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$S1P_1$ receptor overrides regulatory T cell-mediated immune suppression through Akt-mTOR

Guangwei Liu[1], **Samir Burns**[1], **Gonghua Huang**[1], **Kelli Boyd**[2], **Richard L. Proia**[3], **Richard A. Flavell**[4,5], and **Hongbo Chi**[1,5]

[1]Department of Immunology, St Jude Children's Research Hospital, Memphis, Tennessee 38105.

[2]Animal Resources Center, St Jude Children's Research Hospital, Memphis, Tennessee 38105.

[3]Genetics of Development and Disease Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892.

[4]Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, CT 06520.

[5]Department of Immunobiology, Yale University School of Medicine, New Haven, CT 06520.

Abstract

Regulatory T cells (T_{reg}) are critically involved in maintaining immunological tolerance, but this potent suppression must be quenched to allow the generation of adaptive immune responses. Here we report that type 1 sphingosine-1-phosphate (S1P) receptor ($S1P_1$) delivers an intrinsic negative signal to restrain thymic generation, peripheral maintenance and suppressive activity of T_{reg} cells. Combining loss- and gain-of-function genetic approaches, we found that $S1P_1$ blocked the differentiation of thymic T_{reg} precursors and function of mature Treg cells, and affected T_{reg}-mediated immune tolerance. $S1P_1$ induced the selective activation of the Akt-mTOR pathway to impede T_{reg} development and function. Dynamic regulation of $S1P_1$ contributed to lymphocyte priming and immune homeostasis. Thus, by antagonizing T_{reg}-mediated immune suppression, the lipid-activated $S1P_1$-Akt-mTOR pathway orchestrates adaptive immune responses.

Keywords

T cells; tolerance; signal transduction

INTRODUCTION

Regulatory T cells (T_{reg} cells) play a central role in the maintenance of immune tolerance1-5. T_{reg} cells are produced mainly in the thymus and require expression of the

Correspondence should be addressed to H.C. (hongbo.chi@stjude.org.) **Hongbo Chi**, Department of Immunology, St Jude Children's Research Hospital, Memphis, Tennessee 38105, USA. Phone: 901-595-6282; Fax: 901-595-5766 .

AUTHOR CONTRIBUTIONS G.L. designed and performed the experiments with cells and mice, analyzed data, and contributed to writing the manuscript; S.B. performed retroviral transduction of BM cells and reconstitution and managed the mouse colony; G.H. and S.B. contributed to real-time PCR analysis; K.B. analyzed and scored histology data; R.L.P. and R.A.F. provided important animal models; H.C designed experiments, analyzed data, wrote the manuscript, and provided overall direction.

transcription factor Foxp3. Thymic development of T_{reg} cells is dependent upon signals transduced by T cell receptor (TCR) engagement with self-peptide-MHC complexes. In addition, costimulatory factors (such as CD28) and cytokines including interleukin 2 (IL-2) and transforming growth factor-β (TGF-β) contribute to the induction of Foxp3 and thymic development of T_{reg} cells6,7. Following their maturation and release into the periphery, T_{reg} cells employ diverse mechanisms to mediate immune suppression. These mechanisms include production of inhibitory cytokines, modulation of dendritic cell maturation and function and killing or metabolic disruption of target cells8. While T_{reg} activity is essential for immune tolerance and prevention of autoimmunity, the potent T_{reg}-mediated suppression may abrogate adaptive immune responses and render the host susceptible to infection and cancer. How T_{reg} development and activity are controlled to establish protective immunity without pathological anti-self reactivity is an open question. Stimulation of antigen-presenting cells through Toll-like receptors (TLRs) has been implicated in the reversal of T_{reg} suppressive activity9,10. In addition, direct activation of TLR2 and TLR8 expressed by T_{reg} cells can also down-modulate T_{reg} activity11-13. Despite these studies, precisely how T_{reg} cells are regulated to effect immune function and tolerance remains poorly understood.

Sphingosine 1-phosphate (S1P) is a natural lysophospholipid with micromolar concentration in the plasma14-16. S1P signals through five known G protein-coupled receptors ($S1P_1$-$S1P_5$). FTY720, a new class of immunosuppressants in clinical trials for transplantation tolerance and multiple sclerosis, sequestrates T cells in lymphoid organs by acting on four of the five S1P receptors17, 18. Genetic approaches to alter the function of $S1P_1$ (also known as Edg1, http://www.signaling-gateway.org/molecule/query?afcsid=A000813) indicate that $S1P_1$ is the main S1P receptor that regulates T cell trafficking. T cells from $S1P_1$-deficient mice fail to egress from thymus19, 20, while $S1P_1$-transgenic T cells preferentially distribute to the blood rather than lymphoid organs21, 22. Thus, $S1P_1$ is critical for the egress of T cells from lymphoid organs. $S1P_1$ primarily couples with pertussis toxin-sensitive G_i proteins. Major pathways downstream of $S1P_1$ include activation of the kinase cascades involving Ras-Erk and phosphoinositide 3-kinase (PI(3)K)-Akt [http://www.signaling-gateway.org/molecule/query?afcsid=A000249; http://www.signaling-gateway.org/molecule/query?afcsid=A000250; http://www.signaling-gateway.org/molecule/query?afcsid=A000251], calcium mobilization, and actin cytoskeletal rearrangement23.

$S1P_1$ is expressed on T_{reg} cells but its functional significance has not been directly addressed24. Given the limitations of the pharmacological inhibitors, we chose to use two complementary genetic approaches, by eliminating and enhancing $S1P_1$ function selectively in T cells. Our results showed that loss of $S1P_1$ function resulted in enhanced thymic differentiation and suppressive activity of T_{reg} cells. Conversely, increased $S1P_1$ signaling led to reduced development and function of T_{reg} cells *in vitro* and *in vivo*, and more importantly, development of spontaneous autoimmunity due to defects in T_{reg} cells. Thus, $S1P_1$ negatively regulates both thymic generation and suppressive activity of T_{reg} cells. We further demonstrated that the function of $S1P_1$ in T_{reg} cells is mediated by the downstream Akt-mTOR pathway. Finally, $S1P_1$ expression was differentially regulated in T_{reg} cells as compared with conventional T cells (T_{conv} cells), suggesting that $S1P_1$ coordinates the responses of T_{reg} and T_{conv} cells to effect a productive and self-controlled immune response.

