

Foxp3 programs the development and function of CD4⁺CD25⁺ regulatory T cells

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CD4⁺CD25⁺ regulatory T cells are essential for the active suppression of autoimmunity. Here we report that the forkhead transcription factor Foxp3 is specifically expressed in CD4⁺CD25⁺ regulatory T cells and is required for their development. The lethal autoimmune syndrome observed in Foxp3-mutant scurfy mice and Foxp3-null mice results from a CD4⁺CD25⁺ regulatory T cell deficiency and not from a cell-intrinsic defect of CD4⁺CD25⁻ T cells. CD4⁺CD25⁺ regulatory T cells rescue disease development and preferentially expand when transferred into neonatal Foxp3-deficient mice. Furthermore, ectopic expression of Foxp3 confers suppressor function on peripheral CD4⁺CD25⁻ T cells. Thus, Foxp3 is a critical regulator of CD4⁺CD25⁺ regulatory T cell development and function.

The adaptive immune system of higher vertebrates protects organisms from an ever-evolving array of pathogenic microorganisms, while a complex system of thymic and peripheral mechanisms maintain immune tolerance to self. These mechanisms include the active, suppressive function of CD4⁺CD25⁺ regulatory T cells^{1–3}. Lymphopenic mice reconstituted with CD4⁺CD25⁻ T cells develop severe autoimmunity characterized by lymphoproliferation and autoimmune infiltration in multiple organs, whereas cotransfer of CD4⁺CD25⁺ T cells provides long-lived protection from autoimmunity⁴. CD4⁺CD25⁺ T cells do not proliferate following agonistic T cell receptor (TCR) ligation, however they are capable of rapid expansion in lymphopenic host animals⁵. This 'homeostatic proliferation' enhances rather than abolishes *in vitro* suppressor function of CD4⁺CD25⁺ regulatory T cells. These findings suggest that CD4⁺CD25⁺ regulatory T cells maintain a heritable genetic program to function as suppressor T cells. Given their importance in preventing the development of autoimmunity and their therapeutic potential, the molecular mechanisms governing CD4⁺CD25⁺ regulatory T cell development and function are of great interest.

Identification of a mutation in the forkhead/winged helix transcription factor gene *Foxp3* as the defect in the mutant mouse strain scurfy (*sf*) provided an important lead into possible genetic mechanisms underlying the regulation of immune homeostasis⁶. A 2-bp frameshift insertion in the X chromosome gene *Foxp3* results in a truncated gene product lacking the C-terminal forkhead domain. Male mice hemizygous for the X-linked *Foxp3*^{sf} mutation succumb to a CD4⁺ T cell-mediated, lymphoproliferative disease characterized by wasting and multi-organ lymphocytic infiltrates^{7–10}. Similarly, the aggressive, fatal, autoimmune human syndrome X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy (IPEX) has been mapped to multiple mutations in the human homolog of *Foxp3*^{11–13}. In mice, the disease can be transplanted

to T cell-deficient hosts upon transfer of CD4⁺ T cells⁹. Notably, heterozygous female *Foxp3*^{+/sf} carriers display random X chromosome inactivation, yet do not develop lymphoproliferative autoimmunity^{14,15}. We found this phenotype to be consistent with a possible defect in CD4⁺CD25⁺ regulatory T cell development and/or function. Here we explored the role of Foxp3 in development and function of CD4⁺CD25⁺ regulatory T cells. These studies showed Foxp3 to be a unique marker of CD4⁺CD25⁺ regulatory T cells, distinguishing them from activated CD4⁺CD25⁻ T cells. We generated *Foxp3*^{-/-} mice and demonstrated that Foxp3 is specifically required for the thymic development of CD4⁺CD25⁺ regulatory T cells. Additionally, our results indicated that the lethal lymphoproliferative autoimmune syndrome observed in Foxp3-deficient mice results from a deficiency in CD4⁺CD25⁺ regulatory T cells and not from a cell-intrinsic defect of CD4⁺CD25⁻ T cells. Moreover, ectopic Foxp3 expression was sufficient to activate a program of suppressor function in peripheral CD4⁺CD25⁻ T cells.

Results

Regulatory T cells specifically express Foxp3

The study of CD4⁺CD25⁺ regulatory T cells has been hampered by a lack of molecular markers unique to this T cell lineage. Because Foxp3 may represent such a marker, we examined the levels of Foxp3 in CD4⁺CD25⁺ regulatory T cells. Real-time quantitative polymerase chain reaction (PCR) analysis revealed that *Foxp3* mRNA levels were increased ~40-fold in freshly isolated CD4⁺CD25⁺ T cells when compared with CD4⁺CD25⁻ T cells (**Fig. 1a**). Notably, *Foxp3* mRNA was not upregulated upon *in vitro* activation of CD4⁺CD25⁻ T cells. Because many genes are also regulated at the post-transcriptional level, we examined Foxp3 protein expression by western blot analysis. Foxp3 protein was highly expressed in CD4⁺CD25⁺ regulatory T cells and was

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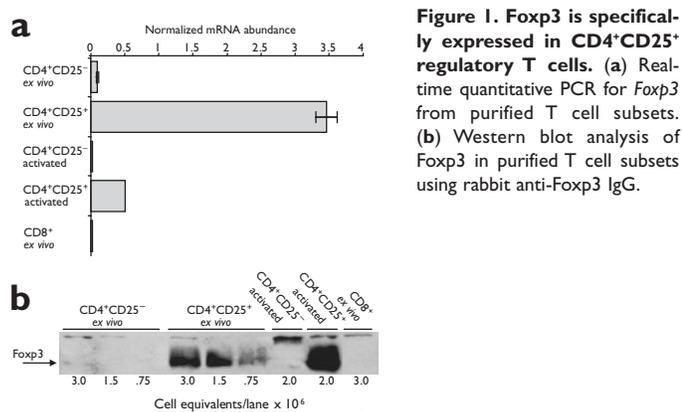


Figure 1. Fxop3 is specifically expressed in CD4⁺CD25⁺ regulatory T cells. (a) Real-time quantitative PCR for *Foxp3* from purified T cell subsets. (b) Western blot analysis of *Foxp3* in purified T cell subsets using rabbit anti-*Foxp3* IgG.

virtually undetectable in both resting and activated CD4⁺CD25⁺ T cells (Fig. 1b). Furthermore, *Foxp3* protein expression was increased upon *in vitro* activation of CD4⁺CD25⁺ regulatory T cells. These results identify *Foxp3* as a molecular marker unique to CD4⁺CD25⁺ regulatory T cells, and suggest a functional role for this molecule in these cells.

