$S1P_1$-mTOR axis directs the reciprocal differentiation of T_H1 and regulatory T cells

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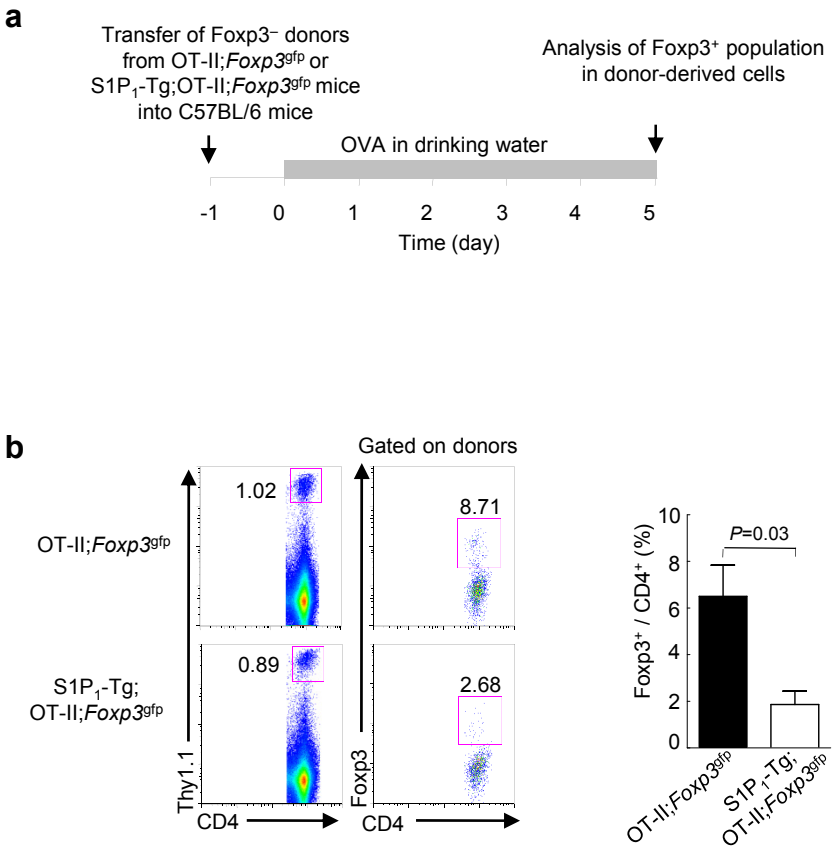
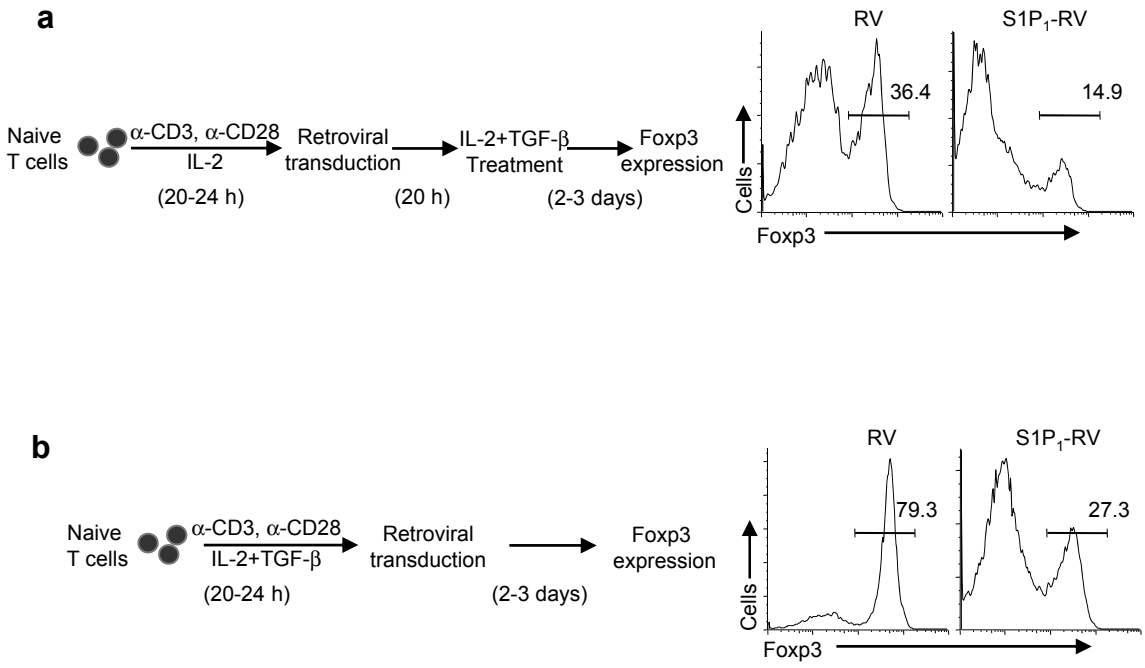
Supplementary Figures 1-13

