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The $S1P_1$-mTOR axis directs the reciprocal differentiation of T_H1 and T_{reg} cells

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Naive CD4+ T cells differentiate into diverse effector and regulatory lineages to orchestrate immunity and tolerance. Here we found that the differentiation of proinflammatory T helper type 1 (T_H1) cells and anti-inflammatory Foxp3+ regulatory T cells (T_{reg} cells) was reciprocally regulated by $S1P_1$, a receptor for the bioactive lipid sphingosine 1-phosphate (S1P). $S1P_1$ inhibited the generation of extrathymic and natural T_{reg} cells while driving T_H1 development in a reciprocal manner and disrupted immune homeostasis. $S1P_1$ signaled through the kinase mTOR and antagonized the function of transforming growth factor-β mainly by attenuating sustained activity of the signal transducer Smad3. $S1P_1$ function was dependent on endogenous sphingosine kinase activity. Notably, two seemingly unrelated immunosuppressants, FTY720 and rapamycin, targeted the same $S1P_1$ and mTOR pathway to regulate the dichotomy between T_H1 cells and T_{reg} cells. Our studies establish an $S1P_1$-mTOR axis that controls T cell lineage specification.

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CD4+ helper T cells are central regulators of adaptive immune responses. In response to antigen stimulation, naive CD4+ T cells proliferate and differentiate into T helper type 1 (T_H1) cells, T_H2 cells and interleukin 17 (IL-17)-producing helper T cells (T_H17) cells to exert specific effector functions. These effector T cells are characterized by distinct patterns of cytokine secretion; interferon-γ (IFN-γ), IL-4 and IL-17 are the signature cytokines for T_H1, T_H2 and T_H17 cells, respectively[1]. Naive precursor cells can also develop into antigen-specific Foxp3+ T_{reg} cells, known as 'induced T_{reg} cells' (iT_{reg} cells), which act in synergy with naturally occurring T_{reg} cells (nT_{reg} cells) to establish immune tolerance and counterbalance 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 on the pleiotropic cytokine transforming growth factor-β (TGF-β (A002271))[4]. Despite extensive progress in this area, how the differentiation of naive precursor cells into opposing regulatory and effector lineages is regulated remains poorly understood.

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, whereas stimulation by TGF-β together with IL-6 or IL-21 promotes the development of T_H17 cells[1]. The differentiation of T cells is further shaped by non-cytokine receptors, especially those of 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 differentiation in a ligand-specific way[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 that signals through five known G protein–coupled receptors ($S1P_1$–$S1P_5$)[10]. FTY720, a new immunosuppressant, induces the sequestration of T cells 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 this debilitating autoimmune disease[11]. However, the 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 $S1P_1$ (A000813) have indicated that $S1P_1$ is the main S1P receptor that facilitates the egress of T cells from lymphoid organs[12,13]. Experiments with gain- and loss-of-function genetic systems have shown a previously unknown role for $S1P_1$ in the negative control of the thymic generation and suppressive activity of nT_{reg} cells in a process dependent on the downstream pathway of the kinases Akt and mTOR (A000094)[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 the 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 on the ability of $S1P_1$ to sustain mTOR activation in T cells. Our studies demonstrate that the differentiation of proinflammatory T_H1 cells and anti-inflammatory T_{reg} cells is reciprocally regulated by $S1P_1$-mTOR and the opposing TGF-β–Smad3 signaling.

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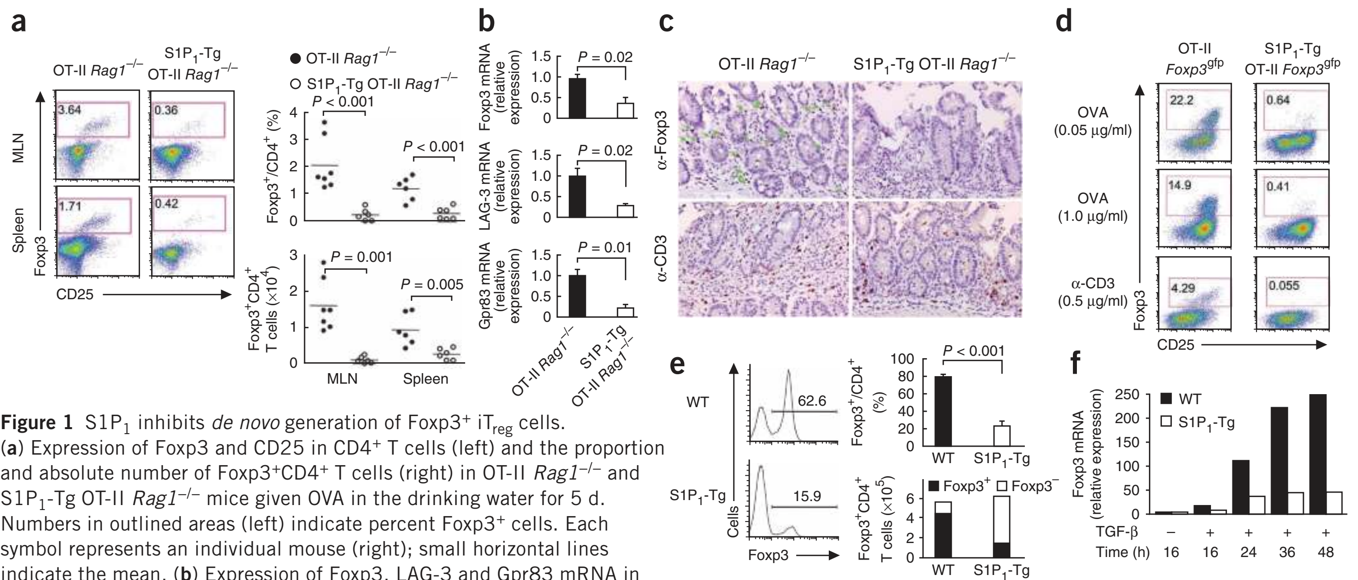

Figure 1 S1P$_1$ inhibits *de novo* generation of Foxp3$^+$ iT$_{reg}$ cells. (**a**) Expression of Foxp3 and CD25 in CD4$^+$ T cells (left) and the proportion and absolute number of Foxp3$^+$CD4$^+$ T cells (right) in OT-II *Rag1*$^{-/-}$ and S1P$_1$-Tg OT-II *Rag1*$^{-/-}$ mice given OVA in the drinking water for 5 d. Numbers in outlined areas (left) indicate percent Foxp3$^+$ cells. Each symbol represents an individual mouse (right); small horizontal lines indicate the mean. (**b**) Expression of Foxp3, LAG-3 and Gpr83 mRNA in CD4$^+$ T cells isolated from MLNs of the mice in **a** (n = 3 per genotype), presented relative to that of wild-type cells. (**c**) Distribution of Foxp3$^+$ and CD3$^+$ cells in the lamina propria of the mice in **a**. Green arrows indicate cells positive for Foxp3 expression. α-, antibody to. Original magnification, ×200. (**d**) Foxp3 expression in naive OT-II *Foxp3*^{gfp} and S1P$_1$-Tg OT-II *Foxp3*^{gfp} T cells stimulated for 5 d with MLN-derived CD103$^+$ DCs and either OVA peptide or anti-CD3. Numbers in outlined areas indicate percent Foxp3$^+$ cells. (**e**) Foxp3 expression (left) in wild-type (WT) and S1P$_1$-Tg naive T cells activated with anti-CD3 and anti-CD28 in the presence of TGF-β (5 ng/ml). Numbers above bracketed lines indicate percent Foxp3$^+$ cells. Right, proportion and absolute number of Foxp3$^+$ and Foxp3$^-$ cells (n = 3 mice per genotype). (**f**) Kinetics of Foxp3 mRNA expression in wild-type and S1P$_1$-Tg naive cells activated for various times with anti-CD3 and anti-CD28 in the presence (+) or absence (–) of TGF-β (5 ng/ml), presented relative to the expression in wild-type cells not treated with TGF-β. *P* values (**a**,**b**,**e**), Student's *t*-test. Data represent three to four independent experiments (error bars (**b**,**e**), s.e.m. of triplicates).

