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Foxp3-deficient regulatory T cells do not revert into conventional effector CD4+ T cells but constitute a unique cell subset[1]

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Abstract

Homeostasis in the immune system is maintained by specialized regulatory $CD4^+$ T cells (T_{reg}) expressing transcription factor Foxp3. According to the current paradigm, high affinity interactions between T cell receptors (TCRs) and class II MHC/peptide complexes in thymus "instruct" developing thymocytes to upregulate Foxp3 and become T_{reg} cells. However, the loss or downregulation of Foxp3 does not disrupt the development of T_{reg} cells but abrogates their suppressor function. Here we show that Foxp3-deficient T_{reg} cells in *scurfy* mice, harboring a null mutation of the Foxp3 gene, retained cellular features of T_{reg} cells including *in vitro* anergy, impaired production of inflammatory cytokines, and dependence on exogenous Il-2 for proliferation and homeostatic expansion. Foxp3-deficient T_{reg} cells expressed a low level of activation markers, did not expand relative to other $CD4^+$ T cells, and produced Il-4 and immunomodulatory cytokines Il-10 and TGF-β when stimulated. Global gene expression profiling revealed significant similarities between T_{reg} cells expressing and lacking Foxp3. These results argue that Foxp3 deficiency alone does not convert T_{reg} cells into conventional effector $CD4^+$ T cells but rather these cells constitute a distinct cell subset with unique features.

Keywords

T cells; Tolerance/Suppression/Anergy; Autoimmunity; Transcription Factors

Natural regulatory T (T_{reg}) cells are produced in the thymus where they initiate expression of the X chromosome linked transcription factor Foxp3 which endows these cells with suppressor function (1–3). Recognition of class II MHC loaded with agonist peptide by the developing thymocytes augmented the generation of T_{reg} cells specific for cognate antigen. This led to the hypothesis that Foxp3 expression and selection of T_{reg} cells is "instructed" by high affinity interaction between TCR and peptide/MHC complexes and further implied that T_{reg} cells express TCRs with higher affinity for self antigens than conventional T cells (4). Foxp3 expression was postulated to decrease sensitivity of TCR stimulation of T_{reg} cells and explained why these cells are anergic *in vitro* and do not become pathogenic *in vivo* despite expressing self-reactive TCRs (5). However, an alternative model, where Foxp3 upregulation may happen

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Disclosures
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regardless of the TCR affinity for the selecting peptide ligand, has never been disproved and the role of self-reactivity in the development of T_{reg} cells remains controversial (6–8).

Decreased function of T_{reg} cells has been associated with various autoimmune disorders in human and mice (9). Reduced level of Foxp3 expression correlated with impaired T_{reg} function and was found in such autoimmune diseases as myasthenia gravis and multiple sclerosis (10, 11). The most conspicuous deficiency of T_{reg} function is observed in the human autoimmune disease IPEX (Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked) and the corresponding disease in *scurfy* mice (12,13). Affected males suffer from fatal, multi-organ, lymphoproliferative disease mediated by $CD4^+$ T cells (14,15). Mutations in the Foxp3 gene affecting its function were found to be the molecular basis of IPEX and *scurfy* diseases.

Recent analyses of mice expressing defective alleles of Foxp3 have shown that Foxp3 deficiency does not impair lineage commitment and development of T_{reg} cells (16,17). Thus, Foxp3 expression might be a concluding, rather than a causal event in the T_{reg} cell lineage differentiation that endows thymocytes that had already initiated the transcriptional program of T_{reg} cells with suppressor function. Foxp3 binds to regulatory regions of hundreds of genes in T_{reg} cells, many of which control the T cell response to antigen stimulation (18,19). The impaired activity of Foxp3 could result in the abrogation of molecular control mechanisms in T_{reg} cells and restoration of $CD4^+$ T cell effector functions. Unfortunately, little is known about the extent of diversity in the level of Foxp3 expression in the T_{reg} cells of healthy subjects and how Foxp3 downregulation affects T_{reg} cellular functions. Investigating the properties of Foxp3-deficient T_{reg} cells could not only reveal cellular functions controlled by Foxp3 but also help better assess the potential of immunotherapy aimed at modulating Foxp3 expression. Since T_{reg} cells may constitute a reservoir of self-reactive $CD4^+$ T cells, uncovering the consequences of Foxp3 downregulation could explain the pathogenesis of multiple autoimmune diseases, in particular, the contribution of Foxp3-deficient T_{reg} cells to autoimmune pathology. $CD4^+$ T cells expressing mutant forms of Foxp3 were found in IPEX patients but their role in autoimmune pathology remains unknown (20–22). These cells could represent thymocytes that attempted T_{reg} cell development and migrated to the periphery but retained at least some properties of functional T_{reg} cells despite losing suppressor function. Alternatively, these cells could represent aggressive, self-reactive T cells that originate from the T_{reg} lineage and significantly contribute to the severity of IPEX disease by producing Il-2 and IFN-γ (22). Since conventional human $CD4^+$ T cells transiently upregulate Foxp3 upon activation, it was not possible to determine the developmental origin of these cells (23).

We have established that Foxp3-deficient T_{reg} cells in sick *scurfy* males, in the absence of functional T_{reg} cells, remained quiescent, did not expand relative to other $CD4^+$ T cells, and expressed a lower level of activation markers compared to effector $CD4^+$ T cells. In *in vitro* assays, *Sf*Foxp3^{GFP+} cells did not produce Il-2 and poorly responded to TCR stimulation. Moreover, *Sf*Foxp3^{GFP+} cells produced much less inflammatory cytokines than effector $CD4^+$ T cells, except Il-4, but were able to produce Il-10 and TGF-β. Gene expression analysis showed that transcription of many T_{reg}-specific genes is similar in *Sf*Foxp3^{GFP+} and functional T_{reg} cells. By comparing genes expression in effector and T_{reg} cells isolated from *scurfy* and healthy mice we defined T_{reg} specific, Foxp3-independent gene signature. Analysis of T cell hybridomas derived from effector and *Sf*Foxp3^{GFP+} cells revealed that the frequency of self-reactive TCRs is similar in both cell subsets. In conclusion, Foxp3 deficiency does not convert T_{reg} cells into conventional, self-reactive effector cells and *Sf*Foxp3^{GFP+} cells retain cellular features of T_{reg} cells in inflammatory environment. Despite poor potential for clonal expansion Foxp3-deficient T_{reg} cells may modulate immune responses by secreted cytokines, especially Il-4 to shift immune response towards Th2 effector cells. At the same time, conventional $CD4^+$ T cells express a highly activated phenotype and produce a very high level of Il-2 and

inflammatory cytokines. Our data suggest that the vast majority of T cells that cause autoimmune pathology in *scurfy* mice originate from conventional $CD4^+$ T cells.

Materials and Methods

Foxp3-GFP transgenic construct

A BAC clone (RP23–446O15, 186.8 kb) isolated from a C57BL6 genomic library and consisting of the Foxp3 gene was purchased from BacPac (Oakland, CA). Three other known genes are located on the BAC DNA, Ppp1r3f, Ccdc22 and Cacna1f, but none of them was reported to be involved in T cell function. GFP followed by polyadenylation signal was introduced in frame with the Foxp3 translation initiation site into exon 1 of the Foxp3 gene using BAC recombineering system (24). BAC modifications were done in *E.coli* strain SW102 by a two-step recombination process with galactokinase (galK) as the selection gene. In the first step galK was inserted into the first exon of the Foxp3 gene by homologous recombination with the targeting plasmid consisting of 5'arm, galK and 3' arm. GalK gene expression was driven by the EM7 promoter. Homologous recombination occurred within the 5' and 3' arms derived from the Foxp3 gene, and the galK gene was inserted into BAC DNA. Bacteria containing the recombinant BAC were selected on minimal media with galactose as the only carbon source. Modified BAC clone was subjected to another recombination event with the second targeting construct consisting of 5' arm, the GFP-polyA cassette, and the 3' arm. Second-step recombinants were selected against galK on minimal medium plates with glycerol as the carbon source and 2-deoxy-galactose as the selecting agent. 2-deoxy-galactose is phosphorylated to toxic 2-deoxy-galactose-1-phosphate by bacteria expressing galK so only bacteria that replaced galK with GFP survive.

The design of the GFP expression cassette ensures that Foxp3 transcript, initiated at exon -2b, about 6.1 kb upstream from the first coding exon, is cleaved downstream of GFP and polyadenylated. The remaining fragment of the transcript is degraded in the nucleus since it can not be capped with methylguanosine and transported to the cytoplasm. This ensures that transcripts originating from the transgene can not be translated into functional Foxp3 protein. Transgenic mice were produced by pronuclear injection of closed circular BAC DNA into oocytes from C57BL5 mice. Founders were genotyped by PCR with primers specific to GFP (forward primer 5'GTGCCCATCCTGGTCGAGCTGGACGG3', reverse primer 5'CTTTGCTCAGGGCGGACTGGGTGCTCAGG3'). Five founders expressed the transgene and transmitted it to progeny. Transgenic founder #90 was selected for further crossing.

Mice

Scurfy and C57BL6 mice were purchased from Jackson Laboratory (Bar Harbor, ME) and crossed with transgenic $Foxp3^{GFP}$ mice. Mice were housed under specific pathogen-free conditions and used according to the guidelines of the Animal Care and Use Committee of the Medical College of Georgia.

Flow cytometry and cell sorting

Single-cell suspensions were prepared from thymi and lymph nodes by mechanical disruption and cells were stained with antibodies available commercially (eBioscience or BD Biosciences). Cells were analyzed using FACSCanto flow cytometer (Becton Dickinson) and FACSDiva or WinList software. Cells were sorted on MoFlo cell sorter (Cytomation). For some experiments, $CD4^+$ T cells were negatively sorted using commercial kit and an AutoMACS magnetic cell sorter (Miltenyi, Auburn, CA). Intracellular staining for Foxp3 and Ki-67 was performed according to the manufacturers' instructions (eBioscience and BD Biosciences respectively).

Proliferation assay

Lymph node proliferation assays was performed with 3–5x10^4 cells isolated from Foxp3^{GFP} or *Sf*Foxp3^{GFP} mice. Cells were sorted directly onto 96-well plates using MoFlo sorter and cultured for 3 days. Wells were coated overnight with anti-CD3 (10 μg/ml) and anti-CD28 (1 μg/ml) antibodies. Proliferation responses were measured by adding 1 μCi/well of ^{3}H-thymidine on day 3 of a 4-day culture.