Supplemental Fig. 1



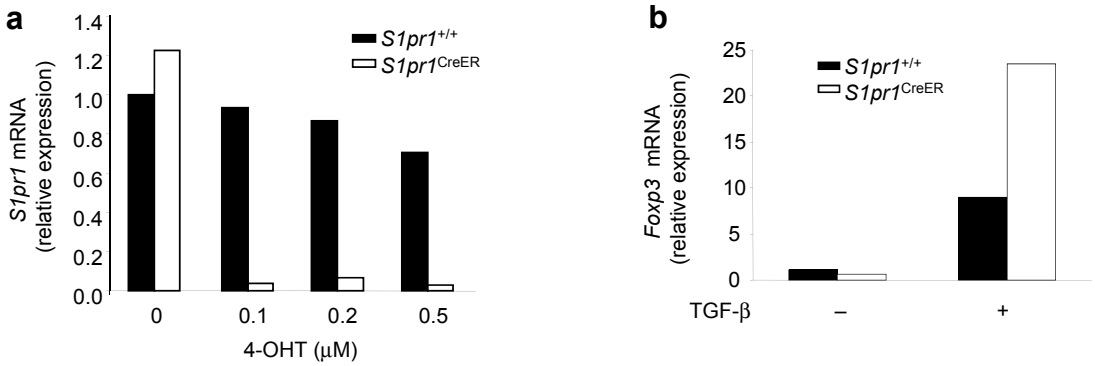
Supplementary Figure 1. Impaired *in vivo* induction of Foxp3$^+$ iT$_{reg}$ population in S1P$_1$-Tg cells following adoptive transfer and Ova feeding. (a) Experimental schematics of the adoptive transfer and Ova feeding. FACS-sorted naive T cells from OT-II;*Foxp3*^{gfp} and S1P$_1$-Tg;OT-II;*Foxp3*^{gfp} (Thy1.1$^+$) mice were transferred to C57BL/6 mice, and 24 h later, fed with Ova in the drinking water for 5 days. **(b)** Foxp3 expression on gated donor cells (Thy1.1$^+$) in the MLN of recipient mice was analyzed. Data are representative of three independent experiments (n=3 mice per group in each experiment).

Supplemental Fig. 2



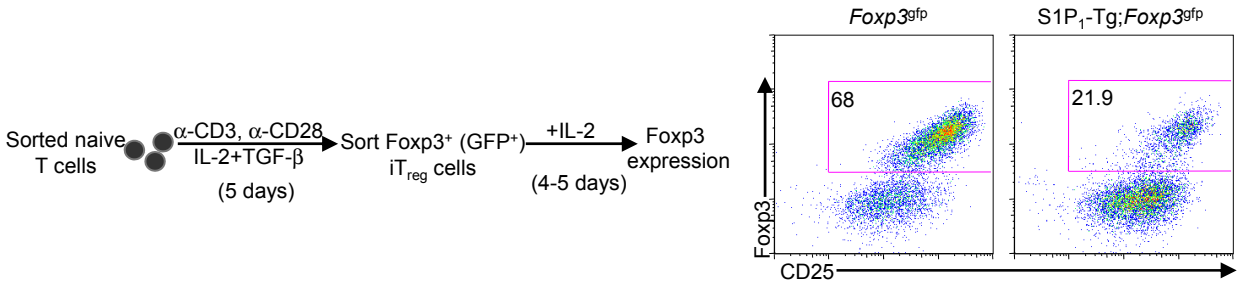
Supplementary Figure 2. Retroviral transduction of $S1P_1$ impairs T cell differentiation into iT_{reg} cells. (a). Wild-type naïve T cells were activated in the absence of TGF-β, and 20-24 h later, transduced with a control retrovirus (RV) or $S1P_1$-expressing retrovirus ($S1P_1$-RV). At 20 h after transduction, cells were washed and stimulated with IL-2 and TGF-β, and Foxp3 expression in the transduced cells was analyzed 2-3 days later. **(b).** Wild-type naïve T cells were activated in the presence of TGF-β, and 20-24 h later, transduced with a control retrovirus (RV) or $S1P_1$-expressing retrovirus ($S1P_1$-RV). Foxp3 expression in the transduced cells was analyzed 2-3 days later. Data are representative of three independent experiments using pooled wild-type mice.

