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Regulatory T cell development in the absence of functional Foxp3

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Although the development of regulatory T cells (T_{reg} cells) in the thymus is defined by expression of the lineage marker Foxp3, the precise function of Foxp3 in T_{reg} cell lineage commitment is unknown. Here we examined T_{reg} cell development and function in mice with a *Foxp3* allele that directs expression of a nonfunctional fusion protein of Foxp3 and enhanced green fluorescent protein (*Foxp3*$^{\Delta EGFP}$). Thymocyte development in *Foxp3*$^{\Delta EGFP}$ male mice and *Foxp3*$^{\Delta EGFP/+}$ female mice recapitulated that of wild-type mice. Although mature EGFP$^+$ CD4$^+$ T cells from *Foxp3*$^{\Delta EGFP}$ mice lacked suppressor function, they maintained the characteristic T_{reg} cell 'genetic signature' and failed to develop from EGFP$^-$ CD4$^+$ T cells when transferred into lymphopenic hosts, indicative of their common ontogeny with T_{reg} cells. Our results indicate that T_{reg} cell effector function but not lineage commitment requires the expression of functional Foxp3 protein.

Naturally arising CD4$^+$CD25$^+$ regulatory T cells (T_{reg} cells) represent a distinct T cell lineage dedicated to maintaining self-tolerance[1,2]. T_{reg} cells develop in the thymus through a process involving the recognition of self peptides in major histocompatibility complex (MHC) molecules on thymic epithelial cells[3–6]. T_{reg} cells express a T cell receptor (TCR) repertoire enriched in self specificities, distinct from that of conventional T cells[7,8]. T_{reg} cells also express a distinct set of both cell surface and intracellular molecules. Most prominent of these is the transcription factor Foxp3, which is expressed only in the T_{reg} cell lineage and is thought to 'program' the suppressor function of T_{reg} cells. Loss-of-function *Foxp3* mutations result in impaired CD4$^+$CD25$^+$ T_{reg} cell development, the lethal X-linked lymphoproliferative disorder of the naturally arising scurfy mouse strain and related genetically engineered murine models[9–12], and the homologous human disorder IPEX syndrome ('immune dysregulation, polyendocrinopathy, enteropathy, X-linked' syndrome)[13–15].

The development of T_{reg} cells in the thymus is thought to be driven by high-affinity interactions between TCRs on developing thymocytes and self peptide–MHC complexes on thymic epithelial cells. The affinity of such interactions is proposed to be somewhere between the affinities of interactions triggering positive and negative selection of thymocytes[3,4,16]. In an 'instructive' or TCR-dependent model of T_{reg} cell differentiation, cell fate is dictated by TCR–peptide–MHC avidity and there is no predetermined selection into the T_{reg} cell lineage. TCR–peptide–MHC interactions of the appropriate avidity would induce expression of Foxp3, which would then specify T_{reg} cell lineage commitment by 'programming' T_{reg} cell effector function. In support of such a model, the earliest stage at which Foxp3 expression is detected is at the late CD4$^+$CD8$^+$ stage of thymocyte development, and Foxp3$^+$ cells are localized mainly in the thymic medulla. Both observations are consistent with the induction of Foxp3 at the time of positive selection or shortly thereafter. In addition, forced expression of Foxp3 converts conventional T cells into cells with a regulatory phenotype[11,17]. These data collectively suggest that Foxp3 is both necessary and sufficient for T_{reg} cell effector function.

However, a purely 'instructive' model has limitations. It does not provide a mechanism by which T_{reg} cells resist negative selection after encounter with high-affinity self ligands[18]. It also fails to account for the limited number of T_{reg} cells that develop in the thymi of TCR-transgenic mice that also express cognate antigen, in which a large increase in T_{reg} cell numbers might otherwise be expected. Such considerations suggest that T_{reg} cell lineage commitment may require additional signals other than those provided by TCR–peptide–MHC interactions during positive selection. It is unclear whether Foxp3 expression is a 'distal' event that is a consequence of the commitment of developing thymocytes to the T_{reg} cell lineage or if it initiates commitment to that lineage. Thus, the position of Foxp3 in the T_{reg} cell developmental pathway and additional factors that specify lineage commitment remain to be determined.

Given the strong bias of the T_{reg} cell repertoire toward self-recognition, questions also arise regarding the fate of cells destined to develop into T_{reg} cells when Foxp3 expression and/or function is adversely affected by deleterious mutations. Are such cells eliminated during development by the process of negative selection? If not, do they contribute to the resulting autoimmune pathology? In favor of such outcomes are the detection of circulating T cells expressing inactive Foxp3 proteins in patients with

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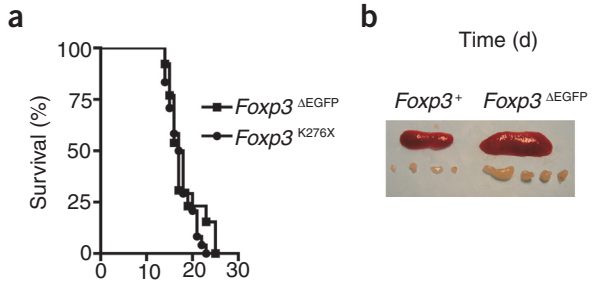

Figure 1 *Foxp3*$^{\Delta EGFP}$ mice develop a fatal lymphoproliferative disease similar to that of Foxp3-deficient mice. (**a**) Kaplan-Meier survival curves of *Foxp3*$^{\Delta EGFP}$ mice ($n = 13$) and Foxp3-deficient *Foxp3*^{K276X} mice ($n = 24$; $P = 0.44$). (**b**) Splenomegaly (top) and lymphadenopathy (bottom) in a *Foxp3*$^{\Delta EGFP}$ male mouse (15 d; right) relative to normal spleen and lymph node size of a littermate control (left).

IPEX syndrome who have missense *FOXP3* mutations and the observation that the autoimmune lymphoproliferative disease associated with Foxp3 deficiency is more aggressive than that in experimental models of acquired T_{reg} cell deficiency induced by neonatal thymectomy or by depletion with antibody to CD25 (anti-CD25)[1,19–21].

To determine the function of Foxp3 in T_{reg} cell lineage commitment and to identify Foxp3-deficient T_{reg} cell precursors exported to the periphery, we generated mice containing a cassette encoding a mutant fusion protein of Foxp3 and enhanced green fluorescent protein (EGFP) in the *Foxp3* locus (*Foxp3*$^{\Delta EGFP}$). EGFP$^+$ CD4$^+$ T cells lacked regulatory function, and *Foxp3*$^{\Delta EGFP}$ male mice developed a fatal autoimmune lymphoproliferative disease indistinguishable from that of other Foxp3-deficient mice. Thymic expression of the mutant Foxp3-EGFP fusion protein occurred at the same developmental stages and in a similar proportion of cells as did that of Foxp3 protein in wild-type mice. EGFP$^+$ CD4$^+$ T cells in *Foxp3*$^{\Delta EGFP}$ mice had a cell surface phenotype and a gene-expression profile that shared many features with those of wild-type T_{reg} cells and were distinct from activated conventional T cells. Overall, our results dissociate the essential purpose of Foxp3 in T_{reg} cell regulatory function from its suggested requirement in initiating T_{reg} cell lineage commitment.

RESULTS

T cell–suppressive function of Foxp3$^{\Delta EGFP}$

We inserted DNA encoding EGFP followed by a stop codon in-frame into the terminal exon 11 of *Foxp3* at codon 397. The resultant allele (*Foxp3*$^{\Delta EGFP}$) encoded a fusion protein expressed under control of the endogenous *Foxp3* promoter and enhancer elements (**Supplementary Figure 1** online). The Foxp3 33–amino acid C-terminal peptide that was replaced with EGFP included residues involved in the binding of Foxp3 to DNA and to the transcription factor NFAT as well as the nucleus-localization sequence, and the nucleotide sequence encoding it is the target of loss-of-function mutations and deletions in patients with IPEX syndrome[22–24]. The Foxp3-EGFP fusion protein was localized to the cytosol, consistent with the loss of the C-terminal nucleus-localization signal (**Supplemental Video 1** online). Male offspring carrying the *Foxp3*$^{\Delta EGFP}$ allele developed a fatal lymphoproliferative disease indistinguishable from that of previously reported mutant mice (*Foxp3*^{K276X}) lacking Foxp3 protein[12] (**Fig. 1**). As is typical of Foxp3-deficient mice, because of random inactivation of X chromosomes, *Foxp3*$^{\Delta EGFP/+}$ females developed normally and were free of disease (data not shown).

Both male and female mice with the *Foxp3*$^{\Delta EGFP}$ allele had EGFP$^+$ lymphocytes restricted to the T cell lineage, mainly the CD4$^+$ T cell lineage with a minor component in the CD8$^+$ T cell population (**Fig. 2a**). Foxp3 localized together with EGFP, as detected by flow cytometry and by real-time PCR analysis (**Fig. 2a**,**b**). Flow cytometry of EGFP$^+$ CD4$^+$ T cells from *Foxp3*$^{\Delta EGFP}$ male mice showed that they expressed several cell surface markers characteristic of T_{reg} cells, including CD25, CTLA-4, GITR and CD44.

