Stony Brook University

An Initial Analysis of the Role of *Sts-1* and *Sts-2* in the Development of Regulatory T cells

A Thesis Presented

by

Nithya Sivaram

to

The Graduate School

in Partial Fulfillment of the

Requirements

for the Degree of

Master of Science

in

Biochemistry and Cell Biology

Stony Brook University

December 2011

Stony Brook University
The Graduate School

Nithya Sivaram

We, the thesis committee for the above candidate for the
Master of Science degree, hereby recommend
acceptance of this thesis.

Dr. Nick Carpino
Associate Professor, Department of Molecular Genetics and Microbiology

Dr. Nancy Reich
Professor, Department of Molecular Genetics and Microbiology

This thesis is accepted by the Graduate School

Lawrence Martin
Dean of the Graduate School

Abstract of the Thesis

An Initial Analysis of the Role of Sts-1 and Sts-2 in the Development of Regulatory T cells

by

Nithya Sivaram

Master of Science

in

Biochemistry and Cell Biology

Stony Brook University

2011

Regulatory T cells (T_{regs}) play a critical role in down-regulating immune responses to self antigens. Here we assess the significance of Sts-1 and Sts-2, two protein phosphatases that negatively regulate TCR signaling, in the development and maintenance of T_{regs}. We show that the number of splenic T_{regs} *in vivo* is elevated in the absence of Sts-1and Sts-2. We also found that Sts-1 and Sts-2 play a redundant role in TGF-β-mediated induction of T_{regs} *in vitro*. These data indicate that the Sts proteins are crucial for the maintenance of normal T_{reg} populations in mice and also in TGF-β-mediated induction of T_{regs} *in vitro*.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

APC	Antigen Presenting Cell
CTLA-4	Cytotoxic T lymphocyte Antigen-4
GITR	Glucocorticoid Induced Tumor Necrosis Factor Receptor
IBD	Inflammatory Bowel Disease
IPEX	Immunodysregulation Polyendocrinopathy Enteropathy X-Linked Syndrome
iT_{regs}	Induced regulatory T cells
nT_{regs}	Natural regulatory T cells
SCID	Severe Combined Immunodeficiency
TGF-β	Transforming Growth Factor-β
T_{regs}	Regulatory T cells

Acknowledgemengts

It gives me great pleasure to thank those who made this thesis possible. First and foremost, I would like to thank my advisor Dr. Nick Carpino for his guidance, support, and encouragement without which this thesis would not have taken shape. I would also like to thank Dr. Nancy Reich for her advice, help, and comments through the course of my work.

I am grateful to Neena Carpino, Boris San Luis, and Ellie Ivanova for teaching me some of the techniques I learnt, and also for their support and company in the lab. I express my gratitude to the members of the Furie lab, Mackow lab, Reich lab, and Ando lab for their assistance with chemicals and other lab equipment.

Finally, I would like to express my indebtedness to my parents, brother, and all my friends for their moral support and constant encouragement that helped me overcome minor set-backs and hurdles throughout the course of my work.

1. INTRODUCTION

The immune system is constantly faced by the challenge of discriminating between self and non-self antigens. The ability of the immune system to elicit immune responses against infection while preventing responses against self-antigens is critical for host survival. The major mechanism by which tolerance to self is established occurs in the early stages of T cell development in the thymus, when cells that have a high affinity to self-antigens are deleted (1). In addition to the process of clonal deletion, self-tolerance is also maintained by a subset of T cells known as regulatory T cells that negatively regulate immune responses and suppress self-reactive T cells.

In 1970, Greshon and Kondo made an important observation that a small population of T cells that were unlike other helper T cells exerts suppressive activity, and coined the term 'suppressor cells' to describe them (2). Research on suppressor T cell activity was pursued extensively until the mid-1980s. However, the existence of these cells became questionable (3) because neither the cells nor the molecules associated with their suppressive function could be characterized (4, 5). As a result, research on suppressor T cells was dropped and the concept of suppressor T cells lay dormant until 1995, when Sakaguchi *et al*. showed that $CD4^+$ T cells that express the high affinity α chain receptor of IL-2 (CD25) control autoreactive T cells *in vivo* (6). Another study reported that antigen-specific T cell clones suppressed the proliferation of $CD4^+$ T cells in response to antigens and prevented colitis in a severe combined immunodeficiency mouse model (7). These and other studies led to the identification and characterization of a population of naturally occurring T cells that are now called $CD4^+CD25^+$ regulatory T cells or T_{regs} (8, 9).

Regulatory T cells or T_{regs}

$CD4^+CD25^+$ regulatory T cells constitute approximately 5–10 % of all the $CD4^+$ helper T cells in the periphery. The best-characterized T_{regs} are the naturally occurring T_{regs} (nT_{regs}) that are produced in the thymus, express CD25 constitutively, and can suppress immune responses in an antigen-dependent fashion (6, 10–12). Sakaguchi and co-workers demonstrated that $CD4^+$

$CD25^+$ T_{regs} exhibited regulatory functions *in vitro* and *in vivo* (6, 13). For example: *in vitro*, they prevented the proliferation of helper CD4 and CD8 T cells and *in vivo*, they prevented colitis that is caused by the injection of $CD4^+$ T cells lacking CD25 (6). Finally, it has been shown that disruption of T_{reg} populations in the thymus or the periphery leads to autoimmunity. In particular, neonatal thymectomy in mice on day 3 after birth leads to severe autoimmune disorders such as IBD (10).

Regulatory T cells develop in the thymus from single positive CD4 cells. In the process of T cell development, $CD4^+$ cells that interact with self peptides/MHC with high affinity develop into $CD4^+CD25^+$ regulatory T cells (14–16). Accessory molecules expressed on the surface of developing T cells also interact with the thymic stromal cells to increase the avidity of the interaction and thereby contribute to the development of regulatory T cells. These cells are called natural T_{regs} and they eventually egress out of the thymus to the periphery. In addition, peripheral $CD4^+$ T cells can also acquire the regulatory phenotype by antigenic stimulation and co-stimulation in the presence of TGF-β (17, 18). The T_{regs} that are induced in the periphery are called induced T_{regs} (iT_{regs}) or adaptive T_{regs}. The cytokine IL-2 is critical for the development of T_{regs} both in the thymus and in the periphery (19).

A cardinal feature of T_{regs} is the expression of Foxp3, a forkhead-winged-helix family transcription factor. Mutations in Foxp3 result in lethal autoimmune disorders. Examples of this are *scurfy* mice and the human X-linked autoimmune disorder IPEX (20–23). Several studies have revealed the significance of Foxp3 in the development and function of T_{reg} cells (24–26). To begin, $CD4^+CD25^+$ cells in the thymus and periphery generally express Foxp3. In addition, introduction of Foxp3 gene into $CD4^+CD25^-$ cells converts them into suppressive T cells similar to T_{regs} (20, 21). This and similar such studies indicate that Foxp3 functions as a master control switch to confer regulatory functions to T cells. Thus, it is currently considered the most specific marker for T_{regs}.