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RESULTS

S1P$_1$ blocks iT$_{reg}$ differentiation

To gain insight into the mechanisms of iT$_{reg}$ differentiation, we determined the role of S1P$_1$ in this process. First, we crossed mice expressing a T cell–specific transgene encoding S1P$_1$ (*S1pr1*; called 'S1P$_1$-Tg mice' here), which expressed two- to threefold more S1P$_1$ than did wild-type mice (data not shown)[13], with OT-II transgenic mice (which express CD4$^+$ T cell antigen receptors (TCRs) specific for ovalbumin (OVA)) on a recombination activating-gene 1–deficient (*Rag1*$^{-/-}$) background. Placing a TCR transgene on the *Rag1*$^{-/-}$ background results in all peripheral T cells' having single antigen specificity without detectable expression of the transcription factor Foxp3, which eliminates the confounding effects of S1P$_1$ function in the nT$_{reg}$ cell compartment[14]. In a model of oral antigen-induced generation of iT$_{reg}$ cells[6,7], Foxp3 induction was much lower in the mesenteric lymph nodes (MLNs) and spleens of S1P$_1$-Tg OT-II *Rag1*$^{-/-}$ mice than in the control OT-II *Rag1*$^{-/-}$ mice (**Fig. 1a**). Expression of the T$_{reg}$ cell–specific factors LAG-3 and Gpr83 was also lower in S1P$_1$-Tg OT-II *Rag1*$^{-/-}$ T cells (**Fig. 1b**). We observed more pronounced effects 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 the similar numbers and distribution of total CD3$^+$ cells in these mice (**Fig. 1c**). To formally exclude the possibility of a contribution of the residual thymus-derived nT$_{reg}$ cells, we crossed S1P$_1$-Tg OT-II mice with mice in which a gene encoding green fluorescent protein (GFP) is knocked into the *Foxp3* allele (*Foxp3*^{gfp} knock-in mice), which marks Foxp3 expression with GFP to distinguish T$_{reg}$ cells and conventional T cells[14]. We purified naive Foxp3$^-$ T cells from the progeny ofthese mice and transferred them into wild-type immunocompetent mice. The administration of oral antigen to the recipients induced a sizable Foxp3$^+$ population from wild-type donors, whereas the Foxp3$^+$ population from S1P$_1$-Tg donors was 70% smaller (**Supplementary Fig. 1**). Thus, S1P$_1$ inhibits new induction of Foxp3-expressing iT$_{reg}$ cells from the peripheral T cell pool.

A specialized subset of dendritic cells (DCs) in the mucosa that expresses CD103 is tolerogenic by inducing Foxp3$^+$ iT$_{reg}$ cells[6,7]. We purified CD103$^+$ DCs from MLNs and cultured them with naive T cells from OT-II *Foxp3*^{gfp} mice (CD62L^{hi}CD44^{lo}Foxp3$^-$ T cells) in the presence of the cognate antigen or antibody to CD3 (anti-CD3). Under these conditions, a substantial population of T cells from OT-II *Foxp3*^{gfp} mice was induced to express Foxp3, whereas S1P$_1$-Tg OT-II *Foxp3*^{gfp} T cells showed 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 ability 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 naive precursor cells by antigen stimulation in the presence of TGF-β[15]. When naive cells were activated in the presence of TGF-β in antigen-presenting cell–free conditions, those expressing the S1P$_1$ transgene were considerably impaired in their ablity to differentiate into Foxp3$^+$ iT$_{reg}$ cells (**Fig. 1e**). Wild-type and S1P$_1$-Tg cells proliferated to a similar degree and had similar expression of apoptotic markers and the antiapoptosis molecule Bcl-2 (**Fig. 1e** and data not shown). Furthermore, crossing S1P$_1$-Tg mice with mice expressing a transgene encoding Bcl-2 failed to rectify the diminished iT$_{reg}$ differentiation (data not shown). Thus, the impaired generation of iT$_{reg}$ cells was not due to altered T cell proliferation or survival, which indicated 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 considerably induced at 24 h after TGF-β stimulation and continued

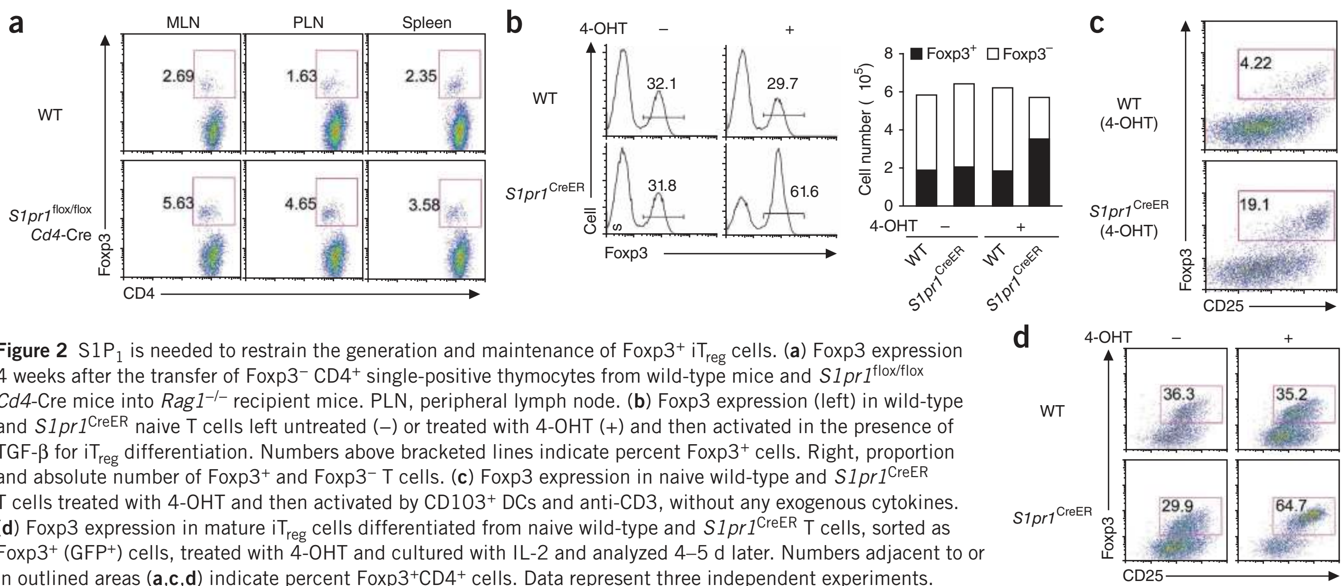

Figure 2 $S1P_1$ is needed to restrain the generation and maintenance of $Foxp3^+$ iT_{reg} cells. (**a**) Foxp3 expression 4 weeks after the transfer of $Foxp3^-$ $CD4^+$ single-positive thymocytes from wild-type mice and $S1pr1^{flox/flox}$ *Cd4*-Cre mice into $Rag1^{-/-}$ recipient mice. PLN, peripheral lymph node. (**b**) Foxp3 expression (left) in wild-type and $S1pr1^{CreER}$ naive T cells left untreated (–) or treated with 4-OHT (+) and then activated in the presence of TGF-β for iT_{reg} differentiation. Numbers above bracketed lines indicate percent $Foxp3^+$ cells. Right, proportion and absolute number of $Foxp3^+$ and $Foxp3^-$ T cells. (**c**) Foxp3 expression in naive wild-type and $S1pr1^{CreER}$ T cells treated with 4-OHT and then activated by $CD103^+$ DCs and anti-CD3, without any exogenous cytokines. (**d**) Foxp3 expression in mature iT_{reg} cells differentiated from naive wild-type and $S1pr1^{CreER}$ T cells, sorted as $Foxp3^+$ (GFP^+) cells, treated with 4-OHT and cultured with IL-2 and analyzed 4–5 d later. Numbers adjacent to or in outlined areas (**a**,**c**,**d**) indicate percent $Foxp3^+CD4^+$ cells. Data represent three independent experiments.

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to increase at 36–48 h. $S1P_1$-Tg cells had much lower Foxp3 expression at 24–48 h (**Fig. 1f**). In addition, transduction of wild-type cells with retrovirus expressing $S1P_1$, followed by TGF-β treatment, resulted in less 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**), which suggested that $S1P_1$ may interfere with sustained TGF-β signaling.

