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Foxp3-Deficient Regulatory T Cells Do Not Revert into Conventional Effector CD4⁺ T Cells but Constitute a Unique Cell Subset¹,²

Michal Kuczma, Robert Podolsky, Nikhil Garge, Danielle Daniely, Rafal Pacholczyk, Leszek Ignatowicz, and Piotr Kraj³

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 TCRs and class II MHC-peptide complexes in thymus “instruct” developing thymocytes to up-regulate Foxp3 and become T_{reg} cells. However, the loss or down-regulation of Foxp3 does not disrupt the development of T_{reg} cells but abrogates their suppressor function. In this study, 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. The Journal of Immunology, 2009, 183: 3731–3741.

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 Ag. 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-Ags 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 up-regulation may happen 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 humans and mice (9). The 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, multiorgan, 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 Ag 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 down-regulation 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 down-regulation 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

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⁶ Address correspondence and reprint requests to Dr. Piotr Kraj, Center for Biotechnology and Genomic Medicine, Medical College of Georgia, CA-4141, Augusta, GA 30912. E-mail address: pkraj@mail.mcg.edu
⁷ Abbreviations used in this paper: T_{reg}, regulatory T; IPEX, immune dysregulation, polyendocrinopathy, enteropathy, X-linked; BAC, bacterial artificial chromosome; galK, galactokinase; Teff, effector T.
autoimmune pathology remains unknown (20–22). These cells could represent thymocytes that attempted Treg cell development and migrated to the periphery but retained at least some properties of functional Treg cells despite losing suppressor function. Alternatively, these cells could represent aggressive, self-reactive T cells that originate from the Treg 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 up-regulate Foxp3 upon activation, it was not possible to determine the developmental origin of these cells (23).

We have established that Foxp3-deficient Treg cells in sick scurfy males, in the absence of functional Treg cells, remained quiescent, did not expand relative to other CD4+ T cells, and expressed a lower level of activation markers compared with effector CD4+ T cells. In in vitro assays, S/Foxp3GFP+ cells did not produce IL-2 and poorly responded to TCR stimulation. Moreover, S/Foxp3GFP+ cells produced much fewer 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 Treg-specific genes is similar in S/Foxp3GFP+ and functional Treg cells. By comparing gene expression in effector and Treg cells isolated from scurfy and healthy mice, we defined Treg-specific, Foxp3-independent gene signature. Analysis of T cell hybridomas derived from effector and S/Foxp3GFP+ cells revealed that the frequency of self-reactive TCRs is similar in both cell subsets. In conclusion, Foxp3 deficiency does not convert Treg cells into conventional, self-reactive effector cells and S/Foxp3GFP+ cells retain cellular features of Treg cells in inflammatory environments. Despite poor potential for clonal expansion, Foxp3-deficient Treg cells may modulate immune responses by secreted cytokines, especially IL-4, to shift the immune response toward 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 bacterial artificial chromosome (BAC) clone (RP23-460015, 186.8 kb) isolated from a C57BL/6 genomic library and consisting of the Foxp3 gene was purchased from BACPac. Three other known genes are located on the BAC DNA, Ppp1r3b, Cdc22, and Ccacna1f, 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 gene using the BAC recombineering system (24). BAC modifications were done in the Escherichia 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. The 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 modified BAC DNA was inserted into the recipient BAC DNA. Bacteria containing the recombineering BAC were selected on minimal medium with galactose as the only carbon source. The modified BAC clone was subjected to another recombination event with the second targeting construct consisting of the 5′ arm, the GFP-poly(A) cassette, and the 3′ arm. Second-step recombinants were selected against galK on minimal medium plates with glycerol as the carbon source and 2-deoxygalactose as the selecting agent. 2-deoxygalactose is phosphorylated to toxic 2-deoxygalactose-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 -26, -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 cannot be capped with methylguanosine and transported to the cytoplasm. This ensures that transcripts originating from the transgene cannot be translated into functional Foxp3 protein. Transgenic mice were produced by pronuclear injection of closed circular BAC DNA into oocytes from C57BL/6 mice. Founders were genotyped by PCR with primers specific to GFP (forward primer, 5′-GTGCCCCATCTGTGAGTGACGG-3′ and reverse primer, 5′-CTTTGCTCAAGGGCAGCTGTTGCTCAGG-3′). Five founders expressed the transgene and transmitted it to progeny. Transgenic founder 90 was selected for further crossing.

Mice
Scurfy and C57BL/6 mice were purchased from The Jackson Laboratory and crossed with transgenic Foxp3GFP 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 thymus and lymph nodes by mechanical disruption and cells were stained with Abs available commercially (eBioscience or BD Biosciences). Cells were analyzed using a FACS Canto flow cytometer (BD Biosciences) and WinList software. Cells were sorted on a MoFlo cell sorter (DakoCytomation). For some experiments, CD4+ T cells were negatively sorted using commercial kit and an AutoMACS magnetic cell sorter (Miltenyi Biotec). Intracellular staining for Foxp3 and Ki-67 was performed according to the manufacturer’s instructions (eBioscience and BD Biosciences, respectively).