Targeted deletion of *Foxp3*

To better characterize the role of *Foxp3* in CD4⁺CD25⁺ regulatory T cell development and function, we used homologous recombination to generate mice with a *Foxp3* allele flanked by *loxP* sites (floxed (lox); Fig. 2a). *Foxp3^{lox}* mice developed normally and showed no signs of autoimmunity (data not shown). Excision of the floxed allele by crossing female *Foxp3^{lox}* carriers to a Cre-deleter transgenic strain produced mice with a deleted *Foxp3* allele (*Foxp3⁻*; Figs. 2b,c,d). Male *Foxp3⁻* mice succumb to an aggressive lymphoproliferative autoimmune syndrome virtually

identical to that of the *Foxp3^{sf}* mutant. Overt signs of disease appear around day 12 and manifest as runting as well as scaly and inflamed skin on the ears and tail. Mice become moribund at approximately 4 weeks of age. Pathological examination revealed extremely enlarged spleen and lymph nodes and lymphocytic infiltrates in multiple organs (Fig. 2e and data not shown). CD4⁺ T cells displayed an activated phenotype, showing increased CD69, CD44, CTLA-4 and GITR expression along with decreased expression of CD62L when compared with wild-type littermate controls (*Foxp3⁺*; Fig. 2f).

Foxp3⁻ mice lack regulatory T cells

Next, we examined male *Foxp3⁻* mice for the presence of CD4⁺CD25⁺ regulatory T cells. Analysis of lymph node cells from 28-day-old mice by flow cytometry revealed a CD4⁺ T cell population with a wide range of CD25 expression (Fig. 3a). This expression pattern was indicative of activated CD4⁺ T cells and was distinct from the discreet CD25⁺CD4^{lo} pattern observed in *Foxp3⁺* mice, which is characteristic of the CD4⁺CD25⁺ regulatory T cell population. To functionally distinguish these CD25⁺ populations, we purified the CD25⁺CD4⁺ T cells from *Foxp3⁻* and *Foxp3⁺* mice and measured their proliferative and suppressive function. Consistent with the phenotype of regulatory T cells, the CD4⁺CD25⁺ T cells from *Foxp3⁺* mice were hyporesponsive to TCR stimulation and suppressed the proliferation of wild-type CD4⁺CD25⁺ responder T cells (Fig. 3b). CD25⁺CD4⁺ T cells from *Foxp3⁻* mice proliferated and showed no suppressor activity. Because the lymphoproliferative disease state of these mice might obscure detection of a CD4⁺CD25⁺ regulatory T cell population, we compared 10-day-old *Foxp3⁻* to *Foxp3⁺* mice. At this age, mice showed no external signs of disease, but exhibited slightly enlarged lymph nodes (data not shown). In contrast to the 28-day-old mice, analysis revealed a diminished CD25⁺CD4⁺ T cell population in both the lymph node and

