Supporting Information

Paiva et al. 10.1073/pnas.1221955110

SI Materials and Methods

Mice. All mice were on a C57BL/6 background, and they were bred and raised in a strict SPF facility. The Forkhead box P3 (Foxp3) -GFP reporter mice (1) were bred to homozygocity with the following animals: Thy1.1 congenic (Jackson), CD4-dnTGF-βRII transgenic (tg) (2), anti- minor histocompatibility Y antigen (HY) T-cell receptor (TCR) tg $Rag^{-/-}$ (3), and anti-ovalbumin TCR tg (OT-II) $Rag2^{-/-}$ (OT-II; Jackson; $Rag2^{-/-}$; CDTA). $TCR\beta^{-/-}$ animals were crossed with IL-$6^{-/-}$ mice (both from Jackson). Nude-$Foxn1^{-/-}$ mice were originally from Taconic. Animals were used between 6 and 12 wk of age unless otherwise indicated. When indicated, mice received i.v. 3×10^5 cells and 100 μg endotoxin-free ovalbumin (endograde ovalbumin; Hyglos). Thymectomies were performed on 5- to 6-wk-old mice under ketamine/xillazine anesthesia. Protocols were approved by the Institutional Ethical Committee and the Portuguese Veterinary General Division.

Human Samples. Thymic specimens were obtained from pediatric corrective cardiac surgery (Hospital de Santa Cruz, Carnaxide, Portugal) after parent's written informed consent. Buffy coats were provided by Instituto Português de Sangue, Lisbon, Portugal. Human studies were approved by the Ethical Board of the Faculty of Medicine, Lisbon, Portugal.

FACS Analysis. Human and mouse antibodies for FACS and cultures are described in Table S1. Mouse lymph node (LN) cells and thymocytes were prepared from pooled thymi or LNs (injections and cultures) or single organs/single mouse (analyses) and preincubated with Fc-block. Human thymocytes were separated on Ficoll-Paque PLUS (GE Healthcare). Human peripheral blood mononuclear cells (PBMCs) were enriched in $CD4^+$ T cells using RosetteSep (StemCell Technologies). Cell sorting and analysis were performed on FACSaria or MoFlo and BD FACScalibur or Cyan ADP, respectively.

Cell Cultures. For mouse cells, plates were coated with 2 μg/mL anti-CD3; antigen-presenting cells (APCs) were either irradiated splenocytes depleted of $Thy1^+$ cells or bone marrow-derived dendritic cells from granulocyte-macrophage colony-stimulating factor cultures. APCs were activated with Lipid A overnight; when indicated, media contained soluble anti-CD3 (2 μg/mL), anti-CD28 (1 μg/mL), TGF-β (0.02 ng/mL; Peprotech), IL-2 (1/250 dilution of X63 supernatant), and/or IL-4 (R&D), IFN-γ (R&D), IL-12 (R&D), IL-6 (R&D), IL-17 (Peprotech), TNFα (R&D), or retinoic acid (Sigma) at 100 ng/mL. For human cells, plates were coated with anti-CD3 at 1 (thymocytes) or 2 (peripheral cells) μg/mL (optimal dose for each cell subset), and media contained anti-CD28 (1 μg/mL), IL-2 (50 U/mL; NIH/AIDS-RRP-Hoffman-La-Roche), and TGF-β (5 ng/mL; Peprotech).

Data Analysis. FACS data were analyzed with FlowJo (Tree Star). Numerical data were processed in Excel (Microsoft) and plotted in Graphpad Prism (Graphpad Software, Inc). Statistical significance was determined using a nonparametric Mann–Whitney test ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$), unless otherwise indicated.

1. Fontenot JD, et al. (2005) Regulatory T cell lineage specification by the forkhead transcription factor foxp3. *Immunity* 22(3):329–341.
2. Gorelik L, Flavell RA (2000) Abrogation of TGFbeta signaling in T cells leads to spontaneous T cell differentiation and autoimmune disease. *Immunity* 12(2):171–181.
3. Lantz O, Grandjean I, Matzinger P, Di Santo JP (2000) Gamma chain required for naïve CD4+ T cell survival but not for antigen proliferation. *Nat Immunol* 1(1):54–58.

