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Transcription factors RUNX1 and RUNX3 in the induction and suppressive function of Foxp3$^+$ inducible regulatory T cells

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Forkhead box P3 (FOXP3)$^+$CD4$^+$CD25$^+$ inducible regulatory T (iT reg) cells play an important role in immune tolerance and homeostasis. In this study, we show that the transforming growth factor-β (TGF-β) induces the expression of the Runt-related transcription factors RUNX1 and RUNX3 in CD4$^+$ T cells. This induction seems to be a prerequisite for the binding of RUNX1 and RUNX3 to three putative RUNX binding sites in the *FOXP3* promoter. Inactivation of the gene encoding RUNX cofactor core-binding factor-β (CBFβ) in mice and small interfering RNA (siRNA)-mediated suppression of RUNX1 and RUNX3 in human T cells resulted in reduced expression of Foxp3. The in vivo conversion of naive CD4$^+$ T cells into Foxp3$^+$ iT reg cells was significantly decreased in adoptively transferred *Cbfb$^{F/F}$ CD4-cre* naive T cells into *Rag2$^{-/-}$* mice. Both RUNX1 and RUNX3 siRNA silenced human T reg cells and *Cbfb$^{F/F}$ CD4-cre* mouse T reg cells showed diminished suppressive function in vitro. Circulating human CD4$^+$ CD25^{high} CD127$^-$ T reg cells significantly expressed higher levels of RUNX3, FOXP3, and TGF-β mRNA compared with CD4$^+$CD25$^-$ cells. Furthermore, FOXP3 and RUNX3 were colocalized in human tonsil T reg cells. These data demonstrate Runx transcription factors as a molecular link in TGF-β–induced Foxp3 expression in iT reg cell differentiation and function.

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Abbreviations used: AML, acute myeloid leukemia; CBF, core-binding factor; ChIP, chromatin immunoprecipitation; iT reg, inducible T reg; NFAT, nuclear factor of activated T cells; nT reg, natural T reg; PEBP2α, polyoma enhancer-binding protein-2α; siRNA, small interfering RNA; T reg, regulatory T; TSS, transcription start site.

Regulatory T (T reg) cells expressing the transcription factor forkhead box P3 (FOXP3, human; Foxp3, mouse) play an essential role in controlling immune responses to autoantigens, allergens, tumor antigens, transplantation antigens, and infectious agents (Hori et al., 2003; Akdis, 2006). Foxp3 is a member of the forkhead/winged-helix family of transcriptional regulators, and its expression in T reg cells is essential for their development and function (Fontenot et al., 2003; Williams and Rudensky, 2007). A spontaneous mutation of the X-linked *Foxp3* gene in *scurfy* mice causes an autoimmune-like disease, whereas the mutation in humans leads to immunodysregulation, polyendocrinopathy, enteropathy, and X-linked syndrome that is also a severe multiorgan autoimmune disease with hyper-IgE (Ziegler, 2006).

Although the essential role of Foxp3 in central and peripheral tolerance has been extensively studied, its regulation, cooperation with other transcription factors, and how it functions in inducible T reg (iT reg) cells to suppress various target genes is mostly not yet understood. It is known that Foxp3 cooperates with the nuclear factor of activated T cells (NFAT) or nuclear factor-kappa B (NF-κB) to regulate the transcription of different target genes (Schubert et al., 2001; Bettelli et al., 2005; Wu et al., 2006). The Th2 cytokine IL-4 inhibits FOXP3 expression during T cell priming. GATA3 binds to the *FOXP3* promoter

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and can repress the FOXP3 trans-activation process directly in Th2 cells (Mantel et al., 2007). It was further demonstrated that both Th1 and Th2 transcription factors T-bet and GATA3 oppose peripheral induction of Foxp3$^+$ T reg cells in mice through STAT1-, STAT4-, and STAT6-dependent pathways (Wei et al., 2007). Although natural T reg (nT reg) cells that differentiate in the thymus are characterized by their stable Foxp3 expression, the generation of iT reg cells specific for allergens, alloantigens, and autoantigens in the periphery has been associated with a transient Foxp3$^+$ phenotype (Fontenot et al., 2003; Hori et al., 2003). The crucial role of TGF-β in their generation has been demonstrated.

The RUNX gene family (Runt-related transcription factor, acute myeloid leukemia [AML], core-binding factor-α [CBFα], and polyoma enhancer-binding protein-2α [PEBP2α]) contains three members, RUNX1 (AML1/CBFA2/PEBP2αB), RUNX2 (AML3/CBFA1/ PEBP2αA), and RUNX3 (AML2/CBFA3/PEBP2αC). They are essential transcriptional regulators of different developmental pathways. RUNX2 is mostly important for bone development and osteoblast differentiation (Komori et al., 1997). RUNX1 plays an important role in hematopoiesis during development, and RUNX3 has important functions in thymogenesis and neurogenesis (Wang et al., 1996; Inoue et al., 2002; Levanon et al., 2002). RUNX1 and RUNX3 also work together in the establishment of lineage specification of T lymphocytes (Taniuchi et al., 2002; Egawa et al., 2007). *RUNX1* is a frequent target for chromosomal translocations associated with leukemias (Look, 1997), and *RUNX3* methylation and silencing is observed in various human epithelial cancers (Blyth et al., 2005).

RUNX family members share the Runt domain, which is responsible for DNA binding (Ito, 1999). The Runt domain-containing protein constitutes the α-chain partner of the heterodimeric CBF complex. RUNX proteins heterodimerize with the non–DNA-binding partner, CBFβ, which increases the affinity for DNA binding and stabilizes the complex by preventing ubiquitin-dependent degradation (Wang et al., 1993). The CBF complexes regulate the expression of cellular genes through binding to promoters or enhancer elements. The effects of the RUNX–CBFβ complex regulation are clearly cell lineage and stage specific. They include the crucial choices between cell-cycle exit and continued proliferation, as well as between cell differentiation and self-renewal (Blyth et al., 2005).

Because of the involvement of RUNX mutations in different autoimmune diseases and the known interaction with TGF-β, we investigated the impact of RUNX1 and RUNX3 on the expression of FOXP3 and subsequently on the development and function of iT reg cells. This study demonstrates that RUNX1 and RUNX3 induced by TGF-β are involved in the development and suppressive function of Foxp3$^+$ iT reg cells.

RESULTS

The role of RUNX1 and RUNX3 transcription factors in TGF-β–mediated iT reg cell generation

To investigate the role of RUNX transcription factors in the development of iT reg cells, we cultured naive CD4$^+$ T cells, isolated from human PBMCs, in conditions that enable the development of iT reg cells. Stimulation with anti-CD2/3/28 mAbs or TGF-β alone resulted in a minimal up-regulation of RUNX1 and RUNX3 mRNA (Fig. 1 A). In contrast, the combination of both TGF-β and anti-CD2/3/28 mAbs induced RUNX1 and RUNX3 mRNAs, as well as FOXP3 mRNA, within 48 h in naive CD4$^+$ T cells. This result suggested further experiments to investigate whether the up-regulation of RUNX1 and RUNX3 might be a feature of iT reg cells during their development or even a prerequisite for their induction.

To test this hypothesis, RUNX1 and RUNX3 expression was knocked down in human naive CD4$^+$ T cells by transfection of small interfering RNAs (siRNAs; Fig. 1 B). Deficiency of RUNX1 or RUNX3 resulted in markedly reduced TGF-β–mediated induction of FOXP3 mRNA in naive CD4$^+$ T cells compared with control cells transfected with scrambled siRNA. The level of FOXP3 mRNA was further reduced when both RUNX1 and RUNX3 were knocked down in naive CD4$^+$ T cells during their differentiation to iT reg cells (Fig. 1 B).

The influence of RUNX1 and RUNX3 on the development of other T cell subsets and their specific transcription factor expression was further investigated. Naive CD4$^+$ T cells were cultured under Th1, Th2, T reg cell, and Th17 differentiation conditions and the mRNA expression of the predominant transcription factor for each cell type was subsequently analyzed. We observed no change in GATA3 expression in Th2 cells, T-bet expression in Th1 cells, or RORC2 mRNA expression in Th17 cells in which RUNX1 and RUNX3 were knocked down compared with control cells. On the contrary, FOXP3 mRNA was significantly decreased in RUNX1- and RUNX3-deficient T reg cells compared with control cells (Fig. 1 C).

The effect of RUNX silencing on the expression level of intracellular FOXP3 during naive CD4$^+$ T cell differentiation to iT reg was evaluated by flow cytometry. FOXP3 was only slightly reduced after RUNX1 silencing. Transfection of siRNA for RUNX3 had a stronger effect. The most striking FOXP3 reduction was observed when RUNX1 and RUNX3 were silenced together (Fig. 1 D). Similar results were obtained in total CD4$^+$ T cells (Fig. S1 and Fig. S2). The increased impact of combined RUNX1 and RUNX3 knockdown implies that RUNX1 and RUNX3 might have redundant functions in the induction of FOXP3. In addition, the levels of IL-4, IL-5, IL-10, IL-13, and IFN-γ in control siRNA-transfected or RUNX1 and RUNX3 siRNA-transfected CD4$^+$ T cells that were cultured with or without anti-CD2/3/28 mAb and TGF-β did not show any significant difference (Fig. S3).

To determine whether RUNX1 and RUNX3 are also expressed in human T reg cells in vivo, we isolated peripheral blood CD4$^+$ CD127$^-$ CD25^{high} T reg cells and compared them with CD4$^+$ CD127$^+$ CD25$^-$ T cells. Circulating T reg cells expressed significantly higher levels of RUNX3 mRNA compared with CD4$^+$CD25$^-$ cells. As expected, IL-10, TGF-β,

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and FOXP3 mRNAs are also expressed in circulating T reg cells (Fig. 2 A). There was no difference in RUNX1 mRNA expression between these two cell subsets. We also performed an analysis of human tonsils, which contain high numbers of FOXP3+ T reg cells (Verhagen et al., 2006). Staining of tonsil sections for FOXP3 and RUNX3 demonstrated in vivo