We analyzed the regulatory function of EGFP$^+$ CD4$^+$ T cells from *Foxp3*$^{\Delta EGFP}$ male mice by *in vitro* suppression assay and compared it with that of T_{reg} cells isolated from male mice expressing a bicistronic cassette encoding both Foxp3 and EGFP under control of the endogenous *Foxp3* promoter (*Foxp3*^{EGFP})[25]. The *Foxp3*^{EGFP} allele enabled specific expression of EGFP in all Foxp3$^+$ T_{reg} cells with no adverse effect on their function; *Foxp3*^{EGFP} males were phenotypically normal (data not shown). EGFP$^+$ CD4$^+$ cells from *Foxp3*^{EGFP} but not *Foxp3*$^{\Delta EGFP}$ male mice suppressed proliferation of splenocytes (**Fig. 2c**). In response to stimulation with CD3- and CD28-specific antibodies, EGFP$^+$ and EGFP$^-$ CD4$^+$ T cells from *Foxp3*$^{\Delta EGFP}$ male mice underwent minimal proliferation due to generalized apoptosis (data not shown). This most likely reflected the intense activation state of both populations *in vivo*, making them primed for apoptosis after restimulation with mitogens *in vitro*. To circumvent this limitation, we examined the proliferative responses of T cells from *Foxp3*$^{\Delta EGFP/+}$ female mice. Whereas EGFP$^+$ CD4$^+$ cells from *Foxp3*^{EGFP} mice did not proliferate in response to stimulation, EGFP$^+$ CD4$^+$ cells from *Foxp3*$^{\Delta EGFP/+}$ female mice showed substantial proliferation after CD3 and CD28 crosslinking (**Fig. 2d**). These results demonstrated that EGFP$^+$ CD4$^+$ T cells from *Foxp3*$^{\Delta EGFP/+}$ mice lacked typical T_{reg} cell suppressor function.

Thymic development in *Foxp3*$^{\Delta EGFP}$ mice

Examination of developing EGFP$^+$ thymocytes from *Foxp3*$^{\Delta EGFP}$ male mice showed that they were similar in several ways to EGFP$^+$ thymocytes isolated from *Foxp3*^{EGFP} controls. In mice of both genotypes, most EGFP$^+$ cells were located in the thymic medulla (**Fig. 3a**). We first detected EGFP expression at the CD4$^+$CD8$^+$ double-positive stage of development; it was mainly in the most mature TCRβ^{hi}CD69$^+$ double-positive population (**Fig. 3b**). EGFP$^+$ CD4$^+$CD8$^-$ single-positive thymocytes in *Foxp3*$^{\Delta EGFP}$ male mice resembled those of their *Foxp3*^{EGFP} counterparts (**Fig. 3c**). They had higher expression of GITR, CD25 and CTLA-4, all markers associated with T_{reg} cell phenotype and function, than did EGFP$^-$ CD4$^+$ single-positive thymocytes. In addition, EGFP$^+$ CD4$^+$ thymocytes from *Foxp3*$^{\Delta EGFP}$ and *Foxp3*^{EGFP} male mice had higher expression of CD44 and CD62L (data not shown) and lower expression of CD69 and CD24 than did EGFP$^-$ CD4$^+$ single-positive thymocytes. All four markers (CD44, CD62L, CD69 and CD24) are associated with the late stages of phenotypic maturation, demonstrating that EGFP$^+$ populations were distinct from EGFP$^-$ thymocytes[26,27]. Expression of CD127 (interleukin 7 (IL-7) receptor-α), reported to be lower in peripheral T_{reg} cells[28,29], was similarly lower in EGFP$^+$ CD4$^+$ thymocytes from *Foxp3*$^{\Delta EGFP}$ and *Foxp3*^{EGFP} male mice. CD103 (the α_E chain of the integrin $\alpha_E\beta_7$) identifies an effector T_{reg} cell subset, mediates T_{reg} cell homing and limits migration[30]. The population expressing CD103 was absent from the thymus but was present in the periphery of *Foxp3*$^{\Delta EGFP}$ male mice (**Fig. 2a**).

The total thymic cellularity of *Foxp3*$^{\Delta EGFP}$ male mice was lower than that of age-matched *Foxp3*^{EGFP} control mice (63×10^6 cells versus 112×10^6 cells, respectively; $P < 0.005$; $n = 9$ mice per group). This lower number was similar to that reported in published studies of Foxp3-deficient mice on the same genetic background (BALB/c) and may relate to the intense stress response and disease severity in these mice[12]. Although overall thymic size was smaller in *Foxp3*$^{\Delta EGFP}$ male mice, the frequency of EGFP$^+$ thymocytes in the relevant subpopulations was higher. *Foxp3*$^{\Delta EGFP}$ male mice had a threefold-greater percent EGFP$^+$ double-positive thymocytes (0.23% ± 0.02% versus 0.07% ± 0.01%;

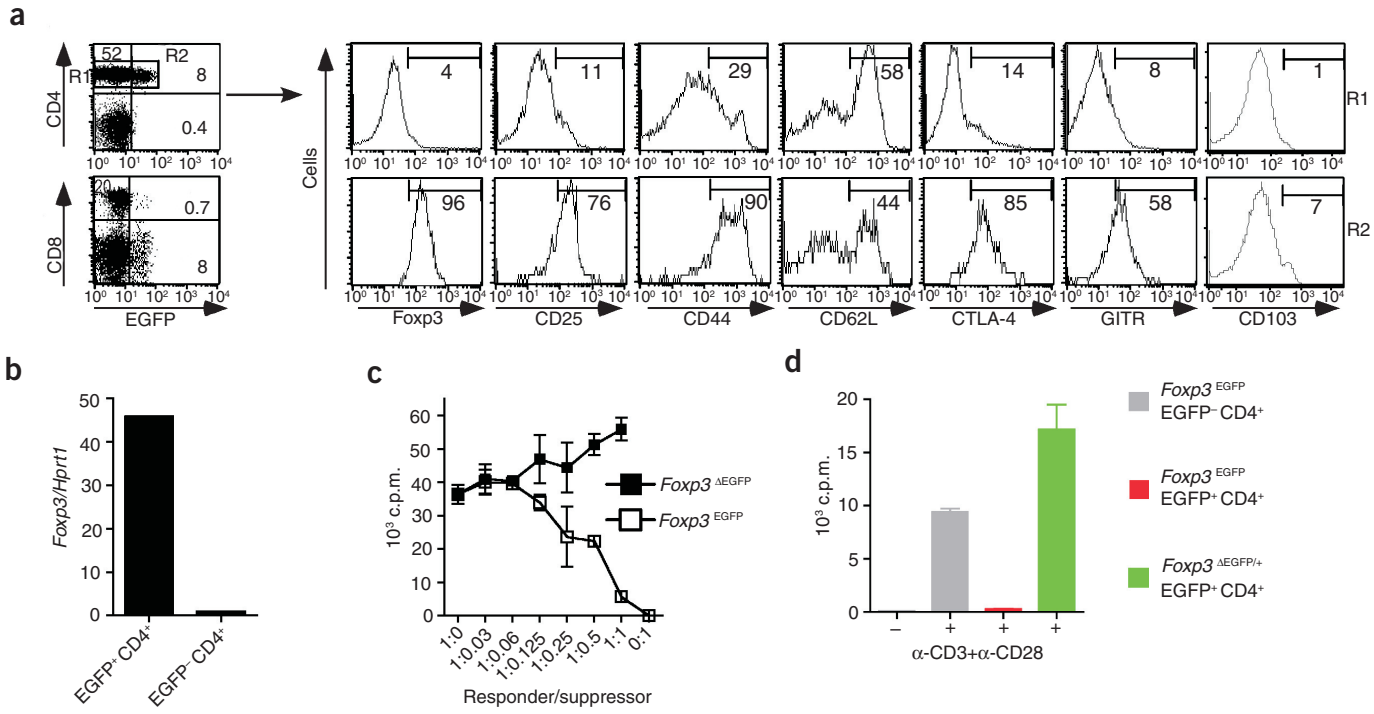

Figure 2 Characterization of peripheral T cells in *Foxp3*$^{\Delta EGFP}$ male mice. (**a**) Flow cytometry of the expression of Foxp3, CD25, CD44, CD62L, CTLA-4, GITR and CD103 on lymph node T cells from *Foxp3*$^{\Delta EGFP}$ male mice. Numbers in quadrants and below bracketed lines indicate percent cells in each gated population. (**b**) Real-time PCR analysis of *Foxp3* mRNA expression in sorted EGFP$^+$ and EGFP$^-$ CD4$^+$ T cells from *Foxp3*$^{\Delta EGFP}$ male mice. *Foxp3* mRNA is normalized to *Hprt1* mRNA (encoding hypoxanthine guanine phosphoribosyl transferase); the amount of *Foxp3* mRNA in EGFP$^-$ CD4$^+$ cells is assigned an arbitrary value of 1. (**c**) Proliferation of 'suppressor' EGFP$^+$ CD4$^+$ cells sorted from *Foxp3*$^{\Delta EGFP}$ or *Foxp3*^{EGFP} mice cultured alone or with 'responder' splenocytes (ratios, horizontal axis); responder cells remained constant at 8×10^4 cells per well. Cells were stimulated with soluble anti-CD3 and proliferative responses were assessed by [^{3}H]thymidine incorporation. (**d**) Proliferation of EGFP$^-$ and EGFP$^+$ CD4$^+$ cells from *Foxp3*$^{\Delta EGFP/+}$ or *Foxp3*$^{EGFP/+}$ female mice stimulated with anti-CD3 and anti-CD28 (α-CD3 + α-CD28), assessed by [^{3}H]thymidine incorporation. Data represent mean ± s.e.m of three replicates (**c**,**d**) and are representative of two to four experiments each (**a–d**).