In addition to Foxp3, T_{regs} also express other activation markers such as CD122, CD132, OX-40, Glucocorticoid Induced Tumor Necrosis Factor Receptor (GITR), and Cytotoxic T Lymphocyte Antigen-4 (CTLA-4). All of these have been evaluated as molecular markers for

the identification of T_{regs} but none are as specific as Foxp3. Also, specific reliable markers to differentiate nT_{regs} and iT_{regs} still remain elusive despite known differences between the two populations (27).

Functionally, T_{regs} are hypo-responsive to *in vitro* TCR stimulation. However, in the presence of CD28 co-stimulation and large amounts of IL-2 T_{regs} proliferate and expand, making it possible to isolate antigen-specific T_{regs} (28). The *in vitro* anergy of regulatory T cells is not reflective of their complex activity *in vivo*. Specifically, adoptive transfer experiments in mice indicate that T_{reg} cells are capable of extensive self-renewal. In fact, transfer of a small number of T_{regs} afforded recipients long-lasting protection against autoimmunity. Thus the initial description of T_{regs} as anergic is misleading.

T_{regs} mediate their suppressive function through various mechanisms. *In vitro* studies suggest that it is important for T_{regs} to establish contact with target cells or APCs in order to suppress effector cells (29–31). This contact is mediated by CTLA-4 through engagement of CD80 and CD86 expressed on the surface of APCs (32, 33). In addition, suppression by T_{regs} may also be cytokine mediated through the secretion of IL-10 and IL-35 (34). Other mechanisms through which T_{regs} suppress effector cells include sequestering IL-2, inhibiting IL-2 synthesis by effector cells, secreting TGF-β, perforin, or granzyme B.

Suppressor of T Cell Receptor Signaling Proteins (Sts-1 and Sts-2)

Activation of T cells is governed by the T cell antigen receptor, the regulation of which is under stringent control. Two proteins belonging to the Suppressor of TCR signaling family, Sts-1 and Sts-2, play an important role in the negative regulation of TCR signaling (35). Sts-1 is ubiquitously expressed while Sts-2 is primarily expressed in hematopoietic cells.

Sts-1 and Sts-2 are enzymes belonging to the superfamily of 2H-phosphatases that have an N-terminal ubiquitin-binding UBA domain, a central SH3 domain that participates in protein-protein interactions, and a C-terminal PGM domain that has phosphatase activity. The SH3 domain of Sts-1 and Sts-2 has been shown to interact with Cbl and prevent Cbl-mediated endocytosis of receptor tyrosine kinases such as epidermal growth factor receptor and platelet

derived growth factor receptor (36). In the context of T cells, Sts-1 and Sts-2 exert their regulatory effect downstream of the TCR by regulating tyrosine phosphorylation of proteins like ZAP-70 (37, 38).

The significant role played by Sts-1 and Sts-2 in controlling TCR signaling was determined from studies on mice in which genes for both proteins have been ablated. When exposed to mitogenic antibodies such as CD3, *Sts-1/2*$^{-/-}$ T cells hyper-proliferate compared to the cells from wildtype, *Sts-1*$^{-/-}$, and *Sts-2*$^{-/-}$ mice. The CD28 pathway has been shown to be unaffected in the *Sts-1/2*$^{-/-}$ T cells (39). Owing to their enhanced sensitivity to TCR stimulation, *Sts-1/2*$^{-/-}$ T cells also produce higher levels of cytokines including IFNγ, IL-2, IL-4, IL-5 and IL-10. Interestingly, the hypersensitive phenotype was not observed in T cells lacking either Sts-1 or Sts-2 alone. (39). Using experimental autoimmune encephalomyelitis as a model, it has been shown that mice lacking Sts-1 and Sts-2 are more susceptible to autoimmune disorders. Thus mice lacking Sts proteins have been used as a model system for autoimmunity.

Aim of the study

This study investigates the role of two proteins, Sts-1 and Sts-2, in the development of T_{regs}. The main focus of the study is on the role of Sts-1 and Sts-2 on TGF-β mediated induction of iT_{regs} development.

2. METHODS

Mice: BL/6 mice were used for all the experiments. *Sts-1*$^{-/-}$, *Sts-2*$^{-/-}$, and *Sts-2*$^{-/-}$ mice were generated by ablating the genes for Sts-1, Sts-2, or both respectively.

Antibodies and Reagents: The monoclonal antibodies used in the experiments included: PE-conjugated anti-mouse CD4 (clone H129.19), FITC-conjugated anti-mouse CD25 (clone 7D4), and APC-conjugated anti-mouse Foxp3 (clone FJK-16s). These antibodies were all purchased for BD biosciences. For induction assay: Purified NA/LE hamster anti-mouse CD3ε (clone 145-2C11) and purified NA/LE hamster anti-mouse CD28 (clone 37.51) from BD Biosciences,

recombinant IL-2 and recombinant human TGF-β1 from PeproTech. For cell culture: RPMI 1640 media containing 10% FCS, 10 mM HEPES, pH 7.0, 2 mM glutamine, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol, 0.1 mM nonessential amino acids, and the appropriate concentration of penicillin/streptomycin and gentamycin, all from Invitrogen, was used.

Flow Cytometry : Spleens from wild-type and mice lacking Sts-1, Sts-2, and both proteins were crushed between two sterile glass slides and suspended in phosphate buffered saline containing 2 % FBS to obtain a single cell suspension. Red blood cells were lysed using ACK lysis buffer, pH 7.2, containing 150 mM NH_4Cl, 1 mM $KHCO_3$, and 0.1 mM EDTA. The debris was removed by filtering through a 70 μm filter and the cells were washed with PBS containing 2 % FBS. The splenocytes were counted using trypan blue staining and 1×10^5 cells were used for analysis. The cells were first stained with the surface markers, CD4 and CD25 followed by permeabilization with cytofix /cytoperm fixation and permeabilization solution to permeabilize the cell wall and cell membrane. The cells were then washed with perm wash buffer and stained for Foxp3 in perm wash buffer. Data was acquired on a FACSCalibur machine and analyzed using CellQuest Pro.

$CD4^+$ T Cell Isolation and Cell Sorting for Induction Assay: $CD4^+$ T cells were isolated from single cell suspensions of splenocytes after RBC hydrolysis by negative staining using a mouse $CD4^+$ T cell isolation kit (Miltenyi) as per the manufacturer's instructions. The $CD4^+$ T cells obtained were stained with PE-conjugated CD4 and FITC-coupled CD25 antibodies for FACS sorting. $CD4^+CD25^-$ cells were collected in gentamycin containing RPMI 1640 medium as the progenitors of T_{regs} to be used in the induction assay.

Induction Assay: 24-well plates were coated with 2 μg/ml of CD3 antibody for 2 hr at 37 °C. The plates were washed and $CD4^+CD25^-$ cells were plated in RPMI medium with 2 μg/ml of CD28, 2 U/ml of IL-2, and 5 ng/ml of TGF-β for varying periods of time. The cells were harvested after 1.5, 2.5, 3.5, 4.5, and 5.5 days and analyzed by flow cytometry as well as qPCR to check for Foxp3 expression at the protein and mRNA level respectively.