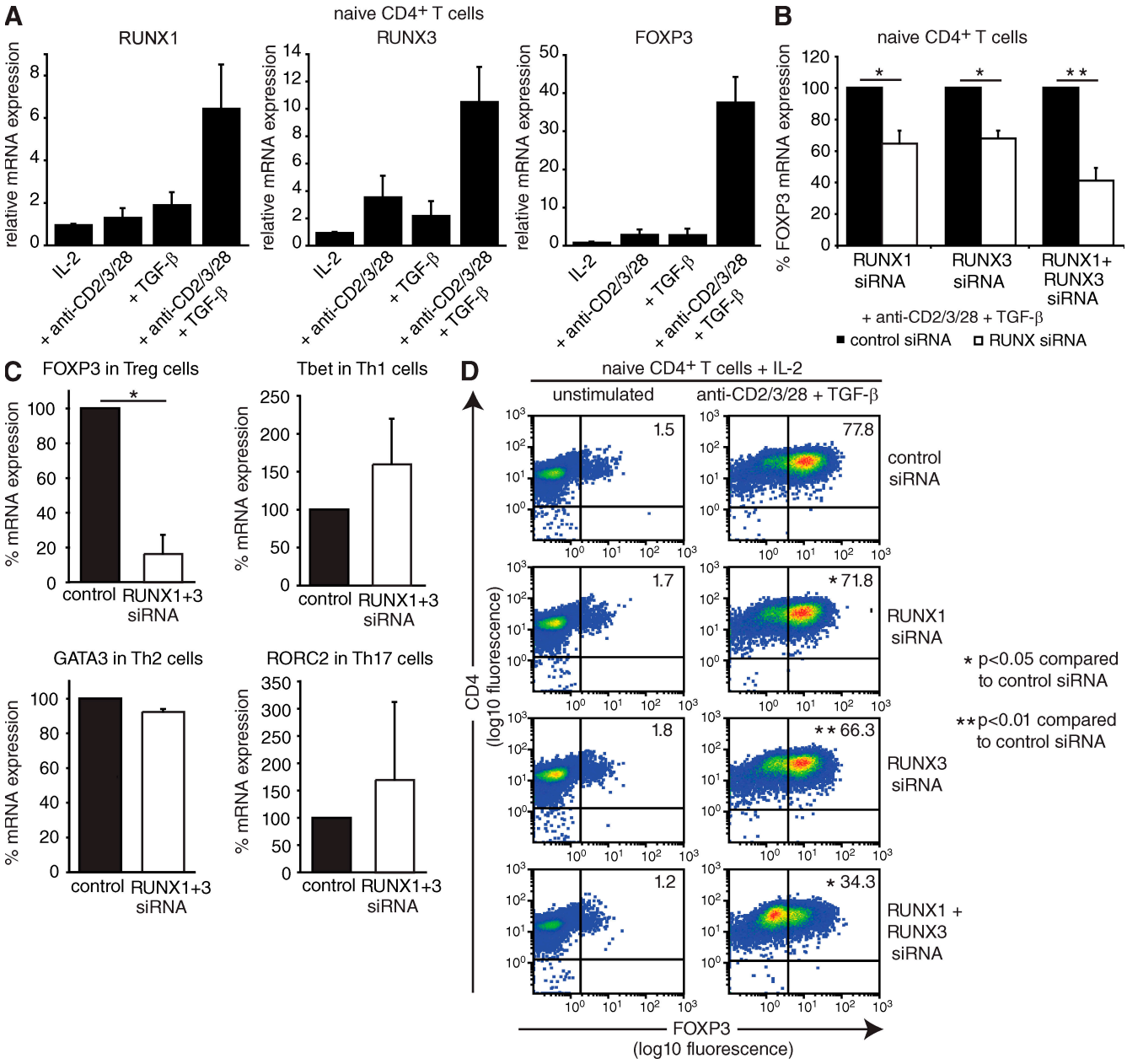

Figure 1. RUNX1 and RUNX3 are involved in the induction of Foxp3 in iT reg cells. (A) RUNX1, RUNX3, and FOXP3 mRNA induction in human naive CD4+ T cells after alone or combined anti-CD2/3/28 mAb and TGF-β stimulation in the presence of IL-2. Real-time PCR of human naive CD4+ T cells after 48 h of culture. Bars show the mean ± SE of three independent experiments. (B) FOXP3 mRNA induction by anti-CD2/3/28 mAb and TGF-β in human naive CD4+ T cells is reduced after siRNA-mediated RUNX1/3 knockdown. Real-time PCR of RNA from human naive CD4+ T cells, transfected with RUNX1 and/or RUNX3 siRNA or with a control siRNA and cultured with anti-CD2/3/28, TGF-β and IL-2. Bars show the mean ± SD of three independent experiments. (C) FOXP3 mRNA is down-regulated in iT reg cells after siRNA-mediated knockdown of RUNX1 and RUNX3. Real-time PCR for FOXP3, T-bet, GATA3, and RORC2 from human naive CD4+ T cells, transfected with RUNX1 and RUNX3 siRNA or with scrambled siRNA (control) and cultured under iT reg, Th1, Th2, or Th17-driving conditions for 12 d. Bars show the mean ± SD of three independent experiments. (D) FOXP3 protein induction in iT reg cells is reduced after siRNA-mediated RUNX1/3 knockdown. Human naive CD4+ T cells were transfected with RUNX1 and/or RUNX3 siRNA or with a control siRNA and stimulated with anti-CD2/3/28 and TGF-β in the presence of IL-2. CD4 and intracellular FOXP3 analysis by flow cytometry after 72 h. One of three independent experiments is shown. Statistical differences were verified by the paired Student's *t* test. *, $P < 0.05$; **, $P < 0.01$.

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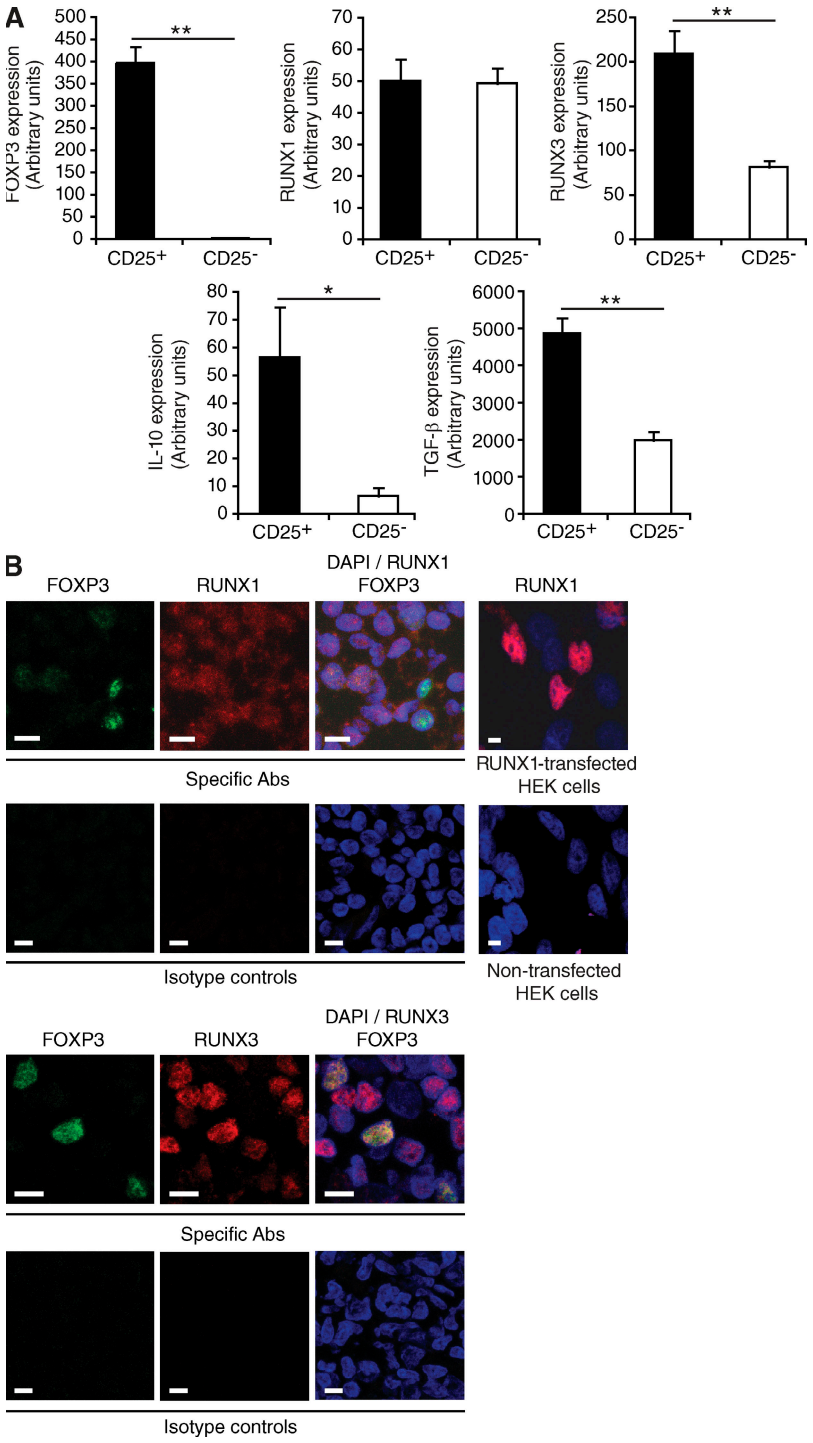

Figure 2. RUNX1 and RUNX3 expression in Foxp3⁺ T reg cells and in human CD4⁺, CD127⁻, CD25^{high} cells. (A) Real-time PCR analysis of CD4⁺, CD127⁻, and CD25^{high} T reg cells and CD4⁺, CD127⁺, and CD25^{neg} T cells isolated from human peripheral blood showed an increased expression of IL-10, TGF-β, FOXP3, and RUNX3 mRNA in CD25⁺ compared with CD25⁻ cells. Bars show the mean ± SE of three independent experiments. (B) Human tonsil sections were analyzed by confocal microscopy. Tissue sections were stained for FOXP3, RUNX1, RUNX3, and DAPI or isotype controls. HEK cells RUNX1-transfected or not transfected served as additional control for RUNX1 staining. Data shown are representative from one of the three tissue samples with similar results. Bars, 5 µm. Statistical differences were verified by the paired Student's *t* test. *, $P < 0.05$; **, $P < 0.01$.

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coexpression of these two molecules in a subset of T reg cells, whereas there was low RUNX1 expression in all tonsil cells (Fig. 2 B).

RUNX1 and RUNX3 bind to the *FOXP3* promoter

Transcription element search system analysis of the human *FOXP3* promoter predicted 3 putative RUNX binding sites at 333, 287, and 53 bp upstream of the transcription start site (TSS). All three binding sites are conserved between human, mouse, and rat (Fig. S4). To verify the putative binding sites in the *FOXP3* promoter, we transiently transfected HEK293T cells with RUNX1 and RUNX3. After the pull-down with oligonucleotides containing the wild-type binding sequences, but not mutant sequences, RUNX binding to the *FOXP3* promoter oligonucleotides was detected by Western blot (Fig. 3 A). To confirm these results and test the ability of single binding site sequences to bind either RUNX1 or RUNX3, we used the promoter enzyme immunoassay. Cell lysates were obtained from HEK293T cells that had been transiently transfected with RUNX1 or RUNX3 expression vectors. The biotinylated *FOXP3* promoter oligonucleotides were linked to a streptavidin-coated microtiter plate, and bound RUNX1 or RUNX3 was detected by using anti-RUNX antibodies and a peroxidase-labeled secondary antibody. We showed binding of RUNX1 and RUNX3 to the mixture of all three oligonucleotides containing the binding sites, whereas there was no binding detectable when a combination of the mutated oligonucleotides was used in the assay (Fig. 3 B). Although there was a similar and high degree of binding to the −333 and −287 sites, a lower degree of binding was detected when the oligonucleotide containing the −53 site in the Foxp3 promoter was used. This effect was observed both for binding to RUNX1 and RUNX3 (Fig. 3 B). The binding to the two single binding sites at −333 and −287 was comparable to the mixture of all three oligonucleotides. Chromatin immunoprecipitation (ChIP) assay results confirmed the binding of RUNX1 and RUNX3 complexes containing CBFβ to *FOXP3* promoter during the differentiation of naive T cells toward T reg cells. Here, naive $CD4^+$ T cells were cultured with IL-2, anti-CD2/3/28 mAb, and TGF-β as a Foxp3-inducing stimulation. Amplification of PCR products from the *FOXP3* promoter region with the predicted RUNX binding sites showed that RUNX1, RUNX3, and CBFβ were immunoprecipitated together with the *FOXP3* promoter (Fig. 3 C). Negative control primer targeting open reading frame-free intergenic DNA, IGX1A did not show any significant change in site occupancy.

Regulation of *FOXP3* promoter activity and FOXP3 protein expression by RUNX1 and RUNX3

To investigate the effect of RUNX1 and RUNX3 binding to the RUNX binding sites in the *FOXP3* promoter, we transfected human peripheral blood $CD4^+$ T cells with a *FOXP3* promoter luciferase reporter vector and RUNX1 or RUNX3 expression vectors. An increase in luciferase activity was observed only when the *FOXP3* promoter (−511 to +176) luciferase construct was cotransfected with RUNX1 or RUNX3 expression vectors (Fig. 4 A). The increase in promoter activity was greater upon cotransfection of RUNX3 compared with RUNX1. Luciferase expression was abrogated when the Runx binding sites in the *FOXP3* promoter (−511 to +176) luciferase construct were mutated (Fig. 4 A). In these experiments, the overexpression of RUNX1 and RUNX3 eliminated the need of TGF-β for FOXP3 promoter activation and PMA/ionomycin stimulation was sufficient.

To examine the role of each of the three RUNX binding sites for the *FOXP3* promoter activity, we mutated each individually or in combination. No reduction in luciferase activity was observed when the −53 site was mutated and only a slight reduction when either the −287 or −333 site was mutated (Fig. 4 B). However, mutating the −53 site in combination with one of the other two sites led to a significant decrease in luciferase activity, with the greatest reduction observed when all three binding sites were mutated (Fig. 4 B), suggesting that the identified binding sites have redundant functions and RUNX binding to more than one site is necessary for the full activation of the *FOXP3* promoter. Supporting these findings, the overexpression of RUNX1 in human primary $CD4^+$ T cells resulted in significantly elevated levels of FOXP3 protein measured by flow cytometry after 48 h. This was achieved without any requirement for anti-CD3, anti-CD28 stimulation, or the presence of TGF-β. Although there was a trend, the transfection of $CD4^+$ T cells with RUNX3 did not lead to statistically significant increase in FOXP3 (Fig. S5).

Role of CBFβ in the induction of Foxp3

CBFβ, a common cofactor of all RUNX proteins, stabilizes and increases the binding of the runt domain to target DNA sequences. To target all Runx proteins that might be involved in the induction of Foxp3, we used mice in which loxP-flanked *Cbfb* alleles were inactivated in T cells through expression of a *CD4-cre* transgene. Retinoic acid and TGF-β synergize in the induction of Foxp3 in naive T cells (Kang et al., 2007). To investigate whether Runx-mediated induction of Foxp3 is dependent on the expression of CBFβ, naive $CD4^+$ $CD8^-$ T cells from $Cbfb^{F/F}$ *CD4-cre* and control $Cbfb^{F/+}$ *CD4-cre* mice were stimulated with anti-CD3/28 mAbs, retinoic acid, and increasing concentrations of TGF-β. After 3 d in culture, the cells were restimulated with PMA and ionomycin and analyzed for intracellular Foxp3 and IFN-γ expression. TGF-β induced Foxp3 in $Cbfb^{F/+}$ *CD4-cre* cells in a dose dependent manner, and this was significantly reduced in $Cbfb^{F/F}$ *CD4-cre* cells. Retinoic acid enhanced Foxp3 expression even in 20 pg/ml of TGF-β and more than 95% of the $CD4^+$ T cells from $Cbfb^{F/+}$ *CD4-cre* mice became $Foxp3^+$ in 100 and 500 pg/ml TGF-β doses. The induction of Foxp3 was again significantly lower in $Cbfb^{F/F}$ *CD4-cre* $CD4^+$ T cells even in the presence of retinoic acid, demonstrating that deficiency in Runx binding to DNA affects the TGF-β induction of Foxp3 in T reg cells (Fig. 5 A). There was no difference in the induction of Foxp3 when endogenous IL-4 and IFN-γ were neutralized (Fig. S6).