$P < 0.005$) and a twofold-greater proportion of EGFP$^+$ CD4$^+$ single-positive thymocytes (5.73% ± 0.34% versus 3.38% ± 0.15%; $P < 0.005$; $n = 9$ mice for both groups) relative to control mice. The percent EGFP$^+$ CD8$^+$ single-positive thymocytes was similar in both groups.

In *Foxp3*$^{\Delta EGFP/+}$ female mice, random X chromosome inactivation resulted in expression of the *Foxp3*$^{\Delta EGFP}$ allele in half of the cells selected into the T_{reg} cell lineage. Thus, we directly compared the development of EGFP$^+$ thymocytes in *Foxp3*$^{\Delta EGFP/+}$ female mice with that in age-matched *Foxp3*$^{EGFP/+}$ female mice. This comparison avoided the confounding effect of massive peripheral T cell activation in *Foxp3*$^{\Delta EGFP}$ male mice on thymocyte development and the T_{reg} cell surface phenotype. The overall size of the thymus and percent EGFP$^+$ cells in the double-positive compartment were similar in *Foxp3*$^{\Delta EGFP/+}$ and *Foxp3*$^{EGFP/+}$ female mice (**Fig. 3d**). Although the percent EGFP$^+$ cells in the CD4$^+$ single-positive compartment was lower in *Foxp3*$^{\Delta EGFP/+}$ than in *Foxp3*$^{EGFP/+}$ female mice (1.46% ± 0.23% versus 2.27% ± 0.25%, respectively; $P = 0.03$; $n = 10$ mice for both groups), the total number of EGFP$^+$ cells in the respective CD4$^+$ single-positive populations was not significantly different ($P = 0.11$; **Fig. 3d**). EGFP$^+$ and EGFP$^-$ CD4$^+$ single-positive thymocytes from *Foxp3*$^{\Delta EGFP/+}$ female mice had higher expression of GITR, CTLA-4, CD25, CD44 and CD62L (**Fig. 3e**). There was also less surface CD127, CD69 and CD24 on EGFP$^+$ and EGFP$^-$ Foxp3$^+$ cells in *Foxp3*$^{\Delta EGFP/+}$ female mice. These results collectively identified EGFP$^+$ thymocytes of *Foxp3*$^{\Delta EGFP}$ and *Foxp3*$^{\Delta EGFP/+}$ mice as being intimately related to developing T_{reg} cells and suggested that in the absence of functional Foxp3 protein, the development of EGFP$^+$ T cells proceeds, at least to the point at which T_{reg} cell effector function is 'programmed'. Implicit in these data is the suggestion that T_{reg} cell lineage commitment occurs independently of Foxp3 expression.

The periphery of *Foxp3*$^{\Delta EGFP/+}$ female mice was depleted of EGFP$^+$ T_{reg} cell precursors relative to EGFP$^-$ T_{reg} cells. The lower percentage of EGFP$^+$ CD4$^+$ single-positive thymocytes in *Foxp3*$^{\Delta EGFP/+}$ female mice suggested that elimination of defective T_{reg} cell precursors might have begun in the thymus. We investigated this possibility by staining thymocytes with annexin V and 7-amino-actinomycin D. We noted a fourfold increase in late apoptotic cells (positive for annexin V and 7-amino-actinomycin D) among EGFP$^+$ CD4$^+$ single-positive thymocytes from *Foxp3*$^{\Delta EGFP/+}$ mice relative to that of *Foxp3*$^{EGFP/+}$ control mice (**Fig. 3f**). These findings are consistent with the hypothesis that lack of Foxp3 results in lower survival of T_{reg} cell precursors in the context of an otherwise T_{reg} cell–sufficient host.

EGFP$^+$ CD4$^+$ cell proliferation and survival

EGFP$^+$ CD4$^+$ T cells in spleens and lymph nodes of *Foxp3*$^{\Delta EGFP}$ males were present in larger absolute numbers and as a larger fraction of the total CD4$^+$ T cell population than those of *Foxp3*^{EGFP} male mice (**Fig. 4a**,**b**). There was much greater population expansion of EGFP$^+$ CD4$^+$ T cells in tissues involved in the autoimmune lymphoproliferative disorder in *Foxp3*$^{\Delta EGFP}$ male mice, including lung and liver (**Fig. 4c**,**d**). To determine whether these observations reflected greater *in situ* proliferation of EGFP$^+$ CD4$^+$ T cells in *Foxp3*$^{\Delta EGFP}$ male mice, we injected 15-day-old *Foxp3*$^{\Delta EGFP}$ male mice with bromodeoxyuridine (BrdU) and assessed BrdU