$S1P_1$ inhibits the generation and maintenance of iT_{reg} cells

We then determined whether $S1P_1$ is needed to suppress iT_{reg} differentiation. Given the fact that the progeny of mice with *lox*P-flanked *S1pr1* alleles ($S1pr1^{flox/flox}$ mice) crossed with mice transgenic for Cre recombinase driven by the *Cd4* promoter (*Cd4*-Cre) have few peripheral T cells[14], we bred those mice onto the $Foxp3^{gfp}$ background and purified $Foxp3^-$ $CD4^+$ single-positive thymocytes. When transferred into $Rag1^{-/-}$ mice, a subset of the donor cells became $Foxp3^+$. Deficiency in $S1P_1$ resulted in an augmented $Foxp3^+$ population (**Fig. 2a**), which indicated the generation of more iT_{reg} cells *in vivo*. To more directly address the requirement for $S1P_1$ in the development of iT_{reg} cells from peripheral T cells, we crossed $S1pr1^{flox/flox}$ mice with mice in which a gene encoding a fusion protein of Cre and the estrogen receptor is introduced into the ROSA26 locus (Rosa26-Cre-ER^{T2} mice; progeny called '$S1pr1^{CreER}$ mice' here). After treatment with 4-hydroxytamoxifen (4-OHT), the *lox*P-flanked *S1pr1* gene was deleted in naive T cells (**Supplementary Fig. 3a**). When these cells were differentiated toward iT_{reg} cells by exogenous TGF-β, they showed a higher frequency of the $Foxp3^+$ population (**Fig. 2b**) associated with greater abundance of Foxp3 mRNA (**Supplementary Fig. 3b**) and normal cell survival (data not shown). Moreover, the $CD103^+$ 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 Foxp3 expression is necessary for T_{reg} cell function. Foxp3 expression in iT_{reg} cells is less stable than that in nT_{reg} cells, and it depends on continuous TGF-β signaling in iT_{reg} cells[16]. To assess 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 the cells with 4-OHT to induce *S1pr1* deletion in mature iT_{reg} cells. After TGF-β withdrawal, control iT_{reg} cells that retained $S1P_1$ expression readily lost Foxp3 expression, whereas iT_{reg} cells deficient in $S1P_1$ encompassed a much larger $Foxp3^+$ population with higher expression of Foxp3 and the cytokine receptor CD25 (**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 the induction and maintenance of iT_{reg} cells prompted us to investigate whether $S1P_1$ diverges naive T cells into alternative lineages. For this we used 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 much larger population (five- to tenfold more abundant) of IFN-γ^+ $CD4^+$ T cells in the various lymphoid organs examined (**Fig. 3a**). Accordingly, quantitative RT-PCR identified higher expression of IFN-γ and the T_H1 transcription factor T-bet in $S1P_1$-Tg cells after oral antigen exposure (**Fig. 3b**). The abundance of IL-4 and IL-17 protein and RNA was low and similar for the two types of mice (**Fig. 3a** and data not shown), which indicated 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 (Thy-1.1^+) into naive recipient mice and immunized the recipients intravenously with OVA. Five days later we detected a much larger IFN-γ^+ population among $S1P_1$-Tg donor–derived cells than among wild-type donor cells (**Fig. 3c**). Also, secretion of IFN-γ, but not of IL-4 or IL-17, was enhanced in the group given transfer of $S1P_1$-Tg cells after *ex vivo* peptide stimulation (data not shown).

Third, we determined the proportion of T cells able to produce IFN-γ in $S1P_1$-Tg mice by intracellular cytokine staining. A higher percentage of $CD4^+$ and $CD8^+$ T cells in the MLNs of $S1P_1$-Tg mice than those from wild-type mice produced IFN-γ (**Fig. 3d**). Quantitative RT-PCR showed that $S1P_1$-Tg $CD4^+$ and $CD8^+$ T cells had seven- to eightfold more IFN-γ than did their wild-type counterparts (**Fig. 3d**). Thus, $S1P_1$ promotes IFN-γ production *in vivo*.

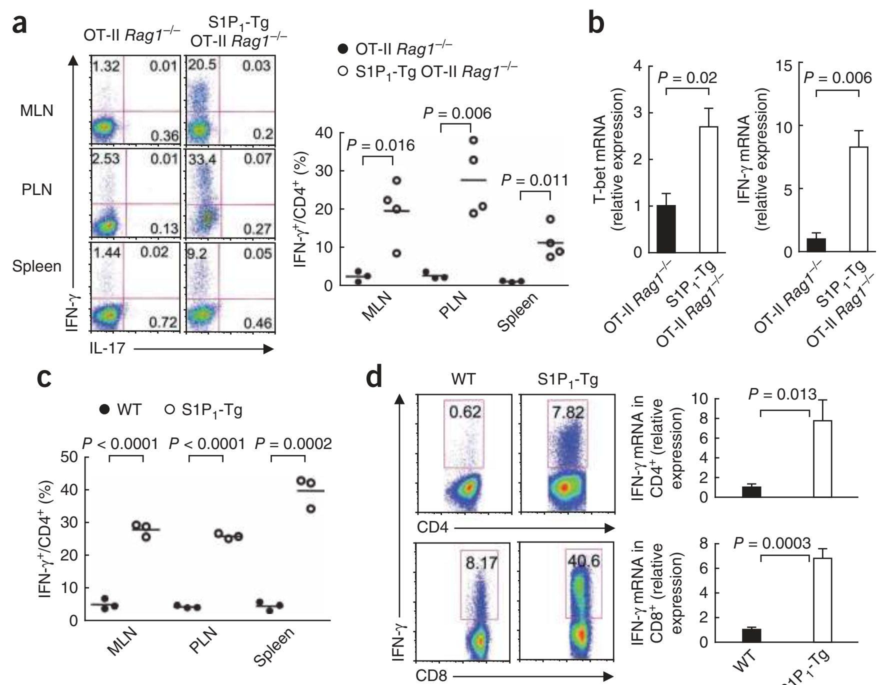

Figure 3 $S1P_1$ drives the differentiation of T_H1 cells. (**a**) Expression of IFN-γ and IL-17 (left) and proportion of IFN-γ+ CD4+ T cells (right) in OT-II *Rag1*−/− and $S1P_1$-Tg OT-II *Rag1*−/− mice fed OVA in their drinking water for 5 d. Numbers in quadrants indicate percent IFN-γ+IL-17− cells (top left), IFN-γ+IL-17+ cells (top right) or IFN-γ−IL-17+ cells (bottom right). (**b**) Quantitative RT-PCR analysis of the expression of T-bet and IFN-γ in CD4+ T cells from the mice in **a** ($n = 3$ per genotype), presented relative to the expression in wild-type cells. (**c**) Proportion of IFN-γ+ CD4+ T cells among gated donor cells from OT-II *Foxp3*gfp and $S1P_1$-Tg OT-II *Foxp3*gfp mice transferred into naive wild-type mice that were subsequently immunized intravenously with OVA. (**d**) IFN-γ expression (left) in total CD4+ and CD8+ T cells from MLNs of wild-type and $S1P_1$-Tg mice, detected by intracellular staining 5 h after stimulation phorbol 12-myristate 13-acetate and ionomycin. Numbers in outlined areas indicate percent IFN-γ+ cells. Right, quantitative RT-PCR analysis of IFN-γ expression after 24 h of stimulation with anti-CD3 and anti-CD28, relative to the expression in wild-type cells ($n = 3$–4 mice per genotype). Each symbol represents an individual mouse (**a**,**c**); small horizontal lines indicate the mean. *P* values, Student's *t*-test. Data represent four independent experiments (error bars (**b**,**d**), s.e.m. of at least three replicates).

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To establish whether $S1P_1$ regulates intrinsic T cell differentiation, we sorted naive T cells from wild-type and $S1P_1$-Tg mice and cultured them under nonpolarizing (T_H0) conditions. A larger proportion of $S1P_1$-Tg cells than wild-type cells spontaneously differentiated into IFN-γ+ cells, associated with higher T-bet expression (**Supplementary Fig. 5a** and data not shown). We also observed higher IFN-γ expression in $S1P_1$-Tg OT-II cells (**Supplementary Fig. 5a**) and in wild-type cells transduced with $S1P_1$-expressing retrovirus than in those transduced with control retrovirus (data not shown). Conversely, deletion of $S1P_1$ resulted in lower 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 $S1P_1$-deficient cells. TGF-β is a pleiotropic cytokine with pronounced effects on the cell-fate determination of many T cell lineages[17], but whether TGF-β directly coordinates the development of T_H1 cells and iT_{reg} cells from naive precursor cells is not well understood. We therefore differentiated wild-type naive cells under T_H0 and T_H1 conditions in the presence of TGF-β and measured the expression of Foxp3 and IFN-γ simultaneously. As expected, a subset of cells expressed IFN-γ, but few expressed Foxp3, under nonpolarizing conditions. The addition of TGF-β induced Foxp3 expression and terminated IFN-γ expression. Under T_H1 conditions, most T cells became IFN-γ+, yet the addition of TGF-β resulted in a smaller IFN-γ+ population and simultaneously induced a Foxp3+ population, which resulted in the coexistence of T_H1 cells and iT_{reg} cells (**Fig. 4a**). The ability of TGF-β to promote Foxp3 expression over IFN-γ expression was lost in $S1P_1$-Tg cells (**Fig. 4b**). In particular, when we added TGF-β to differentiating T_H1 cells to foster the development of both T_H1 cells and iT_{reg} cells, the ratio of IFN-γ+ cells to Foxp3+ cells was reversed for $S1P_1$-Tg cells (**Fig. 4c**). We also observed this reciprocal change for antigen-specific $S1P_1$-Tg OT-II T cells (**Fig. 4c**). Conversely, deletion of $S1P_1$ resulted in the population 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 more $S1P_1$ 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 for $S1P_1$ in directing T_H1 and iT_{reg} lineage commitment.

