FoxP3 Regulates T Follicular Regulatory Cell Function

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This Thesis, FoxP3 Regulates T follicular regulatory cell function, presented by Shenda Hou, and Submitted to the Faculty of The Harvard Medical School in Partial Fulfillment of the Requirements for the Degree of Master of Medical Sciences in Immunology has been read and approved by:

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FoxP3 Regulates T Follicular Regulatory Cell Function

Shenda Hou

A Thesis Submitted to the Faculty of

The Harvard Medical School

in Partial Fulfillment of the Requirements

for the Degree of Master of Medical Sciences in Immunology

Harvard University

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Abstract

T follicular regulatory (Tfr) cells, a subset of regulatory T (Treg) cells, suppress T follicular helper (Tfh) cell mediated B cell responses. However, it is still unclear how Tfr cells function. In this study, we showed that the transcription factor FoxP3 is an essential regulator of the suppressive function of Tfr cells. By using a FoxP3 knockout model (FoxP3^{F/F}, UBC^{ERT2-Cre}), we showed that Tfr cells adopt certain Tfh phenotypes and lose their suppression-related molecules when they lose FoxP3. Interestingly, we found that some Tfr cells naturally downregulate FoxP3 in control mice (FoxP3^{F/F}). This finding inspired us to develop a FoxP3 fate mapper model (Rosa26 lox-stop-lox-Tdtomato, FoxP3-Cre-YFP) which can mark the FoxP3 downregulated Tfr cells with Tdtomato expression. We examined the phenotype of the cells via flow cytometry and assessed the suppressive function of Tfr cells by co-culturing them with B cells and Tfh cells. Our data revealed that Tfr cells that natural downregulate FoxP3 lose the expression of the molecules related to the suppressive function, such as CTLA4, CD25, GITR, ICOS and cannot suppress class switch recombination and antibody production. These findings provide evidence supporting the idea that FoxP3 is critical for Tfr cell suppressive function.
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high CXCR5 (and high CD69) expression follows CXCL13 gradients to the GC. In the GC, Tfh cells interact with GC B cells, stimulating them to undergo activation, class switch recombination, affinity maturation, and differentiation into memory B cells and plasma cells. In the GC, Tfr cells interact with Tfh and GC B cells, leading to suppression of both Tfh and GC B cells. Box B shows a schematic of Tfr suppression of Tfh cells. Tfr cells suppress Tfh cell production of cytokines such as IL21, IL4 and IFNγ that stimulate B cells. Box C shows a schematic of Tfr suppression of GC B cells where positive mediators of Tfr suppression are in green and negative regulators of Tfr suppression are in red².

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**Figure 3.6. The design of FoxP3 Fate Mapper model.** When FoxP3 expressing regulatory cells were generated in FoxP3-IRES-CRE-YFP; Rosa26 Lox-STOP-Lox-Tdtomato mice, FoxP3 was expressing along with CRE recombinase and YFP (left). The cells would be YFP+. Meanwhile, CRE recombinase would cut the Stop cassette and the cells would also be Tdtomato+ (middle). If FoxP3 stops expressing at later time point, YFP which was under the same promoter will stop expressing as well. However, Tdtomato which is in Rosa26 locus with no stop cassette ahead any longer will still be expressing (lower right). If FoxP3 continuously express, the cells will remain YFP+Tdtomato+ (upper right). By detecting the fluorescent proteins, the FoxP3 expression status would be known.

**Figure 3.7. In vivo generated exTfr cells maintain similar phenotype as in vitro exTfr cells.** Immunized FoxP3 Fate Mapper mice with NP-OVA and harvested draining lymph nodes on day 7. A. Flow cytometry of lymphocytes. The representative plots showed the gating strategy for
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**Figure 3.8. In vivo generated exTfr cells cannot inhibit B cell responses and antibody production.** The Tfr, exTfr cells from the immunized FoxP3 Fate Mapper mouse models and Tfh, B cells from immunized FoxP3 reporter mouse models were sorted and cultured in the presence of anti-CD3 (2 µg/ml) and anti-IgM(5 µg/ml) for 6 days. After co-culture, the cells were harvested to do flow cytometry analyses and the supernatants were collected for enzyme-linked immunosorbent assay (ELISA) and cytokine beads array (CBA). A. Schematic figures of the process and co-culture protocol. B. Frequency of IgG1 and GL7 of the B cells. Quantification of secreted antibody and cytokine IL4 in the supernatant. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 (Student’s t-test). Data are from one experiment representative of five independent experiments with similar results.

**Figure 3.9. In the inducible Fate Mapper model, the exTfr cells are similar to those in fate mapper models.** FoxP3 fate mapper model were immunized with NP-OVA for 7d and the frequency of Tdtomato+ B cells was assessed. The FoxP3 Inducible Fate Mapper mice were immunized with NP-OVA and were given tamoxifen treatment on four sequential days. Harvested draining lymph nodes on day 8. A. The frequency of Tdtomato+ B cells in fate mapper model (left) and inducible fate mapper (right). B. The percentage of Tfr and exTfr cells of CD4+CD19-CXCR5+Tdtomato+population C. Frequency of dead cells using the LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit. Quantification of CD25, CTLA4, PD-1,
GITR, and ICOS. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 (Student’s t-test). Data are from one experiment representative of three independent experiments with similar results.
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Chapter 1: Background

1.1 Introduction

The production of high affinity antibodies is essential for pathogen clearance. However, it is still unclear how B cell responses are regulated, preventing excessive inflammation and autoimmunity. Recently, a new subset of regulatory T(Treg) cells-- T follicular regulatory (Tfr) cells were discovered, which can suppress T follicular helper (Tfh) cell-mediated B cell activation and antibody production\(^3\). Understanding Tfr biology sheds light on how humoral immunity is regulated. This knowledge might reveal how to enhance immune responses while avoiding autoimmunity and how to make antibodies more potent for infection and vaccination.

Tfr cells express high levels of Foxp3 like conventional Treg cells. It is reported that Foxp3 serves as a master transcription factor for Treg cell lineages which is indispensable for the ability of Treg cells to suppress fatal inflammation. However, the function of FoxP3 in Tfr cells remains unclear. The goal of this thesis is to investigate the role of FoxP3 in Tfr cells. By using FoxP3 reporter mice, fate mapper mice, and conditional knockout mice, we confirmed that FoxP3 plays an essential role in Tfr function. To provide a context for these studies, this introduction will be divided into several sections. First, I will provide background information on the importance of studying FoxP3. Then I will briefly introduce Treg cells from which Tfr cells differentiate and concisely review B cell responses where Tfr cells function. Last but not least, I will review what is already known about Tfr cells and the molecules that have potential roles in regulating and maintaining their function.
1.2 IPEX

The immune system is of vital importance for defending the human body from foreign invaders. However, the immune system can also malfunction and attack self-tissues, which is known as autoimmune disease. There is a rare autoimmune disease named Immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome which was first discovered in 1982 when Powell et al. described a family with 19 males infants affected by a disease with an X-linked recessive pattern of inheritance. The most common clinical feature is the presence of severe watery or bloody-mucoid diarrhea which is a sign of enteropathy. These infants also suffer from eczematous dermatitis and autoimmune endocrinopathy, which most commonly affects the pancreas and/or thyroid and causes insulin-dependent type 1 diabetes and/or thyroiditis. Generally, affected organs in IPEX patients show lymphocytic infiltration, with or without the presence of autoantibodies and the laboratory tests show elevated serum IgE and IgA, eosinophilia and markedly decreased or absent FOXP3+ Treg cells. With these heterogeneous disorders, patients often die within first two years of life if left untreated. Though it is reported that IPEX syndrome is a rare disorder that affects an estimated 1 in 1.6 million people, some papers suggest the prevalence is more than that because of misdiagnosis and underreporting.

Unfortunately, there are rare treatments for those patients; the only treatment that offers the potential for cure is allogeneic hematopoietic stem cell transplant (HSCT). In recent years, published case reports and outcome studies have shown promising results and improvement or complete resolution of disease symptoms in the presence of full or mixed chimerism. Without HSCT, IPEX patients have to take life-long immune suppressive drugs.
In 2001, Bennett et al. provided genetic evidence to suggest that IPEX is caused by Forkhead box protein 3 (FOXP3) mutation. FOXP3 is an X-linked gene encoding an essential transcription factor for Treg cells. Loss of self-tolerance due to Treg deficiency or malfunction is believed to be the reason for the diverse spectrum of IPEX autoimmune disorders.