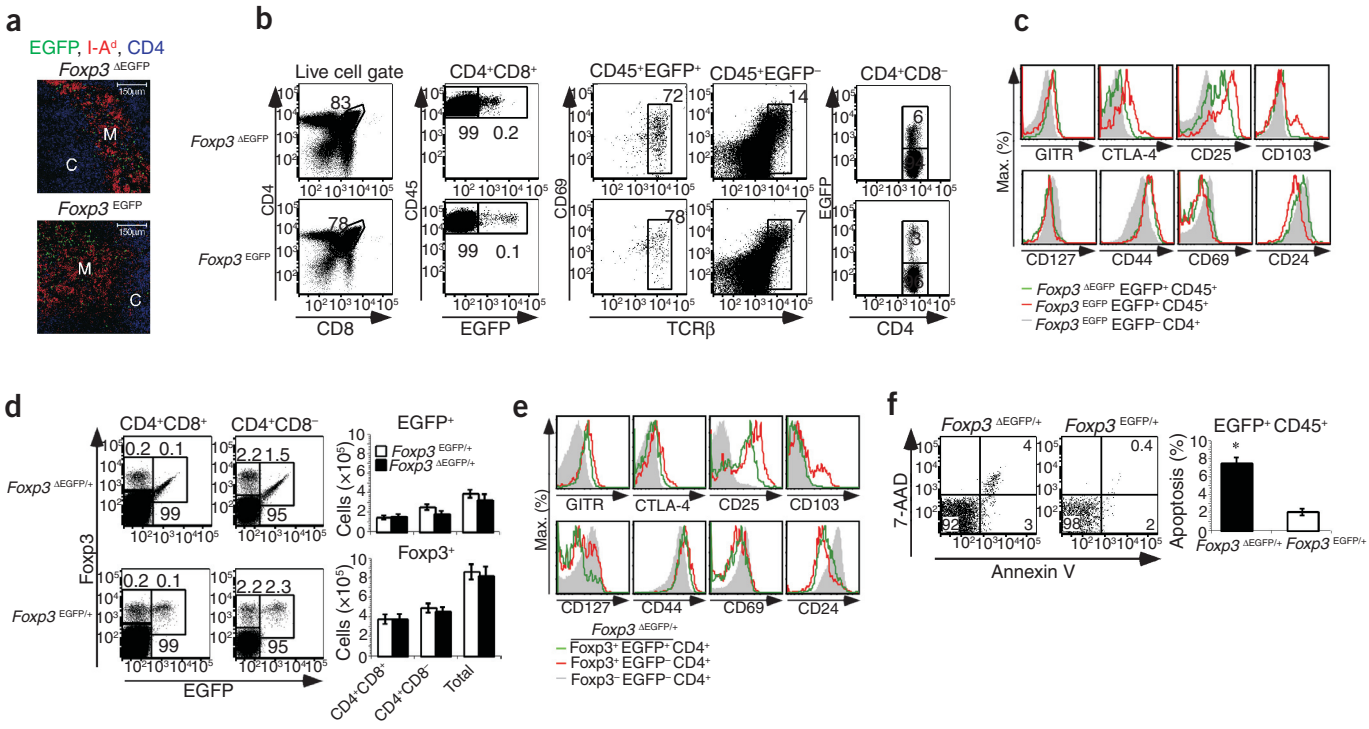

Figure 3 Thymocyte development in *Foxp3*$^{\Delta EGFP}$ mice. (**a**) Localization of EGFP$^+$ cells in thymic sections from *Foxp3*$^{\Delta EGFP}$ and *Foxp3*^{EGFP} male mice, assessed by staining with anti-GFP (green), anti-CD4 (blue) and anti-I-A^d (red). C, cortex; M, medulla. Scale bars, 150 μm. (**b**) Analysis of EGFP expression in the double-positive and CD4$^+$ single-positive thymocyte populations of *Foxp3*$^{\Delta EGFP}$ and *Foxp3*^{EGFP} male mice. Left two plots, EGFP expression in double-positive thymocytes. Middle two plots, CD69 and TCRβ expression on CD45$^+$EGFP$^+$ and CD45$^+$EGFP$^-$ double-positive thymocytes. Right plot, EGFP expression in CD4$^+$ single-positive thymocytes. Numbers in dot plots represent percent of total in each gate. (**c**) Expression of cell surface markers associated with the T_{reg} cell phenotype and with thymocyte maturation on EGFP$^+$ CD4$^+$ thymocytes from *Foxp3*$^{\Delta EGFP}$ mice (green lines) and *Foxp3*^{EGFP} mice (red lines) and EGFP$^-$ CD4$^+$ thymocytes from *Foxp3*^{EGFP} mice (gray shading). (**d**) EGFP and Foxp3 expression in double-positive and CD4$^+$ single-positive populations from *Foxp3*$^{\Delta EGFP/+}$ and *Foxp3*$^{EGFP/+}$ female mice. Dot plots (left) indicate gating; in *Foxp3*$^{\Delta EGFP/+}$ mice, the fusion protein is detected simultaneously with anti-EGFP and anti-Foxp3. Right, mean (± s.e.m.) numbers of EGFP$^+$ cells (top) and Foxp3$^+$ cells (bottom) in double-positive, CD4$^+$ single-positive and total thymocyte populations. (**e**) Expression of cell surface markers associated with the T_{reg} cell phenotype and with thymocyte maturation on Foxp3$^+$ EGFP$^+$ CD4$^+$, Foxp3$^+$ EGFP$^-$ CD4$^+$ and Foxp3$^-$ EGFP$^-$ CD4$^+$ thymocytes from *Foxp3*$^{\Delta EGFP/+}$ female mice. (**f**) Incidence of apoptosis (mean ± s.e.m.) for EGFP$^+$ thymocytes from *Foxp3*$^{\Delta EGFP/+}$ and *Foxp3*$^{EGFP/+}$ female mice, assessed by staining with annexin V and 7-amino-actinomycin D (7-AAD). *, $P < 0.05$. Data are representative of two to four experiments with three to four mice per group for each experiment.

incorporation in their lymphocytes. We found a higher percentage of BrdU$^+$ EGFP$^+$ than BrdU$^+$ EGFP$^-$ T cells in both peripheral lymphoid tissues and infiltrated organs, indicating that EGFP$^+$ CD4$^+$ T cells proliferated more *in situ* (**Fig. 4e**). In contrast, both the EGFP$^+$ and EGFP$^-$ CD4$^+$ T cell populations in *Foxp3*$^{\Delta EGFP}$ male mice had similar frequency of programmed cell death, as detected by staining of freshly isolated lymphocytes for activated caspase 3 (**Fig. 4f**). The staining for activated caspase 3 of both populations was much higher than that of conventional and T_{reg} cells in *Foxp3*^{EGFP} male mice, however, probably reflecting the heightened activation state of T cells in *Foxp3*$^{\Delta EGFP}$ male mice. These findings suggested that the accumulation of EGFP$^+$ T cells in *Foxp3*$^{\Delta EGFP}$ male mice is due to a net proliferative advantage over EGFP$^-$ T cells.

In addition to their heightened proliferation, EGFP$^+$ T cells from the spleen and lymph nodes of *Foxp3*$^{\Delta EGFP}$ male mice had higher production of T helper type 1 (T_H1) and T_H2 cytokines than did their EGFP$^-$ counterparts (**Fig. 4g,h**). Dual staining with monoclonal antibodies specific for IL-4 and interferon-γ (IFN-γ) showed that most EGFP$^+$ T cells produced one or the other cytokine, suggesting that they had already differentiated into T_H1 or T_H2 effector cells (data not shown). In contrast, EGFP$^+$ T cells produced less IL-2 than did EGFP$^-$ T cells (**Fig.4g,h**). These results indicated that EGFP$^+$ T cells might account for a substantial proportion of the exaggerated T_H1 or T_H2 cytokine production characteristic of Foxp3 deficiency[12].

Flow cytometry of EGFP$^+$ CD4$^+$ peripheral T cells from *Foxp3*$^{\Delta EGFP/+}$ females showed that like their counterparts from male mice, they expressed several cell surface markers characteristic of T_{reg} cells, including CD25, CTLA-4, GITR and CD44 (**Fig. 5a**) and produced little IL-2 and more IL-4 and IFN-γ than did EGFP$^-$ CD4$^+$ T cells (**Fig. 5b**). These results confirmed that the phenotypic and functional characteristics of CD4$^+$ T cells expressing the nonfunctional Foxp3-EGFP fusion protein were cell autonomous and were not simply a function of the dysregulated immune environment associated with Foxp3 deficiency. However, the peripheral lymphoid tissues of *Foxp3*$^{\Delta EGFP/+}$ females had many fewer EGFP$^+$ T cells than those of *Foxp3*$^{EGFP/+}$ heterozygous female control mice (**Fig. 5c**). This was more accentuated in nonlymphoid organs (data not shown) and was associated with heightened apoptosis, as demonstrated by more detection of activated caspase 3 in freshly isolated EGFP$^+$ CD4$^+$ T cells from *Foxp3*$^{\Delta EGFP/+}$ females (**Fig. 5c**). Notably, the EGFP$^+$ T cells from *Foxp3*$^{\Delta EGFP/+}$ females underwent more programmed cell death than did EGFP$^-$ T cells from the same mice or EGFP$^+$ and EGFP$^-$ T cells from control *Foxp3*$^{EGFP/+}$ female mice. These results suggested that T cells expressing the nonfunctional Foxp3-EGFP fusion protein were at a survival disadvantage relative to their normal T_{reg} cell counterparts.

Next we examined the capacity of EGFP$^-$ T cells to convert to EGFP$^+$ T cells after transfer of the former into a lymphopenic environment. We transferred equal numbers of EGFP$^+$ and EGFP$^-$ CD4$^+$ T cells from

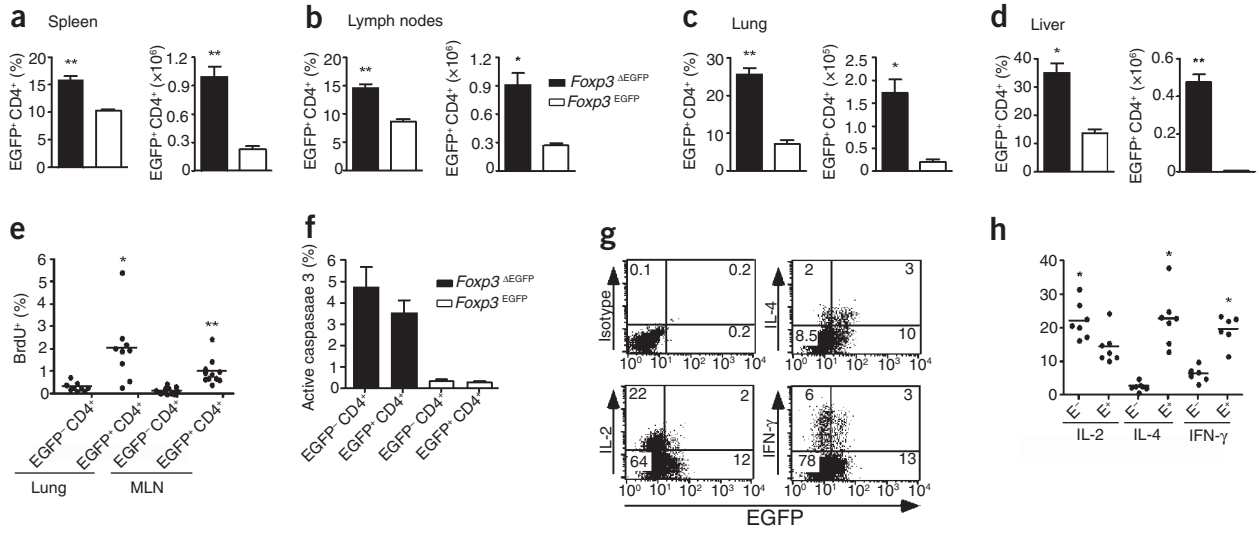

Figure 4 Population expansion of EGFP$^+$ CD4$^+$ T cells from *Foxp3*$^{\Delta EGFP}$ male mice. (**a**–**d**) Frequency and absolute numbers (mean + s.e.m.) of EGFP$^+$ CD4$^+$ T cells in spleen (**a**), lmyph nodes (**b**), lung (**c**) and liver (**d**) of *Foxp3*$^{\Delta EGFP}$ and *Foxp3*^{EGFP} male mice. (**e**) Percent BrdU-labeled cells among EGFP$^+$ and EGFP$^-$ CD4$^+$ T cells in lung and mesenteric lymph nodes (MLN) of *Foxp3*$^{\Delta EGFP}$ male mice. (**f**) Staining for activated caspase 3 (mean + s.e.m.) in EGFP$^+$ and EGFP$^-$ CD4$^+$ T cells freshly isolated from spleens of *Foxp3*$^{\Delta EGFP}$ and *Foxp3*^{EGFP} male mice. (**g**,**h**) Staining (**g**) and percent stained cells (**h**) for lymphocytes isolated from lymph nodes of *Foxp3*$^{\Delta EGFP}$ male mice, then stimulated for 4 h with phorbol 12-myristate 13-acetate plus ionomycin and stained intracellularly with antibodies specific for IFN-γ, IL-2 and IL-4. (**g**) Numbers in quadrants indicate percent cells in each. (**h**) Percent cells staining for IFN-γ, IL-2 and IL-4 among EGFP$^+$ CD4$^+$ cells (E$^+$) and EGFP$^-$ CD4$^+$ cells (E$^-$). In **e**,**h**, small horizontal lines indicate mean; each dot represents one mouse. *, $P < 0.01$; **, $P < 0.0001$. Data represent two to four experiments with three to four mice per group per experiment.

