in the presence of IFN-γ enriches for alloreactive Foxp3$^+$ Treg by inducing preferential apoptosis in responding non-Treg, promoting the expansion of responding nTreg and driving the conversion of non-Treg precursors.\textsuperscript{219} While these results are encouraging, the iTreg cells in these experiments are required to control only a relatively small population of effector cells. An essential next step is to determine whether logistically feasible numbers of iTreg can influence graft outcome in normal mice with an intact T cell repertoire.

A caveat is that there is currently no robust way to generate iTregs \textit{in vitro} in humans,\textsuperscript{112} thus testing of this approach in the clinic will await further basic research. On the
other hand, protocols to generate human iTregs characterized by high expression of IL-10, but not FOXP3, are well established. These so-called Tr1 cells arise when conventional T cells encounter their antigen in the presence of IL-10\cite{222} and mediate antigen-specific suppression via an IL-10-dependent mechanism. In mouse models, Tr1 cells can suppress islet allograft rejection\cite{223} as well as GVHD\cite{224}, leading to the development of clinically-applicable protocols to generate alloantigen-specific Tr1 cells for cellular therapy.\cite{225} A trial is ongoing to test whether delivery of Tr1 cells can control GVHD without affecting responses to other antigens.

### 1.6.4 Generation of Treg cells by ectopic gene expression

As discussed above, Foxp3 over-expression either in vivo in transgenic mice\cite{26} or in vitro using viral transduction\cite{27,28,87} confers regulatory activity on previously non-regulatory T cells. Similarly, over-expression of FOXP3 in human T cells using a vector system which ensures continuous high expression levels allow efficient generation of Tregs from conventional T cells in vitro.\cite{89} A major benefit of this approach is that a relatively large number of T cells could be isolated and reprogrammed into Treg, overcoming the challenge of limiting cell numbers. Furthermore, over-expression of FOXP3 can also reprogram memory T cells into Tregs, allowing for generation of antigen-specific Treg. The generation of Treg by ectopic expression of FOXP3 thus represents an exciting though challenging prospect in clinical transplantation. In the living-donor transplant setting it would theoretically be possible to stimulate recipient T cells with donor APC and then transduce the responding T cell population with FOXP3 for subsequent delivery as a "personalized" Treg cellular therapy.
Another approach involving a combined gene and cell therapy approach is to introduce alloantigen-specific TCRs into Tregs. Tsang et al.\textsuperscript{226} used a mouse model to generate Tregs with both indirect and direct specificity for donor alloantigens. Recipient (B6, H2\textsuperscript{b}) CD25\textsuperscript{+} T cells were repeatedly stimulated with donor (BALB/c, H2\textsuperscript{d}) to generate Treg with direct allo-reactivity, then these cells were transduced with the TCR recognising K\textsuperscript{d} presented by IA\textsuperscript{b}. The resulting cells were then delivered to B6 recipients as a cellular therapy and assessed for their ability to influence the rejection of fully mismatched BALB/c heart allografts. When combined with sub-therapeutic anti-CD8 antibody plus rapamycin, cells with only direct specificity led to \~50\% heart allograft survival at 100 days. In contrast, cells with both direct and indirect specificity led to 100\% graft survival in the same strain combination. This study provided important ‘proof of concept’ data showing firstly that transduction of recipient cells with a TCR of known allospecificity can generate functional Treg and, secondly, that Treg with the capacity to recognize alloantigen via the indirect pathway should not be ignored as protocols are developed for clinical testing. Although these data are striking, it should be noted that the doses of cells used in these mice were not insignificant (1×10\textsuperscript{7} on days -1, +7, 14 and 21). When scaled up from a \~25 g mouse to an 80 kg human, this equates to a total dose approximately 1.2×10\textsuperscript{11} transduced Treg. This number is put into a logistical and possible safety context by the fact that an average 80 kg person has approximately 4×10\textsuperscript{8} circulating CD4\textsuperscript{+}FOXP3\textsuperscript{+} nTreg and perhaps an equivalent number resident in peripheral lymphoid tissues. Thus, such an approach, though technologically elegant would face enormous practical and licensing issues if it were to be considered for clinical transplantation.
1.6.5 The current clinical experience of Treg cell therapy in transplantation

The ‘first-in-man’ study of expanded nTreg to be used as a cellular therapy reported the results of two patients who developed GVHD following bone marrow transplantation.227 One patient developed chronic GVHD (POD 137) and received triple therapy (prednisolone, tacrolimus and MMF) for two years post-transplant, but complications of the immunosuppression prompted the administration of CD4+CD25+ flow-sorted, expanded nTreg from the bone marrow donor at a dose of 1x10^5/kg. This approach allowed MMF withdrawal and a reduction in steroids without overt disease recurrence. The second patient was diagnosed with acute GVHD on day 29 post transplant which was refractory to treatment with steroids, tacrolimus, MMF and ATG. This patient received expanded donor nTreg at a total dose of 3x10^6/kg over three infusions (day+75, +82 and +93) and, although there was a temporary clinical improvement after the first infusion, the patient deteriorated and died of multi-organ failure on day 112.

In late 2010, a much larger Phase I/II study was reported in which 23 patients who received double umbilical cord blood stem cell transplantation were enrolled in a dose escalation Treg trial.206 In each case, CD4+CD25+ T cells were isolated from a third party unit of cord blood partially matched with the recipient. The study design called for delivery of defined doses on day +1 post-transplant, with some patients receiving a second dose on day +15. Doses ranged from 1x10^5/kg to 30x10^5/kg. The rates of GVHD and infectious complications were compared with those from 108 historical controls. Importantly, the study reported no increase in fungal, bacterial or viral infections compared with the control group and although the primary endpoints were safety and feasibility, the authors did report a slight reduction in grade II-IV GVHD in the trial group. It seems highly likely that as various
groups begin planning trials of nTreg in bone marrow and solid organ transplantation, this study will provide an essential reference point for cell doses and cell purity for both study teams and the licensing authorities.

Another study has recently been reported in which expanded donor CD4\(^+\)CD25\(^+\) nTreg were administered to patients who underwent HLA-haploidentical hematopoietic stem cell transplantation.\(^{228}\) Donor Tconv cells were given four days post-transplant with CD34\(^+\) cells in a dose escalation study: 4 patients received 2 x 10\(^6\) Treg/kg plus 0.5 x 10\(^6\) Tconv/kg, the next 17 patients received 2 x 10\(^6\) Treg/kg plus 1 x 10\(^6\) Tconv/kg, and the next 5 patients received 4 x 10\(^6\) Treg/kg plus 2 x 10\(^6\) Tconv/kg. One goal of this study was to deliver Tconv cells in addition to the CD34\(^+\) cells to enhance immune reconstitution and function in the recipient without causing GVHD. Importantly, no GVHD prophylaxis was given. Twenty-six of 28 patients achieved full donor-type engraftment, and of those, no patients developed chronic GVHD at the time of reporting (3.6 to 21.4 months post transplantation). However, two of the 26 patients developed ≥ grade II acute GVHD, but these received the highest dose of Tconv cells emphasising that Treg-mediated control is clearly a dose-dependent phenomenon. The study reported an enhancement of immune cell recovery, and an improved immunity to pathogens as judged by in vitro assays. Furthermore, there was no association with an increased risk of leukemia relapse, indicating that the graft-versus-leukemia response was likely intact. It should be noted that in terms of patient survival, the results of this study appear to be disappointing in that 13/26 patients in the study died, particularly from sepsis, viral or fungal infection. However, some of these patients had fungal infections prior to transplant and importantly, no fatal infections occurred after the first two months post-
transplant indicating a restoration of protective immunity without negative effects of Treg therapy.

The European Union has recently funded a multi-centre Phase I/II study to evaluate various types of immunomodulatory cells in living-donor kidney transplantation. The ‘ONE Study’ involves groups from Regensburg, Berlin, London, Oxford, Milano and Nantes and will develop protocols for the use of expanded recipient nTreg (Regensburg, Berlin, London, Oxford), recipient Tr1 cells (Milano), donor regulatory macrophages, Mregs (Regensburg) and donor tolerogenic DC (Nantes). Critical to the study design is that all centres will use a common immunosuppressive protocol (part of the ‘ONE’ concept), which will be closely based on the recently published Symphony study.229 As with the stem cell trials described above,206,228 the primary endpoints will be safety and feasibility but therapeutic benefit will be examined through immunological monitoring using lessons learned from the European Union-funded RISET and Indices of Tolerance initiatives and from similar studies sponsored by the Immune Tolerance Network.230,231 Each centre will enroll 20 patients in the control arm to receive Symphony-based immunosuppression and 10 patients who will receive the same immunosuppressive regimen (but without anti-CD25 induction) plus cell therapy. Providing the relevant licensing and ethical approval is obtained, the study design calls for the control patients to be transplanted no later than 2013 with the cell therapy groups transplanted in 2014 thus allowing a follow-up period of at least 12 months. The intention with the nTreg group is that recipient nTreg will be isolated, expanded, assessed phenotypically and functionally, then cryo-preserved for delivery at day -1 relative to the time of transplant. Although many details will be subject to modification by the appropriate regulatory and ethical bodies based on the bone marrow transplant experience described
above, it is anticipated that the study will begin with doses of the order of $3 \times 10^6$ expanded nTreg/kg.

1.6.6 Safety concerns of Treg cellular therapy

1.6.6.1 Global immune suppression

One of the main goals of using Treg as a cellular therapy is to decrease the requirement for life-long global immunosuppression which increases the risk of infection and cancer. While Treg have been shown to suppress graft rejection in multiple studies, whether or not they are globally immunosuppressive in the context of cellular therapy has not been extensively studied. However, promising results from transfer of Treg to treat or prevent GVHD show that Treg can suppress GVHD while still maintaining the critical graft versus leukemia effect. Indeed, it has been demonstrated that Treg not only prevent GVHD but also enhance immune reconstitution after bone marrow plus T cell transplant in mice by preventing GVHD-induced damage of the thymus and secondary lymphoid organs, thus allowing protection against lethal cytomegalovirus infection.

There have been fewer investigations of global immune suppression when Treg are used as a cellular therapy in solid organ transplantation. In one study, when antigen-specific Treg are induced in vivo to prevent cardiac allograft rejection in a mouse model, they do not prevent anti-flu responses after challenge with influenza 7 days post-transplant. The same is true when splenocytes from these in vivo tolerized mice are transplanted to naïve mice which receive allografts and virus challenge. Although these data are encouraging, much more work is required to determine whether the potential benefits of expanded nTreg, iTreg,
or FOXP3-transduced cells can be realised in solid organ transplantation without compromising protective immunity.

Limited safety data have been obtained from initial clinical trials. In the phase I clinical trial by Brunstein et al. described above, where nTreg expanded from umbilical cord blood were infused into patients who had undergone double umbilical cord blood transplantation, results indicate that while Treg confer enhanced protection from acute GVHD, they do not increase the incidence of opportunistic infection nor disease relapse.206 In the clinical trial by Di Ianni et al., freshly isolated donor Treg were infused 4 days prior to haploidentical hematopoietic stem cell transplant, and no GVHD prophylaxis was given.228 Infused Treg did not inhibit immune reconstitution, and of 26 patients, no CMV-related deaths were reported, an improvement over 40% of non-leukemic deaths caused by CMV that had previously been reported by this group. Furthermore, seven patients were vaccinated against influenza 3-14 months post-transplant, and five acquired protective antibody titres. These studies provide a basis to move on to larger trials that will shed further light on whether polyclonal and/or adaptive Treg result in global immunosuppression causing relapse or infection.

1.6.6.2 Non-pure populations and plasticity of Treg

Further safety concerns of using Treg as a cellular therapy are the lack of pure populations, where contaminating Tconv cells could cause harm. Even if extremely pure populations can be obtained, there is great debate over the stability of Treg. In inflammatory environments, Treg may lose their suppressive phenotype,134,237-242 but other groups claim that nTreg are a stable lineage, even in the inflammatory conditions of infection and
autoimmunity.\textsuperscript{243} One factor to consider is that different populations of Treg may be more stable than others. In particular, iTreg tend to be more highly methylated at the TSDR while nTreg are demethylated in this region, suggesting that iTreg have less stable FOXP3 expression and therefore less functional stability than nTreg.\textsuperscript{163} Another emerging idea is that the role of Treg may not be limited to suppression of immune responses since a novel role for mouse Treg as helper cells in some environments has been identified.\textsuperscript{244-249} Better understanding of factors that cause Treg to lose or gain suppressive capacity will be required to predict how Treg will behave as cellular therapy for transplantation.

1.6.6.3 Addressing safety concerns

One way to address inevitable safety concerns would be to engineer Treg to express an inducible suicide gene such that these cells can be removed if they become pathogenic.\textsuperscript{250,251} One example of such a strategy would be to generate Treg populations that express a cell fate control gene, such as HSV-thymidine kinase which has been expressed in genetically-engineered conventional T cells delivered in the context of stem cell transplantation so that they can be eliminated by gancyclovir \textit{in vivo} should they cause GVHD.\textsuperscript{252} An example of more advanced cell fate control gene is an enhanced mutant of thymidylate kinase (TK), an enzyme that phosphorylates 3’-azido-3’-deoxythymidine (AZT), converting it into a toxic form. Administration of AZT could efficiently eliminate TK-expressing transferred cells that have become pathogenic or cells that have become malignant as a result of gene integration.\textsuperscript{250} Nonetheless, further advances in gene therapy would be required for this approach to move forward and licensing issues are likely to be less than straightforward. However, it should be recognized that the transplant community is well
accustomed to the use of agents such as Alemtuzumab, Basiliximab and ATG for induction therapy. If adverse events were detected that resulted from Treg cell delivery, there could also be the option to use any one of these antibody preparations to disable and/or deplete the injected population. None of these is Treg-specific but the fact that their transient use does not appear to lead to long-term immunodeficiency suggests that if some form of rescue strategy is required by the regulatory authorities, these agents should be acceptable.

### 1.6.7 Monitoring outcomes

How outcomes are measured will be a critical aspect of clinical Treg cellular therapy studies. As discussed above, initial trials of Treg therapy will see an introduction of Treg into established ‘gold-standard’ immunosuppressive regimens, but by definition, these regimens give good graft outcomes in their own right. Therefore, identifying an additive effect of Treg therapy will be a challenge. Although the most robust confirmation of graft rejection is currently via a biopsy, it does not follow that the same approach can detect reduced alloreactivity. Thus clinical parameters combined with in vitro assays to measure alloantigen-specific effector T cell function, such as the IFN-γ ELISPOT, will be required to identify therapeutic benefit. In addition, simple phenotypic analyses of circulating Treg cells may not give clear results because FOXP3 does not exclusively identify Treg cells in humans\(^\text{108-110,112,113}\) and the presence of Treg cells in the graft rather than the periphery may be a better indicator of outcome.\(^\text{253,254}\)

The most promising methods of monitoring alloantigen-specific tolerance are molecular diagnostics including genetics, epigenetics, transcriptomics, proteomics, and metabolomics.\(^\text{255}\) Biomarkers of operationally tolerant kidney and liver transplant patients
have been identified.\textsuperscript{256,257} Furthermore, investigations using the CDR3-length distribution assay suggest that the TCR repertoire might be a good predictor of graft outcome as investigations suggest the majority of kidney transplant patients with chronic rejection have an accumulation of oligoclonal or monoclonal Vbeta expansions while operationally tolerant recipients have a TCR repertoire like that of healthy individuals.\textsuperscript{258} The best indicators of rejection will probably come from a combination of monitoring techniques. Once the initial series of Phase I/II trials have been completed it will be necessary to conduct large, multi-center trials powered sufficiently to identify a reduced incidence of rejection. Success in defining good ways to measure tolerance would set the scene for subsequent trials in which accelerated drug minimization was the principal aim.

1.6.8 \textbf{Impact of concurrent immunosuppression}

The current success of clinical transplantation depends on immunosuppression, and one of many significant unknowns in the design of Treg-based cell therapy is whether their function will be compromised by immunosuppression. Some data suggest that CNIs can attenuate Treg function \textit{in vivo},\textsuperscript{259} and that rapamycin may preserve or enhance Treg cell function.\textsuperscript{101,102,203,260} While it is easily possible to examine the effect of specific agents on Treg function \textit{in vitro}, extrapolation of results to the \textit{in vivo} setting is problematic because of the difficulty in identifying true dose comparisons. Furthermore, any attempt to use \textit{in vivo} models to ask whether immunosuppressive agents block Treg cell function and result in normal rejection responses are immediately confounded by the fact that the drug therapy will block the rejection responses themselves. While attempts to develop relevant animal models will continue, the most relevant and most direct information regarding the effect of
conventional immunosuppression on Treg function will probably be inferred from the initial clinical trials.

1.7 Research goals

While it is clear that FOXP3 has an important role in maintaining immune tolerance, the mechanisms by which FOXP3 acts in both Treg cells and Tconv cells requires further study for development of methods to manipulate its function for therapeutic benefit. Thus, the aim of my research was to study the role of FOXP3 in Treg and Tconv cells, and also to investigate ways in which FOXP3-expressing T cells could be used as a cellular therapy for transplantation.

Mutation of FOXP3 leads to the severe autoimmune disease IPEX, but it is still unclear how different mutations in FOXP3 affect the cellular function of Treg cells. Thus, the goal of chapter 2 was to further examine the role of FOXP3 in Treg cells by investigating in what ways 3 different mutations in FOXP3 would be defective at re-programming conventional T cells into Treg cells by over-expression of the FOXP3 mutants via a lentiviral vector.

Activation-induced FOXP3 in Tconv cells has, until recently, been considered a human-specific phenomenon, but the role of FOXP3 in these cells is unknown. The goal of chapter 3 was to decipher the role of FOXP3 in different subsets of Tconv cells by comparing FOXP3-deficient with wild type Tconv cells.

The use of regulatory cells as a cellular therapy is an attractive approach to limit immune responses to allografts after transplantation. FOXP3-expressing T cells are one type of regulatory cell that is being actively investigated for this use. The goal of chapter 4 was to
determine whether Tconv cells ectopically expressing FOXP3 could suppress allograft rejection in a humanized mouse model, and whether cynomolgus macaque Tconv cells over-expressing FOXP3 could enhance mixed chimerism after bone marrow transplant as has been observed in mouse studies.
Chapter 2: Point mutants of FOXP3 that cause IPEX have diverse abilities to reprogram T cells into regulatory T cells

2.1 Introduction

Immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) is an X-linked autoimmune disease that is often fatal. IPEX typically presents early in childhood with severe diarrhoea, chronic dermatitis, hyper IgE, and polyendocrinopathies. The disease is caused by mutations in the *forkhead box protein 3* (*FOXP3*) gene, which encodes a transcription factor critical for the function of CD4<sup>+</sup>CD25<sup>+</sup> T regulatory (Treg) cells. IPEX is paralleled in *Scurfy* mice, which also have a mutation in *Foxp3* and develop a lethal autoimmune disorder. Evidence that the *Scurfy* phenotype can be corrected by adoptive transfer of Treg cells led to the hypothesis that IPEX results from a lack of Treg cells. However, IPEX patients generally have circulating CD4<sup>+</sup>FOXP3<sup>+</sup> T cells, suggesting that the disease may not be exclusively attributed to a numerical lack of Treg cells.

FOXP3 is a member of the forkhead family of transcription factors. In addition to its DNA-binding forkhead domain, it contains a leucine zipper that is important for homo- and heterodimerization, a zinc finger, and a repressor domain. Notably, FOXP3 can act both as a transcriptional activator and repressor, for example at the *CD25* (*IL-2R alpha chain*) promoter and the *IL-2* promoter, respectively. Its effects on transcription require coordinated interactions with a variety of proteins including nuclear factor of activated T cells (NFAT), HIV-1 Tat interacting protein, 60kDa (TIP60), Eos, RAR-related orphan receptor gamma (ROR-γt), RAR-related orphan receptor alpha (ROR-α), and Runt-related transcription factor 1 (Runx1). Ectopic expression of FOXP3 is sufficient to reprogram mouse CD4<sup>+</sup> conventional T (Tconv) cells into cells that are phenotypically and
functionally identical to Tregs, and it has been shown that a continuous and high expression of FOXP3 ectopically can also reprogram human CD4+ Tconv cells into Treg cells. In humans, FOXP3 is also expressed transiently in Tconv cells upon activation, although the functional relevance of this observation is undefined. Tconv and Treg FOXP3-expressing cells can be distinguished at the molecular level by analysis of the Treg-specific demethylated region (TSDR). In this region upstream of FOXP3, CpG islands are methylated in Tconv cells and are demethylated in stable Treg lineages.