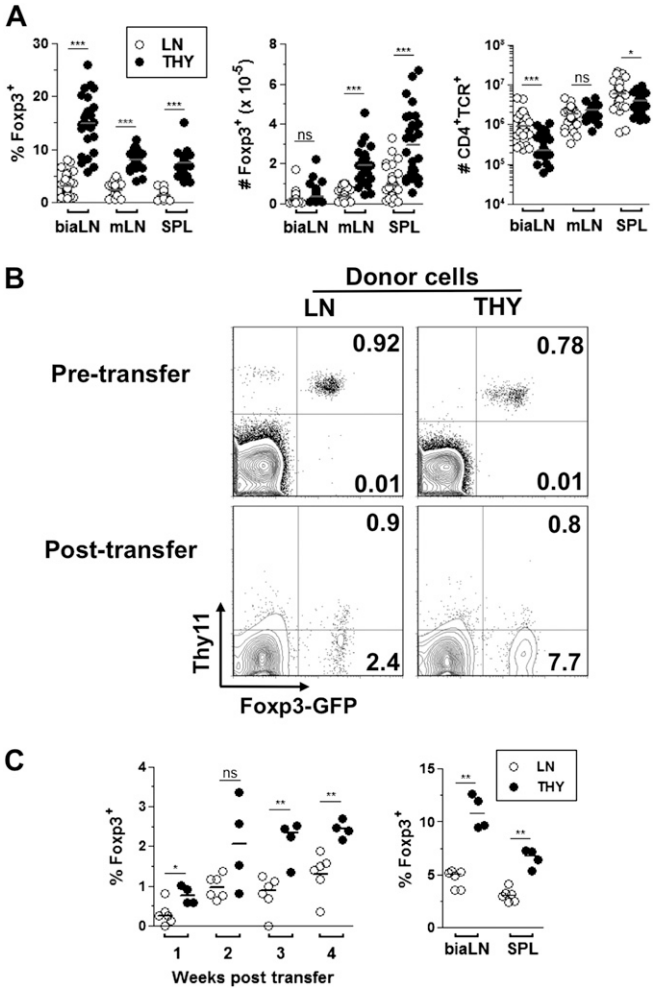

Fig. S1. (*A*) Consistent preferential differentiation of thymocytes to a Foxp3$^+$ phenotype. Pool of six independent experiments conducted as in Fig. 1. Frequency and numbers of Foxp3 and CD4 cells recovered. (*B*) TCRβ$^{-/-}$ recipients received a 90:1 mixture of CD4$^+$CD8$^-$Foxp3$^-$ (Thy1.2) and Foxp3$^+$ (Thy1.1) T cells, both prepared from either thymi or pooled LNs of Foxp3-gfp reporter mice either Thy1.2 or Thy1.1 congenic, respectively (*Upper*). *Lower* shows analysis of recipient mice 4 wk postadoptive transfer after gating on CD4$^+$TCR$^+$ splenocytes. (*C*) As in Fig. 1, except that the recipients were Thy1.1-congenic C57BL/6 mice that had received 400 rad full-body irradiation the day before. In this setting, resident host lymphocytes limited the expansion of the donor cells, which represented 7% of total CD4 lymphocytes in spleen and LNs 4 wk posttransfer. Shown is analysis gated on donor Thy1.2$^+$ CD4$^+$ TCR$^+$ live cells. (*Left*) Kinetic analysis in peripheral blood. (*Right*) Spleen and LN analysis at 4 wk posttransfer. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