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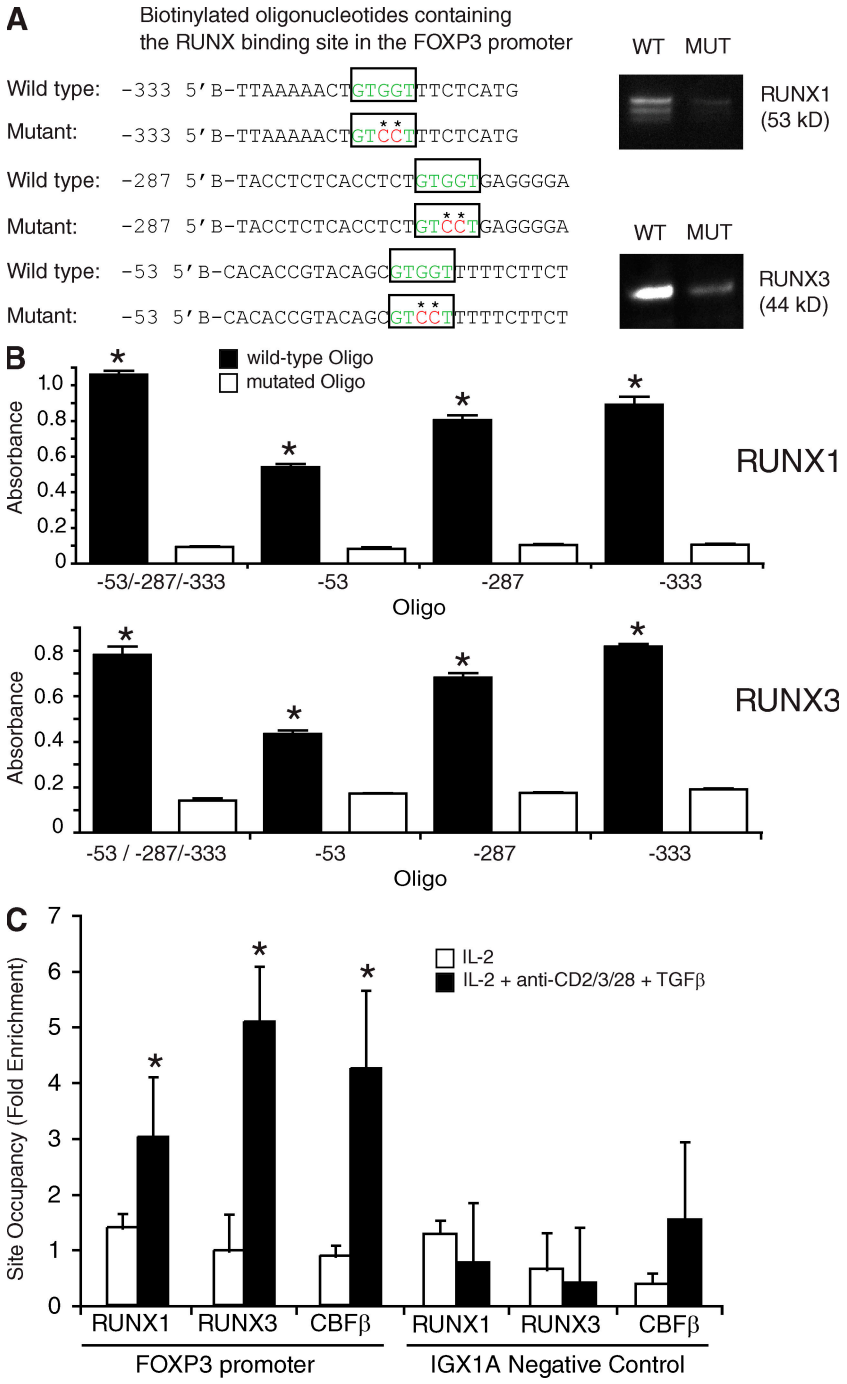

Figure 3. Binding of RUNX1 and RUNX3 proteins to the predicted binding sites in the *FOXP3* promoter. (A) Mutated and wild-type oligonucleotides are shown. The predicted RUNX binding sites are accentuated (boxed and in green letters) and stars mark mutations introduced into the binding site of the control oligonucleotides. Nuclear extracts from HEK293T cells were incubated with biotinylated oligonucleotides. The precipitated oligonucleotide–transcription factor complexes were separated by SDS-PAGE and identified by Western blotting with anti-RUNX1 and anti-RUNX3 antibodies. A mixture of all three oligonucleotides with the predicted binding sites or with the inserted mutation into the predicted sites was used. Data shown are one representative of three independent experiments with similar results. (B) Promoter enzyme immunoassay using wild-type and mutated oligonucleotides within the *FOXP3* promoter. Bars show mean ± SE of three independent experiments. (C) Chromatin immunoprecipitation assay results show binding of RUNX1 and RUNX3 complexes containing CBFβ to the human *FOXP3* promoter in naive $CD4^+$ T cells that were cultured with IL-2 together with anti-CD2/3/28 and TGF-β. There was no change in site occupancy in all immunoprecipitations when IGX1A negative control primers were used. The results are normalized to input and isotype control antibody. Bars show mean ± SE of three independent experiments. Statistical differences were verified by the paired Student's *t* test. *, $P < 0.05$

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The importance of RUNX transcription factors for the in vivo conversion of naive CD4+ T cells into iT reg cells was examined. Control *Cbfb*$^{F/F}$ or *Cbfb*$^{F/F}$ *CD4-cre* naive T cells, harboring a *Foxp3-IRES-GFP* allele were adoptively transferred into *Rag2*$^{-/-}$ mice. 6 wk later, CD4+ T cells in spleen, mesenteric lymph node, and lamina propria of the small intestine were analyzed for Foxp3-GFP expression (Fig. 5 B). There was a consistently lower percentage of CD4+ T cells that had developed Foxp3 expression in the mesenteric lymph node and lamina propria of mice transferred with *Cbfb*$^{F/F}$ *CD4-cre* cells compared with control cells (Fig. 5, B and C). These data affirm the significance of RUNX proteins for the in vivo generation of CD4+ Foxp3+ T cells.

CBFβ, RUNX1, and RUNX3 are important for the suppressive activity of CD25+ Foxp3+ T reg cells

Even though *Cbfb*-deficient CD4+ T cells had impaired induction of Foxp3 after stimulation with anti-CD3/28 mAbs and TGF-β, sufficient numbers of Foxp3+ cells could be generated to permit analysis of their suppressive activity. Purified naive CD4+ T cells from *Cbfb*$^{F/F}$ *CD4-cre* and control *Cbfb*$^{F/+}$ *CD4-cre* mice (*Cd45.2*) harboring a *Foxp3-ires-GFP* allele (Bettelli et al., 2006) were stimulated in vitro with anti-CD3/28 mAbs, IL-2, and TGF-β. After 3 d, Foxp3+-GFP+ cells were sorted by flow cytometry and mixed with CFSE-labeled naive CD45.1+ CD4+ cells at ratios of 1:4, 1:2, and 1:1. The cells were then incubated with inactivated splenocytes and stimulated with anti-CD3 mAb for four more days. CD45.1+ cells were analyzed for CFSE dilution (Fig. 6 A). *Cbfb*$^{F/+}$ *CD4-cre* CD4+ T cells activated in the presence of TGF-β showed a clear suppression of T cell proliferation that became even more apparent when an increased ratio of FOXP3+/CD25− cells was used (Fig. 6 B). The suppression was significantly reduced when cells from *Cbfb*$^{F/F}$ *CD4-cre* mice were used, demonstrating that TGF-β–induced Runx complexes are important for the suppressive activity of Foxp3+ T reg cells (Fig. 6 B). As a control, *Cbfb*$^{F/+}$ *CD4-cre* CD4+ T cells activated in absence of TGF-β were mixed with CFSE-labeled naive CD45.1+ CD4+ cells. No suppression could be observed in all control groups without TGF-β at all tested ratios (Fig. 6 C). The decreased suppression capacity of *Cbfb*$^{F/F}$ *CD4-cre* iT reg cells was unlikely to be caused by decreased survival or proliferation. Foxp3+ and Foxp3− cells generated from both *Cbfb*$^{F/F}$ *CD4-cre* and control cells all displayed similar proliferation rates and cell death as measured by CFSE dilution and annexin V staining, respectively (Fig. S7, A and B).

In addition, we tested the requirement of RUNX1 and RUNX3 for the development of the suppressive capacity in human iT reg cells. We isolated human naive CD4+ T cells and transfected them with a combination of RUNX1 and RUNX3 siRNA, or with a scrambled control siRNA. Cells were cultured under T reg conditions, and then mixed with CFSE-labeled autologous CD4+ T cells and stimulated with anti-CD3 mAb. Cells in which RUNX1 and RUNX3 were knocked down showed markedly lower suppressive activity compared with control iT reg cells at a T reg/CD4+ T responder cell ratio of 1:20, but not when the T reg/CD4+ T responder cell ratio was increased to 1:5 (Fig. 6 D). These results demonstrate the important role of RUNX1 and RUNX3 not only for the induction of FOXP3, but also for the suppressive capacity of iT reg cells both in humans and in mice. The data suggest both quantity and quality of T reg cells are hampered. Reduced intrinsic suppressive capacity of iT reg cells was demonstrated in mice, because Foxp3-GFP+ cells were FACS sorted and same numbers of iT reg cells are included in control experiments. In the suppression experiment with human cells, reduced suppressive activity was caused by reduced FOXP3 expression in T cells by siRNA inhibition of RUNX1 and RUNX3.

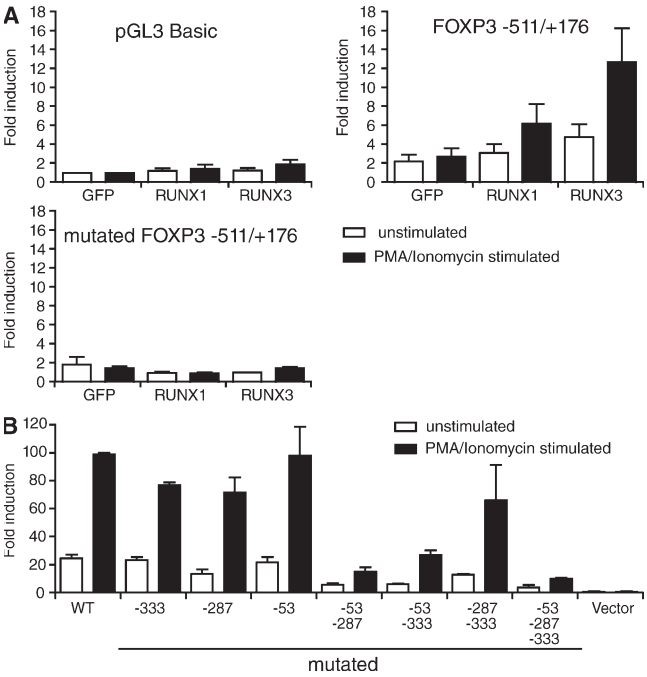

Figure 4. Regulation of *FOXP3* promoter activity by RUNX1 and RUNX3. (A) Human primary CD4+ cells were transfected with an empty vector (pGL3 Basic), a vector containing the wild-type or mutated *FOXP3* promoter region (FOXP3 −511/+176) fused to the luciferase reporter gene together with a GFP, RUNX1, or RUNX3 expression vector. Bars show the mean luciferase activity ± SE measured as arbitrary light units of three independent experiments. (B) Human primary CD4+ cells were transfected with an empty vector (pGL3 Basic), a vector containing the putative *FOXP3* promoter region (FOXP3 −511/+176) fused to the luciferase reporter gene, or with a vector containing the putative *FOXP3* promoter region (FOXP3 −511/+176) with single RUNX binding sites mutated (53, 287, or 333) or with the combination of two or three RUNX binding sites mutated (53, 287, or 333) fused to the luciferase reporter gene. Bars show the mean luciferase activity ± SD measured as arbitrary light units of three independent experiments.

