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Runx-CBFβ complexes control Foxp3 expression in regulatory T cells

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Abstract

Foxp3 plays an indispensable role in establishing stable transcriptional and functional programs of regulatory T (T_{reg}) cells. Loss of Foxp3 expression in mature T_{reg} cells results in a failure of suppressor function, yet the molecular mechanisms ensuring steady heritable Foxp3 expression in the Treg cell lineage remain unknown. Using T_{reg} cell-specific gene targeting we found that Runx-CBFβ complexes were required for maintenance of *Foxp3* mRNA and protein expression in Treg cells. Consequently, mice lacking CBFβb exclusively in the Treg cell lineage exhibited a moderate lymphproliferative syndrome. Thus, Runx-CBFβ complexes maintain stable expression of high amounts of Foxp3 and serve as an essential determinant of Treg cell lineage stability.

Introduction

The regulatory T (T_{reg}) cell lineage is indispensable for suppressing autoimmunity and preventing over-exuberant responses to pathogens1. The X-chromosome encoded forkhead winged-helix transcription factor Foxp3 (http://www.signaling-gateway.org/molecule/query?afcsid=A002750) is a lineage specification factor required for T_{reg} cell differentiation and function2–4. Furthermore, continuous expression of Foxp3 in mature T_{reg} cells is required for the maintenance of suppressor function and of the Foxp3-dependent transcriptional program5. Genome-wide analysis of Foxp3 binding sites coupled to gene expression profiling suggested that Foxp3 can act both as a transcriptional activator and repressor6–8. For example, Foxp3 binding to the promoters and 5′ regulatory regions of *Ctla4* and *Il2ra* genes results in their activation, whereas Foxp3 binding to *Il2* and *Il7ra* promoters facilitates repression of these genes. The dual activity of Foxp3 defines

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characteristic features of T_{reg} cells, which express high amounts of CTLA-4 and CD25 but low amounts of IL-7R, and lack of IL-2 production6,7.

Although the identity of Foxp3 target genes and understanding of their roles in T_{reg} cell biology have began to emerge, the mechanisms regulating expression of the *Foxp3* gene itself remain poorly understood. A number of sequence-specific transcription factors including STAT5, nuclear factor of activated T cells (NFAT), CREB, Smad3, and NF-KB were implicated in transcriptional regulation of *Foxp3* expression, yet questions remain about their redundancy and direct or indirect effect on *Foxp3* transcriptional activation versus survival of T_{reg} cells or their precursors9–12. For example, STAT5 was proposed to be essential for *Foxp3* induction yet forced expression of Bcl2 in T_{reg} precursors rescues Foxp3 expression in the absence of STAT59,10 (Stephen Malin and Meinrad Busslinger, personal communication).

Furthermore, the molecular mechanisms by which Foxp3 controls gene expression in T_{reg} cells appear to be complex. Foxp3 interactions with other sequence-specific transcription factors were proposed to be essential for establishment of the Foxp3-dependent transcriptional program. In this regard, Foxp3 can interact with NFAT; these two proteins cooperatively activate *Ctla4* and *Il2ra* and antagonize NFAT-AP1-dependent *Il2* expression13. More recently, the interaction between Foxp3 and the RUNT domain containing transcription factor Runx1 (http://www.signaling-gateway.org/molecule/query?afcsid=A000523) was suggested to be critical for T_{reg} cell function. Mutations in Foxp3 that abrogate its interaction with Runx1 result in a loss of Foxp3 function. Unlike wild-type Foxp3 protein, the mutant form of Foxp3 is unable to repress *Il2* production or activate *Il2ra* or *Ctla4* when ectopically expressed in $CD4^+CD25^-$T cells14. Using a proteomics approach, we found that Runx1 and its co-factor CBFβ are prominently represented among binding partners of Foxp3 (Rudra et al., in preparation).

Transcriptional activity of Runx proteins is dependent upon heterodimerization with CBFβ (http://www.signaling-gateway.org/molecule/query?afcsid=A000524)15. CBFβ stabilizes Runx protein-DNA interactions and prevents ubiquitin-mediated Runx degradation, thus serving as an indispensable component of Runx transcriptional complexes16,17. The three known Runx factors (Runx1–3) are broadly expressed in hematopoietic cells including T cells. where they play important roles at various stages of differentiation18. Expression of T cell receptor (TCR) genes requires Runx1, which also acts as a transcriptional repressor of the *Cd4* locus in $CD4^-CD8^-$ thymocytes19–23. Runx3 is expressed in high amounts and represses *Cd4* expression in $CD8^+$ T cells23. Runx3 has also been suggested to interact with the T helper type 1 (T_H1) lineage specification factor T-bet and to facilitate *Ifng* activation and *Il4* repression in T_H1 cells24.

The documented importance of Runx in cell fate decisions during T cell differentiation and Runx interactions with Foxp3 prompted us to investigate the role of Runx proteins in T_{reg} cell biology *in vivo* using a genetic approach. We took advantage of the fact that transcriptional activity of all the three Runx proteins is dependent upon CBFβ and the ablation of the *Cbfb* gene results, therefore, in a complete loss of Runx function. To address the role for Runx protein function specifically in the T_{reg} cell lineage we induced deletion of

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a floxed *Cbfb* allele in T_{reg} cells by crossing *Cbfb*^{fl} and *Foxp3*$^{YFP-Cre}$ mice25,26. Unexpectedly, we found that Runx factors were largely dispensable for Foxp3 target gene expression and T_{reg} cell suppressor function. However, Runx protein function was critical for the maintenance of expression the *Foxp3* gene itself. Thus, Runx proteins play an essential role in maintaining high amounts of Foxp3 expression ensuring T_{reg} cell lineage identity.

Results

Lymphoproliferation *in Cbfb*$^{fl/fl}$*Foxp3*$^{YFP-Cre}$ mice

To determine the functional consequence of ablating Runx activity in T_{reg} cells, we crossed mice harboring a floxed *Cbfb* allele (*Cbfb*^{fl}) with mice expressing a YFP-Cre recombinase fusion protein under the control of the *Foxp3* regulatory elements (*Foxp3*$^{YFP-Cre}$)25,26. Efficient Cre-mediated deletion of the *Cbfb*^{fl} allele was confirmed by PCR analysis of genomic DNA isolated from sorted YFP-Foxp3^{+} T_{reg} cells from *Cbfb*$^{fl/+}$*Foxp3*$^{YFP-Cre}$ and *Cbfb*$^{fl/fl}$ *Foxp3*$^{YFP-Cre}$ mice and subsequent reduction in CBFβ mRNA was shown by real-time PCR analysis of RNA isolated from these cells (Supplementary Fig. 1a–d). Furthermore, immunoblot analysis confirmed the absence of the CBFβ protein only in sorted T_{reg} cells derived from *Cbfb*$^{fl/fl}$ *Foxp3*$^{YFP-Cre}$ mice (Supplementary Fig. 1e). DNA, RNA, and protein expression analyses indicated that the deletion of the *Cbfb*^{fl} allele was limited to CD4^{+}YFP-Foxp3^{+} T_{reg} cells and was not observed in the CD4^{+}YFP^{-} "non-T_{reg}" cells.