### 1.3 Treg cells

Negative regulation of the immune system is as important as activation. When microbes invade, the immune system will get stimulated and lots of immune cells and molecules will be produced to defense the body. However, shutting off immune responses is critical for proper function of the immune system. Excessive lymphocyte activation and tissue damage during normal protective responses should be prevented. Immune suppression is required to bring the system to the basal state as an active mechanism in addition to passive mechanism of pathogen elimination. In addition, the suppression of immune responses conserves tissue integrity and organ function at the cost of prolonging virus clearance in certain virus infection such as hepatitis B and cytomegalovirus. Inhibitory mechanisms also mediate self-tolerance which is essential for preventing autoimmunity. During lymphocyte development, immature lymphocytes specific for self-antigens will be eliminated, which is known as central tolerance. However, some self-reactive lymphocytes escape and will be controlled by peripheral tolerance mechanisms. In both infection and peripheral tolerance scenarios, Treg cells play a critical role.

Treg cells suppress the immune system in multiple ways. Firstly, these cells can express some inhibitory cytokines such as IL10, transforming growth factor (TGF-β) and IL35. Treg cells from IL10 knockout mice cannot suppress airway hyperresponsiveness or cytomegalovirus (CMV) related T cell activation unlike normal Treg cells, but IL10 pre-
treatment rescued their suppressive ability in an in vitro assay. By using mice with Treg cell-specific ablation of IL10, Rubtsov et al. showed IL10 produced by Treg cells was not required for controlling autoimmunity but played a role in keeping immune responses in check at environmental interfaces such as the colon and lungs\textsuperscript{17}. Anti-TGF-β treatment reduced regulatory T cell activity when regulating lung allergic responses\textsuperscript{16}. Moreover, blockade of surface-bound TGF-β on Treg cells abrogated suppression of effector T cell function in the tumor microenvironment\textsuperscript{18}. IL35, the Ebi3-IL-12alpha heterodimeric cytokine, is secreted by Treg cells rather than effector T cells. Ectopic expression of IL-35 on naive T cells made them suppress T-cell proliferation, data which suggests that IL35 is a suppressive cytokine that maximizes Treg cell suppressive activity\textsuperscript{19}. Secondly, Treg cells can induce cytolysis by producing granzymes. Granzyme B was not expressed in naive Treg cells but was highly expressed in 5%-30% Treg cells in the tumor environment. Granzyme B deficient mice were better at killing tumor cells whereas wild type Treg cells transfer led to tumor growth\textsuperscript{20}. In a model of Treg-dependent graft tolerance, it was shown that Granzyme B deficient mice were unable to establish long-term tolerance\textsuperscript{21}. Thirdly, Treg cells can consume IL2 which is a critical cytokine for effector T cell survival\textsuperscript{22}. Treg cells also depend on IL-2 for survival and suppressive function\textsuperscript{23}. With high expression of high affinity IL2 receptor, Treg will outcompete effector T cells for IL2 consumption. Effector T cells cocultured with Treg cells underwent apoptosis while addition IL2 made them partially resistant to apoptosis, suggesting a role for IL2 in the Treg suppression function\textsuperscript{24}. Moreover, cAMP is transiently upregulated in T cells during activation, however prolonged high levels of cAMP will lead to immune suppression\textsuperscript{25}. Treg cells transport cAMP to target cells through gap junctions or produce pericellular adenosine which could bind to the A2A receptor on effector T cells and elicit upregulation of cAMP\textsuperscript{26, 27, 28}. Last but not least, Treg cells
can target antigen presenting cells (APCs) such as dendritic cells (DCs). By expressing cytotoxic lymphocyte-antigen4 (CTLA4), which is the high affinity receptor for B7, Treg can compete for binding of B7 to CD28 on effector T cells. The interaction between Treg cells and DCs will cause the downregulation of co-stimulatory molecules and production of indoleamine 2,3-dioxygenase (IDO) of DCs. IDO could generate an immunosuppressive milieu that induces abortive immune responses. Lymphocyte activation gene-3 (LAG-3) is a CD4-related transmembrane protein expressed by Treg cells that binds MHC II on DCs and inhibits their activation. None of the mechanisms of suppression can singly account for Treg cell-mediated control of immunity. Vignali et al. propose possible models for how the different mechanisms are used by Treg cells. One of them is the 'hierarchical' model which suggests that several mechanisms can be used but one or two of them is crucial and consistently important in different regulatory settings. The other one is 'contextual' model which stresses the importance of residency and target cell types.

The execution of these suppressive functions requires the proper regulation of genes within Treg cells, and FoxP3 expression is crucial for the establishment and maintenance of the Treg cell gene expression landscape. Germline deletion of the Foxp3 gene leads to Treg cell deficiency and the development of lethal autoimmune syndrome in mice and human. After Foxp3 deletion, mature Treg cells lost their suppressive function and acquired the ability to produce large amounts of IL-2 and T helper type 1 (TH1) proinflammatory cytokines TNF and IFN-γ. Ectopic expression of Foxp3 in peripheral CD4+CD25- T cells equipped them with the capability to suppress. All the data suggest FoxP3 is the cardinal transcription factor of Treg cells.
FoxP3 can bind to more than 2,800 genomic sites, which corresponds to approximately 700–1,400 genes in developing and established Treg cells\textsuperscript{15}. Some data show that a large number of Foxp3-bound genes are either upregulated or downregulated, suggesting that Foxp3 acts as both a transcriptional activator and repressor\textsuperscript{33}. Foxp3 was bound overwhelmingly to enhancers already accessible in precursor CD4+Foxp3\textsuperscript{−} T cells prior to Foxp3 expression and cofactors are recruited for the function of different epigenetic regulators in different enhancer regions, such as EOS, IRF4, SATB1, LEF1, GATA1 and EZH2\textsuperscript{15,34}. Kwon et al. provided a model for FoxP3’s multimodal operation. They suggested FoxP3 can be alternatively assembled in different complexes. FoxP3 usually potentiates an enhancer when together with RELA-KAT5-EP300, possibly through acetylation-mediated activation via BRD4 and P-TEFb. It is inactive when complexed with IKZF3-YY1-EZH2, which leads to repression by recruitment of the NuRD and polycomb assemblies and displacement away from active regions of the nucleus. The outcome of FoxP3’s activity would result from the balance between these two complexes\textsuperscript{35}. 

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**Regulatory T cell heterogeneity and suppression of distinct classes of the immune response.** Treg cells generated in the thymus or extrathymically can further specialize through upregulation or activation of transcription factors in response to different environmental stimuli. There environmental response factors can cooperate with FoxP3 to confer to Treg cells a transient or lasting cell state, enabling their tailored function under particular environmental or inflammatory conditions; for example, STAT3 activation in response to IL-10 leads to the generation of pSTAT3\textsuperscript{+} Treg cells capable of suppressing Th17 responses, or activation of STAT1 in response to IFN-\(\gamma\) or other STAT1-signaling cytokines leads to generation of Tbet\textsuperscript{+} Treg cells. In a given tissue, Treg cells, upon instruction by the tissue environment, induce expression of tissue-specific transcription factors whose cooperation with FoxP3 results in a distinct tissue-specific Treg cell transcriptional signature and function, and also supports Treg cell subset homeostasis.\textsuperscript{1}
Treg cells can be divided into two subsets due to their origins. The ones developing in the thymus are called natural Treg cells and those that differentiate from peripheral CD4+ T cells in response to T cell receptor stimulation in combination with several other signals including IL-2 and TGF-β are called induced Treg cells. Moreover, Treg cells can also be divided by their different transcription factors. They utilize the lineage determining transcription factors of other cell lineages to equip themselves for their immune regulation, targeting different types of immune responses in various anatomical sites\(^1\). Tbet, a key transcription factor for the differentiation of Th1 cells, is also expressed in Treg cells that inhibit Th1 cells rather than Th2 or Th17 cells\(^36\). Irf4 is expressing in both Th2 and those Treg cells specially suppressing Th2 cells\(^37\). Similarly the Th17 transcription factor STAT3\(^38\) is expressed in Th17 cells and Tregs controlling them. Tregs that express FoxP3 as well as Bcl6, a critical transcription factor for differentiation of Tfh cells, also express CXCR5 and those Treg cells are T follicular regulatory (Tfr) cells that modulate B cell responses\(^39\).