RESULTS

$S1P_1$ signaling reduces the thymic T_{reg} population

To investigate the intrinsic function of $S1P_1$ in T cells, we crossed mice carrying a conditional $S1P_1$ allele (*S1pr1*$^{fl/fl}$)20 with CD4-Cre transgenic mice to delete the floxed *S1pr1* allele specifically in T cells ($S1P_1$-KO mice). Compared with wild-type controls, $S1P_1$-KO mice showed accumulation of mature single-positive thymocytes and substantial reduction of T cells in the periphery (Supplementary Fig. 1a online). Real-time PCR analysis indicated efficient deletion of the *S1pr1* gene in thymocytes (Supplementary Fig. 1b). These findings are consistent with a role for $S1P_1$ in thymocyte egress19, 20. To assess the requirement of $S1P_1$ in the development of naturally occurring Foxp3$^+$ T_{reg} cells, we examined the expression of Foxp3 in mature CD4 single-positive (CD4SP) thymocytes. As compared with wild-type mice, $S1P_1$-KO mice contained elevated numbers of the thymic T_{reg} population expressing Foxp3, CTLA4 and GITR (Fig. 1a and Supplementary Fig. 1c). Thus, $S1P_1$ deficiency causes expansion of the thymic T_{reg} cell population.

Is $S1P_1$ sufficient to affect the thymic T_{reg} population? We analyzed two independent lines of transgenic mice expressing the *S1pr1* gene under the control of the human CD2 promoter-enhancer that results in increased expression and function of $S1P_1$ in T cells ($S1P_1$-Tg mice)21. Transgenic mice had a severe reduction of thymic Foxp3$^+$ CD4SP cells as compared with wild-type controls (Fig. 1b). As a separate gain-of-function approach, we transduced bone marrow (BM) stem cells with a retrovirus expressing $S1P_1$, and implanted them into alymphoid *Rag1*$^{-/-}$ mice. Following reconstitution, Foxp3$^+$ thymocytes expressing $S1P_1$ were one-third the number of those which expressed the empty vector (Fig. 1c). Therefore, increased $S1P_1$ function reduces the thymic T_{reg} population.

The defect in $S1P_1$-expressing cells in the BM chimeras suggests a cell-autonomous effect of $S1P_1$ on T_{reg} cells, because the presence of non-transduced cells in the same host failed to rescue the defect by providing necessary *trans*-acting factors. We further tested this notion by constructing mixed BM chimeras derived from a 1:1 mixture of wild-type BM (CD45.1$^+$) cells and $S1P_1$-KO or $S1P_1$-Tg cells (CD45.2$^+$). Compared with the co-transferred CD45.1$^+$ cells, $S1P_1$-KO and $S1P_1$-Tg BM developed into elevated and reduced thymic Foxp3$^+$ T_{reg} cells, respectively (Fig. 1d). We therefore conclude that $S1P_1$ has a cell-autonomous effect in inhibiting the thymic T_{reg} population.

$S1P_1$ inhibits thymic T_{reg} development

Altered thymic T_{reg} cells in $S1P_1$-KO and $S1P_1$-Tg mice might be due to the effects of $S1P_1$ on T_{reg} egress, differentiation or both. To distinguish these possibilities, we first analyzed the trafficking of $S1P_1$-KO Foxp3$^+$ CD4SP thymocytes relative to Foxp3$^-$ T_{conv} cells. When adoptively transferred, wild-type T_{conv} cells distributed to different lymphoid organs including spleen, lymph nodes and blood, while $S1P_1$-KO T_{conv} cells were able to enter lymphoid organs but unable to exit into blood, as reported19. $S1P_1$-KO T_{reg} cells exhibited a similar defect as $S1P_1$-KO T_{conv} cells (Supplementary Fig. 2 online). Thus, $S1P_1$ is required for egress of both T_{reg} and T_{conv} cells, but this function is unlikely to account for the selective expansion of T_{reg} cells in $S1P_1$-KO thymus. Further supporting this, CD4$^+$CD8$^+$

double-positive thymocytes, which do not egress into the periphery, contained altered Foxp3$^+$ populations in S1P$_1$-KO and S1P$_1$-Tg mice (Supplementary Fig. 1d).

We used two strategies to directly assess an intrinsic role of S1P$_1$ in thymic T$_{reg}$ differentiation. First, we analyzed Foxp3$^+$ thymocytes in mice 3-5 days after birth, a period critical for the initial generation of T$_{reg}$ cells in wild-type mice, before substantial numbers of T$_{reg}$ cells appear in the periphery25. Consequently, the effects of thymocyte emigration are not expected to affect thymic T$_{reg}$ population at this stage. We found the total CD4SP population was not significantly altered in the thymus from neonatal S1P$_1$-KO or S1P$_1$-Tg mice (Supplementary Fig. 3 online), unlike those in adult mice. However, in these neonatal animals, thymic T$_{reg}$ cells were still altered with an increase in S1P$_1$-KO thymus and a reciprocal decrease in S1P$_1$-Tg thymus (Supplementary Fig. 3).

Second, we used fetal thymus organ culture (FTOC), which allowed *de novo* differentiation of thymocytes including T$_{reg}$ cells, to obviate the effects of differential thymocyte egress or peripheral T$_{reg}$ cells homing to the thymus. We cultured thymus isolated from E16.5 embryos *in vitro*, and found no differences in thymocyte numbers or the distribution of CD4 and CD8 expression among different groups. However, the Foxp3$^+$ CD4SP population was significantly increased in S1P$_1$-KO cells and decreased in S1P$_1$-Tg cells (Fig. 2a). These findings collectively demonstrate that S1P$_1$ plays an intrinsic negative role in thymic T$_{reg}$ development.

S1P$_1$ blocks differentiation of CD4$^+$CD25$^+$ Foxp3$^-$ cells

We hypothesized that S1P$_1$ affects thymic T$_{reg}$ development by acting on a precursor population(s). Previous studies suggest that Foxp3$^+$ T$_{reg}$ cells develop from the putative precursors (CD4$^+$CD25$^+$Foxp3$^-$) that are poised to express Foxp3 without TCR engagement, requiring only IL-2 or IL-15 stimulation26, 27. Remarkably, the CD4$^+$CD25$^+$Foxp3$^-$ population was greatly reduced in S1P$_1$-KO thymus but significantly increased in S1P$_1$-Tg thymus (Fig. 2b), suggesting that S1P$_1$ may act on these cells to restrain their further differentiation into mature Foxp3-expressing T$_{reg}$ cells.