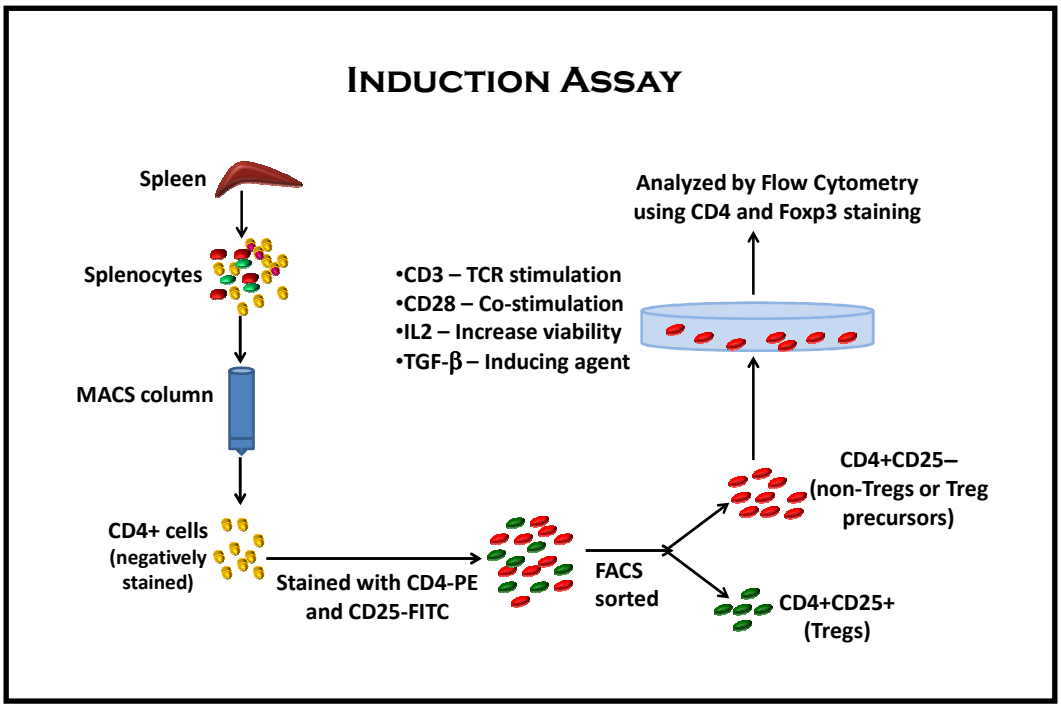

Figure 1. Flow chart showing the steps involved in preparing and plating the cells for induction assay.

Quantitative Real-time PCR: Total RNA was isolated using Trizol (Invitrogen). It was treated with RNase free DNase (Qiagen) and cleaned up using RNeasy column (Qiagen) to ensure the purity of the RNA. cDNA was synthesized from 200 ng of RNA using superScript II (Invitrogen). The cDNA was used for quantitative Realtime PCR in an AB StepOne Plus instrument using Power SYBR green reagents (Invitrogen). AmpErase Uracil N-glycosylase (Invitrogen) was added to prevent carryover contamination. Following incubation and activation at 50 °C for 2 min and 95 °C for 10 min, 40 cycles of 15 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C were carried out. The target genes were normalized against *Hprt* as housekeeper and the data was analyzed using the $2^{-\Delta Ct}$ method. Primers for the assay were: *Foxp3* forward primer 5'-AAT AAA TCA TAA GGC CCT TCT CCA GGA CAG-3', *Foxp3* reverse primer 5'-AAT AAA TCA TAA GCT GAT CAT GGC TGG GTT GT-3', *Hprt* forward primer 5'-ACCA GCC CCA AAA TGG TTA AGG-3', *Hprt* reverse primer 5'-TCT GGG GAC GCA GCA ACT GAC-3'.

3. RESULTS

Increased number of splenic $CD4^+CD25^+Foxp3^+$ T_{regs} in *Sts-1/2*$^{-/-}$ mice

The number of regulatory T cells in wild type and *Sts-1/2*$^{-/-}$ spleens was analyzed using flow cytometry. T_{regs} were identified as $CD4^+$ T cells expressing CD25 as well as Foxp3 markers (Figure 2A). Increased numbers of $CD4^+CD25^+Foxp3^+$ T_{reg} cells were seen in spleens of mice lacking the Sts proteins. A representative of seven experiments is shown in Figure 2A. The number of T_{reg} cells was almost double in *Sts-1/2*$^{-/-}$ spleens compared to the wildtype spleens (Figure 2B) with a mean of 4.818 % ± 0.743806 T_{regs} in wildtype mice and 9.192 % ± 1.041245 in mice lacking Sts-1 and 2.

Sts-1and Sts-2 are required for *in vitro* induction of T_{regs}

To address the question of whether Sts proteins had an effect on the peripheral conversion of T_{regs}, we evaluated progenitors of T_{regs} ($CD4^+CD25^-$) that were isolated from the spleens of *Sts-1/2*$^{-/-}$ mice. Cells from wild type BL/6 mice were used as control. T_{reg} progenitors were isolated from the pool of splenocytes by MACS separation and FACS sorting. Interestingly, the numbers of T_{reg} progenitors sorted were always reduced in *Sts-1/2*$^{-/-}$ mice (53.433 ± 9.569918) in relation to the wildtype mice (82.166 ± 3.262412). The cells were cultured in the presence of TGF-β, which drives the induction of T_{regs} in the periphery. The cells were analyzed by flow cytometry and qPCR to assess the expression of Foxp3.

Unexpectedly, we observed that the absence of the Sts proteins impairs TGF-β driven induction of T_{regs} (Figure 3). The absence of Sts-1 and Sts-2 does not completely inhibit induction but it significantly diminishes the percentage of cells that are induced when compared to the wildtype. In the representative example shown in Figure 3A, the percentage of cells expressing Foxp3 in Sts-1/2$^{-/-}$ cells was a lesser 22% compared to 56 % in the wildtype cells.