1.4 B cell responses

When pathogens invade, their antigens will cross an epithelial barrier and be delivered to the lymphoid organs in their intact and native conformation, can activate specific B cells\(^40\). When B cells get activated, they manifest by their antigen presenting function: migrate to T-B border in the lymph node and present the antigen in the form that can be specifically recognized by T lymphocytes\(^41\). At the same time, the antigens captured by DCs in peripheral will promote their maturation. By processing the antigens, DCs produce large amounts of MHC class II–peptide complexes, express co-stimulatory molecules such as CD40, CD58, CD80, CD86 to help activate T cells, express the chemokine receptor CCR7 to lead the cells to draining lymph nodes and
produce cytokines such as TNF and IL6 to stimulate inflammation\(^{42, 43}\). After encountering the mature DCs in the lymph node, antigen-specific naïve T cells will stop circulating and reside in the lymph node to start to proliferate and differentiate. Higher TCR affinity has been associated with a preference for T follicular helper (Tfh) cells differentiation over non-Tfh differentiation\(^{44}\). Tfh cells upregulate CXCR5 to migrate toward B follicles and express Bcl6 which is a central regulator for Tfh differentiation. At the T-B border, where Tfh cells meet specific activated B cells, both Tfh cells and B cells will undergo further differentiation and form germinal center (GC)\(^{45}\). In the GCs, Tfh cells facilitate B cell activation, proliferation and differentiation, somatic hypermutation, affinity maturation and class switch recombination with the help of follicular dendritic cells (FDCs). FDCs directly sustain the viability, growth and differentiation of activated B cells. They capture antibody–antigen complexes and display the whole complexes, rather than processed antigens, at their surface for long periods. The newborn high affinity B cells can grab antigens from FDC and interact with Tfh cells through TCR-peptide/MHC interaction and costimulatory molecule binding. Then the antigen-equipped B cells can get signals to differentiate into either plasma cells that produce high-affinity antibodies or memory B cells that prepare for another antigen exposure\(^{42}\).

However, autoimmunity and excessive inflammation need to be avoided and the immune system need to ensure Tfh-mediated B cell responses are appropriately controlled. Tfr cells may possibly serve these functions.

### 1.5 Tfr cells

It has been noticed that IPEX patients and scurfy mice (mice with deleterious mutation of FoxP3) have elevated antibody production\(^{46}\), which suggested that Treg cells are essential for
controlling B cell responses. However, it was not clear how Treg cells related to B cell response regulation until 2011 when three independent papers found a subset of effector Treg cells that reside in B follicles and GCs that can suppress antibody production with an RNA profile distinct from regular Treg cells\textsuperscript{39, 47, 48}. Besides high expression of FoxP3, CXCR5 and Bcl6 referred to previously, Tfr cells also have high expression of ICOS and PD1 which are similar to Tfh cells\textsuperscript{2} and CTLA4, GITR, Blimp1 which differs from Tfh cells\textsuperscript{49}. The Tfr cells are a rare population which only occupy 1% of total CD4+ T cells and 6% of all Treg cells in the draining lymph nodes upon activation\textsuperscript{2}. However, they take up a high percentage of T cells in B follicles and GCs. Recent studies have suggested the essential role of Tfr cells in GC reactions.
Tfr cells are differentiated from natural Treg cells and likely go through at least two phases of differentiation: one phase is manifested by interaction with antigen presenting DCs and the other one which includes crosstalk with B cells in the follicles and GCs\textsuperscript{50}. Tfr cells not only exist in lymphoid tissues, but also circulate in the blood. The circulating Tfr cells have memory-like properties and are less suppressive\textsuperscript{51}.

Tfh cocultured B cells were compared with Tfr, Tfh cells cocultured B cells by RNAseq. The data showed the expression of the transcriptional signature genes of effector B cells were not attenuated. However, B cell function-related genes such as those related to GC formation, antibody secretion, class-switch recombination and plasma cell differentiation were downregulated. In addition, multiple metabolic pathways including glycolysis, glutaminolysis, one-carbon metabolism, serine biosynthesis and purine biosynthesis and Myc and mTOR were suppressed. When the Tfh cells in the cultures were analyzed, the Tfh cell transcriptional program was retained while the expression of IL21, IL4 which are critical cytokines for B cell responses were potently suppressed. The data suggested that Tfr cells can suppress the effector molecules and metabolic pathways of B cells and Tfh cells while the global effector programs are maintained\textsuperscript{3}.

Some costimulatory and coinhibitory molecules are important for Tfr cell function and identity. CTLA4 is essential for Tfr cell differentiation and suppressive function. Using the inducible CTLA4 deletion mouse model, Tfr and Tfh cells were expanded after deletion of CTLA4, with relatively greater increases in Tfr cells\textsuperscript{52}. When CTLA4 was deleted after Tfr cell differentiation and the cells then sorted and evaluated using an in vitro suppression assay, there was enhanced GC B cells and more class switching to IgG1 and IgE\textsuperscript{52}. Adoptive transfer of the CTLA4 deleted Tfr cells and Tfh cells into CD28\textsuperscript{−/−} recipients resulted in an overall increase in Tfh cells and
substantial increase in antigen-specific IgG compared to their counterparts. These data suggested CTLA4 inhibited Tfh and Tfr cell differentiation/maintenance, suppresses the effector function of Tfh cells while is required for Tfr cells to fully suppress antigen-specific B cells responses in GCs.

The costimulatory molecule, ICOS regulates Tfr cell differentiation. ICOS is most highly expressed on draining lymph nodes Tfr cells, but is expressed on efferent lymph and circulating Tfr cells at much lower levels and intermediate expression on Peyers patch Tfr cells. Mice lacking ICOS have severe defects in Tfr cells and mice without imR-146a, the ICOS repressor, have increased Tfr cells. PD1 is transiently expressed on activated T cells and declines when the pathogens are cleared. However, during chronic infection, the expression of PD1 stays high and can mediate T cell exhaustion. PD1 inhibits Tfr cell differentiation and function since in PD1 knockout mice, Tfr cell numbers increases and PD-1 deficient Tfr cells suppress antibody production more potently than control Tfr cells in vitro and in vivo. One possible mechanism of PD1 function is by eliciting a prolonged interaction between Tfr cells and B cells, preventing Tfh cells contact with B cells.

CD25, the high affinity receptor for IL2, is essential for both thymic production of Treg cells and their peripheral maintenance since the IL2 signal is of vital importance for Treg cell proliferation and survival. Tfr cells can be subdivided into CD25+ and CD25- subpopulations and the CD25- Tfr cells are suggested to be a mature GC-localized subpopulation with the expression of most suppressive molecules. Glucocorticoid-induced TNFR (GITR), a member of the TNFR superfamily, plays important roles in regulating activities of effector T cells and Treg cells. GITR gene expression is induced by T cell activation and further upregulated in Treg cells. GITR expression is upregulated by an NF-κB-mediated enhancer and FoxP3 can stabilize
binding by anchoring it to the enhancer\(^5\). However, the role of GITR in Tfr cells has not been fully understood.

