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

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Abstract

Naïve $CD4^+$ T cells differentiate into diverse effector and regulatory lineages to orchestrate immunity and tolerance. The differentiation of pro-inflammatory T_H1 and anti-inflammatory Foxp3+ regulatory T cells (Treg) was reciprocally regulated by $S1P_1$, a receptor for the bioactive lipid sphingosine-1-phosphate. $S1P_1$ inhibited extrathymic and natural Treg generation while driving T_H1 cell development in a reciprocal manner and disrupted immune homeostasis. $S1P_1$ signaled through mTOR and antagonized TGF-β function mainly by attenuating sustained Smad3 activity. $S1P_1$ function was dependent upon endogenous sphingosine kinase activity. Remarkably, two seemingly unrelated immunosuppressants FTY720 and rapamycin targeted the same $S1P_1$ and mTOR pathway to regulate the dichotomy between T_H1 and Treg cells. Our studies establish an $S1P_1$-mTOR axis that controls T cell lineage specification.

Keywords

regulatory T cells; T cell differentiation; mTOR; immunosuppressant

INTRODUCTION

$CD4^+$ helper T cells are central regulators of adaptive immune responses. In response to antigen stimulation, naïve $CD4^+$ T cells proliferate and differentiate into T helper type 1 (T_H1) cells, T_H2 cells and T_H17 cells to exert specific effector functions. These effector T cells are characterized by distinct patterns of cytokine secretion: interferon-γ (IFN-γ), interleukin 4 (IL-4) and IL-17 are the signature cytokines for T_H1, T_H2 and T_H17 cells, respectively[1]. Naïve precursors can also develop into antigen-specific $Foxp3^+$ T_{reg} cells, known as induced T_{reg} cells (iT_{reg}), which act in synergy with naturally occurring T_{reg} cells

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AUTHOR CONTRIBUTIONS

G.L. designed and performed *in vivo* and cellular experiments and contributed to writing the manuscript; K.Y. designed and performed biochemical analyses and cellular and molecular experiments; S.B. contributed to *in vivo* and cellular experiments and gene expression analysis; S.S. contributed to cell isolation and gene expression analysis and managed the mouse colony; H.C designed experiments, wrote the manuscript, and provided overall direction.

(nT_{reg}) to establish immune tolerance and counter-balance effector T cell functions[2,3]. Induction of iT_{reg} cells in the peripheral immune compartment is closely related to the generation of T_H17 cells, as the differentiation of both lineages is dependent upon the pleiotropic cytokine transforming growth factor β (TGF-β; http://www.signaling-gateway.org/molecule/query?afcsid=A002271)[4]. Despite extensive progress in this area, it remains poorly understood how the differentiation of naïve precursors into opposing regulatory and effector lineages is regulated.

T cell differentiation is programmed by polarizing cytokines, which signal through cell surface cytokine receptors and intracellular pathways, ultimately leading to the expression of lineage-specific transcription factors and effector cytokines. IL-12, IL-4 and TGF-β drive the differentiation of T_H1, T_H2 and iT_{reg} cells, respectively, while stimulation by TGF-β together with IL-6 or IL-21 promotes T_H17 development[1]. Differentiation of T cells is further shaped by non-cytokine receptors, especially those in the nuclear receptor superfamily that recognize endogenous metabolites, environmental toxins and other immune modulators. Retinoic acid, a vitamin A metabolite, directs reciprocal T_H17 and iT_{reg} differentiation by activating the retinoic acid receptor[5–7]. Aryl hydrocarbon receptor, a transcription factor activated by environmental toxins, regulates T_{reg} and T_H17 cell differentiation in a ligand-specific fashion[8,9]. Aside from the cytokine and nuclear receptors, whether other classes of receptors directly control T cell lineage choices has not been well established.

Sphingosine 1-phosphate (S1P) is a bioactive lysophospholipid enriched in blood[10]. S1P signals through five known G protein-coupled receptors (GPCR): $S1P_1$-$S1P_5$. FTY720, a new immunosuppressant, induces T cell sequestration in lymphoid organs by acting on four of the five S1P receptors. FTY720 is effective in relapsing multiple sclerosis and has become the first oral therapy for the debilitating autoimmune disease[11]. However, mechanism of action for FTY270 is complex, and it remains controversial whether FTY720 acts as an agonist or a functional antagonist or both to regulate lymphocyte trafficking. Genetic approaches to alter the function of $S1P_1$ [http://www.signaling-gateway.org/molecule/query?afcsid=A000813] indicate that $S1P_1$ is the main S1P receptor that facilitates the egress of T cells from lymphoid organs[12,13]. Using gain- and loss-of-function genetic systems, we recently identified a novel role of $S1P_1$ in the negative control of thymic generation and suppressive activity of nT_{reg} cells in a process dependent upon the downstream Akt-mTOR [http://www.signaling-gateway.org/molecule/query?afcsid=A000094] pathway[14].

To investigate the function of $S1P_1$ in the lineage determination of peripheral T cells, we used a combination of genetic systems and pharmacological approaches. We found that $S1P_1$ inhibited differentiation of $Foxp3^+$ T_{reg} cells while promoting the development of T_H1 cells in a reciprocal manner. $S1P_1$ antagonized TGF-β receptor function, through an inhibitory effect on Smad3 activity, to control the dichotomy between these two T cell lineages. Moreover, this regulatory function was dependent upon the effect of $S1P_1$ to sustain mTOR activation in T cells. Our studies demonstrate that differentiation of pro-inflammatory T_H1 and anti-inflammatory T_{reg} cells is reciprocally regulated by $S1P_1$-mTOR and the opposing TGF-β-Smad3 signaling.

RESULTS

$S1P_1$ blocks differentiation of iT_{reg} cells

To gain insight into mechanisms of iT_{reg} differentiation, we determined the role of $S1P_1$ in this process. First, we crossed T cell-specific *S1pr1*-transgenic mice (hereafter called $S1P_1$-Tg), which expressed 2–3 fold more $S1P_1$ than wild-type mice (data not shown);[13], with OT-II transgenic mice ($CD4^+$ T cell receptors (TCR) specific for ovalbumin (Ova)) on a $Rag1^{-/-}$ background. Placing a TCR transgene on the $Rag1^{-/-}$ background results in all peripheral T cells having single antigen specificity without detectable Foxp3 expression, obviating the confounding effects of $S1P_1$ function in the nT_{reg} compartment[14]. In a model of oral antigen-induced iT_{reg} generation[6,7], Foxp3 induction in the mesenteric lymph nodes (MLN) and spleen of $S1P_1$-Tg;OT-II;$Rag1^{-/-}$ mice was markedly reduced as compared with the control OT-II;$Rag1^{-/-}$ mice (Fig. 1a). Expression of T_{reg} cell specific factors LAG-3 and Gpr83 was also decreased in $S1P_1$-Tg;OT-II;$Rag1^{-/-}$ T cells (Fig. 1b). More pronounced effects were observed in the lamina propria, in which an extensive $Foxp3^+$ population was induced in Ova-fed OT-II;$Rag1^{-/-}$ mice but not in $S1P_1$-Tg;OT-II;$Rag1^{-}/^{-}$ mice, despite similar numbers and distribution of total $CD3^+$ cells in these mice (Fig. 1c). To formally exclude the contribution of the residual thymic derived nT_{reg} cells, we crossed $S1P_1$-Tg;OT-II mice with $Foxp3^{gfp}$ knockin mice that marked Foxp3 expression with GFP to distinguish T_{reg} and conventional T cells[14]. We purified naive $Foxp3^-$ T cells from these mice and transferred them into wild-type immunocompetent mice. Administration of oral antigen to the recipients induced a sizable $Foxp3^+$ population from wild-type donors, while the $Foxp3^+$ population from $S1P_1$-Tg donors was decreased by 70% (Supplementary Fig. 1). Taken together, $S1P_1$ inhibits *de novo* induction of Foxp3-expressing iT_{reg} cells from the peripheral T cell pool.

A specialized subset of dendritic cells (DCs) in the mucosa expressing CD103 is tolerogenic by inducing $Foxp3^+$ iT_{reg} cells[6,7]. We purified $CD103^+$ DCs from MLN and cultured them with naïve T cells from OT-II;$Foxp3^{gfp}$ mice ($CD62L^{hi}CD44^{lo}Foxp3^-$) in the presence of the cognate antigen or anti-CD3. Under these conditions, a substantial population of T cells from OT-II;$Foxp3^{gfp}$ mice was induced to express Foxp3, while $S1P_1$-Tg;OT-II;$Foxp3^{gfp}$ T cells exhibited profound defects in Foxp3 induction (Fig. 1d). One key mechanism for $CD103^+$ DC-mediated T_{reg} cell differentiation involves the production of TGF-β[6,7]. Indeed, neutralizing TGF-β abrogated the function of these DCs to induce Foxp3 expression from wild-type and $S1P_1$-Tg cells (data not shown). We conclude that $S1P_1$ inhibits DC-induced iT_{reg} generation.