Supplemental Fig. 3



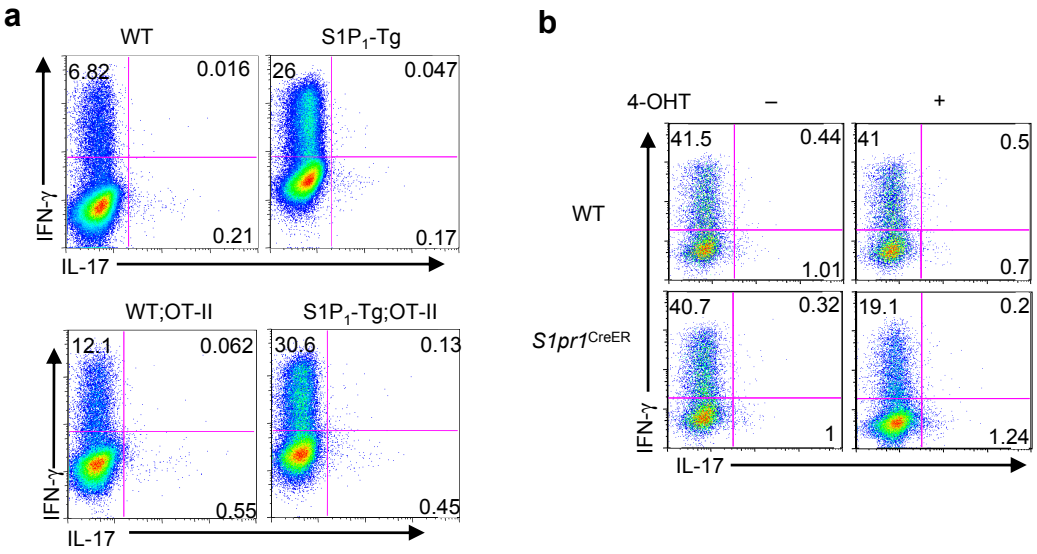
Supplementary Figure 3. Inducible deletion of $S1P_1$ in T cells. (a) T cells from wild-type and *S1pr1*^{CreER} mice were treated with 4-OHT and IL-7 for 2-3 days, and deletion of $S1P_1$ was analyzed by real-time RT-PCR. **(b)** 4-OHT (0.5 μM) treated wild-type and *S1pr1*^{CreER} T cells were activated and then stimulated with TGF-β (0 or 5 ng/ml) for 24 h, and Foxp3 expression was analyzed by real-time RT-PCR. Data are representative of two independent experiments with each experiment consisting of 2-3 mice per group.

Supplemental Fig. 4



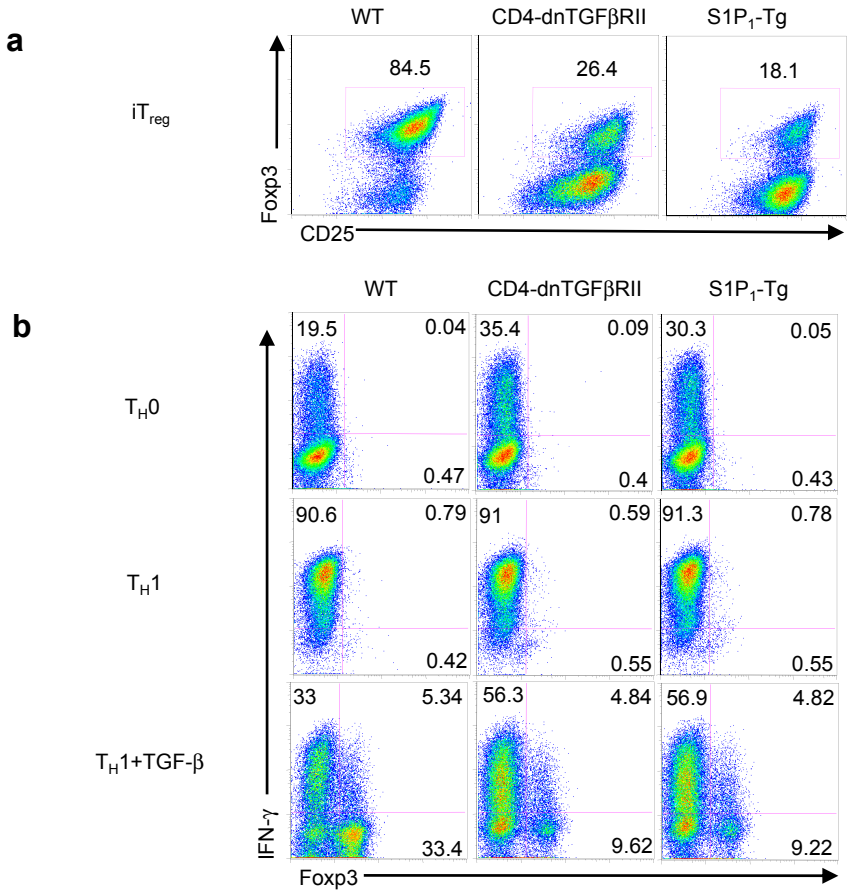
Supplementary Figure 4. Reduced stability of Foxp3 expression in iT_{reg} cells generated from $S1P_1$-Tg mice. Naïve T cells from wild-type and $S1P_1$-Tg mice were differentiated into iT_{reg} cells. $Foxp3^+$ (GFP^+) cells were sorted and cultured with IL-2 alone. Foxp3 expression was analyzed 4-5 days later. Data represent two independent experiments with each experiment consisting of two mice per group.