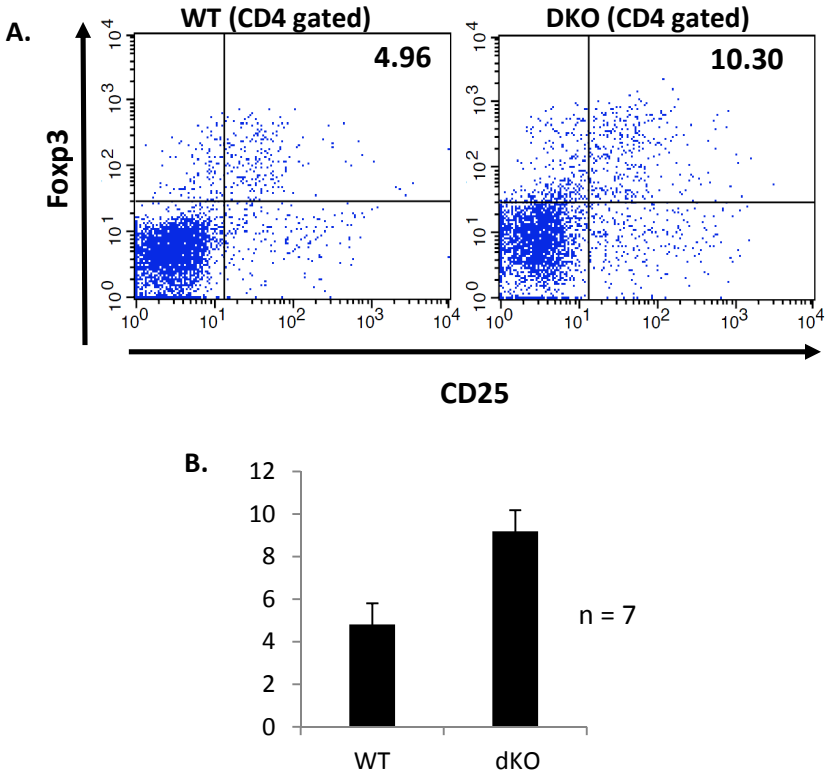

Figure 2. Flow cytometric analysis of splenic T_{regs} in wildtype and *Sts-1/2*$^{-/-}$ mice. **A.** Lack of Sts-1 and Sts-2 proteins leads to an increase in the number of splenic T_{regs}. Percentage of $CD25^{+}Foxp3^{+}$ regulatory T cells gated on $CD4^{+}$ lymphocytes is shown in the upper right quadrant. The dot plots are representative of seven individual experiments. **B.** The difference between regulatory T cell numbers between the wildtype and *Sts-1/2*$^{-/-}$ mice in a cumulative of seven experiments is shown with standard deviations (± 0.743806 and ± 1.041245 respectively).

A similar result was obtained from *Foxp3* mRNA analysis (Figure 3B). The level of *Foxp3* mRNA was determined after 4.5 days of culture with TGF-β and normalized with the mRNA levels of *Hprt* (Figure 3B). We observed a significant difference in the levels of mRNA between the wildtype and the *Sts-1/2*$^{-/-}$ cells after 4.5 days of culture with TGF-β. Similar to the protein expression data (Figure 3A), the mRNA in the *Sts1/2*$^{-/-}$ cells is greatly diminished but not completely absent. The results indicate that *Foxp3* mRNA and protein induction, is impaired in the mice lacking Sts-1 and 2.

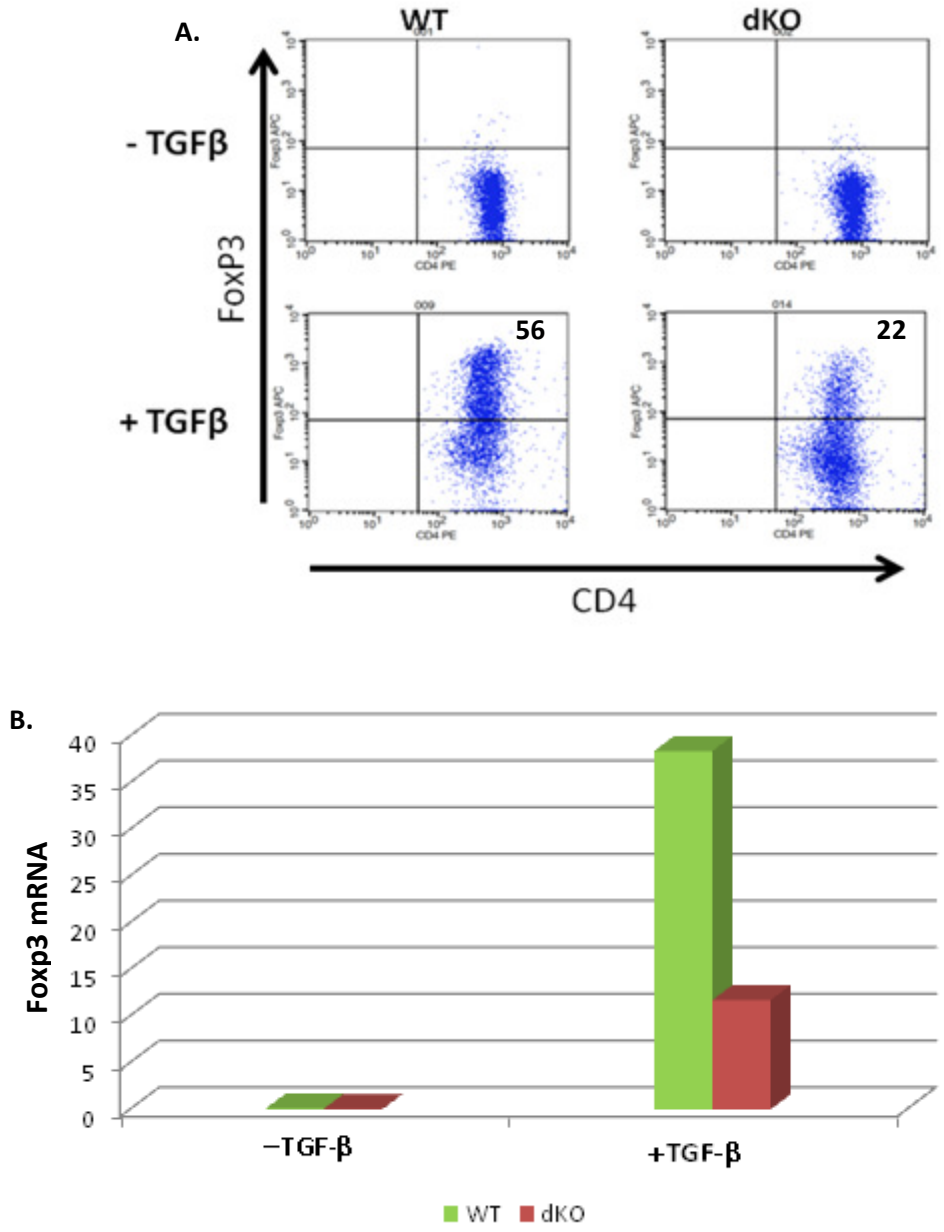

Figure 3. Absence of Sts-1 and Sts-2 impairs TGF-β-mediated induction. **A.** Flow cytometric analysis of Foxp3 protein expression on day 4.5. Dot plots on the left show wild type cells cultured with and without TGF-β and dot plots on the right show *Sts1/2*$^{-/-}$ cells cultured with and without TGF-β. The numbers on the upper right quadrants are the percentages of Foxp3^{+} iT$_{regs}$ after 4.5 days in culture. **B.** qPCR analysis of *Foxp3* mRNA of wild type and *Sts-1/2*$^{-/-}$ cells cultured with TGF-β after culture for 4.5 days.

Time-course analysis of Foxp3 expression

To further investigate the difference in iT_{reg} induction between wildtype and *Sts-1/2*$^{-/-}$ cells, we performed a time-course analysis of Foxp3 expression from 0 to 5.5 days. The results indicate that Foxp3 expression begins after ~40 h of culture. On day 1.5, the percentage of cells expressing Foxp3 is almost equal in the wildtype and *Sts-1/2*$^{-/-}$ cultures. However, by day 2.5, diminished expression of Foxp3 in Sts-deficient cells becomes evident (Figure 4). On day 3.5, although the expression of Foxp3 is maintained in wildtype cells, it drops dramatically in *Sts-1/2*$^{-/-}$ cells (Figure 4). Between days 3.5 and 4.5, the expression levels of Foxp3 also begin to fall in the wildtype but to a much lesser extent than in the Sts-deficient cells. On days 4.5, and 5.5, there continues to be a smaller percentage of cells expressing Foxp3 in the *Sts-1/2*$^{-/-}$ population compared to the wildtype population.