Cbfb$^{fl/fl}$*Foxp3*$^{YFP-Cre}$ mice were born at the expected Mendelian ratio and showed no clinical signs of autoimmunity until 12–14 weeks of age. Nevertheless, examination of the secondary lymphoid organs in 5–8 week old mutant mice revealed lymphadenopathy and splenomegaly (Fig. 1a, b). Importantly, lymphoproliferative syndrome in these mice was relatively minor in comparison to the one observed in Foxp3-deficient mice, or *Foxp3*^{DTR} mice subjected to T_{reg} ablation upon diphtheria toxin treatment2,27. Examination of tissue pathology revealed moderate to marked lymphohistiocytic, and occasionally plasmacytic, inflammation in the affected tissues of the diseased mice (Fig. 2). Notably, the lung had multifocal perivascular and peribronchiolar lymphohistiocytic pneumonitis and arteritis with marked proliferative arteriopathy and bronchiolar goblet cell hyperplasia (Fig. 2).

Next, we examined relative sizes of thymocyte subsets as well as lymphoid and myeloid cell subsets, including CD4 and CD8 T cells, B cells, natural killer (NK) cells, dendritic cells (DCs) and macrophages in the spleen and lymph nodes (LNs), and failed to find marked differences between mutant and wild-type mice (Fig. 1c and Supplementary Fig. 2). To assess the extent of CD4^{+}Foxp3^{-} T cell activation in these mice, we analyzed the expression of a panel of known activation markers including CD25, GITR, ICOS, CTLA-4, and CD62L and observed a modest increase in the proportion of activated T cells in *Cbfb*$^{fl/fl}$*Foxp3*$^{YFP-Cre}$ mice (Fig. 1d). Likewise, the numbers of proliferating cells assessed based on Ki-67 expression were only slightly increased (Fig. 1d), whereas changes in $T_{H}1$ and $T_{H}2$ cytokine production were insignificant in mice harboring CBFβ-deficient T_{reg} cells (data not shown). Thus, the mild dysregulation of T cell responses in the presence of CBFβ-deficient T_{reg} cells contrasted sharply with the highly aggressive and severe immune tissue lesions and T cell activation and population expansion in mice lacking T_{reg} cells27. Thus,

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these observations in *Cbfb*$^{fl/fl}$*Foxp3*$^{YFP-Cre}$ mice implied a partial impairment of T_{reg} cell suppressor function in the absence of CBFβ.

CBFβ controls Foxp3 expression

To directly assess the functional potential of T_{reg} cells in *Cbfb*$^{fl/fl}$*Foxp3*$^{YFP-Cre}$ mice we first examined Foxp3 expression in these cells, as high amounts of Foxp3 are required for T_{reg} cell suppressor function7,28,29. Unexpectedly, intracellular Foxp3 staining combined with flow cytometric analysis revealed a significant reduction in the amount of Foxp3 protein in peripheral T_{reg} cells lacking CBFβ (Fig. 3a). A similar reduction in the amount of YFP-Cre fusion protein was observed (Supplementary Fig. 3), suggesting that down-regulation of Foxp3 expression occurred at a transcriptional level. Indeed, real-time PCR analysis of RNA isolated from sorted $CD4^{+}YFP^{+}$ T_{reg} cells showed a marked reduction in Foxp3 mRNA in the absence of CBFβ (Fig. 3b). In contrast to peripheral T_{reg} cells, amounts of Foxp3 protein were comparable in $Foxp3^{+}$ thymocytes in *Cbfb*$^{fl/fl}$*Foxp3*$^{YFP-Cre}$ and *Cbfb*$^{fl/+}$*Foxp3*$^{YFP-Cre}$ mice likely due to the carryover of CBFβ protein from $Foxp3^{-}$ precursor thymocytes. In agreement with this idea, in *Cbfb*$^{fl/fl}$*CD4-Cre*$^{+}$ mice Foxp3 expression in CD4 SP thymocytes was diminished although to a lesser extent than in the periphery (Supplementary Fig. 4a). A similar cell-intrinsic effect of CBFβ deficiency, characterized by marked reduction in Foxp3 expression primarily in the periphery, was observed upon co-injection of $CD45.2^{+}$ *Cbfb*$^{fl/fl}$*CD4-Cre*$^{+}$ and wild-type C57BL/6 $CD45.1^{+}$ bone marrow transfer into RAG-deficient recipients (Supplementary Fig. 4b). Although it was formally possible that selective outgrowth or survival of $Foxp3^{low}$ cells in the absence of CBFβ accounted for the observed decrease in Foxp3 expression in the CBFβ-deficient T_{reg} cell population, we found that $Foxp3^{high}$ cells lacking CBFβ divide more than their $Foxp3^{low}$ counterparts (Supplementary Fig. 5), in agreement with an earlier finding that high amounts of Foxp3 expression confer proliferative potential to T_{reg} cells7. Furthermore, CBFβ-deficient $Foxp3^{high}$ T_{reg} cells did not exhibit increased apoptosis as compared to $Foxp3^{low}$ cells (data not shown). Together, these results indicate that Runx-CBFβ complexes control Foxp3 expression in T_{reg} cells.

To conclusively demonstrate that the reduced amounts of Foxp3 in *Cbfb*$^{fl/fl}$*Foxp3*$^{YFP-Cre}$ mice were due to the inactivation of the Runx-CBFβ complexes and not a hitherto unknown Runx independent function of CBFβ, we generated *Runx1*$^{fl/fl}$*Foxp3*$^{YFP-Cre}$ mice in which Runx1, which was more prominently expressed than Runx3 in T_{reg} cells, was ablated upon Cre-mediated deletion of a conditional *Runx1*^{fl} allele (Supplementary Fig. 6a). Similarly to the *Cbfb*$^{fl/fl}$*Foxp3*$^{YFP-Cre}$ mice, the *Runx1*$^{fl/fl}$*Foxp3*$^{YFP-Cre}$ mice exhibited a significant reduction in amounts of Foxp3 in peripheral T_{reg} cells (Supplementary Fig. 6b). Additionally, Runx1-deficient $Foxp3^{+}$ thymocytes also showed a modest reduction in Foxp3 amounts on a per cell basis, albeit to a lesser extent than the peripheral T_{reg} cells (Supplementary Fig. 6b). The difference in Foxp3 amounts present in Runx1- and CBFβ-deficient $Foxp3^{+}$ thymocytes in *Cbfb*$^{fl/fl}$*Foxp3*$^{YFP-Cre}$ and *Runx1*$^{fl/fl}$*Foxp3*$^{YFP-Cre}$ mice may be attributed to a shorter half-life and lesser abundance of Runx1 in comparison to CBFβ.