Proliferation assay
Lymph node proliferation assays were performed with 3–5 × 10⁶ cells isolated from Foxp3GFP or S/Foxp3GFP mice. Cells were sorted directly onto 96-well plates using a MoFlo sorter and cultured for 3 days. Wells were coated overnight with anti-CD3 (10 μg/ml) and anti-CD28 (1 μg/ml) Abs. Proliferation responses were measured by adding 1 μCi/well of [3H]thymidine on day 3 of a 4-day culture.

Inhibition assay
Sorted CD4+ Foxp3GFP− cells (5 × 10⁴/well) were incubated on a 96-well plate with irradiated splenocytes (5 × 10⁴/well, 3000 rad) and soluble anti-CD3 (5 μg/ml) Abs. Various numbers of sorted CD4+ Foxp3GFP− cells (1–5 × 10⁵/well) were added. Cells were sorted using a MoFlo sorter. After 3 days culture proliferation was measured by adding 1 μCi/well of [3H]thymidine.

Adaptive transfer
Donor cells for adaptive transfer were isolated by flow cytometry sorting of total lymph node CD4+ T cells or CD4+ GFP+ or CD4+ GFP− cell subsets from S/Foxp3GFP or Foxp3GFP mice. The number of cells indicated in each experiment was transferred i.v. into recipient TCRα chain knockout or scurfy mice. In cotransfer experiments, congenic C57BL/6 or scurfy mice expressing different alleles of CD45 (Ly5) were used as cell donors. Recipient mice were analyzed 4–5 wk after adaptive transfer into lymphopenic mice and 10 days after transfer into scurfy mice.

RT-PCR
RNA was isolated from sorted cells (10⁴ cells/sample) with a RNeasy Mini Kit (Qiagen) and reverse transcribed using a Superscript kit (Invitrogen) according to the manufacturers’ instructions. The quantities of cDNA were normalized for β-actin. Foxp3 CDNA was amplified with sense primer 5′-ATCCAGGCTGCTGCTGACAAAGAC-3′ and reverse primer 5′-GTTTGGTGCTCACTGGACGCACTTGAGG-3′. These primers distinctly 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 (10⁵ cells/sample) CD4+ Foxp3GFP− and CD4+ Foxp3GFP+ cells. Cells were lysed in the gel-loading buffer and resolved on a 10% polyacrylamide gel. Proteins were transferred onto PVDF membrane (Millipore). Membranes were probed with anti-Foxp3 Ab (eBioscience) followed by goat anti-mouse polyclonal Ab coupled with HRP (Bio-Rad). Membranes were developed with an ECL kit (Amersham Biosciences) according to the manufacturer’s instructions.

Cytokine detection
Production of cytokines by CD4+ Foxp3GFP− and CD4+ Foxp3GFP+ T cells was assessed using a Q-Plex mouse cytokine array (Qusyns Biosciences). Cells were sorted onto 96-well plates (5 × 10⁴/well) coated with anti-CD3 (10 μg/ml and anti-CD28 (1 μg/ml). Abs. After 30 h, the supernatant was
collected and used to measure cytokine levels according to the manufacturer’s instruction. The chemiluminescence image was acquired with a Fujiﬁlm LAS-3000 imaging system. Alternatively, cytokine levels were measured by ELISA using commercial kits according to manufacturer’s instructions (E Bioscience). For ELISA, 2 × 10^5 cells were stimulated with anti-CD3 and anti-CD28 Abs and supernatants were collected after 30 h.

Cytokine transcripts were detected in sorted S/Foxp3GFP and S/Foxp3GFP 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′-CCCCCTCAAATGCTGGTGCTGC-3′ and antisense primer 5′-CA TGAGGATCTGTACAGGTC-3′. Cytokine cDNA was ampliﬁed with the following primers: IL-2 sense, 5′-CCTGGCAATACCTTCGACATG-3′ and antisense, 5′-AGGCTCTGAGGTCATTT-3′; IL-4 sense, 5′-CAAGGCGCTGCTGACATTT-3′ and antisense, 5′-ATCC ATGGGCTATTTGCTTC-3′; IL-10 sense, 5′-AGGTTGAGCAAGGGTGA-3′ and antisense, 5′-TTCGGGAAGAGGTACACAG-3′; IL-17 sense, 5′-AGGCCCCAGCAGCTCCTAA-3′ and antisense, 5′-CAGGATCTTCTGCTGATGA-3′; IFN-γ sense, 5′-AATGGGAGCG GTGAAAGTGT-3′ and antisense, 5′-TTGGGAGAGGATCTACAA CG-3′; and TGF-β sense, 5′-GCTACAGGGCCACTTCTGGT-3′ and antisense, 5′-CGTAGTAGACGATGGGCAGT-3′.