The levels of *Foxp3* mRNA were also determined at these time points in an independent experiment. RNA was extracted from cells, total RNA was reverse transcribed, and then used for qPCR analysis. As illustrated in Figure 5, *Foxp3* mRNA begins to increase in both the wildtype and *Sts-1/2*$^{-/-}$ cells once induced by TGF-β to become iT_{regs}. However, lower levels of *Foxp3* mRNA were consistently seen in *Sts-1/2*$^{-/-}$ cells relative to the wildtype cells. On one hand, we observe that *Foxp3* mRNA expression begins at day 1.5, similar to protein expression (Figure 4), and increases drastically between days 2.5 and 3.5 in the wildtype population. The level of mRNA continues to increase in the wildtype cells with the progression of time. On the other hand, the level of *Foxp3* mRNA in *Sts-1/2*$^{-/-}$ cells, although significantly less than that in the wildtype, is seen to steadily increase until day 4.5 and then fall on day 5.5 (Figure 5). The level of mRNA in the wildtype cells peaks at day 5.5 while in *Sts-1/2*$^{-/-}$ cells, it peaks sooner on day 4.5. At each time point, the level of mRNA in *Sts-1/2*$^{-/-}$ population is significantly less than that in the wildtype population.

Sts-1 and Sts-2 play a redundant role in TGF-β driven induction of T_{regs}

To evaluate the role of Sts-1 and Sts-2 individually in the process of induction, $CD4^+CD25^-$ cells from wild type, *Sts-1*$^{-/-}$, *Sts-2*$^{-/-}$, and *Sts-1/2*$^{-/-}$ spleens were cultured in the

presence and absence of TGF-β. Foxp3 expression was analyzed using flow cytometry by CD4 and Foxp3 staining (Figure 6).

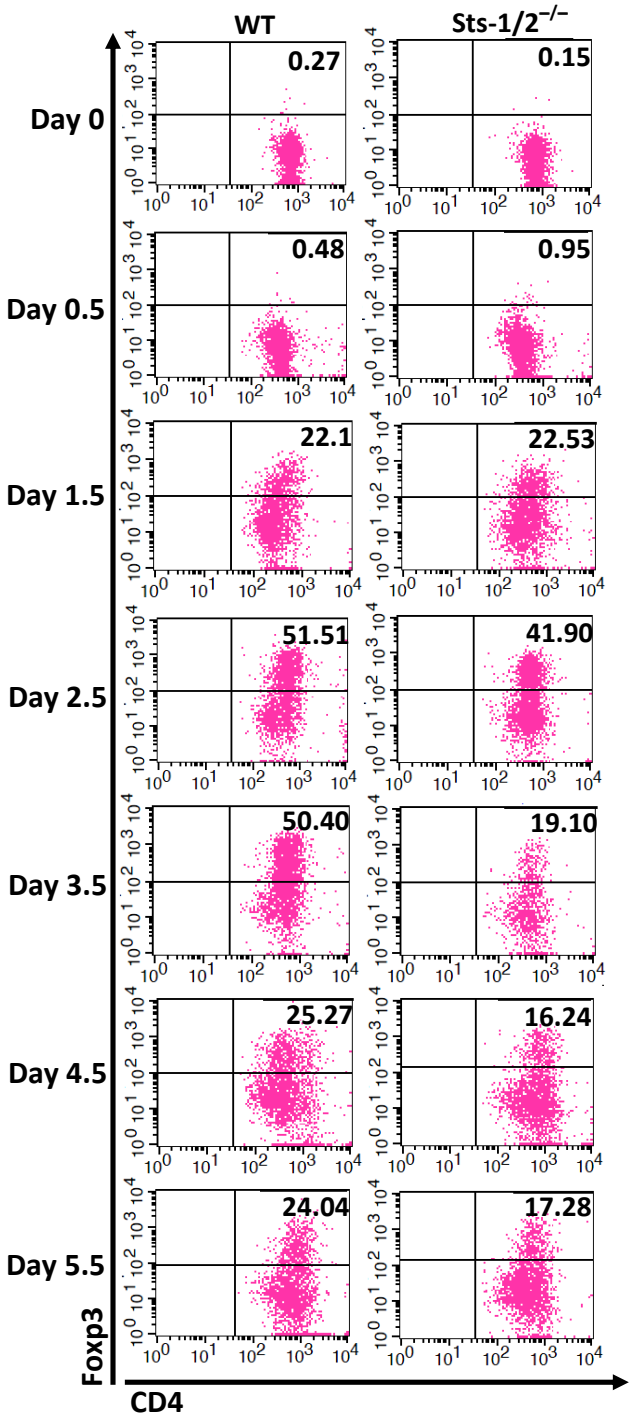

Figure 4. Flow cytometric analysis of cells for expression of Foxp3. The cells were cultured with TGF-β for 0–5.5 Days. The cells were stained for CD4 and Foxp3. Percentage of cells expressing Foxp3 is shown in the upper right quadrant.

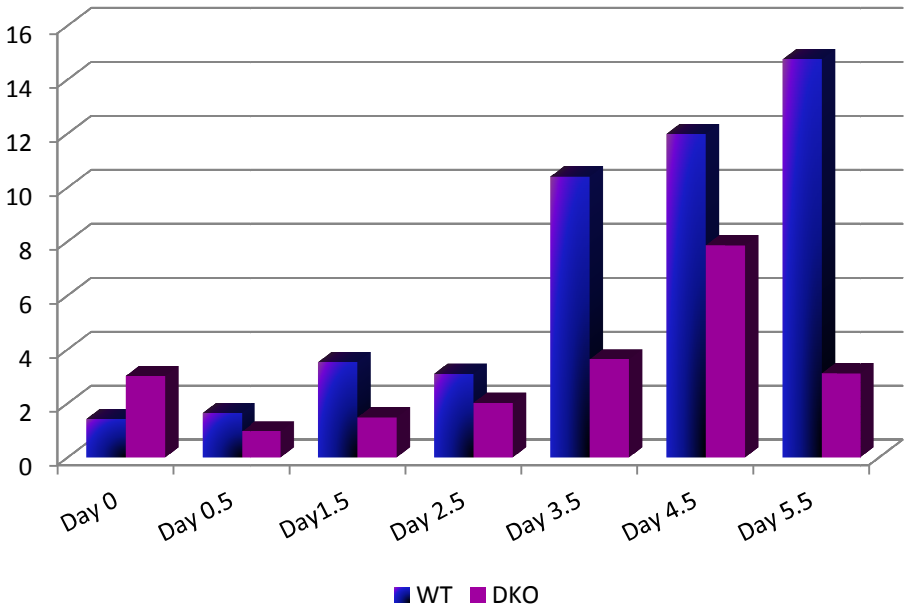

Figure 5. Time course: qPCR analysis of *Foxp3* mRNA expression. RNA was extracted from wildtype and Sts-1/2$^{-/-}$ cells cultured with TGF-β at regular intervals and used for cDNA synthesis. The cDNA was then used for real-time qPCR. The levels of *Foxp3* mRNA presented here have been normalized to the levels of *Hprt*.