CBFβ-deficient T_{reg} cell populations in *Cbfb*$^{fl/fl}$*Foxp3*$^{YFP-Cre}$ mice, but not in healthy littermate *Cbfb*$^{fl/+}$*Foxp3*$^{YFP-Cre}$ mice, contained increased proportions of $GITR^{hi}$, $ICOS^{hi}$, as

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well as Ki-67$^+$ cells, indicative of heightened activation and proliferation likely driven by proliferation and activation of non-T_{reg} cells (Fig. 3c; data not shown). CBFβ-deficient T_{reg} cells suppressed proliferative responses of CD4$^+$ Foxp3$^-$ T cells *in vitro* in a manner comparable to their CBFβ-sufficient counterparts (Fig. 3d). Thus, in agreement with the slowly progressing immune lesions and moderate lymphoproliferative syndrome in *Cbfb*$^{fl/fl}$*Foxp3*$^{YFP-Cre}$ mice, the CBFβ-deficient T_{reg} cell population as a whole does maintain suppressor capacity, likely due to the presence of Foxp3^{hi} T_{reg} cells recently emigrated from the thymus.

To assess competitive fitness of CBFβ-deficient T_{reg} cells in the absence of inflammation we examined relative sizes of CBFβ-sufficient and CBFβ-deficient T_{reg} subsets in healthy heterozygous *Foxp3*$^{YFP-Cre/+}$*Cbfb*$^{fl/fl}$ and *Foxp3*$^{YFP-Cre/+}$*Cbfb*$^{fl/+}$ females. The proportion of peripheral YFP-Cre$^+$ T_{reg} cells in *Foxp3*$^{YFP-Cre/+}$*Cbfb*$^{fl/fl}$ mice was markedly lower than in *Foxp3*$^{YFP-Cre/+}$*Cbfb*$^{fl/+}$ mice (Fig. 3e, f). In these disease-free mice, CBFβ-deficient YFP-Cre$^+$ T_{reg} cells also expressed markedly reduced amounts of Foxp3 on a per cell basis in comparison to CBFβ-sufficient counterparts, in agreement with findings in diseased *Foxp3*$^{YFP-Cre}$*Cbfb*$^{fl/fl}$ mice. Thus, CBFβ-deficiency in T_{reg} cells results in diminished amounts of Foxp3, likely leading to impaired suppressive capacity and diminished competitive fitness in the presence of CBFβ-sufficient T_{reg} cells.

Although the analysis of T_{reg} cells in *Runx1*$^{fl/fl}$*Foxp3*$^{YFP-Cre}$ and *Cbfb*$^{fl/fl}$*Foxp3*$^{YFP-Cre}$ mice made it evident that the Runx-CBFβ deficiency impairs Foxp3 expression, the extent to which Foxp3 expression was affected in these mice was difficult to assess due to aforementioned continuous thymic output. To directly assess the potential loss of Foxp3 expression in a cohort of peripheral T_{reg} cells lacking CBFβ we adoptively transferred sorted CD45.2$^+$CD4$^+$YFP^{hi} cells from *Cbfb*$^{fl/fl}$*Foxp3*$^{YFP-Cre}$ mice or *Cbfb*$^{fl/+}$*Foxp3*$^{YFP-Cre}$ mice mixed with CD4$^+$ effector T cells from CD45.1$^+$*Foxp3*$^-$ mice into *Rag2*$^{-/-}$ recipients. Flow cytometric analysis of transferred cells revealed that in addition to diminished Foxp3 amounts, the CBFβ-deficient T_{reg} population exhibited a more pronounced loss of Foxp3with considerably faster kinetics than the CBFβ-sufficient T_{reg} cell population (Fig. 4a–d). Thus, Runx-CBFβ regulates both the amount and stability of Foxp3 expression in T_{reg} cells.

Runx-CBFβ binds the *Foxp3* locus

Apart from a conserved promoter region, the *Foxp3* locus contains two recently described proximal conserved non-coding sequence (CNS1, CNS2) elements downstream of the transcription start site11,12. Both CNS1, containing a Smad-NFAT response element, and CNS2, containing CREB-ATF and STAT5 binding sites, were proposed to act as enhancers important for *Foxp3* induction in the thymus and in the periphery 11, 12. To test whether Runx-CBFβ complexes bind to the *Foxp3* promoter and CNS elements *in vivo*, we performed CBFβ chromatin immunoprecipitation (ChIP) using nuclear lysates isolated from wild-type T_{reg} cells. We found a marked enrichment of CBFβ bound to CNS2. In addition, we reproducibly observed an enrichment of CBFβ at the *Foxp3* promoter despite a very high background in the control IgG ChIP (Fig. 5). The *Tcrb* enhancer, known to bind Runx1-CBFβ, was used as a positive control in these experiments20.

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To test whether Runx-CBFβ binding to the *Foxp3* promoter and CNS2 directly facilitates *Foxp3* transcription, we performed luciferase reporter assays using primary T_{reg} cells and the EL-4 T cell line. We assessed the role of putative Runx binding sites in the promoter and CNS2 in the direct enhancement of Foxp3 transcription mediated by these elements. In agreement with previous reports, the minimal Foxp3 promoter did not exhibit measurable activity in the reporter assay (Supplementary Fig. 7a,b)11. Although the non-chromatinized DNA constructs containing the minimal Foxp3 promoter and CNS1 (construct B) or CNS2 (construct C) showed an increased luciferase signal in the presence of PMA and ionomycin with and without transforming growth factor-β (TGF-β), mutations of the predicted Runx binding sites had no measurable effect on CNS-mediated enhancement of *Foxp3* promoter activity in EL-4 cells or purified CBFβ-sufficient T_{reg} cells (Supplementary Figure 7c–e). Similarly, the CNS2-containing reporter drove comparable luciferase expression in CBFβ-deficient and CBFβ-sufficient T_{reg} cells (Supplementary Fig. 7e). These results suggested that Runx-CBFβ complexes might regulate Foxp3 expression through epigenetic changes at the *Foxp3* locus; this type of regulation cannot be accounted for in a reporter assay. In support of this idea, we detected a marked decrease in permissive histone H3K4me3 modifications at the *Foxp3* promoter and a concomitant widespread increase in inhibitory histone H3K9me3 modifications at the 5′ end of the *Foxp3* locus (Fig. 6) in CBFβ-deficient T_{reg} cells. In contrast to a pronounced increase in H3K9me3 inhibitory marks, the distribution of another prototypic non-permissive histone modification, H3K27me3, across the *Foxp3* locus was unchanged in CBFβ-deficient T_{reg} cells (Fig. 6). Thus, it seems likely that Runx-CBFβ complexes impart permissive epigenetic modifications and oppose certain inhibitory modifications on the *Foxp3* locus.

