DEFINING THE BIOLOGICAL ROLE OF FOXP3 IN HUMAN CD4$^+$ T CELLS

by

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Abstract

The involvement of regulatory T cells (Tregs) in immune homeostasis is now recognized as one of the fundamental mechanisms of immune tolerance. While several different types of Tregs cooperate to establish and maintain immune homeostasis, much current research is focused on defining the characteristics of the $CD4^+CD25^+$ Treg subset, as these cells can mediate dominant, long-lasting and transferable tolerance in many experimental models.

The aim of this research was to characterize the biological role of a protein known as forkhead box P3 (FOXP3) that was initially identified as an essential transcription factor for the development of mouse $CD4^+CD25^+$ Tregs, in human $CD4^+$ T cells. Following confirmation that, like mouse Tregs, human Tregs also expressed high levels of FOXP3, several approaches were used to investigate the role of this protein in human $CD4^+$ T cells. 1) Characterization of endogenous FOXP3 expression in $CD4^+$ T cell subsets revealed that this protein is not a Treg-specific marker as was previously thought. Instead, low-level and transient expression was found to be typical of highly activated non-regulatory effector T cells. 2) To generate large numbers of Tregs suitable for cellular therapy, the capacity of ectopic FOXP3 expression to drive Treg generation *in vitro* was explored. It was found that high and constitutive expression mediated by a lentiviral vector, but not fluctuating expression driven by a retroviral vector, was sufficient to generate suppressive cells. Over-expression strategies were also used to characterize a novel splice isoform unique to human cells, FOXP3Δ2 (FOXP3b). 3) To further probe the requirements of FOXP3 to induce suppressor function, a system for conditionally-active FOXP3 ectopic expression was developed. These studies established that FOXP3 acts a quantitative regulator rather than a "master switch" for Tregs, and that there is a temporal component to its capacity to direct Treg phenotype and function.

In summary, this research has significantly expanded the understanding of the biological function of FOXP3 in human $CD4^+$ T cells. Based on the potential of these cells to be manipulated for therapy, this work contributes to the field of immunology on both academic and clinical research fronts.

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List of Abbreviations

AIRE, Autoimmune regulator

APC, antigen presenting cell

CCR, chemokine receptor

CFSE, 5- (and 6-) carboxyfluorescein diacetate succinimidyl ester

CTLA-4, cytotoxic T-lymphocyte-associated protein 4

DC, dendritic cell

FMO, fluorescence-minus-one

FoxP3, forkhead box P3

GITR, glucocorticoid-induced tumor necrosis factor receptor

HA, hemagglutinin

IL, interleukin

IFN, interferon

IPEX, immune dysregulation, polyendocrinophathy, enteropathy, X-linked syndrome

MFI, mean fluorescence intensity

MHC, major histocompatibility complex

MOI, multiplicity of infection

NFAT, nuclear factor of activated T cells

NGFR, nerve growth factor receptor

NK, natural killer

TCR, T cell receptor

Teff cell, effector T cell

TGF, transforming growth factor

TNF, tumor necrosis factor

Treg cell, naturally occurring $CD4^+CD25^+$ T regulatory cell

Preface

The sequence of chapters in this thesis is not chronological: work presented in Chapter 3 was carried out prior to that summarized in Chapter 2. However, in order to group viral-mediated expression studies together, data on endogenous FOXP3 expression patterns in Chapter 2 precedes over-expresion studies presented sequentially in Chapters 3, 4, and 5.

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Dedication

I dedicate this work to my parents, who have supported me in more ways than I can count throughout the course of this work; to my sister, who never fails to bring humor to any and every situation; and to my grandparents for believing in me always.

Co-Authorship Statement

I designed and performed all experiments with the exception of the following, which were carried out with guidance provided by me and my supervisor Megan Levings:

Chapter 2

Sarah Q. Crome and Natasha K. Crellin contributed intellectual and technical help for some experiments in Chapter 2. S. Q. C performed the experiments represented by Figure 2.7.

Chapter 3

Laura Passerini carried out the experiments represented by Figure 3.1B. Natasha K. Crellin carried out quantitative polymerase chain reactions (PCRs) shown in the left panel of Figure 3.1A as well as the standard reverse transcriptase PCR to detect different FOXP3 isoforms represented by Figure 3.5A. Minyue Dai carried out the reporter assay data shown in Figure 3.5B, and Lisa Xu assisted with preparation of retrovirus.

Chapter 4

Mario Amendola constructed the backbone of the lentiviral vector subsequently modified to produce an optimal ectopic expression system for $CD4^+$ T cells. Rupinder Dhesi provided technical help in lentivirus production. TGF-β production by FOXP3-transduced cells was determined by Alicia N. Alstad. The analysis of the T cell receptor (TCR) repertoire of transduced cells in Chapter 4, was carried out by Natacha Merindol under the supervision of Hugo Soudeyns.

Chapter 5

Rupinder Dhesi provided technical help in lentivirus production.

Manuscripts were written with the guidance of my supervisor, Megan Levings with intellectual advice and editing provided by co-authors. Paul Orban took part in critical reading of manuscripts represented by Chapters 2, 3, and 4.

1. Introduction

1.1 T cell tolerance

Immune homeostasis is a delicate balance of responses that control infection and tumor immunity, and reciprocal responses that limit inflammation and autoimmune diseases. A major focus of immunological research has been to identify the mechanisms by which the immune system is able to limit responses to self antigens and innocuous antigens, while preserving the ability to mount robust responses to pathogens and tumors. Since Billingham and Medawar first demonstrated immunological tolerance experimentally in 1953 in a model of transplantation (1), scientists have been seeking to understand the mechanisms of this elusive characteristic of the immune system. For many years, it was thought that the body was able to differentiate "self" vs "non-self" antigens (2), a hypothesis that was supported by discovery of major histocompatibility molecules by Gorer, Lyman and Snell in 1948 (3). However, this simple theory could not explain the ability of the healthy immune system to tolerate foreign or development-associated antigens such as food antigens, allergens, or alloantigens expressed by a developing fetus. Similarly, it was not clear how a breakdown in the self/non-self recognition system would result in autoimmunity or allergy. With increased understanding of immune regulation, it has become clear that the self/non-self theory is too simplistic to explain the complexity of the immune system's regulatory control, and that we need to revise our theories about how the body interfaces with internal and external antigens.

Based on their central role in many immune-mediated diseases, there has been a great deal of research on how tolerance is controlled in the T cell compartment. T cell tolerance is generally described as having two components: central and peripheral. Central tolerance is the process by which self-reactive T cells are deleted in the thymus, based on the quantity and quality of their response to self-antigen (4). The expression of a broad range of peripheral antigens within the thymus is largely controlled by the AutoImmune REgulator (AIRE) transcription factor, and enables deletion of the majority of self-specific T cells during thymic development. The importance of central tolerance in immune homeostasis is illustrated by the fact that AIRE-deficient animals have a large

proportion of self-specific T cells in the periphery and subsequently develop autoimmunity in many tissues (5).

Despite the remarkable AIRE-dependent process of central tolerance, a detectable number of self-specific T cells leave the thymus and can be detected in the circulation of normal individuals. Anti-self responses, as well as responses to non-harmful environmental antigens and counter-responses to inflammation, are controlled by a complex network of peripheral tolerance mechanisms. These include T cell deletion, functional inactivation (anergy), clonal exhaustion, ignorance (rarely), and active regulation by specialized immune cell subsets (6). Depending on the antigen in question, the context, the presence of invading pathogens, the tissue location, and the contribution of genetic factors, the involvement of each of these mechanisms in inducing or maintaining peripheral tolerance can differ. In the last decade, it has become clear that one of the most important links in the regulatory circuit of peripheral tolerance is active regulation by T regulatory cells. These cells, by virtue of their essential role in peripheral immune responses, their antigen specificity, and their ability to traffic throughout the body to sites of inflammation, make them a highly attractive target for the development of therapies which seek to enhance or exploit peripheral tolerance mechanisms.

1.2 Subsets of T regulatory cells

The concept of T regulatory cells was initially proposed in the 1970s (7), although their essential role in regulating immune responses was not fully realized until more recently. Early work by Shimon Sakaguchi and colleagues described a population of $CD4^+$ T cells constitutively co-expressing the IL-2Rα chain (CD25) that was critical for normal immune homeostasis, and demonstrated that these cells could be administered as a cellular therapy to induce tolerance to foreign grafts or prevent autoimmunity (8). Since these seminal experiments that defined $CD4^+CD25^+$ T regulatory cells were performed, the pace of research in this field has increased exponentially. In addition to the $CD4^+CD25^+$ T regulatory cells initially characterized by Sakaguchi and colleagues, many other immune cell subsets with regulatory capacity have been identified. These include IL-10-secreting T regulatory 1 (Tr1) cells, TGF-β-secreting Th3 cells, $CD8^+CD28^-$ T cells, $\gamma\delta$ T cells, mast cells, discrete subsets of natural killer (NK) cells, NKT cells, and B cells, as well as several types of immunoregulatory dendritic cells (DCs) (9-12). These

numerous subsets operate together to orchestrate normal immunoregulation, and it is likely that the nature of these interactions differ with the context, tissue location, and severity of inflammation. However, of the many regulatory subsets described, $CD4^+CD25^+$ T cells are the best-characterized subset, and appear to be one of the most promising candidates for development of therapeutic applications.

1.3 Phenotypic & functional characteristics of $CD4^+CD25^+$ Tregs

$CD4^+CD25^+$ T regulatory cells are a distinct T cell subset that develops in the thymus. These cells are present in humans before birth, and can be detected as early as 13 weeks of gestation (13, 14). In addition to development in the thymus, there is increasing evidence that $CD4^+CD25^+$ T regulatory cells can arise from naïve or effector $CD4^+$ T cells in the periphery under certain conditions. The contribution of peripheral conversion to the $CD4^+CD25^+$ T regulatory cell pool under normal conditions seems to be minimal (15, 16); however, situations including infection (17), lymphopenia (18), subimmunogenic antigen stimulation (19, 20), cancer (21, 22), and some pharmacological agents (23-25) promote this process. In most cases, peripherally converted $CD4^+CD25^+$ T regulatory cells are indistinguishable from the thymically-derived subset in terms of phenotype and function. This pool of functionally suppressive $CD4^+CD25^+$ T regulatory cells will hereafter be referred to as Tregs.

In addition to high and constitutive expression of CD25, Tregs also express high levels of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), glucocorticoid-induced tumor necrosis factor receptor (GITR), chemokine receptor 4 (CCR4), CCR5, and low levels of CD127 (26). The expression of other markers previously thought to define Tregs such as HLA-DR, CD45RO, CD62, and CD103 to name a few, have now been shown to be expressed on phentoypically or functionally discrete subsets. For example, HLA-DR was initially thought to be expressed on all Tregs, but it was later revealed that $HLA\text{-}DR^+$ and $HLA\text{-}DR^-$ Tregs had different mechanisms of suppression *in vitro* (27). CD45RO was also thought to mark all Tregs, but more recently it became clear that a functionally distinct subset of human Tregs that had recently emerged from the thymus expressed the CD45RA isoform (28). These naïve Tregs were found to have a more stable phenotype upon *in vitro* expansion, and appear to be distinctly different from CD45RO-expressing

Tregs with respect to their response to apoptotic stimuli. Finally, expression of molecules such as CD62L and CD103 (α_E integrin), and some chemokine receptors are not definitive of the Treg population as a whole, but are important for the homing and trafficking of specific Treg subsets to sites of inflammation (29, 30). These findings help to shed light on the highly heterogeneous nature of the Treg population, and have encouraged immunologists to challenge and broaden their definition of Treg characteristics.

Tregs have traditionally been isolated from mixed populations based on their high CD25 expression. However, because CD25 can also be expressed by activated T cells, this strategy generally leads to impure populations of sorted cells, particularly if the cells are then expanded *in vitro*. Isolation based on a combination of cell-surface markers (such as $CD25^{bright}CD127^{low/-}$ or $CD25^{bright}CD45RA^{+}$) has improved the purity of sorted Tregs, but not to the level that will be required for clinical applications. The identification of a Treg-specific marker will be very valuable as a diagnostic tool to determine quantitative or qualitative measures of Tregs in patient samples, possibly as a marker of tolerance. In addition, a specific marker would greatly facilitate further biological studies and development of new cellular therapy applications. Thus, the search for a unique marker of Tregs continues.

The biological characteristics of Tregs have been widely explored *in vitro* and compared to effector T cells (31-33). In response to T cell receptor (TCR) stimulation, Tregs are profoundly anergic *in vitro*, which is reversed with addition of exogenous IL-2. When stimulated, Tregs make low to undetectable levels of interleukin (IL)-2, interferon (IFN)-γ, IL-4, IL-5, IL-10, tumor necrosis factor (TNF)-α, and transforming growth factor (TGF)-β. While *in vivo* mouse models strongly suggest that IL-10 and TGF-β are important for the suppressive function of Tregs in some contexts (34), these two cytokines are not essential for the *in vitro* suppressor function of human Tregs (35).

1. 4`Suppression mediated by Tregs

Many groups have demonstrated that cell-to-cell contact is required for Tregs to suppress their targets using transwell assays (32). It appears that Tregs can mediate suppression in the absence of antigen presenting cells (APCs) *in vitro* (35), although it is likely that suppression is mediated at the level of APCs *in vivo*, based on evidence from two-photon

live imaging in mice (36, 37). Despite great efforts, the exact mechanism of Treg suppression remains largely unknown. The targets of suppression are numerous and include different subsets of $CD4^+$ T cells (32), $CD8^+$ effector T cells (38, 39), B cells (40), as well as inhibition of innate immune cells (41). The mechanisms by which they potently inhibit their targets are numerous and complex, and can involve cell-contact, secretion or membrane expression of immunoregulatory cytokines (26), induction of IDO by other cells (42, 43) production of the suppressive metabolite adenosine (44, 45) and induction of apoptosis in target cells by a variety of mechanisms (46-48). Finally, Tregs also have the ability to influence the trafficking of pathogenic cells by affecting chemokine receptor expression (49). However, no one mechanism can account for the potent suppressive function of Tregs. What is clear is that Treg development and suppressor function is dependent on a key transcription factor, forkhead box family member P3 (FoxP3).

1.5 FoxP3 and Tregs

In 1949, a spontaneous mutation in the mouse colony at the Oak Ridge National Laboratory occurred, resulting in a mouse strain that suffered from a fatal multi-organ autoimmune disease (50, 51). Studies of these mice, called Scurfy mice, revealed that the disease was X-linked and that the autoimmunity was due to uncontrolled proliferation primarily of $CD4^+$ T cells, which infiltrated many organs to cause autoimmune destruction (52). An X-linked human disease with a similar etiology to Scurfy mice was also described. This disease, referred to as Immune dysregulation Polyendocrinopathy Enteropathy X-linked (IPEX) syndrome, typically presents with a triad of Type 1 Diabetes, severe enteropathy, and dermatitis, but can also include symptoms including hyper IgE, thyroiditis, food sensitivities, and other autoimmune disorders (53). In 2001, genetic linkage analysis was used to define that both Scurfy mice and IPEX patients suffered from a monogenic disease caused by mutations in a previously unidentified forkhead family protein, FoxP3 (52, 54, 55). Scurfy mice have a 2 base pair insertion which results in truncated/absent protein (52), while IPEX patients show varying mutations in the FoxP3 gene, some of which result in lowered expression (53).

Based on the fact that mice deficient in Tregs suffered from severe and fatal autoimmunity similar to that observed in Scurfy mice, immunologists were quick to

uncover that FoxP3 was an essential factor for the development of these cells (56-58). Scurfy mice were found to completely lack Tregs, and infusion of the missing population was sufficient to rescue these mice from autoimmunity. Tregs had emerged as master regulators of the immune system, with the potential to combat autoimmunity in many different tissues and induce tolerance to allografts. The discovery of FoxP3 created quite a stir, as at last it seemed that a specific marker for Tregs had been identified: in mice, only this population was found to express this protein. Furthermore, it was shown that forced expression of FoxP3 was sufficient to convert mouse conventional T cells into Tregs, indicating that this protein was sufficient for their development and could act as a "master switch" for suppressor function (56-58). Immunologists set out to investigate the molecular mechanisms by which a single transcription factor could induce such changes in T cell biology so as to affect the homeostasis of the immune system as a whole.

1.6 Forkhead family transcription factors

Since the description of the first forkhead transcription factor in Drosophila over 10 years ago, over 100 different family members have been identified in organisms ranging from yeast to humans. These proteins have in common a 100 amino acid "winged-helix" DNA binding domain, the forkhead (FKH) domain, with a folded motif made up of three α-helices and two loops, or "wings." Outside of this conserved domain, forkhead proteins are diverse in sequence, structure, and function. Phylogenetic analysis groups Fox proteins into structural (not functional) subclasses, with nomenclature assigned by a standardized committee (59). The convention for naming human FKH proteins has been established as capital letters (eg. FOXP3), mouse proteins with only the first letter capitalized (Foxp3), and with the subclass denoted with a capital letter for all other chordates (FoxP3). To refer to the gene rather than the protein, the convention for the above list is *FOXP3, foxp3, FoxP3*.

FKH proteins are transcriptional activators or repressors, with a broad range of cellular functions including embryonic development, speech and language development, and regulation of the immune system (60). In addition to FoxP3, a handful of other forkhead family members have important roles in the immune system, primarily demonstrated using knockout mice. *Foxj1*$^{-/-}$ mice are embryonic lethal, while fetal-liver chimeric mice suffer from autoimmune pathology due to Th1 cell hyperactivation. Foxn1

has been shown to be critical for differentiation of thymic epithelial cells, as *Foxn1*$^{-/-}$ mice have a "nude" phenotype and lack all T cells. *Foxo3a*$^{-/-}$ mice have abnormal immune homeostasis, lymphocyte proliferation and apoptosis, and suffer from late-onset non-fatal autoimmunity (60).

1.7 FOXP3 functional domains

Human FOXP3 is 431 amino acids long, and is highly homologous to the mouse protein (86% sequence identity) (61). It consists of 11 exons, and the promoter region has been characterized in some detail (62). In mouse cells, the protein does not appear to be alternatively spliced, while in human T cells, two alternatively spliced isoforms, FOXP3a and FOXP3b, are expressed in roughly equal amounts, with the smaller isoform lacking exon 2 (63). Other isoforms have also been reported in human T cells and other cell types (64, 65), although these appear to be present at low levels and/or under pathogenic conditions.

The structure of the FOXP3 protein is unusual compared to other FKH family members. The FKH domain is near the C-terminus, rather than near the N-terminus as for most other FKH family proteins (**Fig. 1.1**). Apart from this conserved DNA-binding domain, FOXP3 does not resemble other FKH proteins to a large degree, save for the few other related FOXP proteins (66). In addition to the FKH domain, the full-length FOXP3 protein contains a C2H2 zinc finger and a leucine zipper in the central region, and a proline-rich region near the N-terminus. Studies of IPEX patients have provided clues as to which regions of FOXP3 are functionally important. Mutations are generally seen in three regions of the protein: the FKH domain, the leucine zipper, and the proline-rich domain (66).

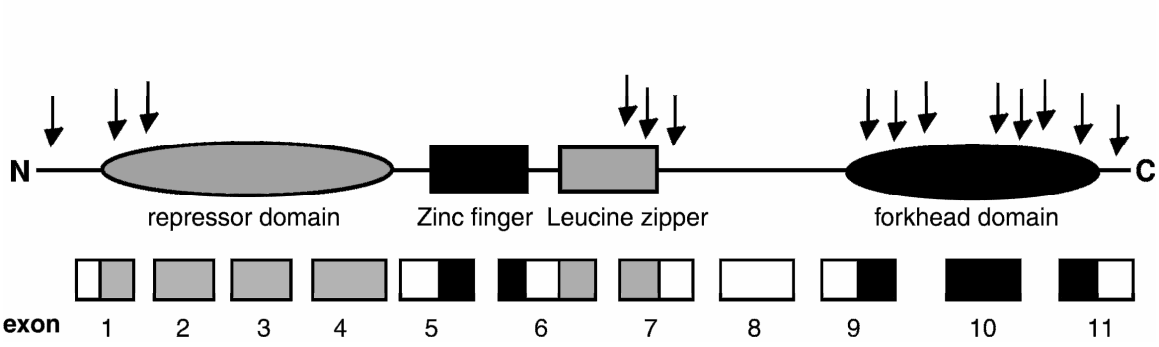

Figure 1.1 Schematic of human FOXP3 and *FOXP3* (adapted from ref (67)). Exons encoding functional domains are coloured according to protein schematic, and missense mutations in FOXP3 that lead to IPEX are marked with arrows.

The FKH domain of FOXP3 is capable of physically interacting with DNA (68, 69). Using investigative strategies based on studies of other FKH proteins (70), it was found that FOXP3 interacts with DNA at consensus sequences found within the promoters of several genes (68). Reporter and chromatin immunoprecipitation (ChIP) assays indicated that FOXP3 binds such sequences in the promoters of IL-2, CD25, CTLA-4 (69), and CD127 (71) genes, to name a few. The FKH domain also contains a nuclear localization sequence. A mutation in the forkhead domain which affects the nuclear localization of FOXP3 leads to severe autoimmunity (66).

Leucine zipper structures are involved in protein-protein interactions, often homo- and hetero-oligomerization. Many FKH proteins have been found to homo- or hetero-oligomerize, including FOXP3. It has been reported that FOXP3 self-associates and can complex with FOXP1, and that the ability to form these interactions is essential for normal protein function (72, 73). IPEX patients with deletions in this region (Δ250, Δ251) suffer from severe disease due to the inability of FOXP3 to self-associate, and/or associate with other forkhead family members such as FOXP1 (72).

While the DNA-binding domain is crucial for FOXP3 to drive normal Treg development and function, the proline-rich region at the N-terminus region is also essential. *In vitro*, the N-terminal region of FOXP3 is sufficient to repress NFAT-mediated transcription, despite lacking the conserved DNA-binding domain. The minimal repressor domain within the N-terminal region was mapped to amino acids 67-132 (66). Although it was previously postulated that FOXP3 inhibited NFAT mediated-

transcription by sterically interfering with NFAT binding at the same or adjacent DNA sequences (68), it was more recently demonstrated that FOXP3 physically interacts with NFAT and inhibits the ability of NFAT to act as a transcriptional activator rather than its ability to bind DNA (69). Even more intriguing, it was found that FOXP3 not only prevents normal NFAT function upon T cell activation, but that these two proteins interact to form a complex with altered transcriptional control specifically required for Treg function. Residues of the FOXP3 protein found to be important for association with NFAT were near or within the FKH domain, but distinct from the residues which actually mediated DNA-binding (69). The NFAT-FOXP3 complex bound to DNA was found to resemble that of the NFAT-AP-1 complex. It is tempting to speculate that the intracellular balance of FOXP3 vs AP-1 components c-jun and c-fos determines the action of NFAT complexes upon TCR stimulation: with preferential pairing of NFAT with FOXP3 rather than AP-1, the molecular consequence of NFAT activation is Treg-directive, rather than Teff cell-directive.

1.8 FOXP3 is part of a supramolecular protein complex and acts as a global regulator of transcription

As mentioned above, many target genes of FOXP3 have been identified using a variety of experimental systems. More recently, investigations employing microarrays and ChIP on ChIP analysis have revealed the extent to which FOXP3 controls the T cell transcriptional programme. Firstly, a study by Zheng *et al* demonstrated that mouse Foxp3 targets approximately 700 genes, and has both transcriptional repressor and activator activity (74). This same study reported distinctly different Foxp3-dependent transcriptional programmes in the thymus and the periphery, suggesting that the molecular function of Foxp3 varies with developmental stage and/or tissue location. Finally, this report indicated that the majority of Foxp3-dependent changes in gene expression were mediated indirectly through other transcription factors (74). A study published in parallel by Marson *et al* reported similar findings, and included an examination of how TCR activation influenced the binding of Foxp3 (75). These investigators found that many Foxp3 target genes are associated with the TCR signaling pathway, and that a major function of Foxp3 is to repress genes normally activated upon T cell activation. In addition, it was found that while Foxp3 readily bound many target

genes in unstimulated cells, the expression of these genes (nearly 1% of the mouse genome) was only altered upon TCR stimulation. The authors postulated that this could be due to the fact that Foxp3 requires other co-factors present in the nucleus following T cell activation (eg. NFAT), or because of increased binding of FoxP3 at target sites upon TCR triggering (75). Evidence from the above papers, as well as that provided in a report by Cobb *et al*, indicate that Tregs have unique microRNA expression patterns that depend on Foxp3 expression (76). Overall, the distinct molecular signature of Treg cells appears to be largely dictated by Foxp3, which involves primarily repression, but also activation, of a broad range of genes. While normal Treg development is dependent on Foxp3, it has both direct DNA-binding activity and the ability to influence gene expression via interaction with other transcription factors.

The finding that the majority of FoxP3-dependent genes were not bound by FoxP3 suggests that FoxP3 mediates much of its activity through other proteins. As mentioned above, FoxP3 has the ability to interact with NFAT, an association which is essential for normal Treg function (69). In addition, a functionally relevant physical association between FoxP3 and many other proteins has also been reported. Bettelli *et al* demonstrated that FoxP3 can associate with and prevent the activity of NF-κB as well as NFAT (77), while Grant *et al* provided evidence that FoxP3 directly or indirectly interferes with the activity of both NF-κB and cAMP-responsive element binding protein (CREB) (78). The ability of FoxP3 to inhibit the activity of these factors suggests that it can negatively regulate several events leading to or enhancing T cell activation. In a report from Sakaguchi's lab, FoxP3 was also shown to physically interact with acute myeloid leukemia 1 (AML-1; also known as runt-related transcription factor-1 [Runx-1]) via a region of the FoxP3 protein between amino acids 278-336 (between the forkhead domain and the leucine zipper domain). Based on this report, Foxp3-AML-1 association is required for repression of IL-2 and IFN-γ genes, activation of CD25, CTLA-4, and GITR genes, and the *in vitro* suppressive activity of Treg cells (79). Because different regions of FoxP3 are involved in associations with NFAT and AML-1, the presence of a tripartite complex has been proposed (80).

Evidence discussed above has characterized FoxP3 as a global regulator of transcription with the ability to influence the expression of a broad range of target genes

directly or via interaction with other transcription factors. Data from the Greene laboratory has also demonstrated that FoxP3 can influence chromatin structure by interaction with histone modifying proteins, adding yet another layer to FoxP3 molecular control. The modification of histones influences chromatin structure by limiting or promoting the accessibility of transcription factors to the DNA. Histone acetyltransferases (HATs) add acetyl groups to histones and open chromatin to permit transcriptional activation, while histone deacetylases (HDACs) remove these residues and tend to close and de-activate chromatin. Greene and colleagues have reported physical associations between FoxP3 and TIP60, a HAT, as well as several different HDAC proteins (81). These investigators found that interactions with TIP60 and HDAC9 in particular were essential for Treg suppression, and that acetylation of FoxP3 regulates its molecular function (82).

Several studies have demonstrated that in addition to an essential role in Treg development, FoxP3 is important for the function of Treg cells in the periphery. Rudensky and colleagues recently demonstrated that ablation of FoxP3 in the Treg cells of adult mice leads to development of autoimmune inflammation equal to that observed in Scurfy mice (83). A study by Wan and Flavell illustrated that reduced expression of FoxP3 in Tregs also led to defective suppressor function and subsequent development of autoimmunity (84). However, other recent reports have cast into question the concept that this transcription factor is truly a "master switch" for Tregs. One such study is that of Gavin *et al*, in which investigators studied female mice carrying an in-frame insertion of GFP in a stop-codon-disrupted FoxP3 locus. In these mice, Treg cells carrying the wild-type Foxp3 allele prevented the development of autoimmunity, while T cells carrying the mutant FoxP3 allele (T_{FN} cells) could be identified based on GFP expression. It was expected that T_{FN} cells would be highly activated and lack typical Treg characteristics. However, T_{FN} cells had an intermediate Treg phenotype, with moderate upregulation of Treg associated molecules including CD25, CTLA-4, and GITR, and a pronounced downregulation of CD127. In addition, their cytokine profile was very similar to that of Foxp3-sufficient Tregs. Like Tregs, T_{FN} cells were hyporesponsive, although anergy was less stable than $Foxp3^+$ Tregs. Despite these characteristics, T_{FN} cells were incapable of suppressing Teff cells *in vitro* and *in vivo*. Similar findings were reported by the Chatila

laboratory, in which many Treg characteristics were still found to develop in the absence of functional FoxP3 expression (85). Finally, an extensive analysis carried out by the Benoist laboratory determined that while much of the Treg signature could be attributed to the effects of FoxP3, other upstream elements appear to be involved in Treg generation and function (86). Thus, although FoxP3 is clearly important in Treg development and function, it seems likely that other molecular features of Treg cells also play a role in determining the phenotype of this lineage.

1.9 Tregs and FOXP3 in human disease

With increasing knowledge of Treg biology and the molecular factors which control their development and function, we are moving steadily towards the goal of applying this knowledge to therapeutic applications. There is mounting evidence that Tregs will be an ideal target for immunomodulatory therapies for applications including autoimmunity, allergy, transplantation, infectious disease, and cancer (37, 87). Increased knowledge about how FOXP3 functions at the molecular level, how its expression may be manipulated to enhance or inhibit Treg function, and how it influences immune homeostasis, will be essential for advancing immune-based therapies.

1.10 Benefits and caveats of strategies developed for ectopic expression of proteins in primary T cells

Over-expression techniques have been widely used to assess the functions of novel proteins in the immune system (88-90). In contrast to cell lines or other primary cell types, primary human T cells cannot easily be transfected *in vitro* using liposome-based, calcium phosphate, or traditional eletroporation protocols. Thus, methods for ectopic expression of proteins in primary T cells are generally limited to either viral-based gene transfer methods or newly developed techniques of nucleofection. While nucleofection has shown to be useful for some applications (78, 91), only viral-mediated gene transfer allows for long-term, stably integrated gene expression without negative effects on cell viability. Methods for viral-mediated gene transfer are summarized in **Table 1.1**. Selection of a gene transfer method, should include consideration of properties such as the size of the gene to be inserted, the need to tranduce differentiated/non-dividing cells, the capacity of the vector to enable long-term gene expression, and the ability to achive high titers.

Table 1.1. Summary of commonly used gene transfer methods (adapted from ref (92)). RV, retrovirus; LV, lentivirus; Ad, adenovirus; HC-Ad, high-capacity, helper-dependent adenovirus; AAV, adeno-associated virus; HSV-1, Herpes simplex virus type 1.

	RV	**LV**	**Ad**	**HC-Ad**	**AAV**	**HSV-1**	**Vaccinia**	**Measles**
Cloning capacity (kb)	~8	~10	7.5	~30	2-4.5	30-130	30	4
Transduction of post-mitotic cells?	No	Yes	Yes	Yes	Yes	Yes	No	No
In vivo	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
In vitro	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Long-term expression	Yes	Yes	No	Yes	Yes	Yes	No	No
Viral titers	10^7	10^9	10^{12}	10^{11}	10^9	10^8	10^6-10^8	10^7

The potential for viral vectors to be used as gene therapy has driven the recent development of safe, highly effective methods for gene transfer studies. The most commonly used viral transfer systems for transducing human T cells include retrovirus- (including lentivirus-), and adenovirus-based expression systems (93). Each of these has benefits and drawbacks, inherent to the expression system itself as well as the experimental or clinical setting in which it is applied. Retroviral vectors are single-stranded RNA viruses and are the most commonly used vectors in clinical trials to date (93). Retroviral-based expression systems are beneficial due to ease of production using stably transfected cell lines; however, retroviruses infect only dividing cells due to the fact that the nuclear membrane must be disrupted during mitosis for the virus to gain access to host chromatin. In contrast, lentiviruses have the ability to infect non-dividing cells, as the import of the lentiviral pre-integration complex into the nucleus depends on active transport mechanisms and not on the mitotic state of the target cells. This, in addition to the option to include internal promoters and additional biosafety afforded by self-inactivating vector design, make lentiviral vectors a more attractive vehicle for T cell gene transfer than retroviral expression systems. Despite these advantages, retroviral or lentiviral vectors can be used to encode genes of limited size, without introns or polyadenylation signals. In addition, the fact that the viral genome inserts randomly into that of target cells means that insertional mutagenesis is a possibility in the clinical setting. Indeed, this has been reported in early clinical trials using retroviral vectors (94).

An alternative to retroviral systems are adenovirus-based systems. Adenoviruses have a double-stranded DNA genome and are advantageous due to the ability to encode much larger inserts in their genome (92, 93). However, the fact that adenoviruses do not integrate into the target genome means that they are not useful for gene expression in proliferating populations. While this would eliminate the potential of insertional mutagenesis occurring *in vivo*, only repeated exposure to adenoviral vectors would be useful for treating chronic problems.

1.11 Hypotheses

This research project was initiated in February 2003, at which time reports describing a role for Foxp3 in mouse Tregs had just been reported (56-58). At this time, it was known that FOXP3 mutations resulted in IPEX, but the link between FOXP3 and human Treg cells had only been hypothesized. Based on the potential for Tregs to be manipulated for therapeutic purposes, the aim of this research was to investigate the phenotype and function of these cells, with a particular focus on the biological role of FOXP3. Hypothesis 1 formed the basis of the project, while Hypotheses 2 and 3 developed over the course of the research presented here.

Hypotheses

1. Human Treg cells express FOXP3, and over-expression of this protein by viral gene transfer is sufficient to drive the generation of human $CD4^+$ Tregs.

2. FOXP3 expression is not limited to the $CD4^+CD25^+$ Treg subset in humans, and may have alternate functions in other cell types.

3. Regulation of the intensity and kinetics of FOXP3 expression are critical to determination of a regulatory phenotype in human $CD4^+$ T cells.

1.11 References

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2. Characterization of FOXP3 expression in human $CD4^+CD25^+$ and $CD4^+CD25^-$ T cell subsets[1]

2.1 Introduction

The forkhead box P3 (FoxP3) transcription factor has a critical role in the development of naturally occurring $CD4^+CD25^+$ T regulatory cells (Tregs), which are required for maintenance of immune tolerance. Mice lacking a functional *FoxP3* gene suffer from systemic autoimmunity, and evidence from adoptive transfer experiments suggests that this is the direct result of a deficit in Tregs. Moreover, ectopic expression of FoxP3 in mouse $CD4^+$ T cells is sufficient to generate Tregs *in vitro* (1-3), leading to the conclusion that expression of this single transcription factor causes a developmental switch to a suppressor cell phenotype. FoxP3 is thus considered a more definitive lineage marker of Tregs than CD25, and recent evidence that, in mice, cells that are $CD4^+FOXP3^+$ but $CD25^-$ are suppressive, supports this concept (4).

Humans lacking a functional FOXP3 gene also suffer from a systemic autoimmune disease known as immune dysregulation, polyendocrinopathy, enteropathy X-linked syndrome (IPEX), and it was hypothesized that this disease was also due to the lack of Tregs (5). Surprisingly, however, we found that, depending on the type of mutation, IPEX patients can have normal numbers of Tregs that are suppressive under conditions of weak activation (6). In addition, retrovirus-mediated expression of full length FOXP3, and/or a form of FOXP3 lacking exon 2, does not consistently convert human $CD4^+$ T cells into potent Tregs, suggesting that, additional factors are required for FOXP3 to cause the full developmental switch to Tregs in humans (7-9).