Foxp3$^{\Delta EGFP}$ male mice into SCID (severe combined immunodeficient) mice and assessed proliferation of the adoptively transferred lymphocytes and development of colitis (precipitated by the lack of T_{reg} cells)[31]. Adoptively transferred EGFP$^-$ T cells expanded their populations in SCID hosts, and SCID recipients of EGFP$^-$ T cells developed colitis, as demonstrated by diarrhea, weight loss and pathological findings within 6–8 weeks (**Fig. 6a** and data not shown). SCID recipients of EGFP$^-$ T cells contained, on average, less than 0.5% EGFP$^+$ T cells in the spleen or mesenteric lymph nodes (**Fig. 6b** and data not shown). In contrast, adoptively transferred EGFP$^+$ CD4$^+$ T cells failed to proliferate in SCID hosts, were undetectable in peripheral lymphoid organs and did not cause colitis. Further analysis showed the presence of very few EGFP$^+$ CD4$^+$ T cells in the lungs of SCID recipients (**Fig. 6b**). A few EGFP$^-$ CD4$^+$ T cells were also present in the lung and spleen, probably representing the population expansion of the few contaminating EGFP$^-$ T cells in the transfer population.

To determine if the failure of EGFP$^+$ CD4$^+$ T cells to expand their populations in a lymphopenic environment reflected a requirement for EGFP$^-$ CD4$^+$ T cells in this process, we gave SCID mice EGFP$^-$ CD4$^+$ T cells alone or together with EGFP$^+$ CD4$^+$ T cells at a ratio of 1:0.2. Only mice receiving EGFP$^-$ CD4$^+$ T cells alone or in combination with EGFP$^+$ CD4$^+$ T cells developed colitis (**Fig. 6c**). The latter group had EGFP$^+$ CD4$^+$ T cells in their spleens at ratios that approached the ratio of EGFP$^+$ CD4$^+$ T cells in the transferred population. However, we did not detect EGFP$^+$ CD4$^+$ in mesenteric lymph nodes draining the colitic lesions. We detected a few EGFP$^+$ CD4$^+$ T cells in the lung (**Fig. 6d**). These data collectively indicated that in a lymphopenic environment, EGFP$^-$ CD4$^+$ T cells acted as distinct population that did not freely convert into EGFP$^+$ CD4$^+$ T cells. They also demonstrate a dependence of EGFP$^+$ CD4$^+$ T cells on EGFP$^-$ CD4$^+$ T cells for survival in a lymphopenic environment.

'Genetic signature' of *Foxp3*$^{\Delta EGFP}$ T cells

To determine whether EGFP$^+$ CD4$^+$ T cells in *Foxp3*$^{\Delta EGFP}$ mice shared a common molecular 'signature' with T_{reg} cells, we did a comparative microarray analysis of four different cell populations derived from spleens and lymph nodes of *Foxp3*^{EGFP} and *Foxp3*$^{\Delta EGFP}$ male mice. We prepared total RNA from purified cells, amplified it and used it to generate probes for Affymetrix 430 2.0 chips. We identified the subset of probe sets whose expression increased or decreased by twofold or more in any comparison. We also compared our results with published gene expression profiles of T_{reg} cells and conventional T cells[32].

We first compared the expression profiles of EGFP$^+$ CD4$^+$ cells from *Foxp3*$^{\Delta EGFP}$ and *Foxp3*^{EGFP} mice with those of conventional EGFP$^-$ CD4$^+$ T cells from *Foxp3*^{EGFP} mice. We identified a total of 514 probe sets corresponding to 413 unique genes as being common to EGFP$^+$ CD4$^+$ cells from *Foxp3*$^{\Delta EGFP}$ and *Foxp3*^{EGFP} mice. Of these, 242 genes were overexpressed and 171 were underexpressed relative to their expression in conventional T cells. 'Interrogation' of the probes using published gene array data[32] with the latter set at 1.5- and 2-fold cutoffs showed concordance rates of 72% and 56%, respectively (**Supplementary Table 1** online).

We then compared EGFP$^+$ and EGFP$^-$ CD4$^+$ cells in each mouse and derived a gene set common to both comparison groups. By incorporating into the analysis comparison of EGFP$^+$ and EGFP$^-$ CD4$^+$ cells from *Foxp3*$^{\Delta EGFP}$ mice, the contribution of the generalized T cell activation state in these mice (which involved both cell populations) to the results was minimized, as many of the genes associated with the T_{reg} cell 'genetic signature' were also expressed in activated T cells. With this approach, we identified a total of 163 probe sets corresponding to 133 unique genes as being common to EGFP$^+$ CD4$^+$ T cells in *Foxp3*$^{\Delta EGFP}$ and *Foxp3*^{EGFP} mice (**Supplementary Table 2** online). Of these, 83 genes were overexpressed and 50 were underexpressed relative to their expression in the respective EGFP$^-$ CD4$^+$ cells. 'Interrogation' of the identified probe sets using the published array data[32] with the latter set at 1.5- and 2-fold cutoffs showed concordance rates of 71% and 49%, respectively. These results indicated that a plurality of the genes identified were constituents of a T_{reg} cell 'genetic signature' shared across different data sets.

Examination of the identified common genes resulted in several findings (**Fig. 7**). First, the shared 'genetic signature' of EGFP$^+$ CD4$^+$

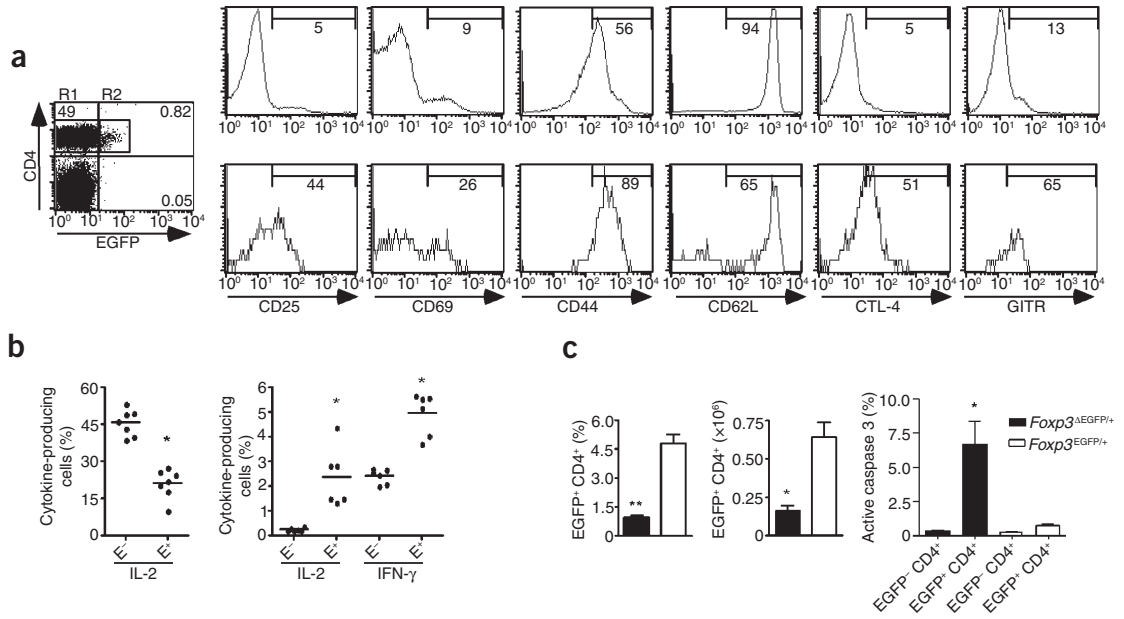

Figure 5 Peripheral depletion of EGFP$^+$ CD4$^+$ T cells in *Foxp3*$^{\Delta EGFP/+}$ female mice. (**a**) Flow cytometry of the expression of CD25, CD69, CD44, CD62L, CTLA-4 and GITR on T cells from lymph nodes of *Foxp3*$^{\Delta EGFP/+}$ female mice. Numbers in quadrants and below bracketed lines indicate percent cells in each gated population. (**b**) Lower production of IL-2 and higher production of IL-4 and IFN-γ by EGFP$^+$ CD4$^+$ T cells in *Foxp3*$^{\Delta EGFP/+}$ female mice. Small horizontal lines indicate mean; each dot represents one mouse. (**c**) Frequency and absolute numbers (mean + s.e.m.) of EGFP$^+$ CD4$^+$ T cells in lymph nodes of *Foxp3*$^{\Delta EGFp/+}$ and *Foxp3*$^{EGFP/+}$ female mice (left and middle), and frequency of activated caspase 3 in EGFP$^+$ and EGFP$^-$ CD4$^+$ T cells freshly isolated from those same lymph nodes (right). *, $P < 0.01$; **, $P < 0.0001$. Data represent two to four experiments with three to four mice per group per experiment.