Competent suppressor function of CBFβ-deficient cells

Reduced and unstable Foxp3 expression may result in attenuated *in vivo* T_{reg} cell suppressor function, which might contribute to the lymphoproliferative syndrome in *Cbfb*$^{fl/fl}$*Foxp3*$^{YFP\text{-}Cre}$ mice. However, it was also possible that Runx-CBFβ–Foxp3 complexes contribute to T_{reg} functionality by cooperatively regulating the T_{reg} cell gene expression signature. To directly distinguish between these two possibilities, we examined expression of the characteristic T_{reg} cell surface molecules CTLA-4, CD25, and GITR upon "forced' Foxp3 expression in CBFβ-sufficient or CBFβ-deficient non-T_{reg} $CD4^+$ cells (Fig. 7). Previous studies showed that retroviral transduction of $CD25^-CD4^+$ T cells with a Foxp3-expressing retrovirus conferred a characteristic T_{reg} cell surface phenotype and suppressor function upon non-T_{reg} cells2,3. It is noteworthy that the amount of *Foxp3* expression in retrovirally transduced cells in these experiments is comparable to, or lower than that observed in T_{reg} cells2,3. We employed the previously described retroviral bi-cistronic vector to express both Foxp3 and GFP, or GFP alone, in activated $CD25^-CD4^+$ T cells from *Cbfb*$^{fl/fl}$*CD4-Cre*$^+$ or *Cbfb*$^{+/+}$*CD4-Cre*$^+$ control mice and examined the aforementioned surface markers and *in vitro* suppressor activity of FACS purified $Foxp3^+GFP^+$ and control GFP^+ T cells. As the retroviral vector expressing Foxp3 cDNA was devoid of the endogenous *Foxp3* regulatory elements that serve as targets of Runx-CBFβ transcriptional complexes, comparable Foxp3 expression was found in transduced CBFβ-deficient and CBFβ-sufficient T cells (Fig. 7a). Furthermore, GITR, CTLA-4, CD25 were present in similar amounts in CBFβ-deficient and CBFβ-sufficient Foxp3-expressing T

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cells (Fig. 7a) and these cells exhibited comparable suppressor activity *in vitro* (Fig. 7b). In contrast, the GFP vector transduced CBFβ-deficient and CBFβ-sufficient T cells demonstrated no suppressive capacity. Instead, they showed enhanced proliferation in comparison to freshly isolated responder T cells, presumably due to their prior activation during the retroviral transduction (Fig. 7c).

To assess *in vivo* suppressor capacity of T_{reg} cells lacking CBFβ but retrovirally transduced with a Foxp3-encoding retrovirus, we isolated $Foxp3^+$ T_{reg} cells from *Cbfb*$^{fl/fl}$Foxp3$^{YFP\text{-}cre}$ or control *Cbfb*$^{fl/+}$Foxp3$^{YFP\text{-}cre}$ mice and transduced them with another bi-cistronic retroviral vector containing the *Foxp3* coding sequence followed by an IRES-driven tailless human CD2 reporter. As a control, cells were transduced with the retroviral vector containing the IRES-driven tailless human CD2 reporter but lacking the Foxp3 insert. FACS sorted transduced T_{reg} cells were co-transferred with effector T cells isolated from *Foxp3*$^-$ mice into *Tcrb*$^{-/-}$ *Tcrd*$^{-/-}$ recipient mice. Transfer of effector *Foxp3*$^-$ T cells alone led to a characteristic lymphoproliferative systemic immune-mediated syndrome associated with the severe weight loss, lymphadenopathy, splenomegaly, and increase in production of T_H1 and T_H2 cytokines (Fig. 7d and Supplementary Fig. 8). Co-transfer of Foxp3- or control vector-transduced control CBFβ-sufficient T_{reg} cells or Foxp3-transduced CBFβ-deficient T_{reg} cells led to an essentially complete rescue of the lymphoproliferative syndrome in the recipient mice. In agreement with relatively mild late-onset autoimmune lesions in unmanipulated *Cbfb*$^{fl/flfl}$*Fox3p*$^{YFP\text{-}cre}$ mice, CBFβ-deficient T_{reg} cells transduced with the control vector exhibited only a mild impairment in suppressor function, reflected in partial rescue of recipient mice from the weight loss (Fig. 7d). It is noteworthy that both Foxp3 and control vector-transduced CBFβ-deficient T_{reg} cells appeared somewhat less efficient at limiting IL-4 production by effector T cells compared to control CBFβ-sufficient T_{reg} cells (Supplementary Fig. 8e). In agreement with the weight loss data, small inflammation foci in the liver, skin and lung found in recipient mice which were co-transferred with effector T cells and control vector-transduced CBFβ-deficient T_{reg} cells were not observed in the presence of Foxp3 transduced CBFβ-deficient or -sufficient T_{reg} cells or control vector transduced CBFβ-sufficient T_{reg} cells (Supplementary Fig. 8f). Thus, $Foxp3^+$ T_{reg} cells in the absence of CBFβ exhibit only modestly diminished *in vivo* suppressor function and ectopic Foxp3 expression largely restores this function. The latter results are in agreement with unimpeded *in vitro* suppression capacity of Foxp3-transduced CBFβ-deficient $CD4^+$ T cells (Fig. 7b). These results suggest that Foxp3 is able to confer T_{reg} cell-specific gene expression and suppressive capacity in the absence of functional Runx-CBFβ complexes and that the moderate decline in suppressor function of T_{reg} cells lacking CBFβ is largely due to progressively diminishing Foxp3 expression.

Discussion

Our studies suggest that in T_{reg} cells Runx-CBFβ activity is required for maintaining the expression of *Foxp3*. The lack of clinical signs of overt autoimmune phenotype in *Cbfb*$^{fl/fl}$*Foxp3*$^{YFP\text{-}Cre}$ mice up to 8–10 months of age was in sharp contrast to the very short 3–5 week lifespan of mice lacking Foxp3. Consistent with the late occurrence of gross clinical manifestations, the lymphadenopathy, splenomegaly, and $CD4^+Foxp3^-$effector T cell activation in *Cbfb*$^{fl/fl}$*Foxp3*$^{YFP\text{-}Cre}$ mice were relatively mild. It is of interest, however,

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that the only pronounced tissue pathology noticeable in mice harboring CBFβ-deficient T_{reg} cells was a marked pulmonary arteriopathy similar to that in two recent studies, which proposed a role for T_{reg} cells in the pathogenesis of this disease30,31. Notably, human lupus patients, who, like aged *Cbfb*$^{fl/fl}$*Foxp3*$^{YFP\text{-}Cre}$ mice, exhibit a diminished size of the T_{reg} cell subset, often develop severe angioproliferative pulmonary hypertension with analogous histopathological lesions32. Except for the lung arteriopathy, immune mediated inflammation in other tissues was far less severe and its progression was much slower than that in mice with a complete loss of T_{reg} function or lack of T_{reg} cells2,27. Of note, ablation of CBFβ in $CD4^+$ T cells in *Cbfb*$^{fl/fl}$*CD4-Cre*$^+$-mice results in asthma-related symptoms due to enhanced T_H2 response presumably due to impaired silencing of *Il4* locus in T_H1 cells25.