DISCUSSION

This study demonstrates that RUNX transcription factors 1 and 3 play an important role in the generation of FOXP3+ iT reg cells by TGF-β. TGF-β mediates RUNX induction and FOXP3 is efficiently up-regulated by RUNX1 and RUNX3

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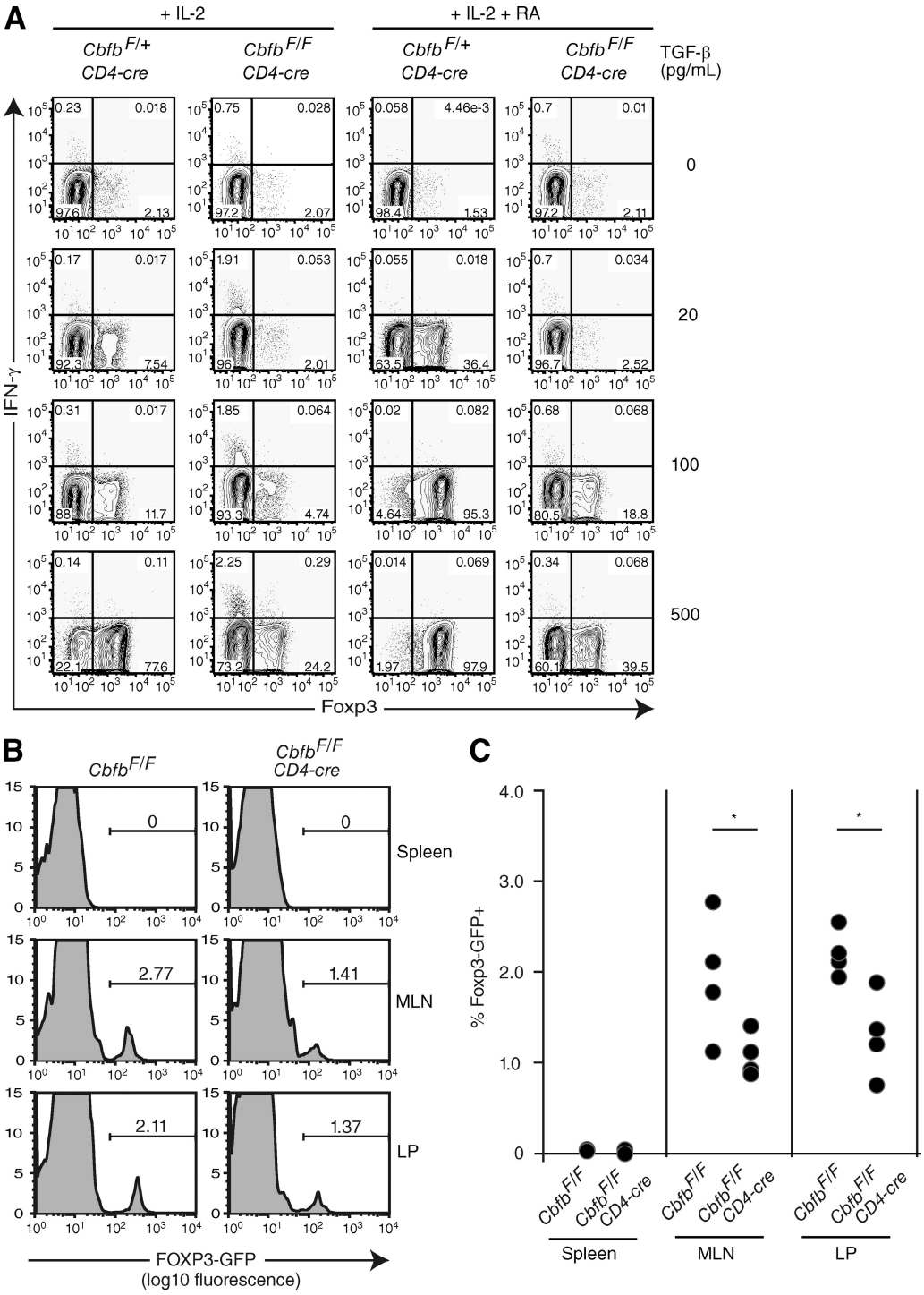

Figure 5. Diminished capacity of *Cbfb*-deficient *CD4-cre* mice T cells in the generation of Foxp3⁺ CD4⁺ T cells. (A) FACS-purified naive CD4⁺ CD8⁻ T cells from *Cbfb*$^{F/F}$ *CD4-cre* and control *Cbfb*$^{F/+}$ *CD4-cre* mice were activated in vitro with anti-CD3/28 mAb, 50 U/ml IL-2, ± 10 nM retinoic acid (RA), and increasing concentrations of TGF-β. After 3 d in culture, the cells were restimulated with PMA + ionomycin, and then analyzed for intracellular Foxp3 and IFN-γ expression. One of five experiments is shown. (B) Naive CD4⁺ T cells from *Cbfb CD4-cre* or control mice (harboring a *Foxp3-IRES-GFP* allele) were adoptively transferred into Rag-deficient mice (5×10^6 cells per transfer). 6 wk later, TCRβ⁺CD4⁺ cells from the spleen, mesenteric lymph node (MLN), and lamina propria of the small intestine (LP) were analyzed for Foxp3-GFP expression. Results from one of four *Cbfb*$^{F/F}$ *CD4-cre* and control *Cbfb*$^{F/+}$ *CD4-cre* mice with same findings are shown. The data from four sets of mice is shown in C. Statistical analysis was performed with Mann-Whitney *U* test. *, $P < 0.05$ between groups.

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in human CD4$^+$ T cells. There are three putative RUNX binding sites in the proximal *FOXP3* promoter. One binding site was predicted as a binding site for RUNX2. Promoter enzyme immunoassay results showed that binding of RUNX1 and RUNX3 also occurred at this site (as well as at the other two), which were initially identified as RUNX1 binding sites. This finding is not surprising because RUNX proteins bind to promoter or enhancer elements of their target genes via the runt domain, which is conserved between members of the RUNX family. The RUNX protein that actually induces the expression of FOXP3 might therefore be dependent on the availability of the specific RUNX family member at certain stages of T cell development. RUNX proteins are able to increase or inhibit transcriptional activity of their target genes depending on the cell type and the target gene (Otto et al., 2003). Mutation of only one of the three binding sites had only a little effect on the promoter activity; however, when two binding sites were mutated, the FOXP3 promoter activity dropped to a greater extent. The most striking effect was observed when all three binding sites were mutated. We therefore assume that these binding sites have partially redundant functions, but binding to at least two sites seems to be necessary for full promoter activation.

TGF-β promotes or inhibits the proliferation, differentiation, and survival of a wide array of different cells. It is also produced in activated T cells and it inhibits T cell proliferation (Kehrl et al., 1986; Siegel and Massagué, 2003). It was shown that TGF-β is mandatory for the maintenance of peripheral T reg cells and their expression of Foxp3 (Marie et al., 2005; Rubtsov and Rudensky, 2007). RUNX transcription factors are targets of the TGF-β superfamily and they are involved in the TGF-β pathway. They interact directly with regulatory SMADs (Miyazawa et al., 2002; Ito and Miyazono, 2003). TGF-β can activate RUNX genes at the transcriptional level, and at the posttranscriptional level through activation or stabilization of RUNX proteins (Jin et al., 2004). It was shown that RUNX2 regulates the expression of TGF-β type I receptor (Ji et al., 2001), suggesting that other mechanisms for their function could be involved. The fusion proteins RUNX1-EVI1 and RUNX1-ETO block TGF-β inhibition of leukemic cell growth. RUNX3 plays an important role in TGF-β–mediated growth control in epithelial cells, as loss of RUNX3 leads to decreased sensitivity to TGF-β and hyperproliferation of the gastric mucosa (Blyth et al., 2005). The present study demonstrates that RUNX3 expression is more dominant in circulating human T reg cells and tonsil T reg cells compared with RUNX1. This could be dependent on the stage of the cells and organ from which they were isolated.

We observed that single siRNA interference of either RUNX1 or RUNX3 alone shows a slight decrease in Foxp3$^+$ T reg cell induction, which could be caused by redundancy of these proteins. For this reason, we decided to use *Cbfβ*$^{F/F}$ *CD4-cre* mice. Foxp3 induction by TGF-β is reduced in CD4$^+$ T cells of *Cbfb*$^{F/F}$ *CD4-cre* mice compared with *Cbfb*$^{F/+}$ *CD4-cre* mice. Retinoic acid is secreted by a subset of dendritic cells in the gut-associated lymphoid tissue. It inhibits the IL-6–driven induction of Th17 cells and facilitates the differentiation of naive T cells to Foxp3$^+$ T reg cells (Mucida et al., 2007). We observed an increased number of Foxp3$^+$ cells by retinoic acid and TGF-β compared with TGF-β treatment alone in *Cbfb*$^{F/+}$ *CD4-cre* mice and *Cbfb*$^{F/F}$ *CD4-cre* mice. In addition, we showed a defective in vivo generation of T reg cells from *Cbfb*-deficient CD4$^+$ T cells in Rag2$^{-/-}$ mice. These data in mice confirm the human data that RUNX proteins play an important role for TGF-β–dependent FOXP3 induction, as well as in the suppressive capacity of iT reg cells. As an additional support for this concept, the overexpression of RUNX1 induced increased FOXP3 protein expression without any requirement of TGF-β and anti-CD3 and anti-CD28 stimulation in human primary CD4$^+$ cells. In both human and mouse systems, reduced Foxp3 expression was associated with reduced T reg cell suppressive activity.

In a recent study, the role of Runx–CBFβ was investigated in nT reg cell development in the thymus (Rudra et al., 2009). It was reported that Foxp3 expression in nT reg cells is unstable in the absence of Runx–CBFβ complexes. *Cbfb*-deficient nT reg cells progressively lose Foxp3 upon division, and there is no evidence of increased death of *Cbfb*-deficient nT reg cells in that study. The experiments in *Cbfb*-deficient *CD4-cre* T cells in mice and the knockdown experiments in humans in this study suggest that the induction of Foxp3 expression is a major contributing factor in the in vivo conversion experiment. Here, we observed that there is a twofold increased Foxp3$^+$ iT reg cell generation in vivo. This is in the same range with previously published studies targeting different mechanisms in Foxp3 induction (Maynard et al., 2007; Sun et al., 2007). Whether the diminished capacity of *Cbfb*-deficient *CD4-cre* T cells in the generation of Foxp3 may be caused by peripheral expansion of *Cbfb*-deficient non–T reg cells or survival problems faced by *Cbfb*-deficient iT reg cells after Foxp3 induction remains to be elucidated.

The involvement of RUNX proteins in autoimmune diseases has been previously suggested (Alarcón-Riquelme, 2003). A mutation in the RUNX1 binding site in the promoter of programmed cell death 1 gene (*PDCD-1*) has been implicated in systemic lupus erythematosus pathogenesis (Prokunina et al., 2002). Polymorphisms that alter RUNX1 binding to other genes have also been described in rheumatoid arthritis linkage at 5q31 in Japanese patients (Tokuhiro et al., 2003) and in a psoriasis linkage at 17q25 (Prokunina et al., 2002; Helms et al., 2003). *RUNX3*-deficient mice spontaneously develop inflammatory bowel disease and hyperplastic gastritis-like lesions (Brenner et al., 2004). These disease symptoms resemble those occurring after depletion of Foxp3-expressing T reg cells (Sakaguchi, 2004). Derepression of Th2 cytokines might also account for some of the observed disease symptoms, as it was shown that T-bet first induces Runx3 in Th1 cells and then partners with Runx3 to direct lineage-specific gene activation. Runx3/Cbfβ are both required for the activation of the *Ifng* gene and silencing of the *Il4 gene* in Th1 cells (Djuretic et al., 2007; Naoe et al., 2007).

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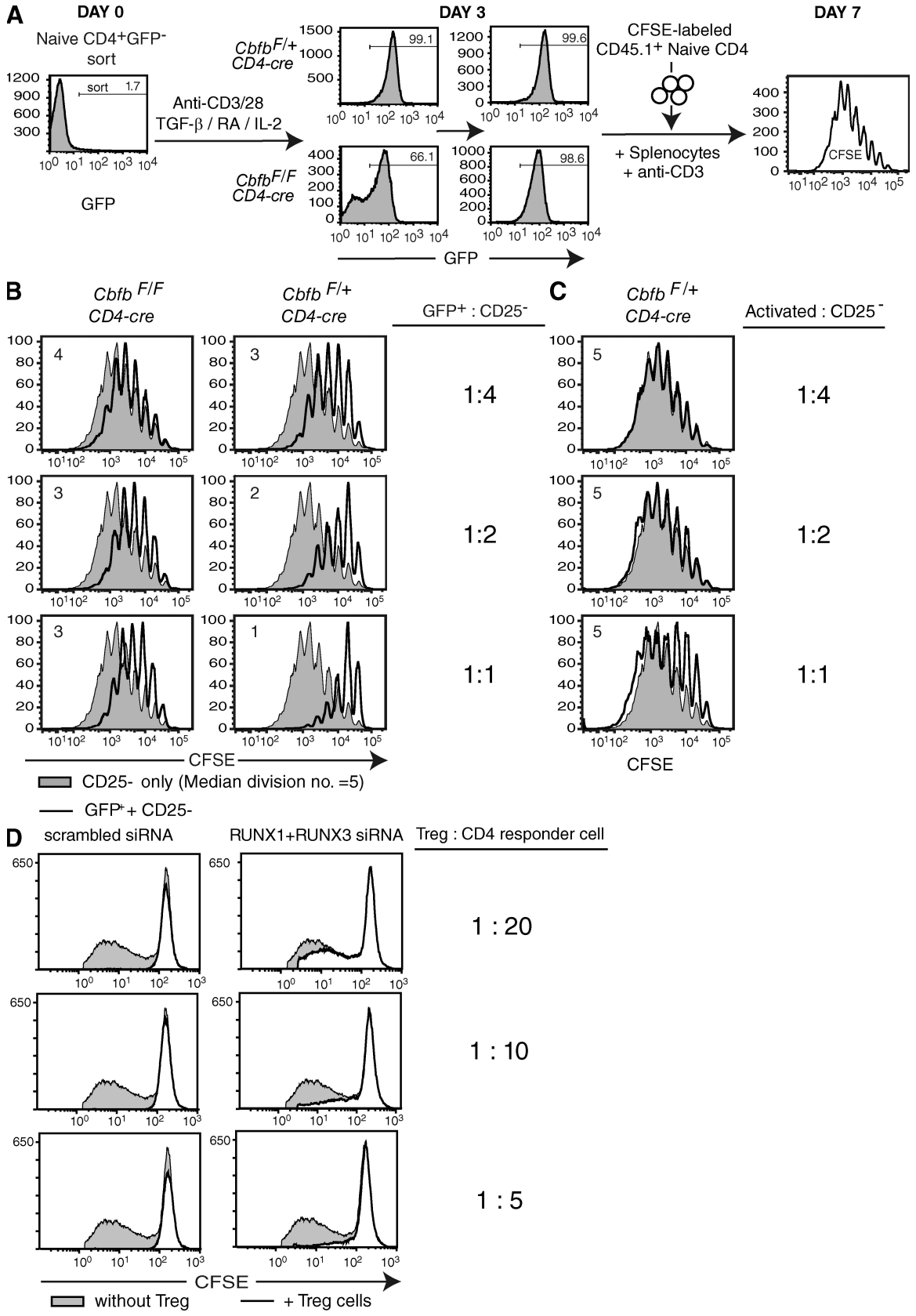

Figure 6. *Cbfb*$^{F/F}$ *CD4-cre* mouse cells and iT reg cells generated from human naive CD4$^+$ T cells undergoing siRNA-mediated RUNX1 and RUNX3 knock down show a diminished suppressive activity. Experimental setup (A) and results of the mouse suppression assay (B), FACS-purified

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Runx proteins also play an essential role during T lymphocyte differentiation in the thymus (Taniuchi et al., 2002). Runx1 regulates the transitions of developing thymocytes from the $CD4^-$ $CD8^-$ double-negative stage to the $CD4^+$ $CD8^+$ double-positive stage and from the DP stage to the mature single-positive stage (Egawa et al., 2007). Runx1 and Runx3 deficiencies caused marked reductions in mature thymocytes and T cells of the $CD4^+$ helper and $CD8^+$ cytotoxic T cell lineages. In addition, inactivation of both Runx1 and Runx3 at the double-positive stages resulted in a severe blockage in the development of $CD8^+$ mature thymocytes. These results indicate that Runx proteins have important roles at multiple stages of T cell development and in the homeostasis of mature T cells, and suggest that they may play a role in nT reg cell development, which remains to be elucidated. Furthermore, it was shown that Runx1 activates *IL-2* and *IFN-γ* gene expression in conventional $CD4^+$ T cells by binding to their respective promoter. RUNX1 interacts physically with Foxp3 protein, and it was demonstrated that this interaction might be responsible for the suppression of IL-2 and IFN-γ production and up-regulation of T reg cell–associated molecules (Ono et al., 2007).

