Supporting Information

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SI Materials and Methods

Mice. *SMAD3*$^{-/-}$, *SMAD2*$^{fl/fl}$, *TAK1*$^{fl/fl}$, *CD4Cre, FGC*, *Rag1*$^{-/-}$, YFP Cre reporter, and CD45.1 mice are on C57BL/6 background and were kept under specific pathogen-free conditions in the animal care facility at the University of North Carolina. All mouse experiments were approved by the Institutional Animal Care and Use Committee of the University of North Carolina.

Flow Cytometric Analysis and Cell Sorting. Lymphocytes were isolated from the various organs (as described) of age- and sex-matched mice. Fluorescence-conjugated antibodies for CD4, CD8, CD25, CD44, CD62L, glucocorticoid-induced TNFR-related protein (GITR), CD45.1, CD45.2, IFN-γ, IL-4, IL-10, IL-17, Foxp3 (eBioscience), and cytotoxic T-lymphocyte antigen 4 (CTLA-4) (BD Bioscience) were purchased. Surface and intracellular staining was performed per the manufacturers' protocols. For intracellular cytokine staining, lymphocytes were stimulated with 50 ng/mL phorbol 12-myristate 13-acetate (PMA) and 1 μM ionomycin for 3–4 h in the presence of Brefeldin A. Stained cells were analyzed on an LSRII station (BD Biosciences) or sorted on a Moflo cell sorter (Dako Cytomation, Beckman Coulter).

Generation of Bone Marrow Chimeras. Bone marrow cells isolated from 3-wk-old CD45.1^{+} *C57BL/6* (WT) mice and 3-wk-old CD45.2^{+} *CD4:DKO* mice were mixed at a 1:1 ratio. Cell mixtures were injected into 8-wk-old *Rag1*$^{-/-}$ mice that were sublethally irradiated (600 cGy) before the transfer. Eight weeks after transfer, lymphocytes were isolated from recipient mice for further analysis.

T-Cell Culture and TH Differentiation. T cells were cultured in Bruff's medium supplemented with 10% (vol/vol) FBS and 1% (vol/vol) penicillin/streptomycin. T cells were activated with soluble 2 μg/mL anti-CD3 and 1 μg/mL anti-CD28 for long-term culturing or with 50 ng/mL PMA and 1 μM ionomycin to stimulate cytokine production. For TH1 and TH2 differentiation, sorted naive CD4^{+}CD25^{-} CD62L^{high}CD44^{low} T cells were activated with plate-bound anti-CD3 and anti-CD28 mAbs for 4 d in the presence of recombinant mouse (m)IL-12 (20 ng/mL) or mIL-4 (20 ng/nl) and mIL-2 (2 ng/mL). Anti–IFN-γ and anti–IL-4 (11B11) mAbs were also used at 20 ng/mL to block TH1 and TH2 differentiation, respectively. TGF-β (2 ng/mL) was used to inhibit TH1 and TH2 differentiation.

In Vitro T-Cell Suppression Assay. CD4^{+}CD25^{+} or GFP^{+} Treg cells were sorted. CD4^{+}CD25^{-}CD45RB^{high} T cells were sorted from WT mice as responder T (Tresp) cells. To assess the efficacy of Treg-mediated immune suppression in vitro, 2×10^4 sorted Tresp cells were labeled with carboxyfluorescein succinimidylester (CFSE) and mixed with varying amounts (as indicated) of Treg suppressor cells. Cell mixtures were stimulated with soluble anti-CD3 antibody (1 μg/mL) in the presence of 1×10^5 irradiated (3,000 cGy) T cell-depleted splenocytes as antigen presenting cell (APC). The proliferation of responder cells was assessed by CFSE dilution detected by flow cytometric analysis 72 h after stimulation. Division indices of Tresp cells were calculated using FlowJo (TreeStar) software.

Treg-Mediated Protection of Naive CD4^{+} T Cell-Elicited Inflammatory Bowel Disease Assay. To assess Treg-mediated protection of naive T cell-elicited inflammatory bowel disease (IBD) in vivo, 1×10^5 sorted GFP^{+} Treg cells were mixed with 2×10^5 naive (CD25^{-}CD45RB^{high}) CD4^{+} T cells sorted from WT *C57BL/6* mice. The cell mixture was transferred into *Rag1*$^{-/-}$ via retro-orbital injection. As controls, 2×10^5 naive CD4^{+} T cells were also transferred alone into *Rag1*$^{-/-}$ mice. To assess IBD development, body weight of the recipient mice was monitored weekly after the transfer.