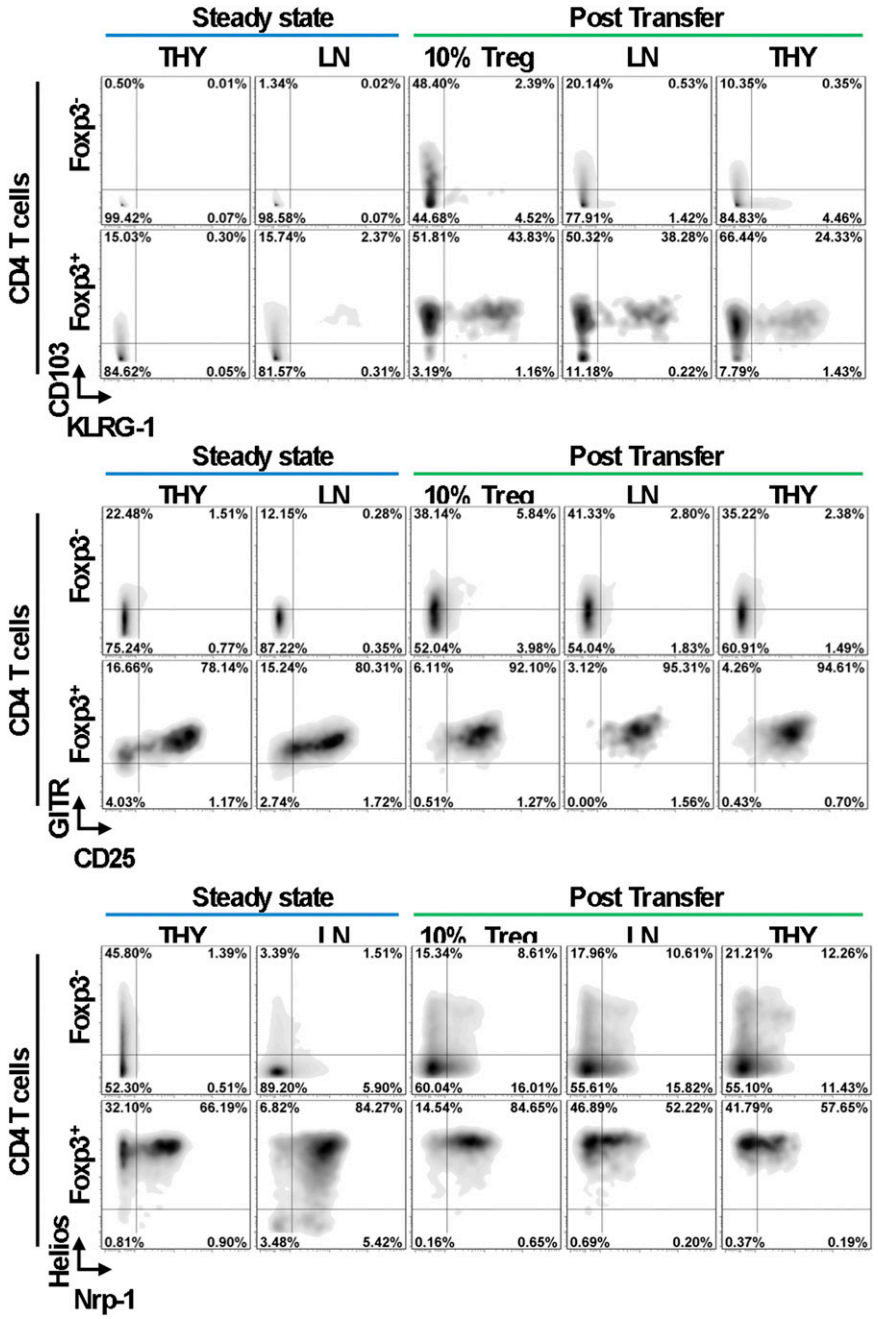

Fig. S2. Pairwise phenotypic analysis of Foxp3^{-} or Foxp3^{+} cells from the spleens of animals treated as in Fig.1*A*. Shown are representative FACS analysis of recovered Foxp3^{-} and Foxp3^{+} cells from thymocytes or LN transfers (columns 4 and 5). Other Foxp3^{-} and Foxp3^{+} cell populations served as references: thymic Foxp3^{+} cells and Foxp3^{-} conventional CD8^{-}CD4^{+} thymocytes as well as peripheral Foxp3^{+} and Foxp3^{-} from LNs were from animals kept at steady state (columns 1 and 2); cells from TCR$\beta^{-/-}$-recipient mice that received a mixture of Thy1.1 Foxp3^{-} and Thy1.2 Foxp3^{+} LN cells at a 9:1 ratio 4 wk earlier (column 3).