Mutations in FOXP3 that cause IPEX are found throughout the gene, but it is unclear whether different mutations similarly impact the development and function of Tregs. In order to better understand the cellular and molecular basis of IPEX, I examined the impact of three different point mutants derived from IPEX patients on the ability of FOXP3 to reprogram Tconv cells into Tregs in vitro. Surprisingly, mutations in the forkhead DNA binding domain of FOXP3 that caused severe IPEX (R347H and F373A) were only partially blocked in their ability to reprogram Tconv cells into Tregs. Moreover, a mutation in FOXP3 that caused mild disease (F324L) did not differ from wild type FOXP3 in any aspect tested. These results challenge the notion that IPEX results solely from a defect in Tregs.
2.2 Materials and methods

2.2.1 Patients

Clinical histories are summarized in Table 2.1.

2.2.2 Construction and production of lentiviral vectors

Point mutant forms of FOXP3 were amplified by RT-PCR from cDNA derived from peripheral blood of the patients, and a hemagglutinin (HA) tag was added at the amino terminal end. The FOXP3b isoform and HA-tagged forms of the full-length FOXP3a isoform (hereafter FOXP3) were cloned into the bi-directional pCCL lentiviral vector.89 Control vector encoded the truncated nerve growth factor receptor (∆NGFR) alone. All vectors were confirmed by sequencing and lentivirus was produced as previously described.89,270 Briefly, VSV-pseudotyped third generation lentivirus was produced by transiently co-transfecting the 2 packaging plasmids (pPKG 12.5 µg; pREV 6.25 µg), envelope plasmid (pENV 9 µg), and transfer vector plasmid (32 µg) into 293T cells plated in a 15 cm dish (20 mL volume). One µM sodium butyrate was added to cultures after 14-16 hours to increase yield, and the virus was concentrated from the culture supernatant of transfected 293T cells after 30 hours by ultracentrifugation. Transforming unit titres were estimated by limiting dilution on 293T cells.

2.2.3 Cell purification, transduction, and culture of T cell lines

Peripheral blood was obtained from healthy volunteers who gave written informed consent in accordance with the protocol approved by the University of British Columbia Clinical Research Ethics Board. Peripheral blood mononuclear cells (PBMCs) were isolated
by ficoll gradient centrifugation. Antigen presenting cells (APCs) were isolated by depleting CD3+ cells from PBMCs with EasySep Human CD3 Positive Selection Kit (StemCell Technologies, Vancouver, BC). CD4+ T cells were isolated from PBMCs with EasySep Human CD4 Negative Selection Kit (StemCell Technologies), and CD4+CD25−CD45RO− (naïve) T cells were isolated by depleting CD25+ and CD45RO+ cells with antibodies conjugated to magnetic beads (Miltenyi Biotec). Naive T cells were transduced at a multiplicity of infection of ten 14-16 hours after activation with autologous APCs and 1 µg/mL anti-CD3 in the presence of 100 U/mL recombinant human IL-2 and 10 ng/mL recombinant human IL-7. IL-7 was maintained in the T cell culture until purification of transduced T cells with antibodies against ΔNGFR conjugated to magnetic beads 7-9 days after activation. To obtain ex vivo Tregs, CD4+ cells were incubated with anti-CD25 magnetic beads and passed over two columns (Miltenyi Biotec). Cells were cultured and re-stimulated every 11-13 days with allogeneic PBMCs, JY cells (an EBV-transformed B cell line), and phytohemagglutinin and were re-purified as necessary.

2.2.4 DNA methylation analysis

DNA methylation analysis was performed by Epiontis GmbH (Berlin, Germany) on genomic DNA extracted from cell pellets by the phenol-chloroform method or using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany).268

2.2.5 Flow cytometric analysis

Analysis of CD25, CD127, CCR4 (all BD Pharmingen) and GITR (R&D Systems) expression was performed on T cell lines in the resting phase (11-14 days after stimulation).
A minimum of 6, 3, 2, or 8 independent experiments were performed for CD25, CD127, GITR, or CCR4, respectively, derived from at least 2 independent donors. To stain for HA (Roche) and FOXP3 (236/AE7, eBioscience), cells were fixed and permeabilized with FixPerm buffer (eBioSciences) prior to addition of monoclonal antibodies. Samples were read on a BD FACSCanto and analyzed with FCS Express Pro Software version 3 (De Novo Software).

### 2.2.6 Determination of cytokine production by transduced T cells

Two hundred thousand T cells/well were stimulated with immobilized anti-CD3 mAbs (10 µg/ml) and soluble anti-CD28 (1 µg/ml) in a final volume of 250µL. Supernatants were collected after 20 hours to measure IL-2 and 48 hours to measure IFN-γ, TNF-α, IL-4, IL-5, and IL-10 using a Th1/Th2 cytometric bead array (BD Biosciences).

### 2.2.7 Proliferation of T cells

To measure the proliferative capacity of T cell lines, 50 000 cells/well were plated with 50 000 irradiated (5000 rads) APCs and soluble anti-CD3 (OKT3, 0.1 or 1 µg/ml). After 72 hours, tritiated thymidine ([³H]-TdR, 1µCi/well, Amersham Biosciences) was added for 16 hours. Proliferation is expressed as percent of control ΔNGFR cell [³H]-TdR counts. Compiled proliferation data are the average of a minimum of 7 independent experiments derived from at least 3 donors.
2.2.8 Suppression of CD4+ responder T cell proliferation by transduced T cells

Suppression of ex vivo CD4+ T responder cells was measured by activating 8 000 CD4+ T cells with anti-CD3/anti-CD28 coated beads (Invitrogen) at a ratio of 1 bead : 8 cells and culturing with increasing numbers of transduced T cells as indicated. After six days, wells were pulsed with [3H]-TdR for 16 hours. Data are expressed as the percent suppression of proliferation of CD4+ responders alone. Compiled data are the averages of a minimum of 3 independent experiments derived from 2 donors for F324L lines or at least 3 donors for wt FOXP3, R347H, and F373A lines.

2.2.9 Suppression of CD8+ T responder cell proliferation by transduced T cells

Suppression of ex vivo CD8+ T responder cells was measured by labeling PBMCs with carboxyfluorescein diacetate succinimidy ester, activating 100 000 PBMCs/well in a 96-well round-bottom plate with anti-CD3/anti-CD28 coated beads (Invitrogen) at a ratio of 1 bead : 32 cells, and culturing with increasing numbers of transduced T cells as indicated. After four days, cells were stained with anti-CD8 (BD Pharmingen). Samples were read on a BD FACSCanto and analyzed with FCS Express Pro Software version 3, gating on CD8+ T cells.

2.2.10 Suppression of CD4+ T cell responder cytokine production by transduced T cells

Suppression of IFN-γ and TNF-α production from CD4+ responders was measured by co-culture of 50 000 CD4+ T cells with 50 000 irradiated (5000 rads) APCs plus soluble anti-CD3 (0.1 or 1 µg/ml) with increasing numbers of transduced T cells. Supernatants were
collected after 72 hours and analysed with a Th1/Th2 cytometric bead array (BD Biosciences). Data are expressed as the percent suppression of cytokine produced by CD4⁺ responders alone. Compiled data are the averages of a minimum of 3 independent experiments derived from 2 donors for F324L lines or at least 3 donors for wt FOXP3, R347H, and F373A lines.

2.2.11 Transduction, culture and analysis of human Th17 cells

CD4⁺CXCR3⁻CCR4⁺CCR6⁺ T cells were sorted from CD4⁺ T cells on a BD FACSARia to >95% purity by staining CD4⁺ T cells with antibodies against CD4, CCR4, CCR6, and CXCR3. CD4⁺CXCR3⁺ cells were sorted as control IL-17⁺IFN-γ⁺ T cells. Cells were activated, transduced and cultured as described above, with the exception that ∆NGFR-positive cells were not purified. Twelve days after transduction, cells were stimulated with phorbol 12-myristate 13-acetate (PMA, 10ng/mL) and ionomycin (100ng/mL) for 6 hours, with brefeldin A (10µg/mL) added for the last 4 hours. Cells were stained for ∆NGFR before being fixed and permeabilized with FixPerm buffer (eBiosciences) for 30 minutes, then stained with anti-IFN-γ (BD Pharmingen) and anti-IL-17A (eBioSciences) in Perm buffer (eBiosciences). Samples were acquired on a BD FACSCanto and analysis was performed on gated ∆NGFR⁺ T cells.

2.2.12 Statistical Analysis

All statistical analyses were performed with the two-tailed Student’s paired t-test, and data were transformed with log (base 10) to account for variability between the cell lines. Error bars represent the standard error of the mean. Stars indicate a significant difference.
compared to wt FOXP3. Stars above a joining line represent significant differences compared to control ΔNGFR-transduced cells. *** p < 0.001, ** p = 0.001 - 0.01, * p = 0.01 – 0.05.
2.3 Results

2.3.1 Effects of IPEX point mutants of FOXP3 on expression of Treg-associated cell surface proteins

To better understand whether the degree of IPEX is correlated with the relative dysfunction of FOXP3 in Tregs, three different point mutant forms of FOXP3 known to cause different severities of disease were selected\(^ {98,105,262,263}\) (Figure 2.1 and Table 2.1). In order to use a previously developed over-expression system in which FOXP3 is expressed at a level similar to that found in nTreg cells, resulting in the reprogramming of naive Tconv cells into Tregs,\(^ {89,270}\) the point mutant forms of FOXP3 were fused to an HA-tag and placed under control of the elongation factor 1 alpha promoter in the bi-directional pCCL lentivirus vector which encodes ∆NGFR as a marker gene. Figure 2.1 shows co-expression of HA and FOXP3 in transduced T cells and that the levels of expression of wt and mutant forms of FOXP3 were equal.

Figure 2.1 Expression of hemagglutinin (HA)-tagged FOXP3 IPEX mutants in Tconv cells.

Naive (CD45RO\(^ {\text{−}}\)) CD4\(^ {\text{+}}\)CD25\(^ {\text{−}}\) T cells were transduced with control (∆NGFR), HA-tagged FOXP3\textsubscript{a}, or one of three HA-tagged IPEX mutant FOXP3-expressing lentiviral vectors. Transduced cells were purified based on expression of the marker gene, ∆NGFR. Expression of HA and FOXP3 following purification of transduced cells is shown for one representative of a minimum of 3 experiments. Locations of IPEX patient mutations are indicated on a schematic representation of FOXP3 which also depicts the NH\(_{2}\) terminal HA tag and exon numbers.
Table 2.1 Summary of the clinical manifestations, treatments, and outcomes of IPEX patients with the FOXP3 mutations investigated.

<table>
<thead>
<tr>
<th>Location of mutation</th>
<th>F324L (Pt 5)*</th>
<th>R347H (Pt 9)**</th>
<th>R347H (Pt 10)**</th>
<th>R347H (Case 1)**</th>
<th>R347H 8</th>
<th>F373A (Pt 12)**</th>
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<tr>
<td></td>
<td>Outside forkhead domain</td>
<td>In forkhead domain</td>
<td>In forkhead domain</td>
<td>In forkhead domain</td>
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<td>Neonatal</td>
<td>&lt; 1 year</td>
<td>&lt; 1 year</td>
<td>&lt; 1 year</td>
<td>Neonatal</td>
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<tr>
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<td>Hyperglycemia, diarrhoea</td>
<td>Recurrent ear infections, high IgE levels</td>
<td>IDDM</td>
<td>Gastritis</td>
<td>Ketoacidosis</td>
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<td>Severe diarrhoea with villous atrophy</td>
<td>Severe chronic gastritis with mucosal atrophy</td>
<td>Diarrhoea</td>
<td>Severe gastritis with eosinophilic infiltration and mucosal atrophy</td>
<td>Severe diarrhoea with villous atrophy</td>
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<td>T1D</td>
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<td>T1D</td>
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<td>Mild eczema</td>
<td>Mild xerosis</td>
<td>-</td>
<td>-</td>
<td>Eczema</td>
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<td>-</td>
<td>-</td>
<td>-</td>
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<td>Coombs negative anemia, food allergy, failure to thrive</td>
<td>Pancreatic exocrine failure, failure to thrive</td>
<td>Failure to thrive, intractable hypertension with encephalopathy</td>
<td>Intestinal metaplasia, failure to thrive, steatorrhea, abnormal sweat test, pancreatic atrophy, hypo-γ-globulinemia</td>
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<td>-</td>
<td>aETC</td>
<td>-</td>
<td>AIA, aETC</td>
<td></td>
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<tr>
<td>Treatment</td>
<td>None; spontaneously recovered</td>
<td>Immuno-suppression (CsA for first 3 years, prednisone)</td>
<td>Immuno-suppression (CsA, methyl-prednisone)</td>
<td>Immunosuppression (CsA, prednisone; later: tacrolimus); HLA-id BMT</td>
<td>Immunosuppression (prednisone, CsA)</td>
<td>HLA-id BMT at 9 months</td>
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<td>Outcome (as of July 2010 unless noted otherwise)</td>
<td>Alive 7 years old</td>
<td>Alive 14 years old</td>
<td>Alive 23 years old</td>
<td>Died at age 14 years 194 days post-BMT</td>
<td>Alive 22 years old as of September 2009</td>
<td>Alive 7.5 years old</td>
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</table>

* This patient has an additional mutation/polymorphism: 543 C>T at intron 4/exon 5 boundary. Pt, patient; TID, type 1 diabetes; GAD, anti-glutamate decarboxylase antibody; AIA, anti-insulin antibody; ICA, anti-islet cell antibody; aETC, anti-enterocyte antibody; IS, immunosuppression; HLA-id BMT, HLA-identical bone marrow transplant.
FOXP3 regulates expression of several cell-surface markers characteristic of Tregs by acting as either a transcriptional repressor or activator.\textsuperscript{118-120} To determine whether IPEX point mutant forms of FOXP3 were defective in transactivation and/or repression, I analyzed the cell surface phenotype upon expression of wild type (wt) or mutant forms of FOXP3. As expected, over-expression of wt FOXP3 resulted in up-regulation of CD25, GITR, and CCR4 and down-regulation of CD127 (Figure 2.2). T cells transduced with F324L did not differ significantly from those transduced with wt FOXP3 in expression of any of these proteins. In contrast, T cells transduced with F373A and R347H failed to up-regulate CD25 to the same extent as those transduced with wt FOXP3 (average fold increase in MFI compared to control was 4.95 ± 0.89 for wt FOXP3 versus 2.36 ± 0.60 for F373A, \( p < 0.0001, n = 12 \), and 3.17 ± 0.53 for R347H, \( p = 0.0075, n = 8 \)). While both F373A and R347H retained their ability to promote expression of GITR and suppress CD127 in transduced T cells, only F373A was defective at upregulating CCR4 (2.43 ± 0.33 average fold increase in mean fluorescence intensity (MFI) for wt FOXP3 compared to 1.62 ± 0.11 for F373A, \( p = 0.0129, n = 10 \)). Although F373A and R347H did not upregulate CD25 to the same extent as wt FOXP3, they caused significant upregulation of CD25 in transduced cells compared with control (ΔNGFR) transduced cells (\( p = 0.0198, n = 12 \), and \( p = 0.0036, n = 7 \) for F373A and R347H, respectively). Likewise, compared to ΔNGFR-transduced cells, F373A induced significant upregulation of CCR4 (\( p < 0.0001, n = 10 \)). These data indicate that mutations in the forkhead domain of FOXP3 do not necessarily abrogate its ability to regulate transcription.
Figure 2.2 Cell surface marker expression on T cells transduced with WT FOXP3 or IPEX mutants.

Expression of the indicated proteins was determined in resting T cell lines. Histograms show one representative experiment gated on ∆NGFR⁺ cells. Bar graphs depict the average fold difference in MFI (ΔMFI) compared to the MFI of control transduced cells. Stars indicate a significant difference compared to wt FOXP3. Stars above a joining line represent significant differences compared to control ∆NGFR-transduced cells. *** p < 0.001, ** p = 0.001 - 0.01, * p = 0.01 – 0.05.

2.3.2 Effects of IPEX point mutants of FOXP3 on cytokine production

A hallmark of Treg cells is their inability to produce IL-2, IFN-γ or TNF-α. To investigate whether point mutations in FOXP3 abrogate its capacity to suppress cytokine production, I compared T cells transduced with wt versus mutant forms of FOXP3. As
expected, expression of wt FOXP3 repressed production of IFN-γ, TNF-α, IL-2, IL-4 and IL-5 (Figure 2.3). T cells expressing F324L suppressed cytokine production to the same extent as those expressing wt FOXP3. Likewise, cytokine production by T cells transduced with the forkhead mutant R347H did not differ significantly from that of cells transduced with wt FOXP3. In contrast, the forkhead mutant F373A was defective at suppressing production of IFN-γ, TNF-α, IL-2, IL-4, and IL-5 (p = 0.0173, n = 4; p = 0.0025, n = 5; p = 0.0073, n = 4; p = 0.0050, n = 4; and p = 0.0083, n = 4, compared with wt FOXP3, respectively). The repressive capacity of F373A, however, was not completely eliminated: compared with ΔNGFR-transduced cells, F373A transduction resulted in a significant decrease in the production of TNF-α and IL-4 (p = 0.0287, n = 5 and p = 0.0200, n = 4, respectively). As was previously reported, expression of wt FOXP3 did not significantly affect IL-10 production, and none of the point mutants differed in this respect. These data show that mutations in FOXP3 which cause IPEX do not necessarily abrogate the capacity of the protein to suppress cytokine production.
2.3.3 IPEX point mutant forms of FOXP3 do not uniformly lose their capacity to reprogram Tconv cells into suppressive Tregs

I next tested whether T cells transduced with the point mutant forms of FOXP3 became hypoproliferative and acquired suppressive function. Although expression of all forms of FOXP3 resulted in decreased proliferation compared with ∆NGFR expression, the ability of the F373A and R347H mutants to induce hyporesponsiveness to polyclonal stimulation was reduced compared to wt FOXP3 (p = 0.0019, n = 13 and p = 0.0034, n = 8,
respectively, Figure 2.4A). All transduced T cell lines proliferated in the presence of IL-2 (data not shown), demonstrating the viability of the cells. As expected, wt FOXP3-transduced T cells significantly inhibited the proliferation and cytokine production of ex vivo responder CD4+ T cells and the proliferation of ex vivo CD8+ T cells in a dose dependent manner (Figure 2.4B-D). Surprisingly, F324L and R347H mutants retained a normal capacity to confer transduced T cell lines with the ability to suppress both proliferation and cytokine production of responders. In contrast, F373A was significantly impaired at reprogramming T cells into suppressive cells compared with wt FOXP3. T cell lines transduced with F373A suppressed CD4+ T cell responder proliferation by an average of 39.3% ± 13.5% at a 1:2 ratio (transduced cells : responder cells) whereas wt FOXP3-transduced lines suppressed by an average of 82.3% ± 6.7% at this ratio (p = 0.0149, n = 6, Figure 2.4B).

Since the contribution of proliferation by CD4+ responders and transduced T cells cannot be distinguished by the tritiated thymidine incorporation assay, suppression assays were also performed by carboxyfluorescein succinimidyl ester (CFSE) dilution in which the proliferation of CD8+ responder T cells after coculture with transduced T cells was determined (Figure 2.4C). A similar trend was observed by CFSE dilution assay as by tritiated thymidine incorporation: F373A-transduced cells were less able to suppress the proliferation of CD8+ responders than wt FOXP3-transduced cells, while R347H and F324L-transduced lines had a suppressive capacity similar to wt FOXP3-transduced lines.
Figure 2.4 Proliferation and suppressive capacity of T cells transduced with WT FOXP3 or IPEX mutants.
Figure 2.4  Proliferation and suppressive capacity of T cells transduced with WT FOXP3 or IPEX mutants.