Published studies have shown 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 naive 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, iT_{reg} cells developed *in situ* from naive donors, together with nT_{reg} donor cells, are needed to control colitis[19]; we used wild-type nT_{reg} cells here to circumvent the confounding effects in $S1P_1$-Tg nT_{reg} cells[14]. Although there was minimal weight loss and inflammation in the group given cotransfer of wild-type cells, transfer of $S1P_1$-Tg naive cells and wild-type T_{reg} cells resulted in substantial weight loss associated with severe colitis and leukocyte infiltration (**Fig. 4g**,**h**). To delineate the underlying mechanisms, we measured the expression of Foxp3 and IFN-γ in cells derived from naive (CD45.2+) cell donors. We observed fewer Foxp3+ iT_{reg} cells from $S1P_1$-Tg donors than their wild-type counterparts and significanty more IFN-γ-expressing T cells (**Fig. 4i**). Therefore, $S1P_1$ controls the reciprocal relationship between iT_{reg} cells and T_H1 cells *in vivo*.

Discrete mechanisms for 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 the generation of iT_{reg}

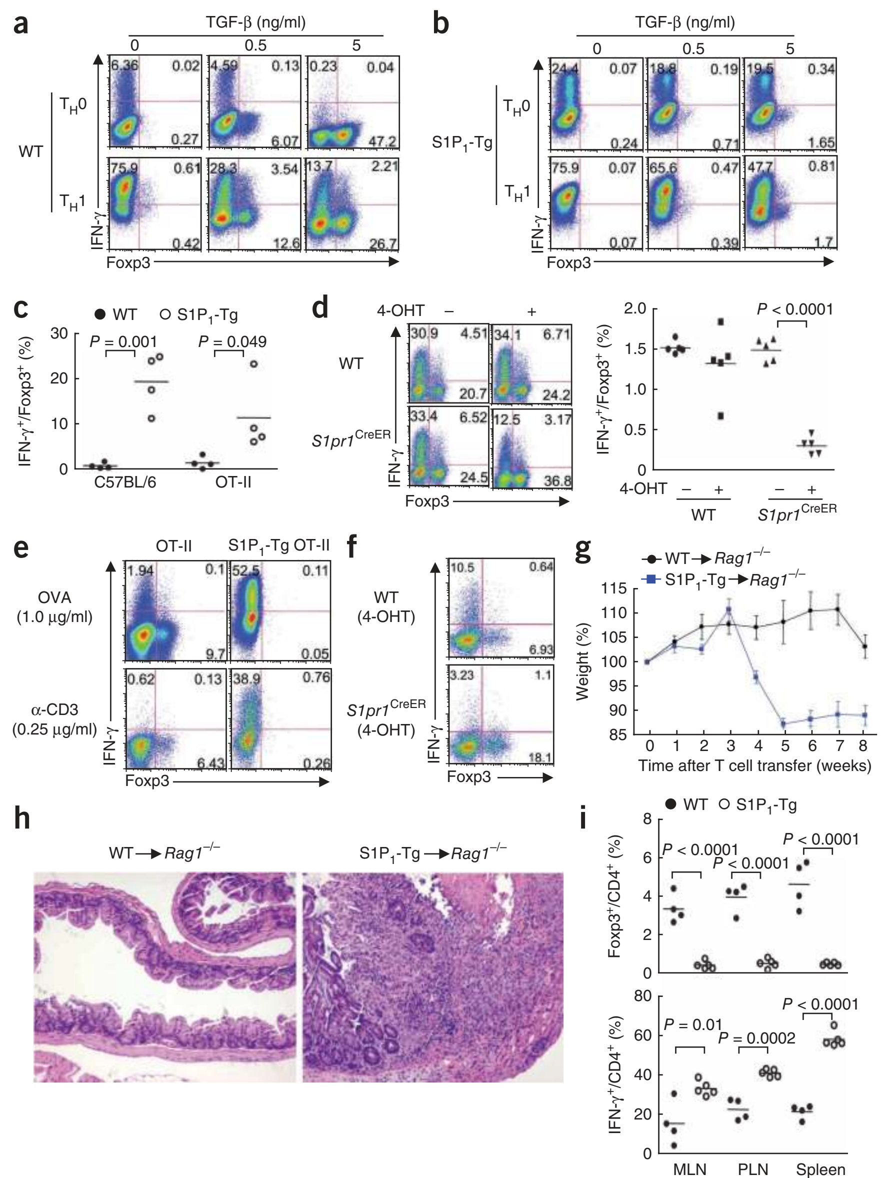

Figure 4 $S1P_1$ regulates reciprocal T_H1 and iT_{reg} differentiation and immune homeostasis *in vivo*. (**a**,**b**) Expression of IFN-γ and Foxp3 in wild-type cells (**a**) and $S1P_1$-Tg cells (**b**) differentiated under T_H0 or T_H1 conditions in the presence or absence of TGF-β. (**c**) Ratio of IFN-γ^+ cells to Foxp3$^+$ cells for wild-type and $S1P_1$-Tg cells differentiated under T_H1 conditions in the presence of TGF-β. (**d**) Expression of IFN-γ and Foxp3 (left) and ratio of IFN-γ^+ cells to Foxp3$^+$ cells (right) for untreated or 4-OHT-treated wild-type and *S1pr1*^{CreER} cells differentiated as in **c**. (**e**,**f**) IFN-γ^+ and Foxp3$^+$ populations of wild-type and $S1P_1$-Tg cells (**e**) and 4-OHT-treated wild-type and *S1pr1*^{CreER} cells (**f**) activated by CD103$^+$ DCs and OVA or anti-CD3 without exogenous cytokines. (**g**) Change in body weight of *Rag1*$^{-/-}$ recipient mice given wild-type or $S1P_1$-Tg naive T cells in combination with wild-type (CD45.1$^+$) T_{reg} cells. (**h**) Intestinal histology of the mice in **g**. Original magnification, ×100. (**i**) Proportion of Foxp3$^+$ and IFN-γ^+ CD4$^+$ T cells derived from naive T cell donors for the mice in **g**. Numbers in quadrants (**a**,**b**,**d**–**f**) indicate percent IFN-γ^+Foxp3$^-$ cells (top left), IFN-γ^+Foxp3$^+$ cells (top right) or IFN-γ^-Foxp3$^+$ cells (bottom right). Each symbol represents an individual mouse (**c**,**d**,**i**); small horizontal lines indicate the mean. *P* values (**c**,**d**,**i**), Student's *t*-test. Data represent three independent experiments (error bars (**g**), s.e.m. of five replicates).

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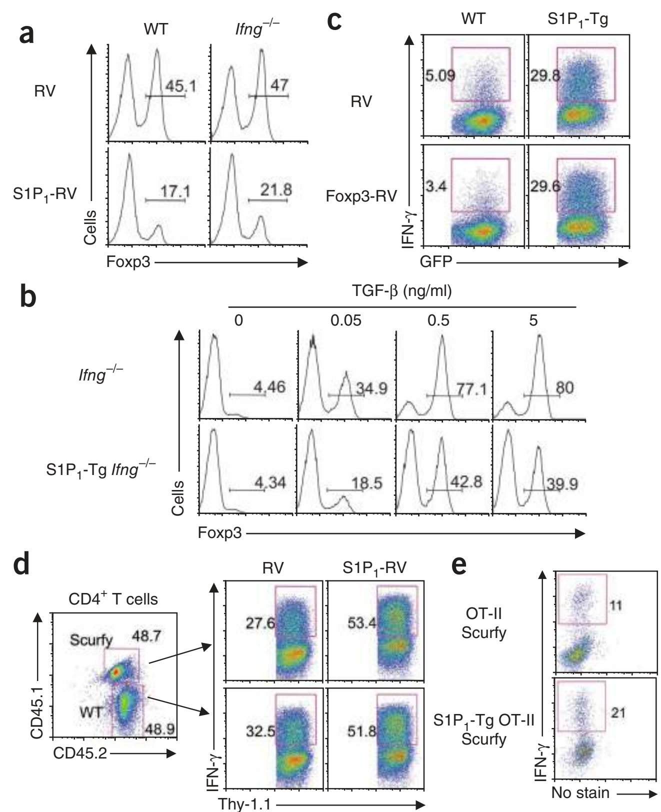
cells[20], although conflicting conclusions also exist[21]. We tested whether inhibition of iT_{reg} differentiation by $S1P_1$ is a secondary consequence of more IFN-γ production. Retroviral transduction of $S1P_1$ into either *Ifng*$^{-/-}$ or wild-type cells resulted in less induction of Foxp3$^+$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