Statistical Analysis. Data from at least three sets of samples were used for statistical analysis. Statistical significance was calculated by the Student *t* test. A *P* value of less than 0.05 was considered significant.

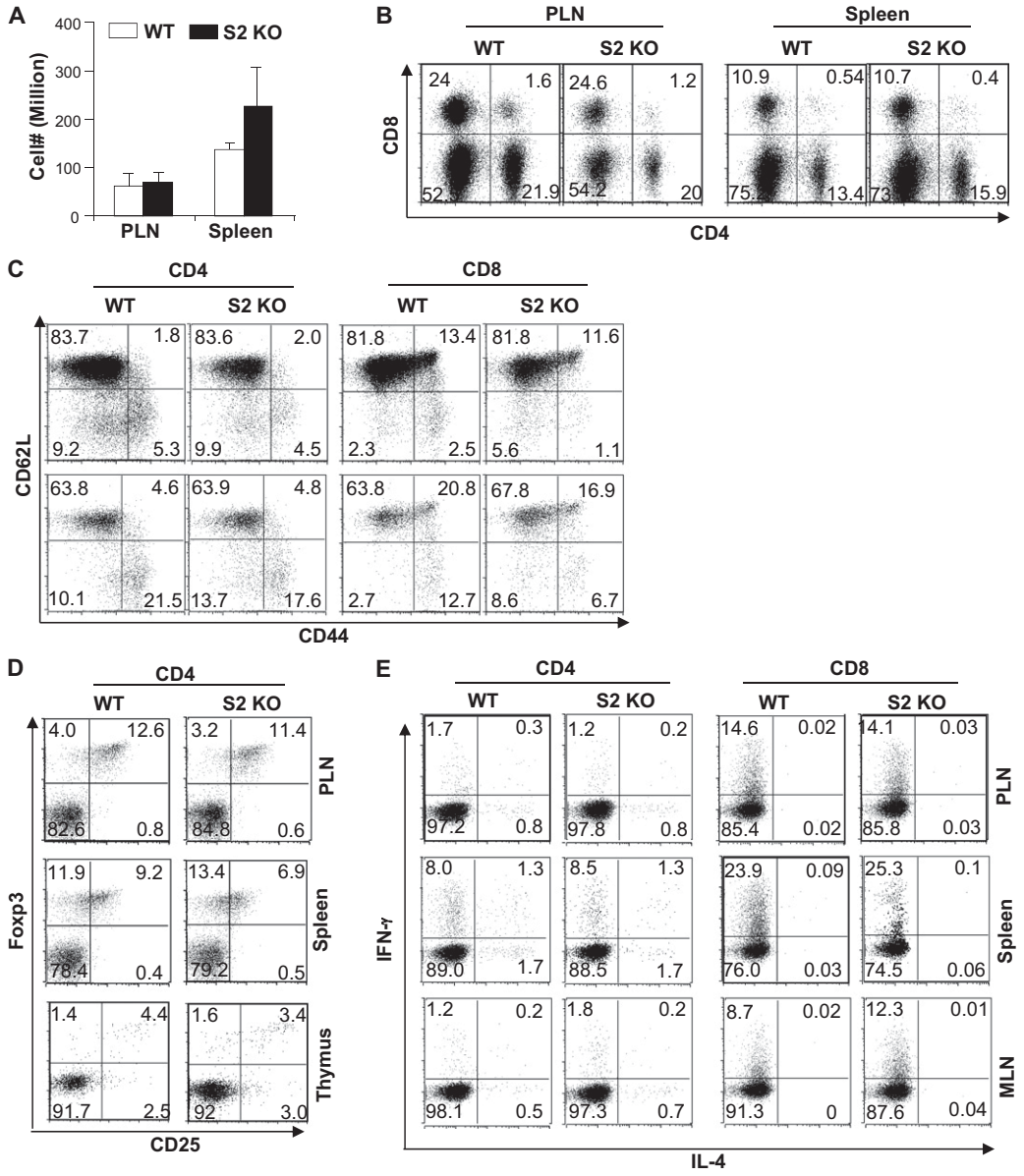

Fig. S1. Normal T-cell phenotypes in the absence of Smad2. Lymphocytes from WT and *CD4Cre:Smad2*$^{fl/fl}$ (S2KO) mice were isolated. The cell numbers (*A*); distribution of CD4 and CD8 T cells in the PLNs and spleens (*B*); T-cell activation (*C*); Treg population in the PLNs, spleens, and thymus (*D*); and effector cytokine production by CD4 and CD8 T cells (*E*) were assessed. Representative results from three experiments are shown.