**Section 1.6 Questions Addressed in the Thesis**

In this thesis, we investigated the role of FoxP3 in Tfr cells. It is already known that FoxP3 plays an essential role in Treg cells and its mutation leads to lethal diseases both in human and mice. RNAseq data from our lab revealed that the transcriptional program of Tfr cells resembles Treg cells and Tfh cells. Based on these findings, we hypothesized that FoxP3 also plays a role in Tfr cell function. It is critical to study the modulators of Tfr cell function, as the B cell response is critical in health and disease, for vaccination, infection and many autoimmune diseases. By using FoxP3 inducible knockout model, fate mapper and inducible fate mapper models as well as the in vitro assays, we found FoxP3 non-expressing Tfr cells cannot suppress as well as Tfr cells, indicating the importance of FoxP3 for Tfr cell suppressive function. This finding suggests that inhibiting FoxP3 expression or related pathways might boost vaccine responses and reinforcing FoxP3 expression or related pathways might ameliorate autoimmune diseases.
Chapter 2: Materials and Methods

Mice. Wild-type C57Bl/6J mice, Rosa26 lox-Stop-lox-Tdtomato mice, Foxp3-Cre-YFP mice, and FoxP3-IRES-CreERT2-eGFP mice were purchased from Jackson Laboratories. Foxp3IRES-GFP (CD45.1.1/CD45.2.2) on the C57Bl/6 background have been published previously.58 FoxP3F/F, UBC ERT2-Cre mice on the C57Bl/6 background were a gift from the Rudensky Lab. The genotypes of the mice were verified by PCR and flow cytometry. All mice were housed in an SPF facility. Each individual experiment contained one sex of mice, but replicates were performed with males or females. All mice were used according to the Harvard Medical School Standing Committee on Animals and National Institutes of Health Guidelines.

Immunization. 100 µg NP18-OVA (Biosearch Technologies) in a 1:1 H37RA CFA (DIFCO) emulsion was injected s.c. on the mouse flanks. Mice were sacrificed at a later time point and inguinal lymph nodes were harvested for analysis.

Antibodies. The following antibodies were used for surface staining at 4 °C: anti-CD4 (BioLegend, 1:200, RM4-5), anti-ICOS (BioLegend, 1:200, 15F9), anti-CD19 (BioLegend, 1:200, 6D5), anti-CXCR5 biotin (BD Biosciences, 1:100, 2G8), GL7 (BD Biosciences, 1:200, GL-7), anti-IA (BioLegend, 1:200, M5/114.15.2), anti-CD25 (BioLegend, 1:200, PC61), anti-GITR (BioLegend, 1:200, DTA-1). For CXCR5 detection, streptavidin-BV421 (BioLegend, 1:400, 405225) also was used at 4 °C. For intracellular staining, samples were fixed with the Foxp3 Fix/Perm buffer set according to the manufacturer’s instructions (eBioscience). Samples were then intracellularly stained with anti-IgG1 (BD Biosciences, 1:200, A85-1), anti-Foxp3 (eBiosciences, 1:200, FJK-16S)12, anti-CTLA4 (BioLegend, 1:200, UC10-4B9) anti-Helios
(BioLegend, 1:200, 22F6), RFP antibody (FITC) (Abcam, 1:400) and LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit (Thermo Fisher Scientific) at 4 °C.

**Sorting.** Single-cell suspensions were diluted in PBS supplemented with 1% FBS with 1 mM EDTA. Tfh cells and Tfr cells were isolated by enriching with CD4+ cells by magnetic positive selection (Miltenyi Biotec). CD4+ enriched cells were then stained and sorted as follows: Tfh (CD4+ICOS+CXCR5+Foxp3−CD19−), Tfr (CD4+ICOS+CXCR5+Foxp3+CD19−). In some cases, an alternative gating strategy was used; Tfh (CD4+ICOS+CXCR5+Tdtomato-YFP−), Tfr (CD4+ICOS+CXCR5+Tdtomato+YFP+), exTfr (CD4+ICOS+CXCR5+Tdtomato+YFP−). B cells were isolated from flow-through from CD4+ selection, which was then positively selected using CD19 beads (Miltenyi Biotec).

**Stability assay.** Foxp3GFP (CD45.1.1) and Foxp3GFP (CD45.2.2) reporter mice were immunized with NP-OVA, and 7 d later, dLN were harvested and CD19+ B cells, CD4+CXCR5+ICOS+ Foxp3−CD19− Tfh and CD4+ICOS+CXCR5+CD19−Foxp3+ Tfr cells were purified by cell sorting. CD45.2.2 B cells and Tfh cells were cultured with CD45.1.1 Tfr cells. 5 × 10^4 B cells, 3 × 10^4 Tfh cells and 1.5 × 10^4 Tfr cells were plated in 96-well plates along with 2 µg/ml anti-CD3 (2c11, BioXcell) and 5 µg/ml anti-IgM (115-006-020, Jackson Immunoresearch). Cultures were harvested 4 d later. For analysis, B cells were gated as CD19+IA+CD4−CD45.2+ cells, Tfh cells were gated as CD4+IA−CD19−CD45.2+Foxp3− cells, and Tfr cells were gated as CD4+IA−CD19−CD45.1+Foxp3+ cells, exTfr cells were gated as CD4+IA−CD19− CD45.1+Foxp3- cells.
Suppression assay. FoxP3<sup>F/F</sup>, UBC<sup>ERT2-Cre</sup> / FoxP3<sup>F/F</sup> control mice and FoxP3 reporter mice were immunized with NP-OVA, 6 d later, FoxP3<sup>F/F</sup>, UBC<sup>ERT2-Cre</sup> / FoxP3<sup>F/F</sup> control mice were given 100ul 10mg/ml tamoxifen i.p. on day 6. On day 7, CD19+ B cells, CD4+CXCR5+ICOS+CD19-FoxP3(GFP)- Tfh cells from FoxP3 reporter mice and CD4+CXCR5+ICOS+CD19-CD25+ Tfr cells from FoxP3<sup>F/F</sup>, UBC<sup>ERT2-Cre</sup>/ FoxP3<sup>F/F</sup> control mice were purified by cell sorting. 5 × 10<sup>4</sup> B cells, 3 × 10<sup>4</sup> Tfh cells and 1.5 × 10<sup>4</sup> Tfr cells were plated in 96-well plates along with 2 μg/ml anti-CD3 (2c11, BioXcell), 5 μg/ml anti-IgM (115-006-020, Jackson Immunoresearch) and 1nM 4-hydroxytamoxifen(H7904-25MG, Sigma-Aldrich). Cultures were harvested 3 d later. For analysis, B cells were gated as CD19+IA+CD4− cells, Tfh cells were gated as CD4+IA− CD19− CD25−Foxp3− cells, and Tfr cells were gated as CD4+IA−CD19−CD25+FoxP3+ cells and FoxP3 knockout Tfr cells as CD4+IA−CD19−CD25+FoxP3− cells.

FoxP3 fate mapper mice were immunized with NP-OVA, and 7 d later, dLN were harvested and CD19+ B cells, CD4+CXCR5+Tdtomato−YFP− Tfh and CD4+CXCR5+Tdtomato+YFP+ Tfr and CD4+CXCR5+Tdtomato+YFP− exTfr cells were purified by cell sorting. B cells and Tfh cells were cultured with or without Tfr cells or exTfr cells. 5 × 10<sup>4</sup> B cells, 3 × 10<sup>4</sup> Tfh cells and 1.5 × 10<sup>4</sup> Tfr cells were plated in 96-well plates along with 2 μg/ml anti-CD3 (2c11, BioXcell) and 5 μg/ml anti-IgM (115-006-020, Jackson Immunoresearch). Cultures were harvested 6 d later. For analysis, B cells were gated as CD19+IA+CD4− cells, Tfh cells were gated as CD4+IA−CD19− Tdtomato−Foxp3− cells, and Tfr cells were gated as CD4+IA−CD19− Tdtomato+Foxp3− cells, exTfr cells were gated as CD4+IA−CD19− Tdtomato+Foxp3− cells. The culture supernatants were assessed for ELISA and Cytokine concentrations measured by Cytometric Bead Array (BD Bioscience).
**ELISA.** MaxiSorp plates (VWR) were coated with anti-mouse Ig (SouthernBiotech, 1010-01) overnight at 4 °C, washed with 0.05 % Triton-X100 (Sigma-Aldrich, T8787-50ML) in PBS, and blocked with 1 % BSA (Sigma-Aldrich: Sigma, A2153) in PBS for 1 h at 37 °C. Cell culture supernatants were diluted 1:50 in 1 % BSA in PBS; add 95 µl per well, and serial dilutions of IgG standard were performed (SouthernBiotech, 1071-01) starting at 500 ng/ml on the same plate as samples. Following a 45 min incubation at room temperature, the plates were washed and anti-mouse IgG AP (SouthernBiotech, 1030-04) added in 1 % BSA to wells and incubated for 45 min at room temperature. The plates were washed again and phosphatase substrate (Sigma-Aldrich, S0942-200TAB) diluted in DEA buffer (Spectrum Chemicals & Laboratory Products, 40420000-1) and added to the wells. After color developed, absorbance at 405 nm was measured on a plate reader (molecular devices).