To directly test this, we crossed S1P$_1$-KO and S1P$_1$-Tg mice with Foxp3^{gfp} knockin mice that express green fluorescent protein (GFP) regulated by the *Foxp3* control elements28. We sorted thymic CD4$^+$CD25$^+$Foxp3$^-$ population and cultured them with IL-2 or IL-15. Under these conditions, no significant differences were observed in the apoptosis of these cells (data not shown). Foxp3 induction was substantially elevated in S1P$_1$-KO cells, while a reciprocal change was observed in S1P$_1$-Tg cells, irrespective of the stimuli and doses used (Fig. 2c). Therefore, S1P$_1$ blocks the differentiation of CD4$^+$CD25$^+$Foxp3$^-$ cells into mature T$_{reg}$ cells.

Altered homeostasis and function of S1P$_1$-KO T$_{reg}$ cells

Our results thus far have identified a negative role for S1P$_1$ in thymic differentiation of T$_{reg}$ cells. We then examined whether S1P$_1$ affects homeostasis and suppressive activity of T$_{reg}$ cells in the periphery. In S1P$_1$-KO peripheral lymphoid organs, there was a selective increase of the T$_{reg}$ population marked by the expression of Foxp3 (Fig. 3a), CD25, GITR

and CTLA4 (Fig. 3b), in line with the thymic alterations. Nonetheless, very few $S1P_1$-KO peripheral cells could be isolated due to blocked thymocyte egress. For functional studies, we sorted $Foxp3^+$ CD4SP thymocytes, which possess suppressive activity similar to peripheral T_{reg} cells2. Although $S1P_1$ deficiency resulted in increased numbers of $Foxp3^+$ CD4SP cells, Foxp3 expression on a per cell basis was comparable between wild-type and $S1P_1$-KO cells (Supplementary Fig. 4 online). In an *in vitro* T cell suppression assay, proliferation of the target $Foxp3^-$ T_{conv} cells from wild-type mice was tested in the presence of wild-type or $S1P_1$-deficient $Foxp3^+$ thymic T_{reg} cells. $S1P_1$-KO T_{reg} cells showed a significantly increased capacity than wild-type T_{reg} cells to suppress T_{conv} cell proliferation and IL-2 production (Fig. 3c). We examined the proliferation of T_{reg} cells alone but no significant proliferation was observed in either wild-type or $S1P_1$-KO $Foxp3^+$ cells (data not shown), suggesting that the difference is not due to the differential proliferation of T_{reg} cells.

Given the altered differentiation of $Foxp3^+$ cells in the $S1P_1$-KO thymus, it remains possible that the increased suppressive activity of $S1P_1$-KO T_{reg} cells is secondary to defective thymic development. Hence, we transduced T_{reg} cells from the periphery of *S1pr1*$^{fl/fl}$ mice with Cre-expressing retrovirus to acutely delete the *S1pr1* gene *in vitro*. *S1pr1*-deleted cells showed a greater suppressive activity than Cre-expressing wild-type controls (Fig. 3d). In contrast, there was no difference in the suppressive activity between the non-transduced (GFP^-) cells of both genotypes (data not shown). Therefore, $S1P_1$ deficiency directly potentiates T_{reg} suppressive activity.

Impaired suppressive activity of $S1P_1$-Tg T_{reg} cells

We next asked whether ectopic $S1P_1$ expression is sufficient to alter T_{reg} suppressive activity. In $S1P_1$-Tg mice, peripheral T_{reg} populations were largely normal (Supplementary Figs. 5a,b online), suggesting that compensatory mechanisms in the periphery might overcome defects in thymic development. However, in the chimeras generated by the mixture of wild-type and $S1P_1$-Tg BM cells, T_{reg} cells derived from $S1P_1$-Tg BM were selectively reduced as compared with co-transferred wild-type counterparts (Supplementary Fig. 5c). Thus, $S1P_1$-Tg T_{reg} cells have a competitive disadvantage in their peripheral maintenance. In T cell suppression assays, $S1P_1$-Tg T_{reg} cells were much less efficient at suppressing the responses of T_{conv} cells. Specifically, proliferation and cell cycle progression of target cells were significantly higher in the presence of $S1P_1$-Tg T_{reg} cells as compared with wild-type T_{reg} cells (Fig. 4a and Supplementary Fig. 6a online). Further, $S1P_1$-Tg T_{reg} cells had a greatly reduced capacity to inhibit the IL-2 production of target cells (Supplementary Fig. 6b). To demonstrate that such an altered T_{reg} activity is a direct result of $S1P_1$ function rather than secondary to defective thymic development, we transduced wild-type T_{reg} cells with $S1P_1$-expressing retrovirus. Expression of $S1P_1$ substantially impaired T_{reg} suppressive activity (Fig. 4b), highlighting an intrinsic inhibitory effect of $S1P_1$ on T_{reg} function.

We next employed two approaches to investigate whether $S1P_1$ controls T_{reg} suppressive activity *in vivo*. First, we used a model of colitis induced by the transfer of T_{conv} cells into lymphopenic hosts; this disease can be prevented by the cotransfer of T_{reg} cells (Supplementary Fig. 7a online)29. Transfer of wild-type T_{conv} cells resulted in severe

weight loss (data not shown), and co-transfer of T_{reg} cells from wild-type, but not $S1P_1$-Tg mice, prevented bodyweight loss (Fig. 4c). Mice were euthanized 10 weeks after transfer and lesions in the colon and cecum were assessed. Recipients of T_{conv} and wild-type T_{reg} co-transferred cells did not develop prominent colitis whereas recipients of T_{conv} alone or those of T_{conv} and $S1P_1$-Tg T_{reg} cell co-transferred cells developed severe colitis (Fig. 4d). The distribution of T_{reg} cells in the colon and lymphoid organs was comparable between wild-type and $S1P_1$-Tg T_{reg} cell transfer groups (Supplementary Figs. 7b,c). Thus, $S1P_1$-Tg T_{reg} cells did not have obvious defects in the expansion and/or homeostasis in this model, suggesting that the inability of these cells to control colitis was most likely due to their impaired suppressive activity.