Microarray data were first normalized using RMA and subsequently quantiﬁed using Microarray analysis. The PCR conditions were: denaturation at 95°C for 2 min. followed by 40 cycles of denaturation at 95°C for 20 s, annealing at 58°C for 10 s, and elongation at 72°C for 20 s. The relative levels of cytokine mRNA were determined. For each sample, the per cell mRNA level of the cytokine gene was determined by normalizing the 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 the 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 S/Foxp3GFP and S/Foxp3GFP cells directly fused to BW thymoma deﬁcient in endogenous αβ TCR as described previously (25). Hybridomas were tested for reactivity to self-Ags using a standard IL-2 release assay (8). Hybridomas produced in two independent experiments (42 and 44 hybridomas from S/Foxp3GFP cells and 25 hybridomas from S/Foxp3GFP cells) were cloned and expressed TCR and CD4 and responded to stimulation with plate-bound Abs by producing IL-2. To determine hybridoma reactivity independent of IL-2 production we assessed CD69 expression on hybridomas stimulated with plate-bound Abs 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 Abs.

Microarray analysis

RNA was prepared from sorted cell subsets using a RNeasy kit (Qiagen). Treg and conventional CD4+ T cells from S/Foxp3GFP and Foxp3GFP mice were analyzed in triplicates. RNA was ampliﬁed using a TargetAMP kit (Epitect). The resulting cRNA was hybridized to Affymetrix GeneChip M430 2.0 Plus. Microarray data were ﬁrst normalized using RNA and subsequently analyzed using Linear Models for Microarray Data (26, 27). We analyzed all data for a factordetermination in which strain (S/F vs wild type) was one factor and cell type (Treg vs efector T (Treg)) 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 response to Foxp3. Genes with no signiﬁcant interaction and no signiﬁcant difference between strains, but having a signiﬁcant difference between Treg and Treg effector cells are those genes that respond to Foxp3 expression equally in both strains, with no differences between strains. The advantage to Linear Models for Microarray Data is that the B statistic (log posterior odds of differential expression) used in this analysis quantiﬁes the evidence for the alternative hypothesis. Since B is on a log scale, a B of 0 indicates that both the alternate and null hypotheses are equally likely. If the B statistic 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 ≥ 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 ~0.01.

Results

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

To facilitate analysis, Foxp3-deﬁcient Treg cells in scurfy males were identiﬁed by expressing GFP reporter. Transgenic mice expressing GFP controlled by the Foxp3 regulatory sequences (Foxp3GFP mice) and scurfy mice were crossed (to produce S/Foxp3GFP mice) and males coexpressing the mutant Foxp3 allele and Foxp3GFP 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 a reporter cassette encoding GFP followed by the STOP codon and the poly(A) 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 Foxp3GFP and C57BL/6 mice as well as scurfy and S/Foxp3GFP 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. 2, A and B, and data not shown). Approximately 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 Foxp3GFP transgene in Treg cells or organs known to contain CD4+ T cells, and production of the Foxp3-GFP fusion protein or truncated Foxp3 (28). Transgenic Foxp3GFP and C57BL/6 mice as well as scurfy and S/Foxp3GFP 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. 2, A and B, and data not shown). Approximately 90% of CD4+ CD25+ T cells in healthy and Foxp3-deficient mice were further inducible by TGF-β treatment (data not shown). In vitro expression of the Foxp3GFP reporter remained stable for >2 days in Ag-stimulated GFP+ cells from healthy and scurfy mice and was further inducible by TGF-β (data not shown). In conclusion, the Foxp3GFP reporter transgene reliably deﬁnes the population of Treg cells.

The loss of suppressor function by S/Foxp3GFP* CD4+ T cells is not accompanied by the acquisition of a high level of activation markers

CD4+ Foxp3GFP+ but not S/Foxp3GFP+ T cells inhibited activation of CD4+ T cells in vitro (Fig. 2D). Adoptively transferred, puriﬁed CD4+ T cells from S/Foxp3GFP, but not Foxp3GFP, mice induced autoimmune disease in T cell-deﬁcient recipients, demonstrating that CD4+ T cells in the former mice lacked functional Treg cells (data not shown). Thus, the in vitro assay and cell transfer studies demonstrate the lack of suppressor function of S/Foxp3GFP* CD4+ T cells. Foxp3GFP+ T cells were preferentially found in the population of activated CD44+ CD62L− cells, consistent with the fact that Treg 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 $\delta$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 $\delta$Foxp3$^{GFP^+}$ and conventional CD4$^+$ T cells (Fig. 3B). $\delta$Foxp3$^{GFP^+}$ cells only modestly up-regulated CD44 and down-regulated CD62L compared with the conventional CD4$^+$ T cells, suggesting that they are less responsive to activation by self-Ags. In summary, expression of activation markers suggests that effector CD4$^+$ T cells are more sensitive to activation by self-Ags than Treg cells that lose Foxp3 expression.