Conversely, we tested whether the lower Foxp3 expression in $S1P_1$-Tg cells was responsible for their enhanced IFN-γ expression. For this we transduced *Foxp3* into wild-type and $S1P_1$-Tg cells and found that forced Foxp3 expression did not lower 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 assessed whether Foxp3 deficiency affected 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 cotransferring bone marrow cells from Foxp3-deficient scurfy (CD45.1$^+$CD45.2$^+$) mice and wild-type (CD45.2$^+$) mice into *Rag1*$^{-/-}$ recipients; the presence of functional T_{reg} cells derived from the wild-type donors prevented autoimmune activation of scurfy T cells[14]. We purified naive T cells from these two donor populations and transduced them with $S1P_1$-expressing retrovirus. $S1P_1$ expression was equally effective in promoting 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]. We stimulated naive T cells from the progeny with OVA peptide and found more T_H1 differentiation in those expressing the $S1P_1$ transgene (**Fig. 5e**). Therefore, either ectopic expression of or deficiency in Foxp3 did not prevent $S1P_1$ from driving T_H1 differentiation, which suggested that $S1P_1$ mediates T_H1 differentiation independently of its effects on the inhibition of Foxp3 expression.

Aged $S1P_1$-Tg mice developed high titers of autoantibodies, concomitant with spontaneous T cell activation[14]. As IFN-γ production is associated with the pathogenesis of systemic autoimmune disease, we assessed whether more IFN-γ in $S1P_1$-Tg cells contributed to the breakdown of immune tolerance. $S1P_1$-Tg *Ifng*$^{-/-}$ mice had titers of antibody to double-stranded DNA 35% as high as those of $S1P_1$-Tg *Ifng*$^{+/+}$ mice (data not shown). In contrast, we observed higher expression of CD44 on $S1P_1$-Tg cells, indicative of their spontaneous activation *in vivo*[14], regardless of the *Ifng*$^{+/+}$ or *Ifng*$^{-/-}$ background (data not shown), consistent with the published observation that spontaneous T cell activation is due mainly to defective T_{reg} cells in $S1P_1$-Tg mice[14]. Therefore, $S1P_1$ regulates immune homeostasis through both T_{reg} cell–dependent and T_{reg} cell–independent mechanisms.

Figure 5 $S1P_1$ mediates iT_{reg} and T_H1 differentiation via discrete mechanisms. (**a**) Foxp3 expression in wild-type and *Ifng*$^{-/-}$ naive T cells transduced with control retrovirus (RV) or $S1P_1$-expressing retrovirus ($S1P_1$-RV) and activated in the presence of TGF-β for iT_{reg} differentiation. (**b**) Foxp3 expression in naive *Ifng*$^{-/-}$ and $S1P_1$-Tg *Ifng*$^{-/-}$ T cells differentiated in the presence of various concentrations of TGF-β. Numbers above bracketed lines (**a**,**b**) indicate percent $Foxp3^+$ cells. (**c**) IFN-γ expression in wild-type and $S1P_1$-Tg cells activated under T_H0 conditions and transduced with control retrovirus (RV) or Foxp3-expressing retrovirus (Foxp3-RV) linked to a GFP reporter; gated GFP^+ cells are presented here. Numbers adjacent to outlined areas indicate percent IFN-γ^+GFP^+ cells. (**d**) IFN-γ expression in Foxp3-deficient cells from sublethally irradiated *Rag1*$^{-/-}$ mice given a 1:1 mixture of wild-type ($CD45.2^+$) and scurfy ($CD45.1^+CD45.2^+$) bone marrow cells; 6 weeks after reconstitution, naive T cells from the two donor populations were purified, activated under T_H0 conditions and transduced with control or $S1P_1$-expressing retrovirus linked to a Thy-1.1 reporter; gated Thy-1.1$^+$ cells are presented here. Numbers adjacent to outlined areas indicate percent $CD45.1^+CD45.2^+$ (scurfy) cells (top left) or $CD45.1^-CD45.2^+$ (wild-type) cells (bottom left), or IFN-γ^+Thy-1.1$^+$ cells (right four plots). (**e**) IFN-γ expression in Foxp3-deficient cells after naive T cells from OT-II and $S1P_1$-Tg OT-II mice bred onto the scurfy background were activated by OVA and irradiated splenic antigen-presenting cells. Numbers adjacent to outlined areas indicate percent IFN-γ^+ cells. Data represent three independent experiments.



$S1P_1$ antagonizes TGF-β–Smad3 signaling

We noticed that the exacerbated colitis after the transfer of $S1P_1$-Tg cells was a phenocopy of the abnormalities of 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 hypothesized that $S1P_1$ mediates reciprocal T_H1 and iT_{reg} differentiation by antagonizing TGF-β receptor signaling. To test our 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, the abnormalities of CD4-dnTGFβRII mice are a phenocopy of the abnormalities of $S1P_1$-Tg mice, which suggests that $S1P_1$ probably affects TGF-β receptor signaling.

We next investigated the molecular mechanisms by which $S1P_1$ and the TGF-β receptor interact. Smad3 is an important transcription

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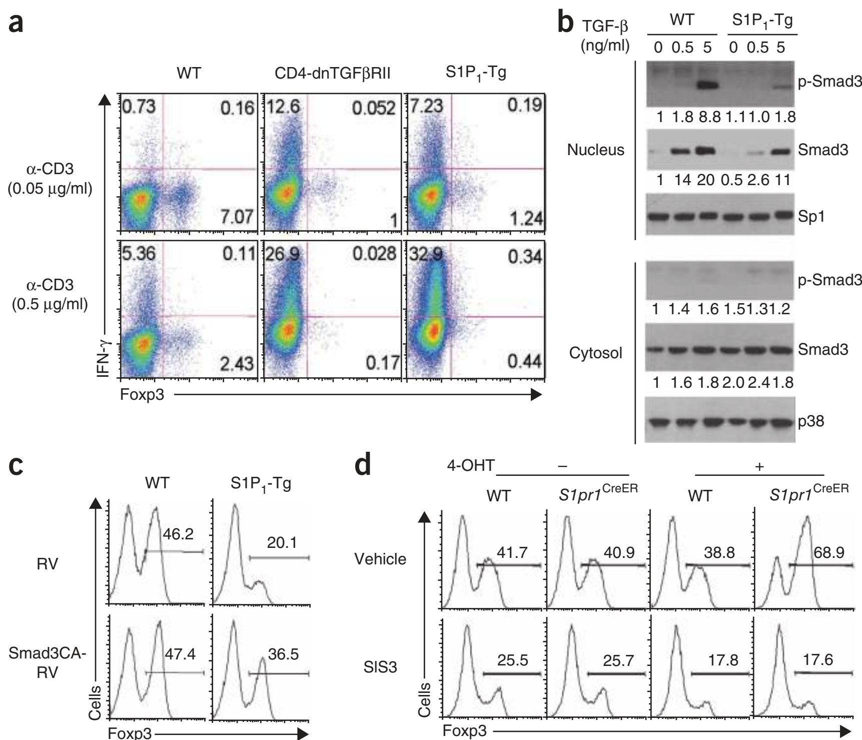

Figure 6 $S1P_1$ attenuates TGF-β-Smad3 signaling. (**a**) Foxp3 expression in wild-type, CD4-dnTGFβRII and $S1P_1$-Tg cells activated by $CD103^+$ DCs and anti-CD3. Numbers in quadrants indicate percent IFN-γ^+Foxp3^- cells (top left), IFN-γ^+Foxp3^+ cells (top right) or IFN-γ^-Foxp3^+ cells (bottom right). (**b**) Nuclear translocation of Smad3 after 2 d of stimulation of wild-type and $S1P_1$-Tg cells with TGF-β, assessed by immunoblot analysis of total Smad3 and phosphorylated Smad3 (p-Smad3). Numbers below lanes indicate band intensity relative to that of loading controls Sp1 (Nucleus) or p38 (Cytosol). (**c**) Foxp3 expression in wild-type and $S1P_1$-Tg cells transduced with control retrovirus (RV) or constitutively active Smad3 retrovirus (Smad3CA-RV) and activated in the presence of TGF-β for iT_{reg} differentiation. (**d**) Foxp3 expression in untreated (–) or 4-OHT-treated (+) wild-type and *S1pr1*^{CreER} cells activated in the presence of TGF-β and the Smad3 inhibitor SIS3 (5 μM). Numbers above bracketed lines (**c**,**d**) indicate percent $Foxp3^+$ cells. Data represent three independent experiments.