The apparently different roles of FOXP3 in mice and humans may be related to the capacity of human non-regulatory $CD4^+$ T effector (Teff) cells to express FOXP3 upon activation (7, 10-14). Early reports suggested that a proportion of human $CD4^+$ Teff cells always express $FOXP3^+$ when activated, and that these cells then become phenotypically and functionally indistinguishable from Tregs (10, 11). More recent

[1] Material in this chapter has been published: Allan, S.E., Crome, S.Q., Crellin, N.K., Passerini, L., Steiner, T.S., Bacchetta, R., Roncarolo, M.G., and Levings, M.K. 2007. Activation-induced FOXP3 in human T effector cells does not suppress proliferation or cytokine production. *Int Immunol* **19**:345-354. Permission granted by Oxford University Press.

studies suggest that expression of FOXP3 in human Teff cells does not necessarily lead to a suppressive phenotype, since $CD4^+FOXP3^+$ T cell clones that are not suppressive can be isolated (13, 14), and production of IL-2 and IFN-γ is not suppressed in all $FOXP3^+$ Teff cells (12).

In order to further investigate the hypothesis that FOXP3 has a distinct role in Teff cells, we characterized the kinetics and levels of FOXP3 expression in activated *ex vivo* Teff cells, Teff cell lines and suppressed Teff cells. Here we report that FOXP3, like other conventional T cell activation markers, is transiently upregulated in all dividing Teff cells, but at levels that are significantly lower than those in Treg. This activation-induced expression in Teff cells was insufficient to uniformly suppress the expression of transcriptional targets of FOXP3, such as CD127, IL-2, and IFN-γ. These data imply that FOXP3 has a role in human Teff cells that is independent of its capacity to promote Treg development and/or that FOXP3 cannot induce regulatory function in the absence of other essential factors that are present in Tregs but not in Teff cells.

2.2 Results

Expression of FOXP3 in resting versus activated $CD4^+$ T cell subsets. We and other groups have reported that in humans, expression of FOXP3 can be induced in Teff cells upon TCR-mediated stimulation (7, 10-16). It is unclear, however, whether this activation-induced FOXP3 is associated with the *de novo* development of Tregs and suppressive activity (10-12) or simply a normal consequence of activation. To further investigate this question, we first performed a detailed comparison of the kinetics and intensity of FOXP3 expression in Tregs and Teff cells. Highly purified Treg and Teff cells were activated with anti-CD3/28-coated beads and expression of FOXP3 was followed over time. The experiments were performed in the absence of exogenous IL-2 in order to determine the innate capacity of Teff cells to express FOXP3. Western Blotting (**Fig. 2.1A**) demonstrated that both full-length FOXP3 (FOXP3a), and the smaller isoform which lacks exon 2 (FOXP3b) (7), were detectable in the nuclear fraction of activated Teff cells, with expression peaking at 72 hours and then gradually declining. In Tregs, expression of FOXP3a/b also increased upon activation to levels greater than those

observed in highly activated Teff cells. Flow cytometric analysis of resting and stimulated Tregs and Teff cells confirmed these results, with a significantly higher mean fluorescence intensity (MFI) of FOXP3 in Tregs than in Teff cells after 72h of activation (**Fig. 2.1B**). The anti-FOXP3 mAbs used for flow cytometric analysis (clone PCH101 or 236A/E7) were found to recognize both isoforms of FOXP3 (data not shown), thus detecting total FOXP3a/b (hereafter referred to simply as FOXP3).

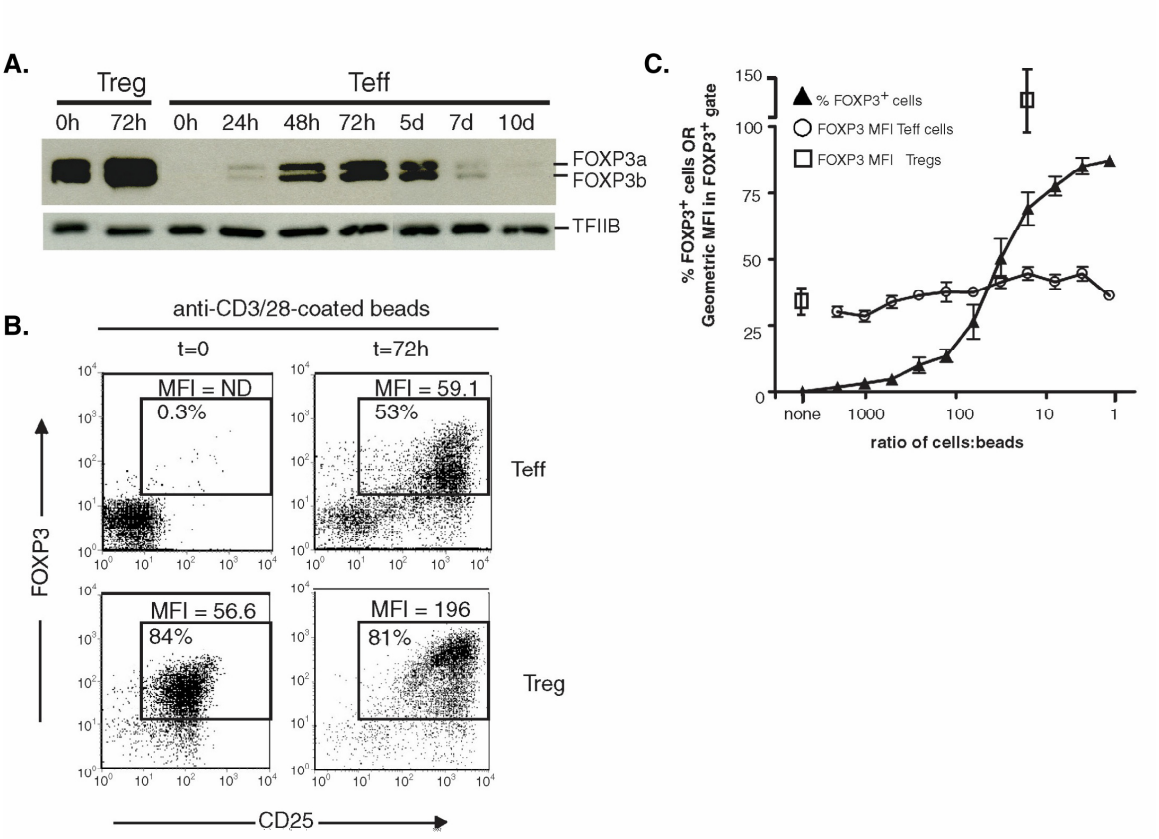

Figure 2.1 Kinetics of activation-induced FOXP3 expression in Teff cells compared to Tregs. **(A)** *Ex vivo* Tregs and Teff cells were activated with anti-CD3/28-coated beads for the indicated times, and nuclear lysates were probed for expression of FOXP3 and TFIIB (as a loading control). **(B)** *Ex vivo* Tregs and Teff cells were activated as in (A) for 72h and analysed for CD25 and FOXP3 expression using anti-FOXP3-PE (clone PCH101). The MFI of FOXP3 was determined by gating on the $CD25^{+}FOXP3^{+}$ cells as indicated. **(C)** *Ex vivo* Teff cells were activated with increasing numbers of anti-CD3/28-coated beads for 72h and analyzed for FOXP3 expression using anti-FOXP3-PE (clone PCH101). The % $FOXP3^{+}$ cells and MFI in the $FOXP3^{+}$ gate are shown. The MFI of FOXP3 expression of resting or activated Tregs is shown for comparison. A is representative of n=2, B of n=8, and C of n=5 experiments.

We next investigated whether all $CD4^{+}$ Teff cells have the potential to express FOXP3, and compared the intensity of expression in Teff cells versus Tregs. Accordingly, Teff cells were activated with increasing numbers of anti–CD3/CD28-

coated beads, and analyzed for expression of FOXP3. Surprisingly, under strong activation conditions, virtually 100% of Teff cells expressed FOXP3 (**Fig. 2.1C**). The total amount of FOXP3 protein induced per cell was similar to that in resting Tregs, but was always significantly lower than levels in Tregs exposed to equivalent activating conditions. These data suggest that in the absence of exogenous factors, Teff cells may have a molecular block that prevents them from expressing high levels of FOXP3, or that Tregs express a unique complement of proteins that drives elevated and sustained expression.

FOXP3 is an activation marker in Teff cells. In addition to FOXP3, Tregs constitutively express CD25, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), and glucocorticoid-induced tumor necrosis factor receptor (GITR), molecules that are also upregulated on activated Teff cells. To investigate how the kinetics of activation-induced expression of FOXP3 in Teff cells compared to these conventional activation markers, we assessed their expression in parallel over a period of 10 days. As expected, *ex vivo* Teff cells were FOXP3$^-$, CD25$^-$, CTLA-4$^-$, GITR$^-$ and CD69$^-$. Following activation, the peak of FOXP3, CTLA-4 and GITR, expression occurred at ~72h (**Fig. 2.2A**), whereas the peak of CD69 and CD25 occurred as early as 24h. After peaking at 72h, FOXP3 expression gradually declined, with a parallel decrease in expression of CTLA-4 and GITR. It is unlikely that this decline in FOXP3$^+$ cells was simply due to cell death since even strongly-activated cultures in which >90% of cells became FOXP3$^+$ (e.g. **Fig. 2.1C**) did not show significant cell death.

In parallel, the kinetics of expression of these markers was also determined in Tregs. *Ex vivo* cells purified on the basis of CD25 expression were typically 65-85% FOXP3$^+$, 30-60% CTLA-4$^+$, GITR^{low} and CD69$^-$. Upon activation, the percentage of Tregs expressing CTLA-4, GITR and CD69 increased. In contrast to Teff cells, the percentage of cells expressing CD25, FOXP3, and CTLA-4 did not significantly decline over a period of 7 days.

To further characterize the pattern of activation-induced FOXP3 in Teff cells, we directly assessed the intensity of FOXP3 expression in purified populations of Teff cells and compared them to Tregs (**Fig. 2.2B**). In parallel, we analysed expression of CD25,

GITR, and CTLA-4 as examples of other Treg-associated markers, as well as CD69 as a control for T cell activation. Levels of expression of CD25, CTLA-4, and GITR increased in both cell types upon activation. The intensity of the activation-induced expression of FOXP3, CD25, CTLA-4, and GITR in Tregs was significantly higher than that in activated Teff cells. After 72 hours, expression of FOXP3 in Tregs was 2.3±0.77 fold higher than in Teff cells ($p=0.026$ $n=6$), that of CD25 1.83±0.42 fold higher ($p=0.014$ $n=4$), that of CTLA-4 3.2±1.1 fold higher ($p=0.001$ $n=5$) and that of GITR 2.24±0.46 fold higher ($p=0.032$ $n=3$). In contrast, CD69 was upregulated to a greater extent in Teff cells than in Tregs (2.0±0.35 fold-higher, $p=0.0001$ $n=4$; at 24h). In addition, while Tregs uniformly co-expressed these molecules (with the exception of CD69), not all $FOXP3^+$ Teff cells were CD25, CTLA-4 or GITR positive (data not shown).

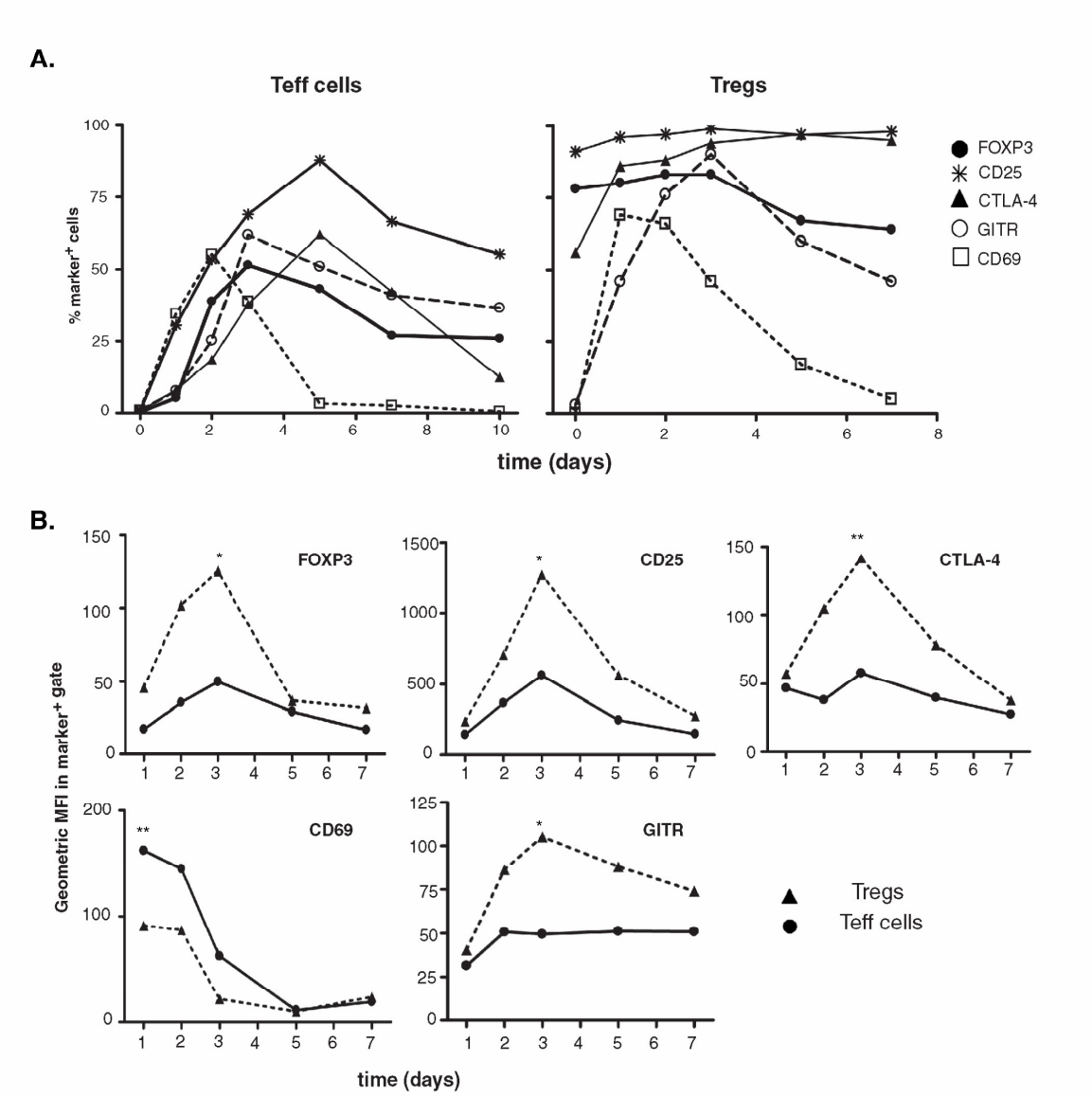

Figure 2.2 FOXP3 is an activation marker in Teff cells. *Ex vivo* Tregs or Teff cells were activated with anti-CD3/28-coated beads and analyzed for expression of (**A**) the percentage of cells positive for indicated molecules or (**B**) the MFI of marker$^+$ fractions over 7 days. Experiments with Tregs were limited to 7d as in the absence of exogenous IL-2 viability was compromised at longer time points. Anti-FOXP3-PE staining was carried out with PCH101 and verified with 236A/E7. Statistical significance was determined by comparing MFI of markers in Teff cells compared to Tregs at indicated time points: *p<0.03, **p<0.001. Plots are representative of at least 3 independent experiments.

Expression of FOXP3 in Teff cells is not dependent on signaling via CD28. It has been shown that co-stimulation via CD28 is necessary for the expression of *Foxp3* in thymocytes and the development of Tregs (17). To investigate the co-stimulatory requirements for activation-induced FOXP3 expression, we stimulated highly purified Teff cells with immobilized anti-CD3 mAbs alone or in the presence of different co-

stimulatory agents (**Fig. 2.3**). As a control, cells were stimulated with anti-CD3/28-coupled beads at a ratio expected to stimulate a moderate amount of FOXP3 expression. After 72h, flow cytometric analysis demonstrated that stimulation via the TCR alone was sufficient to induce a small proportion of Teff cells to upregulate FOXP3, while co-stimulation via CD28, TLR2 (with Pam-3-Cys (18)), or TLR5 (with flagellin (19)) led to an increase in the percentage of $FOXP3^+$ cells (**Fig. 2.3A**). A similar increase was observed in the presence of exogenous IL-2, suggesting co-stimulatory agents may enhance FOXP3 expression due to their capacity to promote autocrine IL-2 production. The proportion of FOXP3-expressing cells correlated with the number of cell divisions in each culture condition, as demonstrated by CFSE dilution after 5 days (**Fig. 2.3B**). Thus, weak stimulatory conditions that promoted minimal proliferation induced a low proportion of cells to express FOXP3, while strong activating conditions promoted significant cell division and FOXP3 upregulation, irrespective of whether CD28 was activated. This observation is in agreement with a report from Gavin *et al*, in which immobilized anti-CD3 alone elicited a small proportion of purified T cells to upregulate FOXP3, and anti-CD28 co-stimulation enhanced this response (12).

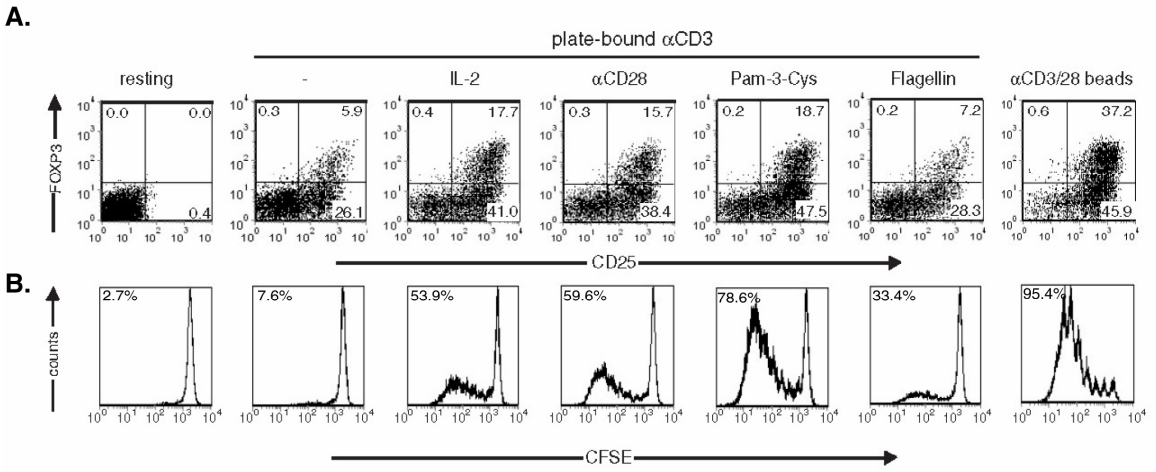

Figure 2.3 Co-stimulatory requirements for activation-induced FOXP3 expression. *Ex vivo* Teff cells were activated for 72h in the presence of 10 μg/ml plate-bound anti-CD3 with or without co-stimulatory molecules (IL-2 100U/ml, anti-CD28 1 μg/ml, Pam-3-Cys 1 μg/ml, Flagellin 100 ng/ml), or with anti-CD3/28-coated beads (16:1 ratio of beads:cells). **(A)** Expression of CD25 and FOXP3 (using 236A/E7, conjugated to PE) was determined after 72h. **(B)** Extent of proliferation was determined by CFSE dilution after 5d. Numbers indicated in (B) indicate % proliferating cells. Results are representative of 3 experiments.

Expression of FOXP3 in Teff cells does not negatively regulate cytokine production. Tregs fail to produce significant amounts of most cytokines, likely due to the ability of FOXP3 to physically interact with and repress transcription from cytokine promoters (7, 20-22). To investigate the possibility that activation-induced FOXP3 might negatively regulate cytokine production in Teff cells, we determined whether induction of FOXP3 was correlated with suppression of IL-2 and IFN-γ. Teff cells were stimulated with anti-CD3/28-coated beads for 72h and then either directly analyzed or restimulated with PMA and Ca^{2+} ionophore to induce maximal cytokine production before analysis of their capacity to produce IL-2 and IFN-γ. As shown in Figure 2.4, irrespective of whether the cells were restimulated, the $FOXP3^+$ Teff cells were the primary producers of IL-2 and IFN-γ. As expected, Treg cells analyzed in parallel produced very low amounts of IL-2 and IFN-γ (data not shown). Thus expression of FOXP3 in Teff cells does not repress production of IL-2 or IFN-γ.

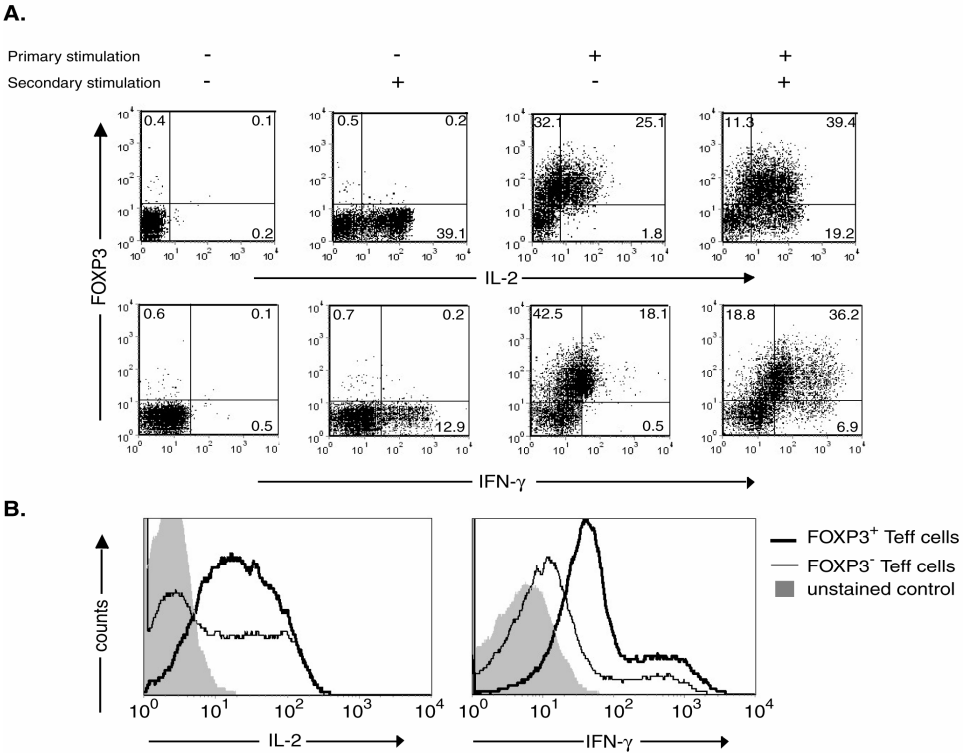

Figure 2.4 $FOXP3^+$ Teff cells produce IL-2 and IFN-γ. **(A)** *Ex vivo* Teff cells were stimulated with anti-CD3/28-coated beads for 72h prior to a secondary 4h stimulation with TPA and Ca^{2+} ionophore. Cells were then stained for IL-2, IFN-γ, and FOXP3 (using 236A/E7, conjugated to APC). Unstimulated cells were analysed in parallel as a control. **(B)** *Ex vivo* Teff cells were exposed to primary and secondary stimulation as in (A) and histogram plots were overlaid by gating on the $FOXP3^+$ or $FOXP3^-$ fractions of Teff cells. Data shown in both (A) and (B) are representative of 4 experiments.

Activation-induced FOXP3 does not suppress CD127 expression. Recently, it was reported that human Tregs can be distinguished from Teff cells on the basis of low CD127 (IL-7Rα) expression (23, 24), possibly due to the capacity of FOXP3 to interact with and repress the CD127 promoter (23). In line with these reports, we found that *ex vivo* Tregs expressed low levels of CD127, and levels remained low over the course of activation (**Fig. 2.5**). Since sorting on the basis of CD25 does not result in a homogeneous population of Tregs (25), both $FOXP3^{hi}$ and $FOXP3^{int}$ cells were observed as early as 24h and at day 7, a small percentage of $FOXP3^{low}CD127^{+}$ T cells was detectable. In contrast to Tregs, expression of CD127 declined immediately after activation of Teff cells, then re-appeared in both $FOXP3^{+}$ and $FOXP3^{-}$ fractions: 33-83% of $FOXP3^{+}$ Teff cells co-expressed CD127 (n=5). These data indicate that activation-induced expression of FOXP3 in Teff cells is not sufficient to uniformly suppress CD127 expression, while high and constitutive FOXP3 expression in Tregs is correlated with their characteristic $CD127^{low/-}$ phenotype. Also of note is the observation that a proportion of early-activated Teff cells are $FOXP3^{+}$, $CD127^{low/-}$, and $CD25^{+}$, and would not be distinguishable from Tregs on the basis of low CD127 expression. Thus, a FACS-sorting strategy based on isolation of $CD127^{low/-}$ cells may not be an infallible method for obtaining populations of pure Tregs constitutively expressing FOXP3, particularly from patients with infection or inflammation (26).

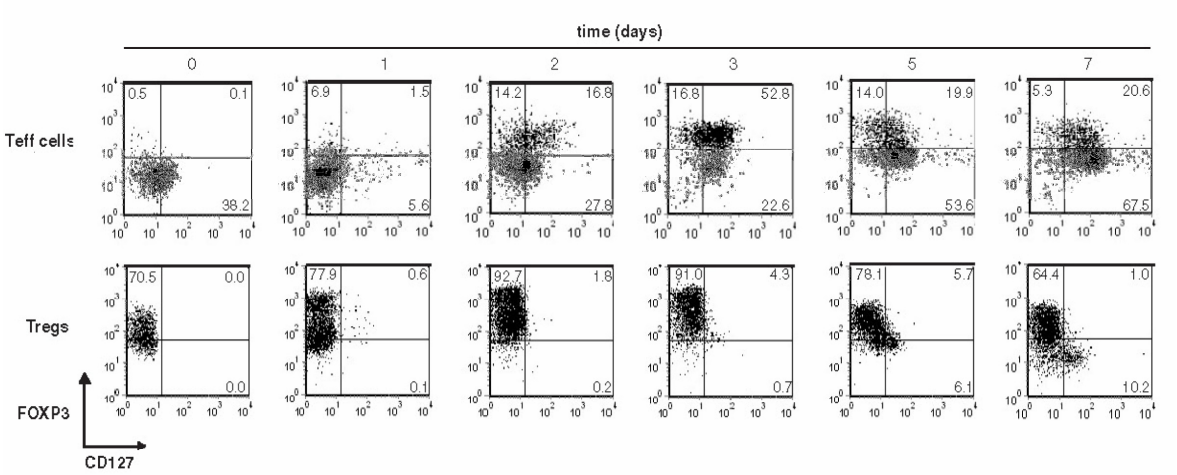

Figure 2.5 Activated Teff cells can co-express FOXP3 and CD127. Purified Teff cells and Tregs were activated with anti-CD3/28-coated beads and analyzed for expression of FOXP3 (using biotin-linked PCH101 and streptavidin-APC) and CD127 at the indicated times. Plots are representative of 4 experiments.

Activation-induced FOXP3 expression is upregulated in dividing but not suppressed cells. Tregs are anergic *in vitro*, and we and others have demonstrated that enforced expression of FOXP3 in human $CD4^+$ T cells induces a hypo-responsive phenotype (7-9). We therefore hypothesized that activation-induced expression of FOXP3 in Teff cells could serve as a negative regulator of T cell activation and proliferation. To address this question, we labeled *ex vivo* Teff cells with CFSE and activated them with anti-CD3/28 coated beads. Interestingly, all proliferating cells upregulated FOXP3, whereas undivided cells comprised a mixture of $FOXP3^+$ and $FOXP3^-$ cells (**Fig. 2.6A**). A similar pattern was observed when expression of CD25 was analyzed. Thus, activation-induced FOXP3 does not prevent progression through the cell cycle and, like CD25, is elevated in all dividing cells. These data also argue against the possibility that the $FOXP3^+$ cells had converted to Tregs since the latter would be expected to be hyporesponsive in the absence of exogenous IL-2.

We further hypothesized that if induction of FOXP3 is a normal consequence of T-cell activation, Tregs should suppress its expression in Teff cells. Alternatively, co-culture with Tregs could amplify the levels of FOXP3 in suppressed cells as a mechanism of infectious tolerance (27, 28). To investigate these possibilities, Teff cells were activated in the absence or presence of Tregs (**Fig. 2.6B**). Activated Teff cells consistently upregulated FOXP3 and CD25 whereas in co-cultures with Tregs, both

proliferation, and expression of these markers, were clearly suppressed. Thus, the anergic state of suppressed Teff cells is not due to induced expression of FOXP3.

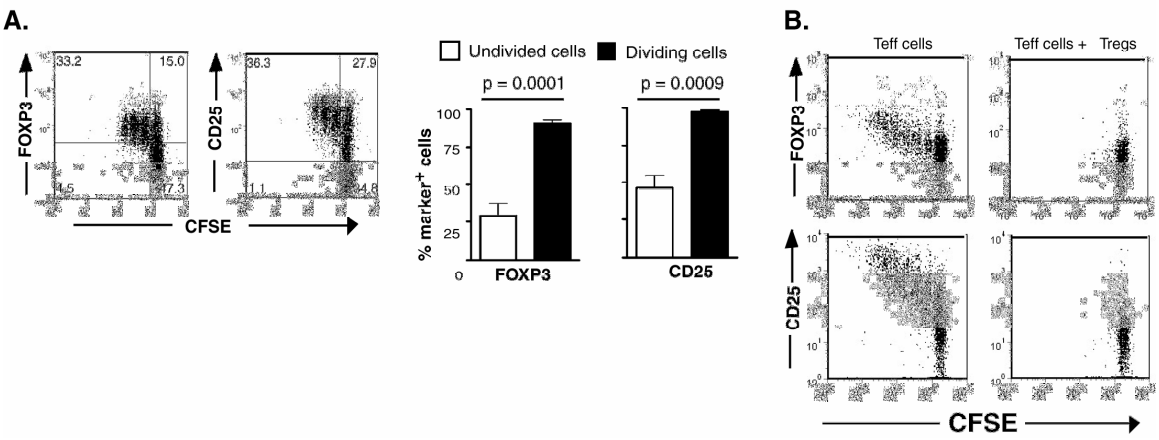

Figure 2.6 All proliferating Teff cells express FOXP3. (**A**) *Ex vivo* Teff cells were CFSE-labeled and levels of CFSE, CD25 and FOXP3 were analysed at 72h. FACs plots are a representative of 6 experiments, which are averaged in graphs shown in middle panels (**B**) *Ex vivo* Teff cells were CFSE-labeled and stimulated with APCs and anti-CD3 in the presence or absence Tregs at a 1:1 ratio. FOXP3 and CD25 expression were analyzed in $CFSE^+$ populations after 96h. FOXP3 staining was carried out using biotin-linked PCH101 and streptavidin-APC. Data are representative of 3 experiments.

Activation-induced expression of FOXP3 in Teff cell lines. From the experiments described above, it appeared that FOXP3 behaved as a classical activation marker in Teff cells, with peak expression levels occurring at the time of maximal activation, and then gradually subsiding upon entry into the resting phase. In order to determine whether this pattern of FOXP3 expression recurred during repeated cycles of activation, we monitored its expression in parallel with other nTreg-markers in Teff cell lines. As shown in Figure 2.7A&B, a significant proportion of cells in the activated Teff cell lines transiently expressed FOXP3 (45.6±16.3% n=3) along with CD25, CTLA-4, and GITR. Similar to our findings with *ex vivo* cells, the intensity of FOXP3 expression and of other nTreg-associated markers (data not shown), in Teff lines was significantly lower than in nTreg lines.

In view of the recent finding that low expression of CD127 may be a more specific cell surface marker for Tregs than CD25, we also investigated whether Teff and nTreg cell lines remained $CD127^+$ and ^{low}, respectively. Upon analysis in the resting phase, nTreg lines displayed significantly lower expression of CD127 (5.1±3.1-fold lower MFI, p=0.036 n=3) than did Teff cell lines, suggesting that the transient FOXP3

expression that occurs in activated Teff cells is not sufficient to repress their CD127 expression in the long term (**Fig. 2.7C**).

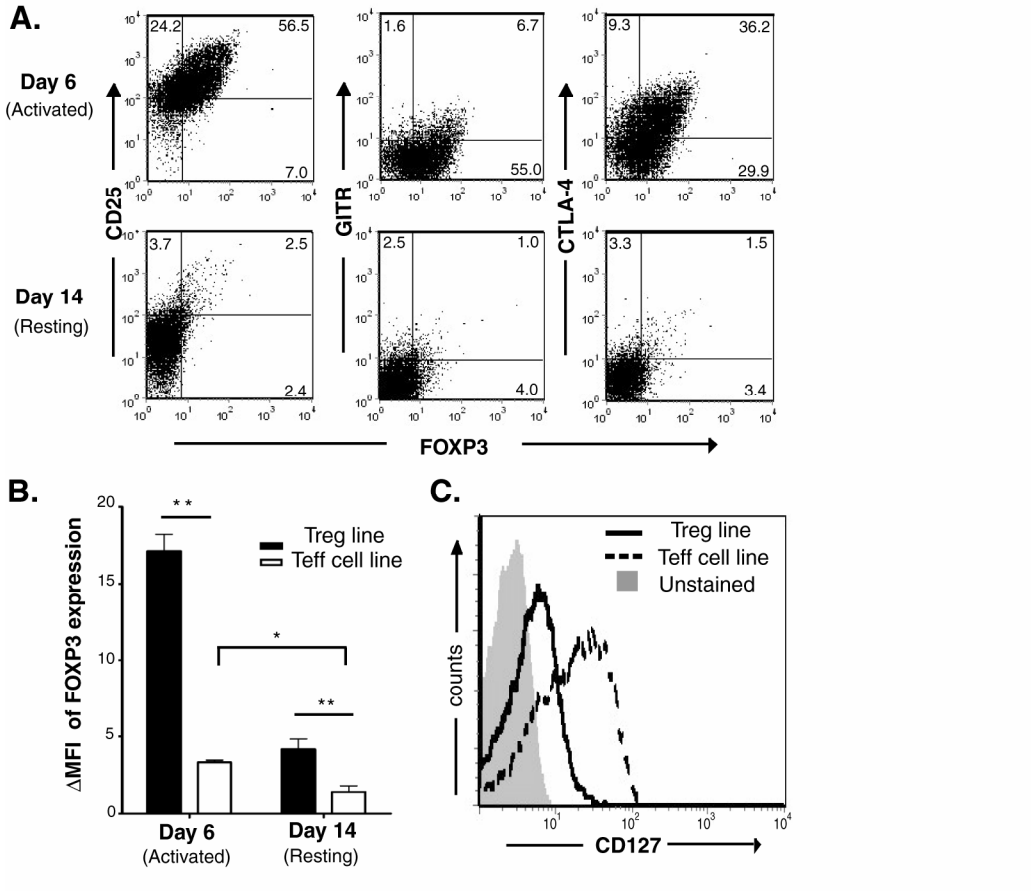

Figure 2.7 Kinetics of expression of Treg-associated markers in Teff cell lines. (**A**) Teff cell lines were analyzed at day 6 (activated) and day 14 (resting) after activation for the indicated markers. (**B**) The average intensity of FOXP3 expression (determined using either PE- or biotin-linked PCH101 and streptavidin-APC) was compared between Teff cell and Treg lines after normalizing to unstained controls for each sample. Statistical significance: *p<0.05, **p<0.01. (**C**) The expression of CD127 was determined in resting nTreg and Teff cell lines. Data are representative of 3 independent cell lines from different donors.