Supplemental Fig. 5



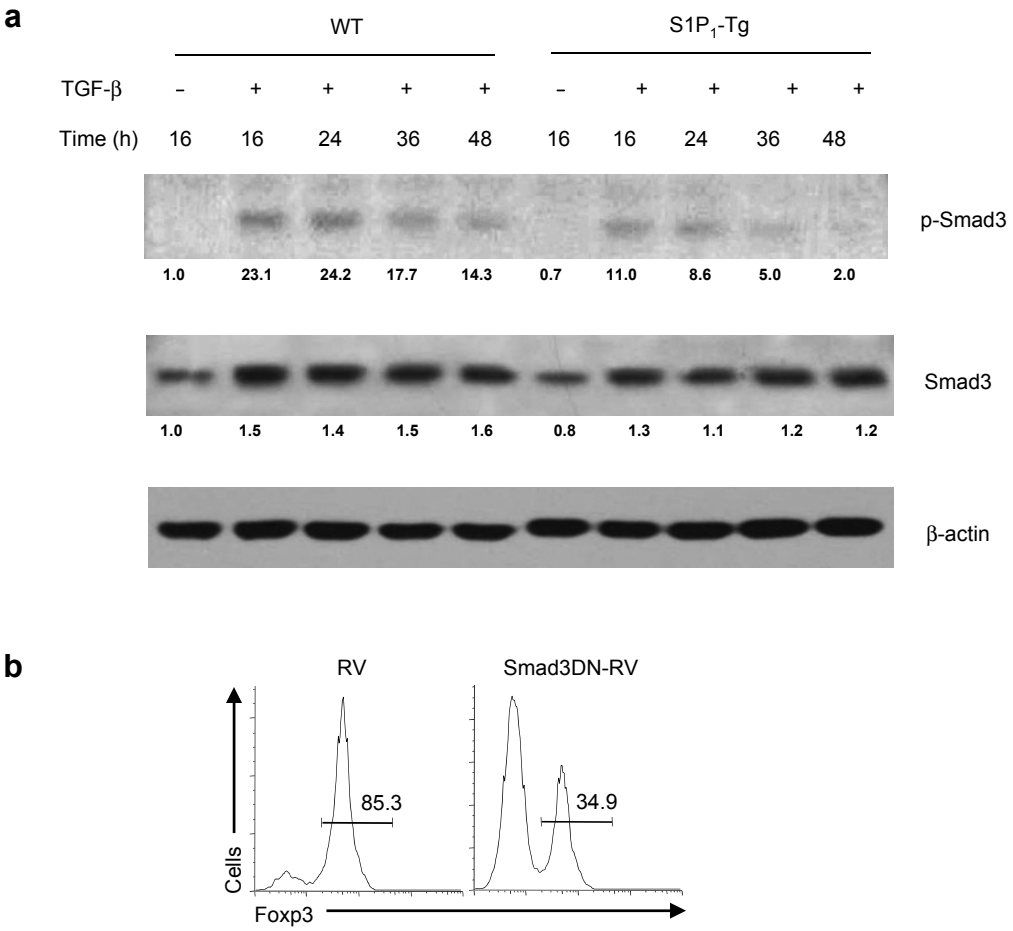
Supplementary Figure 5. $S1P_1$ promotes T_H1 cell differentiation *in vitro.* FACS-sorted naïve wild-type and $S1P_1$-Tg cells (**a**, upper panels), those expressing OT-II TCR (**a**, lower panels), or 4-OHT treated wild-type and *S1pr1*^{CreER} cells (**b**), were differentiated under nonpolarizing conditions (T_H0) for 5 days, followed by analysis of IFN-γ and IL-17 expression. Data represent three to four independent experiments with each experiment consisting of two mice per group.