Consistent with these results, Runx-CBFβ activity was unlikely a prerequisite for T_{reg} function since ectopic expression of Foxp3 in CBFβ-deficient $CD4^+CD25$ T cells derived from *Cbfb*$^{fl/fl}$*CD4-Cre*$^+$mice resulted in a prototypical pattern of Foxp3 target gene expression and conferred suppressive capacity upon these cells. Therefore, it appears that in T_{reg} cells the main function of Runx-CBFβ is to regulate the amount and stability of Foxp3 expression.

Runx-CBFβ nuclear factors may influence *Foxp3* expression in three different yet not mutually exclusive ways. They can bind to *Foxp3* and facilitate its transcription or regulate the locus by modifying the chromatin structure and keeping it in an open configuration. In addition, the effect of Runx-CBFβ on Foxp3 expression in T_{reg} cells can be indirect, if a known or unknown regulator of the *Foxp3* gene is under the control of Runx proteins. Our observation that in T_{reg} cells CBFβ occupies the *Foxp3* promoter and CNS2 supports the first two possibilities. However, the presence or absence of Runx-CBFβ complexes in primary T_{reg} cells had little, if any, effect on expression of a luciferase reporter construct containing *Foxp3* promoter and CNS2 sequences, suggesting the possibility that Runx-CBFβ complexes maintain Foxp3 expression in T_{reg} cells via epigenetic modifications of the *Foxp3* locus. Indeed, ablation of CBFβ in T_{reg} cells was associated with a pronounced decrease in permissive H3K4me3 modifications immediately downstream of and at the *Foxp3* promoter, and a selective increase in non-permissive H3K9me3, but not in H3K27me3 modifications, at the *Foxp3* locus. Thus, these results are consistent with the idea that Runx-CBFβ complexes promote the active state of the *Foxp3* locus via an epigenetic mechanism. Further evidence in favor of this possibility comes from our recent finding that mice lacking CNS2 exhibit a progressive loss of Foxp3 expression in the progeny of dividing $Foxp3^+$ cells similar to that in *Cbfb*$^{fl/fl}$*Foxp3*$^{YFP\text{-}Cre}$ mice (Y. Zheng et al., in preparation).

In contrast to peripheral $Foxp3^+$ T cells, Cre mediated deletion of the *Cbfb*^{fl} allele in Foxp3-expressing cells did not result in a decrease in the amount of Foxp3 expressed in the thymus. This finding could suggest that Runx-CBFβ mediated regulation of *Foxp3* expression is dispensable in the thymus. However, another possible explanation is highlighted by the previously documented carryover of CBFβ protein 25, which lingers in thymocytes long after *Cbfb* allele deletion. In this regard, a recent study showed that even after CD4-Cre mediated deletion of a conditional *Cbfb*^{fl} allele in $CD4^+CD8^+$ thymocytes, CBFβ protein was readily detectable by immunoblotting in $CD4^+CD8^-$ thymocytes, but not in peripheral

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$CD4^+CD8^-$ T cells25. Indeed, in our analysis of *Cbfb*$^{fl/fl}$*CD4*^{Cre} mice we observed that the expression of Foxp3 in CD4 SP thymocytes was diminished in comparison to *Cbfb*$^{fl/fl}$*Foxp3*$^{YFP\text{-}Cre}$ mice, presumably due to earlier deletion of the *Cbfb*^{fl} allele in the former mice. Further support for a role for CBFβ-Runx complexes in regulation of Foxp3 expression in the thymus came from comparison of T_{reg} cell-specific ablation of Runx1 or CBFβ Although T_{reg} cell-specific ablation of Runx1 or CBFβ results in a similar reduction in Foxp3 in the peripheral cells, Runx1-deficient $Foxp3^+$ thymocytes exhibited markedly decreased amounts of Foxp3 likely due to faster turnover or lower amounts of Runx1. Thus, our studies suggest that Runx-CBFβ complexes play an important non-redundant role in the maintenance of Foxp3 expression both in the thymus and in the periphery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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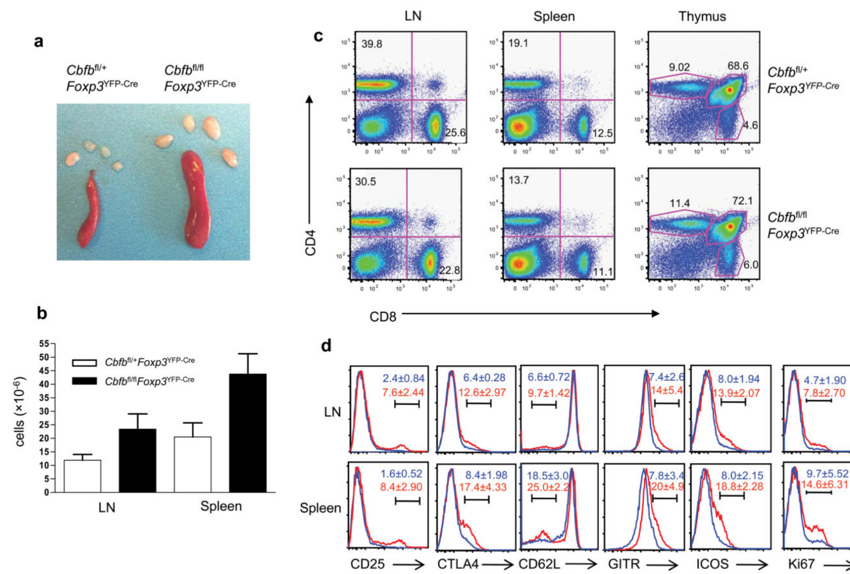

Figure 1.

Characterization of *Cbfb*$^{fl/wt}$*Foxp3*$^{YFP\text{-}Cre}$ mice. (**a**) Splenomegaly and lymphadenopathy and (**b**) total cellularity of LN and spleen in 5–8 week old *Cbfb*$^{fl/fl}$*Foxp3*$^{YFP\text{-}Cre}$ and *Cbfb*$^{fl/+}$*Foxp3*$^{YFP\text{-}Cre}$ littermates. (**c**) Analysis of peripheral and thymic CD4^{+} and CD8^{+} T cell compartments in *Cbfb*$^{fl/fl}$*Foxp3*$^{YFP\text{-}Cre}$ and *Cbfb*$^{fl/+}$*Foxp3*$^{YFP\text{-}Cre}$ mice. (**d**) Expression of activation markers on CD4^{+}Foxp3^{-} T cells from the LN and spleen of 5–8 week old *Cbfb*$^{fl/fl}$*Foxp3*$^{YFP\text{-}Cre}$ (red lines) and littermate *Cbfb*$^{fl/+}$*Foxp3*$^{YFP\text{-}Cre}$ (blue lines) mice. The numbers indicate mean and standard deviation and represent at least three independent experiments.