Second, we examined the importance of $S1P_1$ in controlling systemic autoimmune diseases caused by T_{reg} deficiency. We constructed mixed BM chimeras by transferring BM cells from Foxp3-deficient *Scurfy* (*sf*) mice and $S1P_1$-Tg or wild-type mice into *Rag1*$^{-/-}$ recipients. In the resulting chimeras, only the $S1P_1$-Tg or wild-type BM cells can give rise to $Foxp3^+$ T_{reg} cells (Supplementary Fig. 8a online). In the chimeras that received *sf* BM alone, T cells were overtly activated and there were prominent inflammation and lymphocytic infiltration in the liver, lung and colon. In contrast, chimeras that received *sf* and wild-type BM cells exhibited minimal T cell activation and tissue inflammation. Strikingly, chimeras that received *sf* and $S1P_1$-Tg BM cells showed profound T cell activation and inflammatory diseases that were indistinguishable from *sf* alone chimeras (Supplementary Figs. 8b,c). Thus, $S1P_1$-Tg T_{reg} cells fail to mediate immune tolerance *in vivo*.

Defects in $S1P_1$-Tg T_{reg} cells cause autoimmunity

The impaired suppressive activity of T_{reg} cells from $S1P_1$-Tg mice prompted us to examine whether homeostasis of the immune system was altered in these mice. $S1P_1$-Tg mice had increased numbers of activated $CD62L^{lo}CD44^{hi}$ effector or memory T cells, which became more prominent when the mice aged (Fig. 5a). Further, T_{conv} cells from $S1P_1$-Tg mice, even at young age, were hyper-proliferative to TCR stimulation (Fig. 5b), suggesting a lower threshold for activation. To examine whether this results in altered self-tolerance, we measured amounts of autoantibodies. Increased titers of anti-nuclear and anti-dsDNA antibodies were detected in the sera of aged $S1P_1$-Tg mice (Fig. 5c), indicating autoimmune reactions. Because a predominant T_H1 cytokine response especially interferon-γ (IFN-γ) is associated with the pathogenesis of lupus, a prototypical systemic autoimmune disease30, we determined whether activated T cells in $S1P_1$-Tg mice were differentiated into a T_H1 phenotype. $S1P_1$-Tg cells produced more IFN-γ but less IL-4 as compared to controls (Fig. 5d). Consistently, serum titers of T_H1-dependent IgG2a antibodies, but not T_H2-dependent IgG1 antibodies, were significantly higher in $S1P_1$-Tg mice (Fig. 5e). Together, increased $S1P_1$ signaling in T cells leads to their spontaneous activation and differentiation into a T_H1 phenotype and breakdown of immune tolerance.

We reasoned that autoimmunity in $S1P_1$-Tg mice could be due to an intrinsic defect in T_{conv} cells, or the impaired suppressive activity of T_{reg} cells. To distinguish these possibilities, we generated BM chimeras by transferring wild-type and $S1P_1$-Tg BM cells alone or in combination into *Rag1*$^{-/-}$ mice. As expected, T cells from mice given $S1P_1$-Tg BM alone

were spontaneously activated and hyper-responsive to TCR stimulation (data not shown). In contrast, in the mixed chimeras containing both $S1P_1$-Tg and wild-type cells, $S1P_1$-Tg T_{conv} cells were not spontaneously activated or hyper-responsive to TCR stimulation, and produced normal amounts of IFN-γ and IL-4 (Figs. 5f-h). Therefore, activation of T_{conv} cells in $S1P_1$-Tg mice was non-cell-autonomous and could be rescued by the presence of wild-type cells. Next we determined whether providing $S1P_1$-Tg mice with wild-type T_{reg} cells alone could prevent the T cell activation phenotypes. We injected 3-day old $S1P_1$-Tg mice with wild-type T_{reg} cells, a condition that results in the population of donor T_{reg} cells31. Following neonatal transfer of T_{reg} cells into $S1P_1$-Tg mice, T_{conv} cells in the recipients showed normal homeostasis and proliferation (Supplementary Fig. 9 online). We conclude that the impaired T_{reg} compartment in $S1P_1$-Tg mice accounts for the disrupted immune homeostasis.

$S1P_1$ signals through Akt-mTOR to affect T_{reg} cells

To investigate mechanisms mediating $S1P_1$ function, we stimulated thymic T_{reg} precursors ($CD4^+CD25^+Foxp3^-$) with IL-2, which induced their differentiation into T_{reg} cells (Fig. 2c)26. Activation of IL-2 downstream pathways, including Akt, Erk and STAT5, was examined by flow cytometry for the phosphorylated individual proteins. As expected, IL-2 activated all of the three pathways in wild-type T_{reg} precursors. In contrast, $S1P_1$-Tg cells activated Erk and STAT5 pathways similarly as wild-type cells but showed substantially elevated activation of Akt and phosphorylation of S6 ribosomal protein, a well-established target of the Akt-mTOR pathway (Fig. 6a).

We noted that Akt has recently been shown to inhibit Foxp3 induction *in vitro* and upon thymic injection32, 33. Does increased Akt activity mediate $S1P_1$ functions in T_{reg} differentiation? To this end, we stimulated wild-type and $S1P_1$-Tg thymic T_{reg} precursors with IL-2 in the presence of LY294002 (an inhibitor of PI(3)K, upstream of Akt) or rapamycin (an inhibitor of mTOR, downstream of Akt). LY294002 and rapamycin potentiated Foxp3 induction of $S1P_1$-Tg T_{reg} precursors similar to wild-type cells (Fig. 6b). Treatment with additional PI(3)K or mTOR inhibitors including PI-103 and NVP-BEZ235 (ref.34) had similar effects as rapamycin in restoring the T_{reg} differentiation of $S1P_1$-Tg cells (Supplementary Fig. 10 online), indicating the specific involvement of the Akt-mTOR pathway in $S1P_1$ signaling. In contrast, treatment with U0126 (an inhibitor of Erk) did not affect T_{reg} differentiation (Fig. 6b). Therefore, the increased Akt activity in $S1P_1$-Tg T_{reg} precursors results in their defective differentiation.

