Supporting Information

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SI Materials and Methods

Generation of *Tgfb1*$^{f/f}$ NOD Mice. Mouse genomic DNA of the *Tgfb1* gene was isolated from a nonobese diabetic (NOD) BAC clone 201A23 (Children's Hospital Oakland Research Institute). The targeting vector was constructed by PCR cloning of three genomic fragments into pEasy-FLIRT: a 5′ homology arm (2.7 kb) into the BamHI site, a floxed region containing exon 2 (4.5 kb) into SalI sites, and a 3′ homology arm (1.9 kb) into AscI sites. The targeting vector was electroporated into NOD/129 F1 ES cells. To identify clones whose NOD allele was targeted, the SNP within intron 1 of *Tgfb1* gene (129 allele, T; NOD allele, A) was used. Correctly targeted clones were identified by PCR screening, injected into blastocysts, and implanted into foster mothers. Chimeric mice were bred to NOD mice, and the F_1 generation was screened for germ-line transmission. For detection of the WT, floxed, and deleted alleles, the following PCR primers were used: primer 1, 5′-AAAGAGGCTGGCACTTTCCCGT-3′; primer 2, 5′-TGGATGGCATGGTATCCCTGCT-3′; primer 3, 5′-CCTTGAACTCAAGAGATCCTCC-3′. The *neo* gene was removed by breeding to flippase (FLP) recombinase transgenic NOD mice. The deletion of *neo* gene was analyzed by primer 3 and primer 4; 5′-GGAGCAGACACTTGCAAATCCT-3′.

Mice. *Tgfbr2*$^{f/f}$ (1), *Ifng*$^{-/-}$/NOD (2), and OX40Cre/C57BL/6 (3) mice were kindly provided by H. Moses (Vanderbilt University, Nashville, TN), D. Mathis (Harvard Medical School, Boston, MA), and N. Killeen (University of California, San Francisco, CA), respectively. BDC2.5/NOD, Foxp3-EGFP Cre/NOD (4), and Scid/NOD mice were obtained from Jackson Laboratory. *Tgfb1*$^{f/f}$, *Tgfbr2*$^{f/f}$, OX40Cre, CD4Cre, *Il22*$^{-/-}$ (5), Foxp3-RFP (6), and *Il21r*$^{-/-}$ mice (7) on a C57BL/6 background were backcrossed to NOD mice for at least 10 generations. The use of the animals in this study was approved by the Yale University Institutional Animal Care and Use Committee.

Assessment of Diabetes Development. Diabetes was monitored by measuring either urine glucose level with Diastix (Bayer) or blood glucose levels with One Touch test strips (LifeScan). Mice were considered hyperglycemic when their blood glucose consistently rose above 350 mg/dL. For histology, the pancreas was harvested, fixed in formalin, and stained with H&E.

Cell Isolation and Flow Cytometry. Single-cell suspension from lymph nodes (LNs), spleen, and thymus was generated by mechanical disruption. For pancreas-infiltrating cell isolation, pancreas was cut into pieces and digested in HBSS (Gibco) supplemented with 0.5 mg/mL Collagenase P (Roche). The digested pancreas was resuspended in 80% (vol/vol) Percoll solution (GH Healthcare). Cells were collected from the interface of a 40%:80% Percoll gradient after centrifugation at 800 × *g* for 15 min. For the isolation of regulatory T (Treg) cells from Foxp3Cre-GFP mice, $CD4^+$ T cells were enriched from spleen and LN cells by positive selection with anti-CD4 microbeads (Miltenyi Biotec), according to the manufacturer's protocol. $CD4^+V\beta4TCR^+Foxp3RFP^+$ cells were further purified using a cell sorter (Becton Dickenson). Fluorescent dye–labeled Abs against CD4, CD8α, CD25, CD44, and CD62L were purchased from BioLegend; T-bet, Foxp3, and Helios were from eBioscience; TCRVβ4, IFN-γ, IL-17, and GM-CSF were from BD Biosciences; and TGFβRII and CXCR3 were from R&D Systems. For intracellular cytokine staining, cells were stimulated with phorbol 12-myristate 13-acetate (PMA) (50 ng/mL; Sigma), ionomycin (500 ng/mL; Sigma), and 2 μM Golgistop (BD Biosciences) for 4 h, followed by fixing and permeabilization according to the manufacturer's instructions (BD Biosciences). Foxp3, T-bet, Helios, and cytokine staining were performed according to the manufacturer's instructions (eBiosciences). The cells were analyzed on a LSRII (Becton Dickinson), and data were analyzed with FlowJo software (Tree Star).