$Foxp3^+$ cells can be generated directly from naïve precursors by antigen stimulation in the presence of TGF-β[15]. When naïve cells were activated in the presence of TGF-β in an antigen-presenting cell (APC)-free condition, those expressing the $S1P_1$ transgene were considerably impaired to differentiate into $Foxp3^+$ iT_{reg} cells (Fig. 1e). Wild-type and $S1P_1$-Tg cells proliferated to a similar degree and comparably expressed apoptotic markers and Bcl2 (Fig. 1e and data not shown). Furthermore, crossing $S1P_1$-Tg mice with Bcl2-transgenic animals failed to rectify the diminished iT_{reg} differentiation (data not shown). Thus, the impaired iT_{reg} generation was not due to altered T cell proliferation or survival,

indicating that $S1P_1$ inhibits TGF-β-mediated iT_{reg} differentiation. We next explored the kinetics involved in $S1P_1$-dependent inhibition of Foxp3 induction. In wild-type cells, Foxp3 mRNA was strongly induced at 24 h after TGF-β stimulation and continued to increase at 36–48 h. $S1P_1$-Tg cells showed greatly reduced Foxp3 expression at 24–48 h (Fig. 1f). In addition, transduction of wild-type cells with $S1P_1$-expressing retrovirus, followed by TGF-β treatment, resulted in diminished Foxp3 induction. Moreover, $S1P_1$ exerted its inhibitory effect on Foxp3 induction even when transduction occurred 20–24 h after TGF-β stimulation (Supplementary Fig. 2), suggesting that $S1P_1$ may interfere with sustained TGF-β signaling.

$S1P_1$ inhibits iT_{reg} generation and maintenance

We then tested whether $S1P_1$ is required to suppress iT_{reg} differentiation. Given the marked reduction of peripheral T cells in *S1pr1*$^{flox/flox}$ mice crossed with *Cd4*-Cre transgenic mice[14], we bred these mice onto the *Foxp3*^{gfp} background and purified Foxp3$^-$ CD4 single-positive (CD4SP) thymocytes. When transferred into *Rag1*$^{-/-}$ mice, a subset of donor cells became Foxp3$^+$. Deficiency of $S1P_1$ resulted in an augmented Foxp3$^+$ population, indicating increased iT_{reg} generation *in vivo* (Fig. 2a). To more directly address the requirement of $S1P_1$ in iT_{reg} development from peripheral T cells, we crossed *S1pr1*$^{flox/flox}$ mice with Rosa26-Cre-ER^{T2} (called '*S1pr1*^{CreER} mice' here). After 4-hydroxytamoxifen (4-OHT) treatment, the floxed *S1pr1* gene was deleted in naïve T cells (Supplementary Fig. 3a). When these cells were differentiated toward iT_{reg} cells by exogenous TGF-β, they exhibited a higher frequency of the Foxp3$^+$ population (Fig. 2b), associated with increased *Foxp3* mRNA abundance (Supplementary Fig. 3b) and normal cell survival (data not shown). Moreover, CD103$^+$ DC-induced Foxp3$^+$ population was also enhanced in the absence of $S1P_1$ (Fig. 2c). Therefore, $S1P_1$ deficiency directly potentiates iT_{reg} differentiation.

Continued cells, Foxp3 Foxp3 expression is necessary for T_{reg} function. As compared with nT_{reg} expression in iT_{reg} cells is less stable and depends upon continuous TGF-β signaling[16]. To test the involvement of $S1P_1$ in the maintenance of Foxp3 expression, we generated and sorted Foxp3$^+$ (GFP$^+$) iT_{reg} cells from *S1pr1*^{CreER} mice, and then treated them with 4-OHT to induce *S1pr1* deletion in the mature iT_{reg} cells. Upon TGF-β withdrawal, control iT_{reg} cells that retained $S1P_1$ expression readily lost Foxp3 expression, whereas iT_{reg} cells deficient in $S1P_1$ contained a much greater Foxp3$^+$ population with increased Foxp3 and CD25 expression (Fig. 2d). Conversely, Foxp3$^+$ iT_{reg} cells generated from $S1P_1$-Tg mice were unable to sustain Foxp3 expression in the absence of TGF-β (Supplementary Fig. 4). Therefore, $S1P_1$ negatively regulates the maintenance of Foxp3 expression in iT_{reg} cells.

$S1P_1$ drives T_H1 differentiation

The inhibitory effects of $S1P_1$ on iT_{reg} induction and maintenance prompted us to investigate whether $S1P_1$ diverges naïve T cells into alternative lineages using three *in vivo* systems. First, in the oral antigen model of OT-II;*Rag1*$^{-/-}$ mice, only a small fraction of IFN-γ$^+$ cells was induced in OT-II;*Rag1*$^{-/-}$ mice, as would be expected for T cell responses under the tolerizing conditions. In contrast, $S1P_1$-Tg;OT-II;*Rag1*$^{-/-}$ mice contained a markedly increased population (5–10 fold more abundant) of IFN-γ$^+$ CD4 T cells in various lymphoid organs examined (Fig. 3a). Accordingly, quantitative RT-PCR identified elevated

expression of IFN-γ and the T_H1 transcriptional factor T-bet in $S1P_1$-Tg cells following oral antigen exposure (Fig. 3b). IL-4 and IL-17 protein and RNA abundance were low and comparable between the two types of mice (Fig. 3a and data not shown), indicating selective differentiation of $S1P_1$-Tg cells into the T_H1 lineage.

Second, we adoptively transferred T cells from OT-II or $S1P_1$-Tg;OT-II mice (Thy1.1+) into naïve recipient and immunized recipients with Ova intravenously. Five days later, a substantially increased IFN-γ^+ population was detected among $S1P_1$-Tg donor-derived cells, as compared with wild-type donors (Fig. 3c). Also, secretion of IFN-γ, but not of IL-4 or IL-17, was enhanced in the $S1P_1$-Tg cell transfer group after *ex vivo* peptide stimulation (data not shown).

Third, we determined the proportions of T cells capable of producing IFN-γ in $S1P_1$-Tg mice by intracellular cytokine staining. Compared with MLN cells from wild-type mice, which had few IFN-γ producing $CD4^+$ and $CD8^+$ T cells, a higher percentage of $S1P_1$-Tg cells produced IFN-γ (Fig. 3d). Quantitative RT-PCR revealed 7–8 fold more IFN-γ in $S1P_1$-Tg $CD4^+$ and $CD8^+$ T cells as compared with the wild-type counterparts (Fig. 3d). Taken together, $S1P_1$ promotes IFN-γ production and T_H1 differentiation *in vivo*.

To establish whether $S1P_1$ regulates intrinsic T cell differentiation, we sorted naïve T cells from wild-type and $S1P_1$-Tg mice and cultured them under nonpolarizing conditions (T_H0). A larger proportion of $S1P_1$-Tg cells spontaneously differentiated into IFN-γ^+ cells associated with increased T-bet expression (Supplementary Fig. 5a and data not shown). Higher IFN-γ expression was also observed in $S1P_1$-Tg;OT-II cells (Supplementary Fig. 5a) and wild-type cells transduced with $S1P_1$-expressing retrovirus (data not shown). Conversely, deletion of $S1P_1$ diminished IFN-γ expression (Supplementary Fig. 5b). Therefore, $S1P_1$ promotes IFN-γ expression and T_H1 differentiation.

$S1P_1$ directs reciprocal T_H1 and iT_{reg} differentiation

We next determined the mechanisms for the altered iT_{reg} and T_H1 differentiation in $S1P_1$-Tg and deficient cells. TGF-β is a pleiotropic cytokine with pronounced effects on cell fate determination of multiple T cell lineages [17], but whether TGF-β directly coordinates development of T_H1 and iT_{reg} cells from naïve precursors is not well understood. We therefore differentiated wild-type naïve cells under T_H0 and T_H1 conditions in the presence of TGF-β and measured Foxp3 and IFN-γ expression simultaneously. As expected, a subset of cells expressed IFN-γ but few $Foxp3^+$ cells were detected under nonpolarizing conditions. Addition of TGF-β induced Foxp3 and terminated IFN-γ expression. Under T_H1 conditions, most T cells became IFN-γ positive, yet addition of TGF-β decreased IFN-γ^+ population and simultaneously induced $Foxp3^+$ population, resulting in the co-existence of T_H1 and iT_{reg} cells (Fig. 4a). The effects of TGF-β to promote Foxp3 over IFN-γ expression were profoundly lost in $S1P_1$-Tg cells (Fig. 4b). In particular, when TGF-β was added to differentiating T_H1 cells to foster development of both T_H1 and iT_{reg} cells, the ratio of IFN-γ^+: $Foxp3^+$ cells was reversed in $S1P_1$-Tg cells (Fig. 4c). This reciprocal change was also observed in antigen-specific $S1P_1$-Tg;OT-II T cells (Fig. 4c). Conversely, deletion of $S1P_1$ resulted in the expansion of iT_{reg} cells at the expense of T_H1 cells (Fig. 4d). Therefore, $S1P_1$ mediates reciprocal T_H1 and iT_{reg} differentiation *in vitro*.

As an independent system of T cell differentiation, we primed T cells with tolerogenic $CD103^+$ DCs, without any exogenous cytokines[6,7]. Under these conditions, distinct populations of $Foxp3^+$ and IFN-γ^+ cells were generated from wild-type cells. T cells with increased $S1P_1$ signaling produced IFN-γ instead of Foxp3 (Fig. 4e), whereas those deficient in $S1P_1$ preferentially developed into $Foxp3^+$ T_{reg} cells instead of IFN-γ^+ T_H1 cells (Fig. 4f). These results further indicate an intrinsic role of $S1P_1$ in directing T_H1 and iT_{reg} lineage commitment.