2.3 Discussion

In this report, we investigated the kinetics and expression profile of activation-induced FOXP3 in human Teff cells, and determined its effect on proliferation and cytokine production. We found that in humans, all dividing Teff cells have the potential to become FOXP3 positive, independently of CD28 co-stimulation, and conclude that expression of FOXP3 is a normal consequence of $CD4^+$ T cell activation. Consequently,

simple analysis of FOXP3 expression can no longer be used as a surrogate marker of nTreg cells in humans (12, 15, 16). Rather, like CD25, FOXP3 appears to be strictly associated with the unique phenotype and function of Tregs only when it is expressed constitutively and at high levels. In addition, evidence that expression of FOXP3 is repressed in suppressed Teff cells, indicates that Tregs do not induce anergy or mediate infectious tolerance via induction of this transcription factor. The role of FOXP3 in Tregs appears to be related to its ability to repress a number of different target genes, particularly cytokines (20-23). In order to determine whether activation-induced FOXP3 can repress known transcriptional targets, we investigated the expression of several of these molecules in Teff cells. In contrast to Tregs, we found that $FOXP3^+$ Teff cells produced significantly more IL-2 and IFN-γ compared to $FOXP3^-$ cells in the same culture. Together with our observations that activation-induced FOXP3 was not sufficient to suppress CD127 expression, and that $FOXP3^+$ cells were highly proliferative, these data strongly suggest that the function of FOXP3 in Teff and Tregs may not be equivalent. A differential function may be related to the insufficient and/or unsustained nature of FOXP3 expression in Teff cells. Highly activated Teff cells may also have an imbalance in additional proteins required for suppression of these genes, as FOXP3 exerts its repressor activity in concert with several other co-factors (29).

Some molecules upregulated upon TCR stimulation, such as CD25 and GITR, serve to enhance T cell activation by increasing sensitivity to cytokines or chemokines, or providing co-stimulatory signals (30, 31). In contrast, others, such as CTLA-4, serve to curb T cell activation and dampen the immune response by initiating inhibitory signals within or between cells (32). The kinetics of FOXP3 expression in Teff cells closely resembles those of CTLA-4, as both of these markers are induced later than CD25 and CD69. Interestingly, it has recently been reported that CTLA-4 co-stimulation is required for induction of TGF-β-mediated Foxp3 expression in mouse cells (33). It is therefore possible that CTLA-4 ligation in activated human Teff cell populations contributes to activation-induced FOXP3 expression, and that FOXP3 and CTLA-4 act as co-operative arms in a negative feedback loop that limits T cell expansion.

It has been previously reported that induction of FOXP3 in Teff cells is associated with the development of Tregs and suppression (10, 11). Our data support the conclusion

that the majority of Teff cells that express FOXP3 at the peak of activation do not permanently convert to Tregs. We attempted to directly test whether $FOXP3^+$ Teff cells acquired suppressive capacity, but found that when FOXP3 was maximally expressed (3 days after activation) these experiments were not feasible due to induction of cell death upon reactivation and IL-2 consumption in co-cultures (data not shown). Indirect evidence to support the conclusion that $FOXP3^+$ Teff cells are not Tregs includes the findings that activation-induced expression of FOXP3 neither suppresses cytokine production, CD127 expression nor proliferation. We can speculate, however, that given the appropriate culture conditions, a small subset of these cells may not down-regulate FOXP3 and therefore ultimately develop into functional Tregs. A number of recent reports have described tolerance-promoting agents, such as TGF-β, that can induce suppressor cells from mouse or human Teff cells (34). These exogenous factors may act via enhancing and/or prolonging activation-induced FOXP3 expression, or alternatively, by triggering the assembly of other molecular complexes necessary for suppressor function. Maintenance of sustained FOXP3 expression as T cell activation wanes may be key for acquisition of suppressor function and development of peripherally-induced Tregs.

The role of FOXP3 in Teff cells remains a major outstanding question. Over-expression and molecular studies have clearly shown that viral LTR-driven expression of FOXP3 in human Teff cells limits their ability to proliferate and produce IL-2 and IFN-γ (7-9), suggesting it may have a negative regulatory role. Our data, together with recent evidence from Gavin *et al* (12), suggest, in contrast, that the relatively low levels and transient nature of endogenous FOXP3 expression in activated Teff cells are not sufficient to engender these effects. On the other hand, FOXP3 is also involved in transcriptional activation of genes, and its ability to upregulate CD25 may in fact act to enhance Teff cell activation (20). Thus, it is possible that activation-induced FOXP3 expression may fine tune negative and/or positive aspects of Teff cell proliferation and activation.

Our data from IPEX patients, who, regardless of the site of the FOXP3 mutation, display a defect in the capacity of Teff cells to produce cytokines, further support the concept that the role of FOXP3 is not limited to Tregs (6). In striking contrast to IPEX

patients, Teff cells from mice with mutations in Foxp3 produce higher amounts of inflammatory cytokines than Teff cells from their wild-type littermates (35, 36), indicating this alternate role is likely a species-specific phenomenon. Recent data indicating that FOXP3 prevents the interaction between NFAT and AP1, and co-operates with NFAT to switch T cells into Tregs (20) suggest that the differential role of FOXP3 in Teff versus Treg cells could be related to differential activity/expression of NFAT and/or AP1. Overall, the concept that the exclusive role of FOXP3 is to act as a molecular switch for the development of Tregs needs to be re-examined, and further studies are required to elucidate whether FOXP3 has a positive and/or negative regulatory role in the context of $CD4^+$ T cell activation.

2.4 Materials and methods

Cell purification. Peripheral blood was obtained from healthy volunteers following approval of the protocol by the University of British Columbia Clinical Research Ethics Board and after obtaining written informed consent from individual donors. $CD4^+$ T cells were purified from PBMCs by negative selection (StemCell Technologies Inc.). $CD4^+CD25^+$ Tregs were purified by positive selection over 2 MS columns (purity $\geq$ 90%) and $CD4^+CD25^-$ Teff cells by depletion over LD columns (purity $\geq$ 99%) (Miltenyi Biotec). For generation of T cell lines, the brightest 1% of $CD25^+$ cells and the dimmest 20% of $CD25^-$ cells were FACS-sorted from total $CD4^+$ T cells on a FACS Aria, and expanded *in vitro* as previously described (37). APCs were prepared by depletion of $CD3^+$ cells by positive selection (purity $\geq$ 95%) (StemCell Technologies Inc.).

Activation, proliferation, and suppression experiments. Purified T cells were cultured in X-VIVO15 medium with 5% AB human serum (both Cambrex), 1x Penicillin and Streptomycin, 1x Glutamax (both Invitrogen), in the absence of exogenous IL-2 unless indicated otherwise. For activation and proliferation experiments, purified T cells were plated in 48 or 24 well plates at a density of 1 x 10^6 cells/ml and stimulated with anti-CD3/CD28 coated beads (Invitrogen) in the absence of IL-2. Unless indicated otherwise, a bead to cell ratio of 1:16 was used as based on titration experiments required for each

lot of beads. This ratio provided a moderate stimulation and typically resulted in 25-50% $FOXP3^+$ Teff cells at 72h. Cells were split after 5 days to prevent overgrowth, and every 2 days thereafter. Alternatively, purified T cells were activated with plate-bound anti-CD3 (10 μg/ml OKT3, Ortho Biotech) in the presence or absence of different co-stimulatory molecules: IL-2 (100 U/ml, Chiron); anti-CD28 (1 μg/ml, BD Pharmingen); Pam-3-Cys, (1 μg/ml, Axxora) or *E.coli* flagellin (100 ng/ml, purified as described (19). In some experiments, freshly purified Teff cells were first labeled with 2.5 μM 5- (and 6-) carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes) prior to setting up experiments. For suppression experiments, CFSE labeled Teff cells were activated with irradiated (5000 RADS) autologous APCs at a 1:1 ratio in the presence of anti-CD3 (1 μg/ml OKT3), in the presence or absence of Tregs for 96h.

Western Blotting. A minimum of 3 x 10^6 cells were used to make each T cell lysate. Nuclear and cytoplasmic extracts were enriched using an NE-PER Nuclear and Cytoplasmic Extraction Kit (Pierce Biotechnology Inc.). Protein concentrations were determined using a BCA assay and 7 μg of protein was loaded per lane on 10% SDS-PAGE gels. Nitrocellulose membranes were probed with polyclonal rabbit anti-FOXP3 antiserum (22), followed by goat anti-rabbit–HRP (DakoCytomation). Membranes were stripped and reprobed with anti-TFIIB (Santa Cruz Biotechnology Inc.) to ensure loading equivalency.

Flow cytometric analysis. Staining for cell-surface markers CD69, CD25, CD127 (BD Pharmingen, Mississauga, Canada), and GITR (R&D Systems) was carried out prior to intracellular staining for CTLA-4 (BD Pharmingen) or FOXP3. Staining for FOXP3 was performed with PCH101 or 236A/E7 (eBiosciences) according to manufacturer's instructions. The results with 236A/E7 or PCH101 were equivalent. Based on recommendations from Herzenberg (38) and Roederer (39), gates for FOXP3 positive cells were set based on fluorescence-minus-one controls.

For analysis of intracellular cytokine production, purified T cells were first activated with anti-CD3/28 coated beads (Invitrogen), or left unstimulated for 3 days. Cells were then washed, counted, and left unstimulated or exposed to a secondary

stimulation of PMA (10ng/ml, Sigma) and Ca^{2+} ionophore (500ng/ml, Sigma) for 4 hours, with brefeldin A (10μg/ml, Sigma) added for the final 2 hours. Cells were fixed and stained for intracellular IL-2, IFN-γ (BD Pharmingen) and FOXP3 according to manufacturer protocols for FOXP3 staining. Samples were acquired immediately after staining on a BD FACSCanto and analyzed with FCS Express Pro Software Version 3 (De Novo Software).

Statistics. All analyses for statistically significant differences were performed with 1-tailed paired Student's *t* test. *P* values of less than 0.05 were considered significant.

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3. Retroviral expression of two FOXP3 isoforms in human $CD4^+$ T cells [2]

3.1 Introduction

Immunological homeostasis is maintained by specialized subsets of T cells, known as T regulatory cells. Evidence points to the existence of two types of $CD4^+$ T regulatory cells, those induced by antigen in the periphery (i.e. Tr1 and Th3 cells), and those which arise in the thymus (i.e. $CD4^+CD25^+$ Treg cells [Tregs]) (1-3). Tr1 and Th3 cells typically regulate immune homeostasis via production of cytokines, whereas $CD4^+CD25^+$ Tregs operate via a cytokine-independent mechanism.

Tregs isolated from human peripheral blood are hyporesponsive, and strongly suppress the proliferation of, and cytokine production from, both naive and memory T cells *in vitro* (4-8). Their suppressive activity is related to their ability to inhibit IL-2 production and promote cell-cycle arrest in both $CD4^+$ and $CD8^+$ T cells, via a mechanism that requires direct cell-to-cell contact and remains to be elucidated. Despite their low proliferative capacity, Tregs can be expanded *in vitro* and retain their potent suppressive effects (4, 9, 10). Tregs constitutively express high levels of CD25, CTLA-4 and GITR, and a number of other activation markers (4-8, 11, 12).

Several groups have suggested that there may be a correlation between Tregs and a fatal X-linked autoimmune disorder known as Scurfy in mice and IPEX (immune dysregulation, polyendocrinophathy, enteropathy, X-linked syndrome) in humans (13-16). Analysis of the transcription factor FOXP3, which is mutated in these diseases, demonstrated that mouse Tregs express high levels of *foxp3* mRNA. Importantly, Foxp3 is not induced upon activation of non-suppressive mouse $CD4^+CD25^-$ T cells (14-16). Thus, Foxp3 represents the first truly specific marker for mouse Tregs. Moreover, analysis of genetically-deficient mice shows that Foxp3 appears to be necessary for the generation of suppressive Tregs (16). Remarkably, mouse $CD4^+$ T cells which over-

[2] Material in this chapter has been published: Allan, S.E., Passerini, L., Bacchetta, R., Crellin, N., Dai, M., Orban, P.C., Ziegler, S.F., Roncarolo, M.G., and Levings, M.K. 2005. The role of 2 FOXP3 isoforms in the generation of human CD4+ Tregs. *J Clin Invest* **115**:3276-3284. Permission granted by the Journal of Clinical Investigation.

express Foxp3 following retroviral-mediated gene transfer acquire both the phenotypic and functional characteristics of Tregs (14-16).

The urgent need for homogenous and antigen-specific populations of human Tregs for further biological study, and possibly also clinical applications, prompted us to investigate whether ectopic expression of FOXP3 in human $CD4^+$ T cells acts as a molecular switch to induce Treg cells *in vitro*.

3.2 Results

Expression of FOXP3 in human $CD4^+$ T cells subsets. Recent studies indicating that in the mouse, expression of Foxp3 is restricted to the suppressive Treg subset (15, 16), led us to investigate whether this was also true in human Tregs. As expected, resting human Tregs express significantly higher levels of both FOXP3 mRNA and protein than do $CD4^+CD25^-$ T cells (**Fig. 3.1A**) (17, 18).

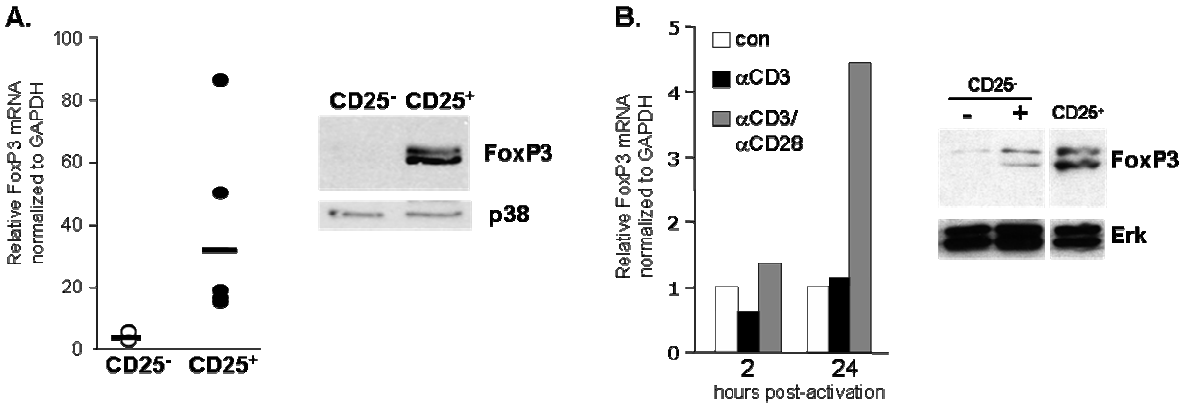

Figure 3.1 Endogenous expression of FOXP3 in human $CD4^+$ T cells. $CD4^+$ T cells were purified and separated into $CD25^-$ and $CD25^+$ cells. (**A**) Expression of FOXP3 was determined by quantitative RT-PCR, with each point representing an individual donor, and Western blotting. (**B**) Highly purified $CD4^+CD25^-$ T cells were activated with immobilized anti-CD3 (1μg/ml) and/or anti-CD28 (1μg/ml) for the indicated times, and assayed for mRNA expression. Levels of FOXP3 protein were determined after 3 days on cells left unstimulated (-) or stimulated (+) with anti-CD3 and anti-CD28. Protein levels were compared to those in *ex vivo* Tregs. Results are representative of 5 experiments.

We also investigated whether expression of FOXP3 could be induced following activation of non-regulatory $CD4^+$ T cells. $CD4^+CD25^-$ T cells which had been highly purified to ensure no $CD25^+$ cells contaminated the cultures, were activated with anti-CD3, with or without anti-CD28 mAbs. After the indicated times, cells were collected

and processed for analysis of FOXP3 mRNA and/or protein levels. *FOXP3* mRNA was consistently induced after 24 hours of activation with anti-CD3 and anti-CD28, and protein was clearly detectable after 3 days (**Fig. 3.1**B). However, the levels of mRNA or protein expression never reached those observed in Tregs. Since Tregs express FOXP3 constitutively at high levels (**Fig. 3.1A**), it seemed unlikely that this induced expression was due to the presence of a pre-existing subset of Tregs within the $CD4^+CD25^-$ T cell population. Further, expression of *FOXP3* in Tregs was found to either remain constant or decrease following activation (data not shown). Therefore, induction of FOXP3 in $CD4^+CD25^-$ T cells could be due to the *de novo* differentiation of Tregs as previously described (17) and/or FOXP3 behaving as an activation marker in human $CD4^+$ T cells as shown by Morgan *et al* (19).

Ectopic expression of FOXP3 in human $CD4^+$ T cells results in T-cell hyporesponsiveness and suppression of cytokines. Given the difficulty of generating homogenous populations of human Tregs (11), ectopic expression of FOXP3 potentially offered a rapid and convenient way to generate functional suppressor cells *in vitro*. Highly purified naive $CD4^+CD25^-CD45RA^+$ T cells were transduced with a control retrovirus (LX) expressing ΔNGFR (nerve growth factor receptor), or a FOXP3- (FP3) encoding retrovirus, co-expressing ΔNGFR as a marker gene (**Fig. 3.2A**). >95% pure populations of transduced cells were obtained by sorting for the ΔNGFR marker with magnetic beads (**Fig. 3.2B**). Note that insertion of a gene of interest in this vector routinely results in lower expression of the marker gene, and the lower mean fluorescence intensity (MFI) of ΔNGFR in the FOXP3-transduced cells does not reflect lower purity or toxic effects. Since existing antibodies did not allow us to reliably detect expression of FOXP3 by flow cytometry in human T cells, we also constructed a retroviral expression vector encoding N-terminally hemagglutinin (HA) tagged FOXP3. Following infection of $CD4^+CD25^-CD45RA^+$ T cells with the HA-FOXP3 encoding virus, flow cytometric analysis demonstrated that 90-95% of $\Delta NGFR^+$ cells co-expressed FOXP3 (**Fig. 3.2C**).

Quantitative RT-PCR revealed that the FOXP3-transduced T cells expressed ~25-fold more FOXP3 mRNA than control (LX) transduced cells (**Fig. 3.2D**). This amount of FOXP3 mRNA is within the lower range of that found in *ex vivo* Tregs (**Fig. 3.1A**), and

compared to the donor in Figure 3.2D is approximately 50% lower. It should be noted that the primers used to quantitate levels of FOXP3 mRNA detect both full length FOXP3 and a naturally occurring second splice isoform (discussed below).

Western blotting confirmed that FOXP3-transduced T cells also over-expressed the protein (**Fig. 3.2E**) at levels comparable to that of *ex vivo* Tregs. Full-length FOXP3 corresponds to the upper band indicated in Figure 3.2E, while the identity of a second, lower band, which is also found in lysates of naturally occurring Tregs, is discussed below. The third band is non-specific, and not present in nuclear fractions. Control LX-transduced T-cells also expressed detectable levels of FOXP3, reflecting the capacity of $CD4^+CD25^-$ T cell populations to upregulate FOXP3 expression following activation and expansion as described in Figure 3.1B.

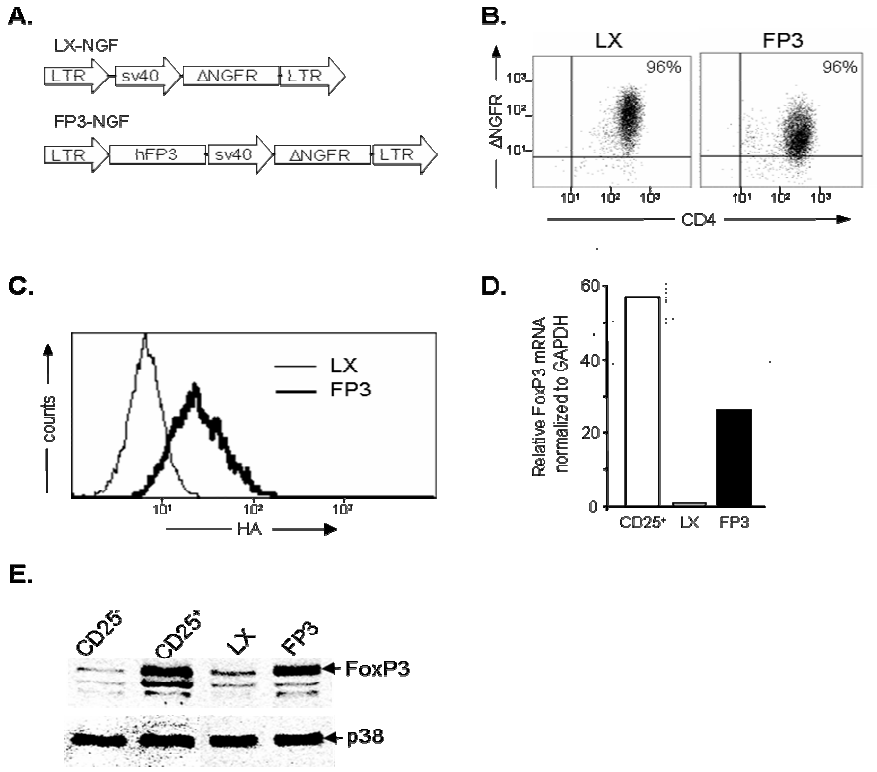

Figure 3.2 Ectopic expression of FOXP3 in human $CD4^+CD25^-CD45RA^+$ T cells. (**A**) Control- (LX) or FOXP3- (FP3) encoding retroviruses expressing ΔNGFR as a marker gene were used for transduction of $CD4^+CD25^-CD45RA^+$ T cells. (**B**) Following purification, transduced T cells were analyzed by flow cytometry. (**C**) In some cases T cells were transduced with HA-tagged FOXP3, and co-expression of ΔNGFR and FOXP3 was monitored by analysis of HA expression in $\Delta NGFR^+$ T cells. (**D**) Quantitative RT-PCR and (**E**) Western Blotting was performed to determine expression levels of FOXP3. *Ex vivo* $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells were included to compare relative levels of expression. Results are representative of at least 8 tests with T cells derived from 3 different donors.

Human Tregs are hyporesponsive and do not produce detectable amounts of most cytokines, including IL-2 and IFN-γ (11). We therefore investigated whether ectopic expression of FOXP3 resulted in a similar phenotype. FOXP3-transduced T cells were hyporesponsive upon TCR-mediated activation (**Fig. 3.3A**). This hyporesponsiveness of FOXP3-transduced T cells, like that of Tregs (4), was reversed by addition of exogenous IL-2 (data not shown).

In addition to being hyporesponsive, FOXP3-transduced T cells had a significantly reduced capacity to produce IL-2 upon activation in comparison to controls ($p \leq 0.006$, n=9) (**Fig. 3.3B**). Moreover, similar to Tregs, they were also impaired in their capacity to produce IFN-γ, although this suppression only bordered on significance since it was found to be variable among donors and less potent than that of IL-2 ($p \leq 0.06$, n=9) (**Fig. 3.3B**).

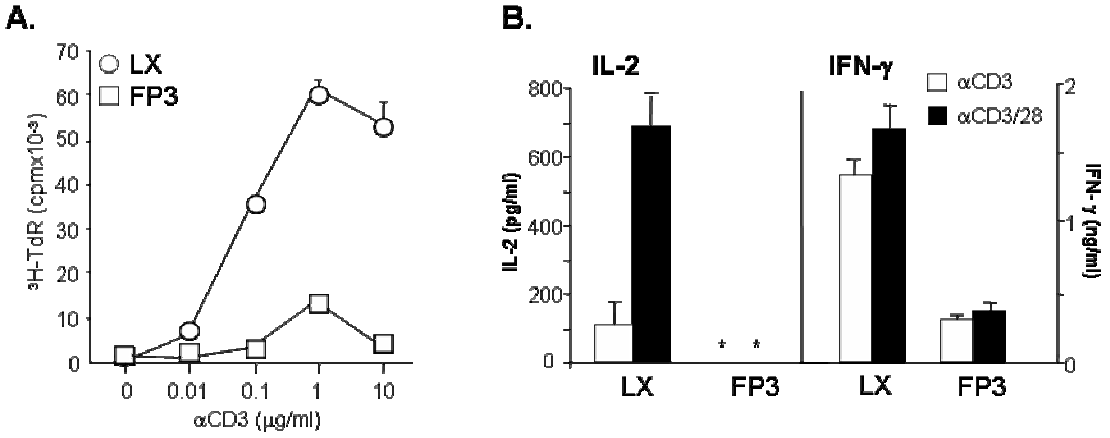

Figure 3.3 Proliferative capacity and cytokine production profile of FOXP3-transduced T cells. (**A**) Transduced T cells were tested for their ability to proliferate in response to increasing concentrations of immobilized anti-CD3. (**B**) T cells were stimulated with anti-CD3 (1μg/ml), with or without anti-CD28 (1μg/ml), for 24 h (IL-2) and 48 h (IFN-γ), * <9 pg/ml. Culture supernatants were analyzed by ELISA. Data are representative of 5 independent experiments for A and 3 for B.

Ectopic expression of FOXP3 in human $CD4^+$ T cells does not result in acquisition of suppressive activity. We next investigated whether ectopic expression of FOXP3 was sufficient to generate a population of cells with suppressive activity *in vitro*. Autologous $CD4^+$ T cells were activated with anti-CD3 and APCs in the absence or presence of control-transduced or FOXP3-transduced $CD4^+$ T cells. FOXP3-transduced $CD4^+$ T cells did not suppress the proliferation of autologous $CD4^+$ T cells when added at a 1:1 or 2:1 ratio (**Fig. 3.4A**). Similar results were obtained in cultures activated with PHA or

alloantigens (data not shown). In contrast, *ex vivo* isolated Tregs potently suppressed proliferation. We also investigated whether the activation conditions used prior to transduction may be important for the development of suppressive T cells. However, parallel experiments involving pre-stimulation in the presence of APCs also failed to induce a population of suppressive T cells upon ectopic expression of FOXP3 (data not shown).

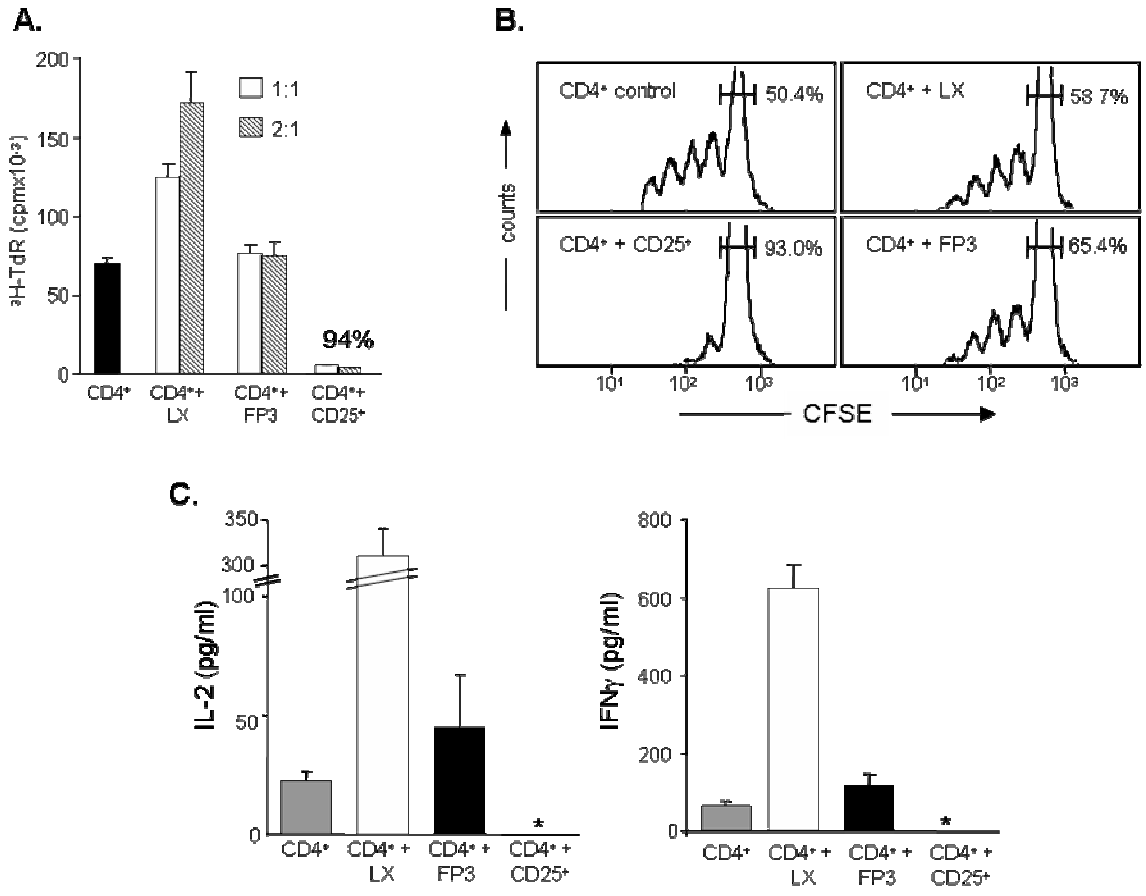

Figure 3.4 Ectopic expression of FOXP3 is not sufficient for suppressive function. (**A**) Autologous $CD4^+$ T cells were stimulated with soluble anti-CD3 (1µg/ml) and APCs, in the presence or absence of control- (LX) or FOXP3-transduced (FP3) T cells, which were generated in the absence or presence of APCs, at a 1:1 or 2:1 (transduced cells:target cells) ratio. *Ex vivo* isolated Tregs ($CD4^+CD25^+$) were added as a positive control. (**B**) In parallel experiments, proliferation of CFSE-labeled autologous $CD4^+$ T cells alone, or in the presence of a 1:1 ratio of control- (LX) or FOXP3-transduced (FP3) T cells, or *ex vivo* Tregs ($CD25^+$), was analyzed by flow cytometry after 96 h. Numbers represent the percentage of undivided cells in the cultures. (**C**) Culture supernatants were collected and analyzed by CBA to determine amounts of IL-2 (after 36 h) or IFN-γ (after 72 h). Results are representative of 4 independent experiments for A, and 3 for B & C * <9 pg/ml.

Interestingly, the net proliferation in co-cultures of $CD4^+$ T cells and FOXP3-transduced T cells was on average ~37% lower ($p \leq 0.013$, n=7) than that in co-cultures with control-transduced T cells. To exclude the possibility that this was due to a combination of suppressed proliferation of the $CD4^+$ T cells and enhanced proliferation of the FOXP3-expressing T cells, we labeled the $CD4^+$ T cells with CFSE and independently assessed their proliferation. Co-culture with control- or FOXP3-transduced T cells did not significantly alter the capacity of the CFSE-labeled $CD4^+$ T cells to divide (**Fig. 3.4B**). In contrast, freshly isolated Tregs potently suppressed their division.

In some cases Tregs may suppress effector functions but not proliferation (20). We therefore tested whether FOXP3-transduced T cells could alter cytokine production by the targets of suppression. As shown in Figure 3.4C, neither control- nor FOXP3-transduced T cells suppressed secretion of IL-2 by $CD4^+$ T cells upon activation with anti-CD3 and APCs. In contrast, freshly isolated Tregs potently suppressed production of IL-2. Similar results were obtained upon analysis of IFN-γ production upon activation with anti-CD3 and APCs (**Fig. 3.4C**). Thus, although FOXP3-transduced $CD4^+$ T cells are hyporesponsive, they do not possess a suppressive activity that is detectable *in vitro*.

Human Tregs co-express two isoforms of FOXP3. During the isolation of the hFOXP3 cDNA by RT-PCR from mRNA from Treg, we noted that several clones encoded a version which completely lacked the second coding exon (FOXP3Δ2) (18, 21). We therefore investigated whether the mRNA for this second, presumably alternatively spliced, isoform was consistently expressed in all donors. As shown in Figure 3.5A, RT-PCR revealed that both isoforms were uniformly expressed in Tregs from all donors tested. Deletion of exon 2 is predicted to result in a ~4 kDa decrease in the MW of FOXP3, offering an explanation for the identity of the second (lower) band detected in lysates from human Tregs (**Fig. 3.1B**).

Exon 2 is in a region of FOXP3 that is relatively uncharacterized and does not contain any known protein domains. We were therefore interested to know whether FOXP3Δ2 acts as a transcriptional repressor protein. Transient transfection assays in Jurkat T cells were performed to assess the relative capacity of FOXP3 and FOXP3Δ2 to suppress activation of the hIL-2 promoter. As expected, co-expression of FOXP3 resulted

in a significant decrease in IL-2 promoter activity following activation with PMA and Ca^{2+} ionophore (**Fig. 3.5B**). The fold decrease in promoter activity, although highly significant, was somewhat less than previously reported (22), possibly because the native hIL-2 promoter was used in the current study rather than a multimer of a nuclear factor of activated T cells (NFAT) regulatory site from the mIL-2 promoter. Importantly, co-expression of FOXP3Δ2 with the IL-2 reporter gene resulted in identical suppression of promoter activity. Thus, the FOXP3Δ2 isoform also appears to be a transcriptional repressor protein.

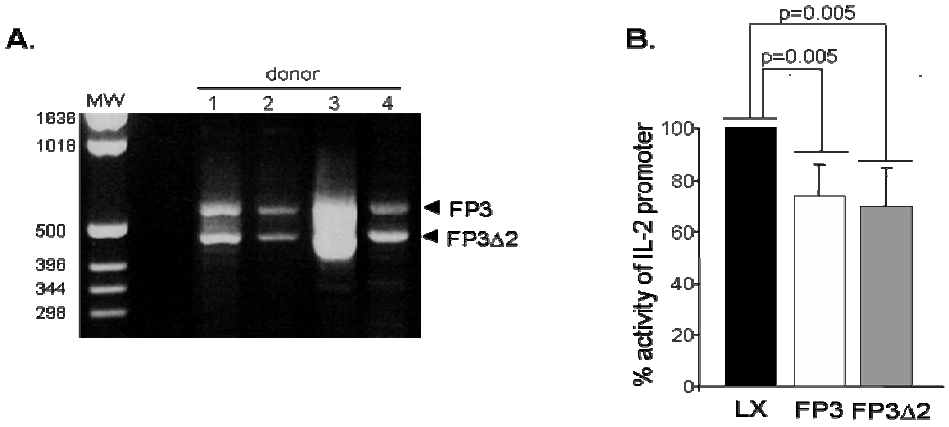

Figure 3.5 Two isoforms of FOXP3 in human Tregs with repressor activity. (**A**) RNA was extracted from purified $CD4^+CD25^+$ cells of normal donors and analyzed by RT-PCR using primers specific for a region of FOXP3 spanning exons 1 and 3. (**B**) Jurkat T cells were co-transfected with a hIL-2-luciferase reporter, β-galactosidase, and either control- (LX), FOXP3- (FP3), or FOXP3Δ2- (FP3Δ2) encoding plasmids. Following stimulation with PMA and Ca^{2+} ionophore, luciferase activity was determined and normalized to amounts of β-galactosidase activity. The graph depicts the % activity of the reporter in comparison to the LX-transfected control. Data represent the average of 5 independent experiments.