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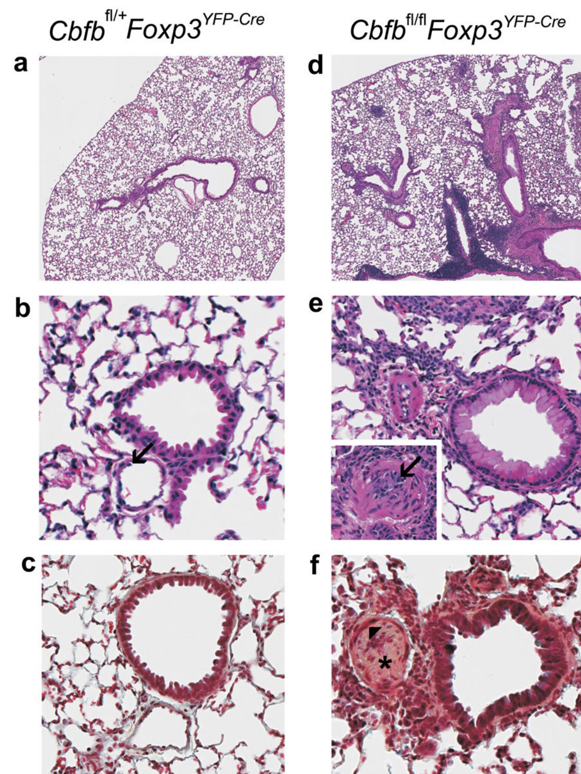

Figure 2.

Histopathology in *Cbfb*$^{fl/fl}$*Foxp3*$^{YFP-Cre}$ mice. Representative histological sections of lungs from female **(a–c)** *Cbfb*$^{fl/+}$*Foxp3*$^{YFP-Cre}$ and **(d–f)** *Cbfb*$^{fl/fl}$*Foxp3*$^{YFP-Cre}$ mice. Sections were stained with (**a, b, d, e**) hematoxylin and eosin or (**c, f**) Movat's pentachrome. Asterisk highlights the thickened vessel wall containing expanded collagen and the arrow points to the thickened layer of smooth muscle cells. Original magnification ×5 (**a,d**) and ×20 (**b, c, e, f**). Data shown is representative of four mice in each group.

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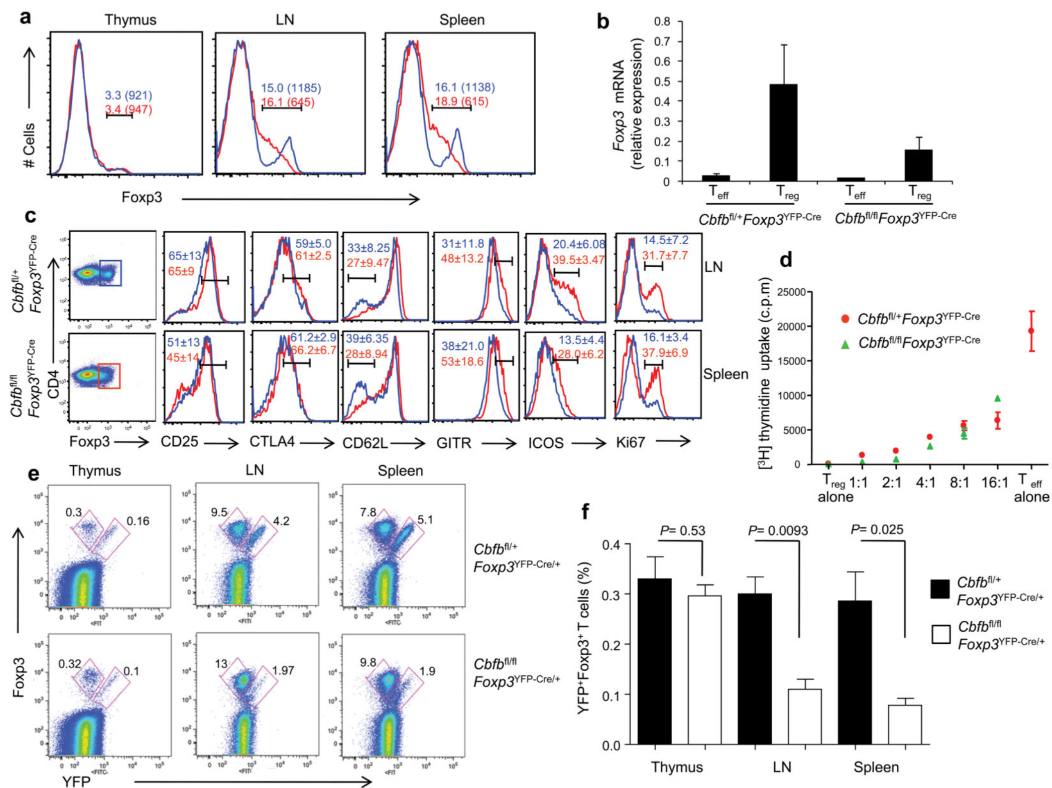

Figure 3.
Characterization of T_{reg} cells from *Cbfb*$^{fl/fl}$*Foxp3*$^{YFP\text{-}Cre}$ mice. **(a)** Flow cytometry of Foxp3 expression in $CD4^+CD8^-$ T cells derived from thymus, LN and spleen of *Cbfb*$^{fl/fl}$*Foxp3*$^{YFP\text{-}Cre}$ (red lines) and littermate *Cbfb*$^{fl/+}$*Foxp3*$^{YFP\text{-}Cre}$ (blue lines) mice. Numbers over bracketed lines indicate percentages of $Foxp3^+$ cells, with the mean fluorescence intensity (MFI) indicated in parentheses. Data represent analyses of at least six different mice in each group. **(b)** Real-time PCR analyses of Foxp3 mRNA normalized against HPRT1 mRNA in sorted $CD4^+YFP^-CD62L^{hi}$ (T_{eff}) or $CD4^+YFP^+$ (T_{reg}) cells from the indicated mice. Data are shown as averages of three independent experiments. **(c)** Expression of activation markers on peripheral $CD4^+Foxp3^+$ T_{reg} cells from *Cbfb*$^{fl/fl}$*Foxp3*$^{YFP\text{-}Cre}$ (red lines) and littermate *Cbfb*$^{fl/+}$*Foxp3*$^{YFP\text{-}Cre}$ (blue lines) mice. The numbers indicate mean and standard deviations and represent at least three independent experiments. Representative $CD4^+Foxp3^+$ gates are shown in the extreme left panel. **(d)** $CD4^+YFP^+$ T cells sorted from a pool of LNs and spleens of the *Cbfb*$^{fl/fl}$*Foxp3*$^{YFP-}$ and *Cbfb*$^{fl/+}$*Foxp3*$^{YFP\text{-}Cre}$ mice were co-cultured with $CD4^+YFP^-$ responder T cells from wild-type mice at the ratios indicated for 72 hours in the presence of anti-CD3 and irradiated T cell-depleted splenocytes. The data are shown as mean [^{3}H]-thymidine incorporation in triplicate cultures and represent one of two independent experiments. **(e)** Random X chromosome inactivation in *Cbfb*$^{fl/fl}$*Foxp3*$^{YFP\text{-}Cre/+}$ mice results in YFP+ and YFP^- populations of T_{reg} cells. Plots show gated $CD4^+$ T cells in *Cbfb*$^{fl/fl}$*Foxp3*$^{YFP\text{-}Cre/+}$ and *Cbfb*$^{fl/+}$*Foxp3*$^{YFP\text{-}Cre/+}$ littermates. **(f)** Bar graphs representing mean frequencies of YFP^+Foxp3^+ cells in indicated groups of mice. Data represent three mice per group.