It is evident from Figure 6 that Foxp3 expression is not impaired in the absence of just one Sts homolog. The percentage of cells expressing Foxp3 after 4.5 days of induction was similar in the wildtype, *Sts-1*$^{-/-}$, and *Sts-2*$^{-/-}$ T cells, while Foxp3 expression in *Sts-1/2*$^{-/-}$ cells was significantly lower. As shown in Figure 6, in the wildtype, *Sts-1*$^{-/-}$, and *Sts-2*$^{-/-}$ populations, more than 50 % of the cells expressed Foxp3 whereas in the case of *Sts-1/2*$^{-/-}$ only 12.69 % of the cells expressed Foxp3.

Inherent variability in induction of T_{regs}

The induction assay was repeated a number of times under similar culture conditions and the results have been presented in Table 1. The results indicate an undisputable role for the Sts proteins in TGF-β-mediated induction of T_{regs}. In all of the experiments conducted, cells from

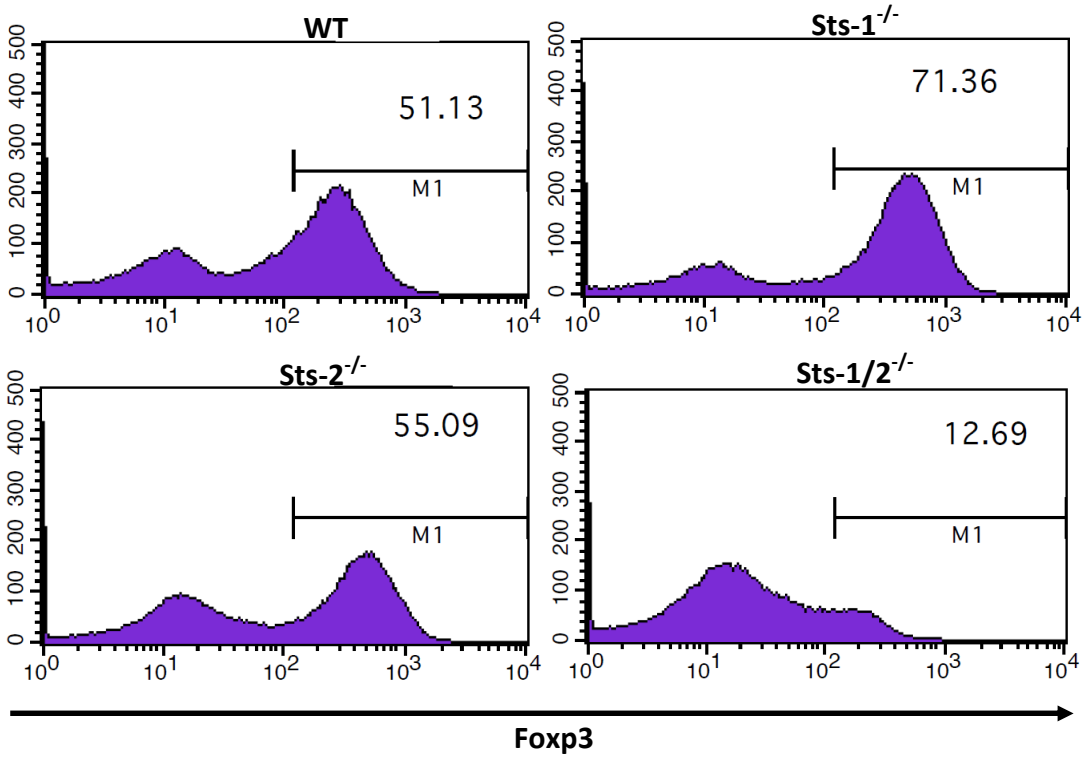

Figure 6. Sts-1 and Sts-2 play a redundant role in TGF-β-mediated induction of T_{regs}. Histograms show the expression of Foxp3 after 4.5 days of culture with TGF-β. Sts-$1^{-/-}$ and Sts-$2^{-/-}$ do not show any impairment in the TGF-β-mediated induction of iT_{regs}. The lack of both proteins on the other hand, impairs the induction of Foxp3 expression.

mice lacking both Sts-1 and Sts-2 showed reduced induction compared to the cells from wild type mice (Table 1). We observed that the percentage of cells induced to express Foxp3 varies considerably in the wildtype and *Sts-1/2*$^{-/-}$ populations. The mean percentage of cells induced to become iT_{regs} was 41.38 % ± 17.42897 in the wildtype cells and 16.04 % ± 13.77106 in the *Sts-1/2*$^{-/-}$ cells respectively. It is worth noting that although there seems to be great variability in the percentage of cells expressing Foxp3, the trend *viz*. reduced levels of Foxp3 expression in the *Sts-1/2*$^{-/-}$ population relative to the wild type population was seen consistently in all the experiments.

Experiment #	% Foxp3 expressing cells	
	Wild type	**Sts-1/2$^{-/-}$**
1.	48.89[a]	23.91 [a]
2.	54.93 [a]	25.81 [a]
3.	63.39	38.10
4.	34.30	0.18
5.	40.79 [a]	28.25 [a]
6.	59.75[a]	38.19[a]
7.	10.20	1.53
8.	16.28	1.50
9.	16.71	0.12
10.	68.13	0.03
11.	47.57	10.99
12.	43.97	6.72
13.	34.81	10.85
14.	50.07	18.68
15.	38.36	8.42
16.	63.80	39.24
17.	27.87	19.79
18.	25.03	16.57
Average ± Std. Dev.	**41.38056 ± 17.42897**	**16.04889 ± 13.77106**

[a]Cells were cultured for 3.5 days

Table 1. Percentage of cells expressing Foxp3 after 4.5 days in culture with TGF-β. The cells were stained with antibodies for CD4 and Foxp3, and analyzed by flow cytometry after 4.5 days of culture unless mentioned otherwise.

4. DISCUSSION

Our results demonstrate that Sts-1 and Sts-2 are required for the maintenance of normal numbers of peripheral T_{regs}. Absence of Sts-1 and Sts-2 leads to an increase in the number of peripheral T_{regs}. This data is consistent with previous unpublished results, indicating a role for Sts-1 and Sts-2 in thymic T_{reg} development. In particular, the number of $CD4^+CD25^+Foxp3^+$ T_{regs} in the thymi of mice lacking Sts-1 and Sts-2 were shown to be elevated in comparison to those in the wildtype mice, implicating a role for the Sts proteins in the T_{reg} development (Carpino, unpublished). As of now it is unclear if the increase in the number of peripheral T_{regs} is due to an increased migration of thymic T_{regs} to the periphery or due to an increased production of iT_{regs} in the periphery or both.