Biological effects of ectopic expression of FOXP3Δ2 in the absence or presence of FOXP3. Given our unexpected finding that ectopic expression of FOXP3 in human $CD4^+CD25^-CD45RA^+$ T cells was not sufficient to induce a potent suppressor cell phenotype, we investigated whether co-expression of the FOXP3Δ2 isoform may be required. A new series of retrovirus-based vectors was generated which encoded FOXP3Δ2, and GFP as a marker gene in place of ΔNGFR (**Fig. 3.6A**). $CD4^+CD25^-$ $CD45RA^+$ T cells were then simultaneously transduced with: LX-GFP and LX-ΔNGFR alone (**LX•LX**); LX-GFP and FOXP3-ΔNGFR (**LX•FP3**); FOXP3Δ2-GFP and LX-ΔNGFR (**FP3Δ2•LX**); or FOXP3Δ2-GFP and FOXP3-ΔNGFR (**FP3Δ2•FP3**). 5-6 days

after transduction, double ΔNGFR$^+$GFP$^+$ cells were sorted by flow cytometry to purities >90% (**Fig. 3.6B**). As above, the lower MFIs of ΔNGFR and GFP in the FOXP3 and/or FOXP3Δ2 cells reflect the presence of a second cDNA upstream of the marker genes.

We confirmed that the various populations of transduced cells expressed high levels of FOXP3 mRNA and protein. Figure 3.6C shows that the FP3Δ2•FP3 T cells express ~140-fold higher levels of mRNA compared to controls. These levels are within the higher range of the amounts of FOXP3 mRNA detected in freshly isolated Tregs (**Fig. 3.1A**). Western blotting revealed over-expression of FOXP3 and/or FOXP3Δ2 protein to levels that appear higher than those in Tregs (**Fig. 3.6D**). Further, the MW of the FP3Δ2 isoform appears identical to that of the second band in Tregs, further supporting our hypothesis regarding its identity. As in Figure 3.2E, the LX•LX control T-cell line also expressed detectable levels of both isoforms of FOXP3 due to activation-induced expression (**Fig. 3.6D**).

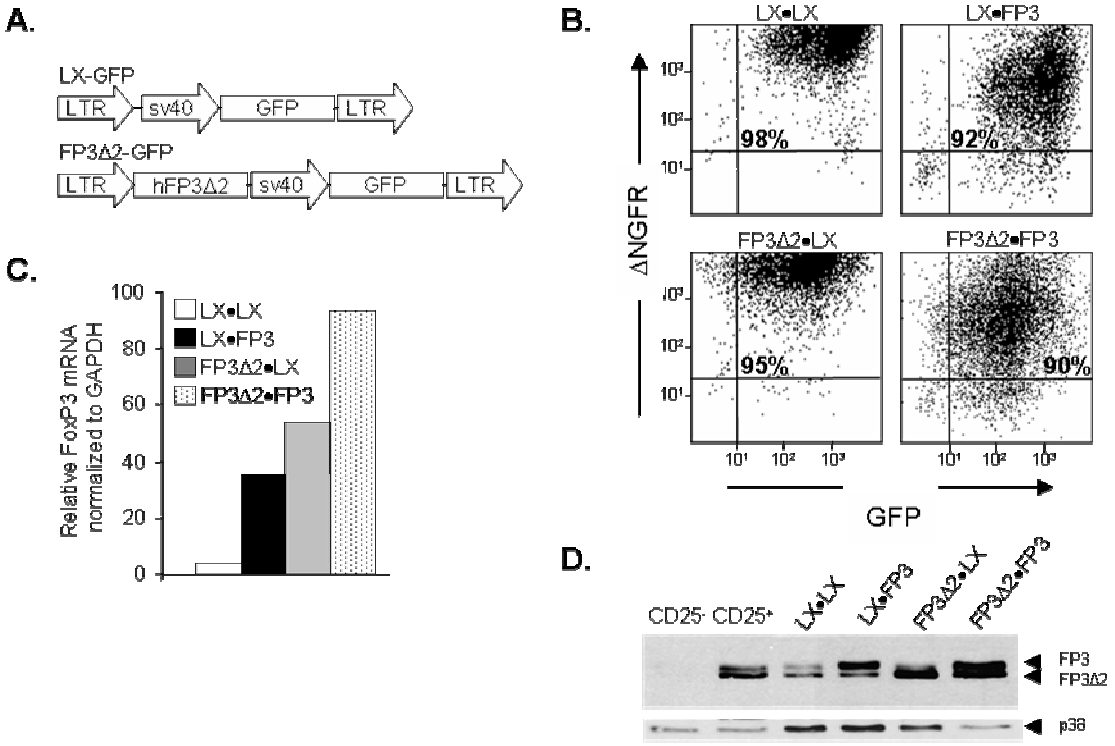

Figure 3.6 Retroviral transduction of CD4$^+$CD25$^-$CD45RA$^+$ T cells with FOXP3Δ2 in the absence or presence of FOXP3. (**A**) Control- (LX) or FOXP3Δ2- (FP3Δ2) encoding retroviral vectors expressing GFP as a marker gene were used in combination with ΔNGFR-expressing retroviral vectors (see Fig 3.2A) for double-transduction of CD4$^+$CD25$^-$CD45RA$^+$ T cells. Following FACs-sorting and expansion, transduced T cells were analyzed by (**B**) flow cytometry, (**C**) quantitative RT-PCR and (**D**) Western Blotting. Results are representative of T cells derived from 3 different donors.

The transduced T-cell lines were next tested to determine their biological phenotype. Although ectopic expression of FOXP3Δ2 also resulted in induction of hyporesponsiveness, the anti-proliferative effect was significantly lower than that in cells over-expressing FOXP3 (**Fig. 3. 7A**). Whereas FOXP3-transduced cells proliferated at a rate of 30±11% compared to controls, FOXP3Δ2-transduced cells proliferated at 64±41% (n=5, 1μg/ml αCD3). In contrast, FP3Δ2•FP3 T cells were found to be profoundly hyporesponsive, and failed to proliferate over the entire range of doses of anti-CD3 tested. A similar pattern was observed upon analysis of IL-2 production. Over-expression of FOXP3Δ2 only moderately reduced IL-2 production in comparison to FOXP3 T cells, whereas FP3Δ2•FP3 T cells displayed a complete block ($p \leq 0.004$ in comparison to LX•LX control, n=7, Figure 3.7B). Further, only when both FOXP3 and FOXP3Δ2 were co-expressed was there consistent and strong suppression of IFN-γ production ($p < 0.009$ compared to control, n=6).

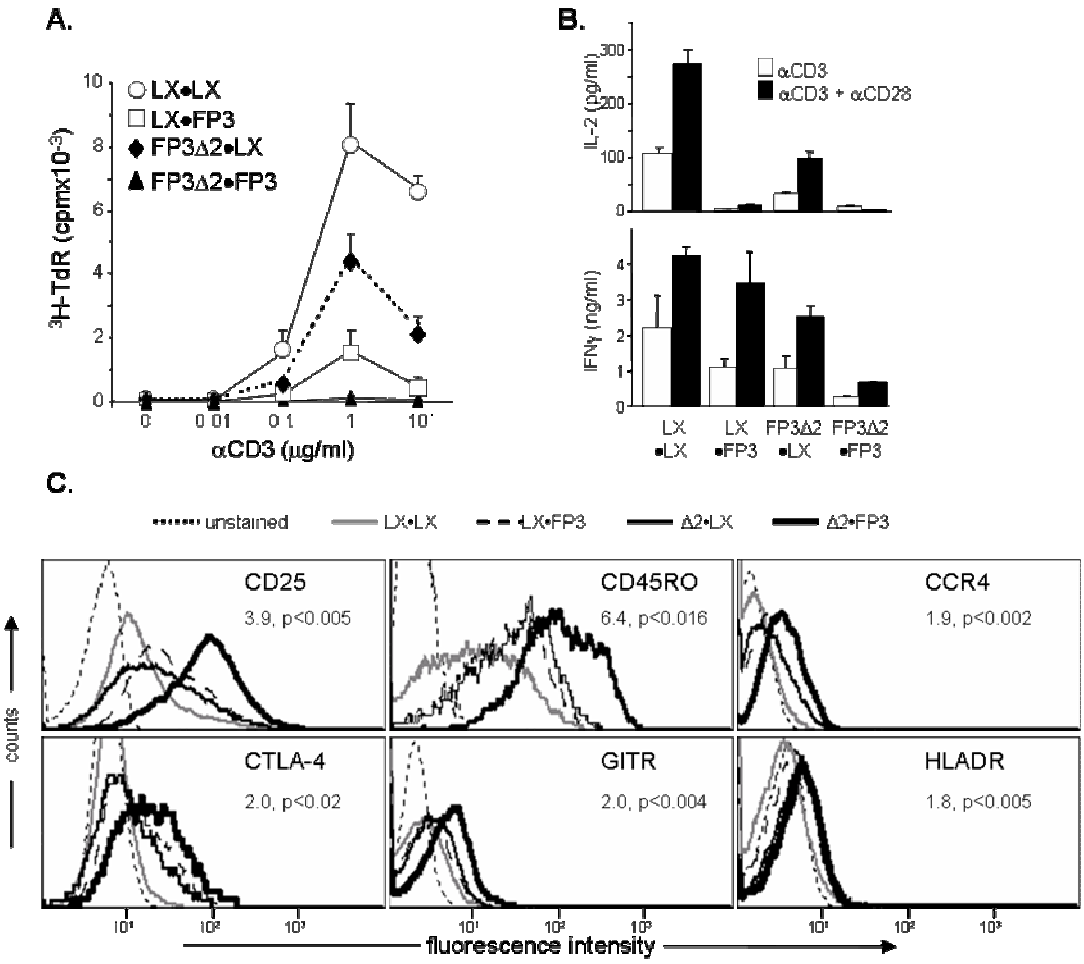

Figure 3.7 Proliferative capacity, cytokine production, and cell-surface phenotype of cells transduced with FOXP3Δ2 in the absence or presence of FOXP3. (**A**) Transduced T cells were tested for their ability to proliferate in response to increasing concentrations of immobilized anti-CD3. (**B**) T cells were stimulated with anti-CD3 (1μg/ml), with or without anti-CD28 (1μg/ml), for 24 h (IL-2) and 48 h (IFN-γ). Culture supernatants were analyzed by ELISA. (**C**) Expression levels of the indicated proteins were determined in the resting phase. Numbers indicate the average fold increase in MFI in the FP3Δ2•FP3 T cells compared to the LX•LX controls. Data are representative of a minimum of 6 independent experiments with T cells derived from 2 different donors.

Finally, we investigated the cell-surface phenotype of double-transduced T-cells in the resting state to determine whether any Treg-associated cell-surface markers were elevated upon over-expression of FOXP3. Although expression of FOXP3 or FOXP3Δ2 alone caused a minor upregulation of some markers, co-expression of both isoforms resulted in a significant elevation of CD25, CD45RO, CCR4, CTLA-4, GITR and HLA-DR (**Fig. 3.7C**). We also investigated CD122 (IL-2Rβ), but found the increase in expression of this protein to be highly variable between experiments (data not shown).

Thus, upon co-expression of FOXP3 and FOXP3Δ2 T cells acquire the *bona fide* cell-surface phenotype of $CD4^+CD25^+$ Treg cells.

Co-expression of FOXP3 and FOXP3Δ2 in human $CD4^+$ T cells results in acquisition of modest suppressive activity. We then tested the transduced T cells for their capacity to suppress T cell responses. Surprisingly, even the FP3Δ2•FP3 T cells failed to consistently and significantly suppress proliferation of $CD4^+$ T cells (**Fig. 3.8A**). We observed suppression at a 1:1 ratio in only 3 out of 7 experiments, and of these 3, the average reduction in proliferation was only 36±10%. In contrast, in all experiments, *ex vivo* Tregs potently suppressed proliferation, on average by 86±7% (n=6).

Finally, we determined whether FP3Δ2•FP3 T cells were capable of suppressing IFN-γ production. Addition of cells over-expressing FOXP3 or FOXP3Δ2 alone did not significantly affect production of IFN-γ by $CD4^+$ T cells. In contrast, a consistent and significant effect was observed in the presence of FP3Δ2•FP3 T cells, and IFN-γ was suppressed in all experiments by an average of 49.5±19%. However, this average suppression of ~50% was less profound than that mediated by highly purified Tregs, which suppressed IFN-γ production by an average of 82±7% (**Fig. 3.8B**).

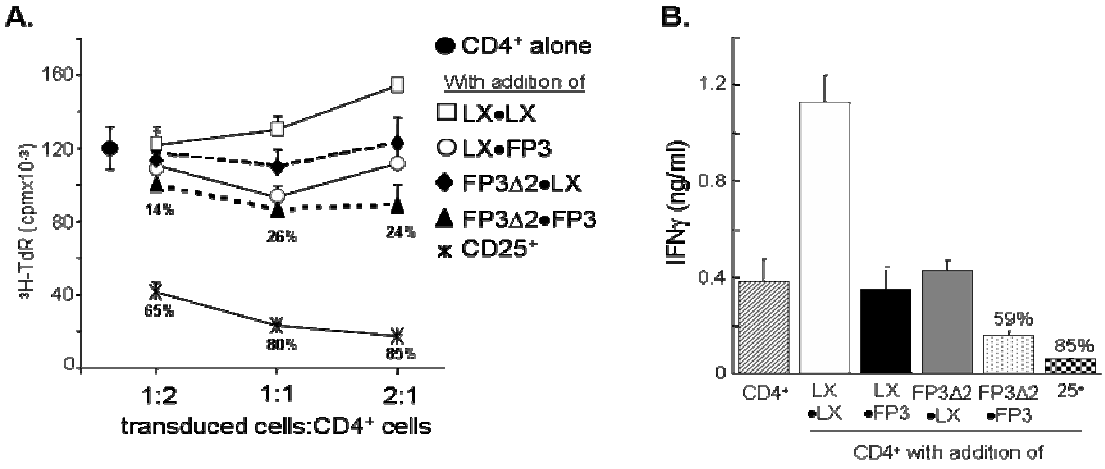

Figure 3.8 Co-expression of FOXP3 and FOXP3Δ2 is not sufficient for acquisition of suppressor activity at levels equivalent to Tregs. (**A**) Autologous $CD4^+$ T cells were stimulated with soluble anti-CD3 (1μg/ml) and APCs, in the presence or absence of double-transduced T cells at increasing transduced cell:target cell ratios. *Ex vivo* isolated Tregs were added as a positive control. (**B**) Culture supernatants were collected and analyzed by CBA to determine amounts of IFN-γ after 72 h. Numbers represent the % suppression compared to $CD4^+$ T cells alone. For A, data are representative of 3 experiments where suppression at a 1:1 ratio was observed (out of a total of 7). B is representative of 4 independent experiments.

3.3 Discussion

We used a retroviral-based over-expression strategy to investigate the capacity of FOXP3 and FOXP3Δ2 to alter the phenotype and function of naive human $CD4^+$ T cells. Although over-expression of FOXP3 alone induced hypo-responsiveness and suppression of cytokine production, it did not result in detectable suppressive capacity *in vitro*. Over-expression of FOXP3Δ2 resulted in a similar phenotype, but with less potent suppression of IL-2 and induction of anergy. Importantly, co-expression of both isoforms of FOXP3, at levels equal to, or greater than, those in Tregs, resulted in profound suppression of IL-2 and IFN-γ production, induction of hyporesponsiveness, and significant upregulation of several Treg-associated markers. However, the suppressive capacity of $FOXP3^+FOXP3\Delta2^+$ T cells was variable and modest in comparison to that of *ex vivo* Tregs. These data, together with the finding that $CD4^+CD25^-$ T cells upregulate FOXP3 following activation, suggest that expression of FOXP3 may be necessary but not sufficient for the generation of *bona fide* human $CD4^+CD25^+$ Treg cells from naive $CD4^+$ T cells *in vitro*.

Unlike mouse Treg cells, we found that human Tregs co-express equal amounts of two isoforms of FOXP3: one corresponding to the canonical full-length sequence; and the other lacking sequence encoded by exon 2 (18, 21). The function of the FOXP3Δ2 isoform remains to be fully defined, but our data indicate that it also possesses transcriptional repressor activity towards the IL-2 promoter (**Fig. 3.5B**). However, the finding that $FOXP3\Delta2^+$ T cells were less hyporesponsive (**Fig. 3.7A**) and produced more IL-2 (**Fig. 3.7B**) than $FOXP3^+$ T cells, suggests that the two isoforms may have distinct functions *in vivo*.

Ectopic expression of FOXP3 and/or FOXP3Δ2 results in T-cell hyporesponsiveness and reduced IL-2 production (**Figs. 3.3&3.7**). The ability of exogenous IL-2 to reverse hyporesponsiveness (data not shown) suggests that defective autocrine production of this cytokine limits proliferative capacity of FOXP3-transduced cells. These findings are consistent with the hypothesis that FOXP3 can directly suppress

IL-2 transcription (**Fig. 3.5B**) by interfering with NFAT activity (22), and support recent findings by Yagi *et al* (18).

Importantly, co-expression of both isoforms of FOXP3 resulted in significantly increased suppression of cytokines and proliferation, and upregulation of several Treg-associated markers to levels comparable to those found on *ex vivo* Tregs (**Fig. 3.7C**) (4, 11, 12). However, since a functional role has not been defined for any of these cell-surface molecules, their increased expression is not necessarily indicative of suppressive capacity. For example, although high CD25 expression is correlated with suppressor activity, it is not sufficient (11). Nevertheless, the fact that co-expression of FOXP3 and FOXP3Δ2 results in the most significant changes in T-cell phenotype suggests that the two isoforms may work in concert at the molecular level to influence transcription of these Treg-associated genes.

We were able to detect regulatory activity in the $FOXP3^{+}FOXP3\Delta2^{+}$ transduced T cells, but their suppressive capacity, as measured by reduction in proliferation, was variable and when present was on average 36% at a 1:1 ratio (**Fig. 3.8A**). When measured by reduction of IFN-γ production, addition of a 1:1 ratio of $FOXP3^{+}FOXP3\Delta2^{+}$ transduced T cells resulted in an average of 50% suppression (**Fig. 3.8B**). Although Yagi *et al*. did not report statistical analyses for their suppression assays, they found that addition of a 1:1 ratio of single-transduced $FOXP3^{+}$ T cells reduced proliferation by ~60% (18). In our hands, only when both isoforms of FOXP3 were over-expressed was a similar suppressive capacity induced. However, this 50-60% suppression is less profound than that mediated by pure populations of *ex vivo* Tregs, which consistently suppress proliferation by an average of 86% and production of IFN-γ by an average of 82% at a 1:1 ratio. Such moderately potent Treg populations would likely not be suitable for cellular therapy. Thus, although it seems likely that FOXP3 is involved in the development of human Tregs, it does not appear to be the sole master switch that can transform human $CD4^{+}$ T cells *in vitro* into suppressor cells which are functionally equivalent to Tregs.

The fact that over-expression of FOXP3 alone was not sufficient to induce significant upregulation of Treg-cell associated-cell-surface markers or detectable suppressive activity was surprising in view of several reports from mouse models (14-16)

and two studies carried out with human cells (18, 23). We considered the possibility that we simply failed to express sufficient FOXP3 to achieve the predicted biological effects. However, Yagi *et al* reported that over-expression of FOXP3 mRNA at levels 70% *lower* than those found in Tregs (protein levels were not directly compared) was sufficient to induce a suppressive phenotype (18). In our system, quantitative RT-PCR and Western Blotting revealed that FOXP3-transduced T cells expressed full-length FOXP3 at levels in the range of those detected in Tregs (**Figs. 3.2D & E, 3.6C & D**) and which appear to be higher than those reported in the study of Yagi *et al*. Moreover, expression levels of ΔNGFR and cell purities seem to be comparable in the two studies. These data, along with our demonstration that 90-95% of ΔNGFR$^+$ T cells co-express FOXP3 (**Fig. 3.2C**), lead us to conclude that the discrepancy between our findings and those of Yagi *et al* (18) and Oswald-Richter *et al* (23) are not the result of insufficient cell purity or expression of FOXP3. Rather, we believe that the overall conclusion from these 3 studies is that although ectopic expression of FOXP3, with or without FOXP3Δ2, results in acquisition of a certain degree of suppressor activity, these cells do not fully recapitulate the function of Tregs, at least *in vitro*. Nevertheless, it remains possible that *in vivo* these cells may be potently suppressive. Evidence that the *in vitro* and *in vivo* phenotype and/or function of Tregs may not be strictly correlated includes the finding that despite their hyporesponsive state *in vitro*, they do proliferate *in vivo*, and a number of discrepancies related to mechanistic dependence on cytokines (24). In addition, in the context of transplantation, Tregs do not detectably alter proliferation or cytokine production *ex vivo*, but clearly induce functional tolerance *in vivo* (25, 26).

In view of recent reports highlighting the importance of costimulatory molecules in Treg cell development (27), we also investigated whether APCs may be required during the Treg cell development process. We therefore performed parallel retroviral transductions in the presence of autologous APCs (18), but found that despite induction of hyporesponsiveness and suppression of cytokine production the resulting FOXP3$^+$ T cells did not acquire significant suppressive activity (data not shown). In addition, the transduction method described by Oswald-Richter *et al*. involved pre-stimulation in the absence of APCs, and regulatory activity in the resulting population was reported (23). However, the function of the transduced cells in the latter study may have been

influenced by expansion with staphylococcal enterotoxin B, an agent that is known to promote the development of suppressive T cells (28, 29). Thus, the presence of APCs during ectopic FOXP3 expression in $CD4^+CD25^-CD45RA^+$ T cells does not appear to be critical for induction of Tregs.

It is possible that peripheral blood $CD4^+$ T cells that are susceptible to retroviral infection are refractory to the full Treg-cell inducing effects of FOXP3. It is also possible that naive $CD4^+$ T cells from adult peripheral blood are in a state of differentiation that prevents them from becoming fully functional upon over-expression of FOXP3 and/or FOXP3Δ2. Alternatively, we favor the hypothesis that activation of human $CD4^+$ T cells prior to transduction, which results in up-regulation of both isoforms of endogenous FOXP3 [**Fig. 3.1**, (17)], may lead to changes in gene expression that modulate the function of FOXP3 to ensure that the majority of cells retain their effector function. Indeed, data suggesting that, in contrast to mouse cells (14-16), FOXP3 is an activation marker in human $CD4^+$ T cells and T cell clones [**Fig. 3.1**, (19, 30)], imply this transcription factor may be subjected to differential regulation in the two species.

Notably, $CD4^+CD25^-$ T cells isolated from *Foxp3* transgenic mice show significantly increased expression of GITR, yet display minimal regulatory activity compared to wild-type Tregs (14). Moreover, Tregs from *Foxp3* transgenic mice are actually less suppressive than their wild-type counterparts on a per cell basis (14). In addition, normal expression of FOXP3 was found in functionally defective Tregs from patients with Autoimmune Polyglandular Syndrome Type II (31). We and others have also found that activated non-suppressive T-cell clones express significant amounts of FOXP3 [our unpublished data and (30)]. Finally, HIV-infected patients who were treated with IL-2 develop a population of cells that express high levels of FOXP3 but exert a weak suppressive function (32). These findings strongly support our interpretation that factors in addition to FOXP3 are required during the process of activation and/or differentiation for the development of *bona fide* Tregs, as also suggested by Fontenot & Rudensky (33).

In conclusion, evidence that human Tregs express high levels of FOXP3 (**Fig. 3.1**) (17, 18), and that mutation of FOXP3 results in the development of severe autoimmunity (13), clearly indicates that this transcription factor has a key role in

immune homeostasis. Analysis of $CD4^+CD25^+$ T cells from patients with IPEX will be important to define the role of FOXP3 in human Treg development and function. Our parallel study of two IPEX patients revealed that functional Tregs *are* present in these children (R. Bacchetta *et al*, submitted), further supporting the conclusion that the role of FOXP3 in mice and humans may not be identical. Characterization of other proteins that interact with FOXP3, and the genes it regulates, will be necessary to understand its true biological function. While ectopic expression of FOXP3 alone, or together with FOXP3Δ2, may not be an effective means of generating large numbers of human Tregs for cellular therapy, this protein is clearly a link in the regulatory circuit which controls immunological tolerance.

3.4 Materials and methods

Cell purification. Peripheral blood was obtained from healthy volunteers following approval of the protocol by the UBC Clinical Research Ethics Board. $CD4^+$ T cells were purified by negative selection from PBMCs with magnetic beads (Miltenyi Biotec). $CD25^+$ cells were purified by positive selection over 2 columns (Miltenyi Biotec) to ensure 85-90% purity. To obtain pure (> 95%) $CD4^+CD25^-$ T cells, the $CD25^-$ fraction was either passed over an LD depletion column (Miltenyi Biotec), or FACS sorted. For isolation of $CD4^+CD25^-CD45RA^+$ T cells, $CD4^+CD25^-$ T cells were incubated with CD45RO beads (Miltenyi Biotech) and passed over an LD depletion column. Purity of $CD4^+CD25^-CD45RA^+$ cells was >95%.

Isolation of hFOXP3 and RT-PCR. hFOXP3 and hFOXP3Δ2 were amplified from cDNA using 5'-GCC CTT GGA CAA GGA CCC GAT G-3' as the sense and 5'-TCA GGG GCC AGG TGT AGG GTT G-3' as the antisense primers. The integrity of the cloned cDNAs was verified by DNA sequencing. For non-quantitative RT-PCR to assess expression of *FOXP3* and *FOXP3Δ2*, the above sense oligo together with the 5'- CAT TTG CCA GCA GTG GGT AGG A-3' antisense oligos were used. For quantitative RT-PCR, amounts of FOXP3 and GAPDH mRNA were determined using Assay on Demand

real-time PCR kits (Applied Biosystems) with TaqMan Master Mix (QIAGEN). Samples were run in triplicate, and relative expression of FOXP3 was determined by normalizing to GAPDH expression in order to calculate a fold-change in value.

Western Blotting. Whole cell lysates were prepared by sonication in lysis buffer containing 1% SDS, 10mM HEPES, and 2mM EDTA (pH 7.4). Alternatively, nuclear lysates were prepared using a NE-PER Nuclear and Cytoplasmic Extraction Kit (Pierce Biotechnology). 10μg of protein was loaded/lane, and membranes were probed with polyclonal rabbit anti-FOXP3 antiserum (22), followed by goat-anti-rabbit-HRP (Dako Cytomation). Membranes were stripped and re-probed with anti-p38 (Santa Cruz Biotechnology, Inc.) or anti-ERK Abs (Cell Signaling Technology, Inc.) to assess loading equivalency.

Retroviral constructs and transduction of $CD4^+$ T-cells. The Moloney mouse leukemia virus-based vector encoding hFOXP3, hFOXP3Δ2, or HA-tagged hFOXP3 amino terminus under control of the LTR, and the truncated low affinity nerve growth factor receptor (ΔNGFR) or GFP under control of the SV40 promoter was generated from the LXSΔN (11) or LXSG vector (a kind gift of C. Traversari, Hospital San Raffaele, Milan, Italy). For production of retrovirus, stable AM12 cell lines expressing retroviral vectors were established and cloned on the basis of high viral titer and expression of both transgenes. Viral supernatants were collected, concentrated using Amicon centrifugation filters (Millipore), and titers of 2 x 10^9/ml were routinely confirmed by infection of 3T3 cells. $CD4^+CD25^-CD45RA^+$ T cells were activated for 48 h with immobilized anti-CD3 (1μg/ml OKT3, Orthoclone), soluble anti-CD28 (1μg/ml, BD Pharmingen), and rhIL-2 (100 U/ml, Chiron) in complete medium (X-VIVO 15, [Cambrex], supplemented with 5% pooled human serum [Cambrex], and penicillin/streptomycin [Invitrogen]). Alternatively, T cells were prestimulated for 48h with a 1:1 ratio of autologous APCs (CD3-depleted PBMCs, irradiated 6000 Rads), soluble anti-CD3 (1μg/ml), and IL-2. Two successive rounds of infection were performed on retronectin-coated plates using 20 virus particles per cell and 8μg/ml polybrene (Sigma) (11). $\Delta NGFR^+$ transduced T cells

(routinely 20-40%) were purified with anti-NGFR magnetic beads following the manufacturer's instructions (Miltenyi Biotec).

For double-transduction experiments, an MOI of 10 for each virus was used for simultaneous infection of activated $CD4^+CD25^-CD45RA^+$ T cells. The efficiency of double-transduction was ~5% and the $\Delta NGFR^+/GFP^+$ T cells were sorted on a BD FACS Aria™ six days following transduction. Miltenyi- or FACs-sorted cells were either used for biological experiments 10-14 days after activation, or re-stimulated and expanded under conditions that preserve Treg activity (4, 11). Untransduced T cells were tested in parallel to ensure the transduction and purification process itself did not alter the phenotype or function (data not shown). Results from untransduced or transduced T cells tested upon entry into the resting phase immediately post-transduction, or following reactivation and expansion, were identical.

Transient transfection reporter assays. Jurkat cells were co-transfected using Lipofectamine 2000 (Invitrogen) with a hIL-2 promoter (-305 to +39) luciferase reporter plasmid (a kind gift of Dan MuellerUniversity of Minnesota Medical School, Minneapolis, USA) and control- (LX), FOXP3-, or FOXP3Δ2- encoding plasmids. A plasmid encoding β-galactosidase under control of an EF1α promoter was also included to normalize for transfection efficiency. After overnight culture, cells were left unstimulated or stimulated with PMA (50ng/ml) and Ca^{2+} ionophore (1μg/ml) for 6 h. Cells were harvested and lysates prepared in 1x reporter lysis buffer (Promega). Luciferase activity was measured using the luciferase assay system (Promega) and the EG&G Berthold Lumat Luminometer. The β-galactosidase activity of each sample was determined using the β-galactosidase enzyme assay system (Promega) and was used to normalize the measured luciferase acivity.

Flow Cytometric Analysis. Analysis of CD25 (Miltenyi Biotec), HLA-DR, CD122, CTLA4, CCR4 (BD Pharmingen), GITR (R&D Systems), CD45RO (Caltag Laboratories) expression was performed on resting (at least 12 days post activation) T-cell lines. Intracellular CLTA-4 staining was performed as previously described (11). For detection of intra-nuclear expression of HA-FOXP3, cells were stained with biotinylated

anti-NGF mAb (ATCC), fixed with 2% formaldehyde at -20°c, permeabilized with 90% MeOH on ice for 30 min, and subsequently incubated with streptavidin-coupled PC5 (Beckman Coulter, Inc.) and FITC-coupled anti-HA (Roche Applied Science, 3F10). Samples were acquired using a BD FACS Canto™ or Calibur™ instrument and analyzed using FCS Express Pro Software Version 3 (De Novo Software).

Determination of cytokine concentration. To determine amounts of IL-2 and IFN-γ, capture ELISAs (BD Pharmingen) were performed on supernatants after activation with immobilized anti-CD3 (1μg/ml), with or without anti-CD28 (1μg/ml), for 24 h (for IL-2) or 48 h (for IFN-γ). To determine cytokine concentrations in suppression assays, Th1/Th2 CBA assays (BD Pharmingen) were used to assay culture supernatants after 36 h (for IL-2) or 72h (for IFN-γ).

Proliferation and Suppression of T cells. Transduced T cells were plated at 50,000 cells/well in 96 well plates coated with anti-CD3 in the presence or absence of IL-2 (100U/ml). Proliferation was assessed after 72 h, after addition of [^{3}H]thymidine (1μCi per well, Amersham Biosciences) for the final 16h. To test for suppressive capacity, autologous $CD4^+$ T cells (50,000 cells/well) were stimulated with either soluble anti-CD3 mAb (1μg/ml) in the presence of APCs (CD3-depleted PBMCs, irradiated 5000 Rads, 50,000 cells/well). Transduced T cells were added, and suppression was assessed by determining [^{3}H]-thymidine incorporation, and measuring the amounts of IL-2 and/or IFN-γ present in supernatants. *Ex vivo* Tregs were included as a positive control in all experiments. In some cases, autologous $CD4^+$ T cells were labeled with CFSE (2.5μM, Molecular Probes) and cultured in the absence or presence of the transduced T cells at a 1:1 ratio of freshly isolated Tregs. After 96 hours, the amount of CFSE-dye dilution was analyzed by gating on $CD4^+$, $7AAD^-$ and $\Delta NGFR^-$ T cells.

Statistical Analysis. All analysis for statistically significant differences was performed with the student's one-tailed paired *t* test. *p* values less than 0.05 were considered significant. All cultures were performed in triplicate and error bars represent the SD.

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4. Generation of potent and stable human $CD4^+$ T regulatory cells by activation-independent expression of FOXP3[3]

4.1 Introduction

$CD4^+CD25^+$ T regulatory cells (Tregs) have a central role in establishing and maintaining immune tolerance. Since defects in these cells are known to underlie autoimmunity, allergy and graft rejection (1-3), it is thought that enhancing the number and/or function of Tregs might be an ideal route to the therapeutic restoration of tolerance to defined antigens *in vivo* (4, 5). The development of methods that rely on the use of Tregs as cellular therapy has been limited, at the technical level, by difficulties in obtaining large numbers of homogeneous populations *in vitro*, and at the theoretical level, by our incomplete understanding of their development and function.

Several strategies for obtaining human Tregs suitable for cell therapy have been explored. We and others have shown that polyclonal or alloantigen-specific Tregs sorted on the basis of high CD25 expression can be expanded *in vitro* in the presence of exogenous IL-2 and strong T cell receptor (TCR)-mediated activation (6-9). Although these methods result in a population of cells which are potently suppressive in the short-term, non-suppressive T effector (Teff) cells outgrow suppressive cells in the long-term (10, 11). Several groups have also attempted to improve sorting strategies based on differential expression of other markers such as CD127, CD45RA, or HLA-DR (10-13). Although these methods may result in more homogeneous populations, they require large numbers of cells for initial sorting and significantly reduce the number of Tregs that can be isolated, requiring subsequent expansion of several-thousand fold in order to generate numbers sufficient for therapy.

Another way to generate large numbers of regulatory cells involves conversion of conventional Teff cells into "inducible" Tregs (iTregs). In humans, TCR-mediated stimulation *in vitro* can lead to the development of a subpopulation of suppressive iTregs (14), a process which is greatly enhanced by the addition of exogenous TGF-β (15-17) or

[3] Material in this chapter has been published: Allan, S.E., Alstad N.A., Merindol, N., Crellin, N.K., Amendola, M., Bacchetta, R., Naldini, L., Roncarolo, M.G., Soudeyns, H., and Levings, M.K. 2007. Generation of Potent and Stable Human T Regulatory Cells by Activation-Independent Expression of FOXP3. *Mol Therapy* **1**:194-202. Permission granted by Nature Publishing Group.

rapamycin (18). Although iTregs resemble *ex vivo* Tregs phenotypically and functionally, the stability of their suppressive capacity is not well-established, and the possibility that they may re-convert to Teff cells remains. Finally, methods based on over-expression of the FOXP3 transcription factor have also been developed. In mice, expression of FoxP3 is necessary and sufficient for the development of Tregs, and retrovirus-mediated over-expression of FoxP3 generates regulatory cells which ameliorate autoimmune disease (19). In human cells, however, the expression patterns and function of FOXP3 are more complex (19), and retrovirus-mediated over-expression of FOXP3 does not consistently result in the generation of potent suppressive T cells *in vitro* (20-22).

Given the limitations discussed above, we sought to define why retroviral over-expression of FOXP3 in human T cells did not lead to robust development of Tregs, and to develop a more efficient protocol for generating a homogeneous population of Tregs *in vitro*. Here we report that lentivirus-mediated expression of FOXP3 under control of the human elongation factor (EF)1α promoter can efficiently convert Teff cells to functional Tregs. Comparison with previously-described retrovirus-based expression systems revealed that Treg conversion requires that expression levels of FOXP3 do not fluctuate with the state of T cell activation. Furthermore, using this method, naïve and memory $CD4^+$ T cells acquire suppressor activity upon FOXP3 transduction. These findings establish for the first time a straight-forward and reliable method for generating large numbers of suppressive $CD4^+$ Treg cells.