Supplemental Fig. 6



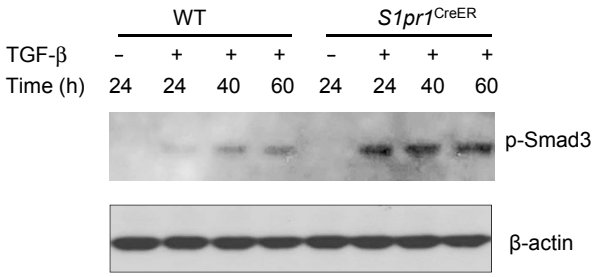
Supplementary Figure 6. CD4-dnTGFβRII T cells phenocopy the altered iT_{reg} and T_H1 differentiation in $S1P_1$-Tg T cells. (a) Naïve T cells from wild-type, CD4-dnTGFβRII, and $S1P_1$-Tg mice were activated in the presence of TGF-β to differentiate toward iT_{reg} cells, followed by analysis of Foxp3 expression. **(b)** Naïve T cells from wild-type, CD4-dnTGFβRII, and $S1P_1$-Tg mice were activated under T_H0, T_H1 or T_H1 plus TGF-β conditions, followed by analysis of IFN-γ and Foxp3 expression. Data are representative of three independent experiments with each experiment consisting of two mice per group.

Supplemental Fig. 7



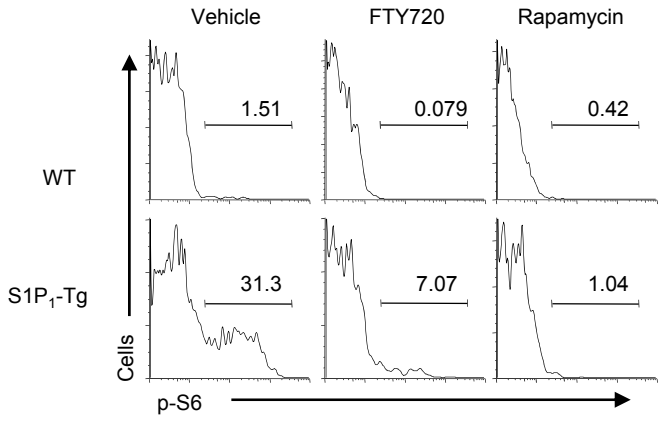
Supplementary Figure 7. Sustained Smad3 activity is required for iT_{reg} generation. (a) Naïve T cells from wild-type and $S1P_1$-Tg mice were activated by anti-CD3, anti-CD28 and IL-2, with or without TGF-β. Phosphorylation of Smad3 was examined at different time points. The numbers below each panel show the quantification of band intensity relative to the loading controls. **(b)** WT naïve T cells were activated under iT_{reg} conditions for 24 h, and transduced with a control retrovirus (RV) or a retrovirus expressing dominant negative Smad3 (Smad3DN-RV). Foxp3 induction was examined 2-3 days later. Data are representative of five (**a**) and three (**b**) independent experiments using pooled mice for each group.