Inhibition assay

Sorted CD4$^+$Foxp3^{GFP-} cells (5x10^4/well) were incubated on a 96-well plate with irradiated splenocytes (5x10^4/well, 3000 Rad) and soluble anti-CD3 (5 μg/ml). Various numbers of sorted CD4$^+$Foxp3^{GFP+} cells (1–5x10^4/well) were added. Cells were sorted using MoFlo sorter. After 3 day culture proliferation was measured by adding 1 μCi/well of ^{3}H-thymidine.

Adoptive transfer

Donor cells for adoptive transfer were isolated by flow cytometry sorting of total lymph node CD4$^+$ T cells or CD4$^+$GFP$^+$ or CD4$^+$GFP$^-$ cell subsets from *Sf*Foxp3^{GFP} or Foxp3^{GFP} mice. The number of cells indicated in each experiment was transferred i.v. into recipient TCRα chain knockout or *scurfy* mice. In co-transfer experiments congenic C57BL6 or *scurfy* mice expressing different alleles of CD45 (Ly5) were used as cell donors. Recipient mice were analyzed 4–5 weeks after adoptive transfer into lymphopenic mice and 10 days after transfer into *scurfy* mice.

RT-PCR

RNA was isolated from sorted cells (10^4 cells/sample) with RNeasy Mini Kit (Qiagen) and reverse transcribed using Superscript kit (Invitrogen) according to the manufacturer's instructions. The quantities of cDNA were normalized for β-actin. Foxp3 cDNA was amplified with sense primer 5'ATCCAGCCTGCCTCTGACAAGAACC 3' and reverse primer 5' GGGTTGTCCAGTGGACGCACTTGGAGC 3'. These primers distinguish between amplification product of the endogenous Foxp3 gene (401 bp) and the transgenic transcript (1357 bp).

Western blotting

Foxp3 protein was detected in sorted (10^5 cells/sample) CD4$^+$Foxp3^{GFP-} and CD4$^+$Foxp3^{GFP+} cells. Cells were lysed in the gel-loading buffer and resolved on 10% polyacrylamide gel. Proteins were transferred onto PVDF membrane (Millipore). Membranes were probed with anti-Foxp3 antibody eBio7979 (eBioscience) followed by goat anti-mouse polyclonal antibody coupled with horseradish peroxidase (BioRad). Membranes were developed with ECL chemiluminescence kit (Amersham) according to the manufacturer's instructions.

Cytokine detection

Production of cytokines by CD4$^+$Foxp3^{GFP-}, and CD4$^+$Foxp3^{GFP+} T cells was assessed using Q-Plex mouse cytokine array (Quansys Biosciences, Logan, UT). Cells were sorted onto 96-well plates (5x10^4/well) coated with anti-CD3 (10 μg/ml) and anti-CD28 (1 μg/ml) antibodies. After 30 hours, the supernatant was collected and used to measure cytokine levels according to the manufacturer's instruction. Chemiluminescence image was acquired with Fujifilm LAS-3000 imaging system. Alternatively, cytokine levels were measured by ELISA using commercial kits according to manufacturer's instructions (eBioscience). For ELISA 2x10^5 cells were stimulated with anti-CD3 and anti-CD28 antibodies and supernatants were collected after 30 hours.

Cytokine transcripts were detected in sorted *Sf*Foxp3^{GFP-} and *Sf*Foxp3^{GFP+} cells without *in vitro* stimulation by real time PCR. cDNA was produced as described above. The quantities of cDNA were normalized for β-actin. β-actin was amplified with the sense primer 5'CCTTCTACAATGAGCTGCGTGTGGC3' and antisense primer 5'CATGAGGTAGTCTGTCAGGTCC3'. Cytokine cDNA was amplified with the following primers: Il-2, sense: 5'CCTTGCTAATCACTCCTCACA', antisense: 5'GAGCTCCTGTAGGTCCATCA3', Il-4 sense: 5'CAAGGTGCTTCGCATATTTT3', antisense: 5'ATCCATTTGCATGATGCTCT3', Il-10, sense: 5'AGTGGAGCAGGTGAAGAGTG3', antisense: 5'TTCGGAGAGAGGTACAAACG3', Il-17, sense: 5'AGGCCCTCAGACTACCTCAA3', antisense: 5'CAGGATCTCTTGCTGGATGA3', IFN-γ, sense: 5'AGTGGAGCAGGTGAAGAGTG3', antisense: 5'TTCGGAGAGAGGTACAAACG3', TGF-β sense: 5'GCTACCATGCCAACTTCTGT3', antisense: 5'CGTAGTAGACGATGGGCAGT3'. cDNA prepared from cells known to produce a particular cytokine was used as a positive control. For Il-2, IFNγ, Il-4, Il-17 cDNA from cells stimulated *in vitro* under neutral, Th2 or Th17 conditions was used. Control cDNA for Il-10 and TGFβ was prepared from T_{reg} cells. Quantitative PCR was performed on the BioRad iCycler using SYBR Green detection. The PCR conditions were: denaturation at 95°C for 2 min. followed by 50 cycles of denaturation at 95°C for 20 s., annealing at 58°C for 10 s., elongation at 72°C for 20 s. The relative levels of cytokine mRNA were determined. For each sample, per cell mRNA level of the cytokine gene was determined by normalizing experimentally determined mRNA level of the cytokine gene of interest to internal control β-actin mRNA level. The mRNA level of the cytokine gene of interest in a sample stimulated in neutral, Th2 and Th17 conditions was arbitrarily set as 1. The relative level of mRNA level of the cytokine gene of interest was calculated and presented in a bar graph.

Production and analysis of CD4$^+$ hybridomas

T cells hybridomas were produced from flow cytometer sorted *Sf*Foxp3^{GFP-} and *Sf*Foxp3^{GFP+} cells directly fused to BW thymoma deficient in endogenous αβ TCR as described (25). Hybridomas were tested for reactivity to self antigens using a standard Il-2 release assay (8). Hybridomas produced in two independent experiments (42 and 44 hybridomas from *Sf*Foxp3^{GFP-} cells and 9 and 25 hybridomas from *Sf*Foxp3^{GFP+} cells) expressed TCR and CD4 and responded to stimulation with plate-bound antibodies by producing Il-2. To determine hybridoma reactivity independent of Il-2 production we assessed CD69 expression on hybridomas stimulated with plate-bound antibodies or autologous splenocytes. The results of both assays were consistent. The fraction of self-reactive hybridomas was calculated by dividing the number of hybridomas responding to splenocytes by the number of hybridomas responding to stimulation by plate-bound antibodies.

Microarray analysis

RNA was prepared from sorted cell subsets using RNeasy kit (Qiagen). T_{reg} and conventional CD4$^+$ T cells from *Sf*Foxp3^{GFP} and Foxp3^{GFP} mice were analyzed in triplicates. RNA was amplified using TargetAmp kit (Epicentre). The resulting cRNA was hybridized to Affymetrix GeneChip M430 2.0 Plus.

Microarray data were first normalized using RMA and subsequently analyzed using LIMMA (26,27). We analyzed all arrays as a factorial experiment in which strain (*Sf* vs wild-type) was one factor and cell type (T_{reg} vs. T_{eff}) was a second factor, along with the interaction of strain and cell type. Genes whose response was Foxp3 dependent were those found significant for the interaction, regardless of significance for the main effects. Genes with no significant interaction and no significant response to Foxp3, but having a significant difference between strains, are those genes that are strain specific regardless of Foxp3 expression. Genes with no

significant interaction and no significant difference between strains, but having a significant difference between T_{reg} and T_{eff} cells are those genes that respond to Foxp3 expression equally in both strains, with no differences between strains. The advantage to LIMMA is that the B statistic (log posterior odds of differential expression) used in this analysis quantifies the evidence for the alternate hypothesis versus the evidence for the null hypothesis. Since B is on a log scale, a B of 0 indicates both the alternate and null hypotheses are equally likely. If the B statistics is positive, then the evidence supports the alternative hypothesis of some difference, while a negative B supports the null hypothesis. The advantage of the B-statistic is that it accurately ranks the genes in order of likelihood of being differentially expressed. Choosing a cutoff for B, however, is just as challenging as using any other statistic. We called all genes with a $B \geq 1.5$ as significant, since the evidence for the alternative would no longer be considered weak. This choice of cutoff also seemed reasonable since the q-values (expected false discovery rates) for those genes we called significant were approximately 0.01.

Results

Expression of GFP reporter defines a population of regulatory CD4$^+$ T cells in healthy and Foxp3-deficient scurfy mice

To facilitate analysis, Foxp3-deficient T_{reg} cells in *scurfy* males were identified by expressing GFP reporter. Transgenic mice expressing GFP controlled by the Foxp3 regulatory sequences (Foxp3^{GFP} mice) and *scurfy* mice were crossed (to produce *Sf*Foxp3^{GFP} mice) and males co-expressing the mutant Foxp3 allele and Foxp3^{GFP} reporter transgene, not located on X chromosome, were examined. To ensure cell-type specific expression of a reporter gene a BAC clone encompassing the whole Foxp3 transcription unit was modified by inserting reporter cassette encoding GFP followed by the STOP codon and the polyA signal sequence into exon 1 in frame with the start codon of the Foxp3 gene (Fig. 1). The design of the expression cassette prevents overexpression of the Foxp3 from the BAC transgene, which is known to alter the function of CD4$^+$ T cells, and production of the Foxp3-GFP fusion protein or truncated Foxp3 (28). Transgenic Foxp3^{GFP} and C57BL6 mice as well as *scurfy* and *Sf*Foxp3^{GFP} mice respectively had equivalent numbers, percentages and cell surface phenotypes of all T and non-T cell subsets, including CD4$^+$CD25$^+$ T cells, in thymus, lymph nodes, and spleen (Fig. 2A, B and data not shown). Around 90% of CD4$^+$CD25$^+$ T cells in healthy mice expressed Foxp3 (29,30). Flow cytometry, RT-PCR, and Western blot analyses show that only GFP$^+$, and not GFP$^-$, CD4$^+$ lymphocytes expressed Foxp3, demonstrating reliable expression of the Foxp3^{GFP} transgene in T_{reg} cells or organs known to contain CD4$^+$ T cells (Fig. 2C, E, F). *Sf*Foxp3^{GFP+} males developed lymphoproliferative disease indistinguishable from the disease in non-transgenic *scurfy* males, and died at about 3–4 weeks of age. Mice displayed morphological symptoms of *scurfy* disease including runting, splenomegaly, dermatitis, malformed ears, and greatly enlarged lymph nodes (data not shown)(13). Heterozygous *scurfy* females remained healthy, and the phenotype of T cell populations was the same as in Foxp3^{GFP} mice (data not shown). *In vitro* expression of the Foxp3^{GFP} reporter remained stable for more than 2 days in antigen-stimulated GFP$^+$ cells from healthy and *scurfy* mice and was further inducible by TGF-β treatment (data not shown). In conclusion, the Foxp3^{GFP} reporter transgene reliably defines the population of T_{reg} cells.