T cells in *Foxp3*$^{\Delta EGFP}$ and *Foxp3*^{EGFP} mice included most of the genes identified as being characteristic of the T_{reg} cell lineage, including (among others) *Il2ra*, *Ctla4*, *Itgae*, *Cd83*, *Gpr83*, *Gzmb*, *Ikzf2*, *Icos*, *Il1r2*, *Il1rl1*, *Klrg1*, *S100a4*, *S100a6*, *Tiam1*, *Tnfrsf4*, *Tnfrsf9* and *Tnfrsf18* (refs. 32–35; **Fig. 7a** and **Supplementary Table 1**). Examination of the relative expression of each gene in EGFP$^+$ CD4$^+$ T cells from *Foxp3*^{EGFP} and *Foxp3*$^{\Delta EGFP}$ mice suggested that the phenotype of cells from the latter mice was closer to that of CD25^{lo} activated T_{reg} cells (lower expression of *Cd25*, *Gpr83* and *Itgae* and higher expression of *Il1rl1* and *Icos*). The subset of genes that remained overexpressed in EGFP$^+$ CD4$^+$ T cells from *Foxp3*^{EGFP} and *Foxp3*$^{\Delta EGFP}$ mice after the contribution of EGFP$^-$ CD4$^+$ T cells from *Foxp3*^{EGFP} and *Foxp3*$^{\Delta EGFP}$ mice was 'filtered out' (**Supplementary Table 2**) included several canonical markers of the T_{reg} cell 'genetic signature', such as *Ctla4*, *Il2ra*, *Itgae*, *Il10*, *Socs2*, *Traf1*, *Nfil3* and *Nrp1*. Another gene of interest that emerged from the second analysis was *Bhlhb2*; deficiency in this gene is associated with autoimmunity and lymphoproliferation[36]. Expression of genes encoding cell signaling regulators, such as *Dusp4*, and those

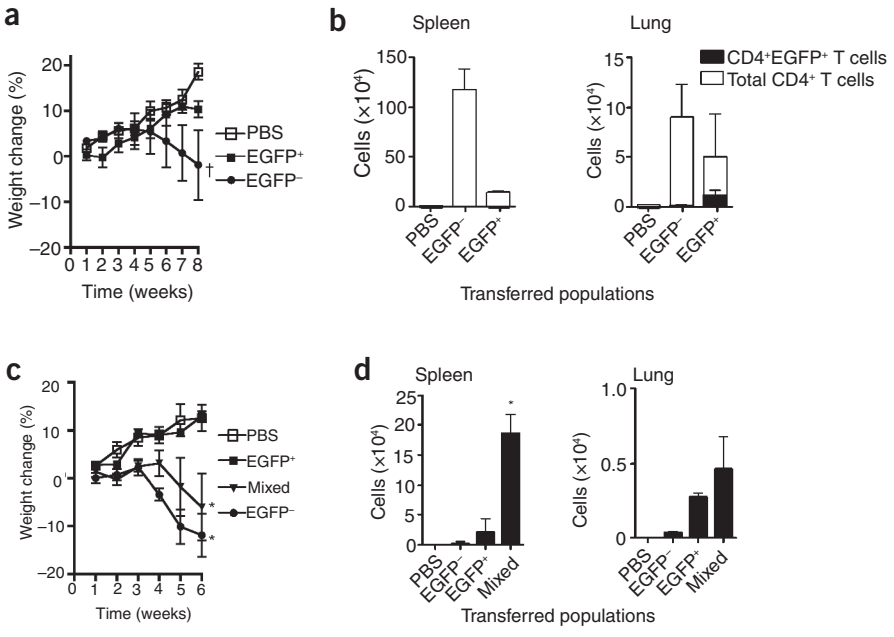

Figure 6 EGFP$^-$ CD4$^+$ T cells from *Foxp3*$^{\Delta EGFP}$ mice do not convert to EGFP$^+$ CD4$^+$ T cells after adoptive transfer into lymphopenic hosts. (**a**,**b**) Weight change (**a**) and cell counts (**b**) after injection of vehicle alone (PBS) or 5×10^5 purified EGFP$^-$ or EGFP$^+$ CD4$^+$ T cells from *Foxp3*$^{\Delta EGFP}$ mice into SCID recipient mice. (**a**) Weight change, tracked over 8 weeks after injection. †, $P = 0.03$, versus PBS group (Mann-Whitney U-test). (**b**) Total (+ s.e.m.) CD4$^+$ T cells or EGFP$^+$ CD4$^+$ T cells in spleens and lungs of recipient mice. Results are pooled from four independent experiments with three to five mice per group per experiment. (**c**,**d**) Weight change (**c**) and cell counts (**d**) after injection of 2×10^5 EGFP$^+$ CD4$^+$ T cells, 1.2×10^6 EGFP$^-$ CD4$^+$ T cells or a combination of 1×10^6 EGFP$^-$ CD4$^+$ plus 2×10^5 EGFP$^+$ CD4$^+$ T cells (Mixed) into SCID host mice; control mice received PBS alone. (**c**) Weight change, tracked over 6 weeks after injection. (**d**) EGFP$^+$ CD4$^+$ T cells (+ s.e.m.) in spleens and lungs of recipient mice. *, $P < 0.01$. Results are representative of two independent experiments with three to five mice per group per experiment.

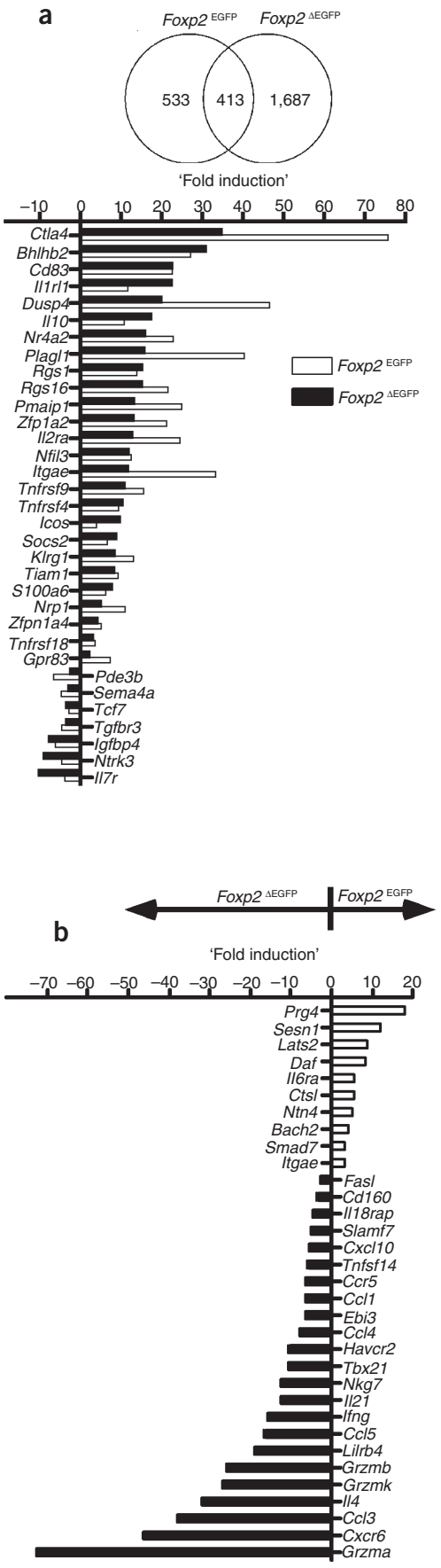

Figure 7 EGFP$^+$ CD4$^+$ T cells from *Foxp3*$^{\Delta EGFP}$ and *Foxp3*^{EGFP} male mice share a common 'genetic signature'. (**a**) Overlap of gene-expression profiles of EGFP$^+$ CD4$^+$ T cells from *Foxp3*$^{\Delta EGFP}$ and *Foxp3*^{EGFP} male mice. Commonly expressed genes are identified by comparison of the expression profiles of EGFP$^+$ CD4$^+$ T cells from *Foxp3*$^{\Delta EGFP}$ and *Foxp3*^{EGFP} male mice with that of EGFP$^-$ CD4$^+$ T cells from *Foxp3*^{EGFP} male mice. Top, comparison groups and number of genes shared. (**b**) Genes differentially expressed in EGFP$^+$ CD4$^+$ T cells from *Foxp3*$^{\Delta EGFP}$ and *Foxp3*^{EGFP} male mice. Data represent mean 'fold change', derived from three independent experiments each representing mRNA pooled from four to ten mice. $P < 0.05$ (Student's unpaired two-tailed T test).

encoding orphan steroid receptors linked the regulation of T cell development, including *Nr4a1*, *Nr4a2* and *Rora*[37], was also enriched in EGFP$^+$ CD4$^+$ T cells from *Foxp3*^{EGFP} and *Foxp3*$^{\Delta EGFP}$ mice. Among the genes underexpressed in EGFP$^+$ CD4$^+$ T cells from *Foxp3*$^{\Delta EGFP}$ and *Foxp3*^{EGFP} mice were *Tcf7*, *Il7r*, *Sema4a*, *Pdlim4* and *Btbd14a* (**Fig. 7a** and **Supplementary Table 1**).