A. Proliferation of T cell lines expressing wt or mutant FOXP3. T cell lines were stimulated with irradiated APC and soluble anti-CD3 (1 µg/mL) and pulsed with tritiated thymidine ([\( ^{3}\text{H} \)-TdR]) 72 hours later. Data are depicted as percent of control ∆NGFR proliferation. B. Suppression of CD4⁺ T cell proliferation by transduced T cells determined by [\( ^{3}\text{H} \)-Tdr] incorporation. Transduced T cell lines were co-cultured with freshly isolated human CD4⁺ T cells at the indicated ratios of transduced cells : responder T cells. Co-cultures were stimulated with anti-CD3/anti-CD28 coated beads and pulsed with [\( ^{3}\text{H} \)-Tdr] after 6 days. Data are shown as average percent suppression of proliferation of CD4⁺ responders alone. C. Suppression of CD8⁺ T cell proliferation by transduced T cells determined by CFSE dilution. Transduced T cell lines were co-cultured with freshly isolated, CFSE-labeled human PBMC at the indicated ratios of transduced cells : responder PBMCs. Co-cultures were stimulated with anti-CD3/anti-CD28 coated beads. Four days later, cultures were stained with anti-CDS to determine CFSE dilution by flow cytometry. D. Suppression of CD4⁺ T cell IFN-\( \gamma \) and TNF-\( \alpha \) production by transduced T cells. Transduced T cell lines were co-cultured with freshly isolated human CD4⁺ T cells at the indicated ratios of transduced cells : responder T cells. Co-cultures were stimulated with irradiated APC and soluble anti-CD3 (1 µg/mL), and supernatants were collected after 72 hours for analysis by cytometric bead array. Data are expressed as the average percent suppression of cytokine produced by CD4⁺ responders alone. For A, B, and D, a representative experiment is depicted on the left and compiled data are depicted on the right. Stars indicate a significant difference compared to wt FOXP3. Stars above a joining line represent significant differences compared to control ∆NGFR-transduced cells. *** p < 0.001, ** p = 0.001 – 0.01, * p = 0.01 – 0.05.

The ability of F373A-transduced lines to suppress production of IFN-\( \gamma \) and TNF-\( \alpha \) by CD4⁺ T cell responders was also significantly diminished (Figure 2.4D). F373A-transduced lines were completely deficient in their capacity to suppress IFN-\( \gamma \): at a 1:2 ratio. At this ratio, IFN-\( \gamma \) production by CD4⁺ responder T cells increased in the presence of F373A-transduced T cells whereas lines transduced with wt FOXP3 suppressed IFN-\( \gamma \) production by CD4⁺ responders by 61.4% ± 8.0 % (F373A compared with wt FOXP3, p = 0.0267, n = 5).

Suppression of TNF-\( \alpha \) by F373A-transduced lines at the 1:2 ratio was 2.1% ± 20.5% compared to 65.3% ± 7.1% by wt FOXP3-transduced lines (p = 0.0015, n = 3). Hence, even though forkhead mutants F373A and R347H both cause severe IPEX, they are not equally defective at reprogramming Tconv into suppressive cells in vitro.
2.3.4 IPEX mutant forms of FOXP3 do not uniformly lose their capacity to inhibit IL-17 production from Th17 cells

Complex interactions between FOXP3 and Th17-associated transcription factors including ROR-γt, ROR-α, and Runx1 have recently been described.\textsuperscript{130,132-135,273-275} Notably, IL-17 expression driven by ectopic expression of Runx1 or ROR-γt in CD4\textsuperscript{+} T cells is inhibited by ectopic expression of FOXP3\textsuperscript{133} indicating that FOXP3 negatively regulates Th17 differentiation. Given the unexpected finding that IPEX point mutants of FOXP3 retained some or all of their ability to convert Tconv cells into Tregs \textit{in vitro}, it was hypothesized that their ability to inhibit IL-17 production from proinflammatory Th17 cells may be impaired. Of particular interest was whether F324L may be defective in regulating IL-17 expression in Th17 cells as this mutation is located within the Runx1-interacting domain of FOXP3 (amino acids 278-336)\textsuperscript{132} and a mutant form of Foxp3 (329VHL) that can bind to ROR-γt but not to Runx1 is defective at inhibiting ROR-γt-induced IL-17 expression.\textsuperscript{133} Due to the difficulties in efficiently differentiating IL-17-producing cells \textit{in vitro}, enriched populations of \textit{ex vivo} Th17 cells were isolated from peripheral blood by sorting CD4\textsuperscript{+}CXCR3\textsuperscript{-}CCR4\textsuperscript{+}CCR6\textsuperscript{+} T cells\textsuperscript{271} and the ability of ectopically-expressed FOXP3 mutants to down-regulate IL-17 production in these cells was examined. Sorted CD4\textsuperscript{+}CXCR3\textsuperscript{-}CCR4\textsuperscript{+}CCR6\textsuperscript{+} T cells were transduced with wt FOXP3, point mutant forms, or the alternately spliced form of FOXP3 (FOXP3b)\textsuperscript{130,135} that is defective at suppressing ROR-γt-driven IL-17 production in 293T cells\textsuperscript{135} because it lacks the ROR-γt and ROR-α interacting domain. Transduced T cells were re-stimulated with PMA and ionomycin twelve days after transduction, and ∆NGFR\textsuperscript{+} cells were analyzed for production of IL-17 and IFN-γ (\textbf{Figure 2.5}). Consistent with its deficiency in blocking ROR-γt-driven
IL-17,\textsuperscript{135} expression of FOXP3b did not suppress IL-17 production by \textit{ex vivo} Th17 cells to the same extent as wt FOXP3 ($p = 0.0088$, $n = 3$). While Th17 cells transduced with F324L or R347H were equivalent to wt FOXP3 in suppression of IL-17 production, Th17 cells transduced with F373A had a deficiency similar to that of FOXP3b and did not suppress IL-17 production to the same extent as cells transduced with wt FOXP3. On average, F373A transduction suppressed IL-17 production by only 26.7% ± 19.2% compared with 49.5% ± 12.1% suppression by wt FOXP3, although this difference did not reach statistical significance ($p = 0.1092$, $n = 3$). These data suggest an impaired ability of F373A to regulate IL-17 expression by fully differentiated \textit{ex vivo} Th17 cells compared with wt FOXP3.

**Figure 2.5 Expression of IL-17 and IFN-γ in Th17 cells expressing wt, wt FOXP3b or IPEX mutant forms of FOXP3.**

A. Data from one representative experiment. B. Summary of percent suppression of IL-17 production based on IL-17 production by control transduced cells. Lines connect individual experiments, comparing percent suppression by wt FOXP3 to suppression by each of the IPEX mutants and to wt FOXP3b. Stars indicate a significant difference compared to wt FOXP3. ** $p = 0.001 - 0.01$. 

Enriched populations of Th17 cells (CD4$^+$CXCR3$^-$CCR6$^+$CCR4$^+$) were sorted and stimulated with irradiated APC and soluble anti-CD3 (1 µg/mL) in the presence of IL-2 for 18 hours, then transduced with IPEX mutants, wt FOXP3, wt FOXP3b, or control (ΔNGFR) lentivirus. Twelve days later, cells were re-stimulated with TPA plus ionomycin for 6 hours, and expression of IL-17 and IFN-γ was analyzed in ΔNGFR$^+$ cells.
2.4 Discussion

Evidence that even IPEX patients with severe disease can have circulating FOXP3 T cells\(^{105,264}\) suggests that the cellular basis for the disease may be due to a functional defect rather than a numerical loss of Treg cells. To further investigate this possibility, whether three point mutant forms of FOXP3 were altered in their ability to reprogram Tconv cells into Treg cells was examined. I found that the mutants were not equally impaired, and none were completely deficient, in their capacity to drive the Treg phenotype. These findings suggest some forms of IPEX may not be caused exclusively by altered Treg cell function.

FOXP3 can act as both a transcriptional activator and repressor of cell surface marker expression.\(^{118-120}\) Upon ectopic expression of wt FOXP3, changes in gene expression characteristic of Treg cells occur, including upregulation of CD25 and CCR4 and downregulation of CD127.\(^{89,276}\) Although F373A FOXP3 was defective at upregulating CD25 and CCR4, it retained a normal capacity to downregulate CD127, suggesting that alternative mechanisms may regulate transcriptional repression versus activation. A critical role for the F373 residue in transactivation of genes by FOX proteins is supported by a study on mutations in FOXL2: a mutation in FOXL2 at F90, which corresponds to F373 in FOXP3, results in defective transactivation of a FoxL2-regulated promoter.\(^{277}\)

It was previously found that the expression of CD25 in \textit{ex vivo} PBMCs from an IPEX patient with the F373A mutation was comparable to that in PBMCs from normal donors, and CD4^+CD25^+ T cell lines generated from this patient maintained high CD25 expression in the resting state.\(^{105}\) These data contrast with the current finding that cell lines transduced with F373A have lower expression of CD25 compared to cell lines transduced with wt FOXP3. This difference could be due to additional Treg-associated transcription factors that
contribute to transactivation of CD25 which are lacking in transduced CD4+ T cells but are present in CD25+ Tregs from IPEX patients. This hypothesis is in line with new evidence that proteins such as the transcription factor Eos148 and the surface receptor glycoprotein A repetitions predominant (GARP)278,279 have a role in defining the Treg phenotype, and that cells with a Treg phenotype can develop in the absence of functional FOXP3.30,72

In addition to their characteristic cell surface marker expression, a hallmark of Treg cells is their lack of cytokine production, which is due to the repressive effects of FOXP3.27,88,272 It was previously observed that, in contrast to Scurfy mice that have elevated cytokine production,107 PBMCs from IPEX patients carrying either the F373A or F324L mutation have decreased cytokine production compared with normal donors, particularly of IFN-γ, TNF-α and IL-2.105 This difference between mice and humans with FOXP3 mutations may exist because in humans, but not mice, FOXP3 is transiently expressed in activated Tconv cells.108-110,112,113 Though the role of FOXP3 in human Tconv is unknown, the surprising phenotype of decreased cytokine production in IPEX patients suggests there could be a dominant negative effect of mutant activation-induced FOXP3 on NFAT-regulated cytokine production in Tconv cells.

Recently, a role for FOXP3 in antagonizing the differentiation of pro-inflammatory Th17 cells via protein-protein interactions with ROR-γt and Runx1 has been recognized.133-135 Since Th17 cells have been implicated in many autoimmune diseases,280 it was speculated that mutant forms of FOXP3 that cause IPEX might inadequately suppress IL-17 production. It has previously been shown that Th17 cells express high levels of CCR4 and CCR6 and not CXCR3.281 Hence, to investigate the abilities of FOXP3 point mutants to suppress IL-17 production by Th17 cells, I developed a novel assay in which sorted CXCR3 CCR4+ CCR6+
T cells were transduced with FOXP3 and showed for the first time that wt FOXP3 can inhibit IL-17 expression in fully differentiated ex vivo Th17 cells. Surprisingly, F324L and R347H were as effective as wt FOXP3 at suppressing IL-17 expression by transduced sorted Th17 cells. By contrast, F373A was less able to suppress IL-17 production, so further investigation is warranted to determine if deregulated IL-17 production may have contributed to disease progression in the patient carrying the F373A mutation.

There is evidence that FOXP3 can bind conserved non-coding sequences and positively regulate its own expression\(^\text{282}\) raising the possibility that ectopically expressed FOXP3 may bind to the promoter of endogenous FOXP3 thereby upregulating wild type FOXP3 which could contribute to a Treg phenotype. Although a possible contribution of endogenous FOXP3 cannot be formally excluded, analysis of the methylation state of the TSDR, the region of the FOXP3 locus which must be demethylated to allow high and stable expression of FOXP3,\(^\text{85,166,167,238,267-269}\) suggested this was unlikely to be the case. Tconv cells transduced with wt FOXP3 had only 3.0% ± 3.1% demethylation of the TSDR (n = 4, data not shown) compared to nearly 100% demethylation in natural Tregs\(^\text{268}\) indicating that ectopic expression of FOXP3 does not result in significant epigenetic remodelling of the endogenous locus. These data are also consistent with previous findings that suggested FOXP3 does not positively self-regulate by demethylating conserved non-coding sequences, but rather by binding those sequences only after they are demethylated.\(^\text{282}\)

Currently, the only curative treatment for IPEX is bone marrow transplantation.\(^\text{98}\) Interestingly, a recent report on chimerism following bone marrow transplant of an IPEX patient showed that after 6 years, while the majority of conventional CD4\(^+\) T cell subsets were of recipient origin, donor chimerism in the CD4\(^+\)CD25\(^{hi}\) T cells subset was 53-91%.\(^\text{283}\)
Importantly, this patient has a FOXP3 null mutation which results in a complete lack of FOXP3 expression, rather than a point mutation where a mutant form of FOXP3 is expressed. Since this patient is in complete remission, these data suggest that, in the complete absence of FOXP3, defective Treg cells may be the sole cause of disease. Nevertheless, we found that F324L, R347H, and F373A were not completely defective at inducing a Treg phenotype, indicating that FOXP3 clearly has additional roles. For example it may play a role either in activated Tconv cells or in Treg cell functions other than direct T cell suppression such as in the inhibitory effects of Treg cells on antigen presenting cells and B cells.284-287

In conclusion, my results suggest that the full clinical manifestations of IPEX may not be exclusively caused by defects in Treg cells in all patients, depending on the mutation. Although both F373A and R347H are mutations located in the forkhead domain that cause severe IPEX, only F373A was significantly, but not completely, impaired in reprogramming Tconv cells into Tregs. Furthermore, F324L, which causes a mild form of IPEX, did not display any significant defect in the best-characterized cellular and molecular functions of FOXP3. The hypothesis that IPEX is not caused by a numerical deficit in Treg cells is supported by a recent study288 in Scurfy mice which found that Foxp3-deficient Treg cells share many characteristics with wt Treg cells including in vitro anergy, decreased cytokine production, and gene expression profiles. Therefore, it seems likely that the immune deficiencies in IPEX patients can be caused by changes in the function of FOXP3 outside of its currently recognized role in regulation of Treg cell development and function. Future investigation into the role of activation-induced FOXP3 in Tconv cells and how functions
may be disrupted in IPEX will be essential to better understand the pathophysiology of this
disease and is examined in chapter 3.
Chapter 3: FOXP3 is highly expressed in activated human Th17 cells and has a role in the regulation of human conventional T cells.

3.1 Introduction

The best-known function of the transcription factor forkhead box P3 is as an extrinsic factor to suppress the activation and function of immune cells in the context of CD4+CD25+ natural T regulatory (Treg) cells. This function was elucidated in 2003 by Sackaguchi, Rudensky, and Ramsdell who showed that overexpression of FOXP3 in mouse Tconv cells via retroviral transduction27,28 or transgene expression26 converts them into suppressive cells with the phenotype of natural Treg cells. Since then, it has been shown that human Tconv cells can also acquire a Treg phenotype upon high and continuous expression of FOXP3 via lentiviral transduction.89,91

The precise mechanisms by which FOXP3 orchestrates the ability of Treg cells to suppress CD4+ and CD8+ T cells, B cells, and antigen presenting cells is unclear, but acquisition of a Treg phenotype is achieved by both repressing and activating genes.119,120 Treg cells do not produce typical T cell derived cytokines, and FOXP3 is able to repress genes including IFN-γ, IL-2, IL-4, IL-5, IL-8 and TNF27,88,272,289,290 while up-regulating Treg cell-associated surface markers including CD25, CTLA-4, and CCR4.27,89,118,290 To mediate its effects, FOXP3 binds to other transcription factors including NFAT118,266 and Runxl132,133 and the Th17 transcription factors ROR-γt135,291 and RORα.130,134 Furthermore, FOXP3 recruits the histone acetyl-transferase TIP60,125 histone deacteylases HDAC7 and HDAC9,125,137 and linker histone H1.5.147

Mice and humans that lack functional FOXP3 develop severe autoimmune disease, known as IPEX in humans.92-94 Some patients with IPEX still have circulating CD25+ T
cells\textsuperscript{105,264} and cells with a characteristic demethylated Treg-specific demethylated region (TSDR)\textsuperscript{106} but, because Treg cells cannot function without FOXP3, the most likely cause of disease in most cases is a lack of functional Treg cells. Nevertheless, my previous studies found that some point mutant forms of FOXP3 were still able to confer suppressive capacity to T cells in which they were over-expressed, suggesting that dysfunctional FOXP3 may contribute to IPEX in a role that is dysregulated outside of Treg cells.\textsuperscript{290} FOXP3 is transiently up-regulated in human conventional T cells upon activation,\textsuperscript{108-114} so dysregulated FOXP3 in Tconv cells may also contribute to the pathogenesis of IPEX. However, the role of activation-induced FOXP3 in Tconv cells has not been determined.

One hypothesis for the role of FOXP3 in Tconv cells is that FOXP3-expressing Tconv cells acquire transient suppressive capacity. Indeed, some groups have reported that FOXP3\textsuperscript{+} Tconv cells are suppressive,\textsuperscript{111,114,115} but others have shown this not to be the case.\textsuperscript{108-110,113,116} These conflicting results are likely due to the difficulty in drawing meaningful conclusions from \textit{in vitro} suppression assays with activated Tconv cells.\textsuperscript{292}

Though results of suppression assays are difficult to interpret, activated, FOXP3-expressing Tconv are not likely to be regulatory cells. Firstly, they rapidly down-regulate FOXP3 three days after activation and produce IL-2 and IFN-\(\gamma\).\textsuperscript{108-110,113} New evidence has emerged that FOXP3 is expressed transiently in mouse Tconv cells and that these FOXP3-expressing mouse Tconv cells, like their human counterparts, produce IL-2 and are not suppressive.\textsuperscript{293} Second, in order for human Tconv cells to acquire the capability of suppressing other T cells, FOXP3 must be expressed at high and stable levels for at least 7-12 days which can be achieved by driving FOXP3 expression from a constitutively active promoter.\textsuperscript{89,91} Over-expression from a viral long terminal repeat that results in fluctuating
levels of FOXP3 did not confer suppressive capacity to Tconv cells\textsuperscript{88,89} and may more closely resemble activation-induced FOXP3. Furthermore, low-level FOXP3 expression in nTregs in a mutant mouse strain resulted in a lack of suppressive capacity and skewing to Th2 effectors despite a preserved anergic phenotype.\textsuperscript{162} Since the mean fluorescence intensity of activation-induced FOXP3 never reaches that seen in similarly activated Treg cells,\textsuperscript{108,112,113} it is unlikely that FOXP3 levels in Tconv cells are high enough or maintained for a long enough period to confer suppressive capacity. Taken together, these observations indicate that the level of FOXP3 expression in T cells plays a major role in the function of FOXP3 and that the role of low-level and transient expression of FOXP3 in activated Tconv cells is not necessarily to reprogram them into Treg cells. Thus, a separate role for FOXP3 in Tconv cells is yet to be uncovered.

In order to investigate the role of FOXP3 in Tconv cells, FOXP3-deficient human Tconv were compared with wild type Tconv cells. It was found that FOXP3-deficient Tconv cells proliferated to a greater extent and produced a greater amount of cytokines than wild type Tconv cells. Furthermore, FOXP3 was highly expressed in Th17 cells during the course of a two-week activation cycle and, in these cells, FOXP3 also demonstrated a role in suppressing IFN-\(\gamma\) production and in up-regulating homing markers.
3.2 Materials and methods

3.2.1 Isolation of blood cells from human subjects

Peripheral blood was obtained from healthy volunteers and from the carrier of a null mutation in FOXP3 who gave written informed consent in accordance with the protocol approved by the University of British Columbia Clinical Research Ethics Board. PBMCs, antigen presenting cells (APCs), CD4+ T cells, and CD4+CD25-CD45RO- (naïve) T cells were isolated, transduced, and expanded as described in section 2.2.3.

3.2.2 Lentiviral vectors

Lentiviral vectors encoding siRNA against FOXP3 or against luciferase were kindly provided by Luigi Naldini.294 Lentivirus was produced as described in section 2.2.2.