The loss of suppressor function by SfFoxp3^{GFP+} CD4$^+$ T cells is not accompanied by the acquisition of a high level of activation markers

CD4$^+$Foxp3^{GFP+} but not *Sf*Foxp3^{GFP+} T cells inhibited activation of CD4$^+$ T cells *in vitro* (Fig. 2D). Adoptively transferred, purified CD4$^+$ T cells from *Sf*Foxp3^{GFP}, but not Foxp3^{GFP}, mice induced autoimmune disease in T cell-deficient recipients demonstrating that CD4$^+$ T cells in the former mice lacked functional T_{reg} cells (data not shown). Thus, the *in vitro* assay and cell transfer studies demonstrate the lack of suppressor function of *Sf*Foxp3^{GFP+} CD4$^+$ T cells.

Foxp3^{GFP+} T cells were preferentially found in the population of activated CD44^{+}CD62L^{-} cells, consistent with the fact that T_{reg} cells express higher levels of CD44 and lower levels of CD62L than conventional CD4^{+} T cells (Fig. 3A). A large proportion of CD4^{+} T cells in *scurfy* mice exhibit an activated phenotype consistent with severity of the disease. Surprisingly, the population of cells expressing an activated CD44^{+}CD62L^{-} phenotype had a smaller fraction of *Sf*Foxp3^{GFP+} cells than the population of naive CD44^{-} CD62L^{+} T cells (data not shown). To further compare the surface phenotype, we analyzed CD44 and CD62L expression on *Sf*Foxp3^{GFP+} and conventional CD4^{+} T cells (Fig. 3B). *Sf*Foxp3^{GFP+} cells only modestly up-regulated CD44 and down-regulated CD62L compared to the conventional CD4^{+} T cells, suggesting that they are less responsive to activation by self-antigens. In summary, expression of activation markers suggests that effector CD4^{+} T cells are more sensitive to activation by self-antigens then T_{reg} cells that lose Foxp3 expression.

The expansion and phenotype stability of SfFoxp3^{GFP+} T cells

The proportion of Foxp3-expressing cells was similar in the lymph nodes of Foxp3^{GFP} and *Sf*Foxp3^{GFP} transgenic mice and did not increase with disease progression, suggesting that these cells do not have a proliferative advantage over effector CD4^{+} T cells (Fig. 3C, D). Tissue infiltrates in peripheral organs were dominated by *Sf*Foxp3^{GFP-} cells demonstrating that *Sf*Foxp3^{GFP+} cells did not migrate and selectively accumulate in peripheral organs affected by the autoimmune disease (Fig. 4 and data not shown).

To compare the proliferative potential of GFP^{+} and GFP^{-} cells, the respective cell subsets were sorted and stained with Ki-67-specific antibody. Ki-67 antigen is expressed in all phases of the cell cycle except G0 so its detection provides an estimate of the fraction of dividing cells in a cell population (31). This analysis showed that in healthy mice the fraction of proliferating T_{reg} cells is about twice as large as the fraction of effector CD4^{+} cells and is consistent with studies showing that steady-state BrdU incorporation in CD4^{+}CD25^{+} cells is higher than in CD4^{+}CD25^{-} cells (Fig. 5A)(32). In contrast, fractions of *Sf*Foxp3^{GFP+} and *Sf*Foxp3^{GFP-} cells expressing Ki-67 were similar in *scurfy* mice. Thus, the major relative increase in the fraction of proliferating cells occurred in the *Sf*Foxp3^{GFP-} population. These findings implied that T cells recruited to the proliferating population preferentially originate from effector *Sf*Foxp3^{GFP-} T cells.

To further evaluate proliferative capacity of *Sf*Foxp3^{GFP+} and *Sf*Foxp3^{GFP-} cells we examined homeostatic expansion of the respective cell populations upon transfer into lymphopenic hosts. This assay is commonly used to compare the potential for clonal expansion of two T cell populations and has been used to estimate the frequency of self-reactive clones in expanded population (5). Analysis of the CD4^{+} T cell population in recipient mice shows diminished proportion of *Sf*Foxp3^{GFP+} cells and suggests that they are outgrown by the effector *Sf*Foxp3^{GFP-} cells (Fig. 5B). In contrast, the proportion of Foxp3^{GFP+} cells in recipient mice reconstituted with CD4^{+} cells from healthy mice was similar to the proportion in the donor population used for transfer. Analysis of Ki-67 in transferred cells shows a much larger fraction of proliferating cells in effector *Sf*Foxp3^{GFP-} cells than in *Sf*Foxp3^{GFP+} cells as well as Foxp3^{GFP-} effector cells from healthy mice (Fig. 5C). This is consistent with more efficient expansion of activated donor CD4^{+} T cells from *scurfy* than from healthy mice. To determine if decreased proportion of GFP^{+} T cells in lymphopenic mice reconstituted with total population of CD4^{+} T cells from *Sf*Foxp3^{GFP} mice is caused by downregulation of the GFP reporter upon homeostatic expansion, we have investigated persistence of *Sf*Foxp3^{GFP+} cells in recipient mice (Fig. 5D). To provide exogenous Il-2 we co-transferred *Sf*Foxp3^{GFP+} cells with total CD4^{+} T cells from healthy mice into lymphopenic mice. Analysis of transferred cells after 4 weeks shows that the predominant fraction of *Sf*Foxp3^{GFP+} cells retained Foxp3 transcription. In another experiment we were able to detect *Sf*Foxp3^{GFP+} cells in recipient mice

a few month after adaptive transfer. Most of these cells retained Foxp3^{GFP+} expression but the fraction of cells derived from Foxp3-deficient T_{reg} cells in the total population of transferred CD4$^+$ T cells declined (data not shown).

It is possible that contribution of Foxp3-deficient T_{reg} cells to autoimmune pathology was concealed by the loss of the GFP expression. To investigate how stable is the phenotype of *Sf*Foxp3^{GFP+} cells in inflammatory environment we have transferred i.p. equal numbers of CD4$^+$Ly5.1$^{+/+}$*Sf*Foxp3^{GFP+} and CD4$^+$Ly5.1$^{+/-}$*Sf*Foxp3^{GFP-} T cells (effector cells) sorted from 17 day old *Sf*Foxp3^{GFP} mice expressing respective allelic markers into 7 day old recipient Ly5.1$^{-/-}$*Sf*Foxp3^{GFP} mice. When recipient mice were analyzed 10 days after cell transfer sufficient numbers of donor cells were only found in abdominal cavity (site of injection) indicating that donor cells did not undergo expansion in the lymphoreplete environment of recipient mice consistent with earlier report (2). In our mouse colony 10 day old *scurfy* mice show first signs of lymphoproliferative disease and die at about 3 weeks of age so a 10 day period of adoptive transfer is appropriate to assess the loss of Foxp3 expression in transferred *Sf*Foxp3^{GFP+} cells. The great majority (80%) of transferred *Sf*Foxp3^{GFP+} T cells retained Foxp3 transcription, however their proportion in the transferred population decreased (Fig. 5E). Thus, this and adoptive transfer experiments described above demonstrate that *Sf*Foxp3^{GFP+} cells are much less capable of expansion than effector CD4$^+$ T cells from *scurfy* mice (see below). Continuous transcription of the Foxp3^{GFP} reporter *in vivo* and its upregulation by TGF-β treatment *in vitro* imply that the regulation of Foxp3 expression in Foxp3-deficient and Foxp3-sufficient T_{reg} cells is similar and complements earlier reports that inflammatory conditions preserve Foxp3 expression in most natural or adoptive T_{reg} cells (data not shown)(2,33). The origin of a small fraction of *Sf*Foxp3^{GFP+} T cells that lost Foxp3 expression is not certain. These cells could represent a subset of genuine, thymus derived T_{reg} cells, with less stable phenotype or they might be adoptive T_{reg} cells that "contaminate" the population of T_{reg} cells. Adoptive T_{reg} cells do not have a stable T_{reg} phenotype and they can be generated, even in inflammatory conditions, from Foxp3-deficient conventional CD4$^+$ T cells (34). In conclusion, adoptive transfer experiment shows that *Sf*Foxp3^{GFP+} T cells that lost reporter expression may constitute only a very small proportion of GFP$^-$ cells in *Sf*Foxp3^{GFP} mice.

SfFoxp3^{GFP+} CD4$^+$ T cells have decreased antigenic response and produce a characteristic pattern of cytokines

Lower expression of activation markers and decreased homeostatic expansion suggested differential reactivity of *Sf*Foxp3^{GFP+} cells and effector CD4$^+$ T cells. When sorted populations of *Sf*Foxp3^{GFP+} and *Sf*Foxp3^{GFP-} cells were stimulated *in vitro*, proliferation of *Sf*Foxp3^{GFP+} cells was very small compared to effector CD4$^+$ T cells and was similar to the proliferation of functional Foxp3$^+$ T_{reg} cells (Fig. 6A). Provision of exogenous Il-2 restored proliferation of both *Sf*Foxp3^{GFP+} and functional T_{reg} cells. Thus, *Sf*Foxp3^{GFP+} cells closely resemble functional T_{reg} cells that are anergic *in vitro* but proliferate and expand *in vivo* (35). *In vitro* activated *Sf*Foxp3^{GFP+} and *Sf*Foxp3^{GFP-} cells produced markedly different cytokine profiles (Fig. 6B–F). Consistent with proliferation assays, *Sf*Foxp3^{GFP+} cells did not produce Il-2. These cells produce low level of inflammatory cytokines with the exception of Il-4 and immunoregulatory cytokines Il-10 and TGF-β. *Sf*Foxp3^{GFP+} cells did not differentiate into notable number of Th1 or Th17 helper T cells and did not secrete Il-6 or Il-12 suggesting that they do not indirectly support differentiation of Th17 or Th1 cells respectively. In contrast, effector CD4$^+$ cells produced much higher levels of inflammatory cytokines IFN-γ, MIP-1α and a very high level of Il-4. In addition *Sf*Foxp3^{GFP-} cells produced broader spectrum of cytokines that included Il-3, Il-5, Il-6, Il-10, GM-CSF and Il-2. The ability to produce high level of Il-2 is the likely reason for efficient expansion of *Sf*Foxp3^{GFP-} cells activated by autoantigens in *scurfy* mice and explains why these cells are able to support homeostatic

expansion of T_{reg} cells expressing defective Foxp3 (17). The earlier reported level of cytokines produced by Foxp3-deficient T_{reg} cells was much higher than the level of cytokines produced by *Sf*Foxp3^{GFP+} cells and lead to the conclusion that Foxp3-deficient T_{reg} cells efficiently produced Th1, Th2 and Th17 cytokines including Il-2 (16). However, the cells used in the reported experiment were treated for a prolonged time with PMA and ionomycin, while we have stimulated cells for a short time only with plate bound antibodies. To determine what cytokines are produced by T cells isolated directly from *scurfy* mice, RNA expression of Il-2, Il-10, TGF-β, IFN-γ, Il-4 and Il-17 was analyzed (Fig. 6E). We detected very low levels of Il-2 transcripts in *Sf*Foxp3^{GFP+} cells and much lower level of IFN-γ than in effector CD4$^+$ T cells consistent with intracellular staining showing that only a small fraction of *Sf*Foxp3^{GFP+} cells produces IFN-γ.