Supplemental Fig. 8



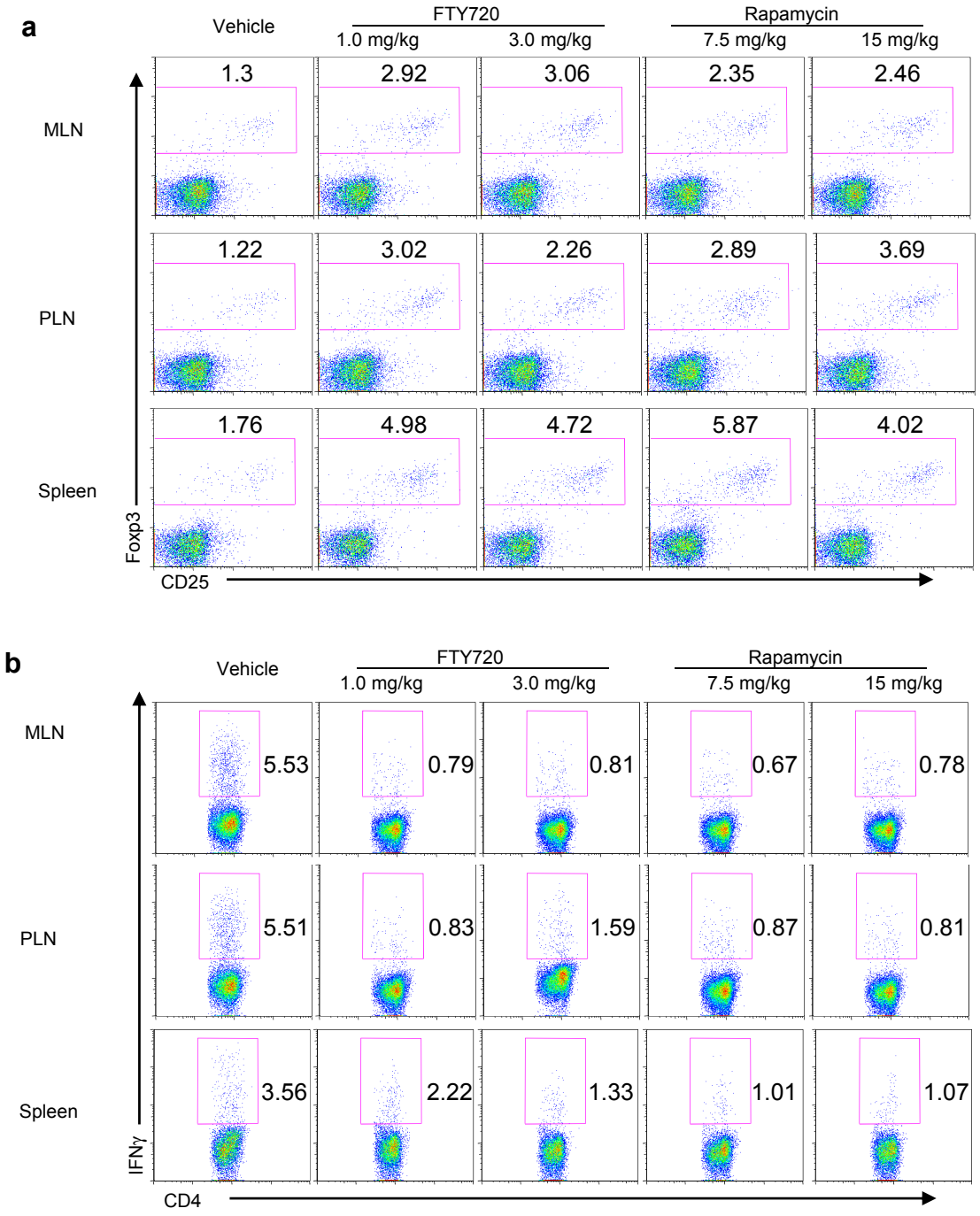
Supplementary Figure 8. Deletion of $S1P_1$ results in enhanced Smad3 activation. 4-OHT treated wild-type and *S1pr1*^{CreER} cells were activated and stimulated with TGF-β. Phosphorylation of Smad3 was examined at different time points. Data are representative of two independent experiments using pooled mice for each group.