Previous studies showed that T_H1 cells and IFN-γ are pathogenic in colitis[18], whereas iT_{reg} cells act in synergy with nT_{reg} cells to establish mucosal tolerance[19]. To determine the *in vivo* relevance of $S1P_1$-mediated T cell differentiation, we transferred naïve T cells from wild-type or $S1P_1$-Tg ($CD45.2^+$) mice in conjunction with wild-type congenic ($CD45.1^+$) T_{reg} cells into *Rag1*$^{-/-}$ mice. In this system, *in situ* developed iT_{reg} cells from naïve donors, together with nT_{reg} donor cells, are required to control colitis[19]; wild-type nT_{reg} cells were used here to circumvent the confounding effects in $S1P_1$-Tg nT_{reg} cells[14]. While there was minimal weight loss and inflammation in the wild-type co-transfer group, transfer of $S1P_1$-Tg naïve cells and wild-type T_{reg} cells resulted in significant weight loss associated with severe colitis and leukocyte infiltration (Fig. 4g,h). To dissect the underlying mechanisms, we measured expression of Foxp3 and IFN-γ in cells derived from naïve cell donors ($CD45.2^+$). As compared with wild-type donor cells, we observed fewer $Foxp3^+$ iT_{reg} cells from $S1P_1$-Tg mice and a significant increase of IFN-γ-expressing T cells (Fig. 4i). Therefore, $S1P_1$ controls the reciprocal relationship between iT_{reg} and T_H1 cells *in vivo*.

Discrete mechanisms in iT_{reg} and T_H1 differentiation

We next determined whether control of T_H1 and iT_{reg} differentiation by $S1P_1$ is interdependent. IFN-γ can inhibit iT_{reg} generation[20], although conflicting conclusions also exist[21]. We tested whether inhibition of iT_{reg} differentiation by $S1P_1$ is a secondary consequence of increased IFN-γ production. Retroviral transduction of $S1P_1$ into either *Ifng*$^{-/-}$ or wild-type cells resulted in diminished $Foxp3^+$ induction under iT_{reg} conditions (Fig. 5a). Also, the $S1P_1$ transgene downregulated iT_{reg} generation in both *Ifng*$^{+/+}$ and *Ifng*$^{-/-}$ backgrounds (Fig. 5b). Thus, $S1P_1$ regulates iT_{reg} differentiation independent of its role in facilitating IFN-γ expression.

Conversely, we tested whether the reduced Foxp3 expression in $S1P_1$-Tg cells is responsible for their enhanced IFN-γ expression. To this end, we transduced the *Foxp3* gene into wild-type and $S1P_1$-Tg cells, and found that forced Foxp3 expression had no effects at reducing the expression of IFN-γ in $S1P_1$-Tg cells (Fig. 5c). To further assess the function of Foxp3 in $S1P_1$-mediated T_H1 differentiation, we examined whether Foxp3 deficiency affects the ability of $S1P_1$ to drive T_H1 differentiation. To prevent the lymphoproliferative autoimmune phenotype due to Foxp3 deficiency, we constructed mixed bone marrow chimeras by co-transferring bone marrow cells from Foxp3-deficient Scurfy mice ($CD45.1^+.2^+$) and wild-type mice ($CD45.2^+$) into *Rag1*$^{-/-}$ recipients; the presence of functional T_{reg} cells derived from the wild-type donors prevented autoimmune activation of Scurfy T cells[14]. Naïve T cells from these two donor populations were purified and transduced with $S1P_1$-expressing retrovirus. $S1P_1$ expression was equally effective to promote IFN-γ expression in wild-type

and Foxp3-deficient cells (Fig. 5d). As an independent approach, we bred OT-II and $S1P_1$-Tg;OT-II mice with Scurfy mice, in which the TCR transgene ameliorated the confounding effects of autoimmune inflammation[22]. Naïve T cells from these mice were stimulated with Ova peptide, and increased T_H1 differentiation was observed in those expressing the $S1P_1$ transgene (Fig. 5e). Therefore, either ectopic expression or deficiency of Foxp3 did not prevent $S1P_1$ from driving T_H1 differentiation, suggesting that $S1P_1$ mediates T_H1 differentiation independent of its effects at inhibiting Foxp3 expression.

Aged $S1P_1$-Tg mice developed high titers of autoantibodies, concomitant with spontaneous T cell activation[14]. Since IFN-γ production is associated with the pathogenesis of systemic autoimmune disease, we tested whether increased IFN-γ in $S1P_1$-Tg cells contributes to the breakdown of immune tolerance. As compared with $S1P_1$-Tg mice, $S1P_1$-Tg;*Ifng*$^{-/-}$ mice contained significantly reduced titers of anti-double stranded (ds) DNA antibody (data not shown). In contrast, enhanced expression of CD44 on $S1P_1$-Tg cells, indicative of their spontaneous activation *in vivo*[14], was observed irrespective of the IFN-γ background (data not shown), consistent with our previous observation that spontaneous T cell activation was mainly due to defective T_{reg} cells in $S1P_1$-Tg mice14. Therefore, $S1P_1$ regulates immune homeostasis through both T_{reg}-dependent and independent mechanisms.

$S1P_1$ antagonizes TGF-β-Smad3 signaling

We noticed that the exacerbated colitis following $S1P_1$-Tg cell transfer phenocopied the abnormalities in T cells with transgenic expression of a dominant negative TGF-β receptor (CD4-dnTGFβRII) in a similar model[23],[24]. Moreover, mice with impaired TGF-β receptor signaling in T cells spontaneously differentiate into T_H1 cells *in vivo*[25],[26]. We hypothesize that $S1P_1$ mediates reciprocal T_H1 and iT_{reg} differentiation by antagonizing TGF-β receptor signaling. To test this hypothesis, we first compared the response of $S1P_1$-Tg cells with that of CD4-dnTGFβRII cells. In response to $CD103^+$ DCs, both $S1P_1$-Tg and CD4-dnTGFβRII T cells were skewed into IFN-γ^+ T_H1 cells rather than Foxp3+ T_{reg} cells (Fig. 6a). Similarly, in response to exogenous TGF-β, CD4-dnTGFβRII and $S1P_1$-Tg cells had similar defects in upregulating Foxp3; instead these cells expressed IFN-γ (Supplementary Fig. 6). Therefore, CD4-dnTGFβRII mice phenocopy the abnormalities seen in $S1P_1$-Tg mice, suggesting that $S1P_1$ likely affects TGF-β receptor signaling.

We next investigated the molecular mechanisms by which $S1P_1$ and TGF-β receptor interact. Smad3 is an important transcription factor to mediate TGF-β effects in iT_{reg} generation[27]. TGF-β stimulation resulted in prolonged activation of Smad3 in wild-type T cells (Supplementary Fig. 7a). Immediately after TGF-β stimulation, Smad3 phosphorylation was comparable between wild-type and $S1P_1$-Tg cells (data not shown). However, $S1P_1$-Tg cells were unable to maintain Smad3 phosphorylation after 16 h of TGF-β treatment, and more pronounced reduction was observed at 24–48 h of stimulation (Supplementary Fig. 7a). Accordingly, nuclear translocation of total and phosphorylated Smad3 was profoundly reduced in $S1P_1$-Tg cells after 2 days of stimulation (Fig. 6b). To understand the importance of sustained Smad3 activation, we transduced a dominant negative Smad3 molecule into wild-type cells at 24 h after initiation of iT_{reg} differentiation, which was effective at inhibiting Foxp3 induction (Supplementary Fig. 7b). Moreover, increased p-Smad3 was

observed in $S1P_1$-deficient cells at and after 24 h of stimulation (Supplementary Fig. 8). To directly determine whether Smad3 mediates $S1P_1$ function, we introduced constitutively active Smad3 into $S1P_1$-Tg cells, which restored Foxp3 expression in $S1P_1$-Tg cells by up to 60% (Fig. 6c). Conversely, treatment of $S1P_1$-deficient T cells with the Smad3 inhibitor reduced iT_{reg} differentiation (Fig. 6d). Therefore, $S1P_1$ antagonizes TGF-β-dependent effects on T cell differentiation mainly by attenuating Smad3 signaling.

The $S1P_1$-mTOR axis is targeted by immunosuppressants

We further explored the signaling pathway utilized by $S1P_1$ to antagonize TGF-β-Smad3 signaling. The Akt-mTOR pathway has recently been shown to restrain iT_{reg} generation[28–31]. However, $S1P_1$ activates mTOR in nT_{reg} cells but not naïve T cells immediately after TCR stimulation[14]. Given the effects of $S1P_1$ on sustained but not early Smad3 activation, we examined mTOR activity at later time points by assessing the phosphorylation of the ribosomal protein S6, a well-established target of mTOR. $S1P_1$-Tg cells exhibited increased amounts of p-S6 after 16 h of TCR stimulation, and more prominent increase was observed at later time points, indicating an important role for $S1P_1$ to sustain mTOR activation (Fig. 7a).