Next, we determined the signaling pathways activated by increased $S1P_1$ expression in peripheral T_{reg} cells. Upon stimulation with IL-2, peripheral T_{reg} cells from $S1P_1$-Tg mice showed normal activation of Erk and STAT5 but increased phosphorylation of Akt and S6 ribosomal protein (Fig. 6c). A similar pattern was observed when $S1P_1$-Tg T_{reg} cells were stimulated with anti-CD3 plus anti-CD28 (Supplementary Fig. 11 online). We then treated peripheral $S1P_1$-Tg T_{reg} cells with pharmacological inhibitors and found that LY294002, rapamycin, PI-103 or NVP-BEZ235, but not U0126, enhanced the suppressive activity of these cells comparable to that of similarly treated wild-type T_{reg} cells (Supplementary Fig. 12 online). Moreover, blocking Akt activation by expression of dominant-negative Akt (dn-

Akt) in $S1P_1$-Tg cells completely restored their suppressive activity (Fig. 6d). We conclude that increased $S1P_1$ function leads to excessive Akt activity which accounts for impaired T_{reg} differentiation and function.

$S1P_1$ is necessary for Akt activation in T_{reg} cells

Whereas the results above indicated that $S1P_1$ is sufficient to activate the Akt pathway to antagonize T_{reg} development and function, it remains unclear whether $S1P_1$ is necessary for Akt activation in T_{reg} cells. To address this issue, we stimulated T_{reg} precursors from wild-type or $S1P_1$-KO mice with IL-2. As compared with wild-type cells, $S1P_1$-KO cells exhibited substantially reduced Akt phosphorylation, but no change on STAT5 or Erk, following IL-2 stimulation (Fig. 7a). Similarly, $S1P_1$ deficiency decreased Akt activation in mature thymic T_{reg} cells (Fig. 7b). Moreover, after acute deletion of $S1P_1$ from peripheral T_{reg} cells *in vitro*, less Akt activation resulted following IL-2 or anti-CD3 plus anti-CD28 stimulation (Supplementary Figs. 13a,b online). Therefore, $S1P_1$ is required to mediate IL-2 and TCR-induced Akt activation in T_{reg} cells.

In addition to $S1P_1$, T_{reg} cells also express all of the other four receptors for S1P24. Exposure of T cells to S1P results in the activation of Akt35, although whether this is mediated by $S1P_1$ is not known. We treated wild-type and $S1P_1$-deficient T_{reg} cells with S1P and examined the downstream signaling pathways. In both thymic $Foxp3^+$ CD4SP cells and peripheral T_{reg} cells after acute deletion of $S1P_1$, activation of Akt, but not Erk, was substantially reduced as compared with wild-type counterparts (Supplementary Fig. 13c). Thus, among the S1P receptors, $S1P_1$ plays an important role in mediating S1P-induced activation of Akt in T_{reg} cells.

We then determined whether the reduced Akt activity accounts for the enhanced suppressive activity of $S1P_1$-KO T_{reg} cells. We transduced T_{reg} cells from *S1pr1*$^{fl/fl}$ mice with Cre-GFP retrovirus to delete *S1pr1*, together with retrovirus expressing constitutively active Akt (Thy1.1 marked). Constitutive activation of Akt modestly reduced the suppressive activity of wild-type T_{reg} cells, but severely decreased that of $S1P_1$-deficient T_{reg} cells (Fig. 7c). Therefore, the reduced Akt activity is responsible for the enhanced suppressive activity of $S1P_1$-KO T_{reg} cells.

Function and signaling of $S1P_1$ in T_{conv} cells

Our data above have delineated that $S1P_1$, by activating Akt, negatively regulates development and function of T_{reg} cells. What are the function and signaling mechanisms of $S1P_1$ in T_{conv} cells? Using mixed BM chimeras and neonatal transfer of T_{reg} cells (Figs. 5f-h and Supplementary Fig. 9), we did not observe an intrinsic defect of T_{conv} cells from $S1P_1$-Tg mice. To further extend this observation, we used retroviral systems to ectopically express $S1P_1$ or delete *S1pr1* in $Foxp3^-$ T_{conv} cells, but did not observe altered proliferation of these cells (Supplementary Figs. 14a,b online). Moreover, anti-CD3 induced similar degrees of proliferation between $Foxp3^-$ CD4SP thymocytes from wild-type and $S1P_1$-KO mice (Supplementary Fig. 14c). Thus, $S1P_1$ does not affect the proliferative response of T_{conv} cells.

To address the signaling mechanisms of $S1P_1$ in T_{conv} cells, we activated wild-type and $S1P_1$-Tg T_{conv} cells with IL-2 or anti-CD3 plus anti-CD28. Activation of Akt, Erk and STAT5 were comparable between these cells (Supplementary Fig. 15 online). Similarly, in $S1P_1$-deficient T_{conv} cells, IL-2 and TCR induced normal activation of Akt (Supplementary Figs. 16a,b online). Thus, unlike in T_{reg} cells, $S1P_1$ is dispensable for TCR or IL-2 induced Akt activation in T_{conv} cells. To ensure that T_{conv} cells have no general defects in $S1P_1$ signaling, we stimulated $S1P_1$-sufficient and deficient T_{conv} cells with S1P. $S1P_1$ deficiency resulted in reduced S1P-induced Akt activation (Supplementary Fig. 16c). Therefore, whereas T_{conv} cells use $S1P_1$ to mediate Akt activation in response to S1P, the activation of Akt in response to TCR or IL-2 occurs independently of $S1P_1$ in these cells.

The differential function of $S1P_1$ in T_{reg} and T_{conv} cells prompted us to examine whether $S1P_1$ expression differs between these T cell subsets. *S1pr1* mRNA can be detected in various immune cells, including T_{reg} and T_{conv} cells (Supplementary Fig. 17 online). Following stimulation with TCR and IL-2, *S1pr1* mRNA was decreased abruptly in T_{conv} cells, as reported21, 22, whereas it was downregulated gradually in T_{reg} cells (Fig. 8a). Expression of KLF2, a transcription factor essential for $S1P_1$ expression in thymocytes36, exhibited a similar pattern as that of $S1P_1$ (Supplementary Fig. 18 online), suggesting that KLF2 contributes to the differential regulation of $S1P_1$ between peripheral T_{reg} and T_{conv} cells. To examine whether this differential regulation of $S1P_1$ occurs *in vivo*, ovalbumin-specific OT-II TCR-transgenic T_{reg} and T_{conv} cells were transferred to wild-type hosts, followed by antigen immunization. Two days after *in vivo* activation, downregulation of *S1pr1* in T_{conv} cells was much more pronounced than that in T_{reg} cells (Fig. 8b). These findings indicate that $S1P_1$ function and expression are differentially regulated between T_{reg} and T_{conv} cells. We propose that dynamic regulation of $S1P_1$ expression contributes to lymphocyte priming and the maintenance of immune homeostasis (Supplementary Fig. 19 online).