**The expansion and phenotype stability of $\delta$Foxp3$^{GFP^+}$ T cells**

The proportion of Foxp3-expressing cells was similar in the lymph nodes of $\delta$Foxp3$^{GFP^+}$ and $\delta$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. 3, C and D). Tissue infiltrates in peripheral organs were dominated by $\delta$Foxp3$^{GFP^-}$ cells, demonstrating that $\delta$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 Ab. Ki-67 Ag is expressed in all phases of the cell cycle except G0; therefore, 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 $\delta$Foxp3$^{GFP^+}$- and $\delta$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 $\delta$Foxp3$^{GFP^-}$ population. These findings implied that T cells recruited to the proliferating population preferentially originate from effector $\delta$Foxp3$^{GFP^-}$ T cells.

To further evaluate proliferative capacity of $\delta$Foxp3$^{GFP^+}$ and $\delta$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 populations (5). Analysis of the CD4$^+$ T cell population in recipient mice shows a diminished proportion of $\delta$Foxp3$^{GFP^+}$ cells and suggests that they are outgrown by the effector $\delta$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 $\delta$Foxp3$^{GFP^+}$ cells than in 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 whether the decreased proportion of GFP$^+$ T cells in lymphopenic mice reconstituted with the total population of CD4$^+$ T cells from $\delta$Foxp3$^{GFP^-}$ mice is caused by down-regulation of the GFP reporter upon homeostatic expansion, we have investigated the persistence of $\delta$Foxp3$^{GFP^-}$ cells in recipient mice (Fig. 5D).

To provide exogenous IL-2, we cotransferred $\delta$Foxp3$^{GFP^+}$ cells with total CD4$^+$ T cells from healthy mice into lymphopenic mice. Analysis of transferred cells after 4 wk shows that the predominant fraction of $\delta$Foxp3$^{GFP^+}$ cells retained Foxp3$^+$ transcription. In another experiment, we were able to detect $\delta$Foxp3$^{GFP^+}$ cells in recipient mice a few months 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 the contribution of Foxp3-deficient Treg cells to autoimmune pathology was concealed by the loss of the GFP expression. To investigate how stable is the phenotype of S/Foxp3<sup>GFP</sup> cells in the inflammatory environment, we transferred i.p. equal numbers of CD4<sup>+</sup>Ly5.1<sup>+</sup> S/Foxp3<sup>GFP</sup> and CD4<sup>+</sup>Ly5.1<sup>+</sup> S/Foxp3<sup>GFP</sup> T cells (effector cells) sorted from 17-day-old S/Foxp3<sup>GFP</sup> mice expressing respective allelic markers into 7-day-old recipient Ly5.1<sup>+</sup> S/Foxp3<sup>GFP</sup> mice. When recipient mice were analyzed 10 days after cell transfer, sufficient numbers of donor cells were only found in the abdominal cavity (site of injection), indicating that donor cells did not undergo expansion in the lymphoreplete environment of recipient mice, consistent with an earlier report (2). In our mouse colony, 10-day-old scurfy mice showed first signs of lymphoproliferative disease and died at ~3 wk of age; therefore, a 10-day period of adoptive transfer is appropriate to assess the loss of Foxp3 expression in transferred S/Foxp3<sup>GFP</sup> cells. The great majority (80%) of transferred S/Foxp3<sup>GFP</sup> 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 S/Foxp3<sup>GFP</sup> cells are much less capable of expansion than effector CD4<sup>+</sup> T cells from scurfy mice (see below). Continuous
transcription of the Foxp3-GFP reporter in vivo and its up-regulation by TGF-β treatment in vitro imply that the regulation of Foxp3 expression in Foxp3-deficient and Foxp3-sufficient T<sub>reg</sub> cells is similar and complements earlier reports that inflammatory conditions preserve Foxp3 expression in most natural or adoptive T<sub>reg</sub> cells (data not shown) (2, 33). The origin of a small fraction of S<sub>Foxp3<sup>GFP</sup></sub> T cells that lost Foxp3 expression is not certain. These cells could represent a subset of genuine, thymus-derived T<sub>reg</sub> cells with less stable phenotype or they might be adoptive T<sub>reg</sub> cells that “contaminate” the population of T<sub>reg</sub> cells. Adoptive T<sub>reg</sub> cells do not have a stable T<sub>reg</sub> phenotype and they can be generated, even in inflammatory conditions, from Foxp3-deficient conventional CD4<sup>+</sup> T cells (34). In conclusion, adoptive transfer experiments show that S<sub>Foxp3<sup>GFP</sup></sub> T cells that lost reporter expression may constitute only a very small proportion of GFP<sup>−</sup> cells in S<sub>Foxp3<sup>GFP</sup></sub> mice.