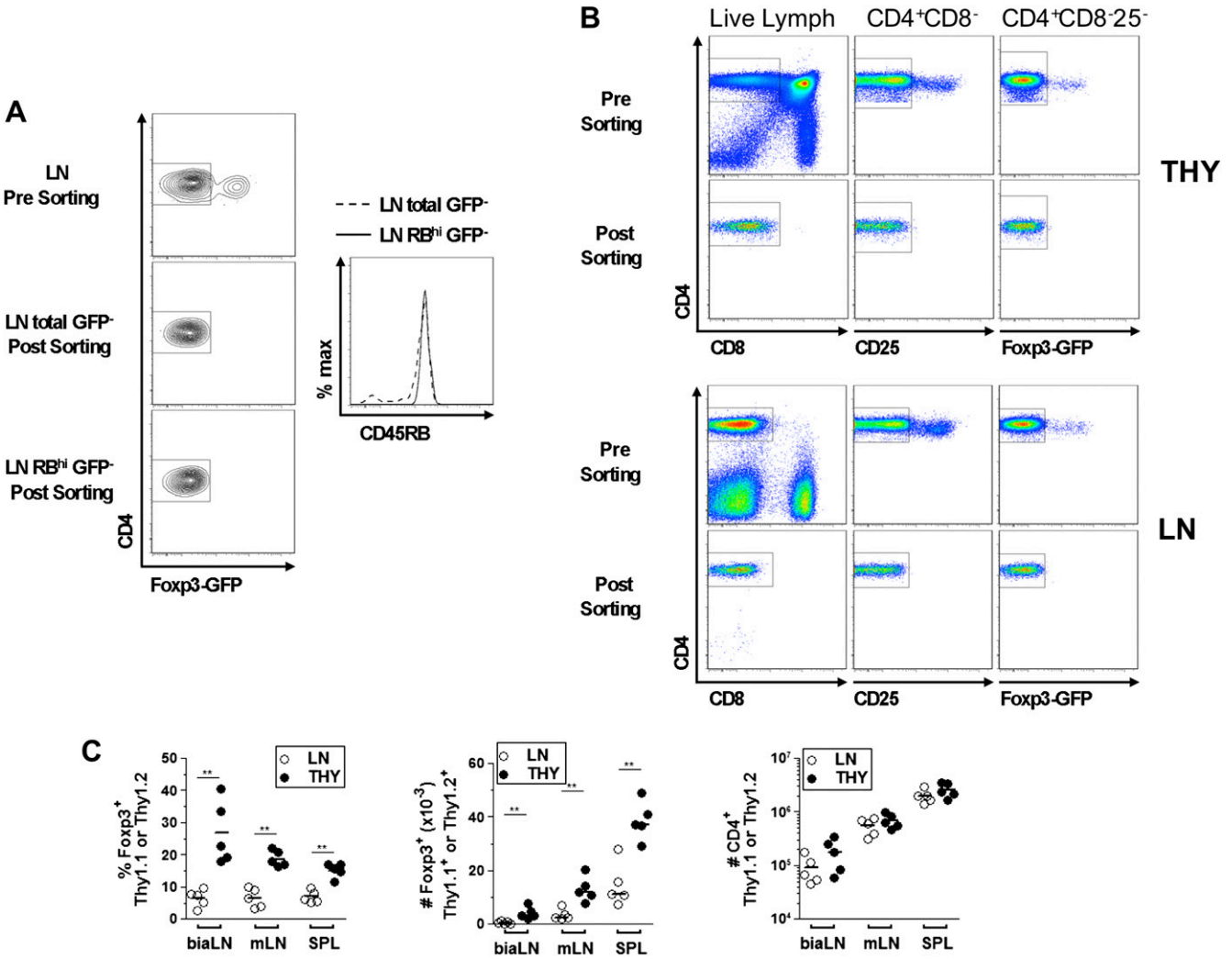

Fig. S3. (*A*) Purification of CD4$^+$Foxp3$^-$CD45RB^{high} LN cells used in experiments presented in Fig. 3*B*. (*B*) Purification of CD8$^-$CD4$^+$Foxp3$^-$CD25$^-$ LN cells and thymocytes used in experiments presented in Fig. 3*D*. (*C*) Preferential differentiation of thymocytes vs. LN cells into regulatory T cells (Tregs) is not a singularity of the Foxp3-gfp allele. CD8$^-$CD4$^+$CD25$^-$ thymocytes and CD8$^-$CD4$^+$CD25$^-$CD45RB^{high} LN cells were sorted from WT Thy1.1 and Thy1.2 mice, respectively, and cotransferred into TCRβ$^{-/-}$ mice. Recipients animals were analyzed as in Fig. 3*C*. $^{**}P < 0.01$.