SfFoxp3^{GFP+} T cells are not enriched in autoreactive CD4$^+$ T cells

Though *Sf*Foxp3^{GFP+} T cells do not exhibit effector functions of activated conventional CD4$^+$ T cells low response to antigenic stimulation *in vitro* and impaired clonal expansion *in vivo* may conceal their self-reactive potential (5,36). To investigate self-reactive T cells we focused on CD4$^+$ T cells that up-regulate activation markers, especially CD25. Up-regulation of CD25 on some *Sf*Foxp3^{GFP+} cells may reflect their dependence on Il-2, or alternatively, denotes their activation status, in particular predisposition to be activated by self-antigens (37). We further investigated the relationship between cells expressing CD25 and the GFP reporter in *Sf*Foxp3^{GFP} mice. Our analysis shows that both CD25$^-$ and CD25$^+$ T cell subsets have a substantial contribution of *Sf*Foxp3^{GFP+} cells (Fig. 7A). The CD25$^+$ T cell subset contained, on average, 2.5–3 times more *Sf*Foxp3^{GFP+} cells than the CD25$^-$ subset. The previously reported greater overlap of TCR repertoire expressed by presumably autoreactive CD4$^+$CD25$^+$ T cells from Foxp3 knockout mice and T_{reg} cells from healthy mice is most likely the consequence of a higher proportion of *Sf*Foxp3^{GFP+} cells in the population of CD25$^+$ than CD25$^-$ cells (5). These results also demonstrate that TCRs isolated from the CD25$^+$ T cell subset may have originated from *Sf*Foxp3^{GFP+} cells instead of autoreactive, expanded, effector CD4$^+$CD25$^+$ T cells.

To estimate the relative frequencies of self-reactive cells in the populations of *Sf*Foxp3^{GFP+} and *Sf*Foxp3^{GFP-} cells, a set of T cell hybridomas prepared from the respective cell subsets was analyzed. This experimental approach allows for analysis of TCR specificity regardless of the cellular context of a T cell. Sorted *Sf*Foxp3^{GFP+} and *Sf*Foxp3^{GFP-} cells were directly fused, and the resulting hybridomas were stimulated with syngenic splenocytes. The frequency of self-reactive TCRs was moderately higher among hybridomas prepared from *Sf*Foxp3^{GFP-} cells (Fig. 7B). This strongly suggests that while Foxp3-deficient T_{reg} cells may express self-reactive specificities, they are not a major reservoir of autoreactive T cells that might become deleterious upon appropriate stimulation or in patients with dysregulated Foxp3 function. Hybridoma analysis complements our recent finding that the vast majority of regulatory CD4$^+$ T cells in healthy mice express T cell receptors specific for non-self ligands (8).

The impact of Foxp3 expression on the gene expression profiles of T_{reg} cells

The data discussed so far strongly suggested that *Sf*Foxp3^{GFP+} cells retained important characteristics of T_{reg} cells despite losing expression of functional Foxp3. To gain further insight into the consequences of Foxp3 deficiency for the transcriptional signature of T_{reg} cells we have compared the gene expression profiles of regulatory and conventional CD4$^+$ T cells isolated from normal and *scurfy* mice. All gene expression data were obtained from highly purified, flow-cytometry sorted cells. Analysis of conventional Foxp3^{GFP-} and regulatory Foxp3^{GFP+} cells from normal mice shows up-regulation of Gpr83, Folr4, Tnfrsf18 (GITR), CTLA4, Foxp3, Dusp4, Il2Rα, Socs2 and Nrp1, genes considered to be a hallmark of T_{reg} cell

transcriptional signature, consistent with previous reports (Fig. 8A)(16,17,30,38,39). Some T_{reg}-specific genes (e.g., Gpr83, Folr4 and, Foxp3) were up-regulated in T_{reg} cells and down-regulated in conventional $CD4^+$ T cells from *scurfy* and C57BL6 mice. This suggests that their expression pattern correlates with cell type, regardless of the ability of T_{reg} cells to produce functional Foxp3 protein and regardless of the activation status of conventional $CD4^+$ T cells. It is then possible that high expression of other T_{reg}-specific genes (e.g., CTLA-4, Socs-2) in the Foxp3-deficient *Sf*Foxp3^{GFP+} cell subset is not necessarily a result of cell activation concomitant with the reversal of the T_{reg} transcriptional program but rather demonstrates their persistent T_{reg} phenotype (40). This interpretation is consistent with a recent analysis demonstrating that expression of some T_{reg}-specific genes overlaps with the TCR response but represents only a subset of the full T cell activation response (41).

Remarkably, transcription of Foxp3 was retained in *Sf*Foxp3^{GFP+} cells consistent with RT-PCR analysis. Thus, expression of the Foxp3 mRNA is regulated by genes located at the higher level of the transcriptional hierarchy of T_{reg} cells and does not require the presence of functional Foxp3 protein. Consequently, the cellular and molecular features common for *Sf*Foxp3^{GFP+} and T_{reg} cells and described in this report are Foxp3-independent. Functional Foxp3 protein is, however, required to regulate Foxp3 dependent genes. This is illustrated by the expression profile of Pde3b (cyclic nucleotide phosphodiesterase 3b)(Fig. 8A). *Sf*Foxp3^{GFP+} cells failed to down-regulate Pde3b expression as reported earlier (16).

To reveal the impact of Foxp3 deficiency on the global gene signature of T_{reg} cells, we conducted a two-factor analysis of variance of gene expression of Foxp3^{GFP-} and Foxp3^{GFP+} populations isolated from normal mice and *Sf*Foxp3^{GFP+} and *Sf*Foxp3^{GFP-} populations isolated from *scurfy* mice. The two factors that influenced the gene expression tested in this analysis were the type of cells (T_{reg} vs. conventional $CD4^+$ T cell) and the ability to express functional Foxp3 protein (*scurfy* vs. C57BL6). Factorial analysis of variance allows us to assess not only the effects of both factors independently of each other but also makes it possible to determine the interaction between factors. In our case, interaction between factors means that the level of gene expression in the examined cell type (conventional or T_{reg} cell) is not independent of the other factor, e.g., the ability to express Foxp3. Thus, the genes differentially expressed in T_{reg} cells from *scurfy* or healthy mice and showing an interaction are the target genes regulated by Foxp3. The result of this analysis is shown in Fig. 8B. Most of the genes presented in the diagram were previously identified as differentially expressed in T_{reg} or effector $CD4^+$ T cells (16,17,30,38,40–42). Heatmaps of these genes are shown indicating where they fall in the Venn diagram.

Of the 1039 differentially expressed genes, 183 genes showed a significant interaction suggesting that they are regulated by Foxp3. This number constitutes 57.7% of all (317) genes differentially expressed in T_{reg} and conventional $CD4^+$ T cells. Of the remaining 722 differentially expressed genes, 134 genes were differentially expressed in T_{reg} versus conventional $CD4^+$ cells regardless of whether the cells were isolated from *scurfy* or healthy mice, and 759 genes were differentially expressed in cells isolated from *scurfy* or healthy mice, regardless of whether they were isolated from T_{reg} or conventional cells. The set of 134 genes not showing an interaction and differentially expressed in T_{reg} versus conventional $CD4^+$ T cells may represent T_{reg} signature genes independent of Foxp3. The expression pattern of a subset of these genes (97) remains constant regardless of whether T_{reg} cells from *scurfy* or healthy mice are analyzed. Another subset of T_{reg}-specific genes (37) is differentially expressed in cells isolated from *scurfy* or healthy mice. These genes represent T_{reg}-specific genes up- or down-regulated by T cell activation or in response to cytokines, but whose pattern of expression is the same in T_{reg} and conventional $CD4^+$ T cells (e.g., they are up- or down-regulated in both subsets). Of 872 genes differentially expressed by $CD4^+$ T cells isolated from *scurfy* or healthy mice, expression of 104 genes was different in T_{reg} and conventional $CD4^+$ T cells. Of these

104 genes, 67 genes showed an interaction indicating dependence on Foxp3. Finally, 722 genes differentially expressed between cells isolated from *scurfy* and healthy mice did not show dependence on the cell type (T_{reg} versus conventional $CD4^+$ cells) in either *scurfy* or healthy mice, consistent with the large differences in the number of activated cells. In summary, our analysis demonstrates that a substantial fraction of the T_{reg} gene signature is controlled by Foxp3, however, the number of these genes is small relative to the number identified using Chip-Chip (18,19). The origin of $CD4^+$ T cells from autoimmune or healthy mice may have a greater quantitative impact on the gene expression profile than the cell type. The list of all genes presented in the Venn diagram is available in Supplemental Material.

Discussion

Recent reports and our own data dissociate the role of Foxp3 in T_{reg} suppressor function from its role in T_{reg} lineage commitment and provoke new and important questions (16,17). What is the scope of T_{reg} cell functions controlled by Foxp3 and what are the characteristics of T_{reg} cells that lose or down-regulate Foxp3? Since Foxp3 is not critical for the fitness of T_{reg} cells, it is conceivable that some T_{reg} cells may lose or down-regulate Foxp3 expression in healthy individuals. The GFP expression in $Foxp3^{GFP+}$ cells differs 100-fold in healthy mice and correlates with the level of Foxp3 protein expression, suggesting that the T_{reg} population is heterogenous (in print). Recent evidence that Foxp3 acts in a dose-dependent, instead of a binary manner, yields further support to the hypothesis that T_{reg} cells may exist in various shades depending on the level of Foxp3 expression (43). Signaling through OX40 may be one of the mechanisms regulating Foxp3 level and T_{reg} suppressor function (44). Here we show that Foxp3-deficient T_{reg} cells do not revert to effector $CD4^+$ T cells but constitute a distinct subset retaining important cellular characteristics of regulatory cells.