We gained insight into Foxp3-dependent gene expression by comparing the 'genetic signatures' of EGFP$^+$ CD4$^+$ T cells from *Foxp3*^{EGFP} and *Foxp3*$^{\Delta EGFP}$ mice. Unexpectedly, few genes characteristic of the T_{reg} cell 'genetic signature' were overexpressed in EGFP$^+$ CD4$^+$ T cells from *Foxp3*^{EGFP} and *Foxp3*$^{\Delta EGFP}$ mice. More typical was the relative enrichment of transcripts not associated before with the T_{reg} cell 'genetic signature', including those encoding a B cell transcription factor (*Bach2*), p53-regulated PA26 nuclear protein (*Sesn1*), large tumor suppressor (*Lats2*), complement protein–regulator decay-accelerating factor (*Daf*) and an inhibitory regulatory Smad protein (*Smad7*). In contrast, EGFP$^+$ CD4$^+$ T cells from *Foxp3*$^{\Delta EGFP}$ mice overexpressed genes encoding many cell cycle regulators, consistent with their intense proliferation (**Supplementary Table 3** online). There was notable overexpression in EGFP$^+$ CD4$^+$ T cells from *Foxp3*$^{\Delta EGFP}$ mice of genes associated with a cytotoxic effector program, including the granzyme-encoding genes *Gzma*, *Gzmb* and *Gzmk*, *Lilrb4* (encoding the inhibitory receptor gp49b), *Slamf7* (encoding Cracc), *Nkg7*, *Lgals3* (encoding galectin 3) and, with lower expression, *Fasl* (encoding Fas ligand) and *Cd160*. Consistent with such a profile is the overexpression in EGFP$^+$ CD4$^+$ T cells from *Foxp3*$^{\Delta EGFP}$ mice of *Tbx21*, encoding the transcription factor T-bet, a key regulator of T_H1 differentiation and IFN-γ production in CD4$^+$ T cells and the cytotoxic effector programs of CD8$^+$, natural killer and natural killer T cells[38]. EGFP$^+$ CD4$^+$ T cells from *Foxp3*$^{\Delta EGFP}$ mice also overexpressed many genes encoding cytokines, most notably *Il4*, *Ifng* and *Il21* and, in smaller amounts, *Ebi3* (encoding IL-27) and *Tnfsf14* (encoding LIGHT), consistent with our functional data (**Figs. 4**,**5**). EGFP$^+$ CD4$^+$ T cells from *Foxp3*$^{\Delta EGFP}$ mice also had a profile of expression of genes encoding chemokines and chemokines associated with tissue-infiltrating inflammatory T cells; the gene encoding the chemokine receptor CXCR6 (*Cxcr6*), linked to the trafficking of T cells that mediate type 1 inflammation, and genes encoding the inflammatory chemokines CCL1 (*Ccl1*), CCL3 (*Ccl3*), CCL4 (*Ccl4*), and CCL5 (*Ccl5*) were overexpressed in EGFP$^+$ CD4$^+$ T cells from *Foxp3*$^{\Delta EGFP}$ mice[39].

DISCUSSION

Here we have shown that in the absence of functional Foxp3 protein, thymocytes destined to develop into T_{reg} cells proceeded to develop into mature T cells that despite lacking regulatory function shared several characteristics with T_{reg} cells, including similar thymic development, exclusive expression of αβTCRs, 'genetic signature' and cell surface phenotype, and a lack of spontaneous conversion from conventional T cells during homeostatic proliferation. The equal number of EGFP$^+$ cells at the double-positive stage of development in *Foxp3*$^{\Delta EGFP/+}$ and

Foxp3$^{EGFP/+}$ female mice indicated that T_{reg} cell lineage commitment occurred before Foxp3 expression. EGFP$^+$ T cells in both *Foxp3*$^{\Delta EGFP}$ male and *Foxp3*$^{\Delta EGFP/+}$ female mice continued down this developmental pathway and displayed a mature cell surface phenotype characteristic of functional T_{reg} cells. Although Foxp3 may have some function in determining the amount of expression of surface molecules, as suggested by other data, the T_{reg} cell–like phenotype was independent of Foxp3. Thymi of *Foxp3*$^{\Delta EGFP/+}$ and *Foxp3*$^{EGFP/+}$ female mice contained equal numbers of Foxp3$^+$ T cells, indicating that Foxp3 did not directly influence either the positive or negative selection of T_{reg} cells. A few mature T_{reg} cell precursors in the thymi of *Foxp3*$^{\Delta EGFP/+}$ female mice showed signs of apoptosis, and there were many fewer of such cells in the periphery. This suggested that the function of Foxp3 in T_{reg} cell survival is linked to the development of T_{reg} cell effector function. Whether this putative survival function is intrinsic to T_{reg} cells or is the result of Foxp3-induced resistance to other mechanisms of tolerance cannot be determined from our studies.

Some of the attributes that distinguished EGFP$^+$ CD4$^+$ T cells in *Foxp3*$^{\Delta EGFP}$ mice from wild-type T_{reg} cells can be directly linked to loss of Foxp3 function. Prominent among these were a lack of suppressive activity and enhanced T_H1 and T_H2 cytokine production; Foxp3 conveys regulatory activity and suppresses cytokine production after its ectopic expression in conventional T cells[40–42]. However, although T_{reg} cells do not produce IL-2 after activation and Foxp3 suppresses *Il2* transcription after its ectopic expression in T cells, activated EGFP$^+$ CD4$^+$ T cells from *Foxp3*$^{\Delta EGFP}$ male mice had much less IL-2 expression than did EGFP$^-$ CD4$^+$ T cells from the same mice. The idea that this was not due to intense activation and cytokine polarization in the context of a dysregulated immune system was indicated by the finding that EGFP$^+$ CD4$^+$ T cells from *Foxp3*$^{\Delta EGFP/+}$ female mice had a similar propensity for less production of IL-2. These results suggested that lower production of IL-2 may be an attribute of cells committed to the T_{reg} cell lineage that may be further accentuated by Foxp3.

Consistent with the 'bias' of the TCR repertoire of T_{reg} cells toward self-reactivity, proliferating EGFP$^+$ CD4$^+$ T cells in *Foxp3*$^{\Delta EGFP}$ male mice were present in tissues targeted by the autoimmune response, including lung and liver. Although antibody-mediated depletion of T_{reg} cells is associated with the precipitation of autoimmunity, the resultant phenotype is less severe than the lethal autoimmune lymphoproliferative process unleashed by Foxp3 deficiency in mice and humans or by toxin-mediated depletion of T_{reg} cells in mice[1,19–21,43]. Our results would suggest that Foxp3 deficiency results in the emergence of a particularly autoaggressive subset of lymphocytes comprising cells originally destined to develop into T_{reg} cells. However, adoptively transferred EGFP$^+$ CD4$^+$ T cells from *Foxp3*$^{\Delta EGFP}$ mice failed to precipitate disease in the target organs of lymphopenic hosts on their own, indicative of a requirement for additional lymphocytic elements to foment autoimmunity and tissue infiltration.

After transfer into lymphopenic hosts, EGFP$^-$ CD4$^+$ T cells from *Foxp3*$^{\Delta EGFP}$ mice did not appreciably convert to EGFP$^+$ CD4$^+$ T cells during homeostatic proliferation. In addition, homeostatic proliferation of transferred EGFP$^+$ CD4$^+$ T cells occurred only in the presence of cotransferred EGFP$^-$ CD4$^+$ T cells. Notably, the population expansion of T_{reg} cells in a lymphopenic environment is also dependent on conventional T cells, reflecting a potential requirement for conventional T cell–derived IL-2 (ref. 44). In contrast to their greater production of IL-4 and IFN-γ, EGFP$^+$ CD4$^+$ T cells from *Foxp3*$^{\Delta EGFP}$ mice were less effective in their production of IL-2, a phenotype also shared by T_{reg} cells. IL-7, a cytokine critical for effective homeostatic proliferation of CD4$^+$ T cells, is dispensable for T_{reg} cell homeostatic population expansion, as the latter expand their populations normally in IL-7-deficient hosts[45]. Consistent with this observation was the finding that EGFP$^+$ CD4$^+$ T cells from *Foxp3*$^{\Delta EGFP}$ mice had very low expression of IL-7 receptor-α.