Supplemental Fig. 9



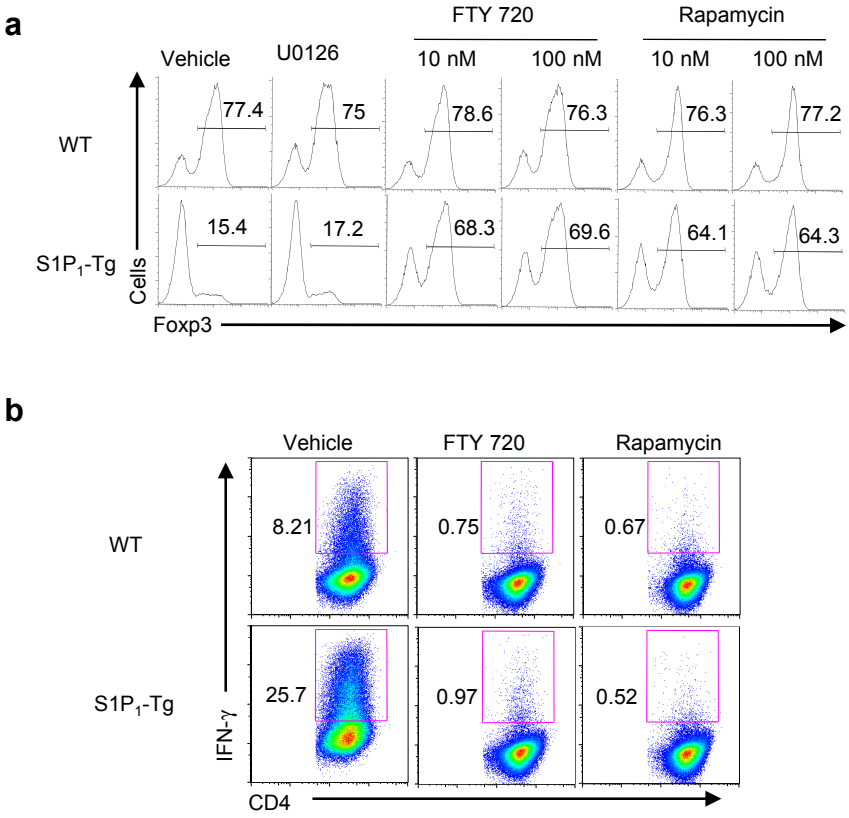
Supplementary Figure 9. $S1P_1$-induced sustained mTOR signaling is targeted by FTY720 and Rapamycin. Naïve T cells from WT and $S1P_1$-Tg mice were pre-treated with FTY720 or Rapamycin, and activated under iT_{reg} conditions. Phosphorylation of S6 ribosomal prootein, a well established target for the mTOR activity, was examined after 36 h of stimulation. Data are representative of two independent experiments with each experiment consisting of two mice per group.

Supplemental Fig. 10



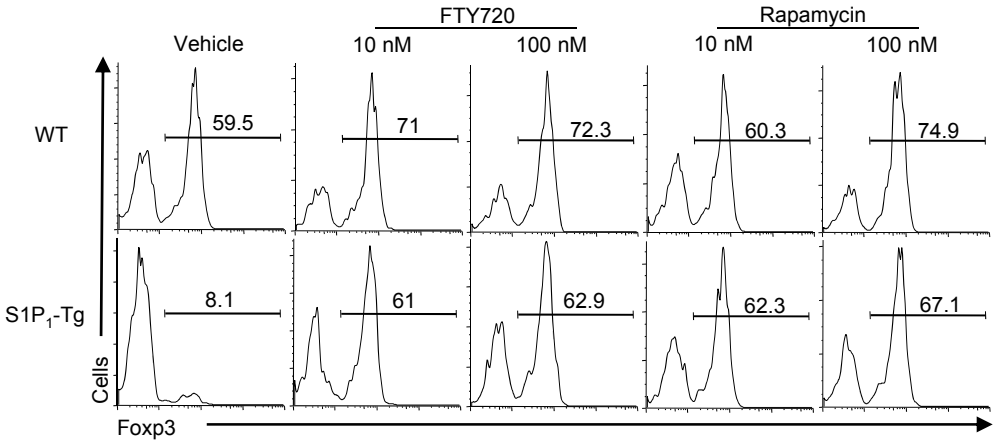
Supplementary Figure 10. FTY720 and Rapamycin modulate reciprocal T_H1 and iT_{reg} differentiation *in vivo*. OT-II;*Rag1*$^{-/-}$ mice were fed with Ova in the drinking water, and FTY720 and Rapamycin were administered by daily i.p. injection. After 5 days, Foxp3 (**a**) and IFN-γ (**b**) expression was examined by intracellular staining. Data are representative of three independent experiments with each experiment consisting of two mice per group.