S<sub>Foxp3<sup>GFP</sup></sub> CD4<sup>+</sup> 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 S<sub>Foxp3<sup>GFP</sup></sub> cells and effector CD4<sup>+</sup> T cells. When sorted populations of S<sub>Foxp3<sup>GFP</sup></sub> and S<sub>Foxp3<sup>GFP</sup></sub> cells were stimulated in vitro, proliferation of S<sub>Foxp3<sup>GFP</sup></sub> cells was very small compared with effector CD4<sup>+</sup> T cells and was similar to the proliferation of functional Foxp3<sup>+</sup> T<sub>reg</sub> cells (Fig. 6A). Provision of exogenous IL-2 restored proliferation of both S<sub>Foxp3<sup>GFP</sup></sub> and functional T<sub>reg</sub> cells. Thus, S<sub>Foxp3<sup>GFP</sup></sub> cells closely resemble functional T<sub>reg</sub> cells that are anergic in vitro but proliferate and expand in vivo (35). In vitro-activated S<sub>Foxp3<sup>GFP</sup></sub> and S<sub>Foxp3<sup>GFP</sup></sub> cells

**FIGURE 4.** Flow cytometry analysis of lymphocyte population infiltrating peripheral organs in S<sub>Foxp3<sup>GFP</sup></sub> mice. Panels show expression of the Foxp3-GFP reporter in CD4<sup>+</sup> T cells isolated from liver, lungs, kidney, intestine, and heart. Bar graph shows average percentage of S<sub>Foxp3<sup>GFP</sup></sub> cells in the respective organs (Li, liver; Lu, lungs; K, kidney; I, intestine, and H, heart).