This newly revealed role of $S1P_1$-mediated reciprocal differentiation of iT_{reg} and T_H1 cells led us to examine possible therapeutic implications. Both rapamycin (Sirolimus), an inhibitor of mTOR, and FTY720 have been shown to modulate iT_{reg} cells[28–30,32]. To address whether FTY720 and rapamycin share an immunosuppressive mechanism, we first examined mTOR activation in $S1P_1$-Tg cells treated with FTY720 or rapamycin. FTY720 and rapamycin both decreased p-S6 (Supplementary Fig. 9). Moreover, both treatments restored p-Smad3 activity in $S1P_1$-Tg cells to that observed in wild-type cells (Fig. 7b). These results identified an $S1P_1$-mTOR axis that acts to interfere with Smad3 signaling and is targeted by immunosuppressive drugs.

To further investigate the effects of the immunosuppressants on immune responses, we administered oral Ova antigen to OT-II;*Rag1*$^{-/-}$ mice and treated them daily with FTY720 or rapamycin. Such treatments increased iT_{reg} cells and reduced IFN-γ^+ T_H1 cells (Supplementary Fig. 10). Treatment of $S1P_1$-Tg;OT-II;*Rag1*$^{-/-}$ mice with FTY720 or rapamycin reversed the altered differentiation of iT_{reg} and T_H1 cells, to similar to untreated wild-type control mice (Fig. 7c,d). Thus, FTY720 and rapamycin modulate reciprocal T_H1 and iT_{reg} differentiation *in vivo*. To ascertain whether this reflects intrinsic effects, we treated T cells with these drugs *in vitro*. Treatment of $S1P_1$-Tg cells with FTY720 or rapamycin increased iT_{reg} and reduced T_H1 differentiation (Supplementary Fig. 11). Taken together, FTY720 and rapamycin directly modulate reciprocal differentiation of iT_{reg} and T_H1 cells by targeting the $S1P_1$-mTOR axis.

We next asked whether FTY720 and rapamycin also target thymic nT_{reg} cells. We treated wild-type and $S1P_1$-Tg mice with FTY720 or rapamycin for 5 days, and assessed the frequency and number of Foxp3$^+$ CD4SP thymocytes. As previously reported, $S1P_1$-Tg mice contained decreased thymic nT_{reg} cells[14]. After FTY720 or rapamycin treatment, nT_{reg} cells in these mice were similar to wild-type mice (Fig. 7e). Accordingly, the diminished *in vitro* differentiation of $S1P_1$-Tg nT_{reg} precursors (CD25$^+$Foxp3$^-$) into mature Foxp3$^+$ cells

was rescued by FTY720 and rapamycin (Supplementary Fig. 12). Therefore, FTY720 and rapamycin affect thymic development of nT_{reg} cells by targeting the $S1P_1$-mTOR axis. To further explore whether Smad3 mediates $S1P_1$ signaling in thymic nT_{reg} cell differentiation, we treated nT_{reg} precursors from wild-type and *S1pr1*$^{flox/flox}$;*Cd4*-Cre mice with the Smad3 inhibitor SIS3. Blocking Smad3 activity ablated the increased nT_{reg} differentiation observed in precursors lacking $S1P_1$ (Fig. 7f). Although TGF-β is required for T_{reg} survival in the thymus[33], SIS3 treatment reduced Foxp3 induction even in Bcl2-Tg mice (data not shown), indicating that the effect of Smad3 in nT_{reg} differentiation is independent of cell survival. Taken together, the drug-sensitive $S1P_1$-mTOR axis interferes with Smad3 signaling to control development of both nT_{reg} and iT_{reg} subsets.

Sphingosine kinases control T cell differentiation

Given an essential role of $S1P_1$ in T_{reg} cell differentiation, we explored mechanisms of $S1P_1$ activation. Supplement of exogenous S1P to naïve T cells did not alter their differentiation into iT_{reg} cells (Fig. 8a). We thus tested whether the endogenously produced S1P is involved. S1P is synthesized by one of the two sphingosine kinases (SphKs) on the substrate sphingosine[34]. In differentiating T cells, SphK1 was strongly upregulated by the treatment of TGF-β (Fig. 8b), suggesting the likely involvement of intrinsic SphK activity in iT_{reg} cell generation. To test this hypothesis, we treated naïve T cells with N,N,-dimethylsphingosine (DMS) or sphingosine kinase inhibitor (SKI), two widely used inhibitors of SphK activity. Blocking SphK activity strongly upregulated Foxp3 induction (Fig. 8c). Treatment of $S1P_1$-Tg cells with DMS or SKI restored their abilities to differentiate into iT_{reg} cells (Fig. 8d). Similarly, blocking SphK activity considerably rectified the defects of $S1P_1$-Tg cells in the reciprocal T_H1 and T_{reg} cell differentiation (Supplementary Fig. 13a). Thus, T cell differentiation mediated by $S1P_1$ is dependent upon intrinsic SphKs, whose expression is upregulated by TGF-β as a feedback mechanism to limit TGF-β responses (Supplementary Fig. 13b).

DISCUSSION

Our results demonstrate that differentiation of T_H1 and T_{reg} cells is reciprocally regulated. $S1P_1$ is a switch factor that drives the development of T_H1 cells at the expense of T_{reg} generation. S1P1 function is dependent upon endogenous SphK activity, and it signals through mTOR and intersects with TGF-β signaling mainly by attenuating the sustained Smad3 activity. Although TGF-β and mTOR have pleiotropic functions in regulating multiple CD4 T cell lineages[17,30], the interplay between $S1P_1$-mTOR and TGF-β-Smad3 signaling with dynamic kinetics selectively controls the reciprocal differentiation of T_H1 and T_{reg} cells. We propose that this novel lineage commitment process controls the balance between immunity and tolerance and contributes to mechanisms of action for immunosuppressive therapy.

The dichotomy of T_H1 and T_{reg} cell fate determination is reminiscent of the reciprocal differentiation of iT_{reg} and T_H17 cells [1,4]. Moreover, the effect on T cell differentiation by $S1P_1$, a G protein-coupled receptor for the endogenous bioactive lipid, is analogous to the control of reciprocal T_{reg} and T_H17 differentiation mediated by non-cytokine receptors such

as retinoid acid receptor and aryl hydrocarbon receptor that recognize certain endogenous metabolites and environmental toxins[5–9]. These results collectively establish a new paradigm of T cell lineage specification controlled by non-cytokine immune modulators. Unlike inhibition of T_H17 cell differentiation by Foxp3[22,35,36], Foxp3 ectopic expression or complete ablation did not prevent $S1P_1$ from driving T_H1 differentiation. Moreover, $S1P_1$ blocked iT_{reg} generation independent of its effects to drive T_H1 differentiation. Thus, $S1P_1$ regulates T_H1 and iT_{reg} differentiation through discrete mechanisms, further supporting the reciprocal nature of these two lineages.

Elevated $S1P_1$ expression is associated with various autoimmune diseases[37]. A direct role for $S1P_1$ in immune homeostasis is highlighted by the development of autoimmunity in aged $S1P_1$-Tg mice[14], which we attributed to defects in both T_{reg} and naïve T cells. Also, transfer of either regulatory or naïve $S1P_1$-Tg cells into *Rag1*$^{-/-}$ mice exacerbated the development of colitis[14]. These observations are reminiscent of TGF-β-dependent immune homeostasis via T_{reg}-dependent and independent mechanisms[25]. Interestingly, premature egress of $CD4^+CD8^+$ thymocytes into peripheral lymphoid organs and tissues has been observed in another strain of $S1P_1$-transgenic mice that utilized an artificial promoter to drive the transgene expression in both T and B cells[38]. We did not observe these alterations in multiple lines of founder mice expressing the $S1P_1$ transgene throuhg the T cell-selective CD2 promoter/enhancer[13] (data not shown). The simplest interpretation for the discordance is the use of different promoters for the transgene expression. Importantly, diminished thymic nT_{reg} cells were apparent in both types of transgenic mice[14,38], highlighting the key function of $S1P_1$ in T_{reg} cell differentiation.

$S1P_1$ attenuates TGF-β-Smad3 function via signaling through mTOR. Even though Smad3 is activated within minutes after TGF-β stimulation, the sustained Smad3 activity, which is antagonized by the $S1P_1$-mTOR axis, is essential for iT_{reg} differentiation. This is in agreement with the observed 48 h delay in Foxp3 expression following TGF-β treatment[16]. The effect of $S1P_1$ on mTOR signaling in naive T cells is distinct from its function in nT_{reg} cells, in which it facilitates immediate mTOR activation[14], suggesting cell context-specific regulation of mTOR signaling. Despite this, the $S1P_1$-mTOR axis negatively regulates the differentiation of both thymic nT_{reg} cells and extrathymic iT_{reg} cells, via antagonizing TGF-β-Smad3 signaling. Although the roles of TGF-β in nT_{reg} development remain controversial[33,39], a requirement for Smad3 signaling in this process is established by the significant reduction of thymic nT_{reg} cells in Smad3$^{-/-}$ mice[40]. This probably reflects the engagement of multiple and potentially opposing signaling pathways by TGF-β, as well as the pleiotropic effects of TGF-β on T cell development and homeostasis[17]. Taken together, while diverse mechanisms promote the generation of nT_{reg} and iT_{reg} cells[3], a common pathway involving $S1P_1$-mTOR and the antagonistic interactions with TGF-β-Smad3 signaling negatively controls the differentiation of both T_{reg} subsets.

mTOR signaling is necessary for the differentiation of multiple effector T cells including T_H1, T_H2 and T_H17 cells while at the same time, inhibiting iT_{reg} generation[30]. Here we found that the $S1P_1$-mTOR axis selectively controls T_H1 and T_{reg} differentiation, suggesting that other receptors may mediate mTOR activation for T_H2 and T_H17 differentiation. Although previous reports showed that a human $S1P_1$ transgene is capable of promoting

T_H2 and T_H17 cell generation *in vitro*[41,42], we found no evidence of $S1P_1$ involvement in these processes upon antigen stimulation *in vivo*. Whether $S1P_1$ regulates these processes under more defined conditions requires further testing. Additional molecules involved in Akt-mTOR signaling, including Rictor (mTORC2), Cbl-b, Foxo1 and Foxo3, has recently been shown to modulate T cell responses[30,43,44]. Given the potent effects of $S1P_1$ on T_{reg} cell induction and maintenance and T_H1 cell generation, $S1P_1$ may engage multiple pathways associated with Akt-mTOR. Furthermore, direct interactions between Smad3 and the Akt, mTOR and Foxo pathways have been observed outside the immune system[45,46], suggesting that Smad3 may integrate these diverse pathways to direct T cell differentiation.