Supplementary Fig. 11



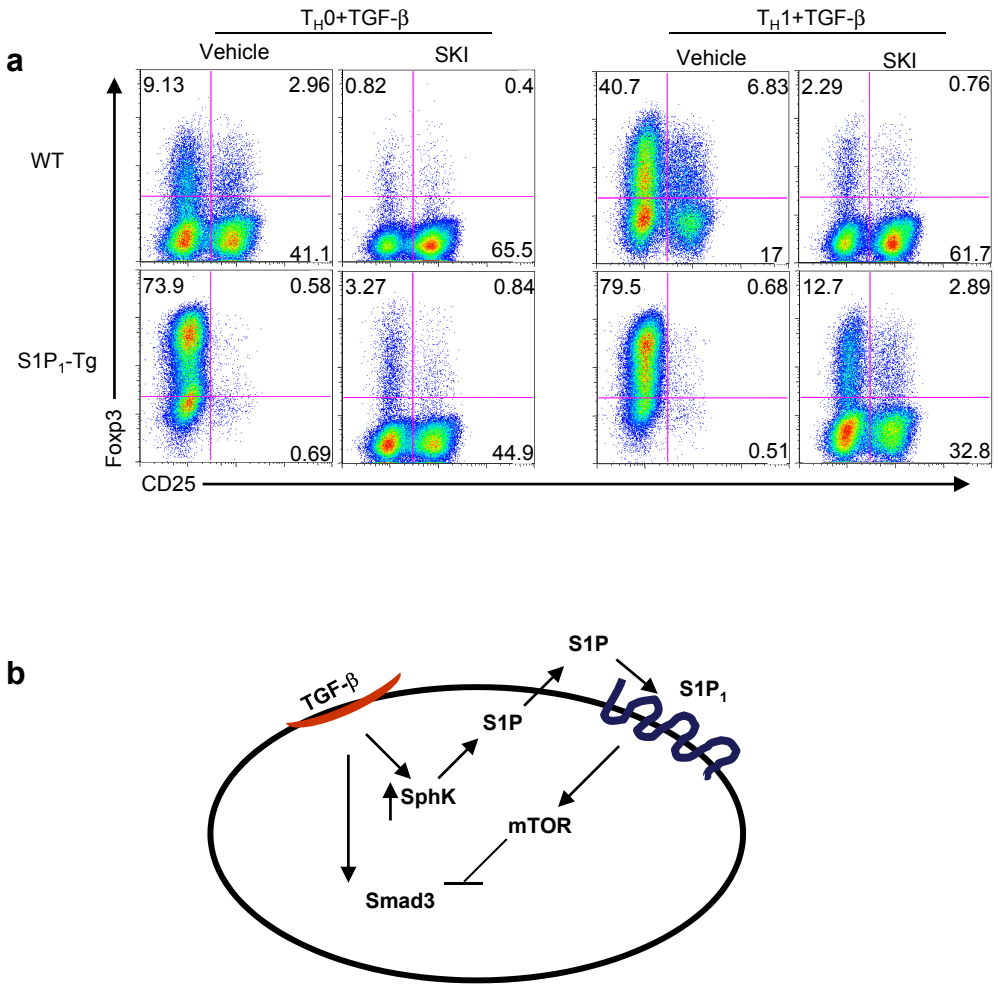
Supplementary Figure 11. FTY720 and Rapamycin treatments rescue the diminished iT_{reg} and enhanced T_H1 cell differentiation phenotypes of $S1P_1$-Tg cells *in vitro*. (a) Naïve T cells from WT and $S1P_1$-Tg mice were treated with vehicle, U0126 (an ERK inhibitor as a control), FTY720 or Rapamycin, and activated under iT_{reg} conditions, followed by analysis of Foxp3 expression. **(b)** Naïve T cells from WT and $S1P_1$-Tg mice were treated as above, and activated for 5 days. IFN-γ expression was detected by intracellular cytokine staining. Data are representative of three independent experiments with each experiment consisting of two mice per group.

Supplemental Fig. 12



Supplementary Figure 12. FTY720 and Rapamycin treatments allow the differentiation of $S1P_1$-Tg thymic nT_{reg} precursors into mature nT_{reg} cells. Thymic T_{reg} precursors ($CD4^+CD8^-CD25^+Foxp3^-$) were sorted from WT and $S1P_1$-Tg mice, and stimulated with IL-2 (50 U/ml) in the presence of FTY720 or Rapamycin. Induction of Foxp3 expression was examined after 20 h. Data are representative of two independent experiments with each experiment consisting of two mice per group.

Supplemental Fig. 13



Supplementary Figure 13. SphK activity regulates reciprocal T_H1 and iT_{reg} cell differentiation. (a) Naïve T cells were pre-treated with SKI (2.5 μM), and differentiated under T_H0 or T_H1 conditions in the presence of TGF-β, followed by analysis of IFN-γ and Foxp3 expression. Similar results were obtained using DMS (data not shown). Data are representative of two independent experiments with each experiment consisting of two mice per group. (b) Model for "inside-out" signaling of S1P and activation of the downstream $S1P_1$-mTOR axis as a feedback mechanism to restrain TGF-β mediated T cell differentiation.