28 days $P=0.23$. “KO-Foxp3” vs “KO-vector” after 24 days $P=0.065$, 26 days $P=0.051$, and 28 days $P=0.125$.

**Online methods**

**Mice.** $Foxp3^{YFP-Cre}$, $Cbfb^{fl/fl} CD4^{Cre}$, and $Runx1^{fl/fl}$ mice were described elsewhere. Experimental mice were age matched, housed and bred under specific pathogen-free conditions, in accordance with guidelines from the University of Washington Institutional Animal Care Committee. For bone marrow transplantation experiments, CD45.1 and $Rag2^{-/-}$ B6 mice were purchased from Jackson Laboratories and Taconic, respectively. All analyses and experiments were performed on animals at 6 to 8 weeks of age. Animals were housed under SPF conditions at the animal facility of the Skirball Institute, and experiments were performed in accordance with approved protocols for the NYU institutional Animal Care and Usage Committee.

**Histology.** Mice were euthanized by CO$_2$ asphyxiation. Necropsies were performed and sections of pancreas, stomach, heart, lungs, kidney and haired skin were fixed in 10% phosphate-buffered formalin. Tissues were processed routinely, stained with hematoxylin and eosin or Movat’s pentachrome and examined by a board certified veterinary pathologist (PT) blinded as to genotype.

**Gene expression analysis.** Total RNA was isolated and prepared from sorted populations of cells using RNA STAT-60 as directed by the manufacturer’s protocol (Tel-Test inc. Catalog# Cs-112), and cDNA was synthesized with oligo dT primers and the Invitrogen SuperScriptII First-Strand Synthesis System. Quantitative Real-time PCR was performed using Power SYBR Green according to the manufacturer's protocol (Applied Biosystems) with the following
primers: Foxp3-s (5'-GGCCCTTCTCCAGGACAGA-3') Foxp3-as (5'-GCTGATCATGGCTGGGTTGT-3'), Cbfb-s (5'-CAGGAAGATGCATTAGCACA-3'), Cbfb-as (5'-CATTTCCTCCCGGTGAGAC-3'), Hprt1-s (5'-AGCCTAAGATGAGCGCAAGT-3'), Hprt1-as (5'-TTACTAGGCAGATGGCCACA-3'). All reactions were done and detected with a 7300 Real Time PCR machine (Applied Biosystems). Relative mRNA quantities are normalized to HPRT1 mRNA quantities.

**Reporter plasmids and luciferase reporter assay.** The reporter construct containing the Foxp3 promoter was kindly provided by M. Tone (University of Pennsylvania)\(^\text{11}\). CNS1 and CNS2 were amplified by PCR of the corresponding regions from a cosmid containing the Foxp3 gene, and PCR products were sub-cloned into BamH1 and Sal1 sites downstream of the luciferase-coding sequence. Mutations of Runx1 binding sites were introduced using the Stratagene QuickChange\(^\text{TM}\) mutagenesis kit. EL4 cells were electroporated with 5µg reporter plasmids and 1µg of phRL-TK as an internal control, rested at 37°C for one hour, and treated with 250 ng/ml ionomycin and 25ng/ml PMA with or without 2.5 ng/ml TGF-β (R&D Systems, Minneapolis, MN). Cells were harvested after 18-24 h and luciferase activity was detected using a dual-reporter assay system (Promega). For luciferase reporter assays in primary T\(_{\text{reg}}\) cells, an Amaxa nucleofection kit was used to electroporate reporter constructs into magnetically purified CD4\(^+\)CD25\(^+\) T\(_{\text{reg}}\) cells.