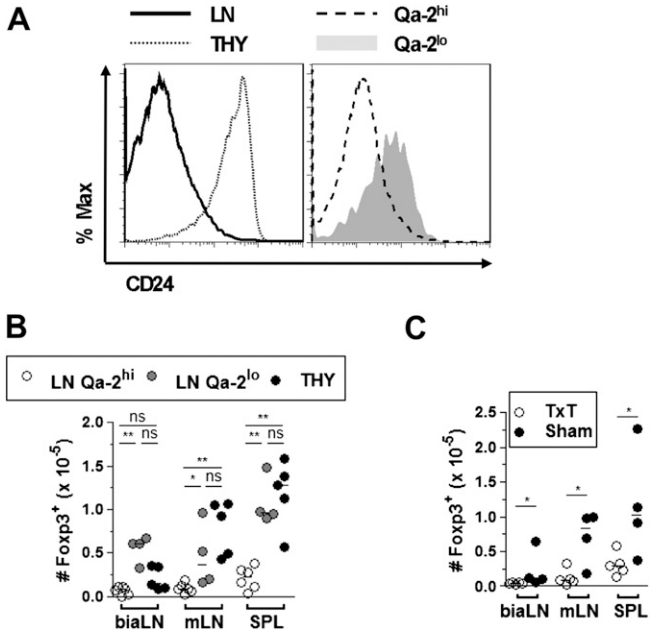

Fig. S4. (*A*) Expression level of surface CD24/heat stable antigen (HSA) on $CD4^+CD8^-Foxp3^-$ cells from LN cells and thymocytes (*Left*) and gated Qa-2^{hi} and Qa-2^{lo} LN cells from 2-mo-old mice. (*B*) Numbers of $CD4^+TCR^+Foxp3^+$cells recovered on adoptive transfer of peripheral resident mature cells (LNQa2^{hi}), recent thymic emigrant (RTE; LNQa2^{lo}), and thymocytes as in Fig. 4*A*. (*C*) Idem for adoptive transfer of total $CD4^+Foxp3^-$ LN cells purified from thymectomized (TxT) or sham operated donor mice as in Fig. 4*B*. $*P < 0.05$; $**P < 0.01$.