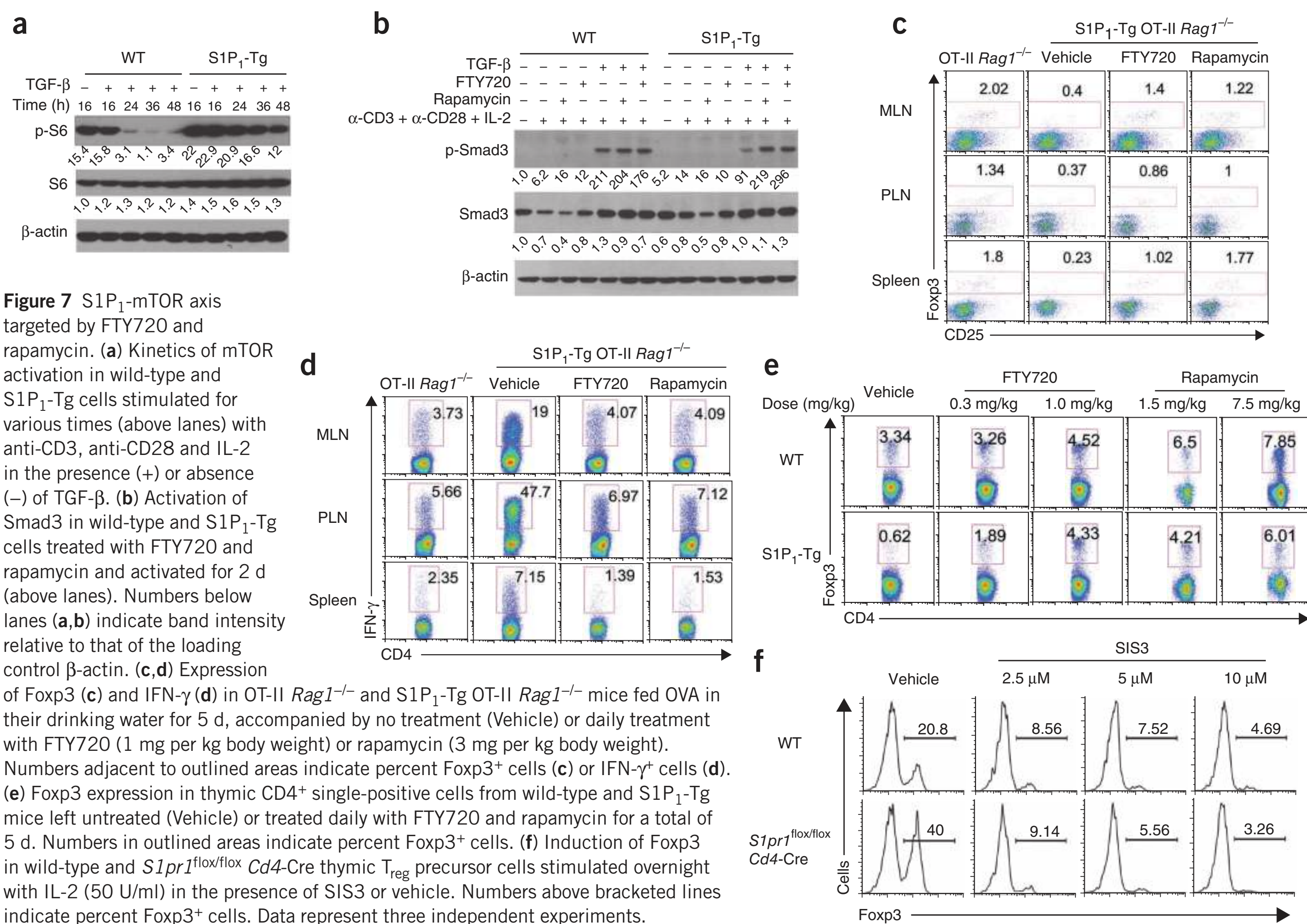

Figure 7 $S1P_1$-mTOR axis targeted by FTY720 and rapamycin. (**a**) Kinetics of mTOR activation in wild-type and $S1P_1$-Tg cells stimulated for various times (above lanes) with anti-CD3, anti-CD28 and IL-2 in the presence (+) or absence (–) of TGF-β. (**b**) Activation of Smad3 in wild-type and $S1P_1$-Tg cells treated with FTY720 and rapamycin and activated for 2 d (above lanes). Numbers below lanes (**a**,**b**) indicate band intensity relative to that of the loading control β-actin. (**c**,**d**) Expression of Foxp3 (**c**) and IFN-γ (**d**) in OT-II *Rag1*$^{-/-}$ and $S1P_1$-Tg OT-II *Rag1*$^{-/-}$ mice fed OVA in their drinking water for 5 d, accompanied by no treatment (Vehicle) or daily treatment with FTY720 (1 mg per kg body weight) or rapamycin (3 mg per kg body weight). Numbers adjacent to outlined areas indicate percent Foxp3$^+$ cells (**c**) or IFN-γ$^+$ cells (**d**). (**e**) Foxp3 expression in thymic CD4$^+$ single-positive cells from wild-type and $S1P_1$-Tg mice left untreated (Vehicle) or treated daily with FTY720 and rapamycin for a total of 5 d. Numbers in outlined areas indicate percent Foxp3$^+$ cells. (**f**) Induction of Foxp3 in wild-type and *S1pr1*$^{flox/flox}$ *Cd4*-Cre thymic T_{reg} precursor cells stimulated overnight with IL-2 (50 U/ml) in the presence of SIS3 or vehicle. Numbers above bracketed lines indicate percent Foxp3$^+$ cells. Data represent three independent experiments.

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factor that mediates the effects of TGF-β in the generation of iT_{reg} cells[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 similar in 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, especially at 24–48 h of stimulation (**Supplementary Fig. 7a**). Accordingly, nuclear translocation of total and phosphorylated Smad3 was much lower in $S1P_1$-Tg cells after 2 d of stimulation (**Fig. 6b**). To understand the importance of sustained Smad3 activation, we transduced a dominant negative Smad3 molecule into wild-type cells 24 h after initiating iT_{reg} differentiation; this was effective in inhibiting Foxp3 induction (**Supplementary Fig. 7b**). Moreover, we observed more phosphorylation of Smad3 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 a Smad3 inhibitor resulted in less iT_{reg} differentiation (**Fig. 6d**). Therefore, $S1P_1$ antagonizes TGF-β-dependent effects on T cell differentiation mainly by attenuating sustained Smad3 signaling.

The $S1P_1$-mTOR axis is targeted by immunosuppressants

We further explored the signaling pathway used by $S1P_1$ to antagonize TGF-β–Smad3 signaling. The Akt-mTOR pathway has been shown to restrain the generation of iT_{reg} cells[28–31]. However, $S1P_1$ activates Akt in nT_{reg} cells but not in naive 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 phosphorylation of the ribosomal protein S6, a well-established target of mTOR. $S1P_1$-Tg cells had more phosphorylation of S6 after 16 h of TCR stimulation and had even more prominent increase at later time points (**Fig. 7a**), which indicated an important role for $S1P_1$ in sustaining mTOR activation.