Peripheral *Sf*$Foxp3^{GFP+}$ cells had a cell surface phenotype distinct from conventional T cells and retained features of T_{reg} cells despite losing suppressor function. These cells remained dependent on exogenous Il-2 for proliferation and were anergic *in vitro*. *Sf*$Foxp3^{GFP+}$ cells produced only small amounts of cytokines compared to conventional T cells with the exception of Il-4. This corresponds well with molecular findings that the Il-4 gene is directly suppressed by Foxp3 (38). The properties of *Sf*$Foxp3^{GFP+}$ cells in our system closely resemble the properties of T_{reg} cells expressing a low level of functional Foxp3 that remained quiescent, produced a Th2-skewed cytokine pattern, and revealed lower homeostatic expansion than T_{reg} cells expressing a normal level of Foxp3 (43). Human T_{reg} cells that down-regulate Foxp3 expression also tend to produce Th2-type cytokines and convert into a Th2 cell type (45).

Sf$Foxp3^{GFP+}$ cells do not differentiate *in vitro* into a notable number of Th1 or Th17 helper T cells and do not secrete high amounts of Il-6 or Il-12, suggesting that they do not indirectly support differentiation of Th17 or Th1 cells. Some cytokines, such as GM-CSF, that are important for pathology in *scurfy* mice, are produced solely by conventional T cells (16,46). Analysis of T cell hybridomas derived from *Sf*$Foxp3^{GFP+}$ or *Sf*$Foxp3^{GFP-}$ $CD4^+$ T cells shows similar frequency of self-reactive T cells, consistent with our recent report that T_{reg} cells in healthy mice do not preferentially express self-reactive TCRs (8). Our findings are consistent with the analysis of T_{reg} expressing a nonfunctional Foxp3 mutant. Foxp3-deficient T_{reg} cells produced little Il-2, could not survive when adoptively transferred into recipient mice, and required $Foxp3^{GFP-}$ cells to promote autoimmunity (17). Since *Sf*$Foxp3^{GFP+}$ cells do not produce Il-2 their expansion is most likely controlled by the level of Il-2 produced by self-reactive conventional $CD4^+$ T cells (47). In conclusion, the features of *Sf*$Foxp3^{GFP+}$ cells do not predispose them to become dominant population of self-reactive T cells mediating the fulminant autoimmune disease in *scurfy* mice. However, the propensity of *Sf*$Foxp3^{GFP+}$ cells to produce Il-4 may affect the course of autoimmune disease in *scurfy* mice by augmenting Th2 type autoimmune response and inhibiting generation of Th1 and Th17 cells. Il-4 was shown

to suppress Il-6 and TGF-β induced generation of Th17 what could explain the absence of these cells in *in vitro* stimulated $CD4^+$ T cells isolated from *scurfy* mice (48,49). The ability to skew the immune response towards Th2 was demonstrated for T_{reg} cells expressing a low level of functional Foxp3 that also have propensity to produce Il-4 (43). Such modulation of the autoimmune response may save *scurfy* mice from the most destructive tissue damage mediated by Th17 cells (50). *Sf*$Foxp3^{GFP+}$-like cells might be also relevant in chronic autoimmune diseases like asthma, or allergic diseases where T_{reg} cell deficiency is associated with activation of Th2 effector cells (51).

Our data suggest more limited role of Foxp3 in the lineage commitment and differentiation of natural T_{reg} cells than suggested earlier (30). Similarly, the scope of cellular functions controlled by the Foxp3 in adoptive T_{reg} cells seems to be limited to their suppressive function. Adoptive T_{reg} cells produced from conventional T cells from Foxp3-sufficient and Foxp3-deficient mice revealed that the gene expression patterns were very similar leading to the conclusion that Foxp3 plays a limited role in the conversion process (34). In a recent report the loss of functional Foxp3 expression led to the reversal of the transcriptional program of T_{reg} cells, Il-2 production and acquisition of the properties of effector $CD4^+$ T cells (40). Foxp3-deficient T_{reg} cells in male mice, lacking functional T_{reg} cells, expanded in the periphery and produced Il-2 and Th1, Th2 and Th17 cytokines. Surprisingly, corresponding cell subset from female mice, having functional population of T_{reg} cells, retained characteristics of T_{reg} cells and remained anergic to TCR stimulation *in vitro*, were dependent on Il-2 for proliferation, and did not produce inflammatory cytokines (16). Such differences between males and females are difficult to reconcile with cell autonomous regulation of T_{reg} cell lineage and suggest that cell-extrinsic factors may convert T_{reg} cells into effector T cells. Differences in the level of produced cytokines may be due to different genetic backgrounds of mice used in the previous studies, distinct stimulation conditions or difficulties of separating T_{reg} cells expressing a low level of GFP from effector T cells. Alternatively, modifications of the endogenous Foxp3 gene to introduce a GFP reporter or deletions of Foxp3 gene fragments may modify the function of the Foxp3-GFP fusion protein and/or affect detection of the Foxp3 transcript.

The properties of *Sf*$Foxp3^{GFP+}$ cells revealed by cellular and immunological analyses correspond well with the analysis of global gene expression using GeneChip technology. The expression pattern of many signature T_{reg} genes (including Foxp3 itself) reported in multiple earlier studies was similar in $Foxp3^{GFP+}$ and in *Sf*$Foxp3^{GFP+}$ cells suggesting that the imprint of Foxp3 on the transcriptional landscape of T_{reg} cells is likely smaller than reported earlier (2,30). To assess the influence of Foxp3 on T_{reg} cell transcription, we not only relied on comparison of gene expression levels in the relevant cell subsets but determined to what extent differential gene expression could be due to the expression of functional Foxp3 protein. This analysis shows that of 317 genes differentially expressed between conventional (GFP^-) and T_{reg} (GFP^+) cells, expression of 183 genes (57.7%) could be regulated by Foxp3. Although a significant fraction of the genes constituting the T_{reg} cell transcriptional profile depends on Foxp3, analysis of expression profile of other genes suggests that they are Foxp3-independent. The existence of a T_{reg}-specific Foxp3-independent set of genes was demonstrated in a recent report (41). The properties of *Sf*$Foxp3^{GFP+}$ cells associated with their commitment to a regulatory lineage are most likely controlled by the set of 97 genes differentially expressed in conventional and T_{reg} cells, not affected by T cell activation, and independent of Foxp3. In conclusion, gene expression profiling supports our findings that Foxp3-deficient *Sf*$Foxp3^{GFP+}$ cells possess a unique phenotype and do not revert to conventional effector $CD4^+$ T cells.

The loss of functional Foxp3 protein by T_{reg} cells has dramatic consequences for the immune system, however, at the level of an individual cell, the transition from Foxp3 expression to Foxp3 deficiency is not associated with a dramatic change in the biology of a T cell committed

to a regulatory lineage manifested by augmented response to antigen, loss of Il-2 dependence, and production of multiple cytokines including Il-2. Rather, T_{reg} cells that lose Foxp3 function become unable to suppress immune responses but retain production of regulatory and Th2 type cytokines. This has important implications for our understanding of autoimmune diseases and immunotherapeutic approaches. T_{reg} cells that down-regulate or entirely lose Foxp3 expression do not revert to effector $CD4^+$ T cells that, due to the high frequency of self-reactive T cell receptors, initiate and subsequently dominate autoimmune disease but rather constitute a cell subset that modulates effector functions of self-reactive conventional $CD4^+$ T cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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The GeneChip expression data were deposited in the GEO database http://www.ncbi.nlm.nih.gov/geo/. The accession number is GSE11775.

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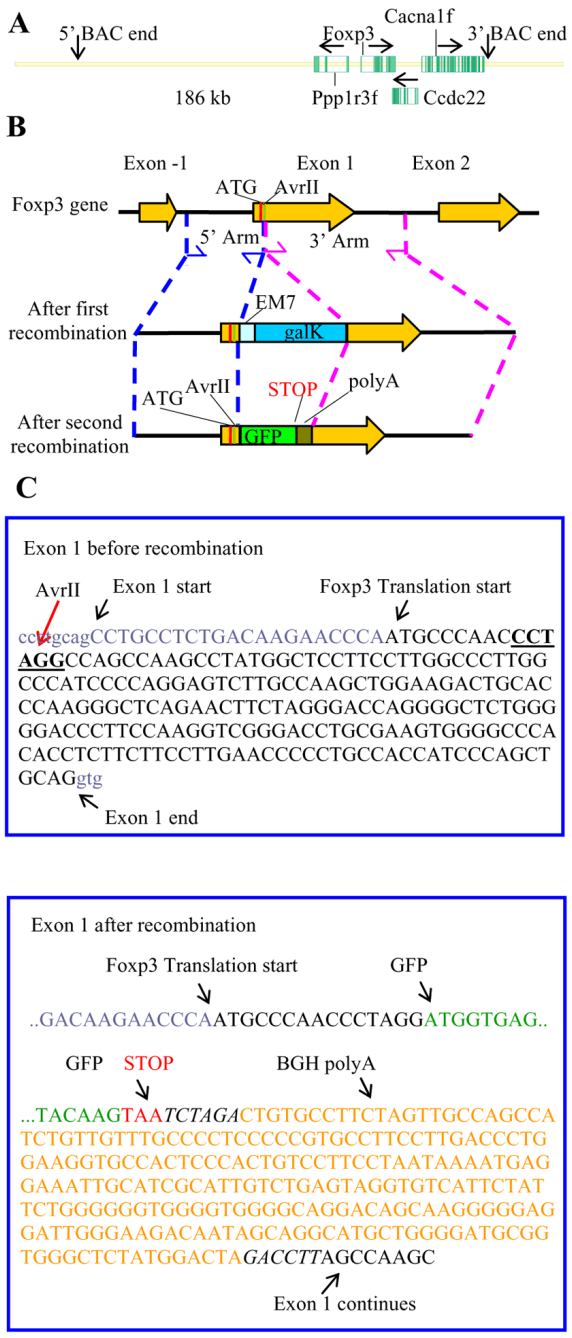


FIGURE 1.

BAC genomic fragment of DNA encompassing Foxp3 gene before (A) and after (B, C) modification to introduce GFP reporter. (A) Genes located on the DNA fragment (between vertical arrows) used to produce transgenic mouse. Genes are depicted by green rectangles, filled segments represent exons and empty segments represent introns. Direction of transcription is shown for each gene by arrows above genes. Ccdc22 gene partially overlaps with untranslated region of Foxp3 and is shown below other genes. (B) Recombination strategy used to introduce GFP reporter into exon 1 of the Foxp3 gene. Purple and pink lines mark DNA segments used as 5' and 3' arms, respectively, in the recombination process. Arrows show location of PCR primers used to amplify and clone DNA segments of 5' and 3' arms.