S1P is maintained at high levels in the blood and lymph by the actions of SphKs but kept at low levels in lymphoid organs by S1P lyase. Separate sources appear to provide S1P to plasma and lymph[47], and a recent study indicates that neural crest-derived pericytes produce S1P to induce thymocyte egress[38.] Combining pharmacological and genetic approaches, we found that $S1P_1$-mediated regulation of T cell differentiation requires endogenous SphK activity. Although exogenously added S1P has no apparent effects on T_{reg} cell generation, we cannot fully exclude the contribution of the exogenous source of S1P in this process, because partioning of S1P distribution might not be recapitulated in the isolated cell cultures. Nonetheless, our results clearly demonstrate a key role for the endogenously produced S1P in T cell differentiation in autocrine and/or paracrine manners. Moreover, SphK1 expression is strongly upregulated by TGF-β stimulation, which likely serves as a feedback mechanism to restrain prolonged TGF-β effects on Foxp3 expression. In growth factor-activated cells, S1P has been found to be produced and secreted out of the cell to activate S1P receptors in its vicinity in a model known as “inside-out” signaling of S1P[34]. To our knowledge, our work represents the first time to identify the similar modes of action for S1P in T cell responses.

We further demonstrated that $S1P_1$ links fundamental processes of T cell lineage commitment to immunosuppressive therapy. FTY720 and rapamycin are thought to affect distinct molecular and cellular pathways in T cells by acting on $S1P_1$ to induce lymph node sequestration and on mTOR to block cell cycle entry, respectively[10,31]. Here we found that these two potent immunosuppressants target the same $S1P_1$-mTOR axis to modulate the balance between pro-inflammatory T_H1 and anti-inflammatory T_{reg} cells *in vivo*. These effects are likely to be T cell intrinsic, as we were able to recapitulate their functions using *in vitro* culture systems. Therefore, regulation of T cell lineage choices by the $S1P_1$-mTOR axis can be further explored to develop novel therapeutics for autoimmunity and transplant rejection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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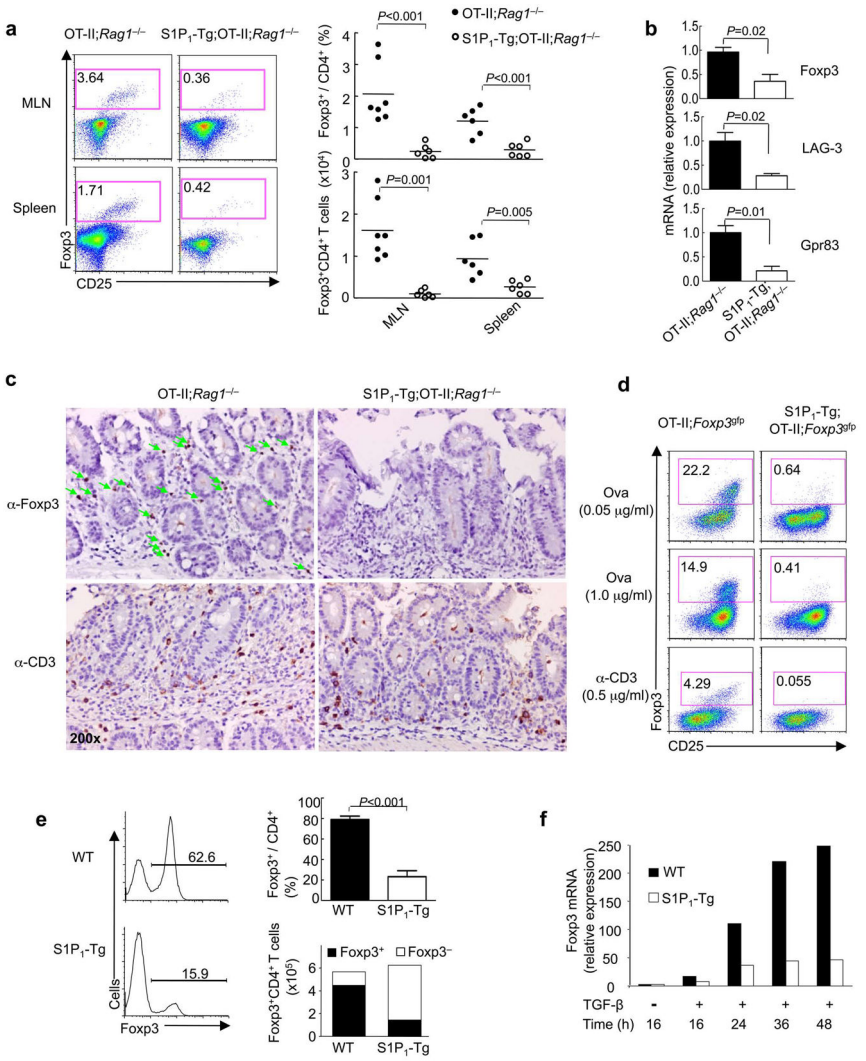

Figure 1. S1P$_1$ inhibits *de novo* generation of Foxp3$^+$ iT$_{reg}$ cells

(a–c) Induction of Foxp3$^+$ iT$_{reg}$ cells after feeding of OT-II;*Rag1*$^{-/-}$ and S1P$_1$-Tg;OT-II;*Rag1*$^{-/-}$mice with Ova in the drinking water for 5 days. **(a)** Foxp3 and CD25 expression in CD4 T cells and proportions and absolute numbers of Foxp3$^+$ CD4 T cells. MLN, mesenteric lymph nodes. **(b)** mRNA expression of Foxp3, LAG-3 and Gpr83 in CD4 T cells isolated from MLN (n=3). **(c)** Distribution of Foxp3$^+$ and CD3$^+$ cells in the lamina propria. **(d)** Analysis of Foxp3 expression after naïve T cells from OT-II;*Foxp3*^{gfp} and S1P$_1$-Tg;OT-II;*Foxp3*^{gfp} mice were stimulated with MLN-derived CD103$^+$ DCs and the Ova peptide or anti-CD3 for 5 days**. (e)** Analysis of Foxp3 expression after wild-type (WT) and S1P$_1$-Tg naïve T cells were activated with anti-CD3 and anti-CD28 in the presence of TGF-β (5 ng/ml). The right panels show proportions and absolute numbers of Foxp3$^+$ and Foxp3$^-$ cells (n=3). **(f)** Kinetics of Foxp3 mRNA expression after wild-type and S1P$_1$-Tg naïve cells were activated with anti-CD3 and anti-CD28 in the presence of TGF-β (5 ng/ml) for various times. Data represent three to four independent experiments.

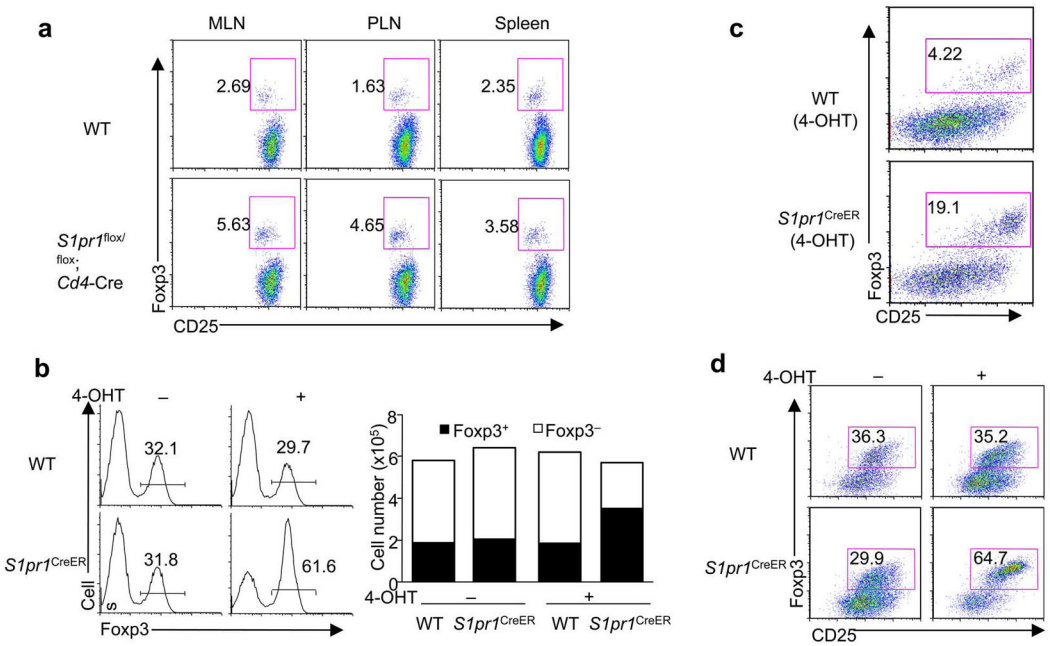

Figure 2. S1P$_1$ is required to restrain the generation and maintenance of Foxp3$^+$ iT$_{reg}$ cells