Real-Time RT-PCR Analysis. Total RNA was extracted with the Qiagen mRNAEasy kit according to the manufacturer's instructions. RNA was reverse transcribed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The cDNA was subsequently used for quantitative PCR on an ABI Prism instrument using commercial primer–probe sets (Applied Biosystems).

Statistics. Unpaired Student *t* tests were used to statistically analyze the results. Differences were considered significant at $P < 0.05$.

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2. Hultgren B, Huang X, Dybdal N, Stewart TA (1996) Genetic absence of gamma-interferon delays but does not prevent diabetes in NOD mice. *Diabetes* 45(6):812–817.
3. Klinger M, et al. (2009) Thymic OX40 expression discriminates cells undergoing strong responses to selection ligands. *J Immunol* 182(8):4581–4589.
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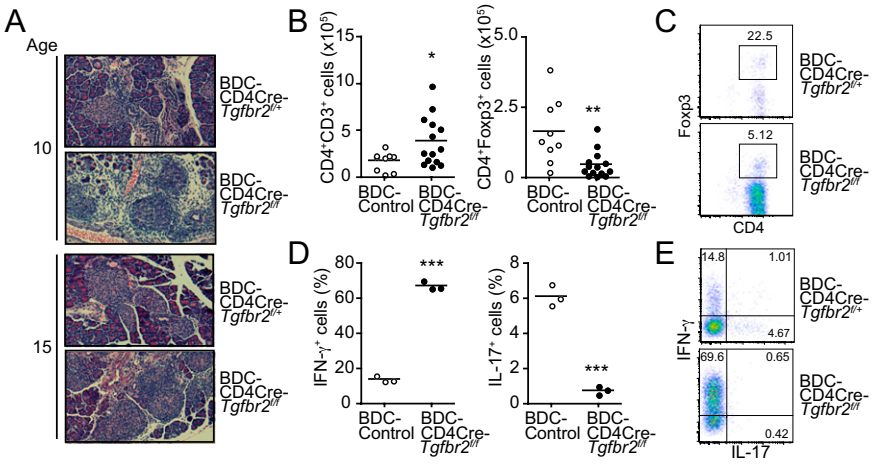

Fig. S1. Diabetes development in the absence of TGF-β signaling in thymic T cells is associated with reduced Treg cell and increased Th1 differentiation in the pancreas. (*A*) Histological sections of pancreas tissue at 10 and 15 d old stained with H&E (original magnification, ×20), representative of four to five mice in each group. (*B*) Absolute number of $CD4^+CD3^+$ or $CD4^+Foxp3^+$ cells in the pancreas of BDC-CD4Cre-*Tgfbr2*$^{f/f}$ mice or littermate controls at 3 wk old. (*C*) Expression of Foxp3 on $CD45^+CD4^+$ cells from the pancreas of BDC-CD4Cre-*Tgfbr2*$^{f/f}$ mice or littermate controls as in *B*. (*D* and *E*) Pancreas-infiltrating $CD4^+$ $V\beta4TCR^+$ cells isolated from the indicated mice at 3 wk old were stimulated in vitro with PMA and ionomycin, and expression of IFN-γ and IL-17 were measured by flow cytometry. Representative plots of the IFN-γ and IL-17 expression are shown in *E*. Each dot represents one mouse. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs. control. Data are representative of two independent experiments.