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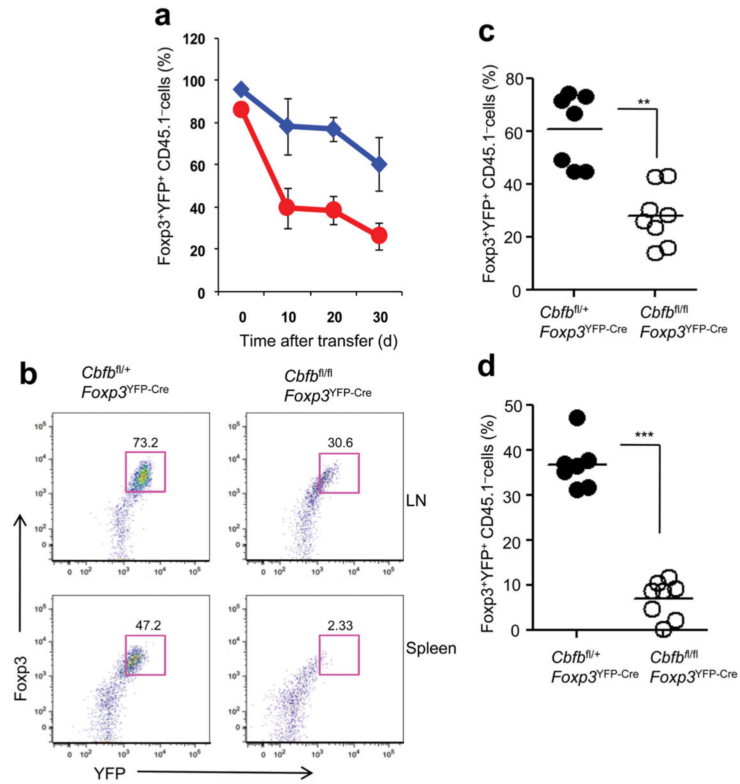

Figure 4.

CBFβ is required for the maintenance of Foxp3 expression in T_{reg} cells. FACS sorted CD4+YFPhi T cells (~90% purity) isolated from *Cbfb*fl/fl*Foxp3*YFP-Cre and *Cbfb*fl/+*Foxp3*YFP-Cre mice were mixed with purified CD4+CD45.1+ *Foxp3*− T cells at a 1:2 ratio and injected into *Rag2*−/− recipients. (**a**) Percentages of Foxp3+CD45.1− cells among CD45.1−CD4+ T cells in peripheral blood were assessed on the indicated days after transfer (red circles, *Cbfb*fl/fl*Foxp3*YFP-Cre donors; blue diamonds, *Cbfb*fl/+*Foxp3*YFP-Cre donors). Data represent one of two independent experiments. (**b**) Representative dot plot gated on CD4+CD45.1− cells in LN and spleen of recipient mice on day 40 after transfer. **(c, d)** Percentages of YFP+Foxp3+CD45.1− cells among CD45.1−CD4+ T cells of the indicated genotypes in LNs ($P = 0.0015$) (c) and spleens ($P<0.0001$) (d) of recipient mice. Each circle represents one recipient mouse. Results represent two independent experiments.

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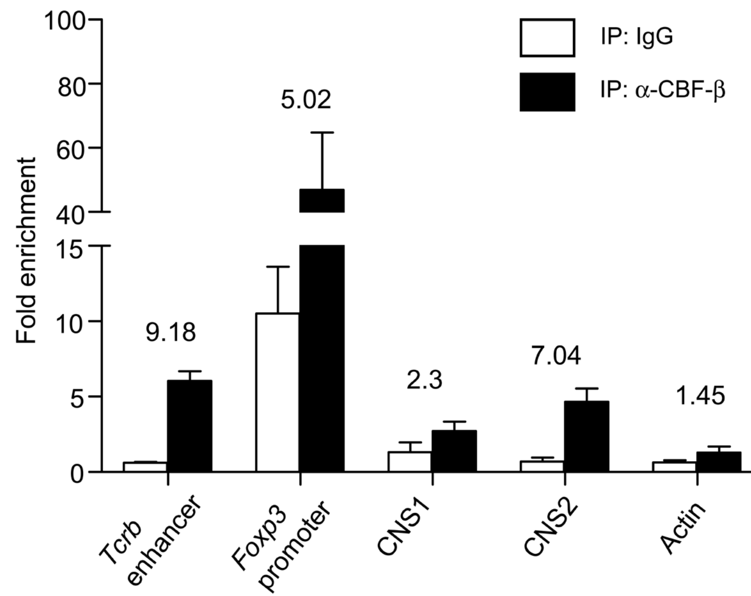

Figure 5.

Runx-CBFβ complexes occupy the Foxp3 promoter and a conserved non-coding sequence element within the Foxp3 locus. Chromatin immunoprecipitation was performed using magnetically purified $CD4^+CD25^+$ T_{reg} cells and rabbit anti-CBFβ or normal rabbit IgG. Data are shown as averages of at least three independent experiments. Numbers on the top of the bar graphs represent the ratio of fold-enrichment between anti-CBFβ and IgG pull-downs.