**Immunofluorescent microscopy.** 10-µm-thick frozen sections of OCT-embedded draining lymph nodes from immunized Wild-type C57Bl/6J mice were fixed with Foxp3 Fix/Perm buffer set (eBioscience), and stained with anti-IgD (BioLegend, 11-26c.2a) conjugated to Pacific Blue, anti-CD3 (BioLegend, 17A2) conjugated to Alexa Fluor 594, anti-FoxP3 (eBiosciences, FJK-16s) conjugated to APC and anti-GL7 (BD Biosciences, 1:200, GL-7) conjugated to Alexa Fluor 488. Samples were imaged on a Nikon spinning-disk confocal microscope using a 10× objective and standard lasers and filters. The z-stacks were converted to projections using ImageJ software. B cells were identified as cyan, Tfr cells were identified as blue and red, germinal center B cells were identified as green, T cells were identified as blue.
Statistics. For single comparisons, unpaired, 2-tailed Student’s t test was used; for multiple comparisons, 1-way ANOVA with Tukey post-test was used. Data represent mean ± SD or SEM as indicated. A P value less than 0.05 was considered significant.
Chapter 3: Results

Section 3.1: FoxP3 expressing Tfr cells exist in the B follicles.

Firstly, we quantified FoxP3-expressing Tfr cells with flow cytometry and confocal microscopy. In wild type mouse models, we found 1.21% in non-immunized mice and 5.05% in NP-OVA immunized mice of CD4+ T cells expressing CXCR5, the chemokine receptor that guides cell migration to B follicles. In the non-immunized mice, the percentage of CXCR5+ CD4+ T cells (combination of Tfh and Tfr cells) among all CD4+ T cells was smaller than immunized ones, which suggested that during inflammation, more B cell regulation was required than homeostasis. We gated Tfr cells as CD4+CXCR5+ICOS+FoxP3+CD19- and Tfh cells as CD4+CXCR5+ICOS+FoxP3-CD19- (Figure 3.1 A B). The ratio of Tfr:Tfh was higher in homeostasis, while during immunization, it decreased as Tfh cells percentages rose to promote antibody production (Figure 3.1 C). In addition, the ratio of Tfr:Tfh varied with different adjuvants. The more potent the adjuvant was, the smaller the Tfr:Tfh ratio was (Figure 3.1 C), which suggested that B cell regulation was dynamic. By confocal microscopy, we saw many FoxP3+ (red) CD3+ (blue) Treg cells in the T cell zones (blue zones of the images) of the lymph nodes and sparse Tfr cells with same colors in B follicle (cyan zones of the images). No germinal centers and rare Tfh and Tfr cells were shown in the slides of non-immunized lymph nodes. There were some small germinal centers in the image of 7d immunization and more Tfr cells in the follicles could be seen. While on day 10, there were some large germinal centers and we found Tfr cells in and outside germinal centers (Figure 3.1 D). All these data confirmed the identification of Tfr cells as a B cell response regulator.
Section 3.2: The phenotype of Tfr cells changes when FoxP3 is deleted.

To study the role of FoxP3 in Tfr cells, we used the FoxP3<sup>F/F</sup>, UBC<sup>ERT2-Cre</sup> mouse model. We compared the phenotype of Tfr cells with FoxP3 deletion with FoxP3<sup>F/F</sup> mice as a control group. In order to trigger Cre recombinase nuclear translocation, tamoxifen treatment was given for six sequential days after NP-OVA immunization and draining lymph nodes harvested on day 7 (Figure 3.2 A). There was about 50% deletion of FoxP3 in CD4+ T cells (Figure 3.2 B). The frequency of high affinity IL2 receptor, CD25, was comparable between groups, which suggested that deletion of FoxP3 within a short period of time did not alter the expression of...
CD25 (Figure 3.2 B). Therefore, it was possible to use CD25 as a gate for Tfr cells to distinguish FoxP3- Tfr cells from FoxP3- Tfh cells. When gated on CD4+CD19-CXCR5+CD25+, there were populations with or without FoxP3 expression (Figure 3.2 C). Within the FoxP3 deletion group (FoxP3\(^{F/F}\), UBC\(^{ERT2-Cre}\)), the FoxP3 deletion efficacy of Tfr cells was similar to Treg cells, approximately 50%. It verified the use CD25 as a gate for Tfr cells. Surprisingly, we found CD25+/FoxP3- cells in the control group (FoxP3\(^{F/F}\)). Those cells were potential FoxP3 naturally downregulated Tfr cells. Comparing FoxP3+ and FoxP3- Tfr cells in both groups, we found that FoxP3- Tfr cells express less CTLA4, GITR and ICOS which are important molecules for Treg as well as Tfr cell function (Figure 3.2 D). Though the frequency of CD25+ Tfr cells maintained, the quantification of CD25 was decreased in FoxP3- groups (Figure 3.2 D). The data

Figure 3.2. FoxP3 deleted Tfr cells showed a different phenotype compared with normal Tfr cells. FoxP3\(^{F/F}\) and FoxP3\(^{F/F}\), UBC\(^{ERT2-Cre}\) mice were immunized with NP-OVA s.c. and given tamoxifen treatment 1mg/mouse/day i.p. for six sequential days. On day 7 the draining lymph nodes were harvested. A. Schematic image of mouse treatment. B. The frequencies of FoxP3+CD25+ cells and CD25+ cells among CD4+ cells were shown in the graphs. Cre- represented data from FoxP3\(^{F/F}\) mice and Cre+ represented data from FoxP3\(^{F/F}\), UBC\(^{ERT2-Cre}\) mice. C. The representative plots for deletion of FoxP3 in Tfr cells gated as CD4+CD19-CXCR5+ cells. The upper outlined area indicated the percentage of FoxP3+ Tfr cells, the lower outlined area indicated the percentage of FoxP3- Tfr cells. D. Quantification of the expression of CTLA4, CD25, GITR, ICOS in Tfr and Tfh cells. NS \(P>0.05\), *\(P<0.05\), **\(P<0.01\), ***\(P<0.001\), ****\(P<0.0001\) (Student’s t-test). Data were from one experiment representative of three independent experiments with similar results.
demonstrated that FoxP3 deleted Tfr cells had a different phenotype from FoxP3 expressing Tfr cells. The decrease of function molecules suggested that the Tfr cells without FoxP3 expression might have impaired suppressive capability.

**Section 3.3: Limited alteration in suppression with partial FoxP3 deleted Tfr cells.**

We next conducted an in vitro suppression assay to investigate the function of FoxP3 deleted Tfr cells. Firstly, we immunized FoxP3<sup>F/F</sup>, UBC<sup>ERT2-Cre</sup> mice and FoxP3<sup>F/F</sup> mice with NP-OVA and gave them tamoxifen treatment on day 6 to start deletion. Then on day 7, we sorted the Tfr cells as CD4+CD19- CXCR5+CD25+, Tfh cells as CD4+CD19- CXCR5+CD25- and B cells as CD4-CD19+. Cells were plated with anti-CD3 and anti-IgM, plus 1 nM 4-OHT to trigger more FoxP3 deletion (Fig 3.3 A). 4-hydroxytamoxifen (4-OHT) is the active metabolite of tamoxifen and has much higher affinity for the estrogen receptor than tamoxifen itself. After a
three-day co-culture, cells were harvested for flow cytometry and ELISA. The 4-OHT induced a small amount of FoxP3 deletion, while the antibody production was comparable between groups (Fig 3.3 B). There are two possible reasons for the equal antibody production. One is that Tfr cells with no FoxP3 expression maintained the capability to suppress B cell responses. The other one is that the FoxP3 deleted Tfr cells have an altered function but the cells are so few to stand out. The in vitro assay failed to move our understanding of FoxP3 step forward.