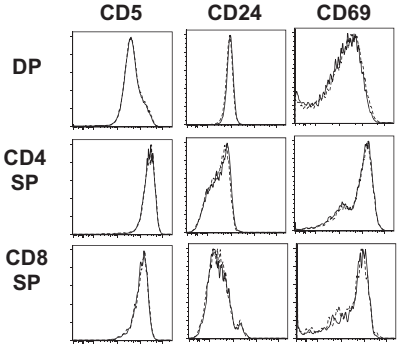

Fig. S2. Normal phenotype of thymocytes in *CD4:DKO* mice. Thymocytes were isolated from WT (solid lines) and *CD4:DKO* (dashed lines) mice. CD5, CD24, and CD69 expression on DP, CD4 single-positive (CD4SP) and CD8SP thymocytes was detected by flow cytometric analysis and compared. Results representative of three experiments are shown.

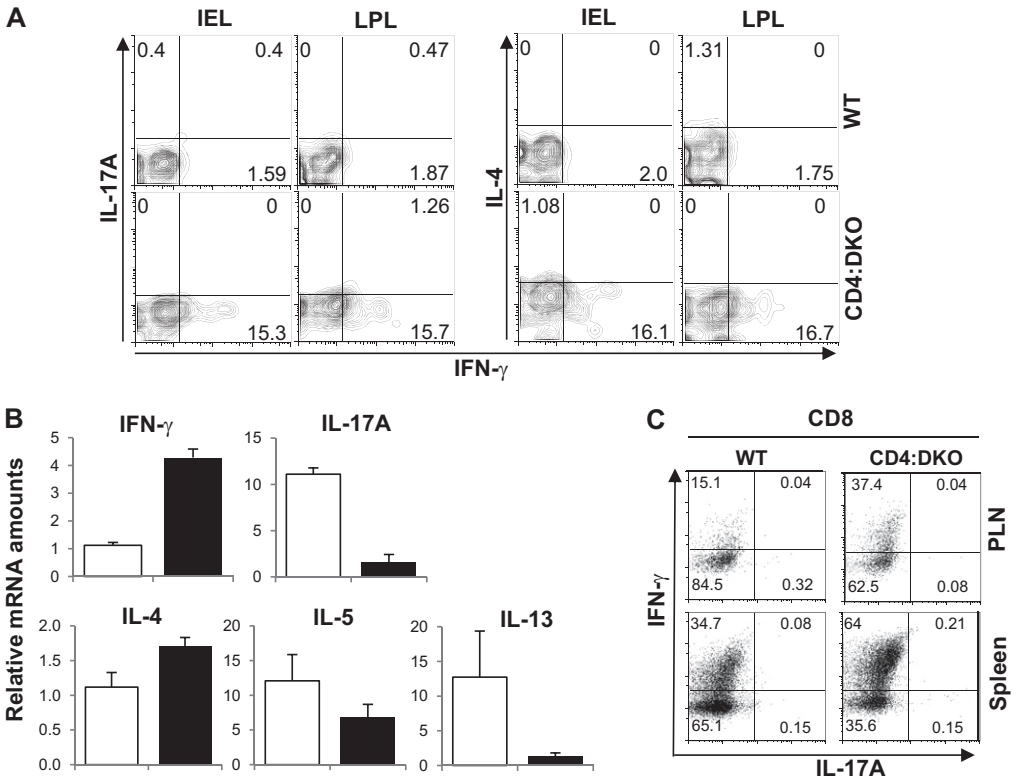

Fig. S3. Cytokine production by T cells from *CD4:DKO* mice. (*A*) Cytokine production by CD4 T cells in the intestines of *CD4:DKO* mice. Intraepithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL) were isolated from WT and *CD4:DKO* mice. IFN-γ, IL-17A, and IL-4 expression in CD4 T cells was detected by intracellular staining and flow cytometric analysis. Results are representative of three experiments. (*B*) Cytokine expression by CD4 T cells from *CD4:DKO* mice. CD4 T cells were isolated from WT (open bar) and *CD4:DKO* (solid bar) mice. mRNA expression of IFN-γ, IL-17A, IL-4, IL-13, and IL-5 was assessed by quantitative RT-PCR. The mean ± SD of three samples in one experiment representative of three are shown. (*C*) Cytokine production by CD8 T cells from *CD4:DKO* mice. CD8 T cells were isolated from the PLNs and spleens of WT and *CD4:DKO* mice. IFN-γ and IL-17A production was detected by cytokine intracellular staining and flow cytometric analysis. Results representative of at least three experiments are shown.