DISSCUSION

Recent work on T_{reg} cell biology has mainly focused on mechanisms of T_{reg}-mediated immune suppression4, 8. How the development and function of T_{reg} cells are regulated remains poorly understood. Here we report that $S1P_1$ is an intrinsic negative regulator of thymic differentiation, peripheral maintenance and suppressive activity of T_{reg} cells, and such functions are mediated by the downstream Akt-mTOR pathway. To our knowledge, $S1P_1$ is the first receptor that negatively regulates these diverse physiological processes of T_{reg} cells. Moreover, among the regulatory mechanisms in T_{reg} cells, the $S1P_1$ pathway is unique in that it couples trafficking and intrinsic development and function of T_{reg} cells, and coordinates the immune responses mediated by T_{reg} and T_{conv} cells.

Using animal models with deficient and enhanced $S1P_1$ functions, we identified an inhibitory role for $S1P_1$ on the thymic T_{reg} population. Although $S1P_1$ facilitates thymic egress of T_{reg} cells, an intrinsic function for $S1P_1$ to block thymic T_{reg} differentiation was revealed by our analyses of neonatal thymi, FTOC and more importantly, of the $CD4^+CD25^+Foxp3^-$ population. Such a population has been postulated to act as precursors for thymic T_{reg} cells, although genetic evidence is lacking26. The reciprocity of the

alterations in $CD4^+CD25^+Foxp3^-$ and $CD4^+CD25^+Foxp3^+$ populations observed in both $S1P_1$-KO and $S1P_1$-Tg mice provides key genetic evidence supporting the definition of $CD4^+CD25^+Foxp3^-$ cells as *bona fide* T_{reg} precursors 26. Collectively, these findings demonstrate that $S1P_1$ delivers a crucial negative signal for thymic development of T_{reg} cells. Previous studies have shown that $S1P_1$ expression is upregulated during thymocyte maturation19. Such upregulation in T_{reg} cells likely serves as a molecular "switch" to restrain T_{reg} cell differentiation (thus maintaining a proper balance between the T_{reg} and T_{conv} populations) and to facilitate their release to the periphery, thereby coordinating the development and egress of T_{reg} cells in the thymus.

Once T_{reg} cells are released from thymus into secondary lymphoid organs, they modulate immunity to both self and foreign antigens. However, excessive T_{reg}-mediated suppression may render the host susceptible to infection and cancer. Signaling through TLRs expressed in T_{reg} or dendritic cells has been implicated in the negative control of T_{reg} suppressive activity9-12, although the mechanisms of action remain unclear. Using multiple *in vitro* and *in vivo* systems, we demonstrated that $S1P_1$ is a critical negative regulator of T_{reg} function. $S1P_1$ is highly expressed in both naïve T_{reg} and T_{conv} cells. At an early stage of immune activation, $S1P_1$ expression is largely maintained in T_{reg} cells, resulting in a low suppressive activity and high mobility of these cells. This serves to prevent premature T_{reg}-mediated suppression in order for an immune response to initiate. In contrast, $S1P_1$ is rapidly and profoundly downregulated in T_{conv} cells to mediate their sequestration in the draining lymph nodes to engage an efficient interaction with antigen-presenting cells19, 21. The overall function of $S1P_1$ in T_{reg} and T_{conv} cells at this stage is to promote a productive immune response. At a late stage of immune activation, $S1P_1$ is downregulated in T_{reg} cells to release T_{reg}-mediated suppression, thereby preventing an exuberant immune response caused by T_{conv} cells. In contrast, $S1P_1$ is slowly recovered in T_{conv} cells to allow their egress into peripheral tissues19, 38. It remains unclear how T_{reg}-mediated suppression is attenuated during acute infection to establish protective immunity9, 11, 39, 40, and our findings suggest that coordination of T_{reg} and T_{conv} responses by $S1P_1$ is crucial for a productive and self-controlled immune response.

To identify the molecular mechanisms mediating $S1P_1$ functions in T_{reg} cells, we examined signaling pathways activated by $S1P_1$. We found a specific role for $S1P_1$ in the activation of Akt, but not Erk or STAT5, in thymic T_{reg} precursors and mature T_{reg} cells after TCR of IL-2 stimulation. Notably, the Akt-mTOR pathway have recently been implicated in blocking T_{reg} differentiation32, 33, but the cellular factors responsible for activating Akt have not been established. Our studies identified that $S1P_1$ is a key receptor that activates Akt-mTOR. Importantly, restoration of proper Akt-mTOR activity corrected the defects caused by $S1P_1$ loss and gain of function. Therefore, activation of Akt-mTOR by $S1P_1$ mediates the negative effects of $S1P_1$ on T_{reg} development and function.

We further identified a fundamental difference in $S1P_1$ signaling between T_{reg} and T_{conv} cells. $S1P_1$ does not affect T_{conv} cell proliferative response, and is dispensable for IL-2 or TCR-induced Akt activation in T_{conv} cells, unlike T_{reg} cells. In contrast, $S1P_1$ is required for Akt activation in response to S1P stimulation in both T_{reg} and T_{conv} cells. It appears that $S1P_1$ is selectively coupled to TCR and cytokine receptors for the activation of Akt

signaling in T_{reg} but not T_{conv} cells. In support of this model, transactivation of $S1P_1$ by growth factor receptors has been observed with distinct mechanisms in a cell context-specific manner41. Notably, T_{conv} and T_{reg} cells have different ability to activate Akt in response to TCR and IL-2 stimulation42, 43, and our studies on $S1P_1$ further highlight the distinct mechanisms in Akt signaling between these two T-cell subsets.