**Section 3.4: The deletion efficacy limitation of the inducible FoxP3 knockout mouse model.** Unfortunately, the deletion efficacy of the tamoxifen induced FoxP3 knockout mouse model was not 100%. We tried to increase the deletion efficacy. Given it is the only mouse model we had available, it is hard to increase the intrinsic CRE efficacy genetically. Then we tested whether gender or age make any difference to the efficacy since hormone and physical condition might impact tamoxifen absorption and metabolism. Comparing the mice of different gender, we found male mice have a slightly higher frequency of FoxP3+ cells among T cells in the background, however, the deletion efficacy was similar in both male and female group (Fig 3.4 A). Then we divided the mice by different ages (7-10 week, 10-12 week,
12-14 week, more than 14 week). The deletion efficacy was comparable among different age groups (Fig 3.4 A). In addition, we tried to promote efficiency by giving more time for tamoxifen to work and DNA repair in vivo. After immunization and six tamoxifen i.p. injections, we held the mice for seven days before harvesting the draining lymph nodes. However, no significant improvement was seen (Fig 3.4 B). In all, the data suggested the low deletion efficiency resulted from some intrinsic reasons.

Section 3.5: In vitro generated FoxP3 naturally downregulated Tfr cells.

In the FoxP3<sup>E/F</sup> (Cre-) mouse model, we found some cells which gated as CD4+CD19-
CXCR5+ICOS+CD25+FoxP3-(Fig 3.2 C). Those cells might be FoxP3 naturally
downregulated Tfr cells. In order to verify FoxP3 natural
downregulation, we conducted an in

![Figure 3.5. In vitro generated exTfr cells resemble FoxP3 deleted Tfr cells in FoxP3 knockout mice. FoxP3+ Tfr, Tfh and B cells were harvested 7d after immunization with NP-OVA from FoxP3 reporter mice. Co-culture CD45.1.1 Tfr cells with CD45.2.2 Tfh and B cells. Data were collected after 4d co-culture (15,000 Tfr cells with 50,000 B cells and 30,000 Tfh cells). A. A representative plot was showed, lines in the plot indicated three technical replicates of Tfr cells in the wells, gated as CD4+CD19-CD45.1+. B. Quantification of the expression of CTLA4, PD-1, GITR, CD25, ICOS in FoxP3+ and FoxP3- Tfr cells. NS P>0.05, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 (Student’s t-test).]
vivo experiment. CD45.2.2 B cells, Tfh cells and CD45.1.1 FoxP3-expressing Tfr cells were sorted from the immunized (CD45.1.1 and CD45.2.2) FoxP3 reporter mice. CD45.1.1 Tfr cells were co-cultured with CD45.2.2 Tfh and B cells along with 2 µg/ml anti-CD3 and 5 µg/ml anti-IgM for 4 days. Then we harvested the cells for flow cytometry. Tfr cells were distinguished from Tfh cells by the congenic markers and a small percentage of Tfr cells were found lose their FoxP3 expression. This finding suggested the existence of FoxP3 naturally downregulated Tfr cells. We termed those FoxP3 naturally downregulated Tfr cells as exTfr cells. Those exTfr cells maintained PD1 expression but had lower expression of CTLA4, GITR, ICOS and CD25 (Fig 3.5 B), a phenotype similar to that seen in FoxP3 inducible knockout Tfr cells.

**Section 3.6: The design of FoxP3 fate mapper.**

From the previous results, we identified exTfr cells in vitro. We next designed a mouse model to verify exTfr cells in vivo. We crossed female knock-in mice harboring a cassette containing an internal ribosome entry site (IRES) followed by DNA sequence encoding a “double” fusion protein of the yellow fluorescent protein (YFP) with Cre recombinase inserted into the 3′ untranslated region (UTR) of the Foxp3 gene (FoxP3-IRES-Cre-YFP) and male reporter mice that express Tdtomato from the Rosa26 promoter only after excision of a loxP-flanked stop cassette (Rosa26-lox-stop-lox-Tdtomato). The male offspring have the only copy of FoxP3-IRES-Cre-YFP in X chromosome which inherited from the female breeder. So all cells of the male offspring that have FoxP3 expressed now or in the past would also transcribe Cre recombinase which cut the stop cassette and make the cell Tdtomato+. We then use Tdtomato to track Treg cells and all their subsets. While YFP is driven by FoxP3, the model is also a reporter for current FoxP3 expression. We can tell whether exTfr cells exist by examining the fluorescent
proteins. If all Tdtomato+ Tfr cells are also YFP+, it suggests that all FoxP3+ Tfr cells continuously express FoxP3 and exTfr does not exist. However, if partial Tdtomato+ Tfr cells do not express YFP, it suggests that some Tfr cells expressed FoxP3 before but stop expressing at a later time point, which provides a strong evidence for the existence of exTfr cells.

![Figure 3.6: The design of FoxP3 Fate Mapper model.](image)

**Figure 3.6. The design of FoxP3 Fate Mapper model.** When FoxP3 expressing regulatory cells were generated in FoxP3-IRES-CRE-YFP; Rosa26 Lox-STOP-Lox-Tdtomato mice, CRE recombinase and YFP would also express (left). The CRE recombinase would cut the Stop cassette and the cells would be both Tdtomato+ and YFP+ (middle). If FoxP3 stops expressing at later time point, YFP which was under the same promoter would stop expressing as well. However, Tdtomato which is in Rosa26 locus with no stop cassette ahead any longer would be expressing independently (lower right). If FoxP3 continuously express, the cells will remain YFP+Tdtomato+ (upper right). By detecting the fluorescent proteins, the FoxP3 expression status would be known.

**Section 3.7: In vivo generated exTfr cells resemble the phenotype of in vitro ones.**

We immunized the FoxP3 fate mapper model with NP-OVA. After 7 days, the draining lymph nodes were harvested. By flow cytometry, we found a certain amount of Tdtomato+FoxP3-exTfr cells by gating on CD4+CD19-CXCR5+ cells (Fig 3.7 A). We also found a very few FoxP3+Tdtomato- cells, which suggested all FoxP3+ cells are confined within the Tdtomato+
population. These data verified the model to some extent that Cre recombinase should be driven by FoxP3 and it should work well in inducing Tdtomato expression. After we confirmed the existence of exTfr cells, we firstly explored whether the cells were dying or dead cells or not, since when cells are dying, they will downregulate the expression of some proteins that might include FoxP3. To exclude the possibility that exTfr cells are dying Tfr cells, we stained the dead cells. The frequency of dead cells in exTfr cells was very low (Fig 3.7 B), suggesting exTfr cells were not dying Tfr cells and it is a bona fide population. Moreover, the quantification of CD25, GITR, ICOS and the frequency of CTLA4 were all decreased in exTfr cells compared with Tfr cells and the expression of the molecules were prone to the level of Tfh cells, while the
expression level of PD1 maintained (Fig 3.7 B). The data suggested exTfr cells have an impaired suppressive ability since they lose their function and identity molecules.

**Section 3.8: ExTfr cells lose their suppressive function.**

In order to verify the function of the exTfr cells, we did in vitro co-culture assays. Firstly, we immunized the fate mapper mice for 7 days. After harvesting the draining lymph nodes, we sorted Tfh cells as CD4+CD19-CXCR5+Tdtomato-YFP-, Tfr cells as CD4+CD19-CXCR5+Tdtomato+YFP+, exTfr cells as CD4+CD19-CXCR5+Tdtomato+YFP-, and B cells as CD4-CD19+. Then we plated the cells into different groups: B cells (50,000), B cells (50,000) + Tfh cells (30,000), B cells (50,000) + Tfh cells (30,000) + Tfr cells (15,000), B cells (50,000) + Tfh cells (30,000) + exTfr cells (15,000). The cells were cultured in R10 media with anti-CD3 (2 μg/ml) and anti-IgM (5 μg/ml) (Fig 3.8 A). After six days of culture, the cells were analyzed by flow cytometry and the supernatant was collected to detect antibodies and cytokines. More B cells got activated within B + Tfh cells group compared to B+ Tfh+ Tfr group. There was a similar frequency of active B cells in the B+ Tfh+ exTfr cells group and B+ Tfh group (Fig 3.8 B). This suggested exTfr cells cannot suppress B cell activation. In addition, the class switch recombination to IgG1 in B cells was high with the presence of Tfh cells while it was totally inhibited by the presence of Tfr cells, while exTfr cells did not suppress class switch recombination (Fig 3.8 B). The secreted IgG antibodies production was facilitated by Tfh cells and was inhibited by Tfr cells rather than exTfr cells (Fig 3.8 B). Unlike Tfr cells, exTfr cells did not suppressed IL4, the cytokine critical for B cell responses (Fig 3.8B). All the data suggested exTfr cells do not maintain the suppressive capacity as Tfr cells do.
Section 3.9: Inducible fate mapper model was used to confirm the results from the fate mapper.