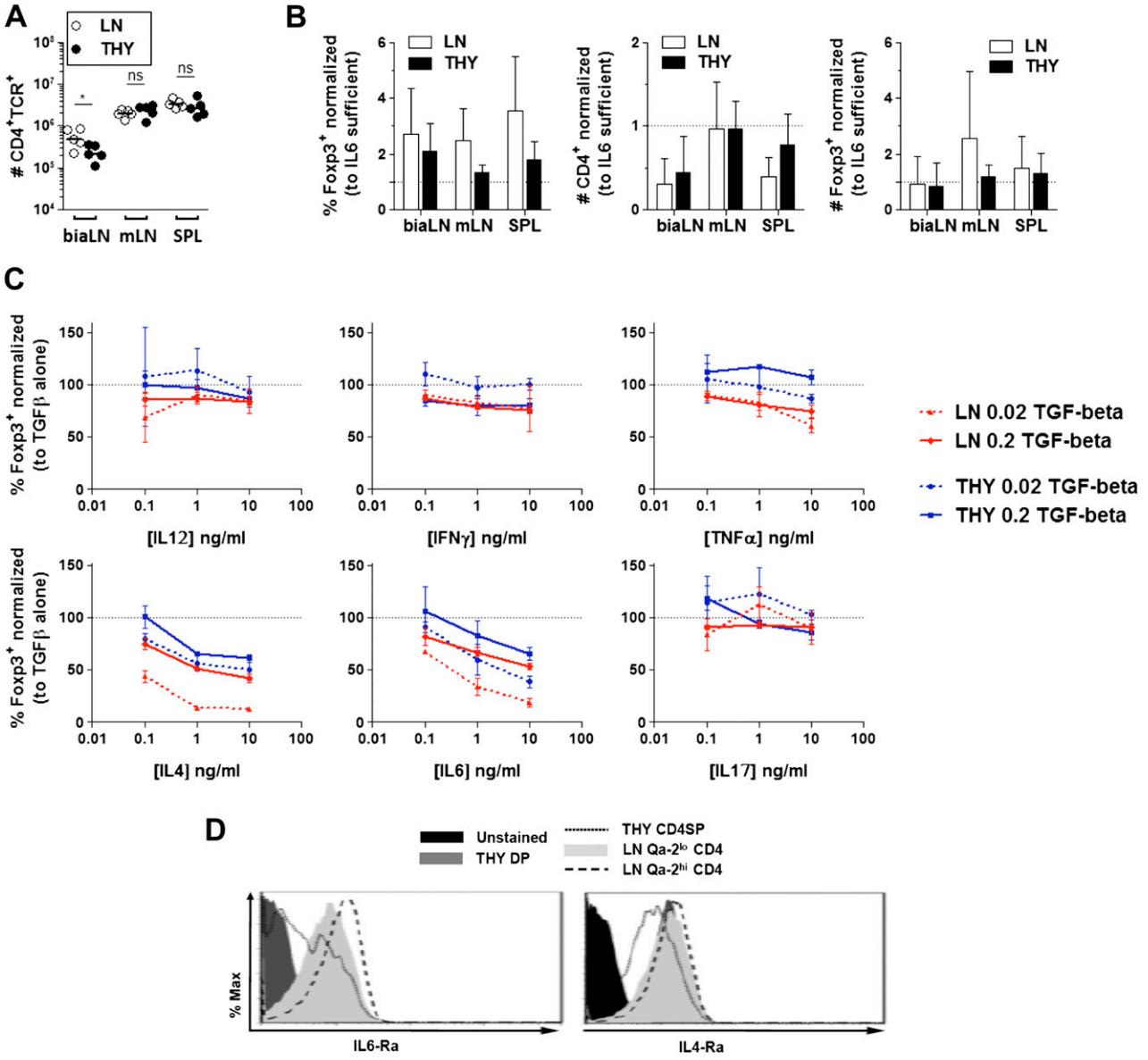

Fig. S5. (*A* and *B*) Number of $CD4^+TCR^+$ cells recovered in $TCR\beta^{-/-}$ IL-$6^{-/-}$ double mutants as in Fig. 5*F* (*A*) and comparison with similar adoptive transfer into IL-6–competent recipient mice (*B*), where the latter are given a value of one. (*C*) Detailed analysis of LN cells and thymocytes sensitivity to candidate inhibitors of Foxp3 induction. Culture conditions were as in Fig. 6*E*: plates were coated anti-CD3 Abs, and media were supplemented with IL-2 and the indicated cytokines. (*D*) Surface expression of IL-6-Rα and IL-4-Rα by the three cell types analyzed in this work. $^*P < 0.05$.