Gene-expression-profile analysis showed that most transcripts associated with the T_{reg} cell 'genetic signature' were present in EGFP$^+$ CD4$^+$ T cells expressing the nonfunctional Foxp3-EGFP fusion protein; these data indicated that expression of such transcripts and, by extension, the T_{reg} genetic program are mostly independent of functional Foxp3 protein. Some T_{reg} cell–associated transcripts, including *Itgae*, had higher expression in T_{reg} cells than in EGFP$^+$ CD4$^+$ T cells from *Foxp3*$^{\Delta EGFP}$ male mice, suggesting that Foxp3 may upregulate the expression of these transcripts. In contrast, many genes involved in cell cycle regulation and in proinflammatory and cytotoxic T cell activity were overexpressed in EGFP$^+$ CD4$^+$ T cells from *Foxp3*$^{\Delta EGFP}$ male mice. This profile can be interpreted as being a further differentiated form of an already existent cytotoxic function of T_{reg} cells that is necessary for their suppressor activity[46–48].

Finally, the development and disposition in normal hosts of T_{reg} cell precursors that fail for some reason to upregulate Foxp3 expression in the thymus is an issue of inherent relevance to the pathogenesis of autoimmunity. The lack of disease in *Foxp3*$^{\Delta EGFP/+}$ female mice or mothers of patients with IPEX syndrome has been interpreted to reflect the capacity of functional T_{reg} cells in such hosts to mediate dominant tolerance[11,13–15]. Our findings extend the understanding of tolerance in carrier females by showing that the periphery is depleted of autoreactive T_{reg} cell precursors with mutated *Foxp3* alleles. Whether such cells could develop and persist in certain conditions and contribute to autoimmune disease requires further investigation.

METHODS

Mice. *Foxp3*^{EGFP} and *Foxp3*^{K276X} mice were generated as described[12,25]. For the generation of *Foxp3*$^{\Delta EGFP}$ mice, *Foxp3* genomic DNA was isolated from a bacterial artificial chromosome clone (Genome Systems) and was subcloned into the plasmid vector pKO (Lexicon). An *Hpa*I restriction site was created by site-directed mutagenesis of codons 396 and 397 in exon 11 of *Foxp3* (from GTGCGA to GTTAAC). EGFP cDNA followed by a stop codon was inserted in-frame after codon 396 (which continued to encode valine) by blunt-ended ligation at the newly created *Hpa*I site. A phosphoglycerate kinase–neomycin-resistance (*PGK-neo*^{r}) cassette was also inserted at the *Eco*RI site in intron 9 of *Foxp3* in the same orientation as *Foxp3* and was flanked by two *lox*P sites to allow excision by Cre-mediated recombination. The targeting construct also included a diphtheria toxin gene for negative selection against randomly inserted targeting constructs. Targeting plasmids were introduced by electroporation into SCC10 embryonic stem cells, which were subjected to G418 selection. Resistant clones were screened by Southern blot analysis and successfully targeted clones were injected into C57BL/6 blastocysts. Chimeric males were mated with 'Cre-deleter' female mice on the BALB/c background that had an allele with constitutive, ubiquitous expression of Cre recombinase[49]. N_1 female mice heterozygous for the *Foxp3*$^{\Delta EGFP}$ allele (minus the *PGK-neo*^{r} cassette) were identified by genotyping. All Foxp3 mutant mouse strains were backcrossed for eight to twelve generations onto the BALB/c background. Mice were housed in specific pathogen–free conditions and were used according to the guidelines of the institutional Animal Research Committees of the University of California in Los Angeles and the Medical College of Wisconsin.

PCR analysis. Screening of the *Foxp3*$^{\Delta EGFP}$ and *Foxp3*^{EGFP} alleles was achieved by PCR amplification with the following EGFP-specific sense primers: forward, 5′-CGGCAAGCTGACCCTGAAGT-3′; reverse, 5′-GGATGTTGCCGTCCTCCTTG-3′. PCR-based discrimination of wild-type and mutated alleles was accomplished by analysis of the presence of residual *lox*P sequence retained in the respective allele after Cre-mediated excision of the *PGK-neo*^{r} cassette. Primers used were 5′-GCGTAAGCAGGGCAATAGAGG-3′ (sense) and 5′-GCATGAGGTCAAGGGTGATG-3′ (antisense). Real-time PCR for *Foxp3* expression was done as described[12]. *Foxp3* expression was normalized to that of the gene encoding hypoxanthine guanine phosphoribosyl transferase.

Flow cytometry. Single-cell suspensions were stained with fluorochrome-conjugated antibodies in PBS containing 5% (wt/vol) BSA (Sigma-Aldrich) and Fc-block (BD Pharmingen), were fixed in 1% (wt/vol) paraformaldehyde and were analyzed on a FACSCalibur or LSRII cytometer (Becton Dickinson). Monoclonal antibodies to the following were obtained from BD Pharmingen: TCRβ (H57), CD3 (2C11), CD4 (L3T4), CD8 (53-6.7), CD11c (HL3), CD25 (PC61), CD28 (37.51), CD62L (MEL14), I-A^d (AMS-32.1), CTLA4 (UC10-4F10-11), CD69 (7E.17G9), GITR (DTA-1), CD103 (M290) and CD45 (2D1), as well as annexin V and anti-BrdU. Intracellular Foxp3 staining was done as described with phycoerythrin-conjugated anti-mouse Foxp3 (ref. 12; clone FJK-16s; eBioscience). Apoptosis was analyzed by intracellular staining with annexin V plus 7-aminoactinomycin D (BD Biosciences) or with activated caspase 3 (BD Biosciences). For intracellular cytokine staining, lymphocytes were activated for 4 h with phorbol 12-myristate 13-acetate (20 ng/ml) and ionomycin (1 μg/ml) in the presence of brefeldin A (BD Pharmingen). Cells were collected and were processed for intracellular cytokine staining with the Cytofix/Cytoperm kit (BD Pharmingen) according to the manufacturer's instructions. For isolation of $EGFP^+$ $CD4^+$ T cells, suspensions of splenic and lymph node lymphocytes were first enriched for $CD4^+$ T cells by magnetic-activated cell sorting (Miltenyi Biotec) or on mouse CD4 columns (R&D Systems). The enriched populations were labeled with phycoerythrin-conjugated monoclonal anti-CD4 and cells were sorted into $EGFP^+$ and $EGFP^-$ $CD4^+$ populations with a FACSAria or FACSVantage SE cell sorter (Becton Dickinson). For assessment of T cell proliferation by incorporation of BrdU, 15-day-old *Foxp3*$^{\Delta EGFP}$ male mice were injected intraperitoneally with 1 mg BrdU. Tissues were collected 2 h after injection and lymphocytes were stained intracellularly with anti-EGFP, anti-CD4 and anti-BrdU.

Suppression assays. Purified splenocytes were cultured in 96-well round-bottomed plates in the presence of 1 μg/ml of anti-CD3. The number of responder splenocytes was kept constant at 8×10^4 cells per well, whereas the number of suppressor cells was 'titrated' to achieve various ratios. Cultures were maintained for 48 h, then were pulsed with 0.4 μCi/well of [^{3}H]thymidine for an additional 18 h, were collected onto fiber filtermats with a Micro96 collecter (Skatron) and were counted.

Immunocytochemistry. Thymic sections were stained as described[25]. Sections were visualized by laser-scanning confocal microscopy with a Leica TCS single-positive II microscope equipped with 488-, 568- and 633-nm lasers. Images were collected with Leica acquisition software. Antibodies and emissions collected were as follows: Alexa Fluor 488–conjugated rabbit anti-GFP IgG fraction (Molecular Probes), collected at 500–545 nm; biotinylated monoclonal anti-I-A^d with streptavidin–Alexa Fluor 568 (Molecular Probes), collected at 615–700 nm; and allophycocyanin-conjugated monoclonal anti-CD4, collected at 650–740 nm.

Gene-expression profiling. $CD4^+$ T cell populations from age-matched *Foxp3*$^{\Delta EGFP}$ and *Foxp3*^{EGFP} male mice (four to ten mice per experiment) were purified by magnetic bead sorting (Miltenyi), and the respective $EGFP^+$ and $EGFP^-$ $CD4^+$ populations were further purified by flow cytometry. Total RNA was isolated with the RNeasy Mini Kit (Qiagen). Aliquots of 2 μg were subjected to single-round probe amplification with the Affymetrix Small Sample Labeling Protocol II. Amplified probes were fragmented and were applied to Affymetrix GeneChip Mouse Genome 430 2.0 arrays. For analysis of gene-expression measures, all Affymetrix data were normalized with the justRMA algorithm from the Bioconductor group[50]. Pairwise comparisons were made for all experimental conditions such that probe sets with a change in expression of at least a twofold were 'selected out' for further analysis. For comparative analysis of gene-expression profiles (**Fig. 7b**), results represent mean 'fold change' values derived from three independent arrays for the cell type, each representing mRNA pooled from four to ten mice, with statistical analysis by Student's unpaired two-tailed *t*-test ($P < 0.05$). Target expression was confirmed by flow cytometry and/or real time PCR. Because the gene-array probes for *Foxp3* are 'distal' to the poly(A) start site of the EGFP cassette in the *Foxp3*^{EGFP} allele, a false-negative result is generated for *Foxp3* in *Foxp3*^{EGFP} T cells.

Accession code. GEO: microarray data, GSE6875.

Note: Supplementary information is available on the Nature Immunology website.

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COMPETING INTERESTS STATEMENT

The authors declare no that they have competing financial interests.

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