The fate mapper model strategy showed the existence of exTfr cells. However, we found that some mice had Tdtomato expression in a small percentage of B cells (Fig 3.9 A), which condition might result from the malfunction of the stop cassette ahead of Tdtomato gene. It is possible that Tdtomato also expresses in some Tfh cells. Tdtomato+ Tfh cells would run into exTfr population since they share the gates CD4+CD19-CXCR5+Tdtomato+FoxP3-. To solve the question above, we used an inducible fate mapper model to reduce the leakiness. In the FoxP3-IRES-CreERT2-eGFP; Rosa26 Lox-STOP-Lox-Tdtomato (FoxP3 inducible fate mapper) model, Cre recombinase can only translocate to nucleus and function in the presence of...
tamoxifen. This model excluded the leakiness accumulation in the long period of development. In addition, inducible labeling of Foxp3 expressing cells with Tdtomato helps avoid the constant incorporation into the labeled population of cells that transiently upregulate Foxp3 and affords assessment of bone fide exTfr cells. After we immunized the mice with NP-OVA, several doses of tamoxifen were given to the mice. After harvest on day 8, we found the leakiness of B cells was much less in the new model (Fig 3.9 A). The majority of Tdtomato+ Tfr cells maintain FoxP3 expression while a small percentage lost FoxP3 (Fig 3.9 B). The phenotype of exTfr cells in

Figure 3.9. The inducible Fate Mapper model. FoxP3 fate mapper model were immunized with NP-OVA for 7 days and the frequency of Tdtomato+ B cells was assessed. The FoxP3 Inducible Fate Mapper mice were immunized with NP-OVA and were given tamoxifen treatment on four sequential days. Harvested draining lymph nodes on day 8. A. the frequency of Tdtomato+ B cells in fate mapper model (left) and inducible fate mapper (right). In the inducible model, B. the percentage of Tfr and exTfr cells from CD4+CD19-CXCR5+Tdtomato+population; C. Frequency of dead cells using the LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit. Quantification of CD25, CTLA4, PD-1, GITR, and ICOS. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 (Student’s t-test). Data are from one experiment representative of three independent experiments with similar results.
the inducible fate mapper was similar to fate mapper model: the exTfr cells were mostly live cells, had less expression of CD25, CTLA4, GITR, ICOS and maintained PD1 expression (Fig. 3.9 C).
Chapter 4: Discussion and Perspectives

There are many papers studying the role of FoxP3 in Treg cells. However, we are only beginning to characterize the new discovered subset, Tfr cells. The findings presented here provide compelling evidence for the essential role of FoxP3 in maintaining the suppressive function of Tfr cells. We used a FoxP3 conditional knockout mouse model. With tamoxifen treatment, half of the Tfr cells delete FoxP3. When compared with FoxP3 expressing Tfr cells, those FoxP3- Tfr cells lost some functional molecules such as ICOS, CD25, GITR, CTLA4. Surprisingly, even in the control mice, we found some CD25+FoxP3- cells. We suspected the existence of natural FoxP3- Tfr cells. By using the fate mapper mouse model and CD45.1/CD45.2 FoxP3 reporter mice, FoxP3-Tfr cells which we termed as exTfr cells were confirmed both in vivo and in vitro and we determined that these exTfr cells have impaired suppressive capacity. In conclusion, using FoxP3 deletion/deregulation mouse models, we are able to show that FoxP3 downregulated Tfr cells have attenuated suppressive capacity and signature genes and that FoxP3 plays an essential role in Tfr cell function.

The Lox/cre system is a widely used DNA modification tool. The Cre protein is a site-specific DNA recombinase that can catalyze the recombination of DNA between specific sites in a DNA molecule. These sites, known as loxP sequences, contain specific binding sites for Cre that surround a directional core sequence where recombination can occur. There are some important genes whose embryonic deletion will lead to lethality. One of the most successful methods to circumvent this problem and study these genes employs Tamoxifen-dependent Cre recombinase that can be used to generate time- and tissue-specific mutant. This utilizes a mutated estrogen receptor (ER) fused to Cre as a transgene (Cre-ER), which only becomes activated and then translocates into the nucleus upon binding of the active tamoxifen metabolite 4-
hydroxytamoxifen (4-OHT). CreERT2 is currently the most successful Cre-ER version, which has very low background Cre activity and robust activation following tamoxifen treatment\textsuperscript{60}. The technology has been used for decades and a big number of gene engineered mouse models have been developed based on this approach. However, sometimes the efficiency can be limited, like our FoxP3\textsuperscript{F/F}, UBC\textsubscript{ERT2-Cre} mice in which the deletion efficacy is about 50%. To promote the efficacy, appropriate targeting and recombination efficiency should be taken into consideration. To check the appropriate targeting, PCR and DNA sequencing can be used. However, we focus more on increasing the recombination efficiency since we have 50% deletion of FoxP3 expression which suggested less chance of faulty targeting. To improve the recombination efficiency, we tested whether the age and gender of mice impact the absorption or metabolism of tamoxifen. DNA methylation, transcriptional activity, chromatin structure at the locus of interest and the distance between the loxP sites can also influence the efficiency of recombination\textsuperscript{61}. We found no differences between varied age and gender groups which suggest the low deletion efficiency might result from some intrinsic reasons.

The existence of FoxP3-Tfr cells in the FoxP3\textsuperscript{F/F} (Cre-) group inspired us to discover exTfr cells. The phenotype and function of exTfr cells provide potent evidence that demonstrate the essential role of FoxP3 in Tfr cells. Moreover, the FoxP3 inducible deletion model helped confirm the importance of loss of FoxP3 in exTfr cells. With merely the fate mapper model, we only learned about the phenotype and function of exTfr cells. However, we did not know whether the alteration resulted from FoxP3 loss or not. It is also possible that some other gene changes that occur simultaneously with FoxP3 directly regulate exTfr cells. The phenotypical similarity between FoxP3 deleted Tfr cells from inducible deletion model and exTfr cells from fate mapper model suggest FoxP3 is the critical element for the changes in exTfr cells.
In 2009, a paper was published to say FoxP3+ T cells could generate follicular B helper T cells in the Peyer’s Patches\(^6^2\). They transferred FoxP3+ T cells into CD3e\(^{−}\) mice and found FoxP3+ cells decreased FoxP3 expression, went into B follicles and facilitated GCs formation in the Peyer’s Patches with a B cell and CD40L dependent manner. Though one possibility is that the unstable Treg cells differentiate into Tfh cells, there is another possibility: the Tfh-functioning cells are exTfr cells. The features of FoxP3 downregulation, differentiation from Treg and the expression of CXCR5, PD-1, ICOS, Bcl6 suggest these cells could be exTfr cells. Moreover, the capacity of these cells to help GC formation is also consistent with our results: exTfr cells phenotypically resemble Tfh cells and they lose their suppressive function. However, we are not sure about the role of exTfr cells in promoting antibody production. To confirm this, in vitro culture of exTfr cells with B cells plus anti-CD3 and anti-IgM and exTfr adoptive transfer assays should be conducted. In addition, the scenario in the paper was only seen in the Peyer’s Patches rather than other lymphoid organ such as spleen and lymph nodes\(^6^2\) while our exTfr cells have been found in the lymph nodes. If the differentiated cells were exTfr cells, then the environment-dependent clue suggests that exTfr cells may vary from different sites. We have found that Tfr cells from different organs have different transcriptional programs and functionality (data not shown). It would be interesting to study exTfr cells in the gut and compare them to those in other second lymphoid organs and the blood.

We showed that FoxP3 downregulated Tfr cells lost Tfr cell phenotype and suppressive function using the fate mapper model. The fate mapper model has been long used in studying the stability of FoxP3. In 2009, Zhou et al. crossed transgenic mice expressing an GFP-Cre controlled by the FoxP3 promoter on a bacterial artificial chromosome with Rosa26-lox-stop-lox-YFP reporter mice, with which they tracked the FoxP3 loss (exFoxP3) T cells with an
activated-memory phenotype and the production of inflammatory cytokines\textsuperscript{63}. However, Rubtsov et al. have argued that FoxP3 BAC transgene-driven Cre expression might not faithfully mirror endogenous FoxP3 expression\textsuperscript{64}. Therefore, FoxP3-IRES-Cre-GFP; Rosa26-lox-stop-lox-RFP mice which were considered a better model were used by Miyao et al\textsuperscript{65}. They suggested those FoxP3 downregulated T cells come from a minor population of nonregulatory FoxP3+ T cells exhibiting promiscuous and transient FoxP3 expression in inflammatory cytokine milieu or in a lymphopenic environment and that there are a few Treg cells that transiently lose FoxP3 expression; such Treg cells retained their memory and robustly re-expressed FoxP3 and suppressive function upon activation\textsuperscript{65}. However, the conventional T cells that transiently express FoxP3 would fall into the population of exFoxP3.

If this is also the case in our model, exTfr cells might be a mixed population of bona fide exTfr cells and Tfh that transient express FoxP3 or conventional T cells that get activated, transiently express FoxP3 and differentiate into Tfh cells or Treg cells that differentiate into Tfh cells. The inducible fate mapper model could exclude the pseudo-population accumulated during development since the tamoxifen treatment will be given to adult mice for a week before harvesting. The pseudo-population generated during activation were still within Tfr population, though some data suggested that the FoxP3 transiently expressing cells are rare\textsuperscript{63}. In order to study the role of FoxP3 in Tfr cells, only those “latent Treg” cells without FoxP3 expression should be studied to assess the phenotype and function. Therefore, we should explore the origin of exTfr cells. Stable Foxp3 expression is accompanied by epigenetic modulation of the Treg-specific demethylated region (TSDR), a CpG-rich, non-coding sequence within the first intron of the Foxp3 gene locus\textsuperscript{66}. The TSDR is demethylated in Tregs stably expressing Foxp3 but is fully methylated in CD4+ conventional T cells. Zhou et al. suggest exFoxP3 T cells maintain a small
percentage of demethylation and partial methylation. If the demethylated TSDR of exTfr cells could be found, then we could further confirm the existence of the Tfr cell-origin of exTfr cells. However, the methylation status of exTfr cells is less predictable since Tfr cells have a different transcription program from Treg cells. Without stable FoxP3 expression, even if exTfr cells maintain methylated TSDR region, we cannot judge the cells’ origin. RNAseq studies of Tfr, exTfr, Tfh and Tconv cells could be useful to analyze the differences and confirm whether exTfr and Tfh cells have same gene expression profile. Although more analysis could be conducted, it is hard to purify the population and do assessment of only “latent” Tfr cells with the fate mapper model.

Though it is difficult to confirm exTfr cells with the in vivo system directly, the in vitro FoxP3 stability assay could not only prove the existence of FoxP3- Tfr cells, but also provide evidence for the origin of exTfr cells. When we sorted FoxP3-expressing Tfr cells and cultured them with Tfh and B cells for 4 days, we found that some Tfr cells lose FoxP3 expression. The in vitro system is purer than the in vivo model without complications from so many immune cell types, so we could confirm that the exTfr cells come from Tfr cells rather than Tfh cells using the congenic marker. The phenotype of the in vitro generated exTfr cells was similar to in vivo ones, which also suggested in vivo exTfr cells be of Tfr origin. We can do RNAseq with the in vitro-generated exTfr cells to study the transcriptional program differences between Tfr, Treg, Tfh cells and exTfr cell, and it would be better than using the in vivo model to study the transcription program since there is no need to concern about blended cells. However, the fate mapper model is still essential to study exTfr cells. We cannot do in vitro suppression assay with the in vitro generated exTfr cells since it is hard to re-sort the cells and culture them with Tfh and B cells since the cells cannot live for a long time in vitro. In the day 4 analysis, we found a large number
of dying T cells. The in vivo model confirms the natural generation of exTfr cells and it broadens our research to explore further about the role of exTfr cells playing in regulating B cell responses.

This thesis demonstrates the importance of FoxP3 in Tfr cells. However, there remain a number of unanswered questions regarding FoxP3 in Tfr cells. For instance, how is FoxP3 working in Tfr cells to control the transcription program and alter suppression? As is known in Treg cells, FoxP3 can bind to a lot of cofactors and regulate large numbers of genes. For instance, FoxP3 binds to Rel family transcription factors, RELA and NFATC2, to inhibit cytokine production and T-cell effector function\(^\text{67}\). Transcriptional repression of IL2 is mediated via its association with histone acetylase KAT5 and histone deacetylase HDAC\(^\text{7}\). FoxP3 can activate the expression of TNFRSF18, IL2RA and CTLA4 and repress the expression of IL2 and IFN\(\gamma\) via its association with transcription factor RUNX1\(^\text{69}\). EZH2 can also bind to FoxP3 and leads to target gene silence\(^\text{35}\). There are sound reasons to speculate that FoxP3 in Tfr cells can bind to some of the cofactors to function.

Our next step would be focus on studying how FoxP3 modulates Tfr suppressive function. Specific genetically engineered mouse models can be used, for example, EZH2 conditional knockout model. We can perform in vivo and in vitro assays to determine whether EZH2 impacts the suppressive function. RNAseq could be conducted to compare the transcriptional profile of EZH2 KO Tfr with FoxP3 KO ones, which would provide a means to investigate the relationship of the two genes.

The other interesting finding of this thesis is the exTfr cells. Here we use exTfr cells to demonstrate the role of FoxP3 in Tfr cells, while the role of exTfr cells in B cell responses is also worth exploring. ExTfr cells cannot suppress antibody production as well as Tfr cells and their phenotype resembles Tfh cells, which suggests they have special modulatory roles in B cell
follicles or germinal centers. For instance, exTfr cells might be a brake for suppression as they might compete with Tfr cells for survival or costimulatory signals. To further study the cells, firstly, we can do immunofluorescence to confirm the location of exTfr cells and answer questions like are they in the T-B border or germinal centers, do they contact Tfh cells, B cells or FDCs. Then we can do a time course assay to check when the exTfr cells are generated. In addition, ex vivo flow cytometry and in vitro assays can be performed to compare exTfr cells with Tfh cells to see if the cells can promote B cell responses. Thirdly, RNAseq can be done to gain an understanding of the transcriptional profile of exTfr cells in order to find more possible functionalities. Last but not least, we can study exTfr cells in the disease models to see if they have any prognostic or diagnostic potentials.

Vaccine responses are closely correlated with antibody production, which suggest Tfr cells play a role in modulating vaccine responses. Autoimmune diseases are usually accompanied with autoantibody production. Reinforcing Tfr function might help ameliorate autoimmunity. FoxP3, as a crucial factor for Tfr cell function, could be a good target for boosting vaccine responses or improving autoimmune therapy.
Chapter 5: Concluding statements

In this thesis, we demonstrated the essential role of FoxP3 in Tfr cell suppressive function. The FoxP3 conditional knockout mouse model suggested Tfr cells downregulate their identity and functional molecules such as CTLA4, ICOS, GITR and CD25 when they lose FoxP3 expression. Interestingly, we found some CD4+CXCR5+CD25+FoxP3-CD19- cells in wild type mice during experiments. These cells can be a population of Tfr cells that naturally downregulated FoxP3. We confirmed the FoxP3 naturally downregulated Tfr cells using an in vitro assay, which cells were termed as exTfr cells. To further explore exTfr cells, we used a FoxP3 fate mapper model to trace exTfr cells. ExTfr cells showed a similar phenotype as FoxP3 knockout Tfr cells and the in vitro suppressive assay suggest exTfr cells cannot suppress B cell responses as well as Tfr cells. To compensate for the limitation of the model, which is the leakiness of red fluorescence, an inducible fate mapper was used. The existence and phenotype of exTfr cells was confirmed. In conclusion, the data in this thesis demonstrated that FoxP3 is of vital importance for Tfr cell suppressive function, a finding that suggests a potential way to regulate Tfr cells to boost vaccine responses or to ameliorate autoimmune diseases.
Chapter 6: References