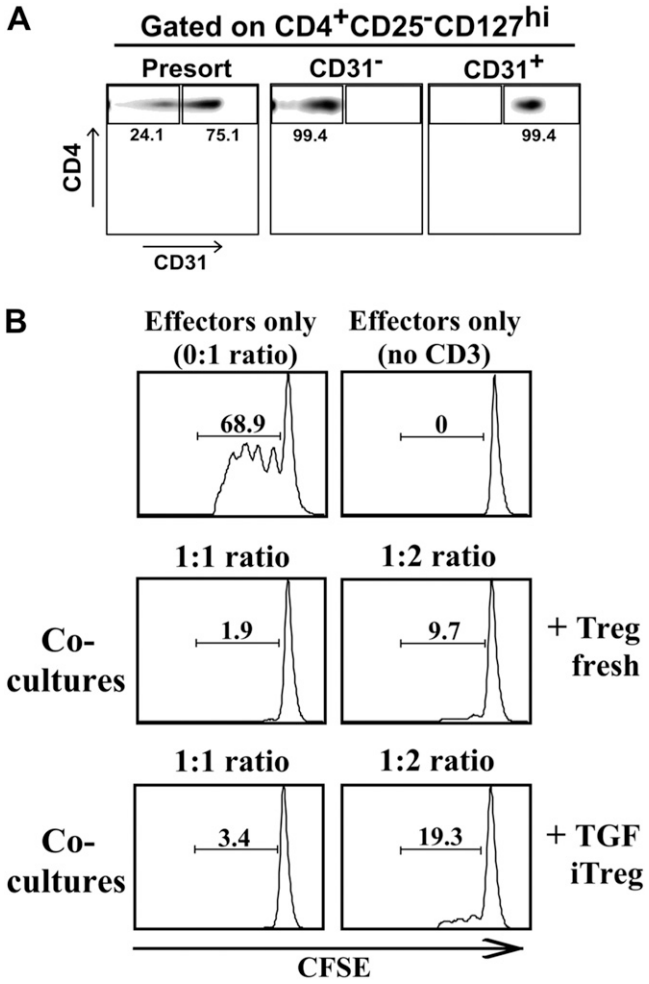

Fig. S6. (*A*) Representative flow cytometric analyses of CD31 expression within naïve $CD4^+CD25^-CD127^{hi}$ PBMCs before and after sorting. (*B*) Proliferation of carboxyfluorescein succinimidyl ester (CFSE)-labeled $CD4^+CD25^-$ cells (targets; 2.5×10^4). As a reference, target cells were stimulated alone with anti-CD3 mAbs (1 μg/mL) using autologous irradiated PBMCs as APCs or left unstimulated (*Top*). As a control, target cells were mixed at the indicated ratio (Treg:target) with freshly isolated $CD4^+CD25^{bright}CD127^{lo}$ cells (*Middle*) or $CD4^+CD25^-CD127^{hi}$ cells previously stimulated for 5 d in the presence of TGF (*Bottom*). Shown is a representative experiment performed with $CD31^+$-induced cells. Proliferation was evaluated at day 4. Numbers indicate the frequency of target cells that have divided at least one time during the culture period.

Table S1. Antibodies used for FACS analysis and cell culture

Reactivity	Clone	Conjugate	Supplier
Mouse FACS analysis			
CD24	M1/69	Cy5	BD PharMingen
IL-4-Ra/CD124	mIL-4R-M1	PE	B.11D PharMingen
CD4	RM4-5	PE/PB/APC	BD PharMingen
CD45RB	16A	PE	BD PharMingen
CD44	IM7	Biot/PB	BD PharMingen
Thy1.2	53–2.1	Biot	BD PharMingen
Thy1.1	Ox7	PE	BD PharMingen
Qa-2	1.1.2	Biot	BD PharMingen
IL-6-Ra/CD126	M182	PE	BD PharMingen
IFN-g-RI/CD119	2E2	PE	eBiosciences
CD103	2-E7	Biot	eBiosciences
KLRG-1	2F1	APC	eBiosciences
GITR	DTA-1	Biot	eBiosciences
Foxp3	FJK-16s	FITC/PE/APC	eBiosciences
CD3e	500 A2-77	APC	In house
IL-2-Ra/CD25	7D4	PE	In house
CD8	YTS169.4	PE/A647	In house
Helios	22F6	PE	Biolegend
Neuropilin-1	Polyclonal	APC	R&D
Streptavidin		A488/PE/A647 PB/APC-Cy7	BD PharMingen
Human FACS analysis			
CD3	OKT3		eBiosciences
CD4	RPA-T4		eBiosciences
CD8α	RPA-T8		eBiosciences
CD31	WM59		eBiosciences
FOXP3	PCH101		eBiosciences
CD25	2A3		BD PharMingen
CD127	40131		R&D
Mouse cell culture			
CD3e	141.2C11		In house, protG purified
Neutralizing–TGF-b	1D11		In house, protG purified
Thy1.2	30H12		In house, protG purified
CD28	37.51		eBiosciences
Human cell culture			
CD3 (OKT3)	OKT3		eBiosciences
CD28	CD28.2		eBiosciences

A, Alexa; APC, allophycocyanin; Biot, biotin; Cy, cychrome; PB, Pacific Blue; PE, phycoerythrin.