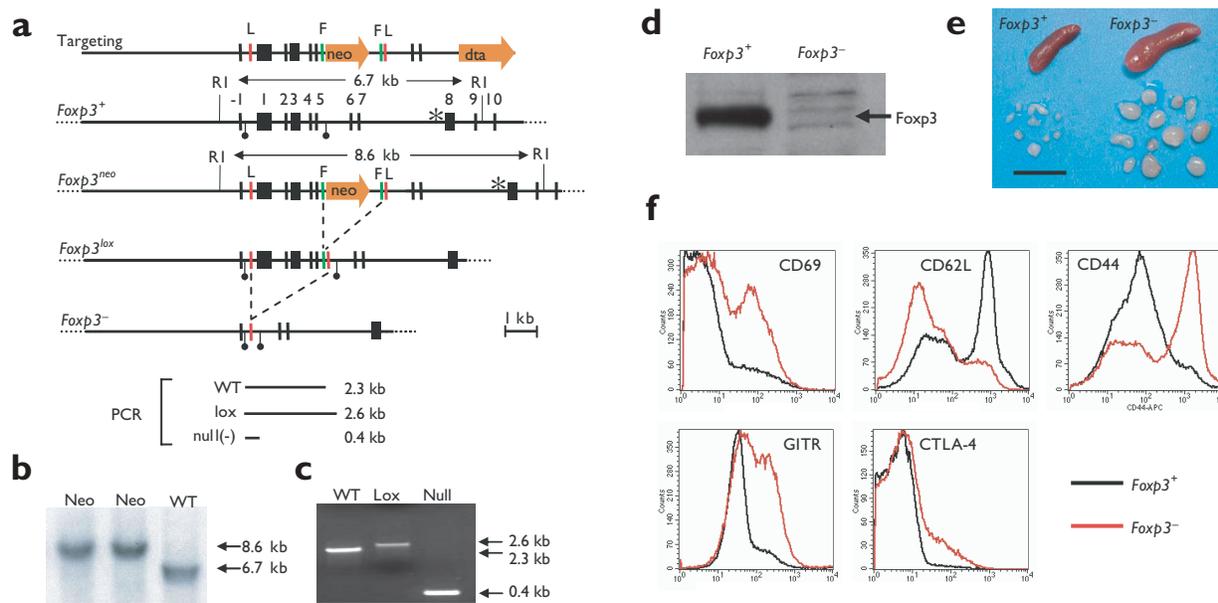


Figure 2. Generation and analysis of *Foxp3* conditional mutant mice. (a) Diagrams of the *Foxp3* targeting construct, the genomic locus (*Foxp3⁺*) and the neomycin-containing (*Foxp3^{neo}*), the *loxP*-flanked (*Foxp3^{lox}*) and the null (*Foxp3⁻*) *Foxp3* alleles. Filled black boxes, exons; red boxes, *loxP* (L) sites; green boxes, FRT (F) sites; orange arrows, sequences for the neomycin gene (neo) and diphtheria toxin (dta). The asterisk marks the location of the probe, and 'RI' marks the *EcoRI* sites used for Southern blot analysis. Filled circles mark the location of PCR primer binding sites. (b) Southern blot analysis of *EcoRI*-digested genomic DNA from the two injected ES cell clones (neo) and a wild-type ES cell clone (wt). (c) PCR of genomic tail DNA from male wild-type (wt), *loxP*-flanked (lox) and null mice. (d) Western blot analysis of *Foxp3* in purified CD4⁺ T cells and (e) spleens and lymph nodes, at 21 d of age from littermate male *Foxp3⁺* and *Foxp3⁻*. Black bar, 1 cm. (f) FACS analysis of CD4⁺ T cells from 28-day-old *Foxp3⁻* and littermate *Foxp3⁺* male mice. Plots are gated on CD4⁺ cells and are representative of >5 mice analyzed.

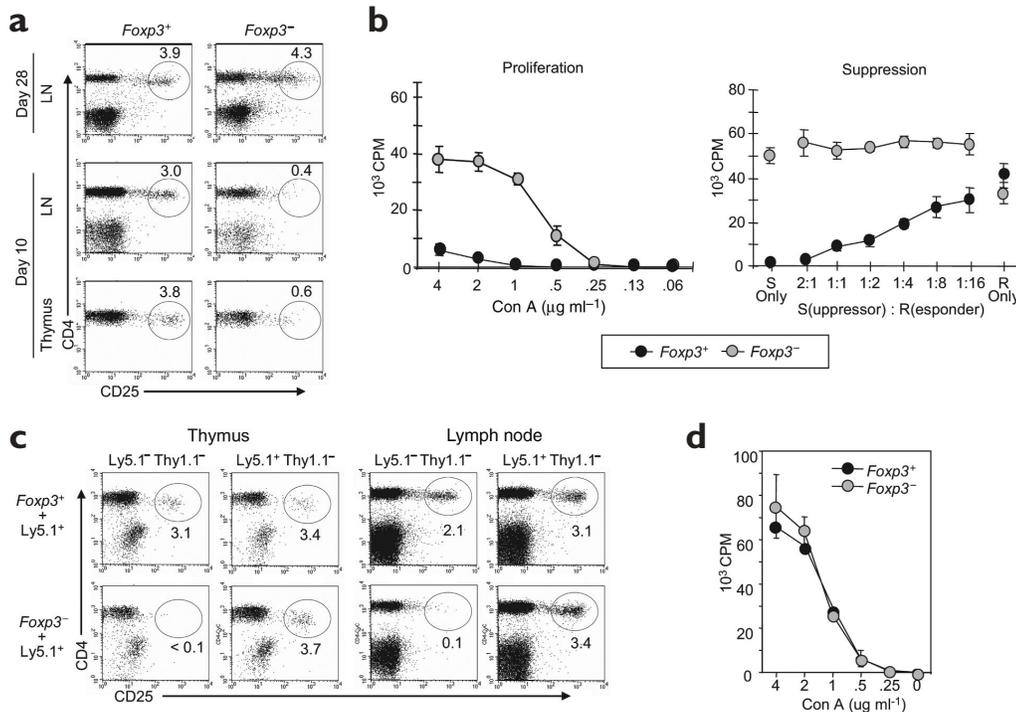


Figure 3. Foxp3 is required for development of CD4⁺CD25⁺ regulatory T cells and does not affect CD4⁺CD25⁻ T cell proliferation. (a) FACS analysis of lymph node cells and thymocytes from male *Foxp3*^{-/-} and littermate *Foxp3*^{+/+} mice. Lymph node cell plots are gated on CD4⁺CD25⁺ cells. (b) Proliferation and suppression by CD4⁺CD25⁺ T cells from 28-day-old male *Foxp3*^{-/-} and *Foxp3*^{+/+} mice. (c) Analysis of lymph node cells and thymocytes from mixed BM chimeras. Lymph node cell plots are gated on TCR β ⁺ cells. Thymocyte plots are gated on CD24^{lo} cells. (d) Proliferation of CD4⁺CD25⁺ T cells from mixed BM chimeras. FACS analysis is representative of two experiments with four mice per group.

thymus of 10-day-old *Foxp3*^{-/-} mice (0.4% and 0.6%, respectively, compared with 3.0% and 3.8%; **Fig. 3a**). Consistent with a loss of Foxp3 function in mutant *Foxp3*^{sf} mice, CD4⁺ T cells from these mice yielded identical results in all of the above experiments (data not shown). These data support a role for Foxp3 in the development of CD4⁺CD25⁺ regulatory T cells.

Regulatory T cell development requires Foxp3

To definitively address the requirement of Foxp3 in CD4⁺CD25⁺ regulatory T cell development, we generated mixed bone marrow (BM) chimeric mice by reconstituting lethally irradiated (1,000 rads) C57BL/6 (B6) Thy1.1⁺ congenic mice with T cell-depleted BM from congenic B6 Ly5.1⁺ mice mixed at a 1:1 ratio with BM from either

Foxp3^{-/-} or *Foxp3*^{+/+} mice. Six weeks after BM reconstitution, mixed BM chimeras were analyzed and showed no phenotypic or pathological signs of lymphoproliferative disease. The CD4⁺CD25⁺ regulatory T cell population in the [Ly5.1⁺B6 + *Foxp3*^{-/-}] chimeras was solely of Ly5.1⁺B6 origin in both the thymus and lymph nodes (**Fig. 3c**). In contrast, both Ly5.1⁺B6 and *Foxp3*^{+/+} BMs contributed equally to the CD4⁺CD25⁺ regulatory T cell compartment in the [Ly5.1⁺B6 + *Foxp3*^{+/+}] chimeras. This result provides clear and direct evidence of a requirement for Foxp3 in the development of CD4⁺CD25⁺ regulatory T cells.