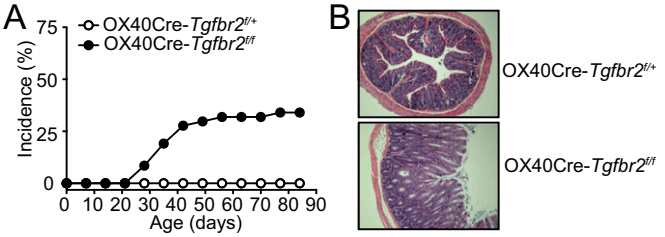

Fig. S2. Development of immunopathology in the absence of TGF-β signaling in effector T cells. (*A*) Diabetes incidence of OX40Cre-*Tgfbr2*$^{f/f}$ ($n = 46$) and OX40Cre-*Tgfbr2*$^{f/+}$ mice ($n = 40$) by positive urine glucose levels. (*B*) Histological sections of colon tissue at 3 mo old stained with H&E (original magnification, ×5), representative of four to five mice in each group.

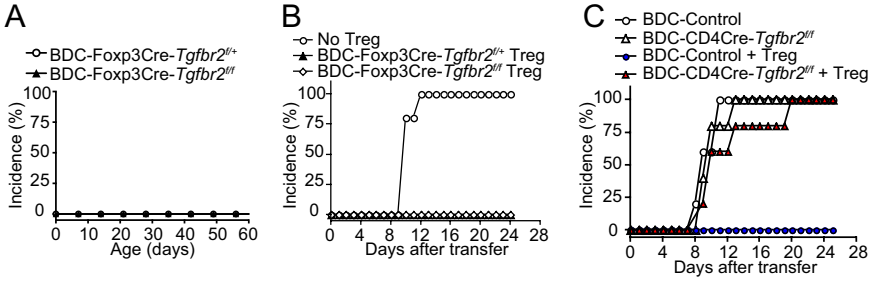

Fig. S3. TGFβRII deficiency in $Foxp3^+$ Treg cells does not impair Treg cell functions. (*A*) Diabetes incidence of BDC-Foxp3Cre-*Tgfbr2*$^{f/f}$ and control littermates ($n = 15$). (*B*) $CD4^+TCRV\beta4^+$ effector T cells were sorted from BDC-WT mice. $CD4^+TCRV\beta4^+Foxp3\text{-}GFP^+$ Treg cells were isolated from BDC-Foxp3Cre-*Tgfbr2*$^{f/f}$ or BDC-Foxp3Cre-*Tgfbr2*$^{f/+}$ mice. $CD4^+TCRV\beta4^+$ effector T cells (2×10^5 cells) were injected into Scid/NOD mice alone or together with $CD4^+TCRV\beta4^+$ $Foxp3\text{-}GFP^+$ Treg cells at a 2:1 ratio, and diabetes incidence was assessed over time ($n = 6$/group). (*C*) $CD4^+TCRV\beta4^+$ effector T cells were sorted from BDC-CD4Cre-*Tgfbr2*$^{f/f}$ mice or BDC control littermates. $CD4^+TCRV\beta4^+CD25^+$ T cells were isolated from BDC-WT mice. $CD4^+TCRV\beta4^+$ effector T cells (2×10^5 cells) were injected into Scid/NOD mice alone or together with $CD4^+TCRV\beta4^+CD25^+$ T cells at a 2:1 ratio, and diabetes incidence was assessed over time ($n = 6$/group). (*B* and *C*) Data are representative of two independent experiments.