It has been shown that Foxp3 also influences Th17 differentiation. Specifically, Foxp3 physically interacts with RORγt, and this interaction inhibits RORγt function (Zhou et al., 2008). This relationship of RORγt and Foxp3 and probably yet unknown mechanisms might be the basis of the observation that the differentiation of Th17 cells and T reg cells is often reciprocal (Bettelli et al., 2006). Recently, data suggests that Runx1 may also be involved in regulating *Il17* transcription, functioning in complex with RORγt to activate transcription (Zhang et al., 2008).

The Runx3-deficient mice develop spontaneous Th2-dominated autoimmune colitis and asthma (Brenner et al., 2004; Fainaru et al., 2005). *Cbfb*$^{f/f}$ *Cd4* mice also show a spontaneous Th2 dominated disease, with increased serum IgA, IgG1, and IgE titers and lymphocyte and eosinophil infiltration of the lung (Naoe et al., 2007). All these phenotypes were previously attributed to a loss of Th2 silencing whereas our findings additionally suggest that loss of T reg function plays a role. We have shown a link between Foxp3 induction in iT reg cells and RUNX1 and RUNX3. RUNX proteins play a central role in pathways regulating cell growth and differentiation, and their interaction with the TGF-β pathway is of particular interest.

Foxp3 protein interacts not only with RUNX proteins but also with several other transcriptional partners, such as NFAT and possibly NF-κB; with histone acetyl transferases, such as TIP60; and histone deacetyl transferase (HDAC) complexes, such as HDAC7 and HDAC9 (Wu et al., 2006; Sakaguchi et al., 2008). NFAT forms a complex with AP-1 and NF-κB and regulates the expression of IL-2, IL-4, IFN-γ, and CTLA4 in conventional T cells, which leads to the activation and differentiation to effector T cells (Dolganov et al., 1996; Hu et al., 2007). The NFAT–AP-1 complex also binds to the *Foxp3* promoter after TCR triggering and regulates its gene expression positively (Mantel et al., 2006). It was shown that NFAT and Smad3 cooperate to induce *Foxp3* expression through its enhancer (Tone et al., 2008), but no TGF-β response element was identified in the *Foxp3* gene or in the surrounding regions. The initial induction of RUNX1 and RUNX3 and the subsequent binding of these transcription factors to the *Foxp3* promoter that we showed here might explain the relatively late induction of *Foxp3* mRNA that peaks 24–48 h after stimulation. The interaction of Foxp3 and NFAT is dependent on their cooperative binding to DNA (Wu et al., 2006). RUNX1 alone, or together with its interacting partners p300 and CREB-binding protein, may cooperate with the NFAT transcription complex to activate the *IL-2* promoter (Sakaguchi et al., 2008). Similar to this interaction, NFAT may also cooperate with RUNX1 or RUNX3 to activate Foxp3, but further studies are necessary to elaborate on this concept.

In conclusion, our findings elucidate the role of RUNX proteins in iT reg cell development and function. The induction of the transcription factors RUNX1 and RUNX3 by TGF-β and the subsequent up-regulation of Foxp3 play a role in iT reg cell generation and its suppressive capacity.

MATERIALS AND METHODS

Mice. *Cbfb*$^{F/F}$ *CD4-cre* and Foxp3^{GFP} mice have previously been described (Bettelli et al., 2006; Naoe et al., 2007). *Cd45.1* and *Rag2*$^{-/-}$ mice were purchased from Jackson ImmunoResearch Laboratories and Taconic, respectively. For the in vivo Foxp3 conversion assay, naive $CD4^+$ T cells from *Cbfb CD4-cre* or control mice (harboring a *Foxp3-IRES-GFP* allele) were adoptively transferred into Rag-deficient mice. 5×10^6 cells were used per transfer. 6 wk later, $TCR\beta^+CD4^+$ gated cells from the spleen, mesenteric lymph node (MLN), and lamina propria of the small intestine were analyzed for Foxp3-GFP expression. All analyses and experiments were performed on animals at 6–8 wk of age. Animals were housed under specific pathogen–free conditions at the animal facility of the Skirball Institute, and experiments were performed in accordance with approved protocols for the New York University Institutional Animal Care and Usage Committee.

naive $CD4^+8^-$ T cells from *Cbfb*$^{F/F}$ *CD4-cre* (left) and control *Cbfb*$^{F/+}$ *CD4-cre* mice (*Cd45.2*; right) were activated in vitro with anti-CD3/28 mAb, 50 U/ml IL-2, and 2.5 ng/ml TGF-β. After 3 d, Foxp3-GFP$^+$ cells were FACS-sorted and mixed with CFSE-loaded naive $CD45.1^+$ $CD4^+$ cells at the indicated ratios. These were then incubated with inactivated splenocytes and anti-CD3 mAb. After a further 4 d, $CD45.1^+$ cells were analyzed for CFSE dilution. (C) As a control, *Cbfb*$^{F/+}$ *CD4-cre* $CD4^+$ T cells activated in absence of TGF-β were mixed with CFSE-loaded naive $CD45.1^+$ $CD4^+$ cells at the indicated ratios. Four days later $CD45.1^+$ cells were analyzed for CFSE dilution. The median division number (of the naive $CD45.1^+$ $CD4^+$ cells in the cultures containing Foxp3-GFP$^+$ cells) is indicated in each of the histograms. One of three experiments is shown. (D) Human naive $CD4^+$ T cells, transfected with RUNX1 and RUNX3 siRNA and cultured under iT reg differentiating conditions were used in an in vitro suppression assay, cultured together with autologous CFSE-labeled $CD4^+$ T cells, and stimulated with anti-CD3 mAb. The CFSE dilution of the $CD4^+$ T cell responder cells was analyzed after 5 d by flow cytometry. The T reg/responder $CD4^+$ T cell ratios used were 1:20, 1:10, and 1:5. One of two experiments is shown.

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Isolation of PBMCs, CD4$^+$ T cells, and culture conditions. Human PBMCs were isolated by Ficoll (Biochrom) density gradient centrifugation and CD4+ T cells were then isolated using the Dynal CD4$^+$ Isolation kit (Invitrogen) according to the manufacturer's instructions. The purity of CD4$^+$ T cells was initially tested by flow cytometry and was ≥95%. Cells were stimulated with the following combination of mAbs to T cell surface molecules (Meiler et al., 2008): anti-CD2 (clone 4B2 and 6G4; 0.5 μg/ml), anti-CD3 (clone OKT3; 0.5 μg/ml), and anti-CD28 mAb (clone B7G5; 0.5 μg/ml; all from Sanquin) and cultured in serum-free AIM-V medium (Life Technologies) with the addition of 1 nmol/liter IL-2 (Roche). TGF-β (R&D Systems) was used at 5 ng/ml, if not stated otherwise. A combination of PMA (25 ng/ml) and ionomycin (1 mg/ml; Sigma-Aldrich) was used.

Human CD4$^+$ CD127$^-$ CD25^{high} and CD4$^+$ CD127$^+$ CD25^{neg} cells were purified by flow cytometry using anti-CD127, anti–CD25-PC5 (Beckman Coulter), and anti–CD4-FITC antibodies (Dako).

Mouse naive (CD62L^{hi}44^{lo}25$^-$) CD4$^+$ T cells were purified by flow cytometry and activated in vitro with 5 μg/ml plate-bound anti-CD3 and 1 μg/ml soluble anti-CD28 antibodies (eBioscience) in RPMI supplemented with 10% FCS, 5 mM β-mercaptoethanol, and antibiotics. Neutralizing anti–IFN-γ and anti–IL-4 mAbs (BD) were used at 1 μg/ml concentrations when indicated.

In vitro T cell differentiation. CD4$^+$ CD45RA$^+$ magnetically sorted (CD45RO depletion with AutoMACS; Miltenyi Biotec) cells were stimulated with immobilized plate-bound anti-CD3 (1 μg/ml; OKT3; IgG1) and anti-CD28 (2 μg/ml). For Th1 differentiation conditions, cells were stimulated with the following: 40 ng/ml IL-2, 5 μg/ml anti–IL-4, and 25 ng/ml IL-12 (R&D Systems). For Th2 conditions, cells were stimulated with the following: 40 ng/ml IL-2, 25 ng/ml IL-4, and 5 μg/ml anti–IL-12 (R&D Systems). For T reg cell conditions, cells were stimulated with the following: 40 ng/ml IL-2, 5 ng/ml TGF-β, 5 μg/ml anti–IL-12, 5 μg/ml anti–IL-4. For Th17 conditions, cells were stimulated with the following: 40 ng/ml IL-2, 20 ng/ml IL-6, 5 ng/ml TGF-β, 20 ng/ml IL-23 (Alexis Biochemicals Corp.), 10 ng/ml IL-1β, 5 μg/ml anti-IL-4, and 5 μg/ml anti–IL-12 were used. Proliferating cells were expanded in medium containing IL-2. The cytokine profile of these cells demonstrated that IFN-γ is the predominant cytokine in Th1 cells, IL-4 and IL-13 in Th2 cells, and IL-17 in Th17 cells (Akdis et al., 2000; Burgler et al., 2009).

Immunohistochemistry. Human tonsils were obtained from tonsillectomy samples of hypertrophic and obstructive tonsils without a current infection. Ethical permission was obtained from Cantonal Ethics Commission, and informed consent was obtained from patients. Paraformaldehyde-fixed tonsil cryosections were stained with unconjugated rabbit IgG polyclonal antibody to human RUNX1 (Santa Cruz Biotechnology, Inc.) or unconjugated mouse IgG1 mAb to human RUNX3 (Abcam). After a washing step, the sections were stained with the corresponding secondary antibodies. RUNX1-binding antibodies were detected by using Alexa Fluor 633–conjugated goat anti–rabbit IgG and RUNX3-binding antibodies were detected by using Alexa Fluor 532–conjugated goat anti–mouse IgG1. Afterward, the sections were washed and in the case of RUNX3 staining a blocking step with an unconjugated mouse IgG1 mAb was used. Finally, the sections were stained with Alexa Fluor 488–conjugated mouse IgG1 mAb to human FOXP3 (eBioscience) or the corresponding isotype control. Tissue sections were stained with DAPI for the demonstration of nuclei and mounted with Prolong antifade (Invitrogen). Images were acquired and analyzed using the confocal microscope DMI 4000B and the TCS SPE system (both from Leica).

In vitro suppression assays. Mouse Foxp3$^+$ CD4$^+$ CD8$^-$ T cells were FACS purified based on GFP expressed from a Foxp3-IRES-GFP knock-in allele (*Foxp3^{GFP}*). Naive (CD62L^{hi}44^{lo}25$^-$) CD4$^+$ T cells (effectors) were FACS-purified from *Cd45.1* mice, then loaded with 5 μM CFSE (Invitrogen). Total splenocytes from C57BL/6 mice inactivated with 50 μg/ml mitomycin C (Sigma-Aldrich) for 45 min were used as APCs. A total of 4×10^5 CD4$^+$ cells (CFSE-loaded CD25$^-$ plus Foxp3-GFP$^+$) was mixed with 10^5 APCs + 1 μg/ml anti-CD3 mAb per well of a 96 well round bottom plate. Proliferation of the effector cells was analyzed by CFSE dilution. Apoptosis of the cells was investigated by annexin V staining and flow cytometry. Positive and negative control gates were made according to T cells cultured only in the presence of IL-2 without anti-CD3/28 stimulation.

Human naive CD4$^+$ T cells were isolated by negative selection by MACS from PBMCs and either transfected with a scrambled siRNA or with a combination of RUNX1 and RUNX3 siRNA. Cells were then cultured under iT reg cell differentiating conditions and mixed with 2×10^5 autologous irradiated PBMCs that were used as APCs and autologous CFSE-labeled CD4$^+$ T cells. T reg cell to responder cell ratio was 1:20, 1:10, and 1:5. To check the proliferation of the CD4$^+$ T cells without suppression, no T reg cells were added in a control group. Cells were stimulated with 2.5 μg/ml anti-CD3 mAb, cultured in a 96-well plate and the proliferation of the effector cells was determined by analyzing the CFSE dilution by flow cytometry after 5 d of culture. Gating on the CD4$^+$CFSE$^+$ T cells enabled the exclusion of APCs and T reg cells.