Intermediate construct harbors galK (galactokinase) gene driven by EM7 prokaryotic promoter. ATG shows Foxp3 translation initiation codon. AvrII restriction enzyme site was used to introduce EM7-galK or GFP into exon 1 of Foxp3. (C) DNA sequence of exon 1 of Foxp3 gene before (upper panel) and after recombination (lower panel). GFP coding sequence (green letters) was inserted into AvrII site (red arrow, upper panel) in frame with the Foxp3. Intron sequences are shown as small gray letters and exons are shown in capital letters, coding fragments are shown in black and non-coding fragments are shown in gray. Bovine growth hormone polyA cassette is shown in orange. Nucleotides shown in italics were added during cloning process.

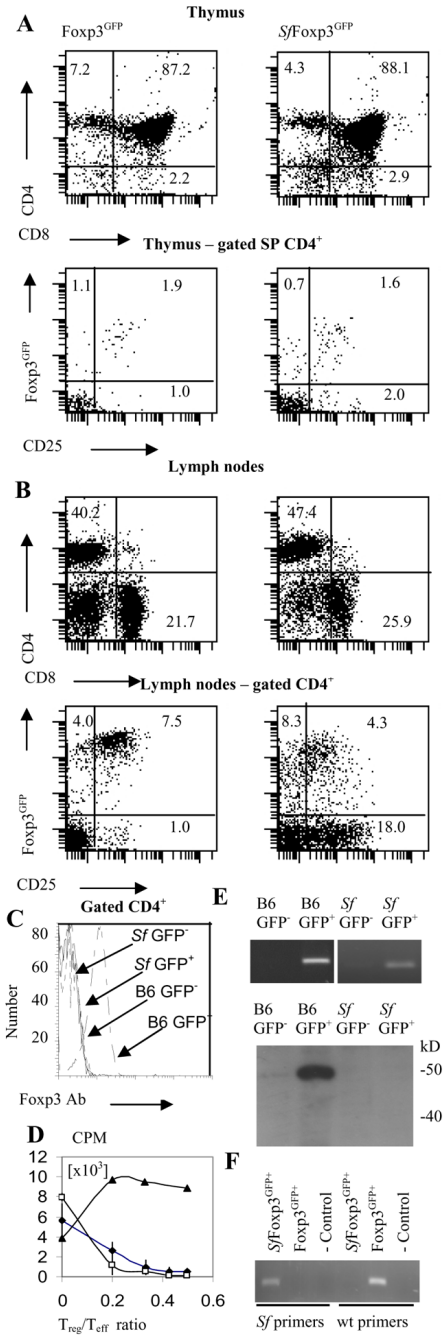


FIGURE 2.

Expression of the Foxp3^{GFP} reporter transgene is confined to cells expressing endogenous Foxp3 gene and does not alter the development of T cells. *A, B,* Flow cytometry analysis of thymocytes (A) and lymph node cells (B) of 2–3 week old healthy Foxp3^{GFP} and sick *Sf*Foxp3^{GFP} mice. At least five mice of each kind were analyzed. *C,* Foxp3^{GFP} transgene is expressed exclusively in cells expressing endogenous Foxp3. *D,* CD4^{+}GFP^{+} (□) or CD4^{+}CD25^{+} (◆) T cells (T$_{reg}$) isolated from Foxp3^{GFP} transgenic mice but not CD4^{+}GFP^{+} (▲) cells from *Sf*Foxp3^{GFP} mice, suppress proliferation of effector CD4^{+}GFP^{-} T cells (T$_{eff}$). *E,* Foxp3 RNA and protein are expressed only in GFP^{+} cells. RT-PCR (upper panel) and Western blot analysis (lower panel) of Foxp3 expression in sorted CD4^{+}GFP^{+} and

$CD4^+GFP^-$ subsets from $Foxp3^{GFP}$ (B6) and *Sf*$Foxp3^{GFP}$ (*Sf*) mice. cDNA was amplified with primers detecting endogenous Foxp3 transcripts. *F*, Truncated transcripts of the $Foxp3^{GFP}$ transgene extending downstream of the polyadenylation site are absent in *Sf*$Foxp3^{GFP}$ transgenic mice. cDNA prepared from T_{reg} cells isolated from *Sf*$Foxp3^{GFP}$ (mutant, lanes 1, 4) and $Foxp3^{GFP}$ (wild type, lanes 2, 5) transgenic males were amplified with primers spanning the DNA segment of *scurfy* mutation, in exon 9 of the Foxp3 gene, specific for mutant (lanes 1, 2) or wild type transcripts (lanes 4, 5). The wild-type Foxp3 transcript in *scurfy* cells could originate only from $Foxp3^{GFP}$ transgene and is not detected (lane 4). Primer specificity is verified by amplification of mutant and wild-type transcripts with the relevant primers (lanes 1 and 5). PCR was done with the sense primers specific for mutant Foxp3 (5'TCAGGCCTCAATGGACAAAA3') or wild-type allele (5'CTCAGGCCTCAATGGACAAG3') and common anti-sense primer (5'CATCGGATAAGGGTGGCATA3'). Negative control is a PCR reaction without added template (lanes 3, 6).

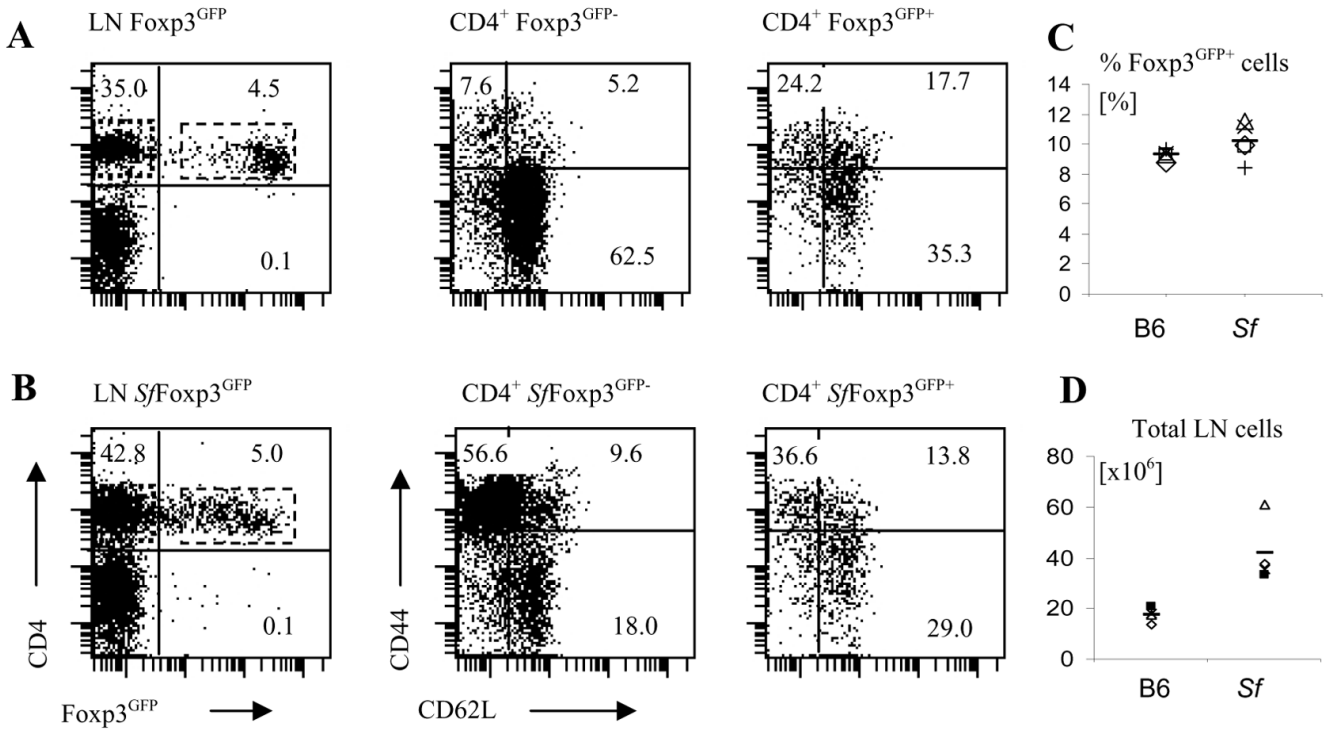


FIGURE 3.

Foxp3-deficient T_{reg} cells do not have proliferative advantage over effector $CD4^+$ T cells and express lower levels of activation markers. *A, B*, Flow cytometry analysis of lymph node cells (left column) of 3 week old $Foxp3^{GFP}$ and *Sf*$Foxp3^{GFP}$ mice. Expression of activation markers CD44 and CD62L on gated conventional (second column) and T_{reg} $CD4^+$ cells (third column) is shown. Analysis gates are shown as rectangles. *C*, Percentage of T_{reg} cells in a population of $CD4^+$ T cells in $Foxp3^{GFP}$ (B6) and *Sf*$Foxp3^{GFP}$ (*Sf*) mice are similar. *D*, The total number of $CD4^+$ lymph node cells is increased in *Sf*$Foxp3^{GFP}$ (*Sf*) mice relative to $Foxp3^{GFP}$ (B6) mice.

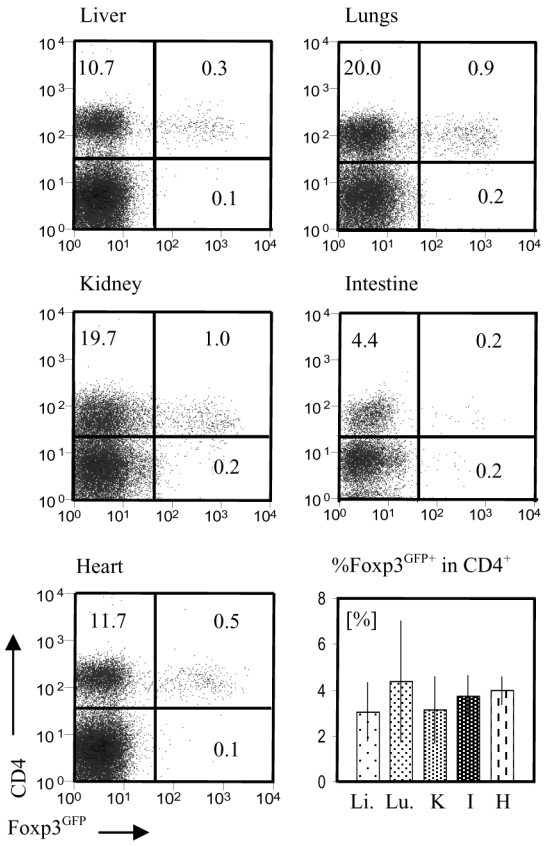


FIGURE 4.