Under physiological conditions or in an *in vivo* setting, T_{regs} can be generated in the periphery by TGF-β-mediated induction. Based on our findings on the splenic T_{reg} numbers in *Sts-1/2*$^{-/-}$ mice (Figure 2), and studies on *Sts-1/2*$^{-/-}$ mice that describe the hyper-sensitive nature of TCR signaling in *Sts-1/2*$^{-/-}$ mice (39), we expected the T_{reg} progenitors from *Sts-1/2*$^{-/-}$ mice to generate greater number of iT_{regs} *in vitro.* The results however were contrary to our expectation. The number of iT_{regs} generated from cells of *Sts-1/2*$^{-/-}$ origin was reduced relative to the iT_{regs} generated from wildtype progenitors. This suggests that the increase in peripheral T_{regs} could be due to an increased egress of nT_{regs} from the thymus to the periphery.

Data from qPCR experiments confirm this impairment in induction at the mRNA level. A closer look at induction over a period of 5 days suggests that Sts-1 and Sts-2 might affect the stability of Foxp3 protein expression in *Sts-1/2*$^{-/-}$ cells. In the absence of Sts-1 and Sts-2, the levels of *Foxp3* mRNA and protein were substantially reduced and the percentage of iT_{regs} generated was diminished. This could indicate that Sts-1and Sts-2 are required not just for induction of T_{regs} but also for the maintenance of Foxp3 expression. We make this interpretation cautiously given the experiment was performed only one time. Nonetheless, we hypothesize that Sts-1 and Sts-2 regulate Foxp3 expression both at the transcriptional and the protein level. Several studies on TGF-β driven induction of T_{regs} have revealed that the continual presence of TGF-β is essential for the maintenance of Foxp3 expression under *ex vivo* conditions (40–42). Drawing inferences based on these studies, we are inclined to think that Sts-1 and Sts-2 may

regulate Foxp3 expression either by altering the availability of TGF-β to the cells or by altering the TGF-β signaling pathway itself. Further, it may also be possible that the absence of Sts-1 and Sts-2 results in rapid degradation of Foxp3 protein.

It has been previously reported that the generation of T_{regs} by TGF-β induction is time dependent (43, 44) and that Foxp3 up-regulation occurs ~2 days after the addition of TGF-β (45). Activation of Smad by TGF-β occurs within minutes, and SMAD-DNA complexes have been observed as early as 10 minutes after TGF-β stimulation (46), suggesting that induction involves a complex series of mechanisms between the formation of SMAD-DNA complexes and the first appearance of Foxp3. The time taken by the cells to respond to TGF-β by upregulating Foxp3 is also dependant on these complex mechanisms. This could also explain the wide variability in the level of T_{regs}. In addition, it is also possible that the variability in the percentage of T_{regs} generated is due to the cells being at different stages of their cell cycle and thus expressing different levels of protein at a particular point in time.

CTLA-4 and Cbl-b have recently been identified as factors necessary for TGF-β-induced Foxp3 expression (47, 48). Emphasizing that Sts-1 and Sts-2 are Cbl-b binding proteins (36) and that CTLA-4 is a negative regulator of TCR signaling, we speculate that Sts-1 and Sts-2 play a role in regulating the complex signaling cascade required for TGF-β-driven induction of Foxp3 expression in a time dependent fashion with or without the involvement of CTLA-4 and Cbl-b.

Finally, we also show that the presence of either one of the proteins compensates for the absence of the other. This data supports the notion that Sts-1 and Sts-2 function redundantly in T cells. This concept has been previously proposed after the demonstration of a redundant role for the Sts proteins in IFNγ production following TCR stimulation (49).

CONCLUSION

In summary, we show that the absence of Sts proteins in mice leads to an increase in the number of splenic T_{regs}. We also show that the lack of Sts-1 and Sts-2 impairs the induction of T_{regs} *in vitro*. This impairment, however, is not observed when one of the two proteins is present,

implying that Sts-1 and Sts-2 compensate for each other in pathways involved in TGF-β signaling that drives the induction of Foxp3 expression on T_{regs}.

REFERENCES

1. Kappler JW, Roehm N, Marrack P. (1987) T cell tolerance by clonal elimination in the thymus. Cell 49:273–280.

2. Gershon, R. K. and Kondo, K. (1970) Cell interactions in the induction of tolerance: the role of thymic lymphocytes. Immunology 18, 723–737.

3. Janeway CA jr (1988) Do suppressor T cells exist? A reply. Scand J Immunol 27:621–623.

4. Green, D. R., Flood, P. M. and Gershon, R. K. (1983) Immunoregulatory T-cell pathways. Annu. Rev. Immunol. 1,439–463.

5. Kronenberg, M., Steinmetz, M., Kobori, J., Kraig, E., Kapp, J. A., Pierce, C. W., et al. (1983) RNA transcripts for I-J polypeptides are apparently not encoded between the I-A and I-E subregions of the murine major histocompatibility complex. Proc. Natl. Acad. Sci.U.S.A. 80, 5704–5708.

6. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M (1995) Immunogic self-tolerance mainained by activated T cells expressing IL-2 receptor (CD25) Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J immunol 155:1151–1164.

7. Grouz H, O'Garra A, Bigler M, Bouleau M, Antonenko S, de Vries JE, et al. (1997) A CD^+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. Nature 389:737–742.

8. Takahashi T, Tagami T, Yamazaki S, Uede T, Shimuzu J, Sakaguchi N et al. (2000) Immunologic self-tolerance maintained by $CD4^+CD25^+$ regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. J Exp Med 192:303–310.

9. Thornton AM, Piccirillo CA, Shevach EM (2004) Activation requirements for the induction of $CD4^+CD25^+$ T cell suppressor function. Eur J Immunol 34:366–376.

10. Sakaguchi S (2004) Naturally arising $CD4^+$ regulatory T cells for immunologic self-tolerance and negative control of immune responses. Annu Rev Immunol 22:531–562.

11. Shevach EM (2002) $CD4^+$ $CD25^+$ suppressor T cells: more questions than answers. Nat Rev Immunol 2:389–400.

12. Shevach EM (2000) Regulatory T cells in autoimmunity. Annu Rev Imunnol 18:423–449.

13. Sakaguchi S, Sakaguchi N, Shimizu J, Yamazaki S, Sakihama T, Itoh M et al. (2001) Immunologic tolerance maintained by $CD25^+$ $CD4^+$ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance. Immunol Rev 182:18–32.

14. Jordan, M. S., A. Boesteanu, A. J. Reed, A. L. Petrone, A. E. Holenbeck, M. A. Lerman, A. Naji, and A. J. Caton (2001) Thymic selection of $CD4^+CD25^+$ regulatory T cells induced by an agonist self-peptide. Nat. Immunol. 2: 301–306.

15. Aschenbrenner, K., L. M. D'Cruz, E. H. Vollmann, M. Hinterberger, J. Emmerich, L. K. Swee, A. Rolink, and L. Klein (2007) Selection of Foxp3_ regulatory T cells specific for self antigen expressed and presented by $Aire^+$ medullary thymic epithelial cells. Nat. Immunol. 8: 351–358.

16. Bensinger, S. J., A. Bandeira, M. S. Jordan, A. J. Caton, and T. M. Laufer. 2001. Major histocompatibility complex class II-positive cortical epithelium mediates the selection of CD4+25+ immunoregulatory T cells. J. Exp. Med. 194: 427–438.