In addition to $S1P_1$, T_{reg} cells also express all of the other four receptors for S1P24, the expression of which was not substantially altered in $S1P_1$-KO and $S1P_1$-Tg T_{reg} cells (data not shown). Whether and how $S1P_1$ interacts with its natural ligand S1P in T_{reg} cells will be an important area of investigation. FTY720, the new generation immunosuppressive drug that targets $S1P_1$, has been shown to enhance T_{reg} activity24, 44, 45. These effects caused by FTY720 appear to be similar as $S1P_1$ deficiency, suggesting that FTY720 may act as an antagonist to inactivate $S1P_1$ in T_{reg} cells. However, mechanisms of action of FTY720 are complex because it can serve as an agonist for four of the five known S1P receptors17, 18. Moreover, FTY720 possesses immunomodulatory activities independent of S1P receptors46. Given the limitation of the pharmacological approaches, we used genetic systems to specifically target the $S1P_1$ pathway in T cells, and unequivocally revealed an intrinsic negative role for $S1P_1$ in T_{reg} development and function.

In summary, $S1P_1$ delivers a key negative signal for the development, maintenance and function of T_{reg} cells. The function of $S1P_1$ is primarily mediated by Akt-mTOR in T_{reg} cells. Our studies highlight that T_{reg} cells are regulated by more than surface expression of TCR and co-stimulatory molecules and limited production of cytokines. Rather, the development and function of these cells are further shaped by an abundant circulatory lipid. Interestingly, FTY720 and Rapamycin, two new promising immunosuppressants for transplantation and autoimmune disease, target $S1P_1$ and Akt-mTOR, respectively. Our studies suggest that a shared mechanism may contribute to immunomodulatory functions of these drugs. The $S1P_1$-Akt-mTOR pathway in T_{reg} cells may be explored to develop novel therapeutics for autoimmunity, cancer and infection.

METHODS

Mice and bone marrow chimeras

Mice of *S1pr1*^{fl} 20, CD4-Cre 47, $S1P_1$-Tg 21 and Foxp3^{gfp} knockin 28 have been described previously, and have been backcrossed to the C57BL/6 background extensively. WT controls for $S1P_1$-KO (CD4-Cre; *S1pr1*$^{fl/fl}$) included Cre$^+$ mice (CD4-Cre; *S1pr1*$^{+/+}$) to account for Cre effects; controls for $S1P_1$-Tg mice were transgene-negative littermates. C57BL/6, CD45.1, Thy1.1, *Rag1*$^{-/-}$, OT-II, and *Scurfy* mice (all on the C57BL/6 background) were purchased from the Jackson Laboratory. Mice at 6-10 weeks old were used unless otherwise noted. Bone marrow chimeras were generated by transferring 1-2 × 10^7 T cell-depleted bone marrow cells into sublethally irradiated (5 Gy) alymphoid *Rag1*$^{-/-}$ mice, as described previously47. All mice were kept in specific pathogen—free conditions in Animal Resource Center at St. Jude. Animal protocols were approved by Institutional Animal Care and Use Committee of St. Jude.

Cell purification and flow cytometry

Lymphocytes were isolated from the thymi, spleens and lymph nodes of mice and sorted on a MoFlow (Beckman-Coulter) or Reflection (i-Cyt). T_{reg} and T_{conv} cells were sorted based on $CD4^+CD45RB^{lo}CD25^+$ and CD4+$CD45RB^{hi}CD25^-$ expression, respectively; alternatively and whenever possible, mice crossed with $Foxp3^{gfp}$ knockin were used from which $CD4^+CD45RB^{lo}GFP^+$ and $CD4^+CD45RB^{hi}GFP^-$ populations were sorted for T_{reg} and T_{conv} cells, respectively. For flow cytometry analysis of surface markers, cells were stained with antibodies (all from eBioscience) in PBS containing 2% (wt/vol) BSA. Flow cytometry analysis of intracellular Foxp3 (FJK-16s; eBioscience), CTLA-4 (UC10-4F10-11), IFN-γ (XMG1.2) and IL-4 (11B11; all three antibodies from BD Biosciences) were performed per manufactures' instructions. For detection of phosphorylated signaling proteins, purified cells were activated with IL-2 or anti-CD3 (145-2C11; BD Biosciences) and anti-CD28 (37.51; BD Biosciences), immediately fixed with Phosflow perm buffer (BD Biosciences), permeabilized with Phosflow lyse/fix buffer (BD Biosciences), and stained with PE or APC directly conjugated antibodies for phospho-Akt (pSer 473) (D9E; Cell Signaling Technology), phospho-Erk (pThr202/pTyr204) (20A; BD Biosciences), phospho-STAT5 (pTyr694) (47; BD Biosciences) and phospho-S6 (pSer235/236) (D57.2. 2E; Cell Signaling Technology). Flow cytometry data were acquired on an upgraded 5-color FACScan (Becton Dickinson), and analyzed using FlowJo software (Treestar). Cell numbers of various populations were calculated by multiplying the total cell number with the percentages of each individual population from the same mouse, and then averaged.

T-cell culture, activation and T_{reg} suppression assays

T cells were cultured in Bruff's medium supplemented with 10% FBS and 1% penicillin-streptomycin as described previously47. For measurement of T cell activation, sorted T_{conv} cells (5×10^4) were cultured in 96-well flat-bottom plates, and stimulated with various doses of anti-CD3 (145-2C11) and/or anti-CD28 (37.51) in the presence of irradiated splenocytes as antigen-presenting cells for 72 h. For *in vitro* T cell suppression assay, sorted T_{conv} (5×10^4) and T_{reg} cells (at different ratios with T_{conv} cells) were cultured in 96-well flat-bottom plates along with 2 μg/ml anti-CD3 (145-2C11) and irradiated splenocytes for 72 h. T cell proliferation was determined by pulsing with [^{3}H]thymidine at 1 μCi per well for the last 12–16 h of culture, or by carboxyfluorescein diacetate succinimidyl diester (CFSE) labeling according to the manufacturer's protocols (Invitrogen-Molecular Probes). IL-2 production was analyzed by bioplex assays (BioRad). For drug treatment, cells were pre-incubated with vehicle, 5 μM U0126, 10 μM Ly294002 or 100 nM rapamycin (all from Calbiochem) for 1 h before stimulation.