Based on the increased proliferative response of CD4⁺ T cells isolated from *Foxp3*^{sf} mice, it has been suggested that the scurfy autoimmune pathology arises from a cell-intrinsic defect in the control of CD4⁺ T cell proliferation^{10,15}. This implies a role for Foxp3 in CD4⁺CD25⁺ T cell

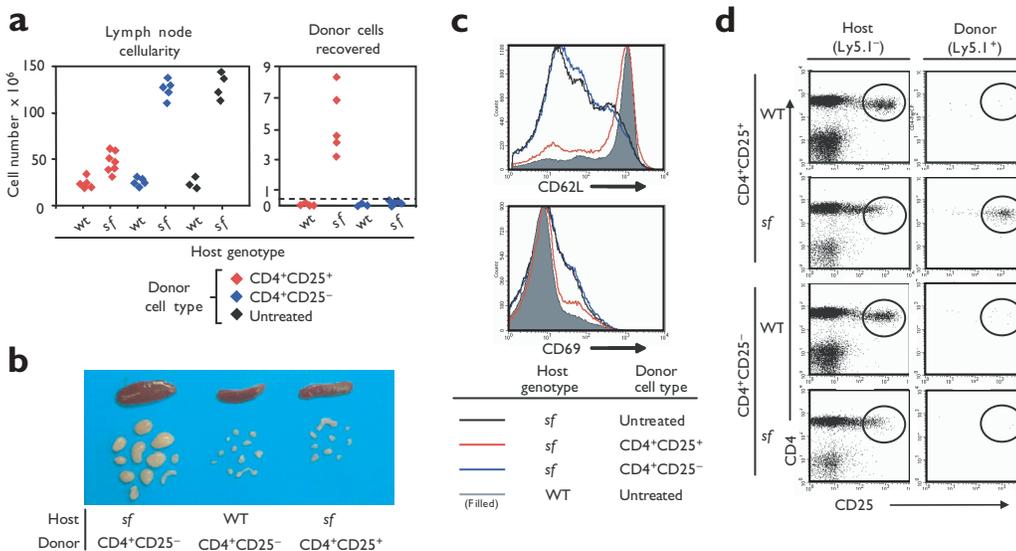


Figure 4. Neonatal transfer of CD4⁺CD25⁺ regulatory T cells rescues the lymphoproliferative syndrome in Foxp3-deficient mice. (a) Lymph node cellularity and total donor cells recovered from 21-day-old mutant and wild-type male mice. (b) Spleen and lymph nodes from 21-day-old male recipient mice. (c) Flow cytometric analysis of activation markers expressed by host CD4⁺ T cells from recipient male mice. Histograms are gated on CD4⁺Ly5.1⁺ cells. (d) Flow cytometric analysis of host- and donor-derived T cell populations. Plots are gated on TCR β ⁺ cells. All data are representative of at least four mice per experimental condition.

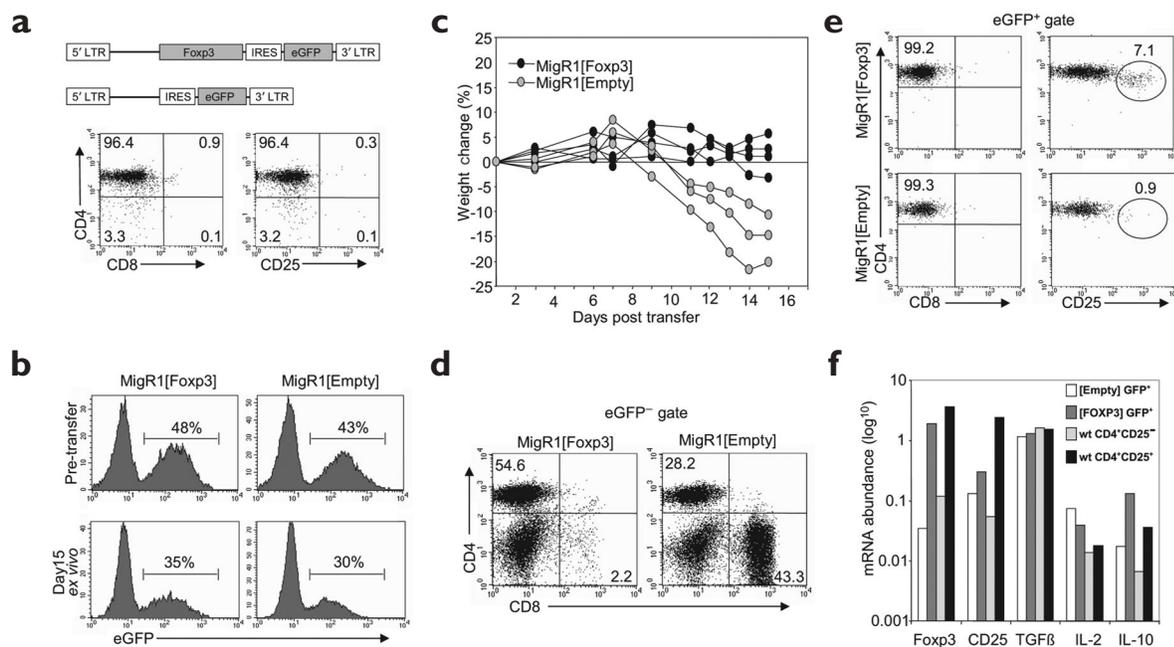


Figure 5. Expression of Foxp3 confers suppressor phenotype and function upon peripheral CD4⁺CD25⁻ T cells. (a) MSCV MigR1 retroviral constructs and representative CD4⁺CD25⁻ T cell population before infection: MigR1 [Foxp3] (top), and MigR1 [empty] (bottom). (b) Retroviral transduction efficiency of transferred cell populations. (c) Suppression of wasting disease and colitis by Foxp3-transduced cells. Percent weight change of RAG1^{-/-} mice injected with retrovirally transduced cell populations. (d) Suppression of CD8⁺ homeostatic proliferation by Foxp3 transduced cells. FACS analysis of untransduced (eGFP⁺) cells upon reisolation from host mice. Plots are gated on GFP⁺ population. (e) Phenotypic characterization of retrovirally transduced cells upon reisolation from host mice. Plots are gated on eGFP⁺ cells. (f) Real-time quantitative PCR analysis of gene expression in sorted retrovirally transduced cell populations, wild-type CD4⁺CD25⁻ and CD4⁺CD25⁺ T cells.

function. However, our analysis of CD4⁺CD25⁻ T cells revealed virtually no Foxp3 expression in these cells. To address a potential role for low Foxp3 expression in CD4⁺CD25⁻ T cells, we compared the proliferative response of purified Ly5.1⁺Thy1.1⁺CD4⁺CD25⁻ T cells from the [Ly5.1⁺B6 + Foxp3⁻] and [Ly5.1⁺B6 + Foxp3⁺] BM chimeric mice. The mixed BM chimeras afforded the analysis of Foxp3-deficient CD4⁺CD25⁻ T cells from otherwise healthy mice. Foxp3⁻ and Foxp3⁺ CD4⁺CD25⁻ T cells showed no significant difference in their proliferative response to *in vitro* TCR ligation (Fig. 3d). This result is consistent with a unique role for Foxp3 in CD4⁺CD25⁺ regulatory T cells.