Flow cytometry analysis of lymphocyte population infiltrating peripheral organs in *Sf*Foxp3^{GFP} mice. Panels show expression of the Foxp3^{GFP} reporter in CD4^{+} T cells isolated from liver, lungs, kidney, intestine and heart. Bar graph shows average percentage of *Sf*Foxp3^{GFP+} cells in the respective organs (Li. – liver, Lu. – lungs, K – kidney, I – intestine, H – heart).

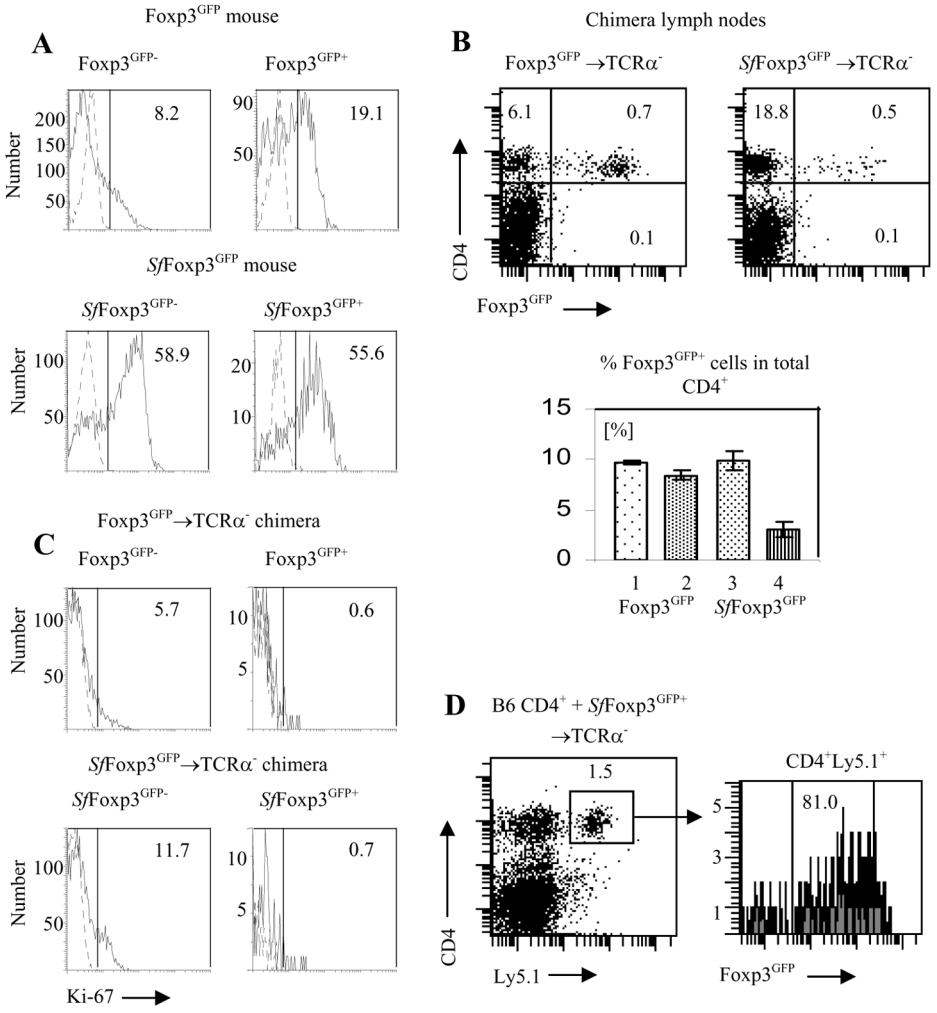
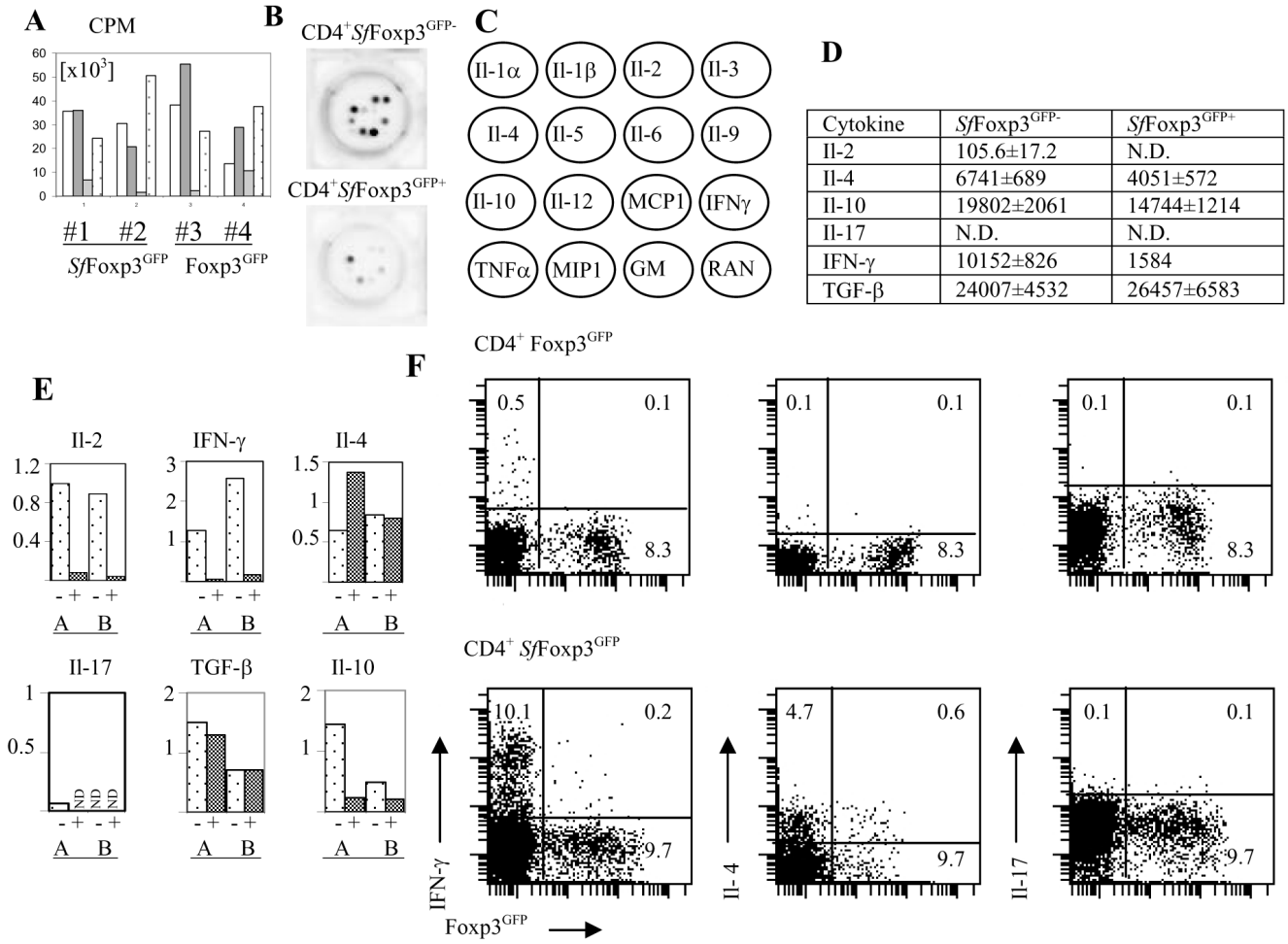


FIGURE 5.

Analysis of the proliferative potential and the stability of the phenotype of T_{reg} and conventional $CD4^+$ T cells from healthy and *scurfy* mice. *A*, The fraction of proliferating $CD4^+$ GFP^- and GFP^+ cells was estimated in $Foxp3^{GFP}$ and *Sf*$Foxp3^{GFP}$ mice by staining sorted cells with Ki-67 specific antibody (continuous line) or isotype-matched control (broken line). *B*, *Sf*$Foxp3^{GFP-}$ $CD4^+$ T cells have proliferative advantage over *Sf*$Foxp3^{GFP+}$ cells. Flow cytometry analysis of lymph node cells of TCR α chain knockout mice adoptively transferred with total $CD4^+$ T cells ($5x10^6$/mouse) from $Foxp3^{GFP+}$ (left panel) and *Sf*$Foxp3^{GFP+}$ (right panel) mice. Mice were analyzed 5 weeks after transfer. Bar graph shows percentage of GFP^+ cells in $CD4^+$ population of $Foxp3^{GFP}$ (1,2) and *Sf*$Foxp3^{GFP}$ (3,4) cells before transfer (1,3) and after (2,4) adoptive transfer into lymphopenic mice. *C*, The fraction of proliferating, adoptively transferred $CD4^+$ GFP^- and GFP^+ cells in recipient mice was estimated by staining cells with Ki-67 specific antibody (continuous line) or isotype-matched control (broken line). The results of one out of three experiments are shown. *D*, Expression of the GFP reporter in adoptively transferred *Sf*$Foxp3^{GFP+}$ $CD4^+$ T cells. Flow cytometry analysis of lymph nodes of TCR α chain knockout recipient mice adoptively co-transferred with sorted lymph node $CD4^+GFP^+Ly5.1^+$ cells ($2x10^5$/mouse) from *Sf*$Foxp3^{GFP}$ mice and total $CD4^+Ly5.1^-$ cells (10^6/mouse) from $Foxp3^{GFP}$ mice. Recipient mice were analyzed 4 weeks after transfer. *E*, Expression of the GFP reporter in adoptively transferred *Sf*$Foxp3^{GFP+}$ and *Sf*$Foxp3^{GFP-}$ $CD4^+$ T cells in the inflammatory environment. $CD4^+Ly5.1^{+/+}$*Sf*$Foxp3^{GFP+}$ and

$CD4^+Ly5.1^{+/-}$*Sf*$Foxp3^{GFP-}$ T cells (effector cells) ($5x10^5$ cells of each subset) sorted from 17 day old *Sf*$Foxp3^{GFP}$ mice expressing respective allelic markers were transferred i.p. into 7 day old recipient $Ly5.1^{-/-}$ *Sf*$Foxp3^{GFP}$ mice. After 10 days recipient mice were sacrificed and cells from abdominal cavity were analyzed. Flow cytometry analysis of gated $CD4^+$ of recipient ($Ly5.1^{-/-}$) and donors' cells ($Ly5.1^{+/-}$ cells - continuous line circle, $Ly5.1^{+/+}$ cells - gate marked by dotted line) is shown (left panel). Expression of the GFP reporter is shown in $Ly5.1^{+/-}$*Sf*$Foxp3^{GFP-}$ (upper right panel) and $Ly5.1^{+/+}$*Sf*$Foxp3^{GFP+}$ $CD4^+$ T cells (lower right panel). Numbers indicate percentages of gated cells. The data show representative data of three recipient mice analyzed.