Retroviral transduction

S1pr1 and Cre cDNAs were cloned into the mouse stem cell virus retroviral vector (MSCV) upstream of an internal ribosome entry site (IRES)-EGFP expression cassette. Retroviral constructs expressing dn-Akt and ca-Akt with linked Thy1.1 marker (MSCV-IRES-Thy1.1, abbreviated as MiT)48 were kindly provided by David Hildeman (U. of Cincinnati). Phoenix-Eco packaging cells were transfected with Lipofectamine (Invitrogen), and

recombinant retroviruses were collected 48 and 72 h after transfection. T_{reg} cells were stimulated with 5 μg/ml of anti-CD3 (145-2C11), 5 μg/ml of anti-CD28 (37.51) and 100 U/ml of IL-2 for 48 h and then were transduced with retroviruses by 'spin inoculation' (650 *g* for 1 h), as described47. Cells were cultured for an additional 5 d before being sorted according to EGFP or Thy1.1 expression, and used for T-cell suppression assays. Deletion of the *S1pr1* gene by Cre expression in *S1pr1*$^{fl/fl}$ cells was confirmed by quantitative PCR analysis (data not shown). In certain experiments, double transduction was used in which T_{reg} cells were transduced with Cre-GFP retrovirus at 48 h, and then with empty MiT vector or MiT expressing ca-Akt retrovirus 6 h later. For transduction of bone marrow stem cells, mice were injected with 5-fluorouracil (0.15 mg/g) and euthanized 2 days later. Bone marrow cells were harvested and expanded with IL-6 (50 ng/ml), IL-3 (20 ng/ml) and SCF (50 ng/ml) (all from R&D Systems) for 2 days before they were transduced with retrovirus as above. At 24 h after transduction, the cells were harvested and injected into irradiated *Rag1*$^{-/-}$ mice.

Measurement of serum antibodies

The titers of autoantibodies and immunoglobulin subclasses were determined with kits from Alpha Diagnostic International and Southern Biotechnology Associates, respectively.

Fetal thymus organ culture (FTOC)

Fetal thymus lobes were dissected from E16.5 embryos, and cultured on sponge-supported filter membranes at an interphase between 5% CO2-humidified air and T cell culture medium for approximately 7 days to induce thymocyte differentiation. The cell culture medium contained undetectable numbers of thymocytes (data not shown).

In vivo migration assays

Purified CD4SP thymocytes from WT or $S1P_1$-KO mice were labeled with CFSE, mixed with approximately same numbers of control WT cells (Thy1.1$^+$), and transferred into C57BL/6 recipient mice (Thy1.2$^+$). The co-transferred Thy1.1$^+$ cells served as an internal control to normalize the transfer and detection efficiencies among different recipients. At 24 h, lymphocytes were prepared from blood, spleen, PLN and Peyer's patches of recipient mice, and stained with Foxp3 (FJK-16s), Thy1.1 (HIS51) and CD4 (RM4-5) antibodies (all from eBioscience). Donor experimental cells, co-transferred internal control cells, and recipient CD4$^+$ T cells were distinguished by CFSE$^+$Thy1.2$^+$, CFSE$^-$Thy1.1$^+$ and CFSE$^-$Thy1.2$^+$, respectively. Donor experimental cells were further divided into Foxp3$^-$ T_{conv} and Foxp3$^+$ T_{reg} cells. The results were expressed as a ratio between WT or $S1P_1$-KO T cells and the cotransferred Thy1.1$^+$ internal control cells, as previously described21.

Neonatal transfer of T_{reg} cells

T_{reg} cells (CD45.1$^+$) were injected intraperitoneally into WT 3-day-old and $S1P_1$-Tg neonatal mice (CD45.2$^+$). At 8 weeks after transfer, the recipient T lymphocytes were analyzed by staining of the congenic markers CD45.1 and CD45.2.

Colitis model

Rag1$^{-/-}$ mice were injected intraperitoneally with 4×10^5 T_{conv} cells (CD45.1$^+$) alone or in combination with 2×10^5 T_{reg} cells (CD45.2$^+$) cells. Mice were weighed and assessed for clinical signs of colitis weekly, and were euthanized 9-10 weeks after transfer. Colons were sectioned, fixed in 10% neutral buffered formalin and processed routinely, and 4-μm sections cut and stained with H&E or Alcian blue/Periodic acid Schiff (PAS). T cells were visualized using a goat anti-CD3 polyclonal antisera (Santa Cruz) and diaminobenzidine chromagen with haematoxylin as a counterstain. T_{reg} cells were visualized with rat anti-Foxp3 clone FJK-16s antibody (eBioscience). Pathology of the colon was scored blindly by an experienced pathologist (K.B.) using a semi-quantitative scale of zero to five. In summary, grade 0 was assigned when no changes were observed; grade 1, minimal inflammatory infiltrates present in the lamina propria with or without mild mucosal hyperplasia; grade 2, mild inflammation in the lamina propria with occasional extension into the submucosa, focal erosions, minimal to mild mucosal hyperplasia and minimal to moderate mucin depletion; grade 3, mild to moderate inflammation in the lamina propria and submucosa occasionally transmural with ulceration and moderate mucosal hyperplasia and mucin depletion; grade 4, marked inflammatory infiltrates commonly transmural with ulceration, marked mucosal hyperplasia and mucin depletion, and multifocal crypt necrosis; grade 5, marked transmural inflammation with ulceration, widespread crypt necrosis and loss of intestinal glands. Blood, spleen, peripheral and mesenteric lymph nodes were also removed, and cells were counted and subsequently stained for CD4 and Foxp3 to determine T_{conv} and T_{reg} numbers in these organs.

Quantitative RT-PCR

RNA was extracted with RNeasy kit (Qiagen), and cDNA was synthesized with Superscript III reverse transcriptase (Invitrogen). An ABI 7900 Real-time PCR system was used for quantitative PCR, with primer and probe sets from Applied Biosystems (*S1pr1*, Mm00514644_m1; *Foxp3*, Mm00475156_m1); results were analyzed with SDS 2.1 software. The cycling threshold value of the endogenous control gene (*Hprt1*) was subtracted from the cycling threshold value of each target gene to generate the change in cycling threshold (ΔCT). The relative expression of each target