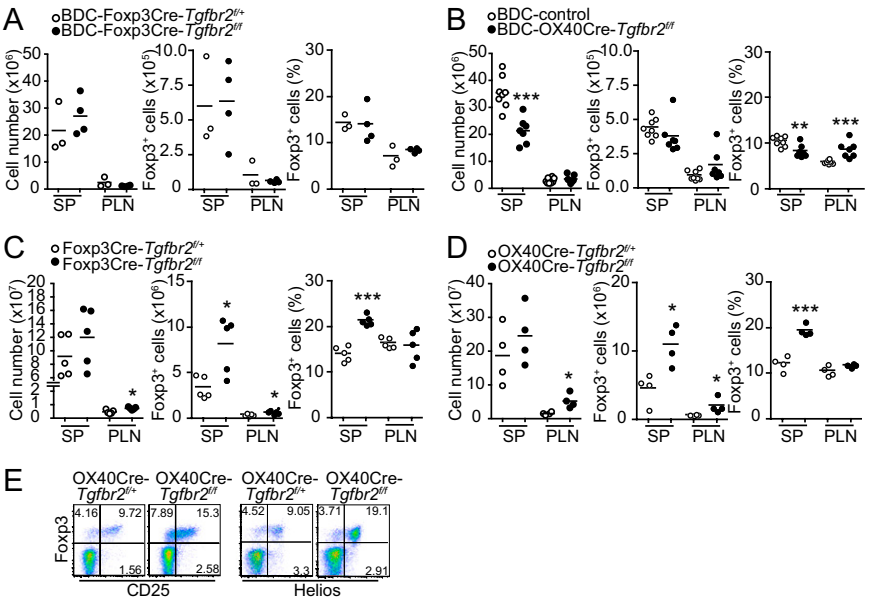

Fig. S4. TGF-β signaling is not required for peripheral Treg cell maintenance. (*A* and *B*) The number of total lymphocytes (*Left*) and the absolute number (*Center*) and percentage (*Right*) of CD4$^+$TCRVβ4$^+$Foxp3$^+$ cells in indicated tissues from BDC-Foxp3Cre-*Tgfbr2*$^{f/f}$ and BDC-Foxp3Cre-*Tgfbr2*$^{f/+}$ mice at 2–3 mo of age or BDC-OX40Cre-*Tgfbr2*$^{f/f}$ and control mice at 3 wk old. Each dot represents one mouse. (*C* and *D*) Number of total lymphocytes (*Left*) and the absolute number (*Center*) and percentage (*Right*) of CD4$^+$Foxp3$^+$ cells in indicated tissues from Foxp3Cre-*Tgfbr2*$^{f/f}$ and Foxp3Cre-*Tgfbr2*$^{f/+}$ mice (*C*) or OX40Cre-*Tgfbr2*$^{f/f}$ and OX40Cre-*Tgfbr2*$^{f/+}$ mice (*D*) at 2–3 mo of age. Each dot represents one mouse. (*E*) Expression of CD25 and Helios on splenic CD4$^+$Foxp3$^+$ cells from same mice as in *D*. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs. control. Data are representative of two independent experiments.

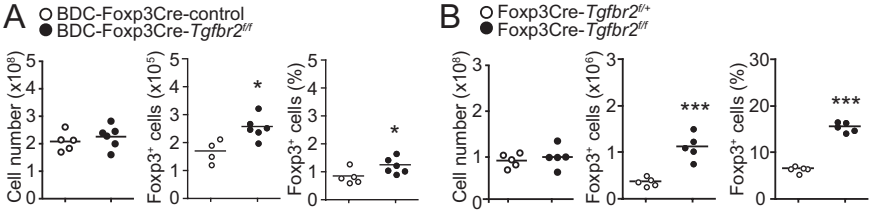

Fig. S5. TGF-β signaling is not required for thymic Treg cell maintenance. (*A*) Number of total lymphocytes (*Left*) and the absolute number (*Center*) and percentage (*Right*) of thymic CD4$^+$Foxp3$^+$ cells from BDC-Foxp3Cre-*Tgfbr2*$^{f/f}$ and control mice at 2–3 mo of age. (*B*) Number of total lymphocytes (*Left*) and the absolute number (*Center*) and percentage (*Right*) of thymic CD4$^+$Foxp3$^+$ cells from Foxp3Cre-*Tgfbr2*$^{f/f}$ and Foxp3Cre-*Tgfbr2*$^{f/+}$ mice at 2–3 mo of age. $*P < 0.05$, $***P < 0.001$ vs. control. Data are representative of two independent experiments.