**Adoptive transfer and bone marrow transplantation.** 10\(^6\) sorted CD4\(^+\)YFP\(^+\) cells from spleen and LN of Cbfb\(^{-}\)/\(^{-}\)Foxp3\(^{YFP-Cre}\) mice or wild Cbfb\(^{+/-}\)Foxp3\(^{YFP-Cre}\) littermate controls were mixed with 2 × 10\(^6\) purified CD4\(^+\) T cells isolated from male CD45.1\(^+\)Foxp3\(^-\) mice, and the mixture was transferred by tail vein injection into Rag2\(^{-}\)/\(^{-}\) mice. The recipient mice were retro-orbitally bled
every 10 days over a period of 30 days and sacrificed after 40 days to analyze the maintenance of Foxp3 in T\textsubscript{reg} cells. For bone marrow transplantations, bone marrow cells for were prepared from the femurs of \textit{Cbfb\textsuperscript{fl/fl} CD4-cre} (CD45.2) and \textit{Cbfb\textsuperscript{+/+}} (CD45.1) mice, mixed in a 1:1 ratio and injected (4×10\textsuperscript{6} cells per mouse) through the retro-orbital plexus into \textit{Rag2\textsuperscript{−/−}} recipient mice that were lethally irradiated (800 Rads). The animals’ drinking water was supplemented with antibiotics for the first two weeks after transplantation. For \textit{in vivo} suppression assay CD4\textsuperscript{+}CD25\textsuperscript{+} Treg cells isolated from \textit{Cbfb\textsuperscript{fl/fl} Foxp3\textsuperscript{YFP-Cre}} or \textit{Cbfb\textsuperscript{fl/+} Foxp3\textsuperscript{YFP-Cre}} control mice were transduced with the hCD2 encoding “empty” MigR2 or MigR2-Foxp3 vector. CD4\textsuperscript{+}YFP\textsuperscript{+}hCD2\textsuperscript{+} cells were FACS sorted two days after transduction, and co-transferred with purified effector CD4\textsuperscript{+}CD45.1\textsuperscript{+} \textit{Foxp3\textsuperscript{−}} T cells at a 1:4 ratio into T cell-deficient \textit{Tcrb/Tcrd\textsuperscript{−/−}} recipients. For the “T\textsubscript{eff only}” group, only CD4\textsuperscript{+}CD45.1\textsuperscript{+} \textit{Foxp3\textsuperscript{−}} T cells were transferred.

\textbf{ChIP.} CBFβ occupancy on the \textit{Foxp3} locus was assayed using the protocol developed by Upstate Biotechnology (Cat# 17-295). Chromatin prepared from 5 × 10\textsuperscript{6} CD4\textsuperscript{+}CD25\textsuperscript{+} cells was subjected to immunoprecipitation overnight at 4°C with rabbit anti-CBFβ or normal rabbit IgG (Santa Cruz Biotechnology sc-20693X and sc-2027 respectively). Quantitative real-time PCR was performed using Power SYBR green PCR master mix (Applied Biosystems) to determine relative abundance of the precipitated DNA fragments of interest. To calculate the fold enrichment of the CBFβ protein on the regions of interest the 2\textsuperscript{−ΔΔCT} method \textsuperscript{33} was applied, using the control \textit{Gmpr} promoter. ChIP analysis to determine histone modifications has been described previously \textsuperscript{6}. Antibodies specific for different histone modifications (H3K4me3, H3K9me3 and H3K27me3) were purchased from Upstate Biotechnology (Catalog numbers 07-443, 07-442 and 07-449 respectively). Specific primer sequences for ChIP-qPCR analyses are listed in \textbf{Supplementary Table 1}.
**Retroviral transduction.** CD4⁺ T cells were isolated from Cbfb^{fl/fl}CD4-Cre⁺ mice or their Cbfb^{+/+}CD4-Cre⁺ littermates using magnetic bead sorting method. CD4⁺ T cells were plated in 24-well plates (2 × 10^6 cells per well) pre-coated with 1 μg/ml anti-CD3 and anti-CD28 in the presence of 50 IU/ml of recombinant IL-2 in DMEM medium supplemented with 10% (volume/volume) FCS, 200 mM L-glutamine, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol and antibiotics. T cell cultures were 'spin-infected' twice over a 48 hour period with viral supernatants collected from MigR1-Foxp3 or MigR1 transfected packaging Phoenix cell lines as described 34. After infection, cells were transferred to new plates and were allowed to expand in number for the indicated number of days before analysis. At a later point during the course of the experiment, the IL-2 concentration in the culture medium was increased to 200 IU/ml since Foxp3 expression confers T_{reg} cell-like properties on CD4⁺ cells.

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