Regulatory T cells rescue Foxp3 deficiency

Because Foxp3 is required for CD4⁺CD25⁺ regulatory T cell development, we reasoned that transfer of CD4⁺CD25⁺ regulatory T cells into newborn Foxp3-deficient mice would rescue the lymphoproliferative disorder observed in these mice. To test this hypothesis, we intraperitoneally (i.p.) injected 4 × 10⁵ Ly5.1⁺B6 CD4⁺CD25⁺ or CD4⁺CD25⁻ T cells into 1- to 2-day-old male mice derived from breeding female B6 Foxp3^{+/-} carriers with B6 males. Use of B6 Foxp3^{+/+} mice allowed us to circumvent potential problems of histoincompatibility arising from the mixed background of the Foxp3^{-/-} mice. Injected mice were monitored for disease development and analyzed at 21 d of age. All Foxp3^{+/+} mice injected with CD4⁺CD25⁻ T cells developed clinical and pathological signs of disease with identical kinetics to uninjected Foxp3^{+/+} controls (Fig. 4a,b and data not shown). In contrast, Foxp3^{+/+} mice receiving CD4⁺CD25⁺ regulatory T cells remained virtually disease-free as determined by gross phenotype and lymph node cellularity.

Analysis of lymphocytes by flow cytometry revealed increased numbers of activated CD69⁺CD62L^{lo} host-derived CD4⁺ T cells in both untreated and CD4⁺CD25⁻ injected Foxp3^{+/+} animals (Fig. 4c). By

contrast, CD4⁺ T cells from Foxp3^{+/+} mice receiving CD4⁺CD25⁺ regulatory T cells expressed CD69 and CD62L at amounts comparable to those of untreated Foxp3^{+/+} mice. Analysis of donor cell engraftment revealed an expansion of CD4⁺CD25⁺ regulatory T cells transferred into Foxp3^{+/+} neonates, but not Foxp3^{+/+} controls. In contrast, CD4⁺CD25⁻ cells showed limited engraftment in both Foxp3^{+/+} and Foxp3^{+/+} hosts (Figs. 4a,d). This observation suggests the existence of a homeostatic niche available for CD4⁺CD25⁺ regulatory T cells in Foxp3^{+/+} mice despite an otherwise full T cell compartment. These data demonstrate that the lymphoproliferative disease in Foxp3-deficient mice is due to lack of CD4⁺CD25⁺ regulatory T cells. This conclusion is further supported by the ability of CD4⁺CD25⁺ regulatory T cells to suppress pathogenic Foxp3^{+/+} CD4⁺ T cells upon cotransfer into lymphopenic hosts. Transfer of 1 × 10⁶ Foxp3^{+/+} CD4⁺ T cells into RAG^{-/-} hosts induced severe wasting disease and colitis in all recipient mice (Supplementary Fig. 1 and data not shown). Cotransfer of 5 × 10⁵ CD4⁺CD25⁺ regulatory T cells prevented the development of wasting disease and colitis based on clinical symptoms and histopathological analysis. In contrast, CD4⁺CD25⁻ T cells were unable to prevent disease development. These results suggest that Foxp3 controls a genetic program of suppressor function in CD4⁺ T cells.

Ectopic Foxp3 expression confers suppressor function

To test whether Foxp3 can induce suppressor function in CD4⁺CD25⁻ T cells, we analyzed the ability of these cells to gain suppressor function upon retroviral-mediated ectopic expression of Foxp3. Purified CD4⁺CD25⁻ T cells were transduced with the MSCV MigR1 retrovirus expressing Foxp3 and a green fluorescent protein (GFP) reporter (MigR1[FoxP3]) or only GFP (MigR1[empty]; Fig. 5a). At 72 h post-infection, RAG^{-/-} mice were injected intravenously (i.v.) with 5 × 10⁶

T cells containing either MigR1[FoxP3]- or MigR1[empty]-transduced cell populations (Fig. 5b). Each group of mice was monitored for development of wasting disease. As expected, mice receiving the T cell population containing MigR1[empty]-transduced T cells developed severe wasting disease with associated colitis as measured by weight loss and organ pathology (Fig. 5c and data not shown). Mice that received the T cell population containing MigR1[Foxp3]-transduced cells displayed no signs of disease for the duration of the experiment. At day 16, pathological analysis revealed severe colitis in all mice that received T cells containing the MigR1[empty]-transduced cells, whereas mice that received the population including MigR1[Foxp3]-transduced T cells were indistinguishable from untreated mice. Furthermore, analysis of the untransduced T cell population revealed an expansion of the few contaminating CD8⁺ donor T cells in the presence of control MigR1[empty]-transduced T cells, whereas homeostatic expansion of CD8 T cells was completely blocked in the presence of MigR1[Foxp3]-transduced T cells (20–48% versus 1.6–2.8% of total lymphoid cells, respectively; Fig. 5d). This observation is consistent with a recent report of CD4⁺CD25⁺ regulatory T cell-mediated suppression of CD8 T cell homeostatic proliferation¹⁶.