4.2 Results

Retroviral LTR-driven, but not EF1α-driven, FOXP3 expression fluctuates depending on the state of T cell activation. We previously demonstrated that although expression of FOXP3 under control of the Moloney Mouse leukemia retroviral LTR caused cell-autonomous blockage of proliferation and cytokine production, it did not consistently result in the differentiation of potent suppressive T cells (20). We hypothesized that this may be because the LTR-driven expression of FOXP3 is influenced by T cell activation (23-26), and may not mimic the pattern of endogenous

FOXP3 expression in human Tregs. Moreover, recent data demonstrating that, in humans, FOXP3 can also be expressed transiently in non-suppressive Teff cells (27-31), suggested that the conditions in which FOXP3 expression would bring about full Treg conversion might be more stringent in humans than in mice. In order to test this possibility, we developed a new bi-directional lentiviral vector (32) in which expression of FOXP3 was placed under control of the EF1α promoter and ΔNGFR was included as a cell-surface marker for tracking and sorting of transduced cells (**Fig. 4.1A**). ΔNGFR was chosen as a marker gene since it is known to be safe and non-immunogenic in humans (33). The pCCL.FP3 vector drives high, co-ordinate FOXP3 and ΔNGFR expression in stably transduced primary human $CD4^+$ T cells (**Fig. 4.2A**).

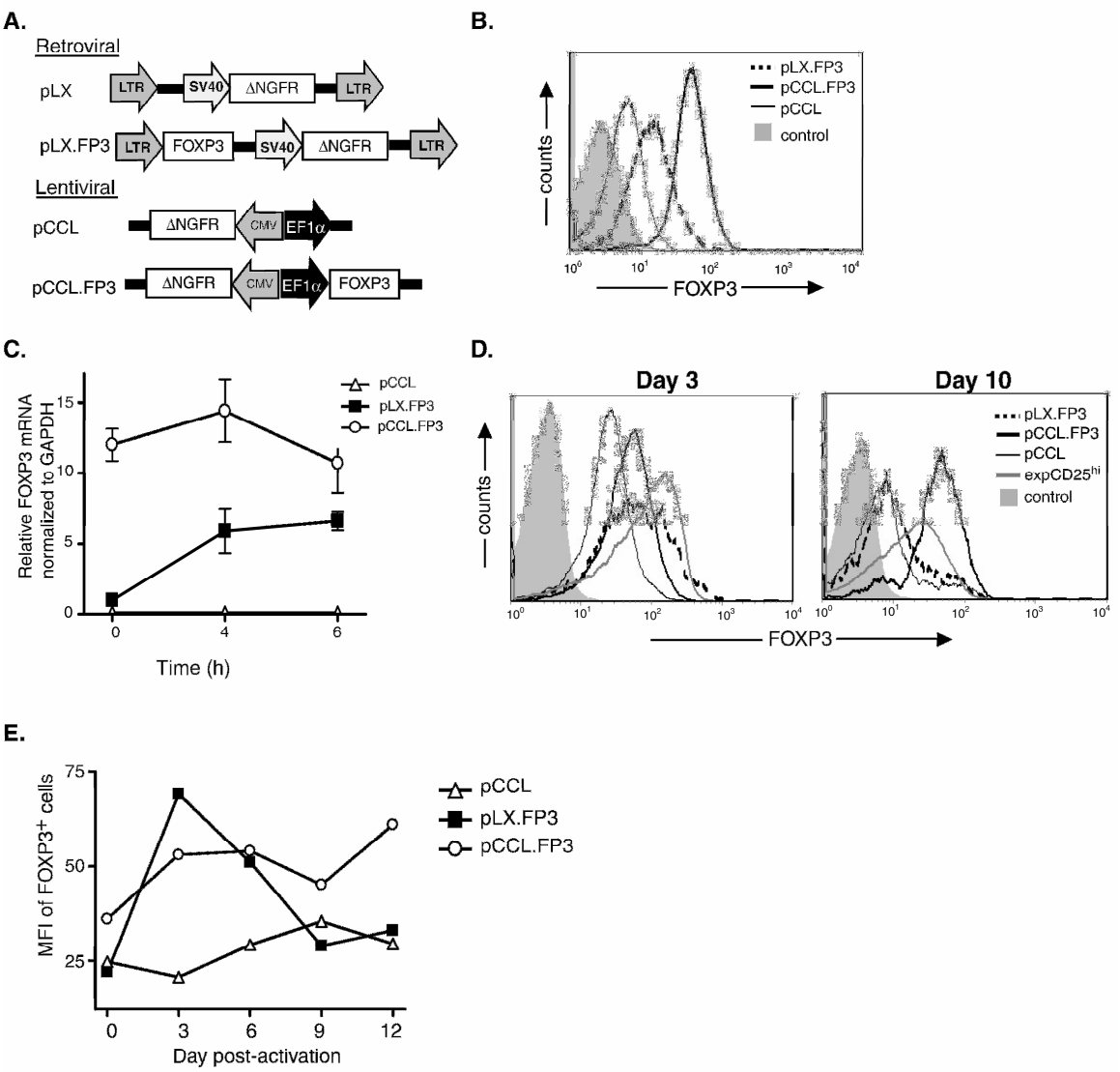

Figure 4.1 Comparison of retrovirus- and lentivirus-based vectors for expression of FOXP3 in human T cells. **(A)** Illustration of control or FOXP3-expressing retro- and lentiviral vector constructs. **(B)** FACS analysis of FOXP3 expression (with clone 236A/E7) in Jurkat cells that expressed ΔNGFR following transduction with retro- or lentiviruses. **(C)** Transduced Jurkat cells were stimulated with TPA (1 ng/ml) and Ca^{2+} ionophore (50 ng/ml) for the indicated times, and amounts of FOXP3 and GAPDH mRNA were determined by qPCR. Normalized levels of FOXP3 mRNA are plotted relative to pLX.FP3-transduced Jurkat cells at time=0. Data represent the average of 3 independent determinations, and error bars indicate the SD. **(D)** $CD4^+CD25^-$ T cells were transduced with pLX.FP3, pCCL.FP3, or pCCL viruses, purified for ΔNGFR expression, and expression of FOXP3 (with clone PCH101) was determined 3 and 10 days after re-stimulation. $CD4^+CD25^{hi}$ Tregs were cultured and analysed in parallel. **(E)** The intensity of FOXP3 expression in retro- vs lentivirally-transduced $CD4^+CD25^-$ T cells during a 12-day expansion period is plotted as the MFI of $FOXP3^+$ cells at the indicated times. D & E are representative of data from 4 different donors. Control indicates unstained or fluorescence-minus-one (FMO)-stained cells.

In order to compare our previous retroviral FOXP3 vector (pLX.FP3) and the new lentiviral FOXP3 vector (pCCL.FP3), we examined the kinetics of FOXP3 expression in Jurkat cells, which do not express endogenous FOXP3. Jurkat cells were transduced with the empty pLX control retroviral vector (data not shown), pLX.FP3, the empty pCCL control lentiviral vector, or pCCL.FP3, and purified on the basis of ΔNGFR expression after 5 days. The intensity of FOXP3 expression was determined by FACS analysis (**Fig. 4.1B**), and these data demonstrated that the pCCL.FP3 lentiviral vector drove higher levels of FOXP3 protein than the pLX.FP3 retroviral vector. The mAb used to detect FOXP3 resulted in some background staining of control pCCL-transduced Jurkat cells, despite the fact that these cells do not express FOXP3 mRNA (**Fig. 4.1C**).

To assess whether vector-driven gene expression was influenced by the state of T cell activation, control and FOXP3-transduced Jurkat cells were stimulated with TPA and Ca^{2+} ionophore, and levels of FOXP3 mRNA were determined by quantitative PCR. In contrast to lentiviral EF1α-driven FOXP3 expression, retroviral-LTR-driven expression was influenced by the activation state of the cells. After 4h, stimulated pLX.FP3-transduced cells upregulated FOXP3 mRNA by an average of 6.4±2.0 fold, in contrast to only 1.5±0.4 fold in pCCL.FP3-transduced cells ($p=0.007$, $n=4$). The difference in expression driven by the retroviral and lentiviral vectors is likely due to the fact that the promoter activity of retroviral LTRs is significantly increased upon T cell activation (24, 26). Control-transduced Jurkat cells were consistently negative for FOXP3 mRNA (**Fig. 4.1C**).

We found a similar difference in expression kinetics between the two vector systems in human $CD4^+$ T cells. At the peak of activation, pLX.FP3- and pCCL.FP3-transduced T cells expressed equal levels of FOXP3 protein, which were comparable to those in $CD4^+CD25^{hi}$ Tregs. At this time point, control pCCL-transduced T cells were also $FOXP3^+$ due to activation-induced expression of this protein (31). As T cell activation waned, however, LTR-driven FOXP3 expression declined while EF1α-driven expression remained consistent (**Figs. 4.1D&E**). To assess the performance of the lentiviral vector independently of endogenous activation-induced FOXP3, we transduced T cells with a lentivirus encoding HA-tagged FOXP3 (**Fig. 4.2B**) and confirmed that high expression of ectopic FOXP3 did not depend on the endogenous protein.

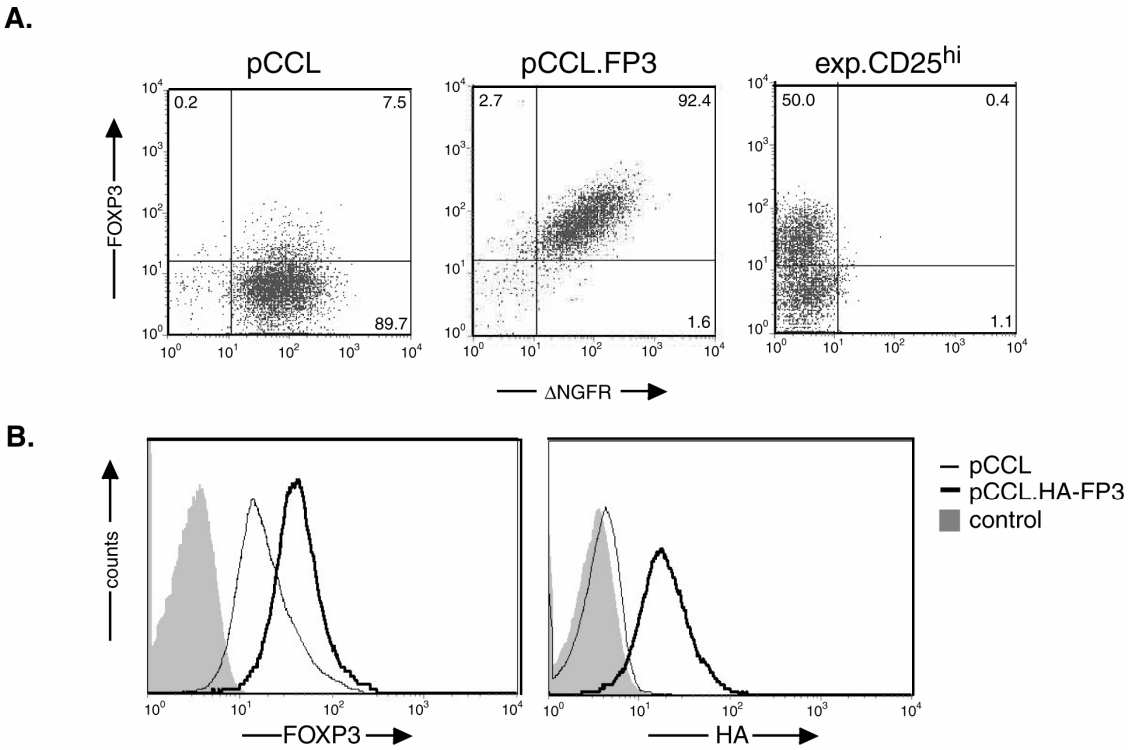

Figure 4.2 Co-ordinate expression of FOXP3 and ΔNGFR by the pCCL.FP3 lentivirus vector. (A) Cell surface ΔNGFR and intranuclear FOXP3 expression (clone 236A/E7) were determined in purified, transduced T cells following 4 weeks of expansion with a T cell feeder. $CD4^{+}CD25^{hi}$ T cells expanded in parallel are shown for comparison. Representative of cells from 9 different donors. **(B)** Intranuclear FOXP3 (clone 236A/E7) or HA expression was determined in ΔNGFR-purified, transduced T cells under activating conditions. Control indicates FMO-stained cells.

Optimized transduction and culture conditions for expression of FOXP3 in $CD4^{+}CD25^{-}CD45RO^{-}$ T cells. Viral supernatants from control pCCL and pCCL.FP3 vectors were used to transduce $CD4^{+}CD45RO^{-}CD25^{-}$ T cells pre-activated with either anti-CD3/28 mAbs for 48h (n=5 donors), or with autologous irradiated APCs and soluble anti-CD3 for 16h (n=4 donors) (**Fig. 4.3A**). Optimal transduction efficiency was achieved upon pre-activation with anti-CD3 mAbs and APCs: 76-97% (mean 86±12%, n=4) of cells were transduced with the control pCCL virus and 28-71% (mean 47±18%, n=4) of cells with the pCCL.FP3 virus (**Fig. 4.3B**). As in other systems (20, 32), the additional sequence (the FOXP3 cDNA) in the pCCL.FP3 vector resulted in a lower transduction efficiency due to less efficient packaging of the larger viral genome.

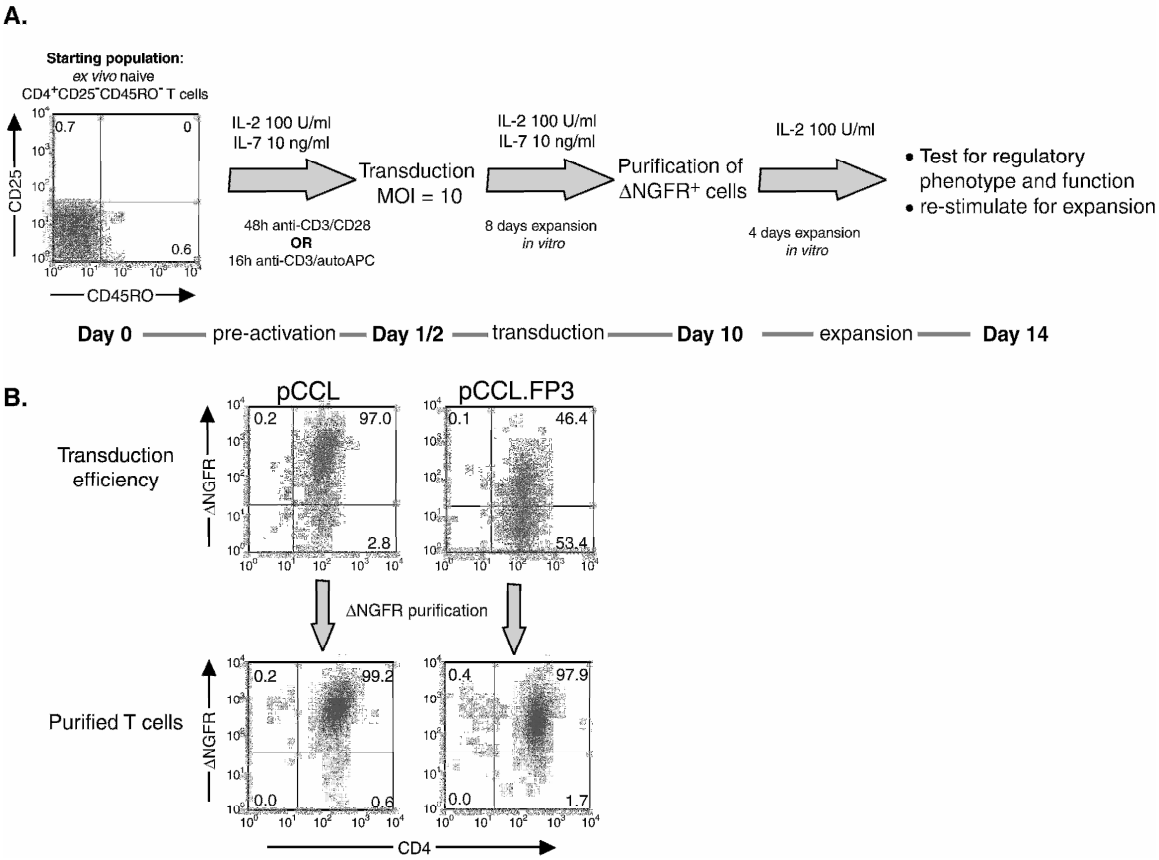

Figure 4.3 Optimized method for lentiviral transduction of human CD4$^+$CD25$^-$CD45RO$^-$ T cells. (A) Schematic diagram of transduction protocol: T cells were pre-activated with either anti-CD3/28 (for 48h) or anti-CD3/APCs (for 16h) in the presence rhIL-2 and rhIL-7, and transduced with concentrated lentiviral supernatants. ΔNGFR$^+$ cells were purified 8 days later and assayed for phenotype and function in the resting phase (~day 14), and re-stimulated for expansion. MOI, multiplicity of infection. **(B)** Transduction efficiencies of pCCL and pCCL.FP3 vectors assessed 6 days following infection of CD4$^+$CD25$^-$CD45RO$^-$ T cells pre-activated with anti-CD3/APCs, and ΔNGFR purity following purification. Representative of a minimum of 4 different donors.

Eight days following transduction, ΔNGFR$^+$ T cells were purified using anti-ΔNGFR magnetic beads and expanded (9). The yield of ΔNGFR$^+$ cells following magnetic sorting ranged from 20-44% (mean 29.5±9.5%) and the resulting cells were always >95% pure. It has been reported that transduction of naïve T cells is optimal following exposure to IL-7 (34), and accordingly we found that addition of IL-2 and IL-7 during T cell pre-activation led to a greater recovery of transduced cells due to increased cell viability, proliferation, and more distinct populations of ΔNGFR$^+$ T cells (data not shown). Although addition of IL-7 improved the overall transduction efficiency and cell growth, it was detrimental to the preservation of the Treg phenotype in the long term.

Culture of *ex vivo* $CD4^+CD25^{hi}$ Tregs in IL-7 completely abrogated their phenotype and function after 4 weeks (**Fig. 4.4**), which could reflect the preferential outgrowth of non-suppressive $CD127^+$ Teff cells in the presence of IL-7 (11, 12). Thus, after purification of $\Delta NGFR^+$ cells, the transduced cells were expanded in the presence of IL-2 alone to minimize the potential for outgrowth of $\Delta NGFR^-$ cells in pCCL.FP3-transduced cultures.

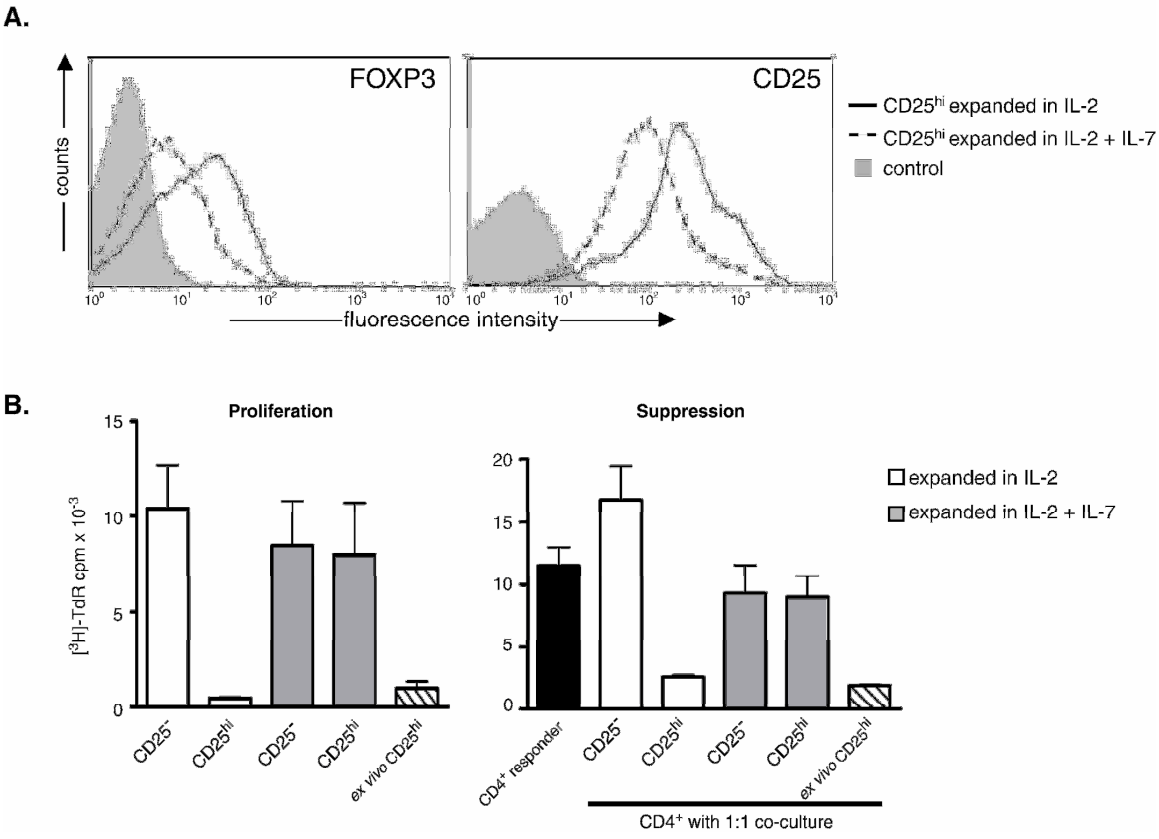

Figure 4.4 Phenotype and function of Tregs expanded for 4 weeks with or without IL-7. $CD4^+CD25^-$ or $CD4^+CD25^{hi}$ T cells were FACS-sorted and expanded *in vitro* with a standard T cell feeder containing IL-2 (100 U/ml) alone or IL-2 and IL-7 (10 ng/ml) for 4 weeks. **(A)** FOXP3 and CD25 expression were determined in $CD4^+CD25^{hi}$ Treg lines in the resting phase by FACS. **(B)** Proliferation of expanded $CD4^+CD25^-$ or $CD4^+CD25^{hi}$ T cells in response to stimulation with soluble anti-CD3 and a 1:1 ratio of irradiated APCs was determined after 4 days. **(C)** $CD4^+$ responder T cells were stimulated with soluble anti-CD3 (1μg/ml) in the presence of APCs with a 1:1 ratio of $CD4^+CD25^-$ or $CD4^+CD25^{hi}$ T cells. Representative of data from T cell lines derived from 2 donors. Control indicates unstained or FMO-stained cells.

EF1α-driven expression of FOXP3 results in upregulation of Treg-associated molecules and hyporesponsiveness. Previous studies reported variable changes in expression of Treg-associated molecules such as CD25, GITR and CTLA4 following FOXP3 transduction (20, 22, 35). pCCL.FP3-transduced T cells expressed significantly

higher amounts of FOXP3, CD25, CTLA4, GITR, and lower amounts of CD127 than control cells (**Fig. 4.5A**). With the exception of CTLA-4, expression levels of these molecules did not significantly differ between pCCL.FP3-transduced cells and $CD4^+CD25^{hi}$ Tregs expanded in parallel (**Fig. 4.5B**). Although CTLA-4 expression was consistently higher in pCCL.FP3-transduced cells than in control cells (1.8±0.5 fold higher MFI, p=0.0005, n=16), it was never expressed as highly as in expanded $CD4^+CD25^{hi}$ Tregs (5.6±2.4 fold higher MFI compared to controls).

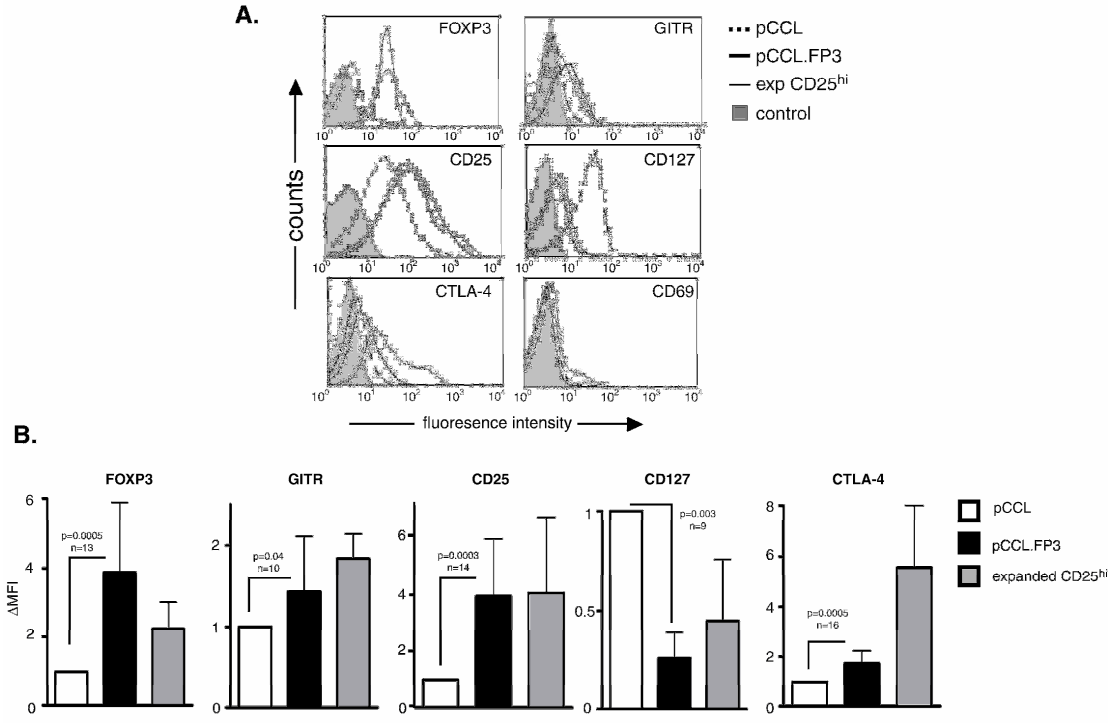

Figure 4.5 Expression of Treg-associated markers in transduced T cells and expanded $CD4^+CD25^{hi}$ T cells. Expression of the indicated markers was determined by FACS analysis of control and FOXP3-expressing T cells in the resting phase. Donor-matched $CD4^+CD25^{hi}$ Tregs (exp $CD25^{hi}$) were expanded in parallel. **(A)** FACS plots are representative of a minimum of 9 independent experiments with cells derived from at least 6 different donors. Control indicates unstained or FMO-stained cells. **(B)** Graphs represent averaged data for ΔMFI, and numbers on each graph indicate significance and number of independent determinations when pCCL- and pCCL.FP3-transduced cells were compared. FOXP3 staining was performed with clone 236A/E7 or PCH101.

In addition to altering the cell-surface phenotype of $CD4^+$ T cells, lentivirus-mediated transfer of FOXP3 induced a hyporesponsive phenotype. Upon stimulation with immobilized anti-CD3 (**Fig. 4.6A**), pCCL.FP3-transduced T cells proliferated significantly less than control T cells (7.6±3.6-fold less [p=0.0001, n=8] at 1 μg/ml anti-

CD3). Hyporesponsiveness was reversed with addition of exogenous IL-2 (**Fig. 4.6B**), and at no point did the FOXP3-transduced cells become growth factor independent.

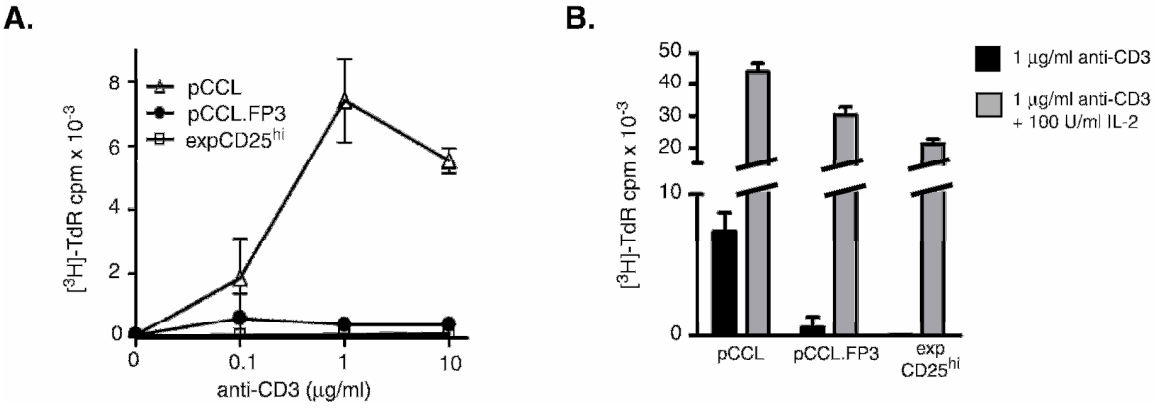

Figure 4.6 Proliferative potential of transduced T cells. (A) Transduced T cells were tested for their ability to proliferate in response to increasing amounts of immobilized anti-CD3. **(B)** The capacity of exogenous IL-2 to induce proliferation of transduced T cells was assessed in the presence of immobilized anti-CD3 (1µg/ml). Error bars indicate SD of values from triplicate wells. Graphs are representative of a minimum of 6 independent experiments performed with transduced T cells derived from at least 4 donors.

EF1α-driven expression of FOXP3 suppresses cytokine production in CD4$^+$ T cells. In agreement with our previous data (20), over-expression of FOXP3 resulted in significantly reduced production of IL-2, IFN-γ, and IL-4 in culture supernatants following TCR-mediated stimulation (**Fig. 4.7A**). IL-10 production by the pCCL.FP3-transduced cells was reduced, although this difference did not reach statistical significance. Expression of TGF-β was equivalent in control and pCCL.FP3-transduced cells. Results for IL-2 and IFN-γ were confirmed by intracellular staining, after re-stimulation with either anti-CD3/28-coated beads or TPA and Ca^{2+} ionophore (**Fig. 4.7B**). The percentage of cytokine-producing cells was significantly lower amongst pCCL.FP3-transduced T cells than amongst control-transduced T cells, irrespective of the activation condition (**Fig. 4.7C**). TPA and Ca^{2+} ionophore stimulation has previously been shown to reverse the anergy and suppressive function of CD4$^+$CD25$^+$ Tregs (36), and in our hands this stimulus was sufficient to overcome a block in IL-2 production. Interestingly, in comparison to the expanded CD4$^+$CD25^{hi} Tregs tested in parallel, the pCCL.FP3-transduced cells consistently showed a more profound suppression of cytokine production, demonstrating superior preservation of a Treg phenotype upon in

vitro expansion. As further evidence that EF1α-driven lentiviral FOXP3 expression enforced a more consistent Treg phenotype than LTR-driven retroviral FOXP3, we compared the proliferative capacity and cytokine production of pCCL.FP3- and pLX.FP3-transduced cells in parallel. As predicted, transduction with pCCL.FP3 led to a more profound suppression of proliferation and cytokine production in $CD4^+$ T cells than with the pLX.FP3 vector (**Fig. 4.8**).

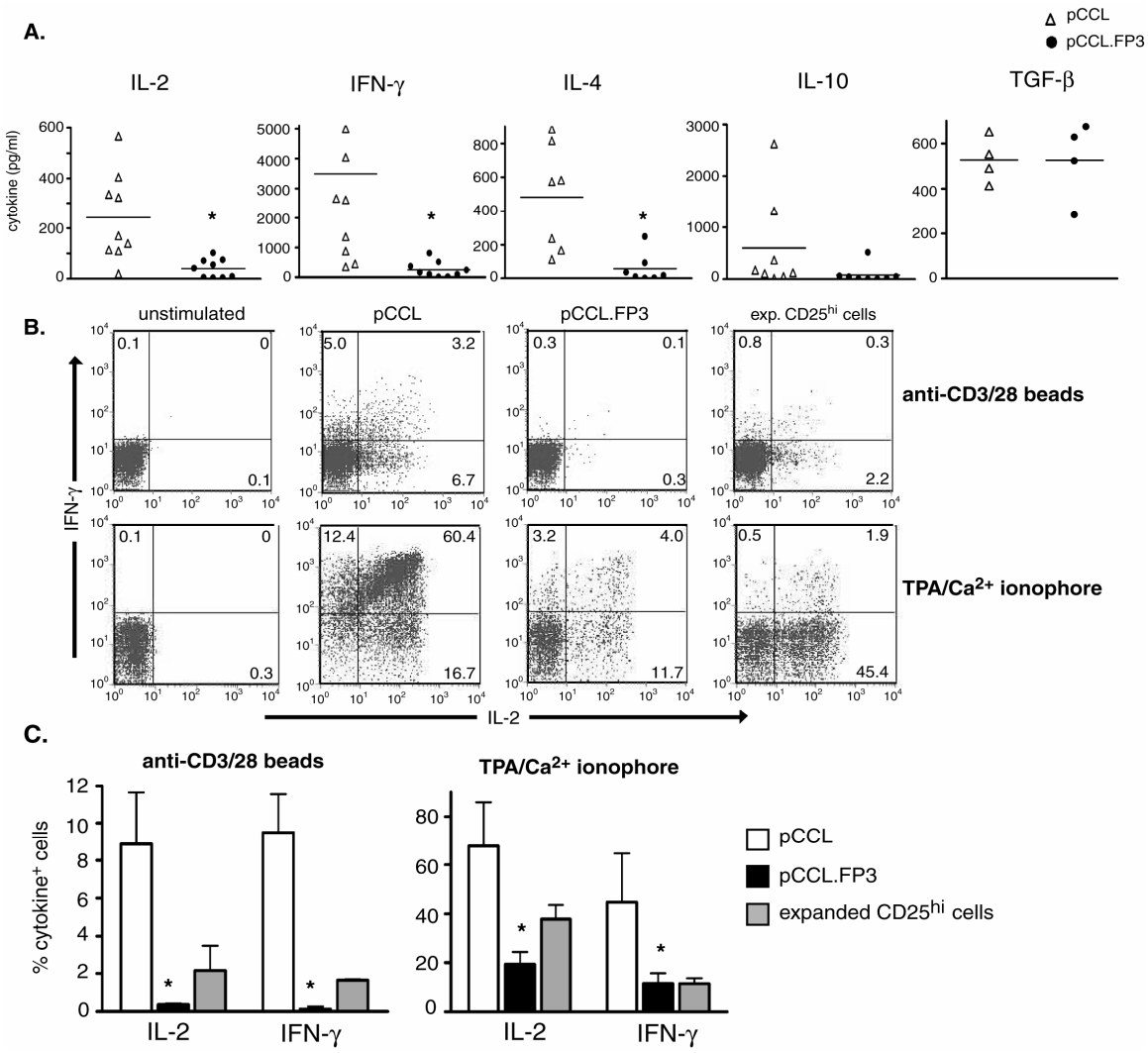

Figure 4.7. Cytokine production by FOXP3-transduced T cells. (A) Cytokine production of transduced T cells was determined by ELISA or CBA 24h (IL-2), 48h (IFN-γ, IL-4, and IL-10), or 72h (TGF-β) after re-stimulating resting T cells with anti-CD3 (10μg/ml) and anti-CD28 (1μg/ml). Each point represents a single determination, * indicates p≤0.02. **(B)** Cytokine production by control or FOXP3-transduced cells was determined by intracellular staining after 6h stimulation with anti-CD3/28 coated beads or 4h TPA and Ca^{2+} ionophore. Data in (A) & (B) represent a minimum of 4 experiments with transduced T cells derived from at least 3 different donors, with results of intracellular cytokine analysis ± SD averaged in (C). * indicates p≤0.04 for comparison of pCCL- and pCCL.FP3-transduced cells.