(a) Analysis of Foxp3 expression 4 weeks after Foxp3$^-$ CD4SP thymocytes from wild-type and *S1pr1*$^{flox/flox}$;*Cd4*-Cre mice were transferred into *Rag1*$^{-/-}$ mice. PLN, peripheral lymph nodes. **(b)** Analysis of Foxp3 expression after naïve T cells from wild-type and *S1pr1*^{CreER} mice were treated with 4-OHT and then activated in the presence of TGF-β for iT$_{reg}$ differentiation. The right panels show proportions and absolute numbers of Foxp3$^+$ and Foxp3$^-$ T cells**. (c)** Analysis of Foxp3 expression after naïve T cells from wild-type and *S1pr1*^{CreER} mice were treated with 4- OHT and then activated by CD103$^+$ DCs and anti-CD3, without any exogenous cytokines. **(d)** Analysis of Foxp3 expression in mature iT$_{reg}$ cells upon acute deletion of S1P$_1$. Naïve T cells from wild-type and *S1pr1*^{CreER} mice were differentiated into iT$_{reg}$ cells. Foxp3$^+$ (GFP$^+$) cells were sorted, treated with 4-OHT and cultured with IL-2. Foxp3 expression was analyzed 4–5 days later. Data represent three independent experiments.

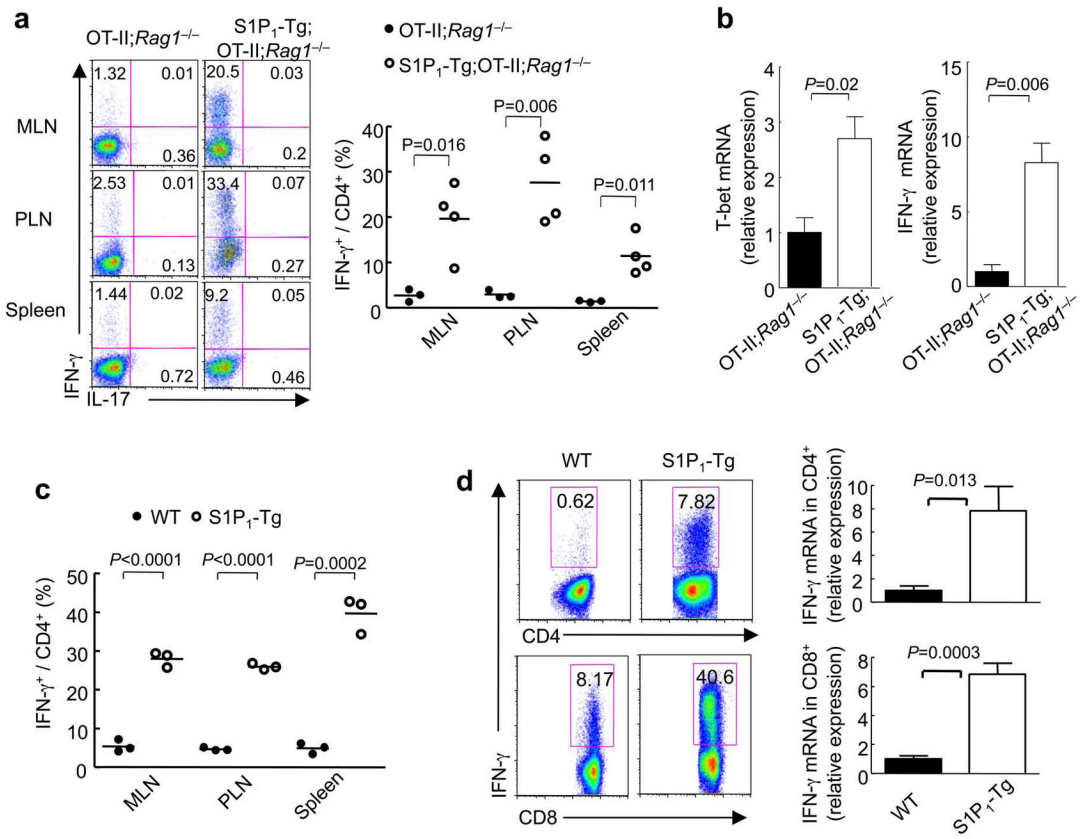

Figure 3. S1P$_1$ drives differentiation of T$_H$1 cells

(a,b) Analysis of T$_H$1 differentiation after OT-II;*Rag1*$^{-/-}$ and S1P$_1$-Tg;OT-II;*Rag1*$^{-/-}$ mice were fed with Ova in the drinking water for 5 days. **(a)** Expression of IFN-γ and IL-17 and proportions of IFN-γ+ CD4 T cells. **(b)** Quantitative RT-PCR analysis of T-bet and IFN-γ expression in CD4 T cells (n=3). **(c)** Proportions of IFN-γ$^+$ CD4 T cells in gated donor cells from OT-II;*Foxp3*^{gfp} and S1P$_1$-Tg;OT-II;*Foxp3*^{gfp} mice that were transferred to naïve wild-type mice and immunized with Ova intravenously. **(d)** IFN-γ expression in total CD4 and CD8 T cells from MLN of wild-type and S1P$_1$-Tg mice, detected by intracellular staining 5 h post PMA/ionomycin stimulation (left), and quantitative RT-PCR after 24 h stimulation with anti-CD3/CD28 (right) (n=3–4). Data represent four independent experiments.

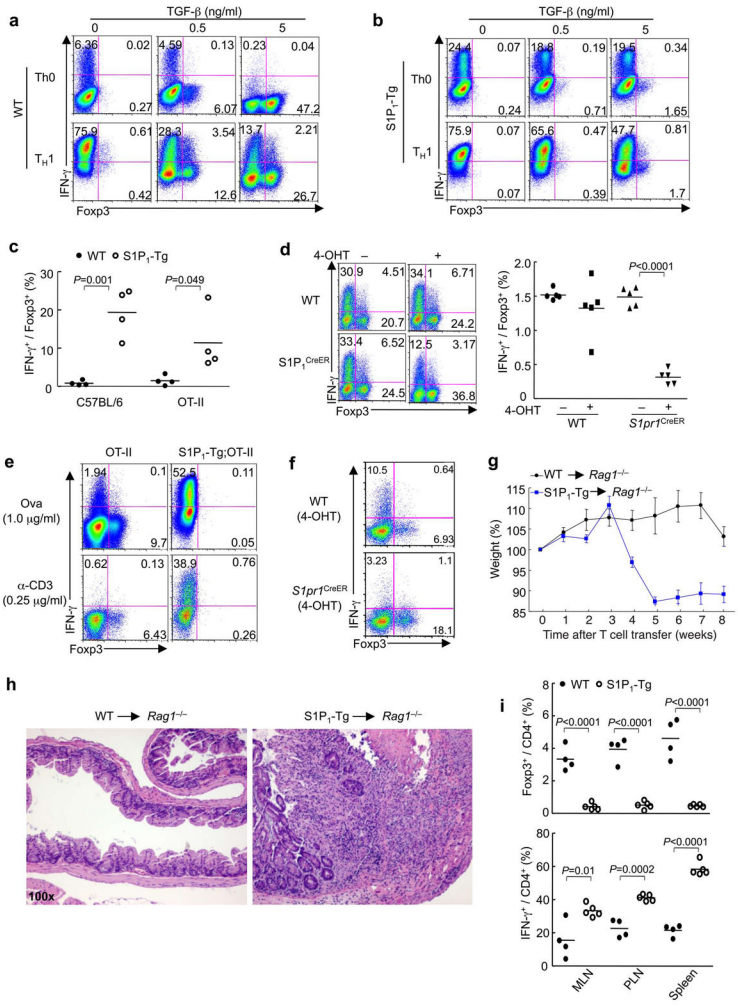

Figure 4. $S1P_1$ regulates reciprocal T_H1 and iT_{reg} differentiation and immune homeostasis *in vivo*

(a,b) Analysis of IFN-γ and Foxp3 expression in wild-type **(a)** and $S1P_1$-Tg **(b)** cells differentiated under the specified conditions. **(c,d)** Ratios of IFN-γ^+ and Foxp3$^+$ populations in wild-type and $S1P_1$-Tg cells **(c)** and 4-OHT treated wild-type and *S1pr1*^{CreER} cells **(d)** that were differentiated under T_H1 conditions in the presence of TGF-β. **(e,f)** Analysis of IFN-γ^+ and Foxp3$^+$ populations in wild-type and $S1P_1$-Tg cells **(e)** and 4-OHT treated wild-type and *S1pr1*^{CreER} cells **(f)** that were activated by CD103$^+$ DCs and Ova or anti-CD3, without any exogenous cytokines. **(g-i)** Analysis of T cell-dependent colitis and *in vivo* differentiation. Wild-type or $S1P_1$-Tg naive T cells were transferred in combination with wild-type Treg cells (CD45.1$^+$) into *Rag1*$^{-/-}$ mice. **(g)** Changes in body weight. **(h)** Representative intestine histology. **(i)** Proportions of Foxp3$^+$ and IFN-γ^+ CD4 T cells derived from naïve T cell donors. Data represent three independent experiments.