To identify the effects of Foxp3 ectopic expression in CD4⁺CD25⁺ T cells, we compared the MigR1[Foxp3]- and MigR1[empty]-transduced populations from each group for CD4 and CD25 expression, by flow cytometry. Ectopic expression of Foxp3 generated a population of cells exhibiting the characteristic CD4⁺CD25⁺ expression pattern typical of CD4⁺CD25⁺ regulatory T cells, whereas the MigR1[empty]-transduced cells were uniformly CD25⁻ (Fig. 5e). CD25 upregulation in some, but not all, MigR1[Foxp3]-transduced cells is likely due to their proliferation in RAG^{-/-} recipients. This observation is consistent with our previous reports of CD25 down-regulation in wild-type CD4⁺CD25⁺ regulatory T cells upon transfer into lymphopenic hosts⁵. To explore potential effector mechanisms for the immunosuppression mediated by the MigR1[Foxp3]-transduced T cells, retrovirally transduced cells from both groups of mice were then purified by FACS sorting and analyzed for expression of cytokines believed to have a role in CD4⁺CD25⁺ regulatory T cell function. Real-time quantitative PCR revealed increased levels of interleukin (IL)-10 mRNA in MigR1[Foxp3]-transduced T cells, whereas levels of transforming growth factor (TGF)- β were equivalent between the two populations (Fig. 5f). IL-10 is required for CD4⁺CD25⁺ regulatory T cell suppression of inflammatory bowel disease¹⁷. Foxp3-transduced T cells expressed Foxp3 and IL-10 at levels comparable to those found in *ex vivo* isolated CD4⁺CD25⁺ regulatory T cells. These data show that ectopic expression of Foxp3 is sufficient to activate a program of immunosuppression in CD4⁺CD25⁺ T cells and is a critical regulator of CD4⁺CD25⁺ regulatory T cell function.

Discussion

In this study, we have demonstrated that Foxp3 is specifically required for the thymic development of CD4⁺CD25⁺ regulatory T cells. Our work has also shown that the lethal lymphoproliferative autoimmune syndrome observed in Foxp3-deficient mice results from a deficiency in CD4⁺CD25⁺ regulatory T cells and not from a cell-intrinsic defect of CD4⁺CD25⁻ T cells. Moreover, ectopic Foxp3 expression is sufficient to activate a program of suppressor function in peripheral CD4⁺CD25⁻ T cells. In contrast to other molecular markers used to identify regulatory T cells, such as GITR, CTLA-4 and CD25, Foxp3 is not up-regulated by activated CD4⁺CD25⁻ T cells. Our analysis revealed a strong correlation between Foxp3 and CD25 expression in the resting CD4⁺ T cell population. However, a very low level of Foxp3 in CD25⁻CD4⁺ T cells was detected. We suggest that Foxp3 expression within the CD25⁻CD4⁺ T cell population will identify a population of CD25⁻ regulatory T cells.

Nonetheless, rescue of lymphoproliferative disease in Foxp3-deficient animals upon provision of CD25⁺, but not of CD25⁻CD4⁺ T cells indicates that the Foxp3⁺CD25⁺CD4⁺ suppressor population is either very small or much less potent¹⁸.

Transfer of regulatory CD25⁺, but not of CD25⁻, CD4⁺ T cells into Foxp3-deficient neonates resulted in an expansion of CD25⁺CD4⁺ T cells, despite the presence of a full lymphoid compartment. In contrast, neither of the two transferred T cell subsets expanded in wild-type littermate controls. Analogous expansion of transferred CD4⁺CD25⁺ regulatory T cells was observed in IL-2R β -deficient mice¹⁹. The deficiency in CD25⁺CD4⁺ regulatory T cells in Foxp3- and IL-2R β -deficient mice may provide a pro-inflammatory cytokine environment supporting the rapid expansion of regulatory CD25⁺CD4⁺ T cells. A homeostatic niche for CD25⁺CD4⁺ T cells may also explain these results²⁰; clearly, these possibilities are not mutually exclusive.

What is the mechanism of Foxp3-mediated suppression by regulatory T cells? In our experiments, retroviral-driven Foxp3 expression in CD4⁺CD25⁻ T cells resulted in complete protection from wasting disease with accompanying colitis and inhibition of homeostatic proliferation of CD8⁺ T cells. Protection from inflammatory bowel disease (IBD) has been a well-established function of normal CD25⁺CD4⁺ T cells and is dependent on expression of IL-10¹⁷. Our results suggest that IL-10 expression is one of the downstream effector mechanisms induced by Foxp3. Notably, our analysis did not reveal a change in TGF- β mRNA levels, another cytokine implicated in regulatory T cell function. Although IL-10 upregulation by Foxp3 can account for protection from wasting disease, the mechanism of Foxp3-dependent suppression of homeostatic proliferation by Foxp3-transduced CD25⁻CD4⁺ T cells is unclear. Nevertheless, this suppression is consistent with the ability of normal CD25⁺CD4⁺ T cells to block homeostatic proliferation of activated CD8 T cells in lymphopenic hosts¹⁶. Identification of additional targets of Foxp3 should yield further insight into this question.

Foxp3 up-regulation upon CD4⁺CD25⁺ regulatory T cell activation and the ability of ectopic Foxp3 expression to confer suppressor function upon peripheral CD4⁺CD25⁻ T cells make a clear argument for a functional role for Foxp3 in regulatory T cells. This is supported by the observation that both CD4⁺CD25⁻ and CD8⁺ T cells from mice bearing a Foxp3 cosmid-derived transgene acquire suppressor activity. Furthermore, the suppressor function of these cells is enhanced after TCR engagement (F. Ramsdell, personal communication). Our data show that, in wild-type CD4⁺CD25⁻ T cells, upregulation of Foxp3 protein occurred despite a detectable decrease in *Foxp3* mRNA, suggesting that post-transcriptional control mechanisms may be important for Foxp3 protein expression. The increase in Foxp3 expression in activated CD25⁺CD4⁺ T cells correlates with a well-established increase in suppressor activity upon TCR ligation in these cells. These data suggest an intimate link between TCR signaling and the level of Foxp3 expression in regulatory T cells. It is our contention that this relationship is central to the development and function of regulatory T cells.