Cloning of the *FOXP3* promoter, construction of mutant *FOXP3* promoter, and RUNX expression plasmids. The *FOXP3* promoter was cloned into the pGL3 basic vector (Promega Biotech) to generate pGL3 FOXP3 -511/+176 (Mantel et al., 2006). Site-directed mutagenesis for the three putative RUNX binding sites in the *FOXP3* promoter region was introduced using the QuickChange kit (Stratagene), according to the manufacturer's instructions and confirmed by sequencing the DNA. The following primers and their complementary strands were used: foxp runx-333, forward 5′-CACTTTTGTTTTAAAAACTGTCCTTTCTCATGAGCCC-TATTATC-3′; foxp runx-333 reverse 5′-GATAATAGGGCTCATGA-GAAAGGACAGTTTTTAAAACAAAAGTG-3′; foxp runx-287 forward 5′-CCTCTCACCTCTGTCCTGAGGGGAAGAAATC-3′; foxp runx-287 reverse 5′-GATTTCTTCCCCTCAGGACAGAGGTGAGAGG-3′; foxp runx-53 forward 5′-GCTTCCACACCGTACAGCGTCCTTTTTCTT-CTCGGTATAAAAG-3′; foxp runx-53 reverse 5′-CTTTTATACCG-AGAAGAAAAAGGACGCTGTACGGTGTGGAAGC-3′.

The human *RUNX1* fragment from the Addgene plasmid 12504 (Biggs et al., 2006) pFlagCMV2-AML1B was sub-cloned in the pEGFPN1 vector (Clontech Laboratories). The *RUNX3* vector pCMV human RUNX3, which was a gift from K. Ito (Institute of Molecular and Cell Biology, Proteos, Singapore) was subcloned into pEGFPN1 vector (Clontech Laboratories; Yamamura et al., 2006).

Transfections and reporter gene assays. T cells were rested in serum-free AIM-V medium overnight. 3.5 μg of the *FOXP3* promoter luciferase reporter vector or a combination together with the *RUNX1*, *RUNX3* pEGFPN1 vector, and 0.5 μg phRL-TK were added to 3×10^6 CD4$^+$ T cells resuspended in 100 μl of Nucleofector solution (Lonza) and electroporated using the program U-15. After a 24-h culture in serum-free conditions and stimuli as indicated in the figures, luciferase activity was measured by the dual luciferase assay system (Promega) according to the manufacturer's instructions. PMA/ionomycin was used to stimulate the cells, because the transfection was only transient and the luciferase assay required a strong and fast stimulation of the cells. To evaluate the effect of overexpression of RUNX1 or RUNX3 on FOXP3 protein levels, CD4$^+$ T cells were preactivated with 2 μg/ml phytohemagglutinin (Sigma-Aldrich) in serum-free AIM-V medium in the presence of 1 nmol/liter IL-2 (Roche) for 12 h, and then transfected with the vector pEGFPN1 containing the *RUNX1* or *RUNX3* fragment using the Nucleofector system (Amaxa Biosystems) and the program T-23. FOXP3 expression was evaluated by flow cytometry after 48 h of culture in AIM-V medium containing 1 nmol/liter IL-2.

RNA interference. CD4$^+$ or naive CD4$^+$ T cells were resuspended in 100 μl of Nucleofector solution (Lonza) and electroporated with 2 μM siRNA using the Nucleofector technology program U-14 (Lonza). Five different Silencer or Silencer Select Pre-designed siRNAs for RUNX1 (Applied Biosystems) and three Silencer Pre-designed siRNAs for RUNX3 (Applied Biosystems) were tested, and the best was selected for all further experiments.

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The Silencer Negative Control #1 siRNA (Applied Biosystems) was used for normalization. Cells were then left unstimulated or were stimulated after 12 h with anti-CD2, anti-CD3, and anti-CD28. Cells were cultured in serum-free AIM-V medium with the addition of 1 nmol/l IL-2 (Roche). Cells were harvested for mRNA detection of the target genes after 24 h and for protein detection after 48 h.

RNA isolation and cDNA synthesis. RNA was isolated using the RNeasy Mini kit (QIAGEN) according to the manufacturer's protocol. Reverse transcription of human samples was performed with reverse-transcription reagents (Fermentas) with random hexamers according to the manufacturer's protocol.

Real-time PCR. PCR primers and probes were designed based on the sequences reported in GenBank with the Primer Express software version 1.2 (Applied Biosystems) as follows: FOXP3 forward primer, 5′-GAAACAGCACATTCCCAGAGTTC-3′; FOXP3 reverse primer, 5′-ATGGCCCAGCGGATGAG-3′; EF-1a forward primer, 5′-CTGAACCATCCAGGCCAAAT-3′; and EF-1a reverse primer, 5′-GCCGTGTGGCAATCCAAT-3′, as previously described (Mantel et al., 2007). GATA3 forward primer, 5′-GCGGGCTCTATCACAAAATGA-3′; and GATA3 reverse primer 5′-GCTCTCCTGGCTGCAGACAGC-3′ (Mantel et al., 2007). T-bet forward primer, 5′-GATGCGCCAGGAAGTTTCAT-3′; T-bet reverse primer, 5′-GCACAATCATCTGGGTCACATT-3′; RORC2 forward primer, 5′-CAGTCATGAGAACACAAATTGAAGTG-3′; and RORC2 reverse primer 5′-CAGGTGATAACCCCGTAGTGGAT-3′. The prepared cDNAs were amplified using SYBR green PCR master mix (Fermentas) according to the recommendations of the manufacturer in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems).

RUNX1 and RUNX3 mRNA was detected by using TaqMan Gene Expression Assays from Applied Biosystems and used according to the manufacturer's instruction using TaqMan master mix using a 7000 real-time PCR system (Applied Biosystems). PCR amplification of the housekeeping gene encoding elongation factor (EF)-1α or by using the 18S rRNA Gene Expression Assay (Applied Biosystems) was performed to allow normalization between samples. Relative quantification and calculation of the range of confidence was performed using the comparative ΔΔCT method (Applied Biosystems). The percentage of FOXP3 mRNA in siRNA-mediated RUNX knockdown cells was calculated in relation to cells, which were transfected with scrambled control siRNA. Arbitrary units show the $2^{-(\Delta ct)}$ values multiplied by 10,000 incorporating the ct values of the gene of interest and the housekeeping gene.

Flow cytometry. For analysis of human FOXP3 expression on the single-cell level, cells were first stained with the monoclonal CD4 mAb (Beckman Coulter), and after fixation and permeabilization, they were incubated with anti–human Foxp3-Alexa Fluor 488 antibody (BioLegend) based on the manufacturer's recommendations and subjected to FACS (EPICS XL-MCL; Beckman Coulter). A mouse IgG1 antibody (BioLegend) was used as an isotype control. Data were analyzed with the CXP software (Beckman Coulter). Cells were cultured with IL-2, and then left unstimulated or stimulated with anti-CD2/-CD3/-CD28 mAb.

Flow cytometry analyses of the mouse cells were performed on an LSRII (BD) and cell sorting was performed on a FACSAria (BD). All antibodies for these experiments were purchased from eBioscience or BD.

Western blotting. For human RUNX1 and RUNX3 analysis on the protein level, 10^6 cells were lysed and loaded next to a protein-mass ladder (Invitrogen) on a NuPAGE 4–12% Bis-Tris gel (Invitrogen). The proteins were electroblotted onto a PVDF membrane (GE Healthcare). Unspecific binding was blocked with 3% milk in TBS Tween, and the membranes were subsequently incubated with a 1:1,000 dilution of rabbit anti-RUNX1 (ab11903; Abcam) or 1:200 dilution of rabbit anti-RUNX3 (H-50; Santa Cruz Biotechnology, Inc.) in blocking buffer containing 3% milk in TBS Tween overnight at 4°C. The blots were developed using an anti-rabbit IgG HRP-labeled mAb (Cell Signaling Technology) and visualized with a LAS-1000 gel documentation system (Fujifilm). To confirm sample loading and transfer membranes were incubated in stripping buffer and reblocked for 1 h and reprobed using anti-GAPDH (6C5; Ambion) and developed using an anti–mouse IgG HRP-labeled mAb (Cell Signaling Technology).

Pull-down assay. HEK293T cells were transfected with RUNX1 or RUNX3 using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instruction. Cells were lysed by sonication in HKMG buffer (10 mM Hepes, pH 7.9, 100 mM KCl, 5 mM $MgCl_2$, 10% glycerol, 1 mM DTT, 0.5% Nonidet P-40) containing a protease inhibitor cocktail (Roche Diagnostics). The cell lysate was precleared using streptavidin-agarose beads (GE Healthcare), incubated with biotinylated double-stranded oligonucleotides containing the wild-type or mutated RUNX binding sites, and polydeoxyinosinicdeoxycytidylic acid (Sigma-Aldrich). A combination of all three oligonucleotides containing the mutated or the wild-type binding sites was used in the assay. DNA-bound proteins were collected with streptavidin-agarose beads, washed with HKMG buffer, and finally resuspended in NuPAGE loading buffer (Invitrogen Life Technologies), heated to 70°C for 10 min, and separated on a NuPAGE 4–12% Bis-Tris gel (Invitrogen Life Technologies). The proteins were electroblotted onto a PVDF membrane (GE Healthcare) and detected using RUNX1 or RUNX3 antibodies described in the previous section.

Promoter enzyme immuno assay. As performed in the pull-down assay, HEK293T cells were transfected with RUNX1 or RUNX3 and subsequently lysed. Insoluble material was removed by centrifugation. 384-well plates, precoated with streptavidin (Thermo Fisher Scientific) were washed 3 times with washing buffer (PBS and 0.05% Tween 20). Biotinylated *FOXP3* promoter/oligonucleotides probes containing the RUNX binding sites were added (1 pmol per well; 50 fmol/μl) and incubated for 1 h at room temperature. Either a combination of all three oligonucleotides containing the mutated or the wild-type binding sites was used or single oligonucleotides were used in the assay. After 3 washing steps with washing buffer, the nuclear extract was added (concentration > 0.2 μg/μl) and incubated overnight at 4°C. The lysates were incubated with 10 μg of poly-deoxyinosinic-deoxycytidylic acid (Sigma-Aldrich). The plate was washed with HKMG buffer and incubated with a 1:1000 dilution of rabbit anti-RUNX1 (ab11903, Abcam) or 1:200 dilution of rabbit anti-RUNX3 (H-50, Santa Cruz Biotechnology, Inc.) at 4°C for 2 h. After three washing steps with HKMG buffer, a secondary antibody (anti–rabbit IgG-HRP, 1:3,000 in HKMG buffer, Cell Signaling Technology) was added, and the plate was incubated for 1 h at 4°C. The wells were washed 4 times with HKMG buffer before adding the substrate reagent (R&D Systems). The colorimetric reaction was stopped by adding 2 M H_2SO_4. Absorbance at 450 nm was measured using a microplate reader (Berthold Technologies).

ChIP. Human naive $CD4^+$ T cells were cultured either with IL-2 only or with IL-2, anti-CD2/3/28, and TGF-β for 72 h, and protein–DNA complexes were fixed by cross-linking with formaldehyde in a final concentration of 1.42% for 15 min. Formaldehyde was quenched with 125 mM glycine for 5 min, and cells were subsequently harvested. The ChIP assay was performed as described in the fast chromatin immunoprecipitation method (Nelson et al., 2006). Cells were lysed with immunoprecipitation buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, NP-40 [0.5% vol/vol]) containing phosphatase (Roche) and protease inhibitors cocktails (Roche), the nuclear pellet was washed, the chromatin was sheared by sonication and incubated with antibodies for RUNX1 (H-65 X; Santa Cruz Biotechnology, Inc.), RUNX3 (H-50 X; Santa Cruz Biotechnology, Inc.), CBFβ (PEBP2β; FL-182 X; Santa Cruz Biotechnology, Inc.), and as controls normal rabbit IgG (Santa Cruz Biotechnology, Inc.), anti-human RNA polymerase II antibody, and mouse control IgG (both from SA Biosciences). The cleared chromatin was incubated with protein A agarose beads and, after several washing steps, DNA was isolated with 10% (wt/vol) Chelex 100 resin. Samples were treated with proteinase K at 55°C for

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30 min. The proteinase K was then inactivated by boiling the samples for 10 min. The purified DNA was used in a real-time PCR reaction. Specific primers for the FOXP3 promoter, spanning the region from −87 to −3, FOXP3 promoter forward primer 5′-AGAGGTCTGCGGCTTCCA-3′, FOXP3 promoter reverse primer 5′-GGAAACTGTCACGTATCAAAAA-CAA-3′, or control GAPDH primer (SA Biosciences) for the RNA polymerase II were used. A negative control PCR for each immunoprecipitation using IGX1A negative control primer targeting ORF-free intergenic DNA (SA Biosciences) was used. The fold enrichment in site occupancy was calculated incorporating IgG control values and input DNA values using the ChampionChIP qPCR data analysis file (SA Biosciences).

