ONLINE METHODS

Mice. Mice of *S1pr1*$^{flox/flox}$, $S1P_1$-Tg, and *Foxp3*^{gfp} knockin have been described previously[13,14], and have been backcrossed to the C57BL/6 background extensively. C57BL/6, CD45.1, Thy1.1, *Rag1*$^{-/-}$, *Ifng*$^{-/-}$, CD4-dnTGFβRII, OT-II and scurfy mice (all on the C57BL/6 background) were purchased from the Jackson Laboratory. Wild-type controls were in the same genetic background and included transgene-negative littermates or where relevant, Cre^{+} mice to account for Cre effects. Mice at 6-10 weeks old were used unless otherwise noted. Bone marrow chimeras were generated by transferring 1-2 $\times$ 10^7 T cell-depleted bone marrow cells into sublethally irradiated (5 Gy) alymphoid *Rag1*$^{-/-}$ mice, as described previously[14]. All mice were kept in specific pathogen–free conditions in Animal Resource Center at St. Jude. Animal protocols were approved by Institutional Animal Care and Use Committee of St. Jude.

Flow cytometry. For analysis of surface markers, cells were stained in PBS containing 2% (wt/vol) BSA and selective antibodies (all from eBioscience, as described previously[13,14]). Foxp3 staining was performed per manufactures' instructions (FJK-16s; eBioscience). For intracellular cytokine staining, T cells were stimulated for 5 h with phorbol 12-myristate 13-acetate (PMA)/ionomycin in the presence of monensin before staining per manufacture's instructions (BD Bioscience). Flow cytometry data were acquired on an upgraded 5-color FACScan (Becton Dickinson), and analyzed using FlowJo software (Treestar).

Cell purification and cultures. Lymphocytes were isolated from the lymphoid organs and naïve T cells sorted on a MoFlow (Beckman-Coulter) or Reflection (i-Cyt). Sorted naïve T cells ($CD4^+CD62L^{hi}CD44^{lo}Foxp3^-$) were used for *in vitro* cultures in Bruff's or Click's medium (plus

β-mercaptoethanol) supplemented with 10% FBS and 1% penicillin-streptomycin as described previously[14]. Briefly, for nonpolarizing (T_H0) condition, naive cells were stimulated with anti-CD3 (2C11), anti-CD28 (37.51) and human IL-2 (100 U/ml); for iT_{reg} differentiation, the culture was supplemented with human TGF-β1 (0.5 – 5 ng/ml); for T_H1 conditions, naive cells were cultured with IL-12 (0.5 ng/ml) and anti-IL-4 (11B11; 10 μg/ml). When OT-II TCR-transgenic mice were used, Ova_{323} peptide (1 μg/ml) was used to replace the anti-CD3/CD28 antibodies. For 4-OHT treatment, cells were cultured in the medium containing IL-7 (10 ng/ml; to maintain cell viability) and 0.1 – 0.5 μM 4-OHT for 2-3 days, and live cells were sorted and used for stimulation. For drug inhibitor treatments, cells were incubated with vehicle, 5 μM U0126, 10 – 100 nM rapamycin, 5 μM SIS3, 1.25 – 10 μM SKI (all from Calbiochem), 10 – 100 nM FTY720, or 0.25 – 1 μM DMS (both from Cayman Chemical) . S1P (Sigma) was added to naïve T cells at the time of stimulation in the X-vivo 15 serum-free medium (BioWhittaker). $CD103^+CD11c^+$ DCs were isolated and purified from MLN according to published protocols[7].

Retroviral transduction. The Foxp3 retroviral construct was a generous gift from D. Littman[35]. *S1pr1* and *Cre* cDNAs were cloned into the mouse stem cell virus retroviral vector (MSCV) upstream of an internal ribosome entry site (IRES)-Thy1.1 marker expression cassette, as previously described[14]. Constitutively active and dominant negative Smad3 constructs (Smad3CA and Smad3DN) were generated by PCR to incorporate the aspartic acid and alanine mutations at the C-terminus of Smad3, respectively, as described[48]. Phoenix-Eco packaging cells were transfected with Lipofectamine (Invitrogen), and recombinant retroviruses were collected 48 and 72 h after transfection. T cells were activated for 24 h and transduced with retroviruses by 'spin inoculation' (650 *g* for 1 h), as previously described[14].

Adoptive transfer and colitis model. Naïve T cells ($CD4^+CD45RB^{high}GFP^-$) were transferred into *Rag1*$^{-/-}$ mice in combination with T_{reg} cells ($CD4^+CD45RB^{low}CD25^+$) from CD45.1 mice, as previously described[14]. Mice were weighed and assessed for clinical signs of colitis weekly, and were euthanized 10 weeks after transfer. Colon and cecum were fixed in 10% neutral buffered formalin, and 4-μm sections cut and stained with H&E, as previously described.

Oral feeding of antigen. Drinking water for OT-II.*Rag1*$^{-/-}$ mice was supplemented with 20 mg/ml Grade VI Ova (Sigma-Aldrich) according to published protocols[6,7]. Alternatively, naïve T cells were purified as above and transferred into C57BL/6 mice, and 24 h later, fed with Ova in the drinking water. T cells in the colon and cecum were visualized using goat anti-CD3 polyclonal antisera (Santa Cruz) and diaminobenzidine chromagen with haematoxylin as a counterstain. T_{reg} cells were visualized with anti-Foxp3 (FJK-16s; eBioscience), as previously described[14].

Quantitative RT-PCR. RNA was extracted with RNeasy kit (Qiagen), and cDNA was synthesized with Superscript III reverse transcriptase (Invitrogen). An ABI 7900 Real-time PCR system was used for quantitative PCR, with primer and probe sets from Applied Biosystems; results were analyzed with SDS 2.1 software. The cycling threshold value of the endogenous control gene (HPRT) was subtracted from the cycling threshold value of each target gene to generate the change in cycling threshold (ΔCT). The relative expression of each target gene is expressed as the 'fold change' relative to that of wild-type unstimulated samples ($2^{-\Delta\Delta C}T$).

Immunoblot analysis. Immunoblot was performed as described previously[49], using the following antibodies: p-S6, S6 and p38 (all from Cell Signaling Technology), p-Smad3 (Millipore/upstate), Smad3 (Abcam), Sp1 (Santa Cruz), and β-actin (Sigma). Nuclear extracts were prepared as described previously[49].

Statistical analysis. P values were calculated using Student's *t*-test. P values of less than 0.05 were considered significant. All error bars in graphs represent SEM calculated from at least 3 replicates.