**FIGURE 5.** Analysis of the proliferative potential and the stability of the phenotype of T<sub>reg</sub> and conventional CD4<sup>+</sup> T cells from healthy and scurfy mice. A, The fraction of proliferating CD4<sup>+</sup> GFP<sup>−</sup> and GFP<sup>+</sup> cells was estimated in Foxp3-GFP<sup>+</sup> and S<sub>Foxp3<sup>GFP</sup></sub> mice by staining sorted cells with Ki-67-specific Ab (continuous line) or isotype-matched control (broken line). B, S<sub>Foxp3<sup>GFP</sup></sub> CD4<sup>+</sup> T cells have proliferative advantage over S<sub>Foxp3<sup>GFP</sup></sub> cells. Flow cytometry analysis of lymph node cells of TCR α-chain knockout mice adoptively transferred with total CD4<sup>+</sup> T cells (5 × 10<sup>6</sup>/mouse) from Foxp3<sup>GFP</sup> (left panel) and S<sub>Foxp3<sup>GFP</sup></sub> (right panel) mice. Mice were analyzed 5 wk after transfer. Bar graph shows percentage of GFP<sup>+</sup> cells in the CD4<sup>+</sup> population of Foxp3<sup>GFP</sup> (bars 1 and 2) and S<sub>Foxp3<sup>GFP</sup></sub> (bars 3 and 4) cells before transfer (bars 1 and 3) and after (bars 2 and 4) adoptive transfer into lymphopenic mice. C, The fraction of proliferating, adoptively transferred CD4<sup>+</sup> GFP<sup>−</sup> and GFP<sup>+</sup> cells in recipient mice was estimated by staining cells with Ki-67-specific Ab (continuous line) or isotype-matched control (broken line). The results of one of three experiments are shown. D, Expression of the GFP reporter in adoptively transferred S<sub>Foxp3<sup>GFP</sup></sub> CD4<sup>+</sup> T cells. Flow cytometry analysis of lymph nodes of TCR α-chain knockout recipient mice adoptively cotransferred with sorted lymph node CD4<sup>+</sup> T cells (5 × 10<sup>6</sup>/mouse) from S<sub>Foxp3<sup>GFP</sup></sub> mice and total CD4<sup>+</sup> Ly5.1<sup>+</sup> cells (10<sup>6</sup>/mouse) from S<sub>Foxp3<sup>GFP</sup></sub> recipient mice. Recipient mice were analyzed 4 wk after transfer. E, Expression of the GFP reporter in adoptively transferred S<sub>Foxp3<sup>GFP</sup></sub> CD4<sup>+</sup> T cells in the inflammatory environment. CD4<sup>+</sup> Ly5.1<sup>+</sup> S<sub>Foxp3<sup>GFP</sup></sub> and CD4<sup>+</sup> Ly5.1<sup>+</sup> S<sub>Foxp3<sup>GFP</sup></sub> T cells (effector cells) (5 × 10<sup>5</sup> cells of each subset) sorted from 17-day-old S<sub>Foxp3<sup>GFP</sup></sub> mice expressing respective allelic markers were transferred i.p. into 7-day-old recipient Ly5.1<sup>−/−</sup> S<sub>Foxp3<sup>GFP</sup></sub> mice. After 10 days, recipient mice were sacrificed and cells from the abdominal cavity were analyzed. Flow cytometry analysis of gated CD4<sup>+</sup> of recipient (Ly5.1<sup>−/−</sup>) and donor cells (Ly5.1<sup>−</sup> cells, continuous line circle; Ly5.1<sup>+</sup> cells, gate marked by dotted line) is shown (left panel). Expression of the GFP reporter is shown in Ly5.1<sup>−</sup> S<sub>Foxp3<sup>GFP</sup></sub> (upper right panel) and Ly5.1<sup>−/−</sup> S<sub>Foxp3<sup>GFP</sup></sub> CD4<sup>+</sup> T cells (lower right panel). Numbers indicate percentages of gated cells. The data show representative data of three recipient mice analyzed.
produced markedly different cytokine profiles (Fig. 6, B–F). Consistent with proliferation assays, S/Foxp3GFP+ T cells did not produce IL-2. These cells produce low levels of inflammatory cytokines with the exception of IL-4 and immunoregulatory cytokines IL-10 and TGF-β. S/Foxp3GFP+ cells did not differentiate into a notable number of Th1 or Th17 helper T cells and did not secrete IL-6 or IL-12, suggesting that they do not directly support differentiation of Th1 or Th17 cells, respectively. In contrast, effector CD4+ cells produced much higher levels of inflammatory cytokines IFN-γ and MIP-1α and a very high level of IL-4. In addition, S/Foxp3GFP− cells produced a broader spectrum of cytokines that included IL-3, IL-5, IL-6, IL-10, GM-CSF, and IL-2. The ability to produce a high level of IL-2 is the likely reason for efficient expansion of S/Foxp3GFP− cells activated by autoantigens in scurfy mice and explains why these cells are able to support homeostatic expansion of Treg cells expressing defective Foxp3 (17). The earlier reported level of cytokines produced by Foxp3-deficient Treg cells was much higher than the level of cytokines produced by S/Foxp3GFP+ cells and led to the conclusion that Foxp3-deficient Treg 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, whereas we stimulated cells for a short time only with plate-bound Abs. 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 S/Foxp3GFP+ cells and much lower levels of IFN-γ than in effector CD4+ T cells, consistent with intracellular staining showing that only a small fraction of S/Foxp3GFP+ cells produces IFN-γ.
S/Foxp3<sup>GFP</sup><sup>+</sup> T cells are not enriched in autoreactive CD4<sup>+</sup> T cells

Although S/Foxp3<sup>GFP</sup><sup>+</sup> T cells do not exhibit effector functions of activated conventional CD4<sup>+</sup> T cells, the 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<sup>+</sup> T cells that up-regulate activation markers, especially CD25. Up-regulation of CD25 on some S/Foxp3<sup>GFP</sup><sup>+</sup> cells may reflect their dependence on IL-2 or, alternatively, denotes their activation status, in particular predisposition to be activated by self-Ags (37). We further investigated the relationship between cells expressing CD25 and the GFP reporter in S/Foxp3<sup>GFP</sup> mice. Our analysis shows that both CD25<sup>+</sup> and CD25<sup>+</sup> T cell subsets have a substantial contribution of S/Foxp3<sup>GFP</sup><sup>+</sup> cells (Fig. 7A). The CD25<sup>+</sup> T cell subset contained, on average, 2.5–3 times more S/Foxp3<sup>GFP</sup><sup>+</sup> cells than the CD25<sup>+</sup> subset. The previously reported greater overlap of the TCR repertoire expressed by presumably autoreactive CD4<sup>+</sup>CD25<sup>+</sup> T cells from Foxp3 knockout mice and T<sub>reg</sub> cells from healthy mice is most likely the consequence of a higher proportion of S/Foxp3<sup>GFP</sup><sup>+</sup> cells in the population of CD25<sup>+</sup> than CD25<sup>+</sup> cells (5). These results also demonstrate that TCRs isolated from the CD25<sup>+</sup> T cell subset may have originated from S/Foxp3<sup>GFP</sup><sup>+</sup> cells instead of autoreactive, effector CD4<sup>+</sup>CD25<sup>+</sup> T cells.