The $S1P_1$-mediated reciprocal differentiation of iT_{reg} and T_H1 cells identified above led us to examine possible therapeutic implications. Both rapamycin, an inhibitor of mTOR, and the immunosuppressant 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 each resulted in less phosphorylation of S6 (**Supplementary Fig. 9**). Moreover, each treatment restored the activity of phosphorylated Smad3 in $S1P_1$-Tg cells to that 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 treatment resulted in more iT_{reg} cells and fewer 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, resembling the differentiation in untreated

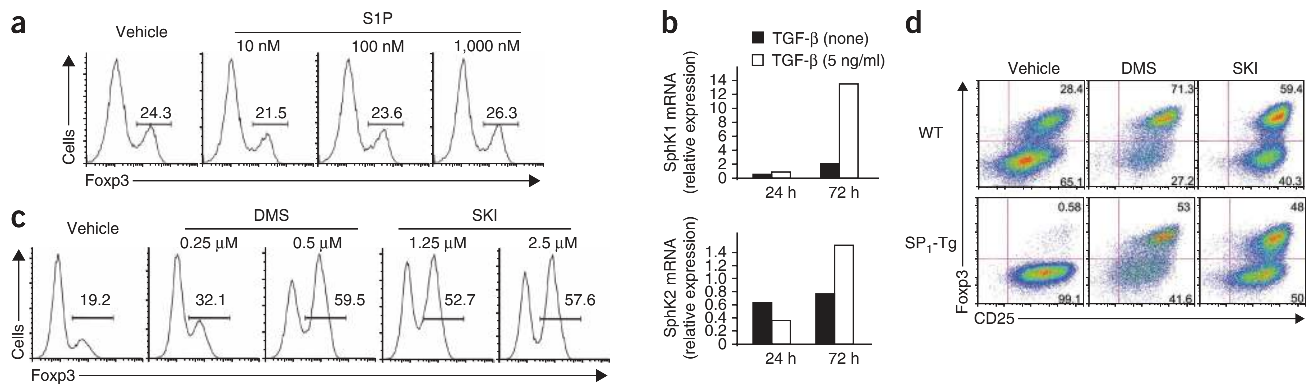

Figure 8 Sphingosine kinase activity regulates T cell differentiation. (**a**) Foxp3 expression in wild-type cells treated with vehicle alone (left) or various concentrations of S1P (above plots) in serum-free medium. Numbers above bracketed lines indicate percent Foxp3$^+$ cells. (**b**) Expression of SphK1 and SphK2 mRNA in cells activated in the presence (5 ng/ml) or absence (none) of TGF-β, presented relative to the expression in naive T cells, set as 1. (**c**) Foxp3 expression in naive T cells pretreated with vehicle, DMS or SKI and activated for 5 d under iT$_{reg}$ conditions. Numbers above bracketed lines indicate percent Foxp3$^+$ cells. (**d**) Expression of Foxp3 and CD25 in wild-type and S1P$_1$-Tg naive T cells pretreated with vehicle, DMS (0.25 μM) or SKI (2.5 μM) and activated for 5 d under iT$_{reg}$ conditions. Numbers in quadrants indicate percent Foxp3$^+$CD25$^+$ cells (top right) or Foxp3$^-$CD25$^+$ cells (bottom right). Data represent three independent experiments.

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wild-type control mice (**Fig. 7c**,**d**). Thus, FTY720 and rapamycin modulate reciprocal T$_H$1 and iT$_{reg}$ differentiation *in vivo*. To ascertain whether this reflected intrinsic effects, we treated T cells with these drugs *in vitro*. Treatment of S1P$_1$-Tg cells with FTY720 or rapamycin resulted in more iT$_{reg}$ differentiation and less T$_H$1 differentiation (**Supplementary Fig. 11**). Thus, FTY720 and rapamycin directly modulate reciprocal differentiation of iT$_{reg}$ and T$_H$1 cells by targeting the S1P$_1$-mTOR axis.

We next sought to determine 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 d and assessed the frequency of Foxp3$^+$ CD4$^+$ single-positive thymocytes. As reported before, S1P$_1$-Tg mice had fewer thymic nT$_{reg}$ cells[14]. After treatment with FTY720 or rapamycin, nT$_{reg}$ cells in these mice were similar to those in wild-type mice (**Fig. 7e**). Accordingly, the diminished *in vitro* differentiation of S1P$_1$-Tg nT$_{reg}$ precursor (CD25$^+$Foxp3$^-$) cells into mature Foxp3$^+$ cells was restored by FTY720 and rapamycin (**Supplementary Fig. 12**). Therefore, FTY720 and rapamycin affect the 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$_{reg}$ precursor cells from wild-type and *S1pr1*$^{flox/flox}$ *Cd4*-Cre mice with the Smad3 inhibitor SIS3. Blocking Smad3 activity ablated the greater nT$_{reg}$ differentiation observed for precursor cells lacking S1P$_1$ (**Fig. 7f**). Although TGF-β is required for the survival of T$_{reg}$ cells in the thymus[33], SIS3 treatment resulted in less Foxp3 induction even in Bcl-2-transgenic mice (data not shown), which indicated that the effect of Smad3 in nT$_{reg}$ differentiation is independent of cell survival. Thus, 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. Supplementing naive T cells with exogenous S1P did not alter their differentiation into iT$_{reg}$ cells (**Fig. 8a**). We thus tested whether endogenously produced S1P is involved. S1P is synthesized by one of the two sphingosine kinases on the substrate sphingosine[34]. In differentiating T cells, the sphingosine kinase SphK1 was considerably upregulated by treatment with TGF-β (**Fig. 8b**), which suggested that intrinsic sphingosine kinase activity was probably involved in the regulation of iT$_{reg}$ differentiation. To test that hypothesis, we treated naive T cells with DMS or SKI, two widely used inhibitors of sphingosine kinase activity. Blocking sphingosine kinase activity resulted in considerable upregulation of Foxp3 induction (**Fig. 8c**). Moreover, treatment of S1P$_1$-Tg cells with DMS or SKI restored their ability to differentiate into iT$_{reg}$ cells (**Fig. 8d**). Similarly, blocking sphingosine kinase activity considerably rectified the defects of S1P$_1$-Tg cells in reciprocal T$_H$1 and T$_{reg}$ differentiation (**Supplementary Fig. 13a**). Thus, T cell differentiation mediated by S1P$_1$ is dependent on intrinsic sphingosine kinases, whose expression is upregulated by TGF-β as a feedback mechanism to limit TGF-β responses (**Supplementary Fig. 13b**).

DISCUSSION

Our results have demonstrated that the differentiation of T$_H$1 cells and T$_{reg}$ cells is reciprocally regulated. We found that S1P$_1$ was a switch factor that drove the development of T$_H$1 cells at the expense of T$_{reg}$ cell generation. S1P$_1$ function was dependent on endogenous sphingosine kinase activity, and it signaled through mTOR and intersected with TGF-β signaling mainly by attenuating sustained Smad3 activity. Although TGF-β and mTOR have pleiotropic functions in regulating many CD4$^+$ T cell lineages[17,30], the interaction between S1P$_1$-mTOR and TGF-β–Smad3 signaling with dynamic kinetics selectively controlled the reciprocal differentiation of T$_H$1 cells and T$_{reg}$ cells. We propose that this previously unknown lineage-commitment process controls the balance between immunity and tolerance and contributes to mechanisms of action for immunosuppressive therapy.

The dichotomy of T$_H$1 and T$_{reg}$ cell-fate determination is reminiscent of the reciprocal differentiation of iT$_{reg}$ cells and T$_H$17 cells[1,4]. Moreover, the effect on T cell differentiation by S1P$_1$, a G protein–coupled receptor for the endogenous bioactive lipid S1P, is analogous to the control of reciprocal iT$_{reg}$ and T$_H$17 differentiation mediated by non-cytokine receptors such as the retinoid acid receptor and aryl hydrocarbon receptor, which recognize certain endogenous metabolites and environmental toxins[5–9]. These results collectively establish a new paradigm of T cell lineage specification controlled by non-cytokine immune modulators. Unlike the inhibition of T$_H$17 differentiation by Foxp3 (refs. 22,35,36), ectopic expression or complete ablation of Foxp3 did not prevent S1P$_1$ from driving T$_H$1

differentiation. Moreover, $S1P_1$ blocked the generation of iT_{reg} cells independently of its ability to drive T_H1 differentiation. Thus, $S1P_1$ regulates T_H1 and iT_{reg} differentiation through discrete mechanisms, in further support of the reciprocal nature of these two lineages.

High $S1P_1$ 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], attributed to defects in both T_{reg} cells and naive T cells. Also, transfer of either regulatory or naive $S1P_1$-Tg cells into *Rag1*$^{-/-}$ mice exacerbates the development of colitis[14]. These observations are reminiscent of TGF-β-dependent immune homeostasis via T_{reg} cell–dependent and T_{reg} cell–independent mechanisms[25]. Notably, premature egress of $CD4^+CD8^+$ thymocytes from the thymus into peripheral lymphoid organs and tissues has been observed in another strain of $S1P_1$-transgenic mice in which an artificial promoter is used to drive transgene expression in both T cells and B cells[38]. We did not observe such alterations in several lines of founder mice expressing the $S1P_1$ transgene via the T cell–selective *CD2* promoter-enhancer[13] (data not shown). The simplest interpretation for this discordance is the use of different promoters for transgene expression. Notably, fewer thymic nT_{reg} cells were apparent in both types of transgenic mice[14,38], which emphasizes the key function of $S1P_1$ in T_{reg} differentiation.