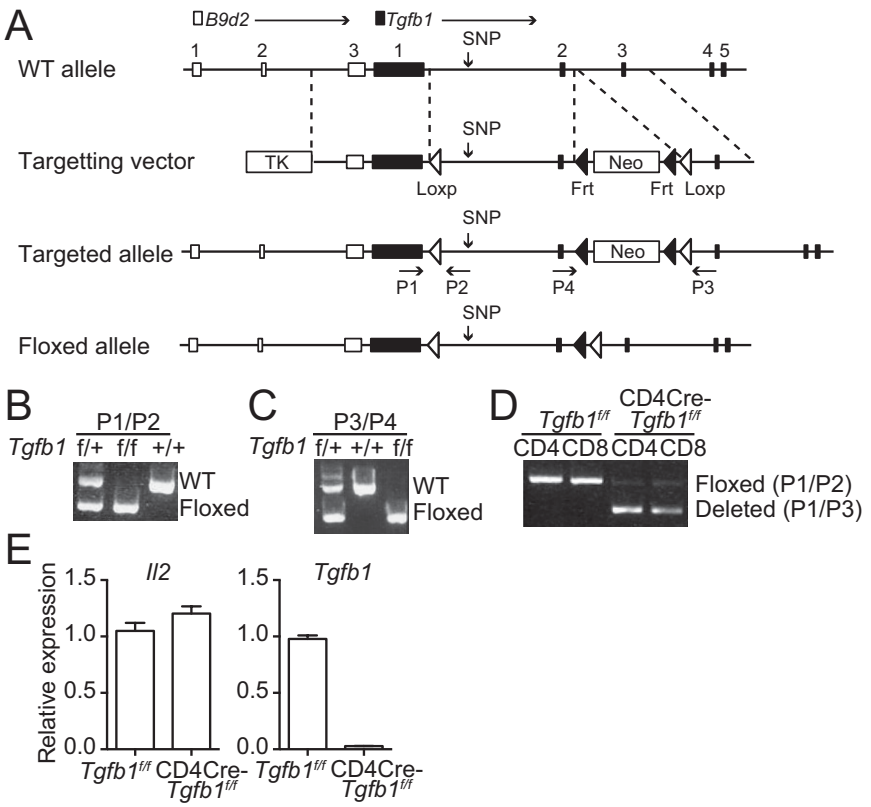

Fig. S6. Generation of *Tgfb1*$^{f/f}$ NOD mice. (*A*) Structure of the mouse *Tgfb1* and *B9d2* locus (WT allele), the TGF-β1–targeting construct (Targeting construct), and the predicted target *Tgfb1* gene locus before (Targeted allele) and after neo deletion (Floxed allele). The regions that are deleted in the floxed allele (Del) are indicated by arrows in WT allele. The locations of primers (P1–P4) used to distinguish floxed and deleted *Tgfb1* allele are indicated by arrows. Exons are represented by boxes. TK, thymidine kinase gene; neo, neomycin resistant cassette. (*B* and *C*) PCR analysis of purified tail DNA from *Tgfb1*$^{+/+}$, *Tgfb1*$^{f/+}$, and *Tgfb1*$^{f/f}$ mice using the primer sets 1 and 2 (*B*) or 3 and 4 (*C*). The upper fragment represents the WT allele, and the lower band represents the floxed allele. (*D*) PCR analysis of purified DNA from splenic CD4$^+$ and CD8$^+$ T cells of a CD4Cre-*Tgfb1*$^{f/f}$ or *Tgfb1*$^{f/f}$ mouse. The floxed and deleted allele are indicated. (*E*) CD4$^+$ T cells isolated from CD4Cre-*Tgfb1*$^{f/f}$ or *Tgfb1*$^{f/f}$ mice were stimulated in vitro with anti-CD3 for 2 d, and mRNA expression of IL-2 and TGF-β1 was determined with real-time RT-PCR. The mRNA expression level in the control cells is defined as 1. Data represent means ± SEM.