Cytokine	*Sf*Foxp3^{GFP-}	*Sf*Foxp3^{GFP+}
Il-2	105.6±17.2	N.D.
Il-4	6741±689	4051±572
Il-10	19802±2061	14744±1214
Il-17	N.D.	N.D.
IFN-γ	10152±826	1584
TGF-β	24007±4532	26457±6583

FIGURE 6.

Functional assays of CD4$^+$ *Sf*Foxp3^{GFP+} T cells. *A*, *Sf*Foxp3^{GFP+} T cells are anergic *in vitro*. Proliferation of T cell subsets sorted from lymph nodes of Foxp3^{GFP} and *Sf*Foxp3^{GFP} mice is shown. Total CD4$^+$ (white bars), CD4$^+$Foxp3^{GFP-} (dark gray bars), and CD4$^+$Foxp3^{GFP+} (light gray bars) T cells were stimulated with anti-CD3ε and anti-CD28 antibodies. Addition of recombinant Il-2 (50 u/ml) restored proliferation of CD4$^+$Foxp3^{GFP+} cells (dotted bars). Data from two individual *Sf*Foxp3^{GFP} (#1, 2) and two Foxp3^{GFP} (#3, 4) mice are shown. The bars show average of duplicate measurements. The data represent one of three experiments. *B, C,* CD4$^+$ *Sf*Foxp3^{GFP+} T cells produce characteristic cytokine pattern. Cytokine levels were measured in supernatants collected from cultures of equal number of sorted CD4$^+$*Sf*Foxp3^{GFP-} (upper image) and CD4$^+$*Sf*Foxp3^{GFP+} (lower image) T cell subsets. Cells were stimulated *in vitro* for 30 hours with plate bound anti-CD3/anti-CD28 antibodies. Chemiluminescence images of wells incubated with culture supernatants are shown in *B*. The arrangement of reagents specific for a particular cytokine is shown in *C*. MIP-1α was abbreviated to MIP1, GM-CSF to GM, and RANTES to RAN. One experiment of three is presented. *D*, Cytokine levels produced by *in vitro* stimulated CD4$^+$ T cell subsets measured by ELISA are shown (cytokine concentration is expressed in pg/ml)(N.D. not detectable). *E*, Analysis of cytokine transcripts present in sorted CD4$^+$ *Sf*Foxp3^{GFP+} and effector *Sf*Foxp3^{GFP-} cell subsets of *scurfy* mice without stimulation *in vitro*. Cytokine mRNA levels were quantitated by real-time PCR. Relative levels of cytokine mRNA expressed by *Sf*Foxp3^{GFP-} (-, dotted bars) and *Sf*Foxp3^{GFP+} (+, dark bars) cells for two mice, A and B are

presented in a bar graph. *F*, Flow cytometry analysis of cytokine production by sorted $CD4^+$ T cells from *scurfy* mice. Cells from healthy $Foxp3^{GFP}$ mice were used as control. Before staining sorted cells were restimulated *in vitro* with plate bound anti-CD3/anti-CD28 antibodies for 4 hours in the presence of monensin.

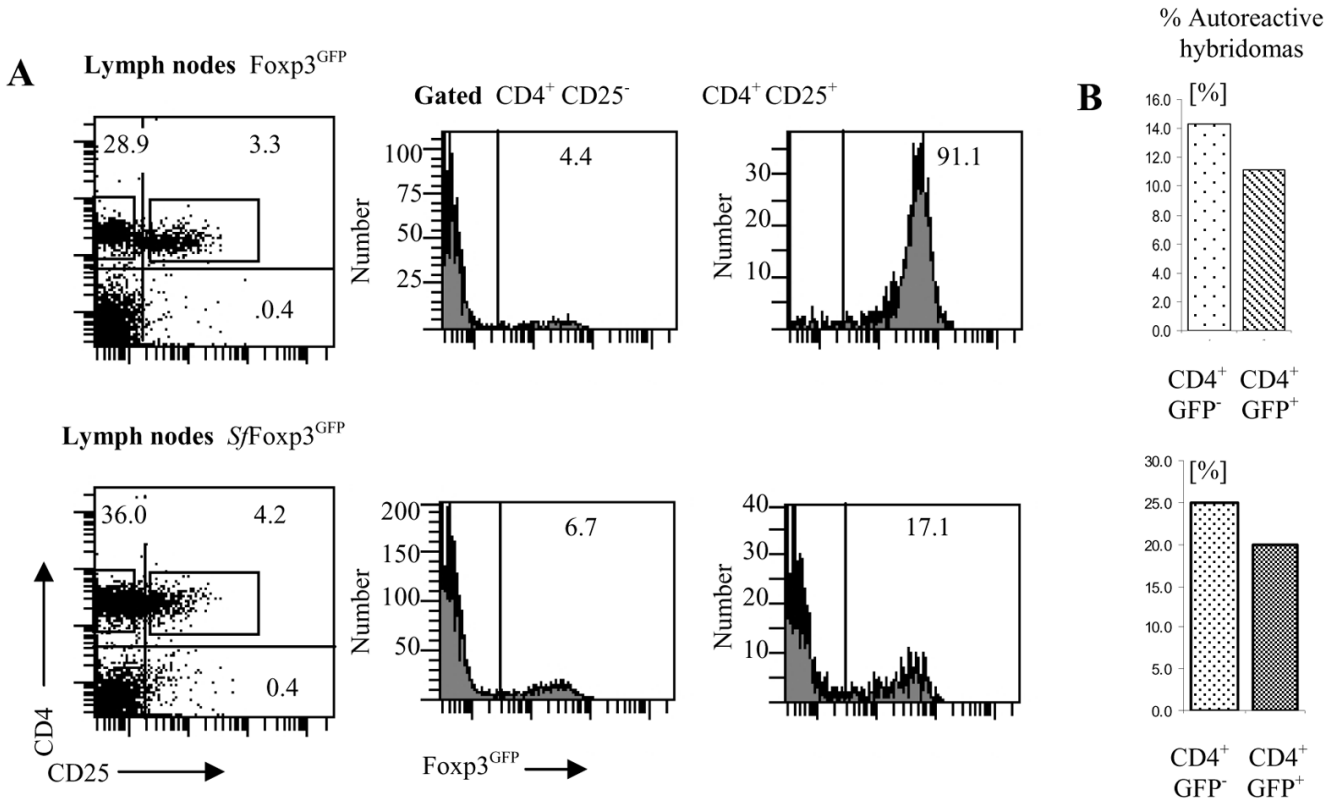


FIGURE 7.

Sf Foxp3^{GFP+} T cells do not express elevated frequency of self-reactive TCRs. *A,* Analysis of the Foxp3^{GFP} expression in CD4^{+}CD25^{-} and CD4^{+}CD25^{+} T cells shows that both cell subsets include *Sf*Foxp3^{GFP+} T cells. Panels in the left column display CD4 and CD25 expression on lymph node cells. Rectangles show gates used to define CD4^{+}CD25^{-} and CD4^{+}CD25^{+} T cell subsets. Foxp3^{GFP} expression on gated CD4^{+}CD25^{-} (middle columns) and CD4^{+}CD25^{+} (right columns) T cells is presented as histograms. *B,* T cell hybridomas produced from *Sf*Foxp3^{GFP-} and *Sf*Foxp3^{GFP+} CD4^{+} T cells express similar frequency of self-reactive TCRs.

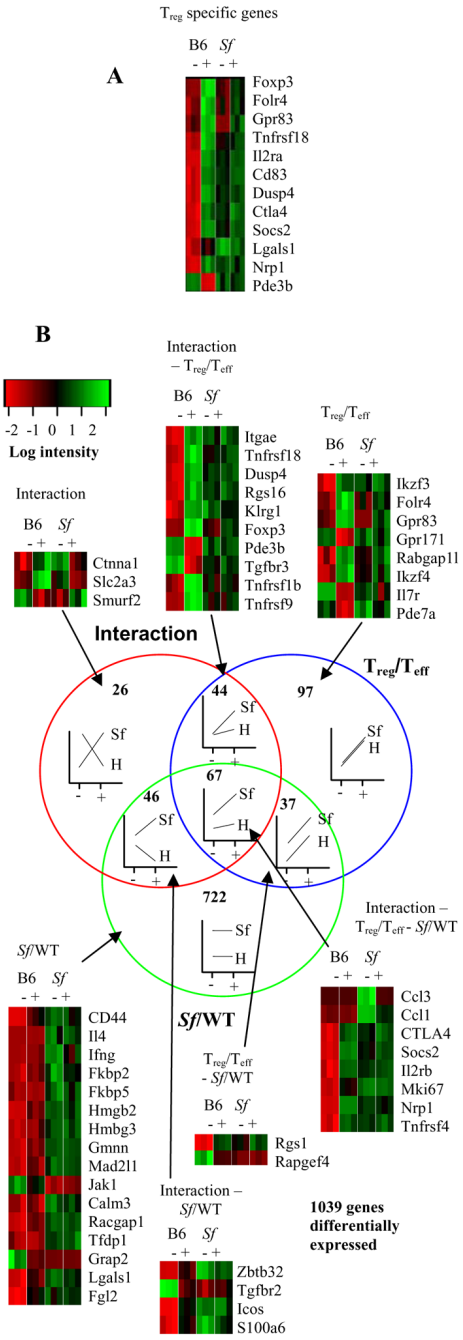


FIGURE 8.

Transcriptional profile of Foxp3-deficient and sufficient T_{reg} cells. *A*, Gene expression of T_{reg} specific genes in Foxp3^{GFP+} and Foxp3^{GFP-} cells from healthy mice and *Sf*Foxp3^{GFP+} and *Sf*Foxp3^{GFP-} cells from *scurfy* mice. *B*, Venn diagram and expression profiles of genes differentially expressed in T_{reg} vs. conventional T cells (blue circle) and CD4^{+} cells from Foxp3-deficient and sufficient mice (green circle). Red circle includes genes showing interaction effect suggesting that they are Foxp3 dependent. Examples of T_{reg}-specific genes are shown. Plots inside Venn diagram show examples of possible gene expression profiles in each section of the diagram. Sf denotes *scurfy* mice, H denotes healthy B6 mice.