To estimate the relative frequencies of self-reactive cells in the populations of S/Foxp3<sup>GFP</sup><sup>+</sup> and S/Foxp3<sup>GFP</sup><sup>−</sup> 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 S/Foxp3<sup>GFP</sup><sup>+</sup> and S/Foxp3<sup>GFP</sup><sup>−</sup> 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 S/Foxp3<sup>GFP</sup><sup>−</sup> cells (Fig. 7B). This strongly suggests that although Foxp3-deficient T<sub>reg</sub> 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<sup>+</sup> 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<sub>reg</sub> cells

The data discussed so far strongly suggest that S/Foxp3<sup>GFP</sup><sup>+</sup> cells retained important characteristics of T<sub>reg</sub> cells despite losing expression of functional Foxp3. To gain further insight into the consequences of Foxp3 deficiency for the transcriptional signature of T<sub>reg</sub> cells, we have compared the gene expression profiles of regulatory and conventional CD4<sup>+</sup> 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<sup>GFP</sup><sup>−</sup> and regulatory Foxp3<sup>GFP</sup><sup>+</sup> cells from normal mice showed that up-regulation of Gpr83, Follr4, Tfrsf18 (GITR), Cli4, Foxp3, Dusp4, IL-2Ra, Scoss2, and Nrp1 genes are considered to be a hallmark of the T<sub>reg</sub> cell transcriptional signature, consistent with previous reports (Fig. 8A) (16, 17, 30, 38, 39). Some T<sub>reg</sub>-specific genes (e.g., Gpr83, Follr4, and, Foxp3) were up-regulated in T<sub>reg</sub> cells and down-regulated in conventional CD4<sup>+</sup> T cells from scurfy and C57BL/6 mice. This suggests that their expression pattern correlates with cell type, regardless of the ability of T<sub>reg</sub> cells to produce functional Foxp3 protein and regardless of the activation status of conventional CD4<sup>+</sup> T cells. It is then possible that high expression of other T<sub>reg</sub>-specific genes (e.g., CTLA4, Scoss2) in the Foxp3-deficient S/Foxp3<sup>GFP</sup><sup>+</sup> cell subset is not necessarily a result of cell activation concomitant with the reversal of the T<sub>reg</sub> transcriptional program but rather demonstrates their persistent T<sub>reg</sub> phenotype (40). This interpretation is consistent with a recent analysis demonstrating that expression of some T<sub>reg</sub>-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 S/Foxp3<sup>GFP</sup><sup>+</sup> 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<sub>reg</sub> cells and does not require the presence of functional Foxp3 protein. Consequently, the cellular and molecular features common for S/Foxp3<sup>GFP</sup><sup>+</sup> and T<sub>reg</sub> cells and described in this report are Foxp3 dependent. 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). S/Foxp3<sup>GFP</sup><sup>+</sup> 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<sub>reg</sub> cells, we conducted a two-factor ANOVA of gene expression of Foxp3<sup>GFP</sup><sup>−</sup> and Foxp3<sup>GFP</sup><sup>+</sup> populations isolated from normal mice and S/Foxp3<sup>GFP</sup><sup>+</sup> and S/Foxp3<sup>GFP</sup><sup>−</sup> populations isolated from scurfy mice. The two factors that influenced the gene expression tested in this analysis were the type of cells (T<sub>reg</sub> vs conventional CD4<sup>+</sup> T cell) and the ability to express functional...
Foxp3 protein (scurfy vs C57BL/6). Factorial ANOVA 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 Treg cell) is not independent of the other factor, e.g., the ability to express Foxp3. Thus, the genes differentially expressed in Treg 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 Treg or effector CD4+ T cells (16, 17, 30, 38, 40–42). Heat maps 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 Treg and conventional CD4+ T cells. Of the remaining 722 differentially expressed genes, 134 genes were differentially expressed in Treg vs 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 Treg or conventional cells. The set of 134 genes not showing an interaction and differentially expressed in Treg vs conventional CD4+ T cells may represent Treg signature genes independent of Foxp3. The expression pattern of a subset of these genes (97) remains constant regardless of whether Treg cells from scurfy or healthy mice are analyzed. Another subset of Treg-specific genes (37) is differentially expressed in cells isolated from scurfy or healthy mice. These genes represent Treg-specific genes up- or down-regulated by T cell activation or in response to cytokines, but whose pattern of expression is the same in Treg 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 Treg 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 (Treg vs 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 Treg 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.5