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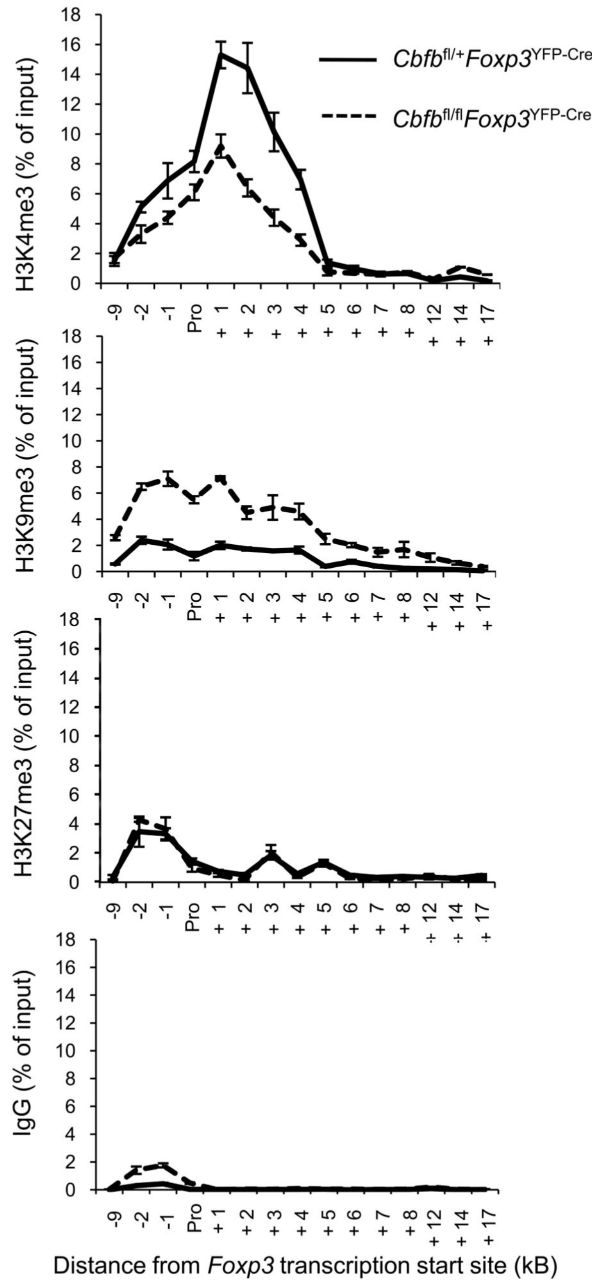

Figure 6.

CBFβ ablation in T_{reg} cells results in a decrease in permissive H3K4me3 and a concomitant increase in non-permissive H3K9me3 chromatin modifications at the Foxp3 locus. Magnetically purified $CD4^{+}CD25^{+}$ T_{reg} cells from *Cbfb*$^{fl/fl}$*Foxp3*$^{YFP-Cre}$ (broken line) and *Cbfb*$^{fl/+}$*Foxp3*$^{YFP-Cre}$ littermate control mice (solid line) were used for ChIP-qPCR analysis of H3K4me3, H3K9me3 and H3K27me3 modifications across the *Foxp3* locus. Chromatin-bound DNA was immunoprecipitated using antibodies specific for H3K4me3 H3K9me3, H3K27me3 or control IgG and probed using primer pairs corresponding to the indicated regions of the *Foxp3* locus. Relative distances (kB) of primer probes from the *Foxp3*

transcription start site (TSS) are indicated on X-axis. TSS is set as 0. Pro: *Foxp3* promoter. Data shown are representative of two or more experiments.

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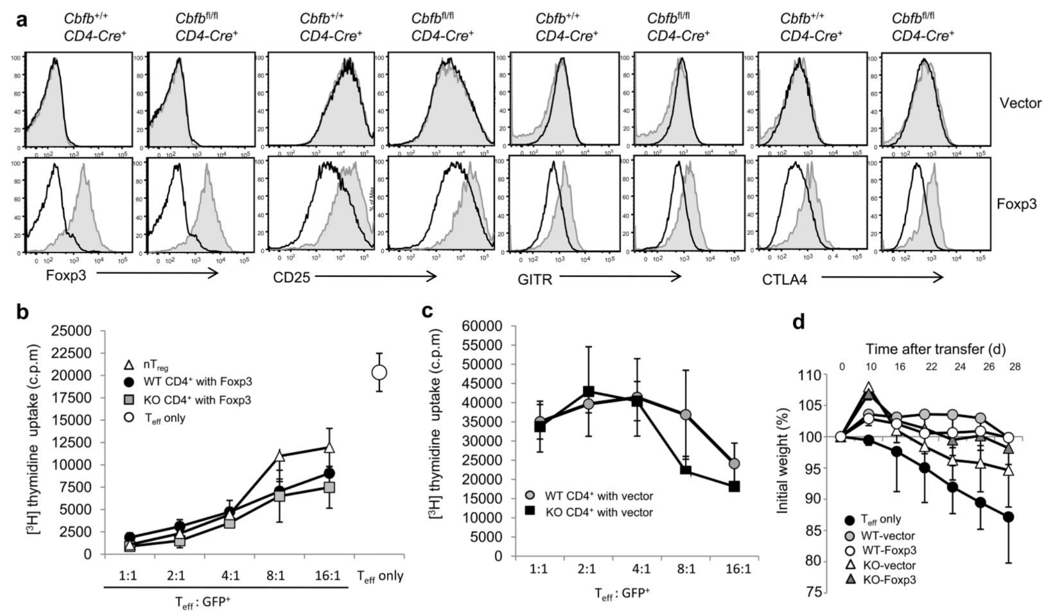

Figure 7.

Ectopic expression of Foxp3 in peripheral CBFβ-deficient T-cells induces a characteristic T_{reg} cell phenotype. $CD4^+$ T cells from *Cbfb*$^{fl/fl}$*CD4-Cre*$^+$ or *Cbfb*$^{+/+}$*CD4-Cre*$^+$ littermate mice were retrovirally transduced with MigR1-Foxp3 or the control vector. **(a)** $CD4^+CD8^-GFP^+$ (shaded) and $CD4^+CD8^-GFP^-$ (open) cells were analyzed for the expression of the indicated markers. Data representative of three independent experiments. **(b, c)** Suppressor capacity of sorted $CD4^+CD8^-GFP^+$ cells from MigR1-Foxp3 (b) or control vector (c) transduced cultures was assessed by co-culturing them with $CD4^+Foxp3^-$ T_{eff} cells at the indicated ratios. Data are shown as mean [^{3}H]-thymidine incorporation in triplicate cultures and are representative of three independent experiments. **(d)** Treg cells purified from *Cbfb*$^{fl/fl}$*Foxp3*$^{YFP-Cre}$ or *Cbfb*$^{fl/+}$*Foxp3*$^{YFP-Cre}$ control mice and transduced with the hCD2 encoding MigR2-Foxp3 or "empty" MigR2 vector were analyzed for their in vivo suppressive capacity as described in "Methods". Percentage change in initial weight of the recipients was monitored over indicated period of time. Each data point is an average change in weight of 4 to 5 recipient mice from two independent experiments. *P* values for difference in weight loss calculated by unpaired one-tailed Student's t-test: "WT-vector" vs "T_{eff} only" after 24 days P= 0.0003, 26 days P= 0.0005, and 28 days P= 0.004. "WT-vector" vs "KO-Vector" after 24 days P= 0.002, 26 days P= 0.0185, and 28 days P= 0.08. "WT-vector" vs "KO-Foxp3" after 24 days P= 0.02, 26 days P= 0.12, and 28 days P= 0.23. "KO-Foxp3" vs "KO-vector" after 24 days P= 0.065, 26 days P= 0.051, and 28 days P= 0.125.