3.2.3 Cloning of CD4+CD25- Tconv from a carrier of a FOXP3 null mutation

CD4+CD25- T cells were sorted from total PBMCs on a BD FACS Aria (BD Biosciences, Mississauga, Ontario, Canada). They were plated at 0.3 T cells/well in 96 well plates and stimulated with irradiated allogeneic PBMCs, JY cells, phytohemagglutinin, and IL-2. Clones were re-stimulated every two weeks hence with allogeneic PBMCs, JY cells, phytohemagglutinin, and IL-2.

3.2.4 X chromosome inactivation analysis

X-chromosome inactivation analysis was performed on each clone to determine which allele of FOXP3, wild type or null, was active.104 The methylation-sensitive restriction enzyme HpaII was used to digest genomic DNA at 37°C for 24 hrs (20 U added at time 0 and
20 U after 6-7 hrs. Digestion with HpaII was improved by addition of 20 U of RSAI restriction enzyme which is not sensitive to methylation. Digested DNA was purified using Millipore Multiscreen plate, and 30 ng of undigested and digested DNA was amplified with the primers HumaraF 5′-6FAM labeled and HumaraR. These primers amplify a fragment of the HAR gene which contains a polymorphic number of CAG repeats. This fragment also contains an HpaII restriction site; thus, after digestion, only the methylated DNA is amplified. DNA from a healthy male was used as an internal control to verify the efficacy of digestion.

Amplicons were diluted in formamide, denatured, and run on the Applied Biosystems 3130xl automated sequencer. Previous work determined the CAG allele co-segregating with the mut-FOXP3 allele, enabling fragment analysis to be performed by GeneMapper software (Applied Biosystems) to evaluate the number of the CAG repeats and thus determine whether the wild type or null allele was active in each clone. Figure 3.1 shows an example of the GeneMapper results for one wild type and one FOXP3 null clone.
Figure 3.1 X chromosome inactivation analysis of CD4⁺CD25⁻ Tconv cell clones.

GeneMapper (Applied Biosystems) results for one FOXP3 null (top panel) and one wild type (bottom panel) CD4⁺CD25⁻ Tconv cell clone. Genomic DNA was digested with the methylation-sensitive restriction enzyme HpaII. The HAR gene, which contains an HpaII restriction site, was amplified, and the size of the amplicon reflects the number of CAG repeats in the amplified, inactive gene. Numbers along the top of the graph indicate the size of the amplicon. The wild type allele of FOXP3 co-segregates with the “shorter” HAR gene; thus, the longer HAR gene is amplified in Tconv cell clones in which the wild type allele is active (bottom panel). Conversely, the FOXP3 null allele co-segregates with the “longer” HAR gene and the shorter HAR gene is amplified when the FOXP3 null allele is active (top panel).

3.2.5 Isolation and expansion of Th1 and Th17 cells

CD4⁺ T cells were depleted of CD25⁺ cells with CD25 microbeads (Miltenyi Biotec Inc., Auburn, California). Th1 (CD4⁺CD25⁻CXCR3⁺CCR4⁻CCR6⁻) and Th17 (CD4⁺CD25⁻CXCR3⁻CCR4⁺CCR6⁺) cells were sorted from CD4⁺CD25⁻ T cells on a BD FACSAria (BD Biosciences) to >95% purity. Cells were stimulated with autologous APCs and soluble anti-CD3 (OKT3, 1 µg/mL) in the presence of 100 U/mL IL-2. After 2 weeks, the Th1 and Th17 cells were rested without IL-2 overnight and then re-stimulated with anti-CD3/anti-CD28 coated beads (Invitrogen, Burlington, Ontario, Canada) at a ratio of 1 bead to 32 cells in the presence of 100 U/mL IL-2 for subsequent analysis of expansion and expression of cytokines, FOXP3 and cell surface markers.
3.2.6 Proliferation assays and expansion curves

CD4⁺CD25-T cell clones or lines were rested overnight without IL-2, labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE), plated at 100,000 / well in 96-well round-bottom plates, and stimulated with different ratios of anti-CD3/anti-CD28 coated beads (Invitrogen). Four days later, T cells were stained with fixable viability dye (eBioscience, San Diego, California), CD4 (eBioscience), and truncated nerve growth factor receptor (ΔNGFR) (BD Biosciences or Miltenyi Biotec Inc.) antibodies. Data were acquired on a BD FACSCanto (BD Biosciences) and analyzed with the FlowJo Proliferation Platform version 7.6 (Treestar Inc., Ashland, Oregon) to determine division index (the average number of divisions undergone by a cell in the starting population).

Expansion curves for Th1 and Th17 cells were determined by counting the number of live (fixable viability dye negative, eBiosciences) ΔNGFR⁺ cells (BD Biosciences or Miltenyi Biotec Inc.) in culture by flow cytometry with counting beads (surfactant-free white sulfate latex beads, 0.5 µM; Interfacial Dynamics Corporation, Eugene, Oregon). Fold expansion was determined by dividing the number of cells at each time point by the number of live, un-stimulated cells counted by the same method.

3.2.7 Determination of cytokine production

IFN-γ and IL-2 production were measured 20 hours after activation and IL-8 and IL-17 production were measured after 48 hours by ELISA for CD4⁺CD25⁻ T cell clones and lines from supernatants of CFSE-proliferation assay cultures.

For Th1 and Th17 cells, IFN-γ, IL-17A, and IL-2 production were measured on day 0, day 8, and day 11 after activation by stimulating with phorbol 12-myristate 13-acetate (10
ng/mL, Sigma-Aldrich) and ionomycin (100 ng/mL, Sigma-Aldrich) for 5.5 hours, with brefeldin A (10 µ/mL, Sigma-Aldrich) added for the last 3.5 hours. Cells were stained with anti-NGFR, fixed and permeabilized, and stained with anti-IFN-γ (BD Biosciences), anti-IL-17A (eBioscience), anti-IL-2 (BD Biosciences), and anti-FOXP3 (236/AE7, eBioscience or 259D/C7, BD Biosciences). In addition, Th1 and Th17 cells were washed and re-plated in fresh media plus 100 U/mL IL-2 at 1 x 10⁶ cells/mL on day 8 after activation and supernatants were collected 48 hours later. Cytokine production was determined by the BD Th1/Th2/Th17 cytometric bead array (BD Biosciences).

3.2.8 Flow-cytometric analysis

Analysis of CD25, CTLA-4, CCR4, CCR6, and ΔNGFR was performed on a BD FACSCanto and data were analyzed with FCS Express Pro Software version 3 (De Novo Software, Los Angeles, California) or with FlowJo (Treestar Inc.). To stain for FOXP3, (236/AE7, eBioscience or 259D/C7, BD Bioscience), cells were fixed and permeabilized with FixPerm buffer (eBioscience) before addition of anti-FOXP3.

3.2.9 Statistical Analysis

All statistical analyses were performed with the 2-tailed Student’s paired (where applicable) or unpaired t test. Data were first transformed with log (base 10) to account for variability between cell lines before the t test was performed. Error bars represent the SEM. Asterisks (*) indicate p > 0.05.
3.3 Results

3.3.1 FOXP3 null human Tconv cell clones proliferate more and produce more IFN-γ and IL-2 than wild type Tconv clones

*FOXP3* is located on the X-chromosome, so it is subject to X-chromosome inactivation.\(^{92-94}\) Previous studies showed that while the wild type allele is exclusively active in the Treg population of healthy female carriers of *FOXP3* mutations, the Tconv cells of these carriers have a random X-inactivation profile.\(^{104}\) This random profile means that in half of the Tconv cells the mutated allele of *FOXP3* is inactivated and the wild type allele is expressed upon TCR stimulation. The other half of the Tconv cells do not express *FOXP3* or express the mutated allele of *FOXP3* upon TCR activation because the wild type allele is inactivated. CD4\(^+\)CD25\(^-\) Tconv cell clones were generated from a female who is heterozygous for a null mutation in *FOXP3* (2 T>C (ATG>ACG)) that causes IPEX\(^{98,105}\) which provided a unique experimental system in which a set of human FOXP3 null Tconv clones could be directly compared to autologous wild type Tconv clones. Figure 3.2A shows examples of FOXP3 expression by flow cytometry 4 days after activation with anti-CD3/anti-CD28 coated beads for two wild type and two FOXP3 null Tconv clones. Whether the mutant or the wild type allele was active in each clone was confirmed by X-chromosome inactivation analysis (data not shown).

The proliferation of Tconv clones expressing wild type FOXP3 was compared with the proliferation of FOXP3 null Tconv clones by labeling the clones with CFSE and activating them with different ratios of anti-CD3/anti-CD28 coated beads in the absence of IL-2. After four days, proliferation was measured by flow cytometry and the division index of the clones was calculated using the FlowJo proliferation platform. Division index is
defined as the average number of divisions undergone by a cell in the starting population. An example CFSE dilution for one wild type and one mutant clone is given in Figure 3.2B. FOXP3 null Tconv clones proliferated to a greater extent than wild type clones (Figure 3.2C). Significant differences between the mean proliferation of wild type and FOXP3 null Tconv clones were found at bead:cell ratios of 1:16 and 1:32. The mean division index at the bead:cell ratio of 1:16 was 0.35 ± 0.096 for wild type compared to 0.79 ± 0.11 for FOXP3 null Tconv clones (p = 0.0173) and the mean division index at the bead:cell ratio of 1:32 was 0.20 ± 0.056 for wild type compared to 0.59 ± 0.11 for FOXP3 null Tconv clones (p = 0.0253).
Figure 3.2 FOXP3 null CD4+CD25- Tconv cell clones proliferate to a greater extent and produce more IFN-γ and IL-2 than wild type Tconv cell clones.

Tconv cell clones were labeled with CFSE and stimulated with different ratios of anti-CD3/anti-CD28-coated beads. After 4 days, Tconv clones were stained with anti-CD4 and anti-FOXP3 antibodies (236A/E7) and read on a BD FACSCanto. A. Activation-induced FOXP3 expression in wild type but not FOXP3 null Tconv cell clones. Two representative wild type and two FOXP3 null Tconv clones are shown after stimulation with 1 anti-CD3/anti-CD28 coated bead per 32 cells. The division indices (DI; the average number of divisions undergone by a cell in the starting population) of FOXP3+ and FOXP3− are given within each plot of wild type Tconv clones, as calculated with the Proliferation Platform in FlowJo Version 7.6 (Treestar Inc.). B. Representative CFSE dilutions for one wild type and one FOXP3 null Tconv cell clone. Numbers in each box represent division index. C. Average division index of 7 different wild type and 11 different FOXP3 null Tconv clones, with the division index of each clone calculated as the average of 1-4 independent experiments. D. Average IFN-γ and IL-2 production for wild type and FOXP3 null Tconv clones (5 x 10⁵ cells/mL). Supernatants from cultures were collected 20h after T cell activation and analyzed by ELISA.
FOXP3 is known to regulate a variety of cytokines including IFN-γ, IL-2, and IL-8, so it was determined whether cytokine production differed between FOXP3 null and wild type Tconv clones. IFN-γ and IL-2 production were determined by ELISA after stimulation of the clones with different ratios of anti-CD3/anti-CD28 coated beads for 20 hours. It was found that FOXP3 null Tconv clones produced more IFN-γ and IL-2 than wild type Tconv clones (Figure 3.2D). There was a trend of higher IFN-γ production from FOXP3 null compared with wild type Tconv clones in all stimulation conditions, with significant differences at the 1:64 and 1:128 bead:cell ratios (wild type 256.1 ± 145.6 pg/mL versus FOXP3 null 1517 ± 453.5 pg/mL, p = 0.0336 for 1:64; and wild type 86.14 ± 41.39 versus FOXP3 null 840.4 ± 231.5 pg/mL, p = 0.0108 for 1:128). At a 1:32 bead:cell ratio and below, wild type Tconv clones were not producing detectable levels of IL-2, unlike FOXP3 null Tconv clones which were still producing detectable IL-2 at a 1:128 bead:cell ratio. At a 1:16 bead:cell ratio, there was a significant difference in IL-2 production between wild type and FOXP3 null Tconv clones, with wild type Tconv clones producing a mean of 41.21 ± 26.33 pg/mL and FOXP3 null Tconv clones producing 572.2 ± 179.5 pg/mL IL-2 (p = 0.0126). IL-17 and IL-8 production were measured after 48 hours by ELISA, but none of the Tconv clones were producing IL-17, and there was no difference between FOXP3 null and wild type Tconv clones in the production of IL-8 (wild type Tconv clones producing a mean of 260.2 ± 139.4 pg/mL and FOXP3 null Tconv clones producing 232.5 ± 107.7 pg/mL of IL-8 at a 1:16 bead:cell ratio, p > 0.05, data not shown).
3.3.2 Knock-down of activation-induced FOXP3 expression in human Tconv cells with siRNA increases their proliferation

In order to confirm the results observed in the Tconv clones, expression of activation-induced FOXP3 in wild type Tconv was knocked down by transducing naïve Tconv cells (CD4^+^CD25^−^CD45RO^−^) with a lentiviral vector encoding a small interfering RNA against FOXP3 (siFP3). This vector encodes an artificial miRNA with a perfectly base-paired 21-bp siRNA within the backbone of miRNA 223, a miRNA that is expressed at high levels in myelomonocytic cells but not in other cell types and has a role in myelopoiesis. The artificial miRNA sequence is located within the elongation factor 1 alpha intron and is thus spliced out to generate the pre-mRNA while still allowing concurrent transcription of ΔNGFR. The signaling-deficient truncated NGFR acts as a cell surface marker of transduced T cells to allow purification with magnetic beads. This vector was previously used to knock down expression of FOXP3 in regulatory T cells by an average of 64% compared with Treg cells transduced with siRNA against luciferase (siLuc). Knock-down of FOXP3 expression in Treg cells with this vector decreased their suppressive capacity and CTLA-4 expression while siLuc had no effect on Treg phenotype or function. Thus, this vector and control were selected to knock down expression of activation-induced FOXP3 in Tconv cells.

Experiments were initially performed after 4 weeks of culture (2 rounds of stimulation) but it was found that the level of FOXP3 induction in siLuc control-transduced Tconv increased with each round of stimulation and was only significantly higher than FOXP3 expression in siFP3-transduced cells after six weeks of culture (3 rounds of stimulation) in vitro (Figure 3.3). Hence, all experiments were performed on T cell lines after at least 6 weeks in culture. After this period in culture, activation-induced FOXP3 was
knocked down by an average of 54.9% ± 3.29% between days 1-4 on subsequent re-
stimulations (Figure 3.4A and B).

Figure 3.3 The level of activation-induced FOXP3 expression increases with each round of TCR
stimulation.

![Graph showing FOXP3 expression levels](image)

Control siLuc-transduced T cell lines were re-stimulated at the end of two-week activation cycle with the
indicated ratio of anti-CD3-anti-CD28 coated beads, and the percent of FOXP3+ T cells was determined 4 days
later. Each marker (circle, square, triangle) represents an individual donor and each line type (solid, long dash,
short dash) represents the time point when the experiment was carried out.

The proliferation and cytokine production of siFP3-transduced Tconv was compared
with siLuc control-transduced Tconv by labeling the cells with CFSE and stimulating them
with different ratios of anti-CD3/anti-CD28 coated beads in the absence of IL-2 and
measuring their proliferation by flow cytometry after 4 days. It was found that there was a
trend of greater proliferation by siFP3-transduced Tconv compared with siLuc control-
transduced Tconv which was significant at bead:cell ratios of 1:16, 1:32, and 1:64 (Figure
3.4C; the mean division index of siFP3-transduced T cells compared with siLuc-transduced T
cells was 1.62 ± 0.25 versus 1.06 ± 0.15, p = 0.0388 for 1:16; 1.22 ± 0.19 versus 0.80 ± 0.14,
p = 0.0155 for 1:32; and 0.85 ± 0.15 versus 0.51 ± 0.12, p = 0.0317 for 1:64). IFN-γ and IL-
2 production were assessed after 20h, but no statistically significant differences were
observed (Figure 3.4D), which could be due to the fact that knock-down of FOXP3
expression was not complete (54.9%) unlike in the FOXP3 null T cells clones which do not
express FOXP3 at all. Nevertheless, there was a trend of greater IFN-γ production by FOXP3-deficient T cells at the 1:16 and 1:32 bead:cell ratios.

Figure 3.4 CD4⁺CD25⁻ T cells transduced with siRNA against FOXP3 proliferate to a greater extent than CD4⁺CD25⁻ T cells transduced with control siRNA against luciferase.

CD4⁺CD25⁻ T cells transduced with siFP3 or siLuc were labeled with CFSE and stimulated with different ratios of anti-CD3/anti-CD28-coated beads. Transduced T cells were stained with anti-CD4 (clone) and anti-FOXP3 antibodies (236A/E7) and read on a BD FACSCanto. A. Average knock-down of activation-induced FOXP3 in NGFR⁺ siFP3-transduced T cells compared with control siLuc-transduced T cells over the course of the 4-day experiment. B. Representative FACS plot of FOXP3 expression in NGFR⁺ siFP3 and siLuc-transduced T cells 3 days after activation at a 1:32 bead:cell ratio. C. Average division index of siFP3-transduced T cells and control siLuc-transduced T cells (n=4). Division index is equal to the average number of divisions undergone by a cell in the starting population and was calculated 4 days after activation with the Proliferation Platform in FlowJo Version 7.6 (Treestar Inc.). D. CD4⁺CD25⁻ T cells transduced with siFP3 or siLuc were stimulated with different ratios of anti-CD3/anti-CD28-coated beads at 0.5 x 10⁶ cells/mL. 20 hours later, supernatants were collected and analyzed for IFN-γ and IL-2 by ELISA.

In addition to expression of cytokines, FOXP3 controls expression of cell surface markers including CD25 and CTLA-4. CD25 and CTLA-4 expression were
analyzed over the course of 4 days of activation with varying strengths of stimulus using anti-CD3/anti-CD28 coated beads, but no difference was found in expression of CTLA-4 (p > 0.05 for days 1-4 after stimulation in all stimulation conditions, n = 3) or CD25 (p > 0.05 for days 1-4 after stimulation in all stimulation conditions, n = 3) between siLucB control-transduced and siFP3-transduced Tconv cells (data not shown).

3.3.3 **FOXP3 is highly expressed in human Th17 cells over the course of a two-week activation cycle**

The balance between FOXP3 and ROR-γt is important in Treg versus Th17 cell differentiation from naïve T cells. Interaction of FOXP3 with ROR-γt can inhibit ROR-γt-driven IL-17 production,\(^{134,135}\) while expression of HIF1α with ROR-γt can lead to repression of FOXP3 expression.\(^{156,171}\) However, the role of FOXP3 in fully differentiated Th17 cells, rather than in their development, was investigated here. The role of FOXP3 in fully differentiated Th1 cells was investigated in parallel.

It was previously shown that FOXP3 is expressed maximally in human CD4⁺CD25⁻ T cells 3 days after TCR activation.\(^{108}\) Activation-induced FOXP3 expression has been demonstrated in human Th1 and Th17 cell clones,\(^{110,297}\) but detailed kinetics of FOXP3 expression in different T cell subsets have not been reported. To gain insight into FOXP3 expression patterns in different T cell subsets, CD4⁺CD25⁻CXCR3⁺CCR6⁺CCR4⁺ (Th17) cells and CD4⁺CD25⁻CXCR3⁺CCR4⁺CCR6⁻ (Th1) cells were sorted from human peripheral blood (Figure 3.5A). Th1 and Th17 cells were expanded with autologous APCs and anti-CD3 in the presence of 100U/mL IL-2 to increase cell numbers. Cytokine expression profiles were determined to confirm the phenotype of the cells following expansion. As
expected, Th17 cells were producing IL-17A but little IFN-γ and Th1 cells were producing IFN-γ but not IL-17A (Figure 3.5B). After resting overnight without IL-2, the Th1 and Th17 cells were stimulated with either allogeneic APCs plus anti-CD3 or with different ratios of anti-CD3/anti-CD28 coated beads to cells (1:8, 1:32, 1:128) in the presence of 100 U/mL IL-2. FOXP3 expression was induced in a proportion of Th1 cells but returned to baseline levels after 4-7 days (Figure 3.5C and D). In contrast, FOXP3 expression in Th17 cells remained high throughout the 14 day activation cycle, with a mean of 31.8% ± 8.4% of Th17 cells compared with 2.9% ± 1.3% of Th1 still expressing FOXP3 on day 14 when stimulated at a bead:cell ratio of 1:32 (p = 0.0171). Importantly, the IL-17A⁺ and IL-17A⁻ subpopulations within the Th17 cell cultures expressed similar levels of FOXP3 12-14 days post activation in all stimulation conditions (Figure 3.5E). These data suggest that activation-induced FOXP3 may play a more prominent role in Th17 cells than Th1 cells.
Figure 3.5 Activated human Th17 cells highly express FOXP3.