Our data demonstrate that Foxp3 serves as a critical regulator of CD25⁺CD4⁺ regulatory T cell thymic development. But what is responsible for Foxp3 induction? Based on evidence suggesting that high-affinity TCR interactions in the thymus favor CD25⁺CD4⁺ regulatory T cell development, we speculated that these same signals may induce Foxp3 expression. Consistent with this hypothesis, we observed upregulation of *Foxp3* mRNA in anergic TCR transgenic CD25⁻CD4⁺ T cells isolated from mice expressing a transgene-encoded cognate peptide ligand (Supplementary Fig. 2). This upregulation, albeit to levels substantially lower than CD25⁺CD4⁺ T cells (2.5- to 4-fold versus 40- to 60-fold), suggests that TCR engagement in the thymus has an essential

role in induction of Foxp3 expression. This idea is consistent with the efficient induction of CD25⁺CD4⁺ TCR transgenic RAG^{-/-} T cells upon coexpression of low levels of a transgene-encoded cognate ligand^{21,22}. We predict that Foxp3 protein levels in T cells from these mice will show a corresponding increase.

Thus, we propose a model of regulatory T cell development wherein Foxp3 is induced in immature thymocytes upon TCR interaction with specific peptide-MHC class II complexes, within an affinity range between positive and negative selection. Strength of TCR signals during thymic selection, perhaps modified by costimulation or the nature of the antigen-presenting cell, may determine Foxp3 expression and, thereby, the regulatory phenotype of the T cell. As our data make clear, Foxp3 deficiency results in the absence of CD4⁺CD25⁺ regulatory T cells. One prediction of this model is that a subset of CD4⁺ T cells bearing self-reactive TCRs are destined to function as regulatory T cells. When this program is not engaged, as in *Foxp3*^{-/-} mice, would-be regulatory T cells leave the thymus to become pathogenic autoreactive T cells in the periphery. This may account for the particularly aggressive nature of the autoimmunity observed in Foxp3-deficient mice.

We have shown Foxp3 to be a unique marker of CD4⁺CD25⁺ regulatory T cells, distinguishing them from activated CD4⁺CD25⁻ T cells. Furthermore, our studies have demonstrated that Foxp3 is specifically required for CD4⁺CD25⁺ regulatory T cell development and is sufficient to activate a program of suppressor function in peripheral non-regulatory CD4⁺ T cells. By these criteria, *Foxp3* is a 'master regulator' gene for this critically important subset of T cells. Furthermore, our findings implicate CD4⁺CD25⁺ regulatory T cell deficiency in a fatal hereditary autoimmune disorder in both mice and humans, and provide a specific genetic mechanism for T cell-mediated immunosuppression. Thus, Foxp3 controls a 'cell extrinsic' dominant mechanism of negative regulation in the immune system. Gain of suppressor function upon ectopic expression of Foxp3 in peripheral CD4⁺CD25⁻ T cells opens up new avenues for cell-based therapies for treatment of autoimmunity and for preventing transplant rejection using auto- and alloantigen-specific, Foxp3-transduced T cell lines. Further insights into the regulation of Foxp3 expression and function will have profound implications for the understanding of immune function in health and disease.

Methods

Mice. All mice were maintained at the University of Washington specific pathogen-free facility. B6.Cg-*Foxp3*^{fl} (B6 *Foxp3*^{fl}), B6.SJL-*Ptprc*^o *Pep3*^{fl}/BoyJ (Ly5.1⁺ B6), B6.PL-*Thy1*^l/Cy (Thy1.1⁺ B6) and C57BL/6J-*Rag1*^{tm1Mmm} (RAG1^{-/-}) mice were from The Jackson Laboratory (Bar Harbor, ME). C57BL/6 mice were from the Charles River Breeding Laboratories (Wilmington, MA). *Foxp3*^{-/-} mice are described below. All mice were used at age 6–16 weeks unless specified in the text. TEa TCR (V_β6^{*}, V_α2^{*}) transgenic B6 mice specific for a peptide derived from MHC class II I-E_α chain (E_α 52–68) presented by I-A^b were previously reported²³. B6 pE_α:Ii mice expressing a human invariant chain transgene, in which the region encoding the CLIP peptide was replaced with I-E_α 52–68 (pE_α), have been described²⁴.

Generation of conditional Foxp3-deficient mice. The Foxp3 targeting vector was constructed from an 8.5-kb *Sph1* fragment of the gene *Foxp3* containing exons –1 through 7 subcloned from a 30.8-kb cosmid containing the complete *Foxp3* gene⁶. A *loxP* site containing a 3' *Nco1* site was cloned into the *DraIII* site 246 bp 5' of exon 1, and the FRT-PGK-NEO-FRT-*loxP* cassette was cloned into the *BsmBI* site 57 bp 3' of exon 5. The PGK-DTA cassette was cloned into the 3' *Sph1* site for negative selection of clones with random integration of the targeting vector. The targeting construct was electroporated into R1 ES cells and neomycin-resistant clones were screened by PCR for evidence of homologous recombination. Positive clones were then screened by Southern blot analysis performed using a 507-bp 3' external probe on *EcoRI*-digested genomic DNA, a 505-bp 5' external probe on *Sap1*-digested genomic DNA, and a 517-bp internal probe on *Nco1*-digested genomic DNA to confirm integration of the 5' *loxP* site (Fig. 2b and data not shown).

Two independently derived embryonic stem (ES) cell clones were injected into C57BL/6 blastocysts. Chimeric male offspring were mated to both Cre-deleter and FLP-deleter²⁵ mice. Transmission of the targeted allele in heterozygous females and hemizygous males was determined by PCR from genomic DNA.

Cell isolations, activation and analysis. Cell populations were purified with an AutoMACS magnetic cell sorter (Miltenyi, Bergisch Gladbach, Germany) as described² and/or using a FACS Vantage cell sorter (Becton Dickinson, Palo Alto, CA). Routinely, purity of all cell preparations was >90% unless otherwise specified in the text. For western blots, T cells were analyzed *ex vivo* or after 48 h of activation using plate-bound anti-TCR β and anti-CD28 in the presence of recombinant IL-2 (100 μ g ml⁻¹). Flow cytometry analysis was performed using a FACSCalibur flow cytometer (Becton Dickinson, Palo Alto, CA) and analyzed using Cell Quest software (Becton Dickinson).