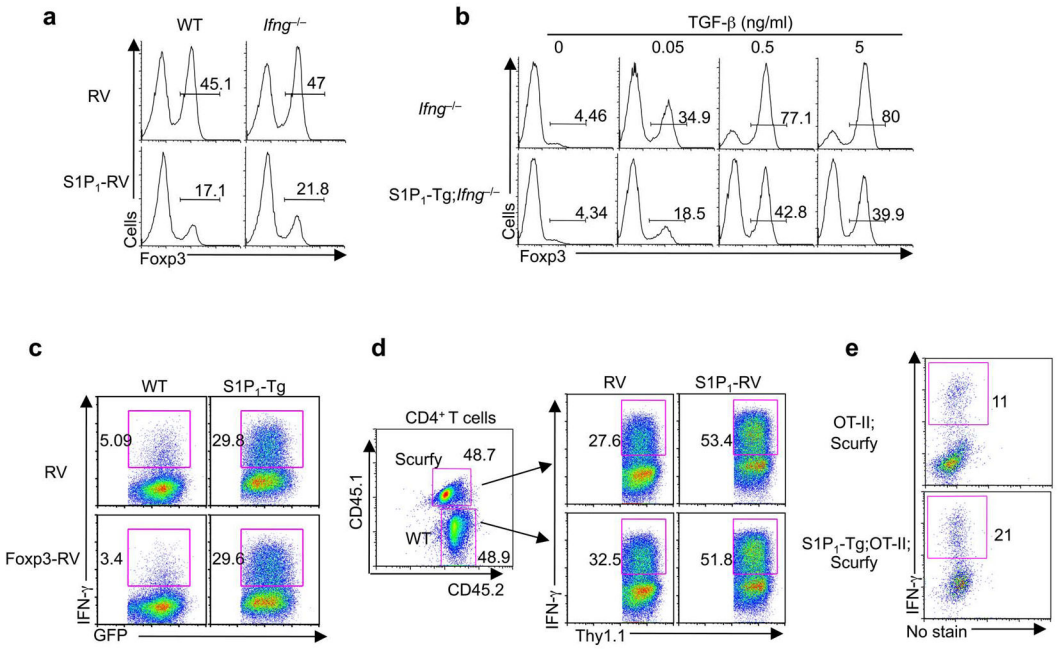

Figure 5. $S1P_1$ mediates iT_{reg} and T_H1 differentiation through discrete mechanisms

(a) Analysis of Foxp3 expression after wild-type and *Ifng*$^{-/-}$ naïve T cells were transduced with control (RV) and $S1P_1$-expressing retrovirus ($S1P_1$-RV) and activated in the presence of TGF-β differentiation. **(b)** Analysis of Foxp3 expression after naïve T cells from *Ifng* $^{-/-}$ and for $iT_{reg}S1P_1$-Tg;*Ifng*$^{-/-}$ mice were differentiated in the presence of different doses of TGF-β. **(c)** Analysis of IFN-γ expression in wild-type and $S1P_1$-Tg cells activated under TH0 conditions and transduced with control (RV) and Foxp3-expressing retrovirus (Foxp3-RV) linked with a GFP reporter; gated GFP$^+$ cells are shown in the FACS plots. **(d)** Analysis of IFN-γ expression in Foxp3-deficient cells. Wild-type (CD45.2$^+$) and Scurfy (CD45.1$^+$.2$^+$) bone marrow cells were mixed at 1:1, and transferred into sublethally irradiated *Rag1*$^{-/-}$ mice. At 6 weeks after reconstitution, naïve T cells from the two donor populations were purified, activated under T_H0 conditions, and transduced with control (RV) and $S1P_1$-expressing retrovirus ($S1P_1$-RV) linked with a Thy1.1 reporter; gated Thy1.1$^+$ cells are shown in the FACS plots. **(e)** Analysis of IFN-γ expression in Foxp3-deficient cells after naïve T cells from OT-II and S1P1-Tg;OT-II mice bred onto the Scurfy background were activated by Ova and irradiated splenic APC. Data represent three independent experiments.

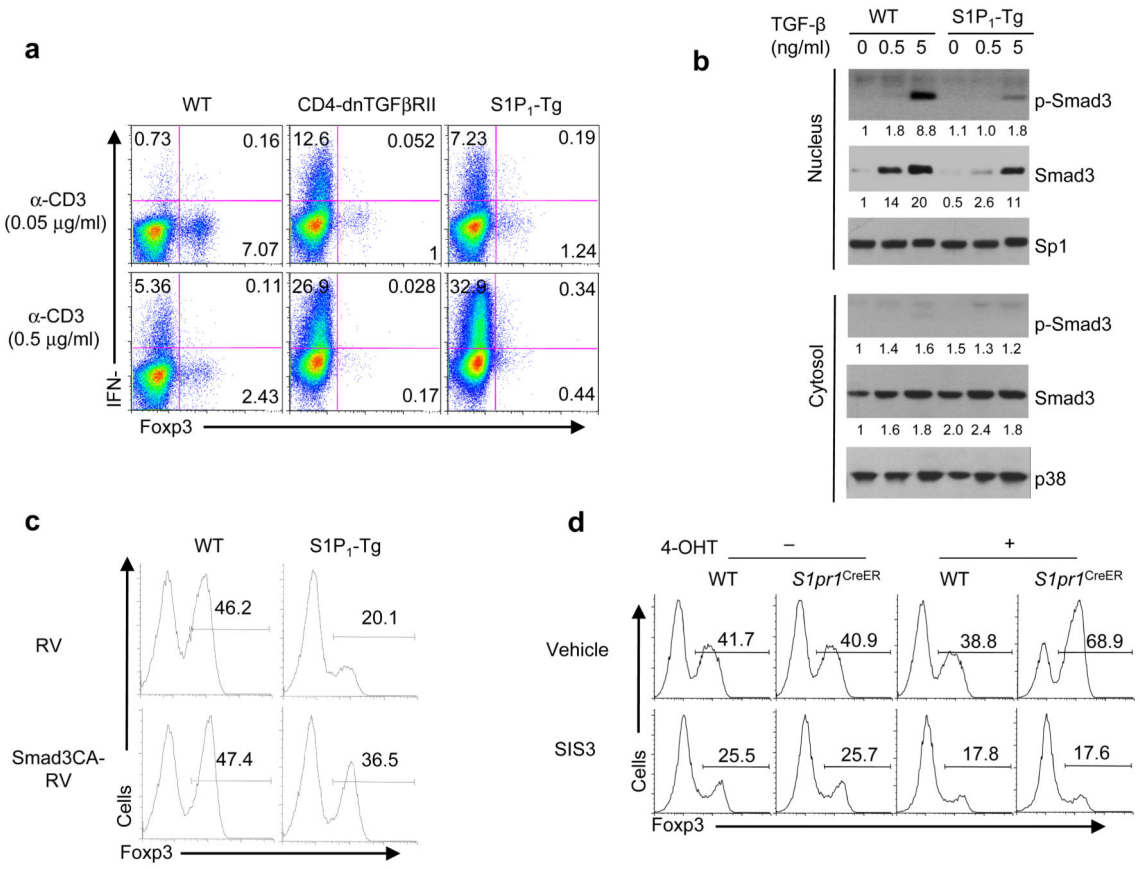

Figure 6. S1P1 attenuates TGF-β-Smad3 signaling

(a) Analysis of Foxp3 expression in wild-type, CD4-dnTGFβRII, and $S1P_1$-Tg cells activated by $CD103^+$ DCs and anti-CD3. **(b)** Analysis of Smad3 nuclear translocation after 2 d stimulation of wild-type and $S1P_1$-Tg cells. The numbers below each panel show the quantification of band intensity relative to the loading controls. **(c)** Analysis of Foxp3 expression in wild-type and $S1P_1$-Tg cells transduced with control (RV) and constitutively active Smad3 retrovirus (Smad3CA-RV) and activated in the presence of TGF-β for iT_{reg} differentiation. **(d)** Analysis of Foxp3 expression in 4-OHT treated wild-type and *S1pr1*^{CreER} cells that were activated in the presence of TGF-β and a Smad3 inhibitor SIS3 (5 μM).