A. Sorting procedure for human Th1 (CD4⁺CD25⁻CXCR3⁺CCR4⁻CCR6⁻) and Th17 (CD4⁺CD25⁻CXCR3⁻CCR4⁺CCR6⁺) cells. CD4⁺ T cells were isolated from human PBMCs and depleted of CD25⁺ cells with CD25 microbeads (Miltenyi) prior to sorting on a BD FACS Aria. B. Phenotype of Th1 and Th17 cells after two weeks expansion with APC + anti-CD3 (1 µg/mL). T cells were re-stimulated with a mixture of phorbol 12-myristate 13-acetate and ionomycin and IL-17 and IFN-γ production were determined by intracellular cytokine staining. The cytokine profiles from one representative donor are shown. C. Time course of FOXP3 expression in Th1 and Th17 cells after activation with different ratios of anti-CD3/anti-CD28-coated beads or APC plus 1 µg/mL anti-CD3. Each time point represents the average FOXP3 expression from 2-6 different donors. D. Representative plots of FOXP3 expression in Th1 and Th17 cells from one donor on days 1, 3, and 12 post activation with 1 bead : 32 cells are shown. E. Similar level of FOXP3 expression in IL-17A⁺ and IL-17A⁻ subpopulations within Th17 cell cultures. Th17 cells were stimulated with anti-CD3/anti-CD28 coated beads at a ratio of 1 bead : 32 cells and FOXP3 and IL-17A expression were determined by intracellular cytokine staining after 12-14 days. The left panel shows a representative plot of FOXP3 and IL-17A expression for one donor and the left panel shows the average % FOXP3⁺ of the IL-17A⁺ and IL-17A⁻ subpopulations for 3 different donors. Similar results were observed when cells were stimulated with different ratios of anti-CD3/anti-CD28 coated beads (1:8 and 1:128 beads:cells) and with APCs plus anti-CD3.
3.3.4 Activation-induced FOXP3 limits expansion, IFN-γ production, and homing marker expression in Th17 cells

To better understand the role that FOXP3 plays in Th1 and Th17 cells upon activation, FOXP3 expression was knocked down in these T cell subsets. Th1 and Th17 cells were sorted as above, transduced with either siFP3 or siLuc, and expanded with autologous APC and anti-CD3 in the presence of 100 U/mL IL-2. After two weeks of expansion and purification of transduced cells, Th1 and Th17 cell lines were rested without IL-2 overnight and then re-stimulated with anti-CD3/anti-CD28 coated beads at a ratio of 1 bead to 32 cells in the presence of 100 U/mL IL-2 and examined for FOXP3 expression and expansion potential during the two-week activation cycle. Activation-induced FOXP3 expression was knocked down by an average of 65.5% ± 2.1% in siFP3-transduced Th1 and 67.3% ± 4.2% in siFP3-transduced Th17 cells compared with siLuc control-transduced T cells over the course of the experiment (Figure 3.6A). FOXP3-deficient Th17 cells had a greater expansion potential than control Th17 cells (Figure 3.6B), with FOXP3-deficient Th17 cells expanding 1.73 ± 0.20 fold times more than control Th17 cells by day 8 after activation (p = 0.0229, n = 4). In contrast, no significant difference in proliferation was observed between FOXP3-deficient Th1 cells and control Th1 cells. It should be noted that the representative experiment of Th1 cell expansion shows slightly greater expansion of siFP3-transduced Th1 cells compared with control siLuc-transduced Th1 cells, but this was the case in 2 of 4 experiments, and siLuc-transduced Th1 cells expanded slightly more than siFP3-transduced Th1 cells in the other 2 experiments. These data indicate that activation-induced FOXP3 limits expansion of Th17 cells.
Figure 3.6 FOXP3-deficient human Th17 cells have a greater expansion potential than control Th17 cells.

Th1 and Th17 cells transduced with siFP3 or control siLucB were stimulated with anti-CD3/anti-CD28-coated beads (1 bead : 32 cells) in the presence of 100 U/mL IL-2. Dotted lines represent siLuc-transduced T cells and solid lines represent siFP3-transduced T cells. A. FOXP3 expression in siFP3 and control siLuc-transduced Th1 and Th17 cells over the course of a two week expansion. B. FOXP3-deficient Th17 cells have a greater expansion potential than control Th17 cells. At each time point, cells were stained with viability dye and anti-NGFR, and live, NGFR^+ cells were counted by flow cytometry with counting beads. Fold expansion was determined by dividing the number of cells at each time point by the number of live, unstimulated cells counted by the same method on day 1. The fold expansion of siFP3-transduced T cells over control siLuc-transduced T cells on each day after activation is shown on the left and one representative experiment is shown on the right.

In parallel to investigating the expansion potential of siFP3-transduced Th1 and Th17 cells, cytokine production and cell surface marker expression were examined. Eight days
after activation, cells were washed and re-plated at 1 x 10^6 cells/mL and supernatants were collected 48 hours later. There was an average of 2.77 ± 0.15 times more IFN-γ in the supernatants of siFP3-transduced Th17 cells than control siLuc-transduced Th17 cells (Figure 3.7A, p = 0.0028, n = 3), but there was no difference in IL-17 or IL-10 production (Figure 3.7A). An increased proportion of IFN-γ-producing IL-17⁺ cells within siFP3-transduced Th17 cells was found compared with siLuc-transduced Th17 cells which was significant on day 11 (21.5% ± 4.3% compared with 15.1% ± 3.0%, p = 0.0033, n = 4; Figure 3.7B and C). There was also an increased proportion of IFN-γ⁺IL-17⁻ cells within siFP3-tranduced Th17 compared with siLuc control-transduced Th17 which was significant on day 8 (9.6% ± 2.6% compared with 7.0% ± 2.6%, p = 0.0436, n = 4; Figure 3.7B and D). There was no difference between siFP3 and control-transduced Th17 cells in the proportion of cells expressing IL-2 (18.5% ± 4.3% for siLuc versus 17.5% ± 3.1% for siFP3 on day 8 and 15.5% ± 4.4% for siLuc versus 16.4% ± 4.9% for siFP3 on day 11, p > 0.05, n = 3, data not shown), and this cytokine was not measured in the supernatants since it was added to the culture media. No differences in IFN-γ or IL-10 expression were found for siFP3-transduced Th1 cells compared with control siLuc-transduced Th1 cells (Figure 3.7E-G, n = 3-4).
Figure 3.7 Cytokine production by FOXP3-deficient Th17 and Th1 cells.

Th1 and Th17 cells transduced with siFP3 or control siLucB were stimulated with anti-CD3/anti-CD28-coated beads (1 bead : 32 cells) in the presence of 100 U/mL IL-2. 

A (Th17) and E (Th1) On day 8 after stimulation, cells were washed and re-plated at 1 x 10^6 cells/mL, and supernatants were collected 48 hours later for analysis by cytometric bead array. Each dot represents the fold difference in cytokine production by siFP3-transduced T cells over control siLuc-transduced T cells. 

B-D (Th17) and F-G (Th1) On days 0, 8, and 11 of the expansion, Th1 and Th17 cells were re-stimulated with phorbol 12-myristate 13-acetate and ionomycin and stained intracellularly for IFN-γ, IL-17, and IL-2. Analysis was conducted on NGFR+ cells. 

B. Representative plot of IL-17 and IFN-γ expression in Th17 cells 11 days after activation. 

C. The average fold difference in the percent of IL-17+ cells producing IFN-γ in siFP3 over control siLuc (n=4). 

D. The average fold difference in the percent of IFNγ+IL-17- Th17 cells in siFP3 over control siLuc over the course of the experiment (n=4). 

F. Representative plot of IL-17 and IFN-γ expression in Th1 cells 11 days after activation. 

G. The average fold difference in the percent of IFNγ+ Th1 siFP3 over control siLuc over the course of the experiment is shown (n=3).
CCR4 expression was significantly decreased in FOXP3-deficient Th17 cells compared to control Th17 cells (Figure 3.8A) on days 5, 8, 11, and 14 post activation (p = 0.0220, 0.0036, and 0.0012, and 0.0227, respectively, n = 3-4). On average, the mean fluorescence intensity of CCR4 in siFP3-transduced Th17 cells on day 8 post activation was 66.1% ± 3.3% that of siLuc-transduced Th17 cells. On the other hand, the intensity of CCR6 in siFP3-transduced Th17 cells on day 8 was not decreased to the same degree, and was only reduced to an average of 81% ± 5.9% that of siLuc-transduced Th17 cells which was not statistically significant (Figure 3.8B, n = 2-3). Furthermore, there was no difference in CTLA-4 expression between FOXP3-deficient and wild type Th17 cells (Figure 3.8C, n = 2-3), and there was also no difference in CTLA-4 expression between FOXP3-deficient Th1 and control Th1 cells (Figure 3.8D, n = 2-3). Not surprisingly, there was no difference in CCR4 and CCR6 expression for Th1 cells since they do not highly express these markers (data not shown). In summary, these data show that FOXP3 has a role in inhibiting IFN-γ expression and in up-regulating CCR4 in Th17 cells and appears to have a more prominent role in differentiated Th17 compared with Th1 cells.
Figure 3.8 Cell surface marker expression in FOXP3-deficient Th1 and Th17 cells.

Th1 and Th17 cells transduced with siFP3 or control siLucB were stimulated with anti-CD3/anti-CD28-coated beads (1 bead : 32 cells) in the presence of 100 U/mL IL-2. Dotted lines represent siLuc-transduced T cells and solid lines represent siFP3-transduced T cells. At the indicated days after activation, cell surface marker expression was determined by flow cytometry. The top panel of A-D shows the average fold difference in mean fluorescence intensity (MFI) of siFP3 compared with siLuc (MFI siFP3/MFI siLuc). The bottom panels of A-D show the MFIs of one representative experiment. A. Th17 CCR4 (n=3-4). B. Th17 CCR6 (n=2-3). C. Th17 CTLA-4 (n=2-3). D. Th1 CTLA-4 (n=2-3).
3.4 Discussion

Investigation of the role of activation-induced FOXP3 in Tconv cells has been overshadowed by the prominent role of FOXP3 in Treg cell function and by the fact that this phenomenon was thought to occur in humans but not mice. However, a better understanding of the role of FOXP3 in Tconv will allow for more accurate interpretation of clinical data where FOXP3 expression is investigated and will also improve our understanding of IPEX pathogenesis. Thus, the role of activation-induced FOXP3 in human CD4⁺CD25⁻ T cells was investigated by comparing wild type Tconv clones with FOXP3 null Tconv clones and by comparing wild type T cells transduced with a siRNA against FOXP3 with autologous T cells transduced with control siRNA against luciferase. Furthermore, the role of activation-induced FOXP3 in Th1 and Th17 cells was investigated by determining the kinetics of FOXP3 expression in these T cells subsets and by knocking down FOXP3 expression with siRNA against FOXP3. It was found that FOXP3-deficient CD4⁺CD25⁻ T cells proliferated to a greater extent and produced more IFN-γ and IL-2 than wild type CD4⁺CD25⁻ T cells. FOXP3 was highly expressed in Th17 cells upon activation and prevented IFN-γ expression while up-regulating CCR4 expression and limiting the expansion potential of Th17 cells. FOXP3 was also induced in Th1 cells upon activation, but levels returned to baseline after 4-7 days as in total CD4⁺CD25⁻ cells. FOXP3 did not appear to inhibit the expansion of differentiated Th1 cells or their IFN-γ or IL-2 expression. Taken together, these data demonstrate that FOXP3 has a role in the regulation of Tconv cell activation and proliferation.

IPEX is a heterogeneous disease caused by over 30 different mutations in FOXP3 known to date. The main cause of disease is a lack of functional Treg cells since FOXP3 is
absolutely required for their suppressive effects. An increase in Th17 cells in the peripheral blood of IPEX patients has recently been described which may arise from conversion of Treg to Th17 cells, contributing to the pathogenesis of IPEX. Now it can be proposed that in addition to these mechanisms, excessive activation and expansion of Tconv cells that are deficient in activation-induced FOXP3 expression may also contribute to the pathogenesis of IPEX.

Since half of the Tconv cells of females that carry IPEX mutations do not express FOXP3 or express mutant forms of FOXP3 upon activation, it might be expected, based on our conclusions, that they may be more susceptible to autoimmune disease. It was previously shown that the wild type allele of FOXP3 is active in 100% of the Treg cells of carriers of FOXP3 mutations. Thus, while no comprehensive study of the health of carriers of FOXP3 mutations has been conducted, presumably their wild type Treg cells are capable of controlling any inappropriate Tconv responses. This hypothesis is supported by the fact that Treg cells from normal donors can control the proliferation of Tconv cells from IPEX patients. It would be interesting, however, to investigate if carriers of IPEX mutations have an increased response to vaccination, but it should be noted that the Tconv cells of many carriers express mutant forms of FOXP3 rather than no FOXP3 at all, so they may behave differently than the FOXP3 null Tconv clones studied here.

While it is reported here that cytokine production is enhanced in FOXP3-deficient Tconv cells, there have been conflicting reports on this matter in IPEX patients. For example, it was previously shown that the PBMCs of some IPEX patients with point mutations in FOXP3 produce less IFN-γ and IL-2 than normal donors upon activation. However, cytokine production by PBMC from a patient with a FOXP3 null mutation was not...
significantly different from normal donors. Furthermore, another patient with a point mutation had increased cytokine production by CD4+ T cells. Hence, different types of mutations in FOXP3 may have different effects on cytokine production, with complete absence of FOXP3 resulting in increases in cytokines but specific point mutants potentially having other effects, possibly caused by dominant negative effects of mutant FOXP3.

FOXP3-deficient Tconv cells had a greater proliferative capacity than WT Tconv cells. Barring the possibility that FOXP3+ Tconv cells acquire transient suppressive capacity, FOXP3-mediated inhibition of proliferation in WT Tconv cells is an intrinsic effect. However, an extrinsic effect, where activation-induced FOXP3 confers Tconv cells with transient suppressive capacity, cannot be ruled out in these studies.

The main difference known so far between FOXP3 expressed in Tconv cells and FOXP3 expressed in Treg cells is in level of expression. This difference is most likely attributed to the fact that in Treg cells, the TSDR is demethylated, allowing for high expression of FOXP3 while in Tconv cells this region is methylated which restricts expression of the gene. Other ways that FOXP3 expression levels can be regulated besides the epigenetic status of FOXP3 is by post-transcriptional regulation by miRNA and post-translational modifications of the protein. miR-31 negatively regulates FOXP3 expression and is downregulated in Treg cells compared to Tconv cells while miR-21 is a positive regulator of FOXP3 and is upregulated in Treg cells. Acetylation is an example of post-translational modification of FOXP3 that increases its stability, possibly by preventing ubiquitination and proteosomal degradation. Treatment of mice in vivo or of cells in vitro with HDAC inhibitors to prevent deacetylation of FOXP3 increases FOXP3 mean fluorescence intensity and Treg stability. Similarly, HDAC9-deficient mice have an
increased number of Treg cells. Thus, differential acetylation of FOXP3 in Treg versus Tconv may contribute to differences in FOXP3 expression level between the two cell types. Whether or not there is a difference in the acetylation state of activation-induced FOXP3 found in Tconv cells and the highly-expressed FOXP3 found in Treg cells remains to be determined. If such a difference exists, investigation of differential expression of acetyltransferases and deacetylases in Treg versus Tconv may explain the difference. The acetyltransferase p300 and the histone deacetylase SIRT1 would be good proteins to investigate since they have been shown to acetylate and deacetylate FOXP3, respectively.

In addition to differential expression of acetylation-modifying enzymes, different expression of binding partners of FOXP3 within Tconv versus Treg cells may result in different functions of FOXP3. Furthermore, it has been shown that mouse FOXP3 can be cleaved at both the NH$_2$ and C terminal ends at proprotein convertase motifs to produce different forms of the protein. Interestingly, the different forms of FOXP3 have different abilities to induce a Treg phenotype in Tconv cells, so different proteolytic processing could also contribute to different roles of FOXP3 in Tconv cells versus Treg cells. Notably, mRNA of proprotein convertases PC1/3 and PC7 that cleave the C-terminal end of FOXP3 are more highly expressed in Treg than Tconv cells. Future investigation of the biochemical differences between FOXP3 protein in activated Tconv and FOXP3 protein in nTreg cells would provide a better understanding of how FOXP3 mediates its functions and may provide insight into how those functions could be modulated in both Treg and Tconv cells.

When proliferation and cytokine production was examined, no differences were observed between siLuc control-transduced and siFP3 transduced CD4$^+$CD25$^-$ Tconv cells that were tested prior to six weeks in culture (data not shown). This was most likely because
FOXP3 induction was lower in control transduced Tconv cells at the earlier time points, so the difference between FOXP3 expression in siFP3 and siLuc-transduced T cells was not significant. The fact that activation-induced FOXP3 expression increased with subsequent stimulations (Figure 3.3) indicates that FOXP3 may have a role in a long-term mechanism to inhibit cell activation and proliferation after multiple rounds of effector T cell stimulation. On the other hand, FOXP3 was induced at high levels immediately in Th17 cells, and knockdown of FOXP3 had immediate consequences, suggesting that FOXP3 has a more prominent role in the regulation of the activation of Th17 cells compared with other CD4⁺ T cells subsets. Indeed, knock-down of FOXP3 expression in Th1 cells did not have any detectable effect. This may be because Th1 cells are more similar to total Tconv cells, so effects of FOXP3-knock down may be more prominent after repeated stimulations.

One possibility is that Th17 cells are turning into regulatory cells. Indeed, Th17 clones have been shown to acquire regulatory capacity after repetitive in vitro stimulation, and even acquire an increasingly demethylated TSDR with each stimulation. The suggestion that high levels of FOXP3 expression observed in Th17 cells throughout the course of the activation cycle could reprogram Th17 cells into suppressive cells is consistent with the idea that high and sustained levels of FOXP3 can mediate Treg conversion. This phenomenon may also occur in vivo since IL-17⁺FOXP3⁺ T cell clones isolated from the peripheral blood of colitic patients were suppressive, although IL-17⁺FOXP3⁺ T cells may also arise from Treg cells that have begun to secrete IL-17. Thus, an inability of Th17 cells to differentiate into FOXP3⁺ suppressive cells may also contribute to IPEX pathogenesis.
In addition to the role that FOXP3 may have in converting Th17 cells into regulatory cells upon repetitive stimulation, FOXP3 may have a more immediate, cell intrinsic role in regulation of Th17 cell activation. CCR4 up-regulation was impaired in FOXP3-deficient Th17 cells, which could lead to aberrant Th17 cell homing in patients with IPEX. It was also found that FOXP3-deficient Th17 cells had increased IFN-γ production, indicating that FOXP3 may limit development of IFN-γ-producing Th17 cells. It has been suggested that FOXP3 acts to suppress the Th1 developmental program during Th17 differentiation, and my data suggest that perhaps this function continues in fully differentiated Th17 cells via activation-induced FOXP3. Nevertheless, IFN-γ-secreting Th17 cells have a role in many disease settings. For example, Th17 cells transduced with a tumour-specific TCR from IFN-γ−/− mice were unable to clear melanoma tumours, and IFN-γ production from Th17 cells acted synergistically with IL-17 to promote CXCL10 production from primary human ovarian cancer cells. Furthermore, IFN-γ, IL-17 double-producing T cells are enriched in inflammatory and autoimmune settings in both mice and humans. However, there is still controversy over whether or not IFN-γ+ Th17 cells are more pathogenic than IFN-γ− Th17 cells, so the consequences of increased IFN-γ expression in FOXP3-deficient Th17 cells in the context of IPEX are unclear.