Antibodies. Biotinylated (BIO), fluorescein isothiocyanate (FITC), phycoerythrin (PE), cychrome (CyC), peridinin chlorophyll protein (PerCP) and allophycocyanin (APC)-conjugated antibodies to TCR- β (H57-597), CD4 (L3T4), CD5 (53-7.3), CD8 α (53-6.7), CD25 (PC-61), CD25 (7D4), B220 (RA3-6B2), CD62L (MEL-14), CD69 (H1.2F3), CD44 (IM7), Thy1.1 (HIS51), Ly5.1 (A20) and CTLA4 (UC10-4B9) were purchased from BD Pharmingen (San Jose, CA) and eBioscience (San Diego, CA). Mouse GITR/TNFRSF18 goat polyclonal antibody was purchased from R&D Systems, (Minneapolis, MN). Anti-Foxp3 IgG was purified on Protein G Sepharose 4 Fast Flow (Amersham Pharmacia Biotech AB, Uppsala, Sweden) from the antiserum of a rabbit immunized with a HIS-tagged recombinant Foxp3 protein purified from *Escherichia coli*.

Proliferation and suppression assays. Proliferation and suppression assays were performed as described². Briefly, for proliferation assays, T cells (2×10^5 cells per well) were stimulated for 72 h with titrated amounts of Concanavalin A (ConA) in the presence of T cell-depleted, irradiated 8×10^4 antigen-presenting cells in 96-well round-bottomed plates, and pulsed with 1 μ Ci per well of [³H]thymidine for the final 8 h. Suppression assays were performed under the same conditions using 2×10^4 CD4⁺CD25⁻ T cells as responders (R), 8×10^4 irradiated antigen-presenting cells and a 1:2 titration of the indicated suppressor (S) T cell population at a starting concentration of 4×10^4 cells per well in the presence of ConA at 2 μ g ml⁻¹ final concentration. All data are shown as mean [³H]thymidine incorporation in triplicate cultures.

Neonatal transfer of CD4⁺ T cells. We injected i.p. 4×10^5 purified wild-type Ly5.1⁺ marked CD4⁺CD25⁺ or CD4⁺CD25⁻ T cells into 1- to 2-day-old pups (Ly5.2^{-/-}) from female *Foxp3*^{fl/fl} mice bred with C57BL/6 males. Male pups were monitored for the development of lymphoproliferative syndrome and analyzed at day 21 of age. Donor cell recovery was calculated based on the total number of lymphocytes recovered multiplied by the percentage of Ly5.1⁺ cells as determined by FACS analysis.

Retroviral infection and analysis of transduced CD4⁺CD25⁺ T cells. MSCV MigR1 retroviral encoding plasmid DNA was transfected into the Φ NX-E packaging cell line using CaPO₄ precipitation as described²⁶. Retrovirus-containing supernatant was collected following 36 h incubation of the transfected packaging cells at 32 °C. Freshly isolated, MACS-purified CD4⁺ T cells were activated using plate-bound anti-TCR β and anti-CD28 and recombinant IL-2 (100 μ g ml⁻¹). After 36 h of activation, CD4⁺ cells were infected by resuspending cells in retrovirus-containing supernatant supplemented with 8 μ g ml⁻¹ Polybrene and recombinant IL-2 (50 μ g ml⁻¹), followed by centrifugation for 90 min at 2,500 r.p.m. and 72 h of incubation at 37 °C.

At 72 h post-infection, 5×10^6 T cells transduced with either MigR1[Foxp3] or MigR1[empty] were intravenously injected into the tail vein of 8-week-old RAG1^{-/-} mice. Host mice were monitored for the development of wasting disease and colitis by weight loss and pathology. At 15 d post-transfer lymph node and spleen cells were isolated from host mice and FACS analyzed. For real-time quantitative PCR analysis of gene expression, retrovirally transduced cells (GFP⁺) were purified by FACS sorting upon re-isolation from host mice, mRNA was isolated and cDNA was generated. mRNA levels of indicated genes were analyzed and compared with mRNA levels from purified wild-type CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells.

Foxp3 expression in anergic CD4⁺ T cells. To generate anergic T cells *in vivo*, TEa TCR transgenic mice were crossed onto pE_α:Ii B6 mice expressing a human invariant chain transgene in which the region encoding the CLIP peptide was replaced with I-E_α 52–68 (pE_α). TEa or TEa-pE_α mice were analyzed by flow cytometry for expression of V_β6^{*}V_α2^{*} transgene encoded TCR on CD4⁺ single-positive and CD4⁺CD8⁺ thymocytes. Pooled lymph node and spleen cells from TEa or TEa-pE_α:Ii mice were sorted for V_β6^{*}V_α2^{*} or V_β6^{*}V_α2⁻ CD4⁺ T cells. Antigen-specific proliferative responses of sorted T cells were tested upon *in vitro* stimulation with irradiated pE_α-expressing or B6 (control) splenocytes as described above. Expression of *Foxp3* and *Il10* mRNA was measured by real-time quantitative PCR with cDNA samples isolated from sorted cell populations.

Real-time quantitative PCR. Real-time quantitative PCR was performed as described². All reported mRNA levels are normalized to the HPRT mRNA level, where HPRT = 1. PCR primer pairs were as follows: Foxp3, 5'-GGCCCTTCTCCAGGACAGA-3', 5'-GCTGATCATGGCTGGGTTGT-3'; CD25, 5'-TGGAAAGGTTGAGGGGTAAG-3', 5'-ACACCTGTAAGCCAGCTCT-3'; TGF- β 1, 5'-ACCATGCCAACTCTGTCTG-3', 5'-CGGGTTGTGTTGGTTGTAGA-3'; IL-2, 5'-TTCAATTGGAAGATGCTGAGA-3', 5'-ATCATCGAATTGGCACTCAA-3'; IL-10, 5'-CCCTTGTGCTATGGTTCCT-3', 5'-TGGTTTCTTCCCAAGACC-3'; HPRT, 5'-AGCCTAAGATGAGCGCAAGT-3', 5'-TTACTAGGCAGATGCCACA-3'.

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

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Competing interests statement

The authors declare that they have no competing financial interests.

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