17. Zheng SG, Gray JD, Ohtsuka K, yamagiwa S and Horwitz DA (2002) generation ex vivo of TGF-beta-producing regulatory T cells from $CD4^+$CD25- precursors. J. Immunol. 169:4183–4189.

18. Chen W, et al (2003) Conversion of peripheral $CD4^+$CD25- naïve T cells to $CD4^+CD25^+$ regulatory T cells by TGF-beta induction of transcription factor Foxp3. J. Exp. Med. 198:1875–1886.

19. Fontenot JD, Rasmussen JP, Gavin MA and Rudensky AY (2005) A function for interlukin 2 in Foxp3-expressing regulatory T cells. Nat. Immunol. 6:1142–1151.

20. Brunkow ME, et al. (2001) Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. Nat. Genet. 27:68–73.

21. Chatila, TA, et al. (2000) JM2, encoding a fork head-related protein, is mutated in X-linked autoimmunity-allergic disregulation syndrome. J Clin Invest. 106:R75–R81.

22. Wildin RS, et al. (2001) X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. Nat. Genet. 27:18–20.

23. Bennett CL et al. (2001) The immune dysregulation, plyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. Nat Genet. 27:20–21.

24. Hori S, Nomura T and Sakaguchi S (2003) control of regulatory T cell development by transcription factor Foxp3. Science 299:1057–1067.

25. Fontenot JD, Gavin MA and Rudensky AY (2003) Foxp3 programs the development and function of $CD4^+CD25^+$ regulatory T cells. Nat Immunol 4:330–336.

26. Khattri R, Cox T, Yasayko SA and Ramsdell F. (2003) An essential role for Scurfin in $CD4^+CD25^+$ T regulatory cells. Nat. Immunol 4:337–342.

27. Bluestone JA, Abbas AK (2003) Natural versus adaptive regulatory T cells. Nat Rev Immunol. 3(3):253–257.

28. Tang Q, et al. (2004) In vitro expanded antigen-specific regulatory T cells suppress autoimmune diabetes. J Exp Med 199:1455–1465.

29. Takahashi, T., Y. Kuniyasu, M. Toda, N. Sakaguchi, M. Itoh, M. Iwata, J. Shimizu, and S. Sakaguchi (1998) Immunologic self-tolerance maintained by $CD25^+CD4^+$ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. Int. Immunol. 10: 1969–1980.

30. Tang, Q., J. Y. Adams, A. J. Tooley, M. Bi, B. T. Fife, P. Serra, P. Santamaria, R. M. Locksley, M. F. Krummel, and J. A. Bluestone (2006) Visualizing regulatory T cell control of autoimmune responses in nonobese diabetic mice. Nat. Immunol. 7: 83–92.

31. Thornton, A. M., and E. M. Shevach (1998) CD4_CD25_ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. J. Exp. Med. 188: 287–296.

32. Onishi Y, Fehervari Z, Yamaguchi T, and Sakaguchi S (2008) $Foxp3^+$ natural regulatory T cells preferentially form aggregates on dendritic cells in vitro and actively inhibit their maturation. Proc. Natl. Acad. Sci. USA 105, 10113–10118.

33. Paust S and Cantor H (2005) Regulatory T cells and autoimmune disease. Immunol. Rev. 204:195–207.

34. Collison LW, Pillai MR, Chaturvedi V, and Vignali DAA (2009) Regulatory T cell suppression is potentiated by target T cells in a cell contact, IL-35 and IL-10 dependent manner. J. Immunol. 182:6121–6128.

35. Tsygankov AY (2009) TULA-family proteins: an odd couple. Cell. Mol. Life Sci. 66:2949–2952.

36. Kowanetz K, Crosetto N, Haglund K, Schmidt MHH, Heldin CH, and Dikic I (2004) Suppressors of T cell receptor signaling Sts-1 and Sts-2 bind to Cbl and inhibit endocytosis of receptor tyrosine kinase. J. Biol. Chem 279(31):32786–32796.

37. Carpino N, Chen Y, Nassar N, and Oh HW (2009) The Sts proteins target tyrosine phosphorylated, ubiquitinated proteins within TCR signaling pathways. Mol. Imunnol. 46:3224–3231.

38. Raguz J, Wagner S, Dikic I, Hoeller D (2007) Suppressor of T-cell receptor signalling 1 and 2 differentially regulate endocytosis and signalling of receptor tyrosine kinases. FEBS Letters, 581(24):4767-4772.

39. Carpino N, Turner S, Mekala D, Takahashi Y, Zang H, Geiger TL, Doherty P, and Ihle JN (2004) Regulation of ZAP-70 activation and TCR signaling by two related proteins, Sts-1 and Sts-2. Immunity. 20:37–46.

40. Gabrysova L, Christensen JR, Wu X, Kissenpfennig A, Malissen B, and O'Garra A (2011) Integrated T-cell receptor and costimulatory signals determine TGF-b-dependent differentiation and maintenance of Foxp3 regulatory T cells. Eur. J. Immunol. 41: 1242–1248.

41. Floess S, Freyer J, Siewert C, Baron U, Olek S, Polansky J, Schlawe K, Chang HD, Bopp T, Schmitt E, et al. (2007). Epigenetic control of the foxp3 locus in regulatory T cells. PLoS Biol. 5, e38.

42. Huehn J, Polansky JK, and Hamann A (2009). Epigenetic control of FOXP3 expression: The key to a stable regulatory T-cell lineage? Nat. Rev. immunol. 9, 83–89.

43. Zheng SG, Gray JD, Ohtsuka K, Yamagiwa S, and Horwitz DA (2002) Generation ex vivo of TGF-β-producing regulatory T cells from CD4+CD25- precursors. J. Immunol. 169:4183–4189.

44. Gray JD, Hirokawa M, and Horwitz DA (1994) The role of transforming growth factor β in the generation of suppression: an interaction between CD8+ T and NK cells. J. Exp. Med. 180:1937–1942.

45. Selvaraj RK, and Geiger TL (2007) A kinetic and dynamic analysis of Foxp3 induced in T cells by TGF-β. J. Immunol. 178:7667–7677.

46. Vindevoghel L, Kon A, Lechleider RJ, Uitto J, Roberts AB, and Mauviel A (1998) Smad-dependent transcriptional activation of hyman type VII collagen (COL7A1) promoter by transforming growth factor-β. J. Biol. Chem. 273:13053–13057.

47. Zheng SG, Wang HK, Stohl W, Kim KS, Gray JD, and Horwitz DA (2004) TGF-β requires CTLA-4 early after T cell activation to induce Foxp3 and generate adaptive CD4+CD25+regulatory cells. J. Immunol. 176:3321–3329.

48. Wohlfert EA, Gorelik L, Mittler R, Flavell RA, and Clark RB (2006) Cutting edge: deficiency in the E3 ubiquitin ligase Cbl-b results in a multifunctional effect in T cell TGF-β sensitivity in vitro and invivo. J. Immunol. 176:1316–1320.

49. San Luis B, Sondgeroth B, Nassar N, and Carpino N (2011) Sts-2 is a phosphatase that negatively regulates zeta-associated protein (ZAP)-70 and T cell receptor signaling pathways. J. Biol. Chem. 286(18):15943–15954.