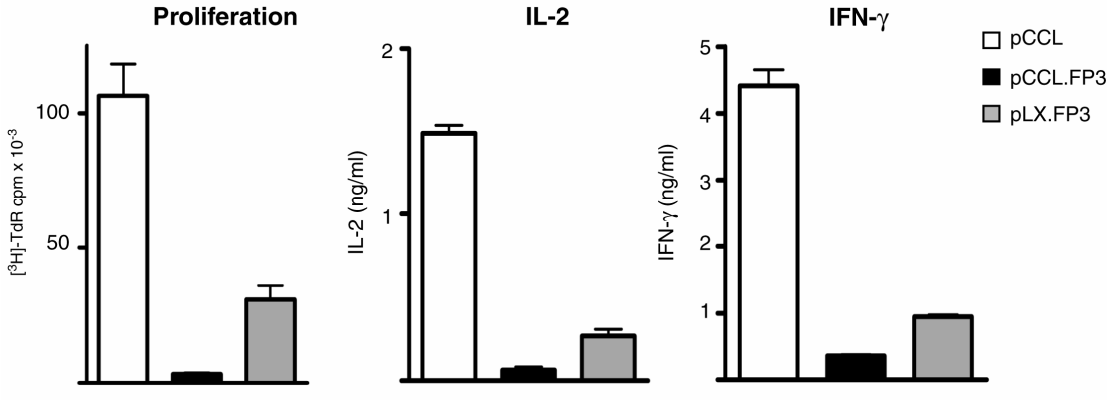

Figure 4.8 Phenotype of T cells transduced with FOXP3-expressing retroviral or lentiviral vectors. pCCL-, pCCL.FP3-, or pLX.FP3-transduced cells were analysed for their potential to proliferate in response to anti-CD3 mAbs in the presence of APCs, or to produce IL-2 (24h) or IFN-γ (48h) in response to stimulation with immobilized anti-CD3 and soluble anti-CD28. Representative of data from transduced T cells derived from 4 donors.

EF1α-driven FOXP3 expression confers potent suppressor function upon both naïve and memory CD4$^+$ T cells. pCCL.FP3-transduced T cells consistently and potently suppressed the proliferation (**Fig. 4.9A**) and cytokine production (**Fig. 4.9B**) of CD4$^+$ responder T cells. Their suppressive capacity was comparable to that of *ex vivo* CD4$^+$CD25^{hi} Tregs. At a 1:1 ratio, pCCL.FP3-transduced T cells suppressed proliferation by an average of 75±8% (n=12) while *ex vivo* CD4$^+$CD25^{hi} Tregs suppressed by 78±11% (p=NS, n=12). Similarly, production of IFN-γ by CD4$^+$ responder T cells was inhibited by an average of 81±15% by pCCL.FP3-transduced T cells in contrast to 83±12% (p=NS, n=8) by *ex vivo* CD4$^+$CD25^{hi} Tregs. Providing the pCCL.FP3-transduced cells remained >90% ΔNGFR$^+$, their capacity to suppress did not decrease over time in culture (tested up to 8 weeks, data not shown). Control-transduced cells did not suppress proliferation or cytokine production by responder CD4$^+$ T cells.

It has been found that ectopic expression of FOXP3 was not capable of converting human memory T cells into Tregs (35). Since our method was more efficient than that described at inducing Tregs from naive cells, we investigated whether EF1α-driven FOXP3 expression would also convert memory Teff cells into Tregs. CD4$^+$CD25$^-$ T cells were expanded in culture for two weeks prior to transduction. Indeed, our method for ectopic expression of FOXP3 was sufficient to induce a suppressor phenotype in

$CD4^+CD45RO^+$ T cells (**Figs. 4.9C, D**), although their suppressive capacity was more variable than when naïve T cells were transduced. Depending on the donor, memory T cells transduced with pCCL.FP3 suppressed proliferation by 32-90% (mean=61.6%, n=5) and IFN-γ by 52-75% (mean=65%, n=3). The reduced and more variable suppressive capacity of pCCL.FP3-transduced memory cells could not be linked to lower FOXP3 expression: when compared to naïve cells transduced with FOXP3 in parallel, the difference in FOXP3 MFI expression between the two populations was not significant (p=0.09, n=4). These data indicate that memory T cells are biologically capable of acquiring suppressor function, provided that they continuously express high levels of FOXP3.

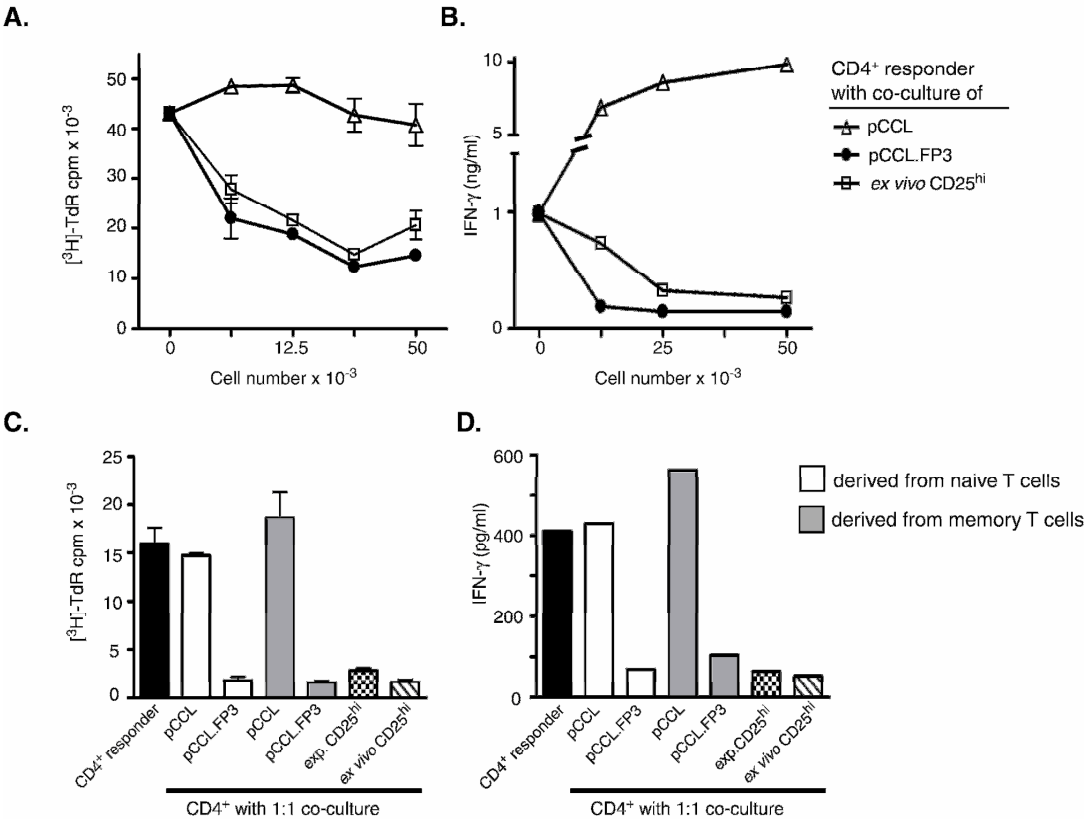

Figure 4.9. Suppressive capacity of transduced T cells. **(A)** $CD4^+$ responder T cells were stimulated with soluble anti-CD3 (1μg/ml) in the presence of APCs with or without the indicated numbers of transduced T cells. **(B)** Culture supernatants from suppression assays were collected and analyzed by the cytometric bead assay for IFN-γ concentrations after 72h. **(C)** The regulatory capacity of transduced cells derived from either naïve (white bars) or memory (grey bars) T cells was determined as in **c** by co-culture of a 1:1 ratio of transduced T cells to $CD4^+$ responder cells. **(D)** Culture supernatants from suppression assays were collected and analyzed by the cytometric bead array for IFN-γ concentrations after 72h. (A) & (B) are representative of a minimum of 8 experiments with transduced T cells derived from at least 6 donors, and (C) & (D) a minimum of 3 experiments with transduced T cells from 3 different donors. Error bars indicate SD between triplicate wells.

Lentiviral vectors co-expressing FOXP3 and ΔNGFR allow long-term maintenance of FOXP3^{hi} suppressive T cell populations. A major limitation of protocols involving *in vitro* expansion of CD4^{+}CD25^{hi} Tregs for the purpose of cellular therapy is that isolation on the basis of CD25 expression does not result in a homogeneous population of FOXP3^{+} cells (11) and that, depending on cell numbers, extensive expansion *in vitro* may be necessary (**Fig. 4.10A**). While pCCL.FP3-transduced T cells maintained a high percentage of FOXP3 expressors over several weeks, expression of FOXP3 in *ex vivo* Tregs sorted on the basis of high CD25 expression declined with each round of expansion (**Fig. 4.10B**). The FOXP3-transduced T cells could be efficiently expanded *in vitro* in the presence of exogenous IL-2: after 12 days, the average fold-expansion of purified FOXP3-expressing cells was 80±48, which was less than that of pCCL-transduced cells (356±133), but comparable to that of CD4^{+}CD25^{hi} Tregs (96±65) (**Figs. 4.10C,D**) (6, 7, 9). The number of Tregs generated in each experiment varied depending on the donor and pCCL.FP3 transduction efficiency. In an average experiment, if 0.5 x 10^{6} T cells were transduced, following purification and one round of expansion (i.e. 3 weeks of *in vitro* culture), approximately 1.5 x 10^{7} Tregs were generated.

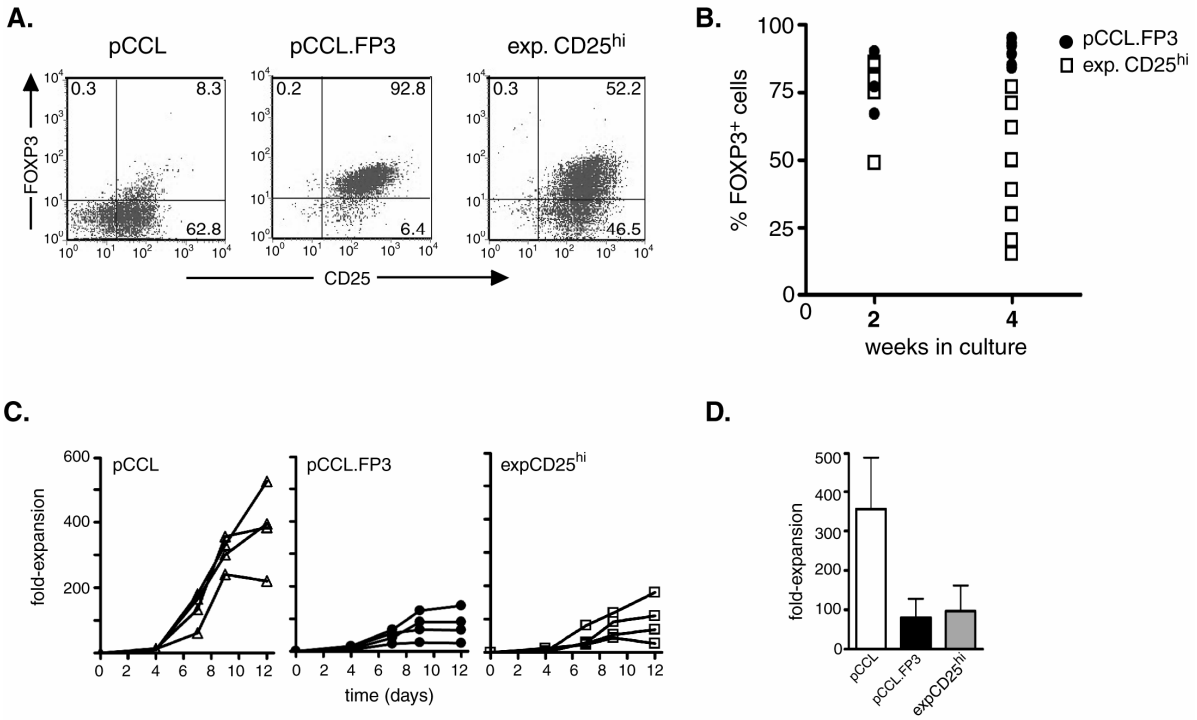

Figure 4.10. Comparison of FOXP3-transduced and CD4$^+$CD25^{hi} T cells during *in vitro* expansion. **(A)** Control or pCCL.FP3-transduced cells were expanded in parallel with CD4$^+$CD25^{hi} T cells for 4 weeks, and expression of CD25 and FOXP3 was determined in the resting phase. **(B)** The % of FOXP3$^+$ cells was compared between pCCL.FP3-transduced T cells and donor-matched CD4$^+$CD25^{hi} Tregs expanded in parallel. Each symbol represents an independent T cell line. **(C)** Fold-expansion of transduced T cells and FACS-sorted CD4$^+$CD25^{hi} T cells was determined over a 12-day culture period. Each line represents T cells derived from a different donor, with averaged results ± SD shown in **(D).**

Expression of FOXP3 does not affect TCR diversity. We also investigated whether the transduction process and/or expression of FOXP3 may affect TCR diversity. Accordingly, the diversity of the TCR repertoire was analyzed *ex vivo* and in expanded control and pCCL.FP3-transduced T cells derived from 2 donors (#207 and #266). Specific subsets of the TCR β chain were amplified, including the CDR3 clonotypic determinant, and multiple (n=27-34) independently-derived clones were sequenced (37, 38) (**Tables 4.1 and 4.2**). As expected, *ex vivo* CD4$^+$ T cells were polyclonal, with 28 unique clonotypes observed in 29 TRBV7 sequences and 31 unique clonotypes (100%) in TRBV30. In contrast, after 4 weeks of culture, both control and pCCL.FP3-transduced T cells from donor #207 showed loss of polyclonality in TRBV7 and TRBV30. Results from donor #266 were similar, with the exception that there was more pronounced oligoclonality, likely due to the fact that the transduced cells were analyzed after 8 weeks

of culture (**Table. 4.2**). These data indicate that while there is a loss of polyclonality with time in culture, there is no apparent difference between control and pCCL-FP3-transduced cells. This loss of TCR diversity is likely related to the process of *in vitro* expansion rather than transduction, as previously reported with retroviral gene transfer (39).

Table 4.1 Diversity analysis of the TCR repertoire in *ex vivo* and transduced CD4$^+$ T cells from donor 207. TCR β chain clones (TRBV7-2/-3 and TRBV30) were amplified from cDNA derived from *ex vivo* CD4$^+$ T cells (***ex vivo***), and control (**NGF**) or pCLL-FP3-transduced (**FP3**) T cells that had been in culture for 4 weeks. * denotes a single TRBV30 clonotype that is shared between control and pCCL-FP3-transduced cells.

Clone	TRBV id	CDR3 sequence	TRBJ id	Length	Frequency
A207exvivo-2	TRBV7-2	CASSFALASGTGELFF	TRBJ2-2	14	2
A207exvivo-1	TRBV7-2	CASSLAETGLSGNTIYF	TRBJ1-3	15	1
A207exvivo-3	TRBV7-2	CASSLPRLQGTYEQYF	TRBJ2-7	14	1
A207exvivo-4	TRBV7-3	CASSLGTSGGVDTQYF	TRBJ2-3	14	1
A207exvivo-8	TRBV7-2	CASSHSGRSGNTIYF	TRBJ1-3	13	1
A207exvivo-9	TRBV7-2	CASRRGGSSYNEQFF	TRBJ2-1	13	1
A207exvivo-10RC	TRBV7-2	CASSPIQGLGTGELFF	TRBJ2-2	14	1
A207exvivo-11	TRBV7-2	CASSLRRETGELFF	TRBJ2-2	12	1
A207exvivo-12	TRBV7-2	CASSLKSGTEAFF	TRBJ1-1	11	1
A207exvivo-14RC	TRBV7-2	CASSLGWQNYGYTF	TRBJ1-2	12	1
A207exvivo-15	TRBV7-3	CASSFGQGAPYEQYF	TRBJ2-7	13	1
A207exvivo-16RC	TRBV7-2	CASSLSYEQYF	TRBJ2-7	9	1
A207exvivo-17	TRBV7-2	CASSLGLTGGTGELFF	TRBJ2-2	14	1
A207exvivo-18	TRBV7-2	CASSLVQLSNQPQHF	TRBJ1-5	13	1
A207exvivo-20	TRBV7-2	CASSLGPNEQFF	TRBJ2-1	10	1
A207exvivo-21	TRBV7-2	CASSDRANSYNSPLHF	TRBJ1-6	14	1
A207exvivo-22	TRBV7-2	CASSLSPGGKAGELFF	TRBJ2-2	14	1
A207exvivo-23	TRBV7-3	CASSLDRDYEQYF	TRBJ2-7	11	1
A207exvivo-24	TRBV7-2	CASSNTRGTENTEAFF	TRBJ1-1	14	1
A207exvivo-26	TRBV7-2	CASSLATGLRGNTIYF	TRBJ1-3	14	1
A207exvivo-28	TRBV7-2	CASSSRGNNSPLHF	TRBJ1-6	12	1
A207exvivo-29RC	TRBV7-2	CASSLGGTRANYGYTF	TRBJ1-2	14	1
A207exvivo-30	TRBV7-2	CASSLDPAGELFF	TRBJ2-2	11	1
A207exvivo-31RC	TRBV7-2	CASSLRGSGATNEKLFF	TRBJ1-4	15	1
A207exvivo-32RC	TRBV7-2	CASSLAASSAKNIQYF	TRBJ2-4	14	1
A207exvivo-33	TRBV7-3	CASSLFSRGLCDNQPQHF	TRBJ1-5	16	1
A207exvivo-34	TRBV7-2	CASSFGGDSPLHF	TRBJ1-6	11	1
A207exvivo-35	TRBV7-3	CASSLEVRGGNEQFF	TRBJ2-1	13	1
A207FP3-2	TRBV7-3	CASSRAVTDTQYF	TRBJ2-3	11	11
A207FP3-5	TRBV7-2	CASSSAGTRNTGELFF	TRBJ2-2	14	2
A207FP3-16	TRBV7-2	CASSSDRAGNTIYF	TRBJ1-3	12	2
A207FP3-1	TRBV7-3	CASSLGGPVSNEQFF	TRBJ2-1	13	1
A207FP3-10	TRBV7-3	CASSLTKNNEQFF	TRBJ2-1	11	1
A207FP3-11RC	TRBV7-2	CASSHTGGGDEQYF	TRBJ2-7	12	1
A207FP3-12	TRBV7-2	CASSFGRGRGAYNEQFF	TRBJ2-1	15	1
A207FP3-19RC	TRBV7-2	CASSFSRRGNHNEQFF	TRBJ2-1	14	1
A207FP3-20	TRBV7-2	CASSLQGVTTGELFF	TRBJ2-2	13	1
A207FP3-21	TRBV7-3	CASSSQGRHTEAFF	TRBJ1-1	12	1
A207FP3-23	TRBV7-2	CASSLDTASGGANTQYF	TRBJ2-3	15	1
A207FP3-25	TRBV7-2	CASSSLTSGVEQFF	TRBJ2-2	12	1

Clone	TRBV id	CDR3 sequence	TRBJ id	Length	Frequency
A207FP3-26	TRBV7-3	CASSPSGTANYGYTF	TRBJ1-2	13	1
A207FP3-29RC	TRBV7-2	CASSLQGNGETQYF	TRBJ2-5	12	1
A207NGF-3	TRBV7-2	CASSLRGIETQYF	TRBJ2-5	11	13
A207NGF-1RC	TRBV7-2	CASSLSWYEQYF	TRBJ2-7	10	3
A207NGF-6RC	TRBV7-3	CASSRGPSYEQFF	TRBJ2-1	11	2
A207NGF-19	TRBV7-2	CASSPQGTGNTIYF	TRBJ1-3	12	2
A207NGF-2	TRBV7-2	CASSLSPGGKAGELFF	TRBJ2-2	14	1
A207NGF-4	TRBV7-2	CASSLSASGGTGELFF	TRBJ2-2	14	1
A207NGF-5	TRBV7-3	CASSMTANNSPLHF	TRBJ1-6	12	1
A207NGF-8	TRBV7-3	CASSPTFGLAGGTLRSYNEQFF	TRBJ2-1	20	1
A207NGF-12RC	TRBV7-2	CASSLRQAQETQYF	TRBJ2-5	12	1
A207NGF-15	TRBV7-2	CASSLTGSYEQYF	TRBJ2-7	11	1
A207NGF-18RC	TRBV7-2	CASSSSGTRDTGELFF	TRBJ2-2	14	1
A207NGF-22	TRBV7-2	CASSLAPGQGSYNEQFF	TRBJ2-1	15	1
A207NGF-25	TRBV7-2	CASSLLRSEQFF	TRBJ2-1	10	1
A207NGF-28	TRBV7-2	CASSLSGQGATEAFF	TRBJ1-1	13	1
A207NGF-30RC	TRBV7-2	CASSLGQLNEKLFF	TRBJ1-4	12	1
A207NGF-31	TRBV7-6	CASSRGPAPGANVLTF	TRBJ2-6	14	1
B207exvivo-1RC	TRBV30	CAWSDTVHSPLHF	TRBJ1-6	11	1
B207exvivo-2RC	TRBV30	CAWSVREEQYF	TRBJ2-7	9	1
B207exvivo-3RC	TRBV30	CAWSTQAYEQYF	TRBJ2-7	10	1
B207exvivo-4RC	TRBV30	CAWSAINRGGNYGYTF	TRBJ1-2	14	1
B207exvivo-5RC	TRBV30	CAWTRARNTIYF	TRBJ1-3	10	1
B207exvivo-6RC	TRBV30	CAWSVGNSPLHF	TRBJ1-6	10	1
B207exvivo-8RC	TRBV30	CALRDTGKGTQYF	TRBJ2-5	11	1
B207exvivo-9RC	TRBV30	CASARGQPPRGFYNEQFF	TRBJ2-1	16	1
B207exvivo-10	TRBV30	CAWSRIIQNTEAFF	TRBJ1-1	12	1
B207exvivo-11RC	TRBV30	CAWSLAVRSGANVLTF	TRBJ2-6	14	1
B207exvivo-13	TRBV30	CAWSRTGGARNSPLHF	TRBJ1-6	14	1
B207exvivo-14RC	TRBV30	CAWSVHNSNNQPQHF	TRBJ1-5	13	1
B207exvivo-15	TRBV30	CAWSWASGAPDTQYF	TRBJ2-3	13	1
B207exvivo-16	TRBV30	CAWSVMGPYGYTF	TRBJ1-2	11	1
B207exvivo-17	TRBV30	CAWSELNTEAFF	TRBJ1-1	10	1
B207exvivo-18	TRBV30	CAWSKFQGDSPLHF	TRBJ1-6	12	1
B207exvivo-19RC	TRBV30	CALRGPYGYTF	TRBJ1-2	9	1
B207exvivo-20RC	TRBV30	CAWSRASRGVLGETQYF	TRBJ2-5	15	1
B207exvivo-21RC	TRBV30	CAWSGSRRGYTF	TRBJ1-2	10	1
B207exvivo-22RC	TRBV30	CAWNEQGAAEAFF	TRBJ1-1	11	1
B207exvivo-23RC	TRBV30	CARSVTDNEQFF	TRBJ2-1	10	1
B207exvivo-24RC	TRBV30	CAWSVVQGGYTF	TRBJ1-2	10	1
B207exvivo-25RC	TRBV30	CAWDRQNYGYTF	TRBJ1-2	10	1
B207exvivo-26RC	TRBV30	CAWSVRGINYGYTF	TRBJ1-2	12	1
B207exvivo-27RC	TRBV30	CAWSVRGRDNQPQHF	TRBJ1-5	13	1
B207exvivo-28RC	TRBV30	CAWTRQTQTGAARDTQYF	TRBJ2-3	16	1
B207exvivo-29	TRBV30	CACPLRLANTGELFF	TRBJ2-2	13	1
B207exvivo-31RC	TRBV30	CAWSPGTRGTDTQYF	TRBJ2-3	13	1
B207exvivo-33RC	TRBV30	CAWSGGGPPVGRYYNEQFF	TRBJ2-1	17	1
B207exvivo-34	TRBV30	CAWSTANTGELFF	TRBJ2-2	11	1
B207exvivo-35RC	TRBV30	CAWSVQGIYGYTF	TRBJ1-2	11	1
B207FP3-7	TRBV30	CAWGIGGSTDTQYF	TRBJ2-3	12	3*
B207FP3-6	TRBV30	CAWLGLLNTGELFF	TRBJ2-2	12	3
B207FP3-17RC	TRBV30	CAWELGRGGTDTQYF	TRBJ2-3	13	3
B207FP3-1RC	TRBV30	CAWSRDPPYEQYF	TRBJ2-7	11	2
B207FP3-2RC	TRBV30	CAWSVQGGNQPQHF	TRBJ1-5	12	2
B207FP3-5RC	TRBV30	CAWSALRWNTEAFF	TRBJ1-1	12	2
B207FP3-13	TRBV30	CAWSGRSSPPEQYF	TRBJ2-7	12	2
B207FP3-3RC	TRBV30	CAWRSAGGYGYTF	TRBJ1-2	11	1
B207FP3-4RC	TRBV30	CAWSVMAGGGYTF	TRBJ1-2	11	1
B207FP3-11RC	TRBV30	CAWNLQRYAEAFF	TRBJ1-1	11	1
B207FP3-14RC	TRBV30	CAWKKPGTSVGYEQYF	TRBJ2-7	14	1
B207FP3-15	TRBV30	CAWSVSALGTQYF	TRBJ2-5	11	1

Clone	TRBV id	CDR3 sequence	TRBJ id	Length	Frequency
B207FP3-19	TRBV30	CAWKRIDSYEQYF	TRBJ2-7	11	1
B207FP3-21RC	TRBV30	CAGSPVTEAFF	TRBJ1-1	9	1
B207FP3-22RC	TRBV30	CAWSVPGRVTQYF	TRBJ2-5	11	1
B207FP3-24RC	TRBV30	CAWTQREGNQPQHF	TRBJ1-5	12	1
B207FP3-26	TRBV30	CAWRFPDRSGYNEQFF	TRBJ2-1	14	1
B207FP3-29	TRBV30	CAWSFVMEGKLFF	TRBJ1-4	11	1
B207FP3-31	TRBV30	CAWSVYGGYNEQFF	TRBJ2-1	12	1
B207FP3-32	TRBV30	CAWTGRNTGELFF	TRBJ2-2	11	1
B207FP3-34	TRBV30	CAWTGPGRTEAFF	TRBJ1-1	11	1
B207NGF-2RC	TRBV30	CAWSVGNSPLHF	TRBJ1-6	10	5
B207NGF-14RC	TRBV30	CAWSTQGRYEQYF	TRBJ2-7	11	2
B207NGF-15RC	TRBV30	CAWRSAGASDTQYF	TRBJ2-3	12	2
B207NGF-17RC	TRBV30	CAWSVATSTDTQYF	TRBJ2-3	12	2
B207NGF-21RC	TRBV30	CAWNLNTGELFF	TRBJ2-2	10	2
B207NGF-6RC	TRBV30	CAWGIGGSTDTQYF	TRBJ2-3	12	1*
B207NGF-1RC	TRBV30	CAWSRIDREQYF	TRBJ2-7	10	1
B207NGF-4	TRBV30	CAWSVRNTEAFF	TRBJ1-1	10	1
B207NGF-9RC	TRBV30	CAWSAGSGAETQYF	TRBJ2-5	12	1
B207NGF-11RC	TRBV30	CAWRPGQGGSYNEQFF	TRBJ2-1	14	1
B207NGF-13RC	TRBV30	CAWSVLYYNSPLHF	TRBJ1-6	12	1
B207NGF-20RC	TRBV30	CAWSQGRQGAERRNQPQHF	TRBJ1-5	17	1
B207NGF-22RC	TRBV30	CAWSGRTLRETQYF	TRBJ2-5	12	1
B207NGF-23RC	TRBV30	CAWSPLGPGPNQPQHF	TRBJ1-5	14	1
B207NGF-25	TRBV30	CARGHRDRGLRNTEAFF	TRBJ1-1	15	1
B207NGF-26RC	TRBV30	CAWTGTAFSYGYTF	TRBJ1-2	12	1
B207NGF-30RC	TRBV30	CAWSKGLPSPRQFF	TRBJ2-1	12	1
B207NGF-33	TRBV30	CAWGAGINTEAFF	TRBJ1-1	11	1
B207NGF-34	TRBV30	CAWSDQINEKLFF	TRBJ1-4	11	1

Table 4.2 Diversity analysis of the TCR repertoire in *ex vivo* and transduced CD4$^+$ T cells from donor 266. TCR β chain clones (TRBV7-2/-3 and TRBV30) were amplified from cDNA derived from *ex vivo* CD4$^+$ T cells (***ex vivo***), and control (**NGF**) or pCLL-FP3-transduced (**FP3**) T cells that had been in culture for 8 weeks.

Clone	TRBV id	CDR3 sequence	TRBJ id	Length	Frequency
A266exvivo-1	TRBV7-2	CASSWGGGADTQYF	TRBJ2-3	12	1
A266exvivo-2	TRBV7-3	CASRFAASYEQYF	TRBJ2-7	11	1
A266exvivo-3	TRBV7-2	CASSFNRGQSEAFF	TRBJ1-1	12	1
A266exvivo-4	TRBV7-3	CASSLRGRWTGELFF	TRBJ2-2	13	1
A266exvivo-5RC	TRBV7-3	CASSLDRANTEAFF	TRBJ1-1	12	1
A266exvivo-6	TRBV7-3	CASSRQPRGLAGGANTGELFF	TRBJ2-2	19	1
A266exvivo-7RC	TRBV7-2	CASSPAGGSRSPLHF	TRBJ1-6	13	1
A266exvivo-10	TRBV7-3	CASASGLSRNTGELFF	TRBJ2-2	14	1
A266exvivo-11	TRBV7-2	CASSLIAVKTGELFF	TRBJ2-2	13	1
A266exvivo-12	TRBV7-2	CASSLTAGLNTGELFF	TRBJ2-2	14	1
A266exvivo-15	TRBV7-2	CASSFAGAYNEQFF	TRBJ2-1	12	1
A266exvivo-16	TRBV7-3	CASSQYAGTGVNTEAFF	TRBJ1-1	15	1
A266exvivo-17	TRBV7-3	CASSHSPAGDEQFF	TRBJ2-1	12	1
A266exvivo-18	TRBV7-2	CASSGLAGGGTGELFF	TRBJ2-2	14	1
A266exvivo-19	TRBV7-2	CASSLAAGSNNSPLHF	TRBJ1-6	14	1
A266exvivo-21	TRBV7-3	CASSLTVDSPLHF	TRBJ1-6	11	1
A266exvivo-22	TRBV7-2	CASSLDPSGSTQYF	TRBJ2-3	12	1
A266exvivo-23	TRBV7-2	CASSLVGSGSGSYNEQFF	TRBJ2-1	16	1
A266exvivo-24RC	TRBV7-3	CASSIGQGYEQYF	TRBJ2-7	11	1
A266exvivo-25	TRBV7-3	CASSWDRASGANVLTF	TRBJ2-6	14	1
A266exvivo-26	TRBV7-3	CASSRTSGSPSEQYF	TRBJ2-7	13	1
A266exvivo-27	TRBV7-2	CASSLPEVAGWYEQYF	TRBJ2-7	14	1
A266exvivo-31	TRBV7-3	CASSLTRAGNTEAFS	TRBJ1-1	13	1
A266exvivo-32RC	TRBV7-3	CASSLEATDTQYF	TRBJ2-3	11	1

Clone	TRBV id	CDR3 sequence	TRBJ id	Length	Frequency
A266exvivo-33	TRBV7-3	CASSYNPPLASSNTGELFF	TRBJ2-2	17	1
A266exvivo-34	TRBV7-2	CAGSNREVGNTGELFF	TRBJ2-2	14	1
A266exvivo-35RC	TRBV7-3	CASSPPSSYNSPLHF	TRBJ1-6	13	1
A266FP3-4	TRBV7-3	CASSPQDPYNEQFF	TRBJ2-1	12	6
A266FP3-5	TRBV7-3	CASSRDVRANTGELFF	TRBJ2-2	14	5
A266FP3-9RC	TRBV7-2	CASSSRDGGTAEAFF	TRBJ1-1	13	2
A266FP3-12	TRBV7-2	CASSPGSFIQQYF	TRBJ2-7	11	2
A266FP3-1	TRBV7-2	CASSLAGGKNTGELFF	TRBJ2-2	14	2
A266FP3-3	TRBV7-3	CASSLQGASGEQYF	TRBJ2-7	12	1
A266FP3-7RC	TRBV7-2	CASSLAAGAFSPLHF	TRBJ1-6	13	1
A266FP3-8RC	TRBV7-3	CASSSLGATSGRLDTQYF	TRBJ2-3	16	1
A266FP3-11	TRBV7-2	CASSFAPISYEQYF	TRBJ2-7	12	1
A266FP3-14	TRBV7-3	CASSTRQGANEQFF	TRBJ2-1	12	1
A266FP3-18	TRBV7-2	CASSLAVGAFSPLHF	TRBJ1-6	13	1
A266FP3-20	TRBV7-3	CASSLLGTSGSQFF	TRBJ2-1	12	1
A266FP3-30	TRBV7-2	CASSLEGAVNTGELFF	TRBJ2-2	14	1
A266FP3-31	TRBV7-2	CASGQREFFHEQYF	TRBJ2-7	12	1
A266FP3-32	TRBV7-2	CASSLQGGASTDTQYF	TRBJ2-3	14	1
A266NGF-1RC	TRBV7-3	CASSPAGYTTEAFF	TRBJ1-1	12	19
A266NGF-16	TRBV7-3	CASSSSSGETQYF	TRBJ2-5	11	4
A266NGF-6	TRBV7-2	CASSLRTGFGELFF	TRBJ2-2	12	2
A266NGF-25	TRBV7-3	CASSLIPAGELFF	TRBJ2-2	11	2
A266NGF-13	TRBV7-2	CASSLGLYSPLHF	TRBJ1-6	11	1
A266NGF-30	TRBV7-2	CASAYPTGGSNNEQFF	TRBJ2-1	14	1
B266exvivo-8RC	TRBV30	CAWRDLAGLGYNEQFF	TRBJ2-1	14	2
B266exvivo-1RC	TRBV30	CAWSVSPDSSTNEKLFF	TRBJ1-4	15	1
B266exvivo-2RC	TRBV30	CAWSVRGGGGYEQYF	TRBJ2-7	13	1
B266exvivo-3RC	TRBV30	CAWSPRYGENTPYEQYF	TRBJ2-7	15	1
B266exvivo-4RC	TRBV30	CAWRSISNTEAFF	TRBJ1-1	11	1
B266exvivo-5RC	TRBV30	CAWSRNRGGQPQHF	TRBJ1-5	12	1
B266exvivo-6RC	TRBV30	CAWTKGGNTEAFF	TRBJ1-1	11	1
B266exvivo-7	TRBV30	CAWRFRSSYNSPLHF	TRBJ1-6	13	1
B266exvivo-9RC	TRBV30	CAWRVRTGSYNEQFF	TRBJ2-1	13	1
B266exvivo-10RC	TRBV30	CAWSVPGASYNEQFF	TRBJ2-1	13	1
B266exvivo-11RC	TRBV30	CAWSPGQGFDTEAFF	TRBJ1-1	13	1
B266exvivo-12RC	TRBV30	CAWRSSGRYPDNEQFF	TRBJ2-1	14	1
B266exvivo-16RC	TRBV30	CAWSSDRGQPQHF	TRBJ1-5	11	1
B266exvivo-17RC	TRBV30	CAGERVNEQFF	TRBJ2-1	9	1
B266exvivo-18RC	TRBV30	CAWQRGSQGNIQYF	TRBJ2-4	12	1
B266exvivo-19RC	TRBV30	CAWSVQIGYTEAFF	TRBJ1-1	12	1
B266exvivo-23	TRBV30	CAWSRPGELFF	TRBJ2-2	9	1
B266exvivo-24	TRBV30	CAWTGGGEQYF	TRBJ2-7	9	1
B266exvivo-25RC	TRBV30	CAWSPVATYGYTF	TRBJ1-2	11	1
B266exvivo-26RC	TRBV30	CAWTGLAGGWLDTQYF	TRBJ2-3	14	1
B266exvivo-27RC	TRBV30	CAWSRAGTAKNIQYF	TRBJ2-4	13	1
B266exvivo-28RC	TRBV30	CAWSVVGSTGEQYF	TRBJ2-7	12	1
B266exvivo-30	TRBV30	CACEGARGNEQFF	TRBJ2-1	11	1
B266exvivo-33	TRBV30	CAWSAAAYEQYF	TRBJ2-7	10	1
B266exvivo-36RC	TRBV30	CAWSANHRETQYF	TRBJ2-3	11	1
B266exvivo-38	TRBV30	CAWKKSGDTNQYKNIQYF	TRBJ2-4	16	1
B266FP3-1RC	TRBV30	CAWSPGFAETGELFF	TRBJ2-2	13	18
B266FP3-7RC	TRBV30	CAWESGLNTGELFF	TRBJ2-2	12	3
B266FP3-10RC	TRBV30	CAWSYPGHPINEKLFF	TRBJ1-4	14	2
B266FP3-26	TRBV30	CAWTTSGRGGVIF	TRBJ2-1	11	2
B266FP3-25RC	TRBV30	CAWSPSSLRDTQYF	TRBJ2-3	12	1
B266FP3-33	TRBV30	CAWSSLMNTEAFF	TRBJ1-1	11	1
B266NGF-2RC	TRBV30	CAWSADSYQPQHF	TRBJ1-5	11	21
B266NGF-29RC	TRBV30	CAWATTTSYEQYF	TRBJ2-7	11	2
B266NGF-3RC	TRBV30	CAWSVQLSRTEAFF	TRBJ1-1	12	2
B266NGF-4RC	TRBV30	CAWRFRQGGQAFF	TRBJ1-1	11	2
B266NGF-1RC	TRBV30	CAWKVDLPLRVGETQYF	TRBJ2-5	15	1

Clone	TRBV id	CDR3 sequence	TRBJ id	Length	Frequency
B266NGF-7RC	TRBV30	CAWSANPYQPQHF	TRBJ1-5	11	1
B266NGF-8RC	TRBV30	CAWTIPRVGNYGYTF	TRBJ1-2	13	1
B266NGF-15	TRBV30	CAWRLASEQFF	TRBJ2-1	9	1
B266NGF-17RC	TRBV30	CASTRADTQYF	TRBJ2-3	9	1
B266NGF-19	TRBV30	CAWCLGQISGNTIYF	TRBJ1-3	13	1
B266NGF-22	TRBV30	CAWSVSSPQGDGYTF	TRBJ1-2	13	1

4.3 Discussion

We describe here the first robust and reliable method for the *in vitro* generation of potently suppressive human $CD4^+$ T cells using lentivirus-mediated gene transfer of FOXP3. In comparison to other methods involving LTR-driven expression of FOXP3, or expansion of *ex vivo* Tregs on the basis of cell-surface marker expression, this method generates a more homogeneous population of cells with stable suppressive capacity equal to that of $CD4^+CD25^{hi}$ Tregs. The phenotype of FOXP3-transduced cells also mimics Tregs with respect to high expression of several Treg-associated molecules, repression of CD127 (11), lack of cytokine production, and hypo-responsiveness to TCR stimulation. Analysis of surface marker expression in FOXP3-transduced cells confirms previous findings that CD127 is a target of FOXP3 repressor activity, but suggests that factors in addition to FOXP3 are required for the high CTLA-4 expression found in naturally occurring Tregs. This method allows functional Tregs to be generated from memory T cells, and does not rely on the preservation of Treg-associated cell-surface markers conventionally used to sort populations of resting or *ex vivo* T cells. A key advantage of our protocol over expansion of $CD4^+CD25^{hi}$ Tregs is the ability to re-sort populations of pCCL.FP3-transduced T cells on the basis of surface ΔNGFR expression, which is transcriptionally coupled to that of FOXP3 (32), to maintain a pure population of suppressive $FOXP3^{hi}$ cells. The numbers of Tregs that will be required for therapeutic applications is currently unknown. Since an unlimited number of $CD4^+CD25^-$ T cells can be transduced, and that once purified they can be expanded by an average of 80-fold in less than two weeks, this protocol should be useful for generating large numbers of Tregs that preserve a diverse TCR repertoire. Future studies will be required to determine the capacity of the pCCL.FP3-transduced cells to survive and expand *in vivo*.

The fact that IL-10 and TGF-β are not produced at high levels by FOXP3-transduced cells indicates that expression of this transcription factor alone is not

sufficient to induce their expression, and that high expression of these cytokines is not required for the *in vitro* suppressive function of these Tregs. Moreover, addition of TGF-β-blocking antibodies to *in vitro* suppression assays does not abrogate the inhibitory function of pCCL.FP3-transduced cells (data not shown), similar to *ex vivo* human Tregs (9, 40). These findings strengthen our conclusion that FOXP3 transduction recapitulates a Treg phenotype and not a Tr1-like phenotype. It is possible, however, that *in vivo* $FOXP3^+$ Tregs may mediate cytokine-dependent suppressive activity.

LTR-driven retroviral expression of Foxp3 in mouse $CD4^+$ T cells efficiently drives the development of Tregs (19). As we have demonstrated here, potently suppressive human Tregs can only be consistently induced by gene transfer when FOXP3 is under the control of an activation-independent promoter. Although retroviral LTR-driven expression was actually higher than that driven by EF1α at the peak of TCR-mediated activation, LTR-driven FOXP3 expression varied with activation state and was less potent than EF1α-driven expression in inducing suppressive cells. Thus stable expression of FOXP3 throughout the cycles of activation, expansion and resting appears necessary to efficiently convert human T cells to suppressor cells. Since the function of FOXP3 is influenced by its ability to physically interact with NFAT (41), the balance of these factors during the initial stages of T cell activation may influence the molecular activities of the protein and determine whether functional suppression is the biological outcome of its expression.

Importantly, our findings indicate that populations of $CD4^+CD25RO^+$ T cells can be transduced with pCCL.FP3 and switched into functional suppressor cells. Thus, memory T cells in the periphery may be biologically capable of acquiring regulatory function provided that high, constitutive FOXP3 expression is induced. Based on this evidence, it is possible that antigen-specific suppressor cells can be generated by *ex vivo* transduction of Teff cells specific for a defined antigen. Small numbers of antigen-specific cells of patient origin could thus be propagated *in vitro* to achieve therapeutically useful populations of antigen-specific $FOXP3^{hi}$ Tregs. Importantly, lentiviral vectors such as pCCL.FP3, which contain self-inactivating LTRs, pose significantly lower risks of genotoxicity than conventional expression vectors, in which transgene expression is driven by a retroviral LTR (42). Our strategy would therefore alleviate concerns related

to insertional mutagenesis and cell transformation, and ensure that engineered Tregs could be used safely for *in vivo* transplantation. Furthermore, ΔNGFR could be replaced with a gene encoding a selective marker, such that pCCL.FP3-transduced cells could be manipulated *in vivo*. Future *in vivo* experiments will be necessary to determine whether Tregs generated by pCCL.FP3 transduction have the *in vivo* suppressive efficacy required for successful cellular therapy applications.

4.4 Materials and methods

Construction and production of retroviral and lentiviral vectors. The Moloney Murine Leukemia Virus-based retroviral vectors encoding FOXP3 and ΔNGFR have been previously described (20). Third generation lentiviral vectors were constructed from a previously characterized vector, MA1, in which two transgenes are efficiently transcribed from a common bi-directional origin: ΔNGFR from the minimal CMV (mCMV) promoter, and a second transgene from the human PGK promoter (32). To construct pCCL.FP3, hFOXP3 was cloned in as the second transgene into MA1, and the PGK promoter was excised and replaced with the human EF1α promoter. The control pCCL vector carries the mCMV-ΔNGFR cassette and the EF1α promoter without a second transgene. Lentiviral vectors were produced by transient 4-plasmid overnight transfection of HEK 293T cells (32). The titers of concentrated virus were determined by limiting dilution on 3T3 or 293T cells, and titers of ~$2x10^9$/ml for retroviral and ~$2x10^8$/ml for lentiviral supernatants were routinely obtained.

Cell purification and expansion of T cell lines. Peripheral blood was obtained from healthy volunteers following approval by the University of British Columbia Clinical Research Ethics Board after obtaining written informed consent. PBMCs were isolated from buffy coats by Ficoll separation, and $CD4^+$ T cells were purified by negative selection (Stemcell Technologies Inc.). *Ex vivo* $CD4^+CD25^{hi}$ Tregs for suppression experiments were purified by positive selection over 2 columns (Miltenyi Biotec) to ensure 85–90% purity, or by FACs sorting for ≥99% purity for *in vitro* expansion. FACs sorted $CD4^+CD25^{hi}$ Tregs which remained >60% $FOXP3^+$ were expanded and tested in

parallel. To obtain pure (≥99%) $CD4^+CD25^-$ T cells, the $CD25^-$ fraction was either passed over an LD depletion column (Miltenyi Biotec) or FACS sorted. For isolation of $CD4^+CD25^-CD45RO^-$ T cells, $CD4^+CD25^-$ T cells were incubated with CD45RO beads (Miltenyi Biotech) and passed over an LD depletion column to achieve purities ≥95%. APCs were purified from PBMCs by depletion of CD3 cells (Stemcell Technologies Inc.). For expansion of T cell lines, a minimum of 200,000 T cells were re-stimulated every ~14 days with a feeder mixture consisting of $1x10^6$ allogeneic PBMCs/ml, $1x10^5$ EBV-transformed JY cells/ml, 1µg/ml PHA, and 100U/ml rhIL-2 (Chiron). In some experiments, 10ng/ml rhIL-7 (BD Biosciences) was added.

Retroviral and lentiviral transduction of primary and Jurkat T cells. Prior to transduction, $CD4^+CD25^-CD45RO^-$ T cells were activated in complete medium (X-VIVO 15 [Cambrex Corp.] supplemented with 5% pooled human serum [Cambrex Corp.], and penicillin/streptomycin [Invitrogen Corp.]), containing rhIL-2 (100U/ml; Chiron) and rhIL-7 (10ng/ml, BD Biosciences). Pre-activation consisted of either 48h exposure to immobilized anti-CD3 (1µg/ml OKT3; Orthoclone) and soluble anti-CD28 (1µg/ml; BD Biosciences), or for optimal transduction efficiency, 16h exposure to soluble anti-CD3 (1µg/ml OKT3) and autologous irradiated APCs, at a 1:5 ratio of T cells to APCs. For retroviral transduction, two successive rounds of infection were performed on retronectin-coated plates using a multiplicity of infection (MOI) of 20 virus particles per cell and 8µg/ml Polybrene (Sigma-Aldrich). For lentiviral transduction, T cells were infected once with lentiviral supernatant (MOI=10). For lentiviral transduction of $CD4^+CD45RO^+$ memory T cells, cells were first expanded *in vitro* with a standard feeder cell mixture for 14 days (see above). T cells were then re-stimulated for 16h with the standard feeder cell mixture prior to a single round of infection with lentiviral supernatant (MOI=10). $\Delta NGFR^+$-transduced T cells were purified 8 days following transduction using ΔNGFR-select beads (Miltenyi Biotec) and expanded in IL-2-containing medium. Prior to testing in functional and phenotypic assays, which was typically performed following 2 rounds of expansion (i.e. after 4 weeks in culture), T cells in the resting phase (12-14 days following activation) were washed and rested in IL-2 free medium overnight.

Untransduced cells tested in parallel were equivalent to control-transduced cells in all aspects of phenotype and function (data not shown).

Proliferation and Suppression Assays. To test proliferative capacity, 50,000 T cells/well were cultured in the presence of immobilized anti-CD3 (1μg/ml OKT3) in the presence or absence of IL-2 (100U/ml; Chiron). Proliferation was assessed after 72h, with [^{3}H]thymidine (1μCi/well, Amersham Biosciences) added for the final 16h of culture. To test for suppressive capacity, *ex vivo* $CD4^+CD25^-$ T cells were stimulated at 50,000 cells/well in the presence of anti-CD3 (1μg/ml OKT3) and 50,000 irradiated APCs. Transduced T cells or $CD4^+CD25^+$ T cells were added at numbers indicated in Figures, and suppression was assessed by measuring the amount of [^{3}H]thymidine incorporation in the final 16h of a 96h culture period, and/or by determination of IFN-γ concentration in culture supernatants after 72h using a Th1/Th2 cytometric bead array (BD Biosciences).

Flow cytometric analysis. Staining for cell-surface markers ΔNGFR (ATCC), CD69, CD25, CD127 (all BD Pharmingen), and GITR (R&D Systems) was carried out prior to intracellular staining for CTLA-4 (BD Pharmingen) or FOXP3 (clone PCH101 or 236A/E7), (eBiosciences), according to manufacturer's instructions. For analysis of intracellular cytokine staining, activated cells were fixed in 2% formaldehyde, permeabilized in 0.5% saponin, and stained with IL-2 and IFN-γ Abs (BD Biosciences). Samples were acquired on a BD FACSCanto and analyzed with FCS Express Pro Software Version 3 (De Novo Software).

Quantitative PCR. For quantitative RT-PCR, amounts of FOXP3 mRNA and GAPDH were determined as previously described (43). All samples were run in triplicate, and relative expression of FOXP3 was determined by normalizing to GAPDH to calculate a fold change in value.

Determination of cytokine concentration. To quantitate amounts of IL-2, IFN-γ, IL-4, and IL-10, capture ELISAs or Th1/Th2 cytometric bead arrays (both BD Biosciences) were performed on supernatants after activation with immobilized anti-CD3 (10μg/ml)

and soluble anti-CD28 (1µg/ml), for 24 hours (for IL-2) or 48 hours (for IFN-γ, IL-4, and IL-10). ELISAs for detection of TGF-β1 (R&D Systems) were used to analyze supernatants after 72 hours of activation with immobilized anti-CD3 and soluble anti-CD28. For analysis of intracellular cytokine production, T cells were activated with 10ng/ml PMA and 500 ng/ml Ca^{2+} ionophore (both Sigma-Aldrich) for 4 hours, or a 1:1 ratio of anti-CD3/CD28 coated beads (Invitrogen) for 6 hours, and brefeldin A (10µg/ml, Sigma-Aldrich) was added half-way through activation.

TCR β-chain cloning and diversity analysis. Total RNA was extracted from a minimum of 2 x 10^6 *ex vivo* $CD4^+$ T cells or transduced T cells using Trizol, and cDNA was synthesized using oligo$(dT)_{12-18}$ primers and reverse transcriptase (all from Invitrogen). TRBV7-3 and TRBV30 TCR β chains were amplified, cloned, and sequenced as described (38). Identification of TRBV and TRBJ segments and determination of CDR3 length and amino-acid sequences were performed using the IMGT/V-QUEST webtool (http://imgt.cines.fr/IMGT_vquest/) (44) and were verified manually. CDR3 length was computed according to (45). Nomenclature of TRBV and TRBJ segments is according to (46).

Statistics. All analyses for statistically significant differences were performed with 1-tailed paired Student's *t* test. *P* values of less than 0.05 were considered significant.

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5. Temporal and quantitative requirements for FOXP3 to induce regulatory phenotype and function in human $CD4^+$ T cells[4]

5.1 Introduction

FoxP3 is a forkhead family transcription factor that is involved in the development of $CD4^+CD25^+$ T regulatory cells (Tregs) (1-3) and regulation of immune homeostasis. Scurfy mice have a mutation in *FoxP3* which prevents expression of the full-length protein, resulting in the absence of Tregs in the periphery and subsequent rapid and fatal autoimmunity (4). The analogous human disease, immune dysregulation polyendocrinopathy X-linked (IPEX) syndrome, is caused by a variety of *FOXP3* mutations (5), and the pathology of other autoimmune diseases has been linked to defects Treg number and/or function (6). In addition to these data suggesting an essential role for FOXP3 in the development of Tregs, expression of this transcription factor also appears to be necessary for controlling their function. For example, ablation of *Foxp3* in the Treg cells of adult mice leads to development of autoimmune inflammation similar to that observed in Scurfy mice (7). Furthermore, attenuated expression of *Foxp3* in mouse Tregs causes abrogation of suppressor function, but leaves other aspects of the Treg phenotype, such as anergy and lack of cytokine production, intact (8).

While these data suggest that FoxP3 is essential for normal Treg development and function, other findings indicate that additional factors are required for commitment to, and stabilization of, the Treg lineage (9-11). For example, it was recently reported that several genes previously thought to be direct Foxp3 targets are not transactivated by, but instead are co-regulated with, this transcription factor (10). In addition, the data of other groups indicates that Foxp3 is important for normal Treg function but that other upstream events are important for defining and shaping the Treg lineage (9, 11). Thus, the precise roles of FoxP3 in driving the Treg phenotype and function remain to be clarified, particularly in the human system.

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[4] A version of this chapter has been accepted with minor alterations in *Eur J Immunol* as of April 16th, 2008. Allan, S. E., Song-Zhao, G. X., Abraham, T., Alstad, A., and Levings, M.K. Inducible reprogramming of human T cells into T regulatory cells by a conditionally active form of FOXP3.

We recently found that in order to convert human $CD4^+$ T cells into potent and stable Tregs, FOXP3 must be expressed constitutively at high levels that do not fluctuate depending on the state of T cell activation (12, 13). In addition, transient and low-level FOXP3 expression is not sufficient to impart Treg characteristics in human effector T (Teff) cells (14, 15). Thus, the temporal and quantitative requirements of FOXP3 to control the molecular and biological characteristics of human Tregs appear to be stringent. In order to further investigate whether FOXP3 acts as a quantitative regulator rather than a "master switch" for the development of Tregs, we developed a lentiviral vector encoding a conditionally-active FOXP3 protein, and investigated the consequences of inducing its activity in human $CD4^+$ T cells.

5.2 Results

Expression of a conditionally-active FOXP3 protein in human $CD4^+$ T cells. In order to develop a system for expression of a conditionally-active FOXP3 protein, we fused human FOXP3a to the steroid-binding domain of the estrogen receptor (ER). It has previously been shown that ER fusion proteins are conditionally active (16-19), and upon exposure of ER fusion proteins to the estrogen analogue 4-hydroxytamoxifen (4HT), there is a rapid and dose-dependent induction of protein functions. The activating effects of 4HT are reversed within 16h of 4HT withdrawal, as the drug is rapidly metabolized and broken down *in vitro* (18). The FOXP3-ER cDNA was transferred into a bi-directional lentivirus which also encodes a truncated version of the nerve growth factor receptor (ΔNGFR) as a marker gene (12) (**Fig. 5.1A**).

To determine the capacity of the FOXP3-ER lentiviral vector to drive high levels of protein expression in human $CD4^+$ T cells, we transduced naïve $CD4^+CD25^-CD45RO^-$ T cells with lentiviral supernatants and analyzed ΔNGFR-purified populations for FOXP3 expression by flow cytometry. Control ΔNGFR-transduced cells expressed a moderate amount of endogenous activation-induced FOXP3 (12, 14), while FOXP3-ER-transduced cells expressed levels of FOXP3 that were equivalent to those in T cells transduced with

wildtype FOXP3 (**Fig. 5.1B**). Thus, the FOXP3-ER-encoding lentivirus was suitable for over-expression of a stable fusion protein in naïve human $CD4^+$ T cells.

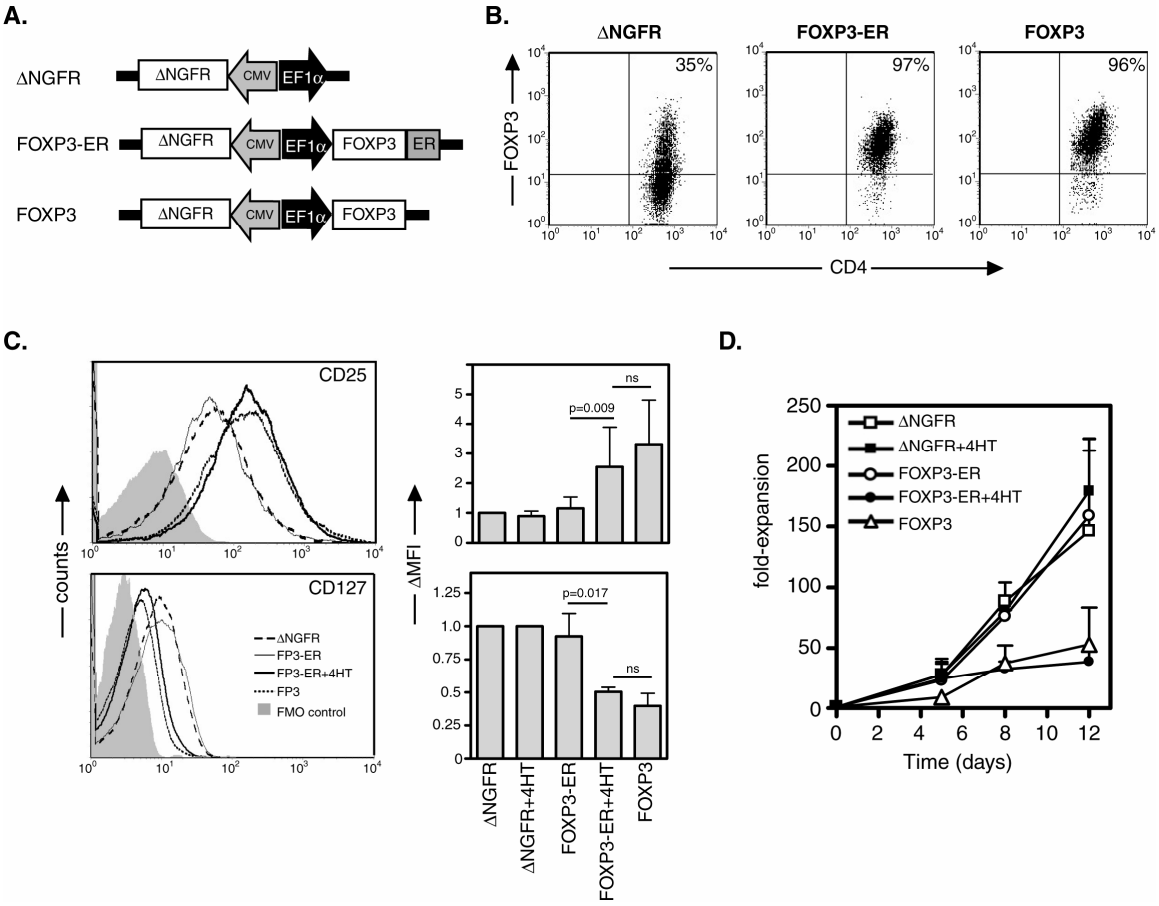

Figure 5.1 Characterization of transduced human $CD4^+$ T cells expressing conditionally-active FOXP3. (A) Illustration of control ΔNGFR, FOXP3-ER, and FOXP3 expressing lentiviral vector constructs. **(B)** Flow cytometric analysis of intracellular FOXP3 expression in T cells following transduction and purification on the basis of ΔNGFR expression. **(C)** Flow cytometric analysis of cell-surface CD25 and CD127 expression in transduced, purified T cells in the resting phase. Histograms show representative experiments, and graphs show averaged data obtained from transduced T cells of 4 different donors. ΔMFI indicates the fold-difference in the MFI of CD25 or CD127 expression of indicated populations compared to that of ΔNGFR-transduced T cells. **(D)** The fold-expansion of transduced, purified T cells over a 12-day culture period was determined by cell counting. Averaged data from 4 experiments is shown. Error bars in (C) & (D) indicate SD. FMO, fluorescence-minus-one; ns, non-significant; MFI, mean fluorescence intensity.

To determine if the conditionally-active FOXP3-ER protein was functional, we tested whether over-expression of FOXP3-ER upregulated expression of Treg-associated markers known to be direct transcriptional targets of FOXP3 (20, 21). ΔNGFR-purified T cell lines were expanded for 12 days in the presence or absence of 4HT and assayed for cell-surface CD25 and CD127 expression in the resting state (**Fig. 5.1C**). In the absence

of 4HT, FOXP3-ER- and ΔNGFR-transduced cells expressed equivalent levels of CD25 and CD127, confirming that fusion of the ER domain to FOXP3 prevents FOXP3 from acting as a transcription factor. In the presence of 4HT, FOXP3-ER-transduced cells upregulated CD25 and downregulated CD127 to levels that were equivalent to those in cells transduced with wildtype FOXP3, while the phenotype of ΔNGFR-transduced cells was unaffected by culture in 4HT.

In addition to altering the expression of cell-surface molecules, over-expression of FOXP3 results in induction of hyporesponsiveness (12). To further test the functionality of the conditionally-active FOXP3-ER protein, we determined the expansion of the transduced T cell lines in the presence or absence of 4HT over a 12 day time course. In the absence of 4HT, the expansion of the FOXP3-ER-transduced T cells was comparable to that of ΔNGFR-transduced cells (**Fig. 5.1D**). In the presence of 4HT, however, the expansion of the FOXP3-ER-transduced T cells was significantly less than in the absence of the drug ($p=0.037$, n=4 donors, t=12 days) and was similar to that of T cells transduced with wildtype FOXP3. Thus, FOXP3-ER acts as a conditionally-active protein, and, when activated by 4HT, can convert naive T cells into cells that are phenotypically similar to Tregs.

FOXP3 suppresses Th1 and Th2 cytokines in a time and dose-dependent manner. We and others have demonstrated that expression of FOXP3 in $CD4^+$ T cells is correlated with suppression of cytokine production (12, 13, 22, 23), and FOXP3 is known to directly interact with and repress the promoters of several cytokine genes (24). In order to define whether the amount of active FOXP3 is directly correlated with the degree of cytokine suppression, and to investigate the temporal nature of this effect, we stimulated ΔNGFR and FOXP3-ER transduced cells with anti-CD3/28 in the absence or presence of 4HT for different amounts of time and at different concentrations. As shown in Figure 5.2A, FOXP3-ER-transduced cells displayed a dose-dependent decrease in production of IL-2, IFN-γ, TNF-α, IL-10, IL-4, and IL-5 upon addition of 4HT at the time of TCR activation. To examine whether induction of FOXP3-ER activity prior to TCR activation augmented its inhibitory capacity, we also analysed cytokine production following either short-term (16h) or long-term (12 days) pre-treatment with 4HT. Pre-incubation with 4HT increased

the degree to which cytokine production was inhibited in FOXP3-ER-transduced T cells, with the most potent effects observed after long-term culture (**Figs. 5.2A & B**), while the cytokine profile of ΔNGFR-transduced cells was unaffected by exposure to the drug. In contrast to the effect of FOXP3 on the production of Th1 and Th2 cytokines tested, and in agreement with our previous findings (12), addition of 4HT to FOXP3-ER transduced T cells had no effect on TGF-β production (data not shown). These data imply that the full capacity of FOXP3 to inhibit cytokine production requires molecular events in addition to simple promoter binding, and likely also involves molecular and/or transcriptional changes that occur over longer periods of time. Moreover, the strict dose dependence of this effect supports our previous conclusion that high and constitutive levels of active FOXP3 are required to convert a naive T cell into a Treg (12).

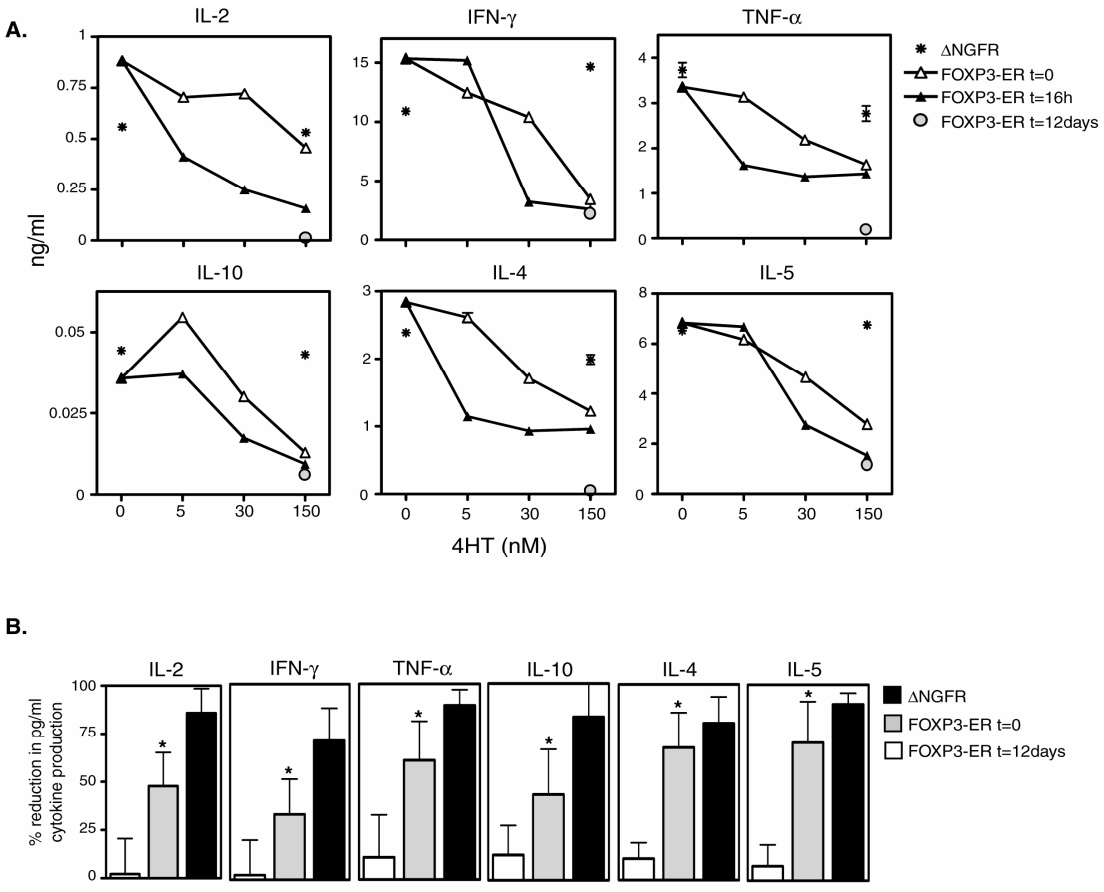

Figure 5.2 Inhibition of cytokine production by FOXP3 is dose- and time-dependent. (A) Transduced T cells were exposed to the indicated concentration of 4HT either at the time of the assay (t=0), 16h, or 12 days prior to stimulation with anti-CD3/28. Supernatants were collected after for 24h (IL-2) or 48h (all other cytokines) and analyzed using a Th1/Th2 cytometric bead assay. Plots are representative of data obtained from transduced T cells from a minimum of 3 donors. **(B)** % reduction in cytokine production in

ΔNGFR- or FOXP3-ER-transduced T cells upon addition of 150nM 4HT at the time of the assay (t=0) or for 12 days (t=12 days) as indicated. Data are averaged from a minimum of 5 different experiments. Error bars indicate SD. Statistics compared % reduction when 4HT was added at t=0 *vs* t=12d: * indicates p<0.015, ** indicates p<0.035.

Induction of hyporesponsiveness by FOXP3 is time and dose-dependent. As shown in Figure 5.1D, in the presence of 4HT, FOXP3-ER-transduced T cells had a reduced proliferative capacity over a 12-day expansion period. To determine whether the capacity of FOXP3 to induce hyporesponsiveness was dose- and time-dependent, we first assayed the ability of transduced T cells to respond to immobilized anti-CD3 over a 72h period in the presence of increasing concentrations of 4HT added at the time of stimulation (t=0 in **Fig. 5.2A**). While control ΔNGFR-transduced cells were unaffected by 4HT, FOXP3-ER-transduced T cells showed a dose-dependent decrease in proliferation, which at 150nM 4HT was suppressed to a level equivalent to that of T cells transduced with wildtype FOXP3 (**Fig. 5.3A**).

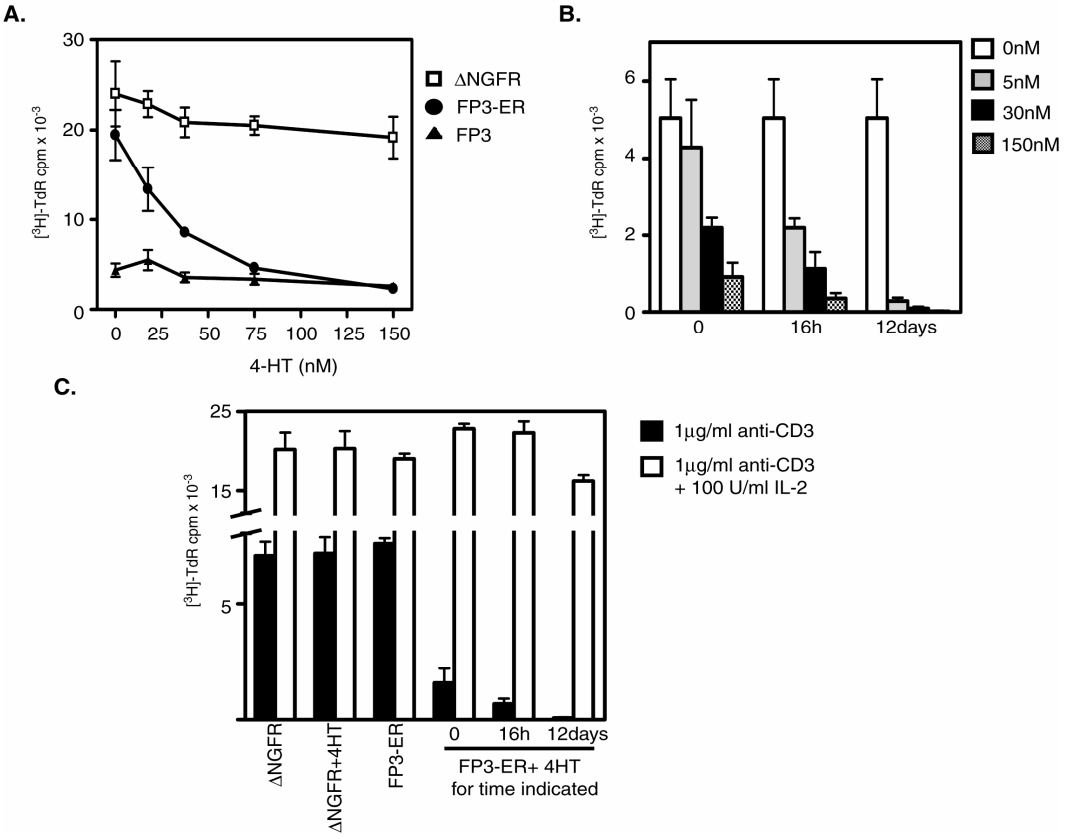

Figure 5.3 Induction of T cell hypo-responsiveness by FOXP3 is dose- and time-dependent. (A) Transduced T cells were tested for their ability to proliferate in response to immobilized anti-CD3 (1μg/ml) in the presence of increasing amounts of 4HT added at the time of the assay (t=0). **(B)** FOXP3-ER-transduced T cells were stimulated with a 1:1 ratio of irradiated APCs and soluble anti-CD3 (1μg/ml) and were exposed to increasing amounts of 4HT either at the time of the assay (t=0), 16h, or 12 days prior. **(C)** Transduced T cells were tested for their ability to respond to immobilized anti-CD3 (1μg/ml) in the

presence or absence of IL-2. 150nM 4HT was added at the time of the assay (t=0) or pre-incubated as indicated.

To further investigate the kinetic requirements for this effect, we compared the proliferative capacity of FOXP3-ER-transduced T cells pre-incubated with 4HT (for either 16h or 12 days), or exposed to 4HT at the time of the experiment. As observed for cytokine production, pre-incubation of FOXP3-ER-transduced cells with 4HT resulted in a more marked level of hyporesponsiveness, which increased with long-term exposure to the drug (**Fig. 5.3B**). Interestingly, addition of a low dose of 4HT (5nM) at the time of the assay had a minimal effect on proliferation, whereas long term culture in the same concentration of the drug resulted in potent hyporesponsiveness. The anti-proliferative effects of active FOXP3-ER did not lead to compromised viability, as anergy was fully reversed upon addition of 100 U/ml IL-2 (**Fig. 5.3C**). Thus, FOXP3 induces T cell hyporesponsiveness in a time- and dose-dependent manner.

High levels of FOXP3 activity are required prior to TCR activation to induce maximum suppressive capacity. We next determined whether expression of FOXP3-ER resulted in cells with inducible suppressive capacity. Addition of 4HT at the time of an *in vitro* suppression assay caused FOXP3-ER-transduced T cells to inhibit the proliferation of *ex vivo* $CD4^+CD25^-$ responder T cells in a dose-dependent manner (**Fig. 5.4A**). Importantly, pre-activation of FOXP3-ER for 72h prior to the assay resulted in a greater capacity of FOXP3-ER-transduced cells to inhibit T cell proliferation (**Fig. 5.4B**). Similar results were also obtained upon analysis of IFN-γ production: FOXP3-ER-transduced cells inhibited IFN-γ production by responder cells by only 13±22% when 4HT was added at the time of the assay, whereas pre-incubation with 4HT for 12 days resulted in 76±9% inhibition ($p=0.007$, $n=4$) (**Fig. 5.4C**). 4HT did not affect the proliferation of *ex vivo* $CD4^+CD25^-$ responder T cells, induce suppressor function in control ΔNGFR-transduced T cells, nor affect the suppressor function of *ex vivo* $CD4^+CD25^+$ Tregs (**Fig. 5.4A**). Thus, FOXP3-induced suppressor function has both quantitative and temporal requirements, and maximum regulatory capacity is induced only upon long-term and high expression of functional FOXP3.

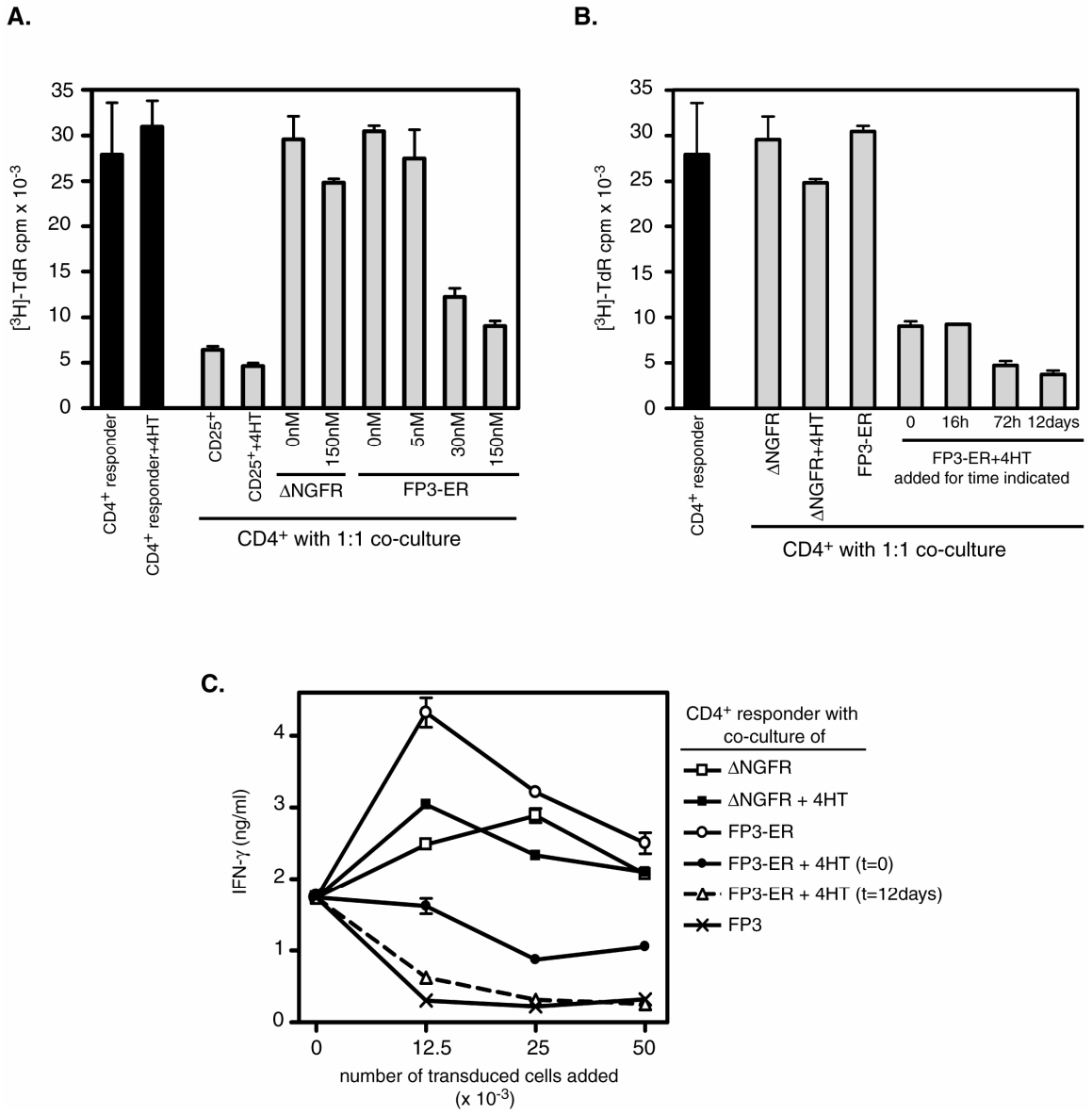

Figure 5.4 Maximum suppressor function requires high and long-term FOXP3 activity. 50,000 *ex vivo* $CD4^+CD25^-$ responder T cells were stimulated with a 1:1 ratio irradiated APCs and anti-CD3 (1μg/ml) in the presence or absence of transduced T cells as indicated. 4HT was added at the time of the experiment at the concentrations indicated in **(A)** & **(C)** and added at 150nM for the times indicated in **(B)**. All data are representative of a minimum of 3 experiments.

5.3 Discussion

We developed a conditionally-active form of FOXP3 to investigate temporal and quantitative requirements for this transcription factor to induce several of the key characteristics of human Tregs. Using lentivirus-mediated over-expression of FOXP3-ER, we demonstrate that the capacity of this transcription factor to repress the production of several Th1 and Th2 cytokines, induce T cell hyporesponsiveness, and promote suppressor function is influenced by the amount of FOXP3, as well as the time-frame of its activity, in human $CD4^+$ T cells.

The role of FOXP3 in regulating the production of inflammatory and regulatory cytokines has been a major topic of study. Here, we confirm our previous results that FOXP3 directly inhibits production of several Th1 and Th2 cytokines, and demonstrate that repression by FOXP3 is dose-dependent. In general, IL-10 and TGF-β are not involved in the suppressive mechanism of human Tregs (25), while in several mouse models these cytokines are essential *in vivo* (26). Our results indicate that neither IL-10 nor TGF-β are induced by FOXP3 activity, indicating that other factors may be required for Tregs to produce these cytokines, or that transduced T cells lack specific signals *in vitro* that may trigger their production an *in vivo* environment. Due to the fact that these cytokines are important in several disease models, it will be important to assess the mechanism of suppression of FOXP3-transduced cells *in vivo* to determine if these cytokines are involved.

FOXP3 was originally thought to be exclusively expressed in functional Tregs cells, and all $FOXP3^+$ cells were thought to be suppressive. We have shown here that the full acquisition of the Treg phenotype and function is dose-dependent. In addition, the phenotypic alterations induced by FOXP3 are only fully expressed following prolonged induction of protein activity, suggesting that transcriptional reprogramming likely requires new protein synthesis and molecular changes that enhance or reinforce the Treg phenotype. Such events could include physical interactions with other transcription factors (21) or alteration of chromatin structure by interaction with histone acetyltransferases and histone deacetylases (28) as previously reported. Our data support previous findings that activation-induced expression of FOXP3 is not sufficient to re-

program human T cells into Tregs (14, 15) and that only upon high and constitutive expression of FOXP3 is the Treg phenotype and function recapitulated (12).

The fact that the level of FOXP3 activity is directly correlated with suppressor function suggests that even small reductions in FOXP3 expression or activity would markedly affect the action of Tregs *in vivo*. Thus, measuring the intensity of FOXP3 protein expression on a per-cell basis may a useful indicator of Treg function in patients suffering from immune-mediated diseases. Indeed, it has been reported that IPEX can be caused by mutations in the promoter region of FOXP3 which cause reduced expression (29). In addition, a recent study of multiple sclerosis patients found a significant decrease in the FOXP3 expression and suppressive function in the $CD4^{+}CD25^{high}$ subset of patients compared to healthy controls (30). Our data also indicate that enhancing FOXP3 expression or molecular function could enhance the suppressive functions of Tregs *in vivo*. In line with this idea, it has recently been demonstrated that augmenting the acetylation state, and thus molecular function, of Foxp3 by treatment with the histone deacetlyase inhibitor Trichostatin A leads to enhanced Treg function *in vivo* (31). Thus reduced FOXP3 expression may contribute to the development of autoimmune diseases, while targeted manipulation of its expression will be a useful tool to modulate the immune responses.

In conclusion, using a drug-inducible FOXP3 we have clearly defined that the capacity of FOXP3 to reprogram T cells into Tregs is dose- and time-dependent. These data indicate that simply classifying T cells as $FOXP3^{+}$ or $FOXP3^{-}$ is not sufficient to make conclusions regarding suppressive function. Expression levels of FOXP3 must be carefully quantified and compared to *ex vivo* Tregs over the course of the T cell activation cycle before extrapolations on the functional status of cells can be made. The conditionally-active FOXP3-ER protein will also be a valuble tool to further study the cellular and molecular phenotype of Tregs and to regulate their function in vivo.

5.4 Materials and methods

Construction and production of lentiviral vectors. The third generation bi-directional lentiviral ΔNGFR control and FOXP3-encoding lentiviral vectors have been previously described (12). The lentiviral backbone efficiently transcribes two different transgenes from a common bi-directional origin: ΔNGFR from the minimal CMV promoter, and a second transgene from the human EF1α promoter. To construct an expression system for conditionally-active FOXP3, cDNA encoding FOXP3 fused at the C-terminus to the steroid-binding domain of the mouse estrogen receptor (ER) domain was cloned into the lentiviral backbone to construct FOXP3-ER. The added region of the ER domain been modified such that it does not bind endogenous estrogen but is capable of interacting with the estrogen analog 4-hydroxytamoxifen (4HT) (19). Lentiviral vectors were produced by transient 4-plasmid overnight transfection of HEK 293T cells (32). The titers of concentrated virus were determined by limiting dilution on 293T cells, and titers of ~$2x10^9$/ml were routinely obtained.

Cell purification and expansion of T cell lines. Peripheral blood was obtained from healthy volunteers following approval by the University of British Columbia Clinical Research Ethics Board after obtaining written informed consent. Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats by Ficoll separation, and $CD4^+$ T cells were purified by negative selection (Stemcell Technologies Inc.). *Ex vivo* $CD4^+CD25^{hi}$ Tregs for suppression experiments were purified by positive selection over 2 columns (Miltenyi Biotec) to ensure 85–90% purity. For isolation of $CD4^+CD25^-$ $CD45RO^-$ T cells, $CD4^+$ T cells were incubated with CD45RO and CD25 beads (Miltenyi Biotech) and passed over LD depletion columns to achieve purities ≥95%. APCs were purified from PBMCs by depletion of CD3 cells (Stemcell Technologies Inc.). For expansion of T cell lines, a minimum of 200,000 T cells were re-stimulated every ~14

days with a feeder mixture consisting of $1x10^6$ allogeneic PBMCs/ml (irradiated 5000 RADS), $1x10^5$ EBV-transformed JY cells/ml (irradiated 7500 RADS), 1μg/ml PHA, and 100U/ml rhIL-2 (Chiron).

Lentiviral transduction and culture of primary $CD4^+$ T cells. Prior to transduction, $CD4^+CD25^-CD45RO^-$ T cells were activated in complete medium (X-VIVO 15 [Cambrex Corp.] supplemented with 5% pooled human serum [Cambrex Corp.], and penicillin/streptomycin [Invitrogen Corp.]), containing rhIL-2 (100U/ml) and rhIL-7 (10ng/ml, BD Biosciences). Pre-activation consisted of 16h exposure to soluble anti-CD3 (1μg/ml OKT3) and autologous irradiated APCs, at a 1:5 ratio of T cells to APCs (irradiated 5000 RADS). T cells were infected once with lentiviral supernatant (MOI=10). $\Delta NGFR^+$-transduced T cells were purified 8 days following transduction using ΔNGFR-select beads (Miltenyi Biotec) and expanded in IL-2-containing medium. Prior to testing in functional and phenotypic assays, which was typically performed following 2 rounds of expansion (i.e. after 4 weeks in culture), T cells in the resting phase (12-14 days following activation) were washed and rested in IL-2 free medium overnight. Untransduced cells tested in parallel were equivalent to control-transduced cells in all aspects of phenotype and function (data not shown). In some cases, 4-hydroxytamoxifen (4HT) (Sigma-Aldrich) solubilized in ethanol was added to cultures at time points indicated in Figures, or for long-term expansion was replenished every 2 days. The concentration of 4HT was 150nM unless otherwise indicated, and ethanol in cultures never exceeded 0.2%.

Proliferation and Suppression Assays. To test proliferative capacity, 50,000 T cells/well were cultured either in the presence of immobilized anti-CD3 (at concentrations indicated in Figures) or a 1:1 ratio of APCs (irradiated 5000 RADs) with 1μg/ml soluble anti-CD3 in the presence or absence of IL-2 (100U/ml). Proliferation was assessed after 72h (immobilized anti-CD3 assay) or 96h (APC/anti-CD3 assay) by [^{3}H]thymidine (1μCi/well, Amersham Biosciences) incorporation, added for the final 16h of culture. To test for suppressive capacity, *ex vivo* $CD4^+CD25^-$ T cells were stimulated at 50,000 cells/well in the presence of anti-CD3 (1μg/ml OKT3) and 50,000 irradiated

APCs (5000 RADS). Transduced T cells or *ex vivo* $CD4^+CD25^+$ Tregs were added at numbers indicated in Figures, and suppression was assessed by measuring the amount of [^{3}H]thymidine incorporation in the final 16h of a 96h culture period, and/or by determination of IFN-γ concentration in culture supernatants after 72h using a Th1/Th2 cytometric bead array (BD Biosciences).

Flow cytometric analysis. Staining for cell-surface markers ΔNGFR, CD25, CD127 (all BD Pharmingen) was carried out prior to intracellular staining for FOXP3 (clone 236A/E7, eBiosciences), according to manufacturer's instructions. Samples were acquired on a BD FACSCanto and analyzed with FCS Express Pro Software Version 3 (De Novo Software).

Determination of cytokine concentration. To quantitate amounts of IL-2, IFN-γ, TNF−α, IL-10, IL-4, and IL-5, Th1/Th2 cytometric bead arrays (BD Biosciences) were performed on supernatants after activation of T cells with immobilized anti-CD3 (10µg/ml) and soluble anti-CD28 (1µg/ml) for 24 hours (for IL-2) or 48 hours (all others). ELISAs for detection of TGF-β1 (R&D Systems) were used to analyze supernatants after 48 or 72 hours of activation with immobilized anti-CD3 (10µg/ml) and soluble anti-CD28 (1µg/ml).

Statistics. All analyses for statistically significant differences were performed with 1-tailed paired Student's *t* test. *P* values of less than 0.05 were considered significant.

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6. Discussion & Conclusions

6.1 Overview, speculations, and future perspectives

The aim of this research was to investigate the role of a newly identified transcription factor, FOXP3, in driving the generation and function of human Tregs. Based on early mouse studies (1-3), this protein appeared to be a critical checkpoint for control of immune homeostasis. Therefore, more information on its biological function was in great demand. The scope of the research carried out included 1) investigation of activation-induced FOXP3 in human T effector (Teff) cells 2) description and characterization of a novel FOXP3 splice isoform 3) development of a robust method for the *in vitro* generation of Tregs by lentiviral mediated gene transfer of FOXP3 and 4) elucidation of quantitative and temporal requirements for this protein to induce full regulatory functions in human $CD4^+$ T cells.

As discussed in Chapter 2, $CD4^+CD25^-FOXP3^-$ human $CD4^+$ T cells are capable of upregulating FOXP3 mRNA and protein following TCR stimulation. Based on the fact that $FOXP3^+$ Tregs are anergic and do not produce cytokines, it was initially hypothesized that the function of activation-induced FOXP3 in non-Teg cells could be to negatively regulate these pathways. However, the data presented in Chapter 2 demonstrates that Teff cells that become $FOXP3^+$ proliferate at least as much, and make elevated levels of cytokines, compared to $FOXP3^-$ Teff cells. Thus, FOXP3 appears to mark highly activated cells, does not directly suppress effector functions, nor induce a Treg phenotype in Teff cells. The transient nature of this expression in Teff cells is in marked contrast to the high and constitutive pattern of FOXP3 expression found in Tregs, suggesting that this transcription factor could be differentially regulated in the two cell types.

Importantly, it has been demonstrated that polyclonal as well as antigen-specific activation of Teff cells leads to FOXP3 upregulation (4). Our data indicates that upregulation of FOXP3 occurs independently of CD28 activation or ligation of other co-stimulatory molecules, and instead correlates with the degree of endogenous IL-2 produced by the cells. Indeed, our subsequent studies investigating the role of IL-2 and other cytokines that signal through the common gamma chain suggested that this pathway

is essential for upregulating IL-2 (5). It is likely that cytokine-mediated activation of STAT5 is involved in activating the FOXP3 gene, based both on molecular data of other groups (6-8) as well as our own experiments. Interestingly, lentiviral over-expression of a constitutively active form of STAT5a resulted in a significant and sustained upregulation of FOXP3 in $CD4^+CD25^-$ T cells, but without concomitant induction of suppressor function (5). This is in agreement with our own data (9) and that of others (10, 11) that activation-induced FOXP3 in Teff cells does not necessarily endow them with suppressor function.

One report characterizing activation-induced FOXP3 suggested that a transient state of suppressor function was induced in $FOXP3^+$ Teff cells. Wang *et al* found a variation in the ability of Teff cells to suppress *in vitro*: in 3 of 9 experiments, inhibition of proliferation was exhibited by $FOXP3^+$ Teff cells (10). While these authors suggested that inter-individual differences were due to a donor-intrinsic capacity of FOXP3 to induce suppressor function in Teff cells, it is more likely that intersecting pathways or other regulatory mechanisms operating in activated Teff cells are involved. Based on these studies and the data presented in Chapter 2, it is not possible to demonstrate a mechanistic link between FOXP3 and suppressor function in Teff cells, but only to correlate the two events. Indeed, it is technically difficult to assess the biological capacity of highly activated cells due to the phenomenon of activation-induced cell death upon re-stimulation through the TCR, and thus other approaches will be necessary to examine the role of FOXP3 in Teff cells. Studies in progress aim to investigate the effector functions of T cell clones isolated from a female carrying a *FOXP3* mutation in the initiating ATG codon (which prevents FOXP3 protein expression). This carrier was identified following the death of her male child shortly after birth due to severe IPEX. Based on random X-inactivation, Teff cell clones isolated from the carrier mother should express either the normal or mutated *FOXP3* gene. Preliminary data indicates that approximately 50% of isolated Teff cell clones express the mutant allele, suggesting that this transcription factor is not essential for development of Teff cells in the periphery. Interestingly, a panel of $FOXP3^-$ and $FOXP3^+$ Teff cell clones tested *in vitro* were found to have comparable proliferative capacity and cytokine production, supporting our conclusions that activation-induced FOXP3 is not a direct negative regulator of these pathways. Recent

data from the Benoist laboratory indicates that Foxp3 expression in mouse T cells is important for responsiveness to TGF-β (12). Thus, future experiments on $FOXP3^+$ *vs* $FOXP3^-$ human Teff cells and Teff cell clones will address whether FOXP3 influences the ability of T cells to respond to TGF-β. Additional follow-up studies will be useful to identify whether there is an essential and/or alternate role for this transcription factor in regulating Teff cell responses.

Based on our initial studies it was clear that, like mouse Tregs, human Tregs expressed very high levels of FOXP3. However, in contrast to mouse studies and as discussed in Chapter 3, we found that over-expression of FOXP3 in naïve human $CD25^-$ T cells using a retroviral vector was not sufficient to induce potent regulatory function. Our finding that human Tregs and activated Teff cells expressed two different isoforms of FOXP3 (formerly FOXP3 and FOXP3Δ2; more recently FOXP3a and FOXP3b) prompted us to test whether simultaneous expression of both isoforms was required for induction of full regulatory function, but these experiments revealed that retroviral expression of both isoforms was also insufficient.

This result was somewhat of a surprise, as high homology of FoxP3 between the two species suggested an analogous role. Based on our observations that endogenous FOXP3 expression was both dynamic and differentially regulated in Treg vs Teff cells, a working hypothesis developed that human $CD4^+$ T cells might require specific patterns of FOXP3 expression for induction of full regulatory function. To probe this hypothesis, we set out to examine more closely the kinetics of ectopic expression by our retroviral vector. We developed an improved lentiviral vector designed for transduction of a broader range of cell types, and compared its ability to drive FOXP3 expression to that of the retroviral vector. As discussed in Chapter 4, this investigation revealed a significant difference between the two vector systems, and subsequently a major difference in the phenotype of cells transduced with each vector type. Here, results demonstrated that only high and constitutive ectopic FOXP3 expression (such as that driven by the lentiviral vector encoding an internal EF1α promoter) was sufficient to induce regulatory function, while transient and/or fluctuating expression (such as that driven by the retroviral activation-dependent LTR) was insufficient. These findings were in line with the data in

Chapter 2 demonstrating that transient FOXP3 expression in activated Teff cells did not lead to acquisition of regulatory activity.

Studies of endogenous patterns of FOXP3 expression (Chapter 2), as well as the consequences of ectopic FOXP3 expression (Chapters 3 & 4), suggested that both the dose and the kinetics of FOXP3 expression could be related to its capacity to promote regulatory phenotype and function in human $CD4^+$ T cells. To more closely examine these aspects, a system for ectopic expression of a conditionally-active FOXP3 protein was developed. By fusing a modified version of the steroid-binding domain of the estrogen receptor to FOXP3, we created a protein that was functional only in the presence of the drug 4-hydroxytamoxifen (4HT). In studying $CD4^+$ T cells transduced with conditionally-active FOXP3, it was possible to address questions related to both the dose and timing of FOXP3 by fine-tuning the activity of the fusion protein with controlled addition of 4HT. In the experiments summarized in Chapter 5, both a quantitative and temporal effect of FOXP3 on several different Treg characteristics was confirmed including hyporesponsiveness, suppression of cytokine production, and induction of suppressive capacity. Overall, these results suggest that 1) the presence of active FOXP3 in the nucleus for a significant period prior to TCR-activation is critical for maximal suppressive capacity to be induced and 2) that higher levels of functional FOXP3 are correlated with a stable *bona fide* Treg phenotype.

6.2 FoxP3 in mice and men

Initial analyses of human $CD4^+$ T cells revealed two important findings distinct from results of mouse T cells: 1) that human T cells expressed two isoforms of FOXP3 and 2) that non-Treg cells could express FOXP3 under activating conditions, indicating species-specific differences in regulation and/or function of this protein. In addition to the species-specific differences revealed in the course of this research, other distinctions in FoxP3 in mice and men should prompt scientists to use caution when translating knowledge gained from using animal models to human applications.

As demonstrated in Chapter 3, FOXP3Δ2 (now known as FOXP3b) is less potent than full-length FOXP3 (FOXP3a) at inducing aspects of a Treg phenotype. The mechanism for the difference between FOXP3a and FOXP3b was not further explored,

although we speculated that the region lacking in FOXP3b (exon 2) may have an important functional role in the generation or function of Tregs. Indeed, it was later reported that exon 2 is a functionally important region of the N terminal repressor domain. It has been shown to mediate physical interactions with other proteins (13), and in particular is involved in the repression of another transcription factor named retinoic acid receptor-related orphan receptor C (RORC) (S. Zeigler, World Immune Regulation Meeting; Davos, Switzerland, April 2007). RORC is thought to be a key transcription factor for the differentiation of human IL-17-producing T cells (14), which have recently been found to be major drivers of autoimmune and chronic pathological inflammation in mice (15). It has been hypothesized that FOXP3a but not FOXP3b is able to repress RORC, and that expression levels of the two isoforms may influence Th17 differentiation (D. Littman, International Congress of Immunology; Rio de Janeiro, Brazil, August 2007). Mouse T cells, with only the full-length repressive form of Foxp3, would lack this ability. Interestingly, other mammals including monkeys (16) and dogs (S. Ziegler, pers. comm.) also express both isoforms of this protein. Future experiments may reveal why mice T cells lack the smaller isoform, and define its precise function in the T cells of animals that express it.

A second major species-specific difference is that FoxP3 is not a specific marker for Treg cells in humans, as it can be expressed outside of this cell subset. In contrast, mouse Foxp3 expression seems to be largely limited to Treg cells and is a reliable indicator of regulatory activity. In the mouse system, the induction of Foxp3 expression in non-Treg cells appears to be dependent on the presence of both TGF-β and IL-2 (17), and the resulting cells are suppressive both *in vivo* and *in vitro*. However, the phenotype of these cells is not stable, and in the absence of the factors which induced them, their Foxp3 expression and suppressive capacity are rapidly lost (12). Results defining the reliance on TGF-β for activation-induced FOXP3 expression in human T cells is less conclusive. While data from Ethan Shevach's laboratory demonstrated a strict requirement for endogenous TGF-β (11), in our hands, blocking this cytokine has only a partial effect in preventing activation-induced FOXP3. Regardless of this controversy, it is clear is that FOXP3 expression in human Teff cells does not confer suppressive function, even transiently (5, 9-11).

6.3 Therapeutic relevance

The finding that human Teff cells can express FOXP3 is of major clinical relevance, as it has recently been proposed that this protein can be used as a diagnostic indicator of tolerance in settings such as autoimmune disease and organ transplantation. It is likely that this would be an inaccurate measure of tolerance, as immune activation is characteristic of such patients, and essentially all Teff cells are capable of expressing FOXP3 under activating conditions. Thus, FOXP3 expression *in vivo* likely marks Tregs as well as activated Teff cells. Until a more specific marker of Tregs is revealed, functional assays are the most reliable means by which to test for the presence of Tregs in patients.

Experimental models have demonstrated that Tregs have great potential to be manipulated to benefit clinical applications including autoimmune diseases, allergy, transplantation, and cancer (18, 19). Before Treg knowledge can be translated to the clinic however, immunologists face many challenges. In addition to increased information regarding the *in vivo* development, function, and survival of these cells, a major caveat to developing cellular therapy applications is the generation of sufficient numbers of Tregs to treat patients. Because these cells make up only a small fraction (<5%) of circulating $CD4^+$ T cells, it is not possible to purify Tregs from a single donor in numbers adequate for therapy. Great efforts are currently being made to develop methods for expansion of Tregs *in vitro* without affecting their regulatory phenotype, while other groups are working on methods to convert non-Treg cells into Tregs. The method of FOXP3 lentiviral transduction described here can potentially be developed for clinical applications, as an unlimited number of Teff cells could be transduced with FOXP3 to yield large numbers of suppressive T cells. As discussed in Chapter 4, Tregs generating using this system are stable and can be expanded as a homeogenous population. In the mouse system, it has been shown that Foxp3-transduced cells have the ability to limit autoimmune disease, inflammation, and transplant rejection (2, 20-25).

Several of these studies, as well as a large amount of data on naturally occurring Tregs in a variety of pre-clinical models (19), suggests that antigen-specificity will be an important factor in designing cellular therapy applications. As well as specific activation

of Treg functions upon antigen-specific triggering (26), infusion of Tregs specific for an organ- or disease-specific antigen appears to promote trafficking of infused Tregs to the site of inflammation, increasing the efficacy of the treatment. Importantly, our ongoing experiments demonstrate that antigen-specific human T cell populations can be "switched" into cells with regulatory properties upon FOXP3 transduction, suggesting that it might be possible to generate pools of antigen-specific Tregs by this method. It will be essential to determine the *in vivo* behavior of these cells, including whether their potent suppressive abilities are preserved, whether the cells can traffic efficiently to sites of inflammation, and how long the cells can persist and act as immune regulators. Experiments in progress will make use of a *trans vivo* model of mouse islet xenograft rejection to address these questions and assess the utility of FOXP3-transduced cells as a candidate for human cellular therapy applications.

A broad range of immune-mediated diseases have recently been linked to altered FOXP3 expression or defects in Tregs (27-31), highlighting its potential importance in a range of organ-specific diseases in addition to the pathology of IPEX. Furthermore, characterization of activation-induced FOXP3 in human Teff cells has opened up the possibility that this transcription factor may have an important and/or alternate role outside of the Treg subset. Indeed, the recent interesting finding that FOXP3 can act as a tumor suppressor in breast epithelial cells (32, 33) indicates that this protein has distinct functions even outside of the hematopoeitic system. Identification of endogenous pathways or exogenous factors that specifically activate the FOXP3 gene will be of great therapeutic potential, while further development of cellular therapy applications with *in vitro* expanded Tregs or FOXP3-transduced T cells will open up the field of cellular therapy for immune-mediated diseases.

6.4 In closing

The fact that $FOXP3^+$ Teff cells do not acquire regulatory functions suggests that FOXP3 is not a "master switch" for regulatory function – that is, induction of FOXP3 expression alone is not sufficient to endow a T cell with Treg properties. Instead, this protein might work in concert with other Treg-inducing factors and/or have an alternate role outside of the Treg subset. Nevertheless, this protein clearly has a central and

essential role in controlling immune homeostasis, as individuals and animals with FOXP3 mutations suffer from devastating autoimmunity. As discussed in Chapter 4 and 5, ectopic FOXP3 expression is sufficient to re-program both naïve and mature $CD4^+$ T cells to acquire a regulatory phenotype, provided that its expression is high and constitutive. This pattern of expression is the limiting factor for the induction of regulatory function, and thus might be sufficient to enable the other molecular events that are crucial for induction of a Treg phenotype. Therefore, although FOXP3 might not be the "master switch" for Tregs as was originally hoped, it is certainly an essential (and possibly the most important) link in the circuit that controls Treg-mediated immune homeostasis.

Overall, the research presented here has contributed significantly to understanding the biological role of FOXP3 in human cells. In addition, this work has established a robust method for the *in vitro* generation of human Tregs suitable for further biological study, and potentially for the development of antigen-specific cellular therapy applications.

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A.1: UBC Ethics Approval

The University of British Columbia
Office of Research Services
Clinical Research Ethics Board – Room 210, 828 West 10th Avenue, Vancouver, BC V5Z 1L8

ETHICS CERTIFICATE OF EXPEDITED APPROVAL: AMENDMENT

PRINCIPAL INVESTIGATOR:	DEPARTMENT:	UBC CREB NUMBER:
Megan K. Levings		H03-70062

INSTITUTION(S) WHERE RESEARCH WILL BE CARRIED OUT:

Institution	Site
Vancouver Coastal Health (VCHRI/VCHA)	Vancouver General Hospital

Other locations where the research will be conducted:
N/A

CO-INVESTIGATOR(S):
Rosa Garcia
Alicia Alstad
Raewyn Broady
Megan Himmel
Sarah E. Allan
Sarah Crome

SPONSORING AGENCIES:
British Columbia Transplant Society
Canadian Institutes of Health Research (CIHR) - "Immunomodulation of Regulatory Mechanisms in Mucosal Immunity: A multi-disciplinary bench-to-bedside approach to the study and treatment of IBD "
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Vancouver Coastal Health Authority

PROJECT TITLE:
Extracorporeal photopheresis: a novel approach to induce transplantation tolerance?

REMINDER: The current UBC CREB approval for this study expires: February 18, 2009

AMENDMENT(S):			AMENDMENT APPROVAL DATE:
Document Name	Version	Date	**February 26, 2008**
Addition of Funding DD 20 February 2008			

CERTIFICATION:
In respect of clinical trials:
1. The membership of this Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Division 5 of the Food and Drug Regulations.
2. The Research Ethics Board carries out its functions in a manner consistent with Good Clinical Practices.
3. This Research Ethics Board has reviewed and approved the clinical trial protocol and informed consent form for the trial which is to be conducted by the qualified investigator named above at the specified clinical trial site. This approval and the views of this Research Ethics Board have been documented in writing.

The amendment(s) for the above-named project has been reviewed by the Chair of the University of British Columbia

Vancouver Coastal Health Research Institute
Healthier lives through discovery

Bernard H. Bressler, PhD

Vice President, Research, Vancouver Coastal Health
Executive Director, Vancouver Coastal Health Research Institute

Assistant Dean Research, Faculty of Medicine
The University of British Columbia

February 28, 2008

Dr. Megan Levings
Jack Bell Research Center
2660 Oak Street
Vancouver, B.C.

Vancouver Coastal Health Authority Research Study #V03-0053

RENEWAL CERTIFICATE OF APPROVAL

TITLE: "Extracorporeal Photopheresis: A Novel Approach to Induce Transplantation Tolerance?"; "Generation of Antigen Specific T-Regulatory Cells for Prevention of GVHD through Induction of Transplantation Tolerance"; "Immunomodulation of Regulatory Mechanisms in Mucosal Immunity: A Multi-Disciplinary Bench-to-Bedside Approach to the Study and Treatment of IBD"

SPONSOR: Canadian Institutes of Health Research (CIHR), BC Transplant Society, VGH & UBC Hospital Foundation, National Cancer Institute of Canada; Roche Organ Transplant Research Foundation; Vancouver Coastal Health Authority

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This is to inform you that your project has been renewed for one year. Approval has been granted until **February 18, 2009** based on the following:

1. UBC Ethics Committee Certificate of Renewal Approval – H03-70062
2. VCHA Request for Renewal Reply

Yours truly,

For:
Dr. Bernard Bressler
Vice-President Research

Clinical Research Ethics Board and the accompanying documentation was found to be acceptable on ethical grounds for research involving human subjects.

Approval of the Clinical Research Ethics Board by:

Dr. Bonita Sawatzky, Associate Chair