**Discussion**

Recent reports and our own data dissociate the role of Foxp3 in Treg suppressor function from its role in Treg lineage commitment and provoke new and important questions (16, 17). What is the scope of Treg cell functions controlled by Foxp3 and what are the characteristics of Treg cells that lose or down-regulate Foxp3? Since Foxp3 is not critical for the fitness of Treg cells, it is conceivable that some Treg cells may lose or down-regulate Foxp3 expression in healthy individuals. The GFP expression in Foxp3GFP+ cells differs 100-fold in healthy mice and correlates with the level of Foxp3 protein expression, suggesting that the Treg population is heterogeneous (Kuczma, M., I. Pawlikowska, M. Ko-pij, R. Podolsky, G. A. Rempala, and P. Kraj, manuscript in preparation). Recent evidence that Foxp3 acts in a dose-dependent,
instead of a binary manner yields further support to the hypothesis that Treg cells may exist in various shades depending on the level of Foxp3 expression (43). Signaling through OX40 may be one of the mechanisms regulating the Foxp3 level and Treg suppressor function (44). In this study, we show that Foxp3-deficient Treg cells do not revert to effector CD4+ T cells but constitute a distinct subset retaining important cellular characteristics of regulatory cells.

Peripheral SFFoxp3GFP+ cells had a cell surface phenotype distinct from conventional T cells and retained features of Treg cells despite losing suppressor function. These cells remained dependent on exogenous IL-2 for proliferation and were anergic in vitro. SFFoxp3GFP+ cells produced only small amounts of cytokines compared with 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 SFFoxp3GFP+ cells in our system closely resemble the properties of Treg cells expressing a low level of functional Foxp3 that remained quiescent, produced a Th2-skewed cytokine pattern, and revealed lower homeostatic expansion than Treg cells expressing a normal level of Foxp3 (43). Human Treg cells that down-regulate Foxp3 expression also tend to produce Th2-type cytokines and convert into a Th2 cell type (45).

SFFoxp3GFP− 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 SFFoxp3GFP+ or SFFoxp3GFP− CD4+ T cells showed a similar frequency of self-reactive T cells, consistent with our recent report that Treg cells in healthy mice do not preferentially express self-reactive TCRs (8). Our findings are consistent with the analysis of Treg expressing a nonfunctional Foxp3 mutant. Foxp3-deficient Treg cells produced little IL-2, could not survive when adoptively transferred into recipient mice, and required Foxp3GFP− cells to promote autoimmunity (17). Since SFFoxp3GFP+ 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 SFFoxp3GFP+ cells do not predispose them to become the dominant population of self-reactive T cells mediating the fulminant autoimmune disease in scurfy mice. However, the propensity of SFFoxp3GFP+ cells to produce IL-4 may affect the course of autoimmune disease in scurfy mice by augmenting the 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 toward Th2 was demonstrated for Treg cells expressing a low level of functional Foxp3 that also have the 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). SFFoxp3GFP−like cells might be also relevant in chronic autoimmune diseases like asthma or allergic diseases where Treg cell deficiency is associated with activation of Th2 effector cells (51).

Our data suggest a more limited role of Foxp3 in the lineage commitment and differentiation of natural Treg cells than suggested earlier (30). Similarly, the scope of cellular functions controlled by the Foxp3 in adoptive Treg cells seems to be limited to their suppressive function. Adaptive Treg 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 Treg cells, IL-2 production, and acquisition of the properties of effector CD4+ T cells (40). Foxp3-deficient Treg cells in male mice lacking functional Treg cells expanded in the periphery and produced IL-2 and Th1, Th2, and Th17 cytokines. Surprisingly, the corresponding cell subset from female mice having a functional population of Treg cells retained characteristics of Treg cells and remained anergic to TCR stimulation in vitro, was 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 Treg cell lineage and suggest that cell-extrinsic factors may convert Treg cells into T eff 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 Treg cells expressing a low level of GFP from Treg 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 SFFoxp3GFP+ 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 Treg genes (including Foxp3 itself) reported in multiple earlier studies was similar in Foxp3GFP+ and in SFFoxp3GFP− cells, suggesting that the imprint of Foxp3 on the transcriptional landscape of Treg cells is likely smaller than reported earlier (2, 30). To assess the influence of Foxp3 on Treg 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 Treg (GFP+) cells, expression of 183 genes (57.7%) could be regulated by Foxp3. Although a significant fraction of the genes constituting the Treg cell transcriptional profile depends on Foxp3, analysis of the expression profile of other genes suggests that they are Foxp3 independent. The existence of a Treg-specific Foxp3-independent set of genes was demonstrated in a recent report (41). The properties of SFFoxp3GFP− cells associated with their commitment to a regulatory lineage are most likely controlled by the set of 97 genes differentially expressed in conventional and Treg cells, not affected by T cell activation, and independent of Foxp3. In conclusion, gene expression profiling supports our findings that Foxp3-deficient SFFoxp3GFP− cells possess a unique phenotype and do not revert to conventional effector CD4+ T cells.

The loss of functional Foxp3 protein by Treg 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 Ag, loss of IL-2 dependence, and production of multiple cytokines including IL-2. Rather, Treg 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. Treg 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.
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