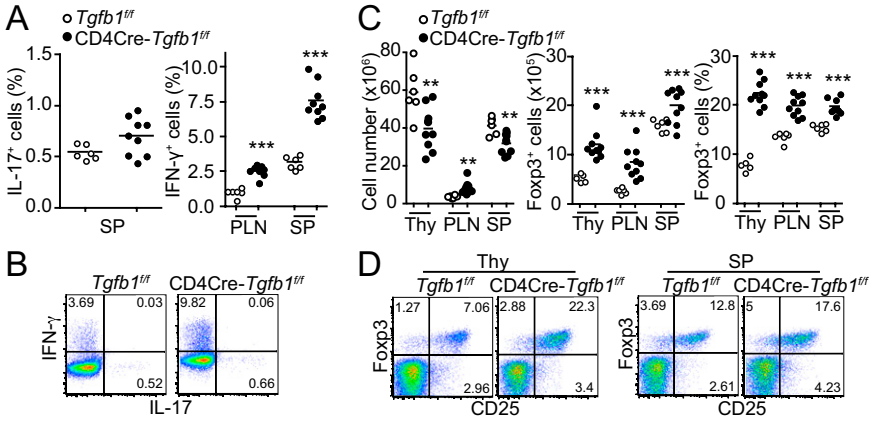

Fig. S7. T cell–derived TGF-β1 suppresses Th1 cell differentiation and Treg cell expansion in NOD mice. (*A*) CD4$^+$ T cells isolated from the indicated tissues of CD4Cre-*Tgfb1*$^{f/f}$ and *Tgfb1*$^{f/f}$ mice at 3–4 mo of age were stimulated in vitro with PMA and ionomycin, and expression of IFN-γ or IL-17 was measured by flow cytometry. Each dot represents one mouse. (*B*) Representative dot plots of the expression of IL-17 and IFN-γ on splenic CD4$^+$ T cells from same mice as in *A*. (*C*) Number of total lymphocytes (*Left*) and the absolute number (*Center*) and percentage (*Right*) of CD4$^+$Foxp3$^+$ cells in indicated tissues from CD4Cre-*Tgfb1*$^{f/f}$ and *Tgfb1*$^{f/f}$ mice at 3–4 mo of age. Each dot represents one mouse. (*D*) Expression of Foxp3 and CD25 on thymic or splenic CD4$^+$ T cells from same mice as in *C*. ***$P < 0.001$ vs. control. Data are representative of two independent experiments.

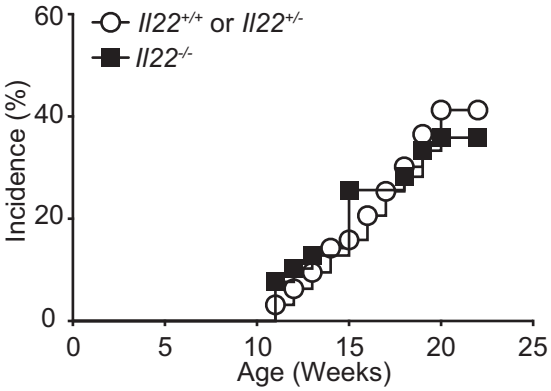

Fig. S8. IL-22 is not required for diabetes development in NOD mice. Diabetes incidence of female *Il22*$^{-/-}$ ($n = 42$) and *Il22*$^{+/+}$ or *Il22*$^{+/-}$ littermates ($n = 63$) by positive urine glucose levels.