FOXP3 expression was thought to be exclusively expressed in Treg cells in mice, but recent evidence suggests that FOXP3 is also expressed transiently in activated mouse Tconv cells. This phenomenon was observed in vitro when naive Tconv cells were TCR activated in the presence of IL-2 but in the absence of memory T cells and APCs. This differs from FOXP3 expression in human Tconv cells in that FOXP3 induction in human Tconv occurs readily in the presence of APCs. It is interesting to note, however, that FOXP3
induction in human Th17 cells was not as high when they were activated with APCs as when they were activated with anti-CD3/anti-CD28 coated beads, suggesting that APCs may modulate FOXP3 expression in Th17 cells. Indeed, different types of APCs may have different effects on activation-induced FOXP3 expression as evidenced by the observation that activation-induced FOXP3-expression was reduced in naïve T cells that were stimulated in the presence of mature DCs compared with immature DCs or anti-CD3-depleted PBMCs. Notably, the most significant differences in proliferation between FOXP3 null and wild type Tconv cell clones were observed with weaker TCR stimuli. Together, these data allow the speculation that FOXP3 may be especially important for controlling autoreactive T cells that have escaped negative selection in the thymus and encounter weak TCR stimulus by their cognate antigen in the periphery in the absence of pro-inflammatory antigen-presenting cells.

In conclusion, FOXP3 is an important regulator of T cell activation and proliferation that acts extrinsically by conferring Treg cells with the ability to suppress conventional T cells but can also be up-regulated in human Tconv cells to limit T cell proliferation and activation. CTLA-4 plays an intrinsic role in the negative regulation of T cell proliferation, but FOXP3 acts in parallel to CTLA-4 instead of upstream of CTLA-4 in Tconv, as knocking down FOXP3 had no effect on CTLA-4 expression. The role of FOXP3 as a negative regulator of T cell activation may be especially relevant in differentiated Th17 cells that highly express FOXP3 after activation.
Chapter 4: Development of methods to use FOXP3⁺ Treg cells as a cellular therapy for transplantation.

4.1 Introduction

Organ transplantation has been developed over the last 60 years and is now a feasible treatment for end-stage organ disease.⁸⁰⁹,⁸¹⁰ Despite advanced surgical techniques that made physical transplantation possible in the 1950s and 1960s, successful preservation of transplanted organs was hindered by acute immune rejection of the grafts. Development of cyclosporine A in the 1970s significantly improved transplant outcomes. Nevertheless, ways to further improve transplant success and improve the quality of life of organ recipients are being investigated. With current transplantation protocols, patients must take expensive immunosuppressive drugs for the remainder of their lives to prevent rejection of the foreign tissue. Though these drugs are successful at preventing acute rejection, chronic graft rejection can occur, so methods to induce long-term tolerance to grafts would significantly improve graft longevity. Furthermore, immunosuppressive drugs have many side effects, including global immunosuppression resulting in increased instances of infection and cancer, so minimization of these drugs while maintaining graft function is a high priority in transplant research.

One way to establish transplant tolerance without maintenance immunosuppression is through developing mixed chimerism by transplanting the solid organ recipient first with donor hematopoietic stem cells following non-myeloablative conditioning. This protocol does not deplete all of the host immune cells, but allows space for donor cells to co-exist with the host cells in a “mixed chimera.” Thus, both host and donor antigen-presenting cells are present in the thymus, and donor-reactive T cells undergo negative selection by interaction
with donor APCs.\textsuperscript{311} This procedure has allowed kidney transplant recipients to discontinue immunosuppressive therapy,\textsuperscript{66,312-314} but similar results have not been obtained for islet, heart, or lung transplants in cynomolgus macaques. Furthermore, animal models of durable mixed chimerism have required recipient irradiation, cytotoxic drug/antibody treatment, or clinically unobtainable doses of bone marrow which is not routinely feasible in the clinic due to medical risks associated with irradiation and cytotoxic drugs.\textsuperscript{315} Co-injection of Treg cells with bone marrow cells has been shown to increase mixed chimerism, presumably by preventing a host versus graft response, and promotes skin graft tolerance in mouse models.\textsuperscript{315}

Besides boosting mixed chimerism after bone marrow transplant, there is much interest in harnessing the ability of Treg cells to suppress immune responses for use as a cellular therapy to prevent graft rejection of other tissues and organs and to prevent GVHD after bone marrow transplant. Many animal models demonstrate the ability of Treg cells to suppress graft rejection,\textsuperscript{316} and the first clinical trials have been conducted to evaluate the safety of this approach, with more underway.\textsuperscript{206,227,228,317}

Limitations to translation of Treg cellular therapy to the clinic include the relatively low abundance of Treg cells in human peripheral blood and the lack of specific cell surface markers with which to isolate pure Treg cell populations. This means that\textit{ex vivo} Treg cells need to be expanded\textit{in vitro} before infusion into recipients. The challenge of\textit{in vitro} Treg expansion is that these cells are anergic\textit{in vitro}, and contaminating populations of Tconv cells rapidly outgrow the Treg cell population. Thus, methods to expand pure populations of Treg cells are greatly desirable to minimize the potential negative effects of infusing a large population of effector cells.
One way to address the limiting numbers of Treg cells and lack of suitable purification markers is to generate Treg cells in vitro by over-expressing FOXP3. Using this strategy, large numbers of Tconv cells can be reprogrammed into regulatory cells and effectively purified with a cell surface marker co-expressed with FOXP3. It was previously shown that human Tconv cells over-expressing FOXP3 (FP3 T cells) are suppressive in vitro, but whether these cells will recapitulate the function of Treg cells in vivo remains to be determined. This question was addressed in two different ways: 1. A humanized mouse model of islet allograft rejection was developed in which immunodeficient mice are transplanted with human islet tissue and the ability of in vitro generated Treg cells to suppress graft rejection caused by allogeneic human PBMCs was assessed; and 2. Whether cynomolgus Tconv cells could also be reprogrammed into regulatory cells upon over-expression of FOXP3 and whether these in vitro-generated cynomolgus macaque Tconv cells could increase chimerism and establish donor acceptance of a kidney graft without maintenance immunosuppression was examined. Simultaneously, a method to expand large, pure populations of Treg cells was developed in order to compare the efficacies of in vitro-generated Treg cells with ex vivo expanded Treg cells in both of these models.
4.2 Materials and methods

4.2.1 Human tissues

Peripheral blood was obtained from healthy volunteers who gave written informed consent in accordance with the protocol approved by the University of British Columbia Clinical Research Ethics Board. Human islets isolated from cadaveric organ donors were received from the Human Islet Transplant Program at Vancouver General Hospital (Vancouver, Canada). Islets were cultured in CMRL culture medium containing BSA, 11.1 mmol/l glucose, 50 U/mL penicillin, 50 µg/mL streptomycin, and 50 µg/mL gentamycin at 37°C, 5% CO₂ until they were transplanted into mice.

4.2.2 Isolation of human cells

PBMCs and CD4⁺ T cells were isolated from peripheral blood as previously described in section 2.2.3. CD25⁺ T cells were enriched from CD4⁺ T cells with CD25 magnetic beads (Miltenyi Biotec) before staining with antibodies against CD4, CD25, and CD45RA. Treg cells were sorted on a BD FACS Aria to >95% purity.

4.2.3 Isolation of cynomolgus macaque cells

PBMCs were separated from 10-20 mL of whole blood by layering diluted buffy coat over 60% Percoll. Naïve CD4⁺CD45RO⁻ T cells were isolated from PBMCs by first depleting the CD45RO⁺ cells by labeling with anti-CD45RO-PE and anti-PE magnetic beads and passing over a magnetic column. CD4⁺ T cells were then positively selected by labeling with anti-CD4 and anti-mouse IgG1 magnetic beads and passing over a second column.
(Miltenyi Biotec). Treg cells were isolated from PBMCs by staining with CD3, CD4, and CD25 antibodies and sorting on a BD FACSaria.

### 4.2.4 Lentiviral vectors and transduction of human and cynomolgus macaque T cells

The lentiviral vectors encoding human FOXP3 and empty control vector and the methods to produce them were previously described. Transduction of naïve human T cells was also carried out as previously described, but cynomolgus macaque naive T cells were transduced 48 hours after activation with coated anti-CD3 (10 µg/mL) and soluble anti-CD28 (5 µg/mL) in the presence of 100 U/mL IL-2 at a multiplicity of infection of 30. Alternatively, naïve T cells were transduced 18 hours after activation with L cells (mouse fibroblasts expressing human CD80, CD58, and CD32) at a 1:1 ratio with 100 ng/mL anti-CD3 in the presence of 100 U/mL IL-2 at a multiplicity of infection of 30.

### 4.2.5 Analysis of cytokine production

T cells or splenocytes were stimulated with phorbol 12-myristate 13-acetate (10 ng/mL, Sigma-Aldrich) and ionomycin (100 ng/mL, Sigma-Aldrich) for 5 hours, with brefeldin A (10 µ/mL, Sigma-Aldrich) added for the last 3 hours. Cells were stained for surface markers, fixed and permeabilized (eBioscience), and stained with anti-IFN-γ (BD Biosciences), anti-IL-2 (BD Biosciences), and anti-FOXP3 (236/AE7, eBioscience or 259D/C7, BD Biosciences).
4.2.6  *In vitro* suppression assays

Suppression of cynomolgus macaque T cells was measured with a thymidine-based assay after resting the cells overnight without IL-2. Ten thousand freshly isolated PBMCs were stimulated with anti-CD2/anti-CD3/anti-CD28-coated beads at a 1:1 ratio (Non-human primate T cell activation/expansion kit, Miltenyi Biotec) in 96-well round bottom plates. Different ratios of Treg or FOXP3-transduced T cells were added to the cultures, and cells were incubated for a total of 5 days at 37ºC. Wells were pulsed with [³H]-TdR for the last 16 hours.

4.2.7  Preparation of human PBMCs, FOXP3 T cells, and *ex vivo* expanded Treg cells for injection into immunocompromised mice

Two hundred millilitres of whole blood from a donation of 450 mL was processed immediately for isolation of PBMCs. PBMCs were depleted of red blood cells by ammonium chloride lysis, and were frozen in liquid nitrogen in 10% DMSO in fetal bovine serum. The viability of frozen cells was tested prior to injection into mice by thawing an aliquot and staining with trypan blue. One million thawed PBMCs were plated in 1 mL X VIVO 15 media in a 24-well plate and were re-stained with trypan blue the next day. PBMCs were considered suitable for injection into mice if less than 5% of cells stained positive for trypan blue immediately after thawing and less than 15% of cells stained positive for trypan blue after overnight culture.

The remaining blood from the donation was left overnight at room temperature, and Treg cells and naive CD4⁺ T cells were isolated as described. *Ex vivo* Treg cells were expanded for 11-13 days, checked for FOXP3 expression, cytokine production, and *in vitro*
suppressive function, and frozen in liquid nitrogen. Naive CD4\(^+\) T cells were transduced with FOXP3-encoding or control lentiviral vector, purified, and expanded for 20-24 days before freezing in liquid nitrogen. FOXP3-transduced T cells and \textit{ex vivo} expanded Treg cells were injected into mice with autologous PBMCs, in a maximum volume of PBS of 300 µL.

4.2.8 Humanized mouse model of islet allograft rejection

Diabetes was induced in 7-10 week old male NOD/SCID (NOD.CB17-Prkdc\(^{scid}\)/J) or NSG (NOD.Cg-Prkdc\(^{scid}\)IL2rg\(^{pm1Wj}\)/SzJ mice (The Jackson Laboratory) by a single intraperitoneal injection with streptozotocin (NOD/SCID: 200 mg/kg; NSG: 180 mg/kg). Three days later, mice were transplanted under the kidney capsule with 500 hand-picked human islets. Blood glucose levels and body weight were monitored at least twice a week throughout the experiment. Approximately 2-3 weeks after islet transplantation, mice were injected intravenously or intraperitoneally with thawed human PBMCs, plus or minus FP3 T cells or \textit{ex vivo} expanded Treg cells. Seventy microlitre blood samples were taken every two weeks starting at week 1 or 2 via saphenous veins for analysis by flow cytometry. Mice were considered diabetic and were sacrificed after two consecutive blood glucose readings of 15 mM or higher. A drop in body weight of 20%, hunched posture, and inactivity were signs of GVHD, and mice were sacrificed upon GVHD development in accordance with the animal care protocol. Upon sacrifice, blood, graft, and spleen samples were collected for flow cytometric analysis and histology.
4.2.9 Flow cytometry of humanized mouse blood and splenocytes

Red blood cells were depleted from samples by ammonium chloride lysis. Blood cells and splenocytes were blocked with anti-CD16/anti-CD32 (2.4G2, BD Biosciences) and stained with mouse CD45, human CD45, CD3, CD4, CD8, FOXP3, and ΔNGFR antibodies. Samples were read on a BD FACSCanto (BD Biosciences). Counting beads (surfactant-free white sulfate latex beads, 0.5 µM; Interfacial Dynamics Corporation) were added to samples to determine the absolute number of cells per µL of blood or mg of spleen.

4.2.10 Immunohistochemistry of islet graft and spleen samples

Paraffin-embedded sections (5 µM) were deparaffinized, rehydrated with alcohol, and steamed in sodium citrate buffer (pH 6.0, 30 min) to retrieve antigens. Endogenous peroxidase activity was blocked (3% H2O2, 10 min) and sections were incubated with DAKO protein blocker for 30 min. Sections were stained with anti-CD45 (eBioscience) or anti-FOXP3(eBioscience), followed by biotinylated secondary antibody and streptavidin-HRP. Staining was developed with 3,3’-diaminobenzidine (BioGenex) and sections were counterstained with hematoxylin, dehydrated with alcohol, and mounted.

4.2.11 Bone marrow and kidney transplantation of cynomolgus macaques

Blood was drawn 4, 3, and 2 week prior to bone marrow transplant from the recipient to isolate and expand Treg cells. Expanded Treg cells were frozen in liquid nitrogen until re-injection into the recipient. Whole body irradiation (1.5 Gray) was given 6 and 5 days before and horse anti-thymocyte globulin (ATGAM, 50mg/kg IV) was given 2 and 1 day before and on the day of bone marrow transplantation (200 x 10⁶/kg). Anti-CD154 monoclonal antibody
(American Type Culture Collection, catalogue number 5c8.33) was given IV on days 0, 2, 5, 7, 9, and 12 (10 mg/kg except days 0 and 2 20 mg/kg), and cyclosporine A (15 mg/kg) was given continuously until day 28 post bone marrow transplant. Thawed expanded Treg cells were infused on day 0, 2, 5, 7, and 50. Chimerism was determined by detection of MHC Bw6 by flow cytometry. Kidney transplantation was performed on day 126, and the remaining kidney was removed 100 days later. A control monkey received the same treatments without infusion of Treg cells.
4.3 Results

4.3.1 Expansion of Treg cells from human peripheral blood with maintenance of FOXP3 expression and Treg phenotype

To compare the in vivo efficacy of in vitro generated Treg cells with ex vivo expanded Treg cells, a method to isolate and expand human Treg cells was developed. Since naïve (CD45RA⁺) Treg cells represent the most homogeneous starting population and maintain FOXP3 expression and TSDR demethylation longer in vitro than Treg cells sorted on the basis of CD25 and lack of CD127 expression,¹⁸⁴,¹⁸⁷ CD4⁺CD25⁺CD45RA⁺ T cells were sorted from human PBMCs (Figure 4.1). CD4⁺CD25⁺ T cells were enriched by CD4⁺ negative selection followed by CD25⁺ positive selection with magnetic beads prior to sorting by flow cytometry. This pre-enrichment step was done to reduce the number of cells put through the flow cytometer, decreasing the total cost by minimizing antibody consumption and reducing sorting time. The reduction in sorting time is also beneficial for improving the health of the sorted Treg cells. The sorting gate was set to be as stringent as possible, sacrificing cell number for cell purity. From 200-300 mL of peripheral blood, an average of 5.8 x 10⁶ ± 1.1 x 10⁶ CD4⁺CD25⁺ T cells were sorted with magnetic beads and an average of 47 000 ± 9500 CD4⁺CD25⁺CD45RA⁺ Treg cells were obtained after FACS.

Figure 4.1 Sorting strategy to isolate naïve Treg cells.

CD4⁺CD25⁺ T cells were first enriched by magnetic bead separation before FACS.
To develop a protocol for Treg cell expansion, previous expansion protocols established by the groups of Edinger, Bluestone, and Riley were referenced. Treg cells were activated with either a 1:1 ratio of anti-CD3/anti-CD28-coated beads or artificial antigen-presenting cells plus 100 ng anti-CD3 in the presence of 300 U/mL IL-2. Preliminary experiments determined that restimulation of Treg cells after 1 week greatly improved the expansion potential (data not shown), similar to findings of Golovina et al. Furthermore, Treg cells were expanded in the presence of 100 ng/mL rapamycin for the first 7 days, discontinuing its addition after day 7 because while rapamycin improves the purity of Treg cell cultures, it also impedes their expansion potential.

In order to expand sorted Treg cells, some groups have used anti-CD3/anti-CD28-coated beads while other groups have used artificial antigen-presenting cells (aAPCs). There are different types of aAPCs, which are non-immune cells stably expressing different stimulatory molecules on their surface. I made use of mouse L 929 fibroblasts that stably express human CD32, CD58, and CD80. CD32 is the Fc gamma receptor II which binds the Fc portion of soluble anti-CD3 and presents the antibody to T cells for CD3 signaling. CD80 provides costimulation by binding CD28 on the T cell, and CD58 (LFA-3) binds CD2, also providing costimulation. The expansion of Treg cells stimulated with beads versus aAPCs was compared. Figure 4.2 shows 3 independent experiments with 3 different donors in which greater expansion was achieved with aAPCs than beads. In one experiment, a new medium, OpTMizer T cell Expansion Medium was tested. It was found that the cells proliferated to a greater extent in serum-free OpTMizer T cell Expansion Medium compared with X Vivo 15 plus 5% human serum (data not shown), so OpTMizer T cell Expansion Medium was used for future experiments.
Human naïve Treg were stimulated as indicated and the fold expansion over two weeks was determined.

For all future experiments, the IL-2 concentration was increased from 300 U/mL to 1000 U/mL based on the expansion protocol of the Wood group. Thus, the final expansion protocol is described in Figure 4.3A. With this expansion procedure, an average of 907-fold ± 179-fold expansion was achieved with an average of 88.0% ± 3.0% FOXP3 purity (Figure 4.3B and C, n = 6). Expanded Treg cells maintained their Treg phenotype as they had high expression of CD25, low expression of CD127 and did not produce IFN-γ or IL-2 (Figure 4.3C and D).
Figure 4.3 Expansion of human naive Treg cells with maintenance of a Treg phenotype.

A. Naïve Treg cells were stimulated with L cells (1:1) plus 100 ng/mL anti-CD3 in the presence of 100 ng/mL rapamycin and 1000 U/mL IL-2 in OpTmizer T cell Expansion Medium (Invitrogen). Fresh IL-2 was given every 2-3 days, assuming consumption, and rapamycin was added to the culture when new media was given at a concentration of 100 ng/mL until day 7. On day 7, Treg cells were washed, counted, and re-stimulated with L cells (1:1) plus 100 ng/mL anti-CD3 in the presence of 1000 U/mL IL-2 (no rapamycin). Treg cells were frozen between day 12-14. B. Expansion of naïve Treg cells is shown for 5 different donors. C-D. Treg cells were rested overnight without IL-2 to check FOXP3 expression (C), cytokine production (C), and surface marker expression (D).

4.3.2 Development of a humanized mouse model of islet allograft rejection to compare in vitro generated Treg cells with ex vivo expanded Treg cells

Human Treg cells have been shown to prevent graft rejection in humanized mouse models.209,210,319 A humanized mouse model of islet allograft rejection in NOD/SCID mice was chosen to compare the suppressive capacities of ex vivo expanded Treg cells versus FOXP3-over-expressing Tconv cells in vivo. In this model, diabetes was induced in immunodeficient mice by injection with streptozotocin to destroy the insulin-producing beta
cells. Mice were then transplanted with 500 hand-picked human islets under the kidney capsule to restore normal blood glucose levels. Approximately two weeks later, after blood glucose levels normalized, mice were injected with human PBMCs to cause rejection of the allogeneic islet graft, which was indicated by increased blood glucose levels. *Ex vivo* expanded Treg cells or FP3 T cells autologous to the PBMCs were injected in parallel to test their ability to prevent graft rejection (Figure 4.4).

Figure 4.4 Humanized mouse model of islet allograft rejection.

A. Obtaining sets of autologous PBMCs and FP3 T cells and *ex vivo* expanded Treg cells for injection into mice. From each peripheral blood donor, PMBCs are frozen on the day of collection from 150 mL whole blood. APCs, naïve (CD45RO-) CD25- T cells, and naïve (CD45RA+) Treg cells are isolated from the remaining blood. Naïve CD25- cells are activated with autologous APC and 1 µg/mL anti-CD3 and transduced the next day with FOXP3. These cells are expanded for a total of 20 – 26 days, purified, and frozen. Naïve Treg cells are expanded as described in section 4.3.1 and frozen. B. Humanized mouse model of islet allograft rejection, as described in the text.

Initial experiments were aimed at establishing the model of islet allograft rejection in NOD/SCID mice. These mice have a mutation in a DNA repair enzyme necessary for V(D)J recombination, so they do not have mature T or B cells. They also have a low level of innate immunity, and low but present NK-cell activity. While human PBMCs could engraft in
these mice, the engraftment was highly variable (Figure 4.5A and B). When Treg cells were injected together with PBMCs in these mice, rejection of islet grafts was prevented in some instances and FOXP3+ T cells were enriched in islet grafts, but in other instances, Treg injection seemed to have no effect (Figure 4.5C). Notably, in the experiment depicted in Figure 4.5, the mouse that was protected from graft rejection had more FOXP3+ cells within its graft than the mouse that received Treg cells but rejected its graft (Figure 4.5B). This finding is compatible with the hypothesis that when Treg cells are present in the graft they are able to prevent graft rejection. It is unclear why Treg cells from the same preparation were found in the graft of one mouse but not the other, but this could be due to variable engraftment of human cells in the mice. Without consistent engraftment of PBMCs and Treg cells, it was impossible to make a definitive conclusion about the suppressive capacity of ex vivo expanded Tregs in this model.
Figure 4.5 Humanized mouse model of islet allograft rejection in NOD/SCID mice.

One representative experiment of two in which the ability of \textit{ex vivo} expanded Treg cells to suppress islet allograft rejection was tested. On day 31, $15 \times 10^6$ PBMCs were injected plus or minus $2 \times 10^6$ autologous \textit{ex vivo} expanded Treg cells into NOD/SCID mice harbouring 500 allogeneic hand-picked human islets. A. Variable engraftment of human cells within NOD/SCID mice. Percent human CD45$^+$ and CD3$^+$ cells are shown in the blood of 2 mice 14 days after injection (day 45). B. Immunohistochemistry of the islet grafts of mice. The top panel shows human CD45 staining (brown stain, magnification x200) and the bottom panel shows FOXP3 staining (brown stain, magnification x400), with arrows pointing to FOXP3$^+$ cells. C. Blood glucose levels during the course of the experiment.

It was therefore decided to switch to a more robust model, replacing NOD/SCID mice with NSG mice. Some NOD/SCID mice may still have some T and B cells through spontaneous recombination of T and B receptors. NSG mice, on the other hand, have an additional mutation in the IL-2 receptor gamma chain which completely prevents development of T and B cells. Furthermore, NSG mice do not have any NK cells and have
further reduced innate immune function, allowing human PBMCs to engraft in these mice with a higher efficiency.\textsuperscript{208}

The aim of the first experiment performed in NSG mice was to determine whether human PBMCs could robustly reject human islet grafts in these mice. Ten million PBMCs were injected intraperitoneally (IP) or intravenously (IV) after blood glucose levels normalized in mice following human islet transplantation. As seen in Figure 4.6A, all mice receiving human PBMCs rejected their grafts as measured by rising blood glucose levels, while the control mouse that received PBS maintained graft function. Human CD45\textsuperscript{+} cells were seen in the grafts of the mice, and human CD45\textsuperscript{+} from the spleens of rejecting mice produced IFN-\(\gamma\) (Figure 4.6B and C). Graft rejection occurred at a faster rate when PBMCs were injected IP than when they were injected IV. To ensure graft rejection occurred before GVHD and since IP injections are used by others, this injection route was chosen for further experiments.

**Figure 4.6 Model of islet allograft rejection in NSG mice.**

On day 14, 10 x 10\textsuperscript{6} human PBMC were injected IP or IV into NSG mice that had been transplanted with 500 allogeneic hand-picked human islets. A. Blood glucose levels of the mice over the course of the experiment. B. Examples of human cell infiltration (brown stain, human CD45\textsuperscript{+} cells) of the graft in rejecting mice at the time of sacrifice. C. Splenocytes isolated from mice upon rejection of islet allografts include human CD45\textsuperscript{+} cells that produce IFN-\(\gamma\) upon stimulation with PMA and ionomycin.
In the next experiment, the ability of $5 \times 10^6$ FP3 T cells to suppress islet allograft rejection caused by $10 \times 10^6$ PBMCs (1:2 Treg:PBMC ratio) was tested. Mice that received PBMCs alone quickly rejected their grafts (Figure 4.7A). However, mice that received FP3 T cells also rejected their grafts. It was observed that the ratio of NGFR$^+$ T cells was significantly lower than expected after 2 weeks based on the numbers injected (only 1.19% and 0.92% of human CD3$^+$ T cells, Figure 4.7B), so it could be that FP3 T cells are not surviving well in the mice. Other groups have used higher ratios of Treg:PBMCs in humanized mouse models of graft rejection,$^{209,210}$ so the Treg:PBMC ratio was increased to 1:1 for the next experiments.

Figure 4.7 Low engraftment of transduced T cells in NSG mice.

A

![Blood glucose levels over the course of the experiment.](image)

B

![Low engraftment of transduced T cells in NSG mice. Plots shown are from the blood of mice 2 weeks after injection and are gated on human CD45$^+$CD3$^+$ cells.](image)

On day 18, $10 \times 10^6$ PBMC were injected into mice that had been previously transplanted with 500 hand-picked allogeneic human islets plus or minus $5 \times 10^6$ autologous FP3 or NGFR T cells. A. Blood glucose levels over the course of the experiment. B. Low engraftment of transduced T cells in NSG mice. Plots shown are from the blood of mice 2 weeks after injection and are gated on human CD45$^+$CD3$^+$ cells.

The ability of ex vivo expanded Treg cells to suppress islet allograft rejection in NSG mice was tested next. Ten million PBMCs plus or minus $10 \times 10^6$ ex vivo expanded Treg
cells were injected into NSG mice harboring human islet grafts. Autologous sets of PBMCs and Treg cells from two different donors were injected IP. Unfortunately in this experiment, PBMCs did not cause rejection of the islet grafts before causing xenogeneic GVHD (Figure 4.8A and B). One reason for this could be that the PBMCs did not engraft as efficiently as in other experiments. Three weeks after injection, the percent of human CD45+ cells in the blood of the mice was only between 0.16 – 9.7% in this experiment (Figure 4.8C) whereas in the first two experiments, the percent of human CD45+ cells was 7 -20% in the blood after only 2 weeks (data not shown). Importantly, ex vivo expanded Treg cells were able to engraft and survive in the NSG mice when injected at the 1:1 ratio of Treg:PBMCs as shown by the enrichment of FOXP3+ T cells of total CD3+CD4+ T cells in the blood of NSG mice 1-3 weeks after injection (Figure 4.8D). These cells did not persist at high levels past 5 weeks, similar to other reports in the literature. Notably, though there was no rejection for the Treg cells to inhibit, the ex vivo expanded Treg cells appeared to prevent the expansion of human PBMCs within NSG mice (Figure 4.8C), and mice that received Treg cells had a delayed onset of GVHD (Figure 4.8B). These data indicate that ex vivo expanded Treg cells can survive in the NSG mouse and prevent expansion of mouse-reactive PBMCs.
Figure 4.8 *Ex vivo* expanded Treg cells survive in NSG mice and prevent development of xenogeneic GVHD.

NSG mice were transplanted with 500 hand-picked human islets, and 10 x 10⁶ allogeneic human PBMC plus or minus 10 x 10⁶ autologous *ex vivo* expanded Treg cells were injected IP 19 days later. A. Blood glucose levels of the mice during the course of the experiment. B. Weight of the mice during the course of the experiment. Mice receiving PBMCs only lost weight indicating development of GVHD and were sacrificed along with mice that received PBMCs plus Treg cells in donor sets (1 sacrifice for mice injected with cells from donor 1, and one sacrifice for mice injected with cells from donor 2). C. Engraftment of human CD45⁺ cells in the blood of NSG mice. D. Top panel shows percent FOXP3⁺ of CD3⁺CD4⁺ T cells in the blood of NSG mice during the course of the experiment. Bottom panel shows FACS plots gated on human CD45⁺CD3⁺CD4⁺ of FOXP3⁺ cells found in the blood of mice 21 days post injection.

The final experiment performed in the humanized mouse model was to test the ability of FP3 T cells to suppress islet allograft rejection at the 1:1 ratio. In this experiment, mice were transplanted with 600 islets instead of 500 because they were smaller than usual.
Human cells did not engraft efficiently in two mice (one that received PBMCs alone and one that received PBMCs plus NGFR T cells). The percent human CD45\(^+\) cells of total CD45\(^+\) cells in these two mice was still below 1% at 50 days post injection. In mice that did engraft with human cells, the complication of mice developing xenogeneic GVHD before rejecting their grafts was encountered again, and even in these mice, the engraftment rate of human PBMC was low (Figure 4.9A and B). Transduced cells (NGFR\(^+\)) were detected in the blood of mice 1 week after injection, but had significantly decreased by 3 weeks (Figure 4.9C). Unlike \textit{ex vivo} expanded Treg cells, FP3 T cells did not appear to prevent the expansion of mouse-reactive PBMCs and did not delay the onset of GVHD in this experiment.

\textbf{Figure 4.9} Humanized mouse model of islet allograft rejection with a 1:1 ratio of FP3 T cells : PBMCs

Twenty-two days after transplant with 600 hand-picked human islets, NSG mice were injected with 10 x 10\(^6\) human PBMCs plus or minus 10 x 10\(^6\) FP3 or NGFR T cells. A. Blood glucose levels of the mice over the course of the experiment. B. Percent human CD45\(^+\) cells of total CD45\(^+\) cells in the blood of mice during the course of the experiment. C. Percent NGFR\(^+\) cells of total human CD3\(^+\)CD4\(^+\) T cells in NSG mice over the course of the experiment.

While \textit{ex vivo} expanded Treg cells can inhibit the onset of GVHD in humanized mice, it is unclear whether FP3 T cells will have the same capability. Future experiments will
focus on further testing of FP3 T cells in a xenogeneic GVHD model due to the scarcity of human islet sources and the complication of xenogeneic GVHD occurring before allograft rejection.

4.3.3 Generation of cynomolgus macaque regulatory cells by lentiviral transduction of Tconv cells with human FOXP3

In addition to testing FP3 T cells in a humanized mouse model, it was also of interest to test their in vivo suppressive capacity in a non-human primate model. The first step in developing the model was to establish whether cynomolgous macaque Tconv cells could be reprogrammed into suppressive T cells in vitro by over-expressing human FOXP3 via a lentiviral vector.

First, the transduction of cynomolgus macaque Tconv cells with lentivirus encoding human FOXP3 was optimized. An attempt was made to transduce cynomolgous macaque Tconv cells 48 hours after activating them with anti-CD3 (10 µg/mL) and anti-CD28 (5 µg/mL) as has been previously done for transduction of human Tconv cells.\textsuperscript{89} However, the transduction efficiency was very low. In an effort to improve the transduction efficiency, transduction after activation with anti-CD3 and anti-CD28 was compared with transduction after activation with L cells and anti-CD3. It was first confirmed that L cells could activate cynomolgus Tconv cells by examining CD25 expression after 4 days (Figure 4.10A). Indeed, essentially all of the Tconv cells stimulated with L cells were expressing CD25 compared to 90% of Tconv cells expressing CD25 under standard activation conditions.

It was also confirmed that human Tconv cells could be transduced efficiently following activation with L cells, since transductions had not been previously performed
under these activation conditions. Tconv cells were transduced with control NGFR or FP3 vector 18 hours after activation and it was found that the Tconv cells were transduced at similar efficiencies as previously described (Figure 4.10B; 93% for NGFR and 67% for FP3).

Next, lentiviral transduction of cynomolgus Tconv cells after standard activation was directly compared with transduction after activation with L cells. Figure 4.10C shows an example of transduction of one donor while Table 4.1 summarizes the data. The average transduction efficiency with NGFR control vector was 12.0% with a range from 3.1% to 23.6% with standard activation and an average of 20% with a range from 11.6% to 32.5% with L cell activation. For the FP3 vector, the average transduction efficiency with standard activation was 6.8% with a range from 1.3 to 16.9% and with L cell activation the average was 3.3% with a range from 2.0% to 6.0%. These data suggest that there is no difference in transduction efficiency after standard activation compared with L cell activation, and that the transduction efficiency after either type of activation is significantly less than that observed for human Tconv cells. However, L cell stimulation generally resulted in a greater number of total cells, so a greater absolute number of total transduced cells can be achieved with L cell activation.
Figure 4.10  Cynomolgus macaque T cells can be activated with aAPCs and transduced with lentiviral vectors following aAPC activation.

A. Cynomolgus macaque CD4^+CD25^- T cells were activated with either a 1:1 ratio of L cells plus 100 ng/mL anti-CD3 or 10 µg/mL immobilized anti-CD3 plus 5 µg/mL soluble anti-CD28. Four days later, CD25 expression was determined by flow cytometry.  

B. Human CD4^+CD45RO^- T cells are efficiently transduced with control NGFR and FOXP3-encoding (FP3) lentiviral vectors following activation with L cells and anti-CD3.  

C. Cynomolgus macaque CD4^+CD45RO^- T cells are not as efficiently transduced with lentiviral vectors as human T cells.  In B and C, human or cynomolgus macaque T cells were either activated with L cells at a 1:1 ratio plus 100 ng/mL anti-CD3 and were transduced 18 hours after activation or were activated with anti-CD3 (10 µg/mL) and anti-CD28 (5 µg/mL) and were transduced 48 hours after activation. A multiplicity of infection of 30 was used, and transduction efficiencies were determined 9 days after activation by determining the percent NGFR^+ cells by flow cytometry. Fold expansion depicted in C is after 9 days expansion.

Table 4.1  Transduction efficiencies of cynomolgus macaque CD4^+CD45RO^- T cells with HIV-based lentiviral vectors.

<table>
<thead>
<tr>
<th>Stimulation</th>
<th>N</th>
<th>Average efficiency NGFR vector</th>
<th>Range of efficiency NGFR vector</th>
<th>Average efficiency FP3 vector</th>
<th>Range of efficiency FP3 vector</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-CD3/anti-CD28</td>
<td>8</td>
<td>12.0%</td>
<td>3.1-23.6%</td>
<td>6.8%</td>
<td>1.3-16.9%</td>
</tr>
<tr>
<td>L cell + anti-CD3</td>
<td>5</td>
<td>20.0%</td>
<td>11.6-32.5%</td>
<td>3.3%</td>
<td>2.0-6.0%</td>
</tr>
</tbody>
</table>

Despite the limited number of FOXP3-transduced cynomolgus Tconv cells obtained, their *in vitro* suppressive capacity and Treg characteristics were evaluated. Transduced T cells were purified with NGFR magnetic beads 6 days after activation and were re-stimulated with allogeneic PBMCs and soluble anti-CD3 on day 7 and every 10-12 days thereafter. Nine to eleven days after re-stimulation, the transduced T cells were tested for Treg function and phenotype. Figure 4.11A shows that FOXP3 was expressed at levels equal to that of
freshly isolated, autologous CD4^+CD25^+ Treg cells. CD25 was up-regulated on FOXP3-transduced Tconv compared with control ΔNGFR-transduced and untransduced Tconv (Figure 4.11B). Furthermore, IFN-γ and IL-2 production were reduced in FOXP3-transduced Tconv compared with control NGFR-transduced Tconv cells (Figure 4.11C). Lastly, the ability of FOXP3-transduced cynomolgus Tconv cells to suppress the proliferation of autologous PBMCs was tested. Compared with control NGFR transduced Tconv and untransduced Tconv cells, FOXP3-transduced Tconv efficiently suppressed PBMC proliferation (Figure 4.11D). These data indicate that over-expression of human FOXP3 in cynomolgus macaque Tconv cells can effectively reprogram them into T cells with the phenotype and function of Treg cells.

Figure 4.11 Cynomolgus macaque Tconv cells can be re-programmed into regulatory cells by over-expression of human FOXP3.

A. Cynomolgus macaque FP3-transduced T cells express FOXP3 at equal levels to ex vivo CD4^+CD25^+ Treg cells. B. FP3-transduced Tconv cells have increased expression of CD25 compared to control NGFR-transduced Tconv cells. C. IFN-γ and IL-2 production is suppressed in FP3-transduced Tconv cells compared to control NGFR-transduced Tconv cells. D. FP3-transduced Tconv cells suppress proliferation of autologous PBMCs. Freshly isolated PBMCs were co-cultured with different ratios of ex vivo CD4^+CD25^+ Treg, FP3-transduced Tconv, NGFR-transduced Tconv, or untransduced Tconv cells and activated with a 1:1 ratio of anti-CD2/anti-CD3/anti-CD28-coated beads. Cells were incubated for 5 days and tritiated thymidine was added for the last 16 hours of the culture.
4.3.4 Expanded Treg cells promote mixed chimerism in cynomolgus macaques following bone marrow transplant leading to acceptance of kidney grafts without maintenance immunosuppression

The main aim of using FOXP3 transduction to generate Treg cells is to quickly obtain large numbers of regulatory cells that would be useful in the clinic for cellular therapies. Despite the successful reprogramming of cynomolgus macaque Tconv cells into regulatory cells by over-expression of human FOXP3, the numbers of suppressive cells generated using this protocol were not ideal due to the low transduction efficiency of cynomolgus macaque T cells. Thus, it was investigated whether my previously developed method of expanding large, homogeneous populations of human ex vivo Treg cells could be applied to the expansion of cynomolgus macaques Treg cells for use in a non-human primate model of Treg cellular therapy.

It was found that ex vivo CD4+CD25+ cynomolgus macaque Treg cells could be expanded efficiently with initial stimulation with L cells and anti-CD3 and subsequent weekly restimulations with allogeneic PBMCs and anti-CD3. After 3 weeks expansion, Treg cells from one donor could be expanded up to 1000-fold with maintenance of FOXP3 expression and in vitro suppressive function (Figure 4.12). Expanded Treg cells were frozen after 3 weeks expansion for future use in in vivo experiments.
Figure 4.12 *In vitro* expansion of cynomologus macaque Treg cells with maintenance of FOXP3 expression and suppressive capacity.

**A.** CD4+CD25\textsuperscript{hi} T cells were isolated from the peripheral blood of one donor 3 different times and were expanded for 1 week with L cells plus 100 ng/mL anti-CD3 in the presence of 100 ng/mL rapamycin and 200 U/mL IL-2. The Treg cells were re-stimulated on days 7 and 14 with allogeneic PBMCs plus 1 µg/mL anti-CD3 in the presence of 200 U/mL IL-2. FOXP3 expression (**B**) and *in vitro* suppressive capacity (**C**) were determined after 3 weeks expansion, and Treg cells were frozen in liquid nitrogen. *In vitro* suppressive capacity was determined by stimulating autologous PBMCs for 5 days with a 1:1 ratio of anti-CD3/anti-CD28/anti-CD2-coated beads and titrating fresh or thawed Treg or Tconv cells at the indicated ratios. Cultures were pulsed with tritiated thymidine ([\(\text{H}^{-}\text{TdR}\)] for the last 16 hours of culture.

It was investigated whether *ex vivo* expanded Treg cells from cynomologus macaques could increase mixed chimerism and allow acceptance of donor kidney grafts without maintenance immunosuppression. Expanded Treg cells were infused into the autologous monkey who received a bone marrow transplant from an MHC Class I mismatch (Donor Bw6 positive, recipient Bw6 negative), Class II haplomatch donor. The monkey was conditioned with whole body irradiation and anti-thymocyte globulin before receiving the
bone marrow transplant (200 x 10⁶/kg). Treg cells were thawed and infused on day 0 (75 x 10⁶), 2 (100 x 10⁶), 5 (75 x 10⁶), 7 (200 x 10⁶), and 50 (140 x 10⁶). Anti-CD154 was given on days 0, 2, 5, 7, and 9, and 12 and cyclosporine A was given continuously from days 1 to 28. Multi-lineage donor chimerism (up to 95% myeloid and 14% lymphoid) was detected to day 335 post bone marrow transplant (Figure 4.13A). In contrast, the maximal mixed chimerism of a control animal receiving the same treatment without Treg infusion was 6% and was undetectable after 50 days. Kidney transplant from the same donor was performed 4 months after bone marrow transplant, and the remaining kidney was removed 100 days later. Renal function was stable without maintenance immunosuppression 246 days post kidney transplant (Figure 4.13B) while the transplanted kidney of a control monkey failed after one month. These data support the notion that ex vivo expanded recipient Treg cells can promote mixed chimerism following allogenic bone marrow transplant that can subsequently allow acceptance of solid organs from the same donor.
Figure 4.13 Ex vivo expanded Treg cells promote mixed chimerism and tolerance to an allogeneic kidney graft without maintenance immunosuppression.

A. Peripheral blood chimerism (lymphocyte, granulocyte, and monocyte) as determined by percent donor cells. The control animal underwent the same procedure as the Treg recipient, but did not receive any Treg cells. B. Stable kidney graft function without maintenance immunosuppression in the Treg recipient but not the control animal as measured by creatinine level.
4.4 Discussion

Treg cell therapy is a promising approach to induce allograft tolerance and limit immunosuppressive drug regimens. While the *in vivo* efficacy of human Tconv cells over-expressing FOXP3 was not demonstrated, large numbers of pure Treg cells from both humans and cynomolgus macaques that showed *in vivo* suppressive capacity were successfully expanded *in vitro*.

The ability to isolate and expand large numbers of pure human Treg cells is essential for the study of their function and also for their use in the clinic but is hindered by the lack of Treg-specific cell surface markers with which to sort pure starting populations and by the limited *in vitro* expansion potential of Treg cells and the rapid outgrowth of contaminating Tconv cells. I developed a method to routinely obtain pure populations that expanded an average of 900-fold in two weeks using artificial antigen presenting cells and a high dose of IL-2. These *in vitro* expanded Treg cells maintained a Treg phenotype in that they expressed high levels of FOXP3 and other Treg-associated markers and did not produce IFN-γ or IL-2. This Treg expansion protocol allowed for the investigation of the potential of Treg cell therapies in two pre-clinical models. Importantly, *ex vivo* expanded cynomolgus macaque Treg cells demonstrated *in vivo* function by enhancing mixed chimerism after bone marrow transplant. *Ex vivo* expanded human Treg cells were also suppressive *in vivo*, delaying onset of xenogeneic GVHD in NSG mice.

The ability of *in vitro* generated or *ex vivo* expanded Treg cells to suppress islet allograft rejection in a humanized mouse model was not effectively evaluated. One reason for this is because graft rejection was not observed in half of the experiments before onset of xenogeneic GVHD. The Greiner group has successfully demonstrated islet allograft
rejection in this model with rejection occurring after 10-20 days; however, they injected $20 \times 10^6$ PBMCs while half that amount was injected in my experiments, which could impact the rate at which the islets are rejected. It would be difficult to increase the numbers of PBMCs injected in this model because the number of Treg cells would concurrently need to increase. Even with the optimized expansion procedure, this would not be routinely feasible. Other differences between the models include the time and mode of PBMC injection, with the Greiner group injecting cells intravenously at the time of transplant instead of two weeks later intraperitoneally after normalization of blood glucose levels. Since delayed GVHD was observed in mice receiving ex vivo expanded Tregs, future experiments could continue in this vein, testing whether FP3 T cells have the same effect. Switching to a skin model may also be beneficial as ex vivo expanded cells have been shown to be suppressive in this model, rejection occurs rapidly so GVHD is less likely to occur first, and skin tissue is more readily available than islet tissue, allowing a greater frequency of experiments to be conducted.

Another way the model could be improved is with the use of new humanized mouse models that are currently being developed. Instead of injecting PBMCs after islet transplant to cause rejection of the graft, a more physiologically relevant model would be to have a human immune system already present and functioning in the mouse prior to the islet transplant. This can be achieved by transplanting neonatal NSG mice with fetal liver and thymic tissue under the kidney capsule and injecting CD34$^+$ stem cells isolated from fetal liver. However, lack of human cytokines and growth factors to regulate hematopoietic development and homeostasis limit the types and numbers of immune cells that can be found in these mice. Improvements have been made to this model with mice that express a variety
of human factors. For example, transgenic expression of human thrombopoetin prolongs human hematopoietic stem cell survival in the bone marrow,\textsuperscript{323} expression of signal regulatory protein alpha on mouse macrophages prevents them from phagocytosing human cells.\textsuperscript{324} IL-3/GM-CSF transgene expression allows human alveolar macrophage development and innate immune responses in the lung,\textsuperscript{325} and M-CSF expression increases the frequency of human macrophages.\textsuperscript{326}

With these new models, or in a model of xenogeneic GVHD, it would be worthwhile to continue investigating whether FP3 T cells have \textit{in vivo} suppressive properties. If these cells can suppress graft rejection in these models, and with advances in the safety of gene therapy, this approach could one day be used in the clinic. Additional safety precautions can be taken by including a suicide gene in the vector construct for quick removal of the cells from the body in the case they become pathogenic.\textsuperscript{250} Furthermore, engineered Treg cells could be co-transduced with a chimeric antigen receptor\textsuperscript{327} for the purpose of targeting them to specific tissues for a localized immunosuppressive effect to avoid the potential of global immune suppression currently associated with immunosuppressive drugs.

Cynomolgus macaque Treg cells were successfully generated by over-expression of human FOXP3. However, the transduction efficiency with the lentiviral vector was extremely low. This could be due to restriction factors that inhibit infection by non-native lentiviruses. For example, tripartite motif 5 alpha (TRIM5α) recognizes viral capsids and prevents reverse transcription and integration. This restriction is enhanced when cyclophilin A binds the capsid. In rhesus macaques, cyclophilin A can bind the HIV capsid, but not the SIV capsid, so HIV infection of rhesus macaque cells is restricted while SIV infection is permitted.\textsuperscript{328} Since the lentiviral vector is HIV-based, the hypothesis of HIV restriction in
the cynomolgus macaque T cells was tested by infecting them with an SIV-based vector encoding GFP. A slight increase in transduction efficiency was observed (data not shown) with standard activation conditions, but the improvement was not enough to pursue developing an SIV-based vector encoding FOXP3 since other unknown factors also likely play a role in the decreased transduction efficiency.

The first clinical trials of Treg cell therapy have been performed to treat or prevent GVHD following bone marrow transplantation. Three studies demonstrated the safety of polyclonal Treg cell therapy as no increase in infection or cancer relapse was observed.\textsuperscript{206,227,228} These results will pave the way for trials in solid organ transplantation. Even with initial successes, development of Treg isolation or generation and expansion procedures that are good manufacturing practice compatible for transfer to the clinic will be essential for routine application of Treg cell therapy.
Chapter 5: Conclusion

5.1 Summary

The importance of FOXP3 to immune homeostasis has been known since its discovery, but there is still much to learn. Research presented here aimed to investigate the role of FOXP3 in human CD4+ Treg cells and Tconv cells including the specific T cell subsets Th1 and Th17.

The best-defined role of FOXP3 is in CD4+ natural Treg cells. Here, FOXP3 plays a key role in tolerance by mediating the ability of Treg cells to suppress immune responses including controlling autoreactive T cells circulating in the periphery and limiting responses to infection. However, the presence of Treg cells can also hinder the ability of the immune system to respond appropriately to cancerous cells. Manipulation of FOXP3+ Treg cells in each of these settings either to boost or inhibit their function would therefore be extremely useful therapeutically. Of particular interest is the potential for FOXP3+ Treg cells to be used as a cellular therapy in the context of organ transplantation to prevent graft rejection. I set out to investigate the potential of cynomolgus macaque and human Tconv cells ectopically expressing high levels of FOXP3 to be used as a regulatory cellular therapy. In addition, ways to expand large, homogenous populations of both cynomolgus Treg cells and human Treg cells were also optimized.

The Treg expansion protocol that was developed was extremely successful at expanding large, pure populations of both human and cynomolgus macaque Treg cells with maintenance of a Treg phenotype. Furthermore, these ex vivo expanded Treg cells demonstrated in vivo suppressive capacity, supporting the use of ex vivo Treg cells for cellular therapy.
Unfortunately, it was not possible to conclusively deduce whether human Tconv cells over-expressing FOXP3 could suppress graft rejection in a humanized mouse islet allograft rejection model. The main barrier to this model was the development of xenogeneic GVHD in the mice before allogeneic rejection of the grafts. Nevertheless, these in vitro-generated Treg cells can be tested in other ways, such as in humanized mouse models of allogeneic skin rejection which should occur at a faster rate and in which ex vivo expanded Treg cells have been shown to prevent graft rejection, or simply in a model of xenogeneic GVHD.

While cynomolgus macaque Tconv cells could be successfully converted into regulatory cells, poor transduction efficiencies prevented testing of the in vivo function of these cells to promote mixed chimerism after non-myeloablative bone marrow transplant. The great expansion potential achieved for ex vivo Treg cells from cynomolgus macaques prompted the continuation of experiments with these cells rather than further pursuit of FOXP3-transduced Tconv cells at this point. However, if human FOXP3-transduced Tconv cells show in vivo suppressive capacity in future experiments in new models of rejection, then it would be worthwhile to return to FOXP3-transduction of cynomolgus macaque Tconv cells to evaluate their in vivo suppressive capacity in this system.

Study of mutations in FOXP3 that cause IPEX offer insight into disease pathogenesis and the role of FOXP3 in Treg cells. The abilities of 3 different point mutant forms of FOXP3 were compared with the ability of wild type FOXP3 to reprogram Tconv cells into regulatory cells in order to decipher what cellular characteristics of Treg cells are altered by different FOXP3 mutations and how those alterations may contribute to disease pathogenesis. Surprisingly, two of the mutant forms of FOXP3 were not completely defective in their ability to reprogram Tconv cells into Treg cells, and no defect at all could be found for one
mutant form of FOXP3 that causes a more mild form of the disease. This suggested that FOXP3 may have a role outside of regulatory T cells, the dysregulation of which contributes to disease in IPEX patients. Alternatively, it is possible that Tconv cells over-expressing mutant forms of FOXP3 might be defective in important ways that we currently do not understand and do not test for in vitro. Perhaps the current methods of evaluating human Treg cells in vitro by cell surface marker expression, cytokine production, and in vitro suppression assays do not accurately reflect their in vivo function, highlighting the need for more extensive characterization of Treg suppressive mechanisms and the part that FOXP3 plays in orchestrating them.

The role of FOXP3 in human Tconv cells has not been extensively studied. FOXP3 is expressed at much lower levels in Tconv cells than Treg cells, and is down-regulated 4-5 days after activation. Some groups have proposed that FOXP3-expressing Tconv cells transiently acquire suppressive capacity, but work showing that FOXP3 must be expressed continuously at high levels for a period of at least 7 days to confer suppressive capacity argues against this suggestion. The role of FOXP3 in human Tconv cells was examined by comparing FOXP3-deficient Tconv cells with wild type Tconv cells and it was found that FOXP3-deficient Tconv cells had a greater expansion potential and produced more cytokines than wild type Tconv cells, demonstrating a role for FOXP3 in human Tconv cells that has not been previously appreciated.

The approach that was taken in knocking down FOXP3 expression with a small interfering RNA against FOXP3 has its limitations. First, FOXP3 expression was knocked down by only ~50-60%, so some transduced T cells still expressed low levels of FOXP3. Second, since the control siRNA against luciferase does not have an endogenous target
within the cell, the rates of siRNA turnover and processing could differ between control and test cells which could differently affect the biology of the cells. Lastly, a standard control used in RNA interference systems is re-expression of the target gene to rescue the pre-knock-down phenotype. However, it would be impossible to mimic the activation-induced FOXP3 expression pattern with a re-expression vector, with the result that Treg cells would most likely be generated instead of a re-capitulation of the function of activation-induced FOXP3.

Despite these limitations, the results were confirmed and strengthened by the fact that a parallel, unique approach was set up with CD4⁺CD25⁻ Tconv clones from an individual who is heterozygous for a null mutation in FOXP3. Thus, Tconv cells that express wild type FOXP3 upon activation were compared with autologous Tconv cells that do not express FOXP3 upon activation, and results were observed that were similar to the siRNA approach, supporting that the observed effects are truly due to differences in FOXP3 expression.

In addition to the analysis of FOXP3 in bulk CD4⁺CD25⁻ Tconv cells, the role of FOXP3 in Th1 and Th17 Tconv cell subsets was investigated. Recent work demonstrates that FOXP3 can interact with the Th17 transcription factors ROR-γt and RORα and has a reciprocal role in the differentiation of Treg versus Th17 cells. The role of FOXP3 in Th17 cells can now be extended to fully differentiated human Th17 cells based on the observation of high expression of FOXP3 upon activation throughout a two-week activation cycle. Whether or not these FOXP3-expressing Th17 cells are differentiating into suppressive cells remains to be determined, but suppressive FOXP3⁺IL-17⁺ T cells have been described so the role of FOXP3 in Th17 cells warrants further study. By knocking down activation-induced FOXP3 in Th17 cells with siRNA, it was determined that FOXP3 may
have a role in Th17 cells to limit expansion and IFN-γ production and up-regulate the cell surface marker CCR4 in these cells. FOXP3 was also induced in Th1 cells upon activation, but with similar kinetics to total Tconv cells, highlighting a more prominent role for activation-induced FOXP3 in Th17 cells compared to other Tconv cell subsets.

5.2 The future of FOXP3

Together, these studies contribute to the larger body of literature that demonstrates the power of FOXP3 in immune cell function. The prospect of using FOXP3+ regulatory cells as a therapy for transplantation could have enormous benefit for transplant patients if this type of cellular therapy allows discontinuation or reduction in immunosuppressive drug regimens. Even with the first clinical trials of Treg cell therapy already underway, a better understanding of how FOXP3 mediates its functions at both the cellular and molecular level will be of utmost importance to harnessing the power of Treg cells for therapies and manipulating both Treg and Tconv cells in disease settings.

We do not yet know how exactly how Treg cells suppress immune responses, though there are many hypotheses including secretion or expression of inhibitory molecules including IL-35, IL-10, and galectin-1 and cAMP; down-regulation of APC function via CTLA-4 and CD39; cytotoxic activity via granzyme and perforin secretion, and physical blocking of Tconv access to APCs by strong Treg interaction with APCs via LAG-3 and neuropillin-1.\textsuperscript{50,331} We understand even less about how FOXP3 might be orchestrating these mechanisms. Recent evidence shows that Treg cells display characteristics of the cells they suppress and this phenomenon is in fact required for their ability to suppress those specific immune responses.\textsuperscript{332,333} I have tested the ability of FOXP3-transduced T cells to suppress
bulk CD4+ or CD8+ T cell responses in vitro and am currently trying to determine their in vivo suppressive capacity in allograft rejection, but future experiments could include investigating whether or not FOXP3-overexpressing Tconv cells can suppress different types of immune cells such as Th1, Th17, and antigen-presenting cells, and if they operate by all the same mechanisms as natural Treg cells. These studies could be initially conducted in vitro, but would ultimately benefit from the use of humanized mouse models that support a fully functional human immune system which are currently being developed.\textsuperscript{323-326} Data from gene microarrays show that a Treg genetic signature can develop even in the absence of FOXP3.\textsuperscript{30,72} If FOXP3-over-expressing Tconv cells are defective at any aspect of immune suppression compared to natural Treg cells, what other factors are involved for these processes? This kind of future work will help to illuminate the role of FOXP3 and the inner workings of Treg cells.

A more thorough understanding of the molecular mechanisms by which FOXP3 activates and represses gene transcription would also be useful for development of therapies to interfere with or enhance these processes. We are beginning to understand how FOXP3 epigenetically modifies target genes, but much work is still to be done in this regard. The epigenetic changes described at target genes occur immediately upon FOXP3 binding,\textsuperscript{145,146} so this does not explain why expression of FOXP3 is required for prolonged periods to effectively reprogram Tconv cells into Treg cells. Is it simply the degree of histone acetylation and methylation that determines gene expression, with active marks increasing over time and leading to a stable Treg phenotype, or is FOXP3 involved in maintaining other epigenetic changes that encode a more long-term program? Advances in the field of epigenetics will be required to fully address this question.
Lastly, enhancing our knowledge of the mechanisms by which FOXP3 is regulated itself would also provide options for therapeutic intervention. A demethylated TSDR found in Treg cells but not Tconv cells\textsuperscript{163} is the most obvious cause for the different levels of FOXP3 protein expression in these two cell subsets, but this is not necessarily the only difference. Post-translational modifications of FOXP3 may affect the stability of FOXP3 expression in Treg versus Tconv cells, and it is already known that FOXP3 is stabilized by acetylation.\textsuperscript{334} Is there a difference in acetylation state of FOXP3 in Treg versus Tconv cells? Furthermore, differences in proteolytic processing of FOXP3 may lead to different functions in Treg versus Tconv cells.\textsuperscript{155} This question can be extended to the difference in FOXP3 expression in Th1 versus Th17 cells as well – why is FOXP3 protein expressed more highly in Th17 than Th1? Do different post-translational modifications of FOXP3 between these two cell types account for the difference? An exciting new area of research is post-transcriptional regulation by microRNA, and a human Treg miRNA signature has been investigated.\textsuperscript{298} Thus, differences in miRNA expression between T cell types may also affect FOXP3 protein levels.

Advances in our understanding of the molecular mechanisms governing FOXP3 expression and function will propel the future of FOXP3 research in both Treg and Tconv cells. Future work will include optimizing Treg cellular therapies and further characterizing the role of FOXP3 in Tconv cells, especially in the Th17 cell subset.
References


123 Li, B. & Greene, M. I. FOXP3 actively represses transcription by recruiting the HAT/HDAC complex. Cell Cycle 6, 1432-1436 (2007).

Li, B. et al. FOXP3 interactions with histone acetyltransferase and class II histone deacetylases are required for repression. *Proc Natl Acad Sci USA* **104**, 4571-4576 (2007).


citation

text


190 Tran, D. Q. et al. Selective expression of latency-associated peptide (LAP) and IL-1 receptor type I/II (CD121a/CD121b) on activated human FOXP3+ regulatory T cells allows for their purification from expansion cultures. *Blood* **113**, 5125-5133 (2009).


211 Powrie, F., Correa-Olivera, R., Mauze, S. & Coffman, R. L. Regulatory interactions between CD45RB<sup>high</sup> and CD45RB<sup>low</sup> CD4<sup>+</sup> T cells are important for the balance


Oldenhove, G. et al. Decrease of Foxp3+ Treg cell number and acquisition of effector cell phenotype during lethal infection. Immunity 31, 772-786 (2009).


Cohn, M. Whither T-suppressors: if they didn't exist would we have to invent them? Cell Immunol 227, 81-92 (2004).

Sharma, M. D. et al. Reprogrammed foxp3(+) regulatory T cells provide essential help to support cross-presentation and CD8(+) T cell priming in naive mice. Immunity 33, 942-954 (2010).