Quantification of cytokine levels. IL-4, IL-5, IL-6, IL-10, IL-13, IL-17, and IFN-γ secretion was assessed using fluorescent bead-based technology. The Bio-Plex-hu Cytokine Panel, 17-Plex Group 1 was used according to the manufacturer's instructions (Bio-Rad Laboratories). Fluorescent signals were read and analyzed using the Bio-Plex 200 System (Bio-Rad Laboratories).

Online supplemental material. Fig. S1 shows the induction of RUNX1, RUNX3, and FOXP3 mRNA in human $CD4^+$ T cells after anti-CD2/3/28 mAb and TGF-β stimulation. Fig. S2 shows decreased RUNX1 and RUNX3 mRNA and protein expression after siRNA-mediated knockdown and decreased FOXP3 expression in human $CD4^+$ T cells after RUNX1 and RUNX3 knockdown. Fig. S3 shows the quantification of IL-4, IL-5, IL-10, IL-13, and IFN-γ levels in control siRNA transfected or RUNX1 and RUNX3 siRNA transfected human $CD4^+$ T cells. Fig. S4 shows the putative RUNX binding sites in the *FOXP3* core promoter sequence of human, mouse, and rat. Fig. S5 shows the induction of FOXP3 protein after overexpression of RUNX1 and RUNX3 in human CD4+ T cells. Fig. S6 shows that endogenous IL-4 and IFN-γ do not effect Foxp3 expression in naive $CD4^+$ T cells of $Cbfb^{F/F}$ *CD4-cre* and $Cbfb^{F/F}$ control mice, which were stimulated with anti-CD3 and anti-CD28 mAbs, IL-2 and TGF-β in the absence or presence of anti-IL-4 and anti-IFN-γ neutralizing mAbs. Fig. S7 shows similar cell death (A) and proliferation (B) of $Foxp3^+$ and $Foxp3^-$ cells in $Cbfb^{F/F}$ *CD4-cre* and $Cbfb^{F/F}$ control mice cultures. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20090596/DC1.

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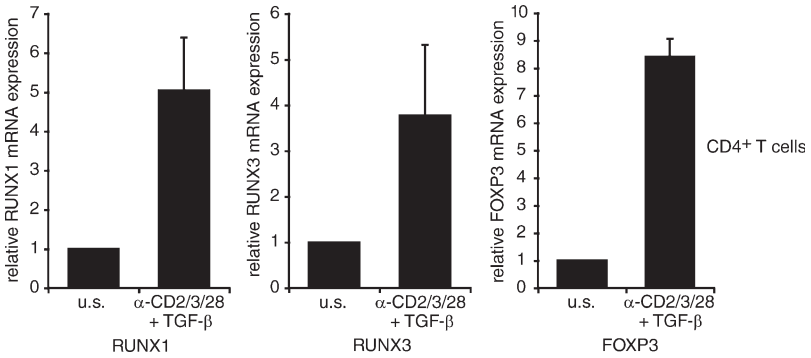

Figure S1. Induction of RUNX1, RUNX3, and FOXP3 mRNA in human CD4+ T cells after anti-CD2/3/28 mAb and TGF-β stimulation. Human primary CD4+ T cells, isolated from PBMCS, and cultured with IL-2, anti-CD2/3/28 mAb and TGF-β. Cells were harvested after 48 h, RNA was isolated and reverse transcribed, and real-time PCR was performed. Bars show the mean ± SE of three independent experiments.

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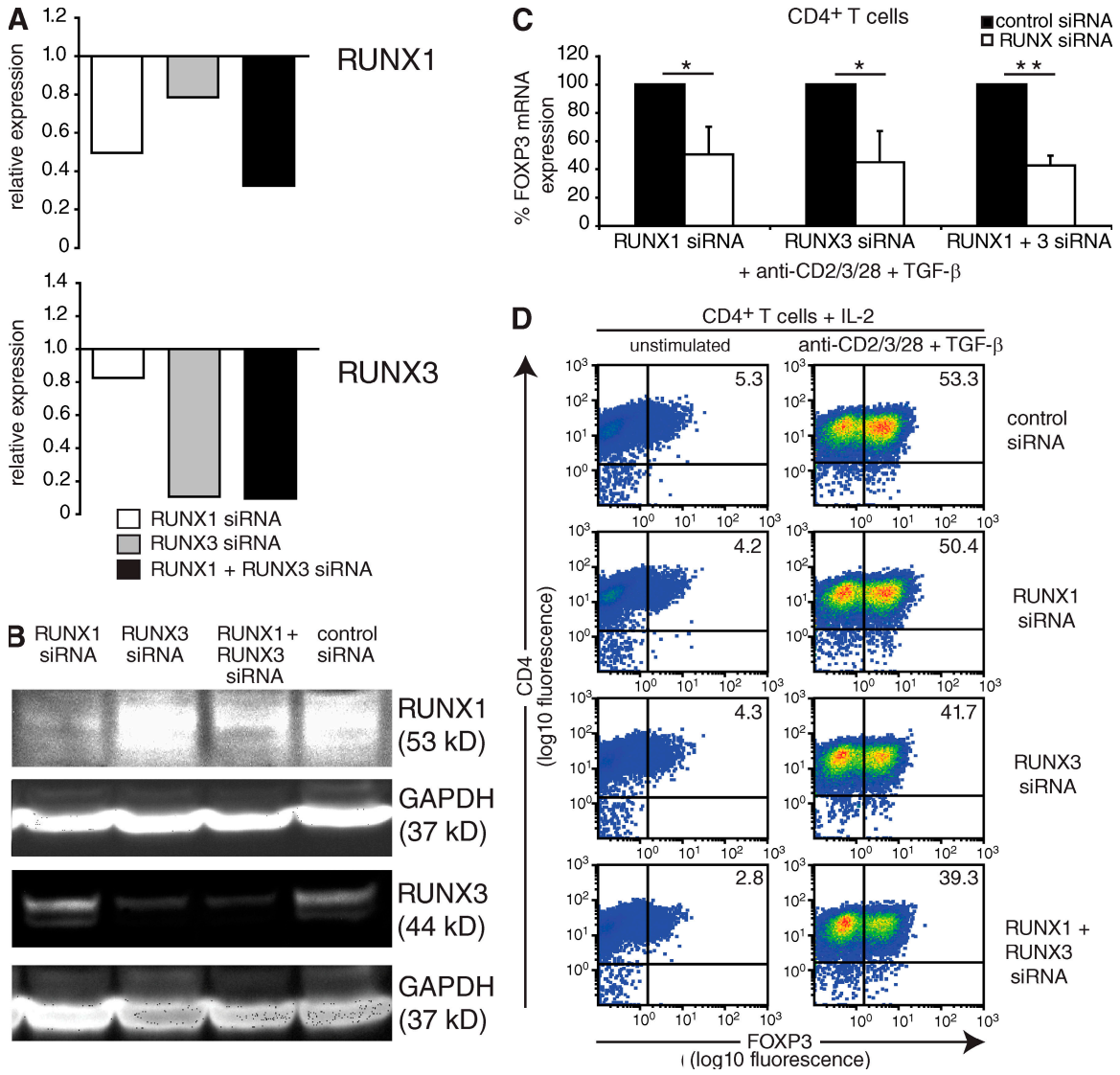

Figure S2. Decreased FOXP3 expression in human CD4+ T cells after siRNA-mediated RUNX1 and RUNX3 knockdown. (A) RUNX1 and RUNX3 mRNA is reduced after siRNA-mediated knockdown. Human CD4+ T cells, isolated from PBMCs, were transfected with RUNX1 and/or RUNX3 siRNA or with a control siRNA. Real-time PCR for RUNX1 and RUNX3 after 24 h of culture. One of three independent experiments is shown. (B) RUNX1 and RUNX3 protein is reduced after siRNA-mediated knockdown. Cell lysates from human CD4+ T cells 48h after transfection with RUNX1 and/or RUNX3 siRNA or with control siRNA were separated by SDS-PAGE and specific RUNX1, RUNX3, or GAPDH protein was detected by Western blot. One of three independent experiments is shown. (C) FOXP3 mRNA induction by anti-CD2/3/28 mAb and TGF-β in human CD4+ T cells is reduced after siRNA-mediated RUNX1/3 knockdown. Real-time PCR from human naive CD4+ T cells, transfected with RUNX1 and/or RUNX3 siRNA or with a control siRNA and cultured with anti-CD2/3/28 mAbs, TGF-β, and IL-2. Bars show the mean ± SD of three independent experiments. Statistical analysis was performed with paired Student's *t* test. *, $P < 0.05$; **, $P < 0.01$. (D) FOXP3 protein induction by anti-CD2/3/28 mAb and TGF-β in human CD4+ T cells is reduced after siRNA-mediated RUNX1 and RUNX3 knockdown. Human CD4+ T cells were transfected with RUNX1 and/or RUNX3 siRNA or with a control siRNA and stimulated with anti-CD2/3/28 mAb and TGF-β in the presence of IL-2. Cells were harvested after 72 h and stained for CD4 and intracellular FOXP3 and analyzed by flow cytometry. One of three independent experiments is shown.

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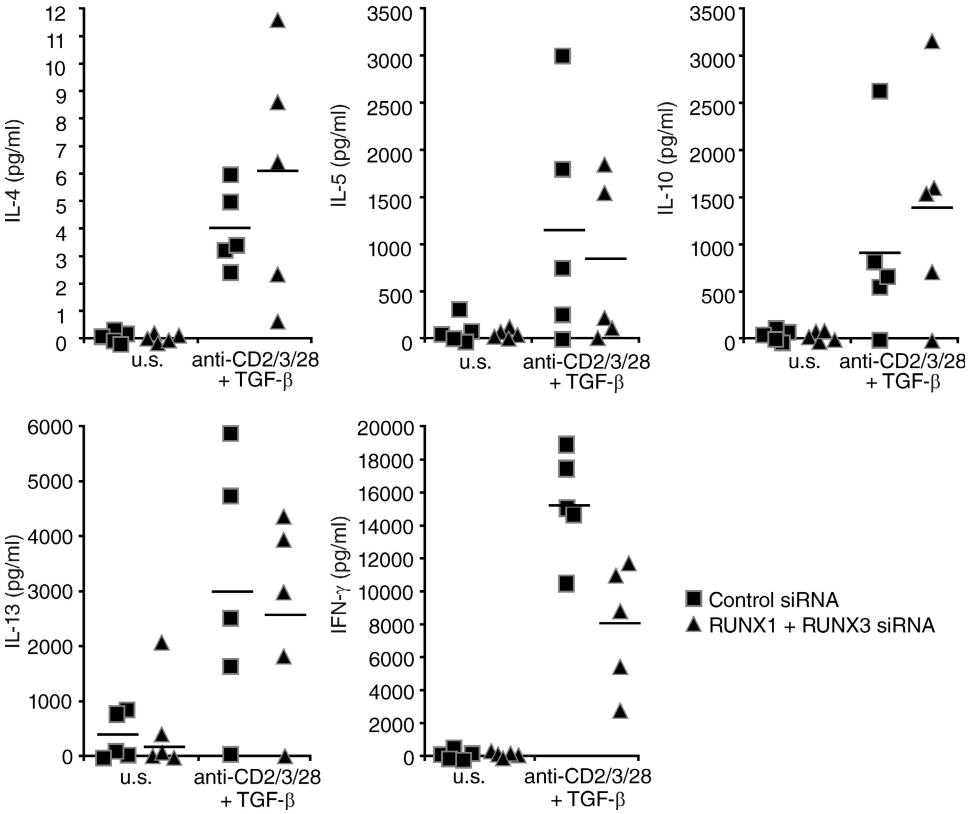

Figure S3. Quantification of cytokine levels after siRNA-mediated RUNX1 and RUNX3 knockdown. IL-4, IL-5, IL-10, IL-13, and IFN-γ secretion was assessed in unstimulated and anti-CD2/3/28 and TGF-β–stimulated $CD4^+$ T cells transfected with RUNX1 and RUNX3 siRNA or scrambled siRNA using fluorescent bead-based technology. Bars show the mean ± SE of five different donors. Statistical differences were verified by the Wilcoxon Signed Ranks test.