$S1P_1$ attenuates TGF-β–Smad3 function via signaling through mTOR. Although Smad3 is activated within minutes of TGF-β stimulation, 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-hour delay in Foxp3 expression after 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], which suggests cell context–specific regulation of mTOR signaling. Despite that, the $S1P_1$-mTOR axis negatively regulates the differentiation of both thymic nT_{reg} cells and extrathymic iT_{reg} cells by 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 has bveen established by the finding of fewer 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]. Thus, whereas diverse mechanisms promote the generation of nT_{reg} and iT_{reg} cells[3], a common pathway involving $S1P_1$-mTOR and antagonistic interactions with TGF-β–Smad3 signaling negatively controls the differentiation of both T_{reg} subsets.

Signaling by mTOR is necessary for the differentiation of many effector T cells, including T_H1, T_H2 and T_H17 cells, while at the same time it inhibits the generation of iT_{reg} cells[30]. Here we found that the $S1P_1$-mTOR axis selectively controlled T_H1 and T_{reg} differentiation, which suggests that other receptors may mediate mTOR activation for T_H2 and T_H17 differentiation. Although published reports have shown that a human $S1P_1$ transgene is able to promote the generation of T_H2 and T_H17 cells *in vitro*[41,42], we found no evidence for the involvement of $S1P_1$ in these processes after antigen stimulation *in vivo*. Determining whether $S1P_1$ regulates these processes under more defined conditions will require further testing. Additional molecules involved in Akt-mTOR signaling, including Rictor (mTORC2), Cbl-b, Foxo1 and Foxo3, have been shown to modulate T cell responses[30,43,44]. Given the potent effects of $S1P_1$ on the induction and maintenance of T_{reg} cells and the generation of T_H1 cells, $S1P_1$ may engage diverse pathways associated with Akt-mTOR. Furthermore, direct interactions between Smad3 and the Akt, mTOR and Foxo transcription factor pathways have been observed outside the immune system[45,46], which suggests that Smad3 may integrate these pathways to direct T cell differentiation.

High concentrations of S1P are maintained in the blood and lymph by the actions of sphingosine kinases, but low concentrations are induced in lymphoid organs by S1P lyase. Separate sources seem to provide S1P to plasma and lymph[47], and a study has indicated that neural crest–derived pericytes produce S1P to induce thymocyte egress[38]. Combining pharmacological and genetic approaches, here we found that $S1P_1$-mediated regulation of T cell differentiation required endogenous sphingosine kinase activity. Although exogenously added S1P had no apparent effects on T_{reg} cell generation, we cannot completely exclude the possibility of a contribution by the exogenous source of S1P in this process, because S1P distribution might not have been recapitulated in the isolated cell cultures. Nonetheless, our results have demonstrated a key role for endogenously produced S1P in T cell differentiation in an autocrine and/or paracrine way. Moreover, SphK1 expression was upregulated substantially by TGF-β stimulation, which probably serves as a feedback mechanism to restrain prolonged effects of TGF-β on Foxp3 expression. In growth factor–activated cells, S1P has been found to be produced and secreted from 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 identification of 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 targeted the same $S1P_1$-mTOR axis to modulate the balance between proinflammatory T_H1 cells and anti-inflammatory T_{reg} cells *in vivo*. These effects are probably T cell intrinsic, as we were able to recapitulate their functions with *in vitro* culture systems. Therefore, the regulation of T cell lineage 'choices' by the $S1P_1$-mTOR axis can be further explored to develop new therapeutics for autoimmunity and transplant rejection.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureimmunology/.

Accession codes. UCSD-Nature Signaling Gateway (http://www.signaling-gateway.org): A002271, A000813 and A000094.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

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

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COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

Mice. *S1pr1*$^{flox/flox}$ mice, $S1P_1$-Tg mice and *Foxp3*^{gfp} knock-in mice have been described[13,14] and were backcrossed extensively to the C57BL/6 background. C57BL/6, CD45.1, Thy-1.1, *Rag1*$^{-/-}$, *Ifng*$^{-/-}$, CD4-dnTGFβRII, OT-II and scurfy mice (all on the C57BL/6 background) were from the Jackson Laboratory. Wild-type controls were on the same genetic background and included transgene-negative littermates or, where appropriate, Cre^+ mice to account for Cre effects. Mice 6–10 weeks of age were used unless noted otherwise. Bone marrow chimeras were generated by the transfer of T cell–depleted bone marrow (1×10^7 to 2×10^7 cells) into sublethally irradiated (5 Gy) alymphoid *Rag1*$^{-/-}$ mice as described[14]. All mice were kept in specific pathogen–free conditions in the Animal Resource Center at St. Jude Children's Research Hospital. Animal protocols were approved by the Institutional Animal Care and Use Committee of St. Jude Children's Research Hospital.

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

Cell purification and culture. Lymphocytes were isolated from the lymphoid organs and naive T cells were sorted on a MoFlow (Beckman-Coulter) or Reflection (i-Cyt). Sorted naive T cells ($CD4^+CD62L^{hi}CD44^{lo}Foxp3^-$) were used for *in vitro* culture in Bruff's or Click's medium (plus β-mercaptoethanol) supplemented with 10% (vol/vol) FBS and 1% (vol/vol) penicillin-streptomycin as described[14]. For nonpolarizing (T_H0) conditions, naive cells were stimulated with anti-CD3 (2C11; Bio X Cell), anti-CD28 (37.51; Bio X Cell) and human IL-2 (100 U/ml); for iT_{reg} differentiation, cultures were 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 (10 μg/ml; 11B11; Bio X Cell). When OT-II mice were used, anti-CD3 and anit-CD28 were replaced with OVA peptide (amino acids 323–339; 1 μg/ml). For 4-OHT treatment, cells were cultured for 2–3 d in medium containing IL-7 (10 ng/ml; to maintain cell viability) and 0.1–0.5 μM 4-OHT, and live cells were sorted and used for stimulation. For drug-inhibitor treatment, cells were incubated with vehicle, 5 μM U0126 (Calbiochem), 10–100 nM rapamycin (Calbiochem), 5 μM SIS3 (Calbiochem), 1.25–10 μM SKI (sphingosine kinase inhibitor; Calbiochem), 10–100 nM FTY720 (Cayman Chemical) or 0.25–1 μM DMS (N,N,-dimethylsphingosine; Cayman Chemical). S1P (Sigma) was added to naive T cells at the time of stimulation in X-VIVO 15 serum-free medium (BioWhittaker). $CD103^+CD11c^+$ DCs were isolated and purified from MLNs according to published protocols[7].

Retroviral transduction. The Foxp3 retroviral construct was a gift from D. Littman[35]. *S1pr1* cDNA and *Cre* cDNA were cloned into a mouse stem cell virus retroviral vector upstream of an internal ribosome entry site–Thy-1.1 marker expression cassette, as described[14]. Constitutively active and dominant negative Smad3 constructs were generated by PCR for incorporation of the aspartic acid and alanine substitutions at the C terminus of Smad3, respectively, as described[48]. Phoenix-Eco packaging cells were transfected using Lipofectamine (Invitrogen), and recombinant retroviruses were collected 48 and 72 h after transfection. T cells were activated for 24 h and were transduced with retroviruses by spin inoculation (650 *g* for 1 h) as described[14].

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

Oral feeding of antigen. Drinking water for OT-II *Rag1*$^{-/-}$ mice was supplemented with grade VI OVA (20 mg/ml; Sigma-Aldrich) according to published protocols[6,7]. Alternatively, naive T cells were purified as described above and transferred into C57BL/6 mice that were fed OVA in the drinking water 24 h later. T cells in the colon and cecum were visualized with goat polyclonal anti-CD3 antiserum (sc-1127; Santa Cruz) and diaminobenzidine chromagen with hematoxylin as a counterstain. T_{reg} cells were visualized with anti-Foxp3 (FJK-16s; eBioscience) as described[14].

Quantitative RT-PCR. RNA was extracted with an 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 (*Hprt1*, encpding hypoxanthine guanine phosphoribosyl transferase) was subtracted from the cycling threshold value of each target gene to generate the change in cycling threshold (ΔC_T). The expression of each target gene is presented as the 'fold change' relative to of the expression in wild-type control samples ($2^{-\Delta\Delta CT}$).

Immunoblot analysis. Immunoblot analysis was done as described[49] with the following antibodies: antibody to phosphorylated S6 (2F9; Cell Signaling Technology), anti-S6 (5G10; Cell Signaling Technology), anti-p38 (9212; Cell Signaling Technology), antibody to phosphorylated Smad3 (07-1389; Millipore/Upstate), anti-Smad3 (ab28379; Abcam), anti-Sp1 (sc-14027; Santa Cruz) and anit-β-actin (AC-15; Sigma). Nuclear extracts were prepared as described[49].

Statistical analysis. *P* values were calculated with Student's *t*-test. *P* values of less than 0.05 were considered significant.

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