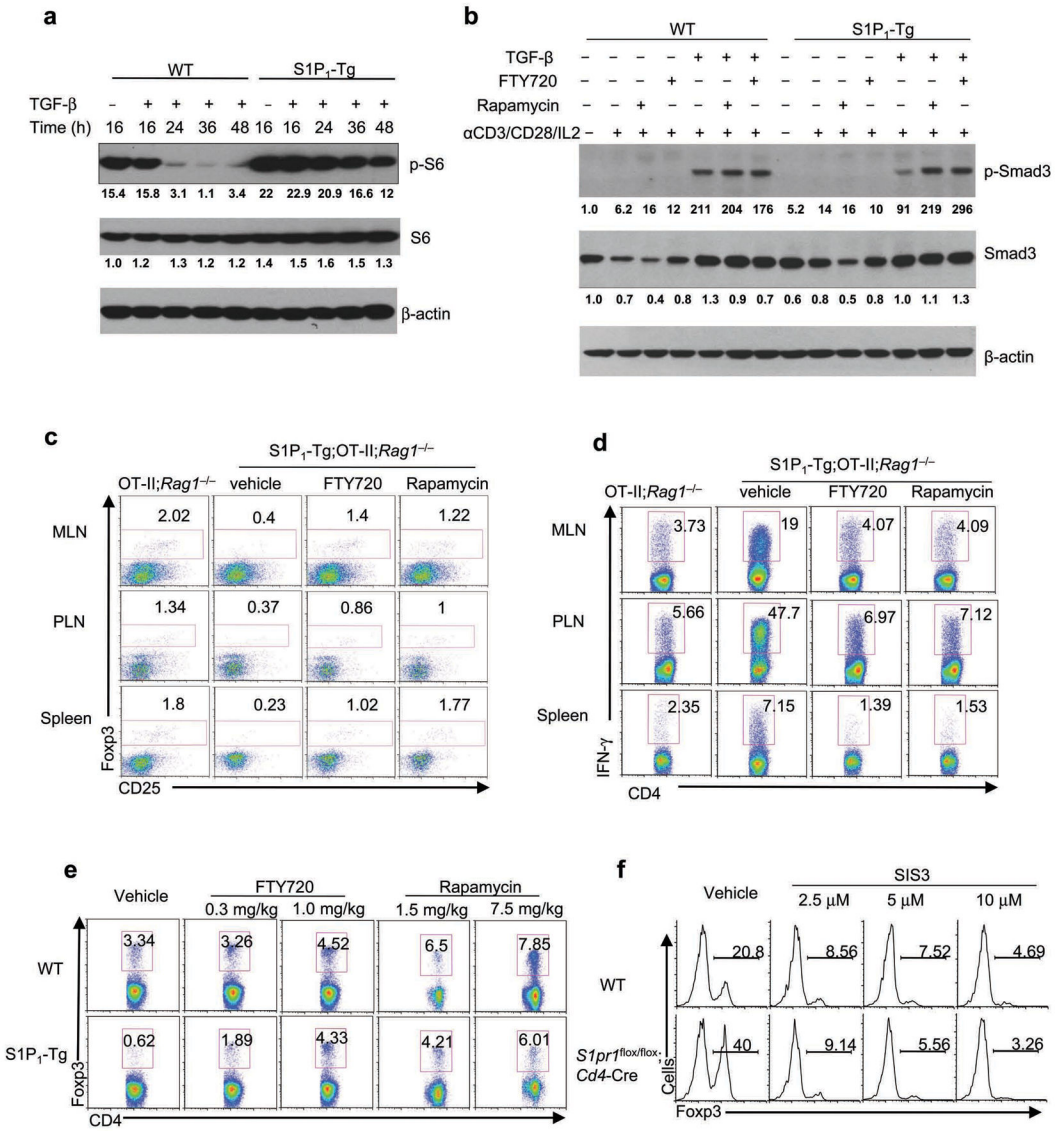

Figure 7. S1P$_1$-mTOR axis is targeted by FTY720 and rapamycin

(a) Kinetics of mTOR activation in wild-type and S1P$_1$-Tg cells stimulated for various times. **(b)** Analysis of Smad3 and S6 activation in wild-type and S1P$_1$-Tg cells treated with FTY720 and rapamycin and activated for 2 days. **(c,d)** Analysis of Foxp3 **(c)** and IFN-γ expression **(d)** in OT-II;*Rag1*$^{-/-}$ and S1P$_1$-Tg;OT-II;*Rag1*$^{-/-}$ mice fed with Ova in the drinking water for 5 days, accompanied by daily FTY720 (1 mg/kg) or rapamycin (3 mg/kg) treatment. **(e)** Analysis of Foxp3 expression in thymic CD4SP cells from wild-type and S1P$_1$-Tg mice treated daily with FTY720 and rapamycin for a total of 5 days. Data represent three independent experiments. **(f)** Induction of Foxp3 in thymic T$_{reg}$ precursors from wild-type and *S1pr1*$^{flox/flox}$;*Cd4*-Cre mice stimulated with IL-2 (50 U/ml) in the presence of SIS3.

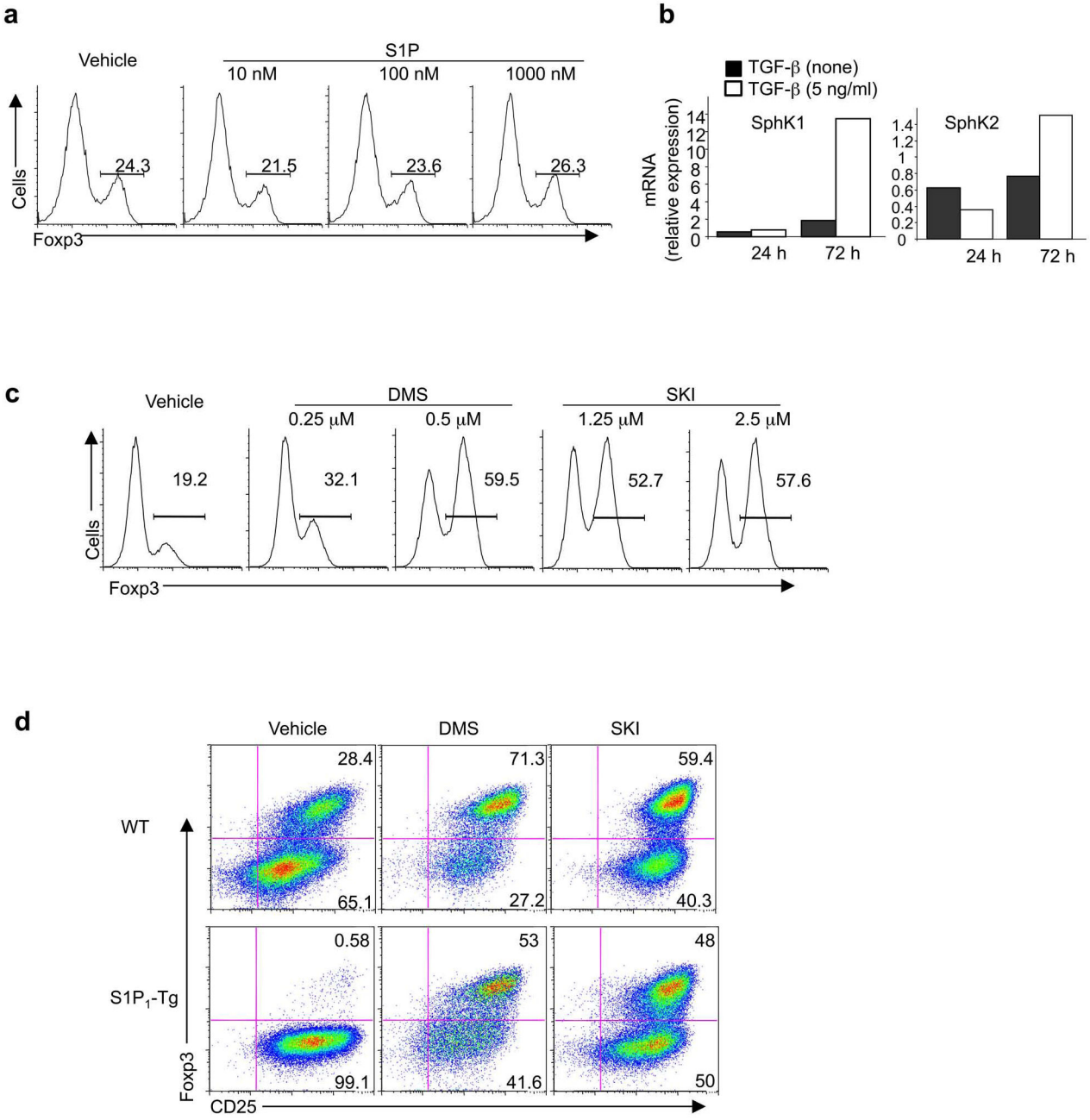

Figure 8. SphK activity regulates T cell differentiation

(a) Analysis fo Foxp3 expression in wild-type cells treated with various concentrations of S1P in a serum-free medium. **(b)** Analysis of *SphK1* and *SphK2* mRNA expression in cells activated in the absence or presence of TGF-β. Levels in naïve T cells were set to 1. **(c)** Analysis of Foxp3 expression after naïve T cells were pre-treated with DMS or SKI, and activated under iT_{reg} conditions for 5 days. **(d)** Analysis of Foxp3 and CD25 expression in wild-type and S1P1-Tg naïve T cells were pre-treated with DMS (0.25 μM) or SKI (2.5

μM), and activated under iT_{reg} conditions for 5 days. Data represent three independent experiments.