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                  -592       -582       -572       -562       -552       -542       -532       -522
Homo sapiens      CCATATCAAT TCCATCTATA GGGAAACTGA GGCCTGGAGT AGGGCGAGGC CTCTGGGAAC CCAGCCCTAT TCTGTCTCTT
Mus musculus      TCATATGCAT GCTTGCTAAG GAAATACTGA GGTTTGGAGC AGAAGGAAGC CTCTGGAGAC AGAGCACTAC CCCACCTCTC
Rattus norvegicus TCATGTTAAC GCTCCCTAGG GAAGCACCGA GGCTTGGAGC AGAATGAAGC CTCTGGAGAC CAAGCACTAT CCCATGTCAC

                  -512       -503       -493       -483       -475       -467       -457       -447
Homo sapiens      TCCCTGGCAT -TTCCCATCC ACACATAGAG CTTCAGATTC --TCTTTCTT TC--CCCAGA GACCCTCAAA TATCCTCTCA
Mus musculus      -CCCTGGCTG CTTCCCATTC ACATGGCAGG CTTCAGATCC CTTCTTCTGT TCAACCCAGC GATCCTCCAA ---CGTCTCA
Rattus norvegicus -CCTTGGCTG CTTCTCATTC ACATGTCAGG CTTCAGATCC CTTCTTCTGT TCAACCCAGC AATCCTCAAA T---ATCTCC

                  -437       -427       -417       -407       -497       -387       -377       -367
Homo sapiens      CTCACAGAAT GGTGTCTCTG CCTGCCTCGG GTTGGCCCTG TGATTTATTT TAGTTCTTTT CCCTTGTTTT TTTTTTTTTC
Mus musculus      CAAACACAAT GCTGTCTCTA CCTGCCTCGG GATGCCTTTG TGATT----T GACTTATTTT CCCTCAGTTT TTTTTTTCTG
Rattus norvegicus CGCACAGAAT GGTGTCTCTA CCTGCCTCGG AATGCCTTTG TGATT----T GACTTATTTT CCCTCAGTTT TTTTTCT---

                                     -337                                                          -287
                  -357       -347       RUNX       -327       -317       -307       -297       RUNX
Homo sapiens      AAACTCTATA CACTTTTGTT TTAAAAACTG TGGTTTCTCA TGAGCCCTAT TATCTCATTG ATACCTCTCA CCTCTGTGGT
Mus musculus      --ACTCTACA CACTTTTGTT TAAGAAATTG TGGTTTCTCA TGAGCCCTGT TATCTCATTG ATACCTTTTA CCTCTGTGGT
Rattus norvegicus -AACTCTACA CACTTTTGTT TAAGAAATTG TGGTTTCTCA TGAGCCCTGT TATCTCATTG ATACCTTTTA CCTCTGTGGT

                  -277       -267       -257       -247       -237       -227       -217       -207
Homo sapiens      GAGGGGAAGA AATCATATTT TCAGATGACT CGTAAAGGGC AAAGAAAAAA ACCCAAAATT TCAAAATTTC CGTTTAAGTC
Mus musculus      GAGGGGAAGA AATCATATTT TCAGATGACT TGTAAAGGGC AAAGAAAAAA -CCCAAAATT TCAAAATTTC CGTTTAAGTC
Rattus norvegicus GAGGGGAAGA AATCATATTT TCAGATGACT TGTAAAGGGC AAAGAAAAAA -CCCAAAATT TCAAAATTTC CGTTTAAGTC

                  -197       -187       -177       -167       -157       -147       -138       -128
Homo sapiens      TCATAATCAA GAAAAGGAGA AACACAGAGA GAGAGAAAAA AAAAACTATG AGAACCCCCC CCC-ACCCCG TGATTATCAG
Mus musculus      TCATAAGAAA AGAATAAACA AAGTAAGAGA GCAAAGAAAA AAAAACTACA AGAACCCCCC CCCCACCCTG CAATTATCAG
Rattus norvegicus TCATAAGAAA AGAATAAACA AAGCGAGAGA GCGAAGAAAA AAAAACTACG AGAACCCCCC C---ACCCTG CGATTATCAG

                                                                                   -58
                  -118       -108       -98        -88        -78        -68        RUNX       -49
Homo sapiens      CGCACACACT CATCGAAAAA AATTTGGATT ATTAGAAGAG AGAGGTCTGC GGCTTCCACA CCGTACAGCG TGGTTTTT-C
Mus musculus      CACACACACT CATCAAAAAA AAATTGGATT ATTAGAAGAG CGAGGTCTGC GGCTTCCACG CC-------G TGGTTTTT-C
Rattus norvegicus CACACACACA CTCATAAAAA AAAATGGATT ATTAGAAGAG AGAGGTCTGC GGCTTCCACA CC-------G TGGTTTTTTC

                                                   TSS 0
                  -39        -29        -19        -10                   11         21         31
Homo sapiens      TTCTCGGTAT AAAAGCAAAG TTGTTTTTGA TAC-GTGACA GTTTCCCACA AGCCAGGCTG ATCCTTTTCT GTCAGTCCAC
Mus musculus      TTCTCGGTAT AAAAGCAAAG TTGTTTTTGA TAATGTGGCA GTTTCCCACA AGCCAGGCTG ATCCCCCTCT AGCAGTCCAC
Rattus norvegicus TTCTCGGTAT AAAAGCAAAG TTGTTTTTGA TAATGTGGCA GTTTCCCACA AGCCAGGCTG ATCCCTCTCT GTCAGTCCAC

                  41         47         57         67         77         87         97         107
Homo sapiens      TTCACCAAGG TGAG----TG TCCCTGCTCT CCCCTACCAG ATGTGGGCCC CATTGGAGGA GATGGCAGGG AGGTAGGCAC
Mus musculus      TTCACCAAGG TGAGCGAGTG TCCCTGCTCT CCCCCACCAG ACACAG-CTC TGCTGGCGAA AGTGGCAGAG AG--------
Rattus norvegicus TTCACTCAGG TGAG----TG TCCCTGCTCT CCCCCACCAG ACACAG-CTC TGCTGGAGAA AGTGGCAGGG AAGGAGTGTC
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RUNX binding site

Figure S4. Putative RUNX binding sites in the *FOXP3* core promoter sequence of human, mouse, and rat. The *FOXP3* promoter was analyzed for transcription factor binding sites using the program MatInspector. The program identified three putative RUNX binding sites. Although two of them (−333 and −287 upstream of the TSS) were identified as binding sites for RUNX1, the third was predicted as binding site for RUNX2 (−53 bp upstream of the TSS). All three binding sites had GTGG as a core sequence. RUNX transcription factor binding sites in the *FOXP3* promoter region of human (GenBank/EMBL/DDBJ accession no. AF235097), mouse (AF277994) and rat (NW_048035) are indicated by boxes. TSS, Transcriptional start site.

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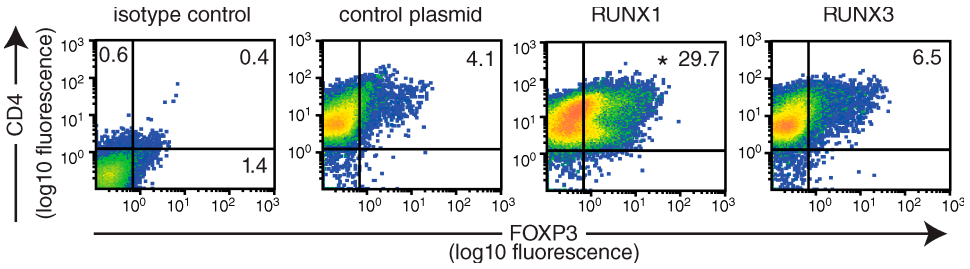

Figure S5. Induction of FOXP3 protein by RUNX1 and RUNX3. Human primary CD4+ T cells were transfected with RUNX1 or RUNX3 expression vectors or the corresponding control plasmid. The expression of FOXP3 was determined by flow cytometry after 48 h. One of four independent experiments is shown. Statistical analysis was performed with paired Student's *t* test. *, $P < 0.05$

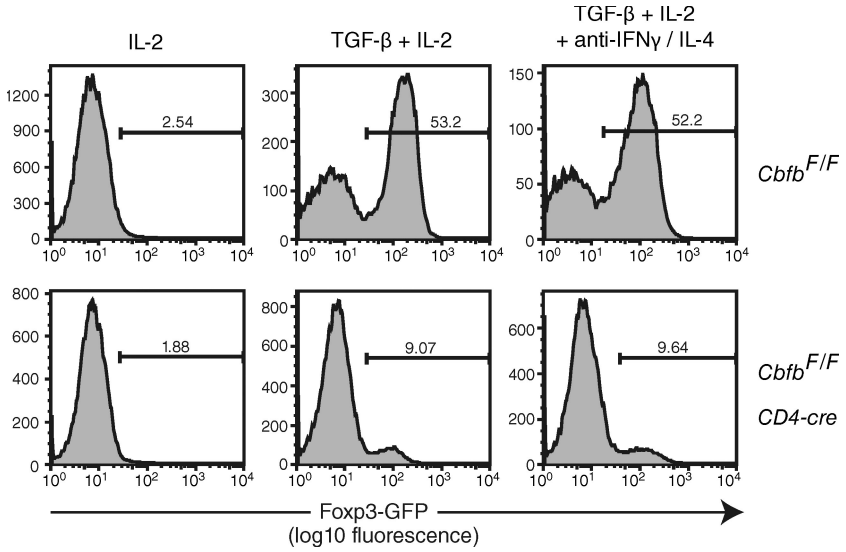

Figure S6. Endogenous IL-4 and IFN-γ do not effect Foxp3 expression in *Cbfb*$^{F/F}$ *CD4-cre* and *Cbfb*$^{F/F}$ control mice. Naive CD4+ T cells from *Cbfb*$^{F/F}$ *CD4-cre* and *Cbfb*$^{F/F}$ control mice were stimulated with anti-CD3 and anti-CD28 mAbs, 50 U/ml IL-2 and 2 ng/ml of TGF-β. 1 μg/ml of anti-IL-4 and anti-IFN-γ neutralizing mAbs were used. After 3 d, cells were analyzed for Foxp3 expression by intracellular staining. One of three independent experiments is shown.

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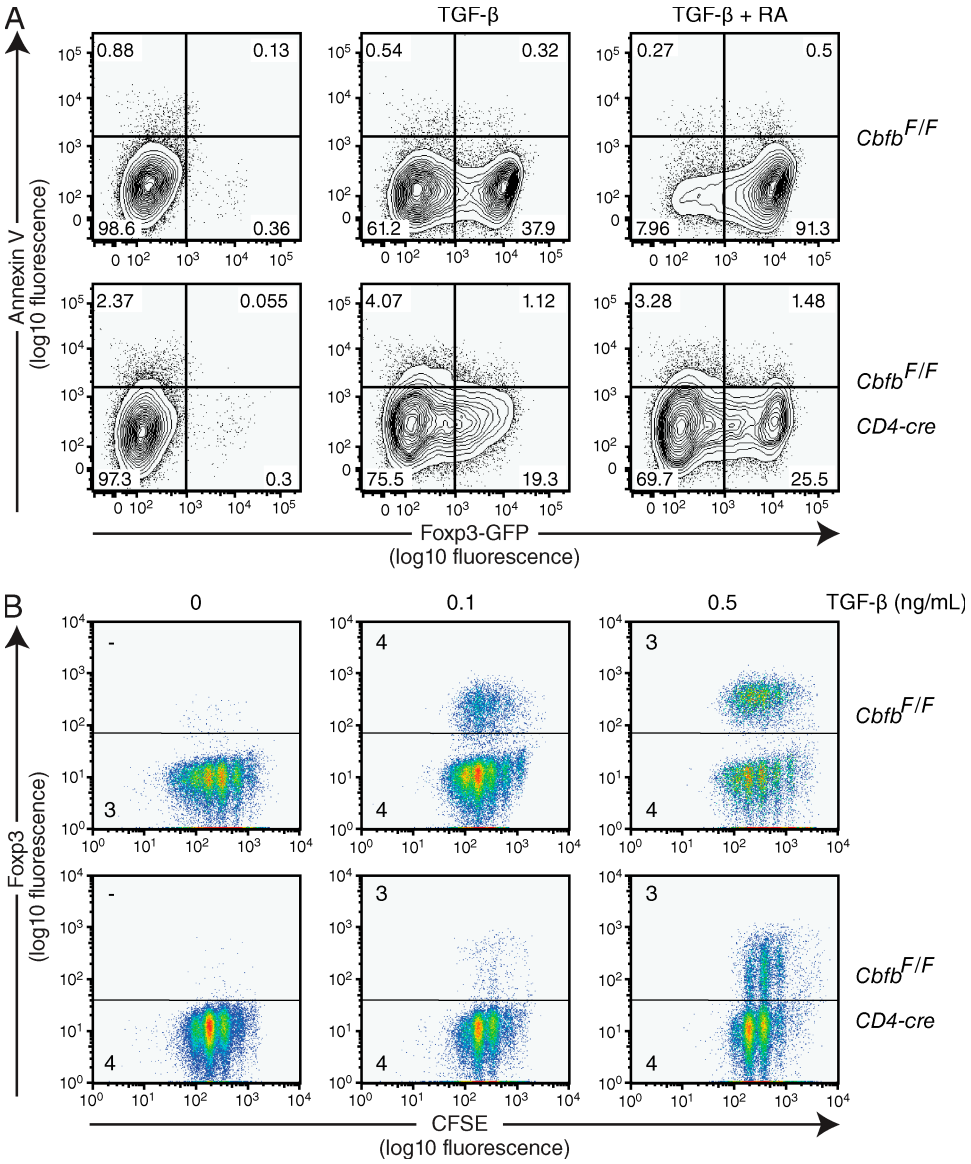

Figure S7. Foxp3⁺ and Foxp3⁻ cells in *Cbfb*$^{F/F}$ *CD4-cre* and *Cbfb*$^{F/F}$ control mice cultures show similar cell death and proliferation. (**A**) Naive CD4⁺ T cells from *Cbfb*$^{F/F}$ *CD4-cre* and *Cbfb*$^{F/F}$ control mice (harboring a *Foxp3-IRES-GFP* allele) were activated with anti-CD3/28 and 50 U/ml IL-2 in the presence or absence of 0.5 ng/ml TGF-β and 10 nM retinoic acid (RA). After 4 d, the cells were analyzed for Foxp3-GPF expression and Annexin V staining. One of two independent experiments is shown. (**B**) Naive CD4⁺ T cells from *Cbfb*$^{F/F}$ *CD4-cre* and *Cbfb*$^{F/F}$ control mice were loaded with CFSE and stimulated with anti-CD3/28, 50 U/ml IL-2 and the indicated concentration of TGF-β. After 3 d, the cells were analyzed for CFSE dilution and Foxp3 expression by intracellular staining. Median division of each cell subset is indicated in the dot plots. One of two independent experiments is shown.

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