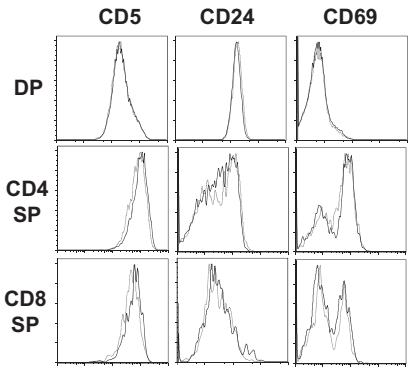

Fig. S4. Normal phenotype of Smad2/Smad3 double-deficient thymocytes from bone marrow chimeric mice. Thymocytes were isolated from bone marrow chimeric mice reconstituted with bone marrow cells from WT (solid lines) and *CD4:DKO* (dashed lines) mice. CD5, CD24, and CD69 expression on DP, CD4 single-positive (CD4SP), and CD8 (CD8SP) thymocytes was detected by flow cytometric analysis and compared. Results representative of at least three experiments are shown.

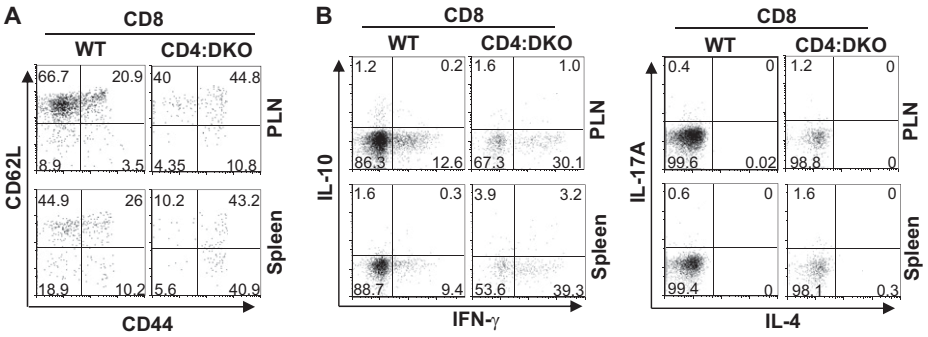

Fig. S5. Activation of Smad2/Smad3 double-deficient CD8 T cells in bone marrow chimeric mice. CD8 T cells were isolated from bone marrow chimeric mice reconstituted with bone marrow cells from WT (CD45.1$^+$) and *CD4:DKO* (CD45.2$^+$) mice. T-cell activation (*A*) and effector cytokine production (*B*) of CD8 T cells were assessed by flow cytometry analysis. Results are representative of at least three experiments.

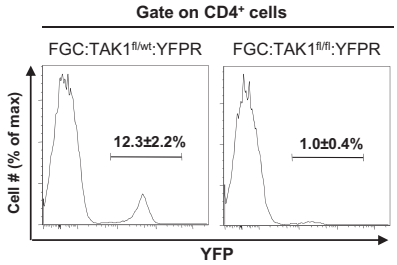

Fig. S6. Remaining Treg cells in *FGC:TAK1$^{fl/fl}$* mice did not express functional Cre. *FGC:TAK1* mice were crossed with YFP Cre-reporter (YFPR) mice, wherein YFP is produced when cells express functional Cre. The percentages of CD4 T cells that expressed functional Cre (YFP$^+$) were determined in the splenocytes of *FGC:TAK1$^{fl/+}$:YFPR* and *FGC:TAK1$^{fl/fl}$:YFPR* mice by flow cytometry analysis. The mean ± SD of three experiments are shown.

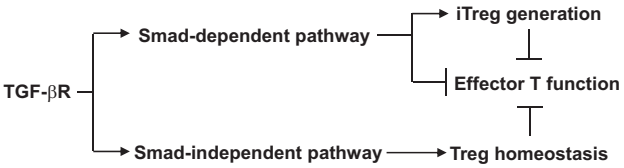

Fig. S7. Schematic illustration of how TGF-β controls non-Treg and Treg functions through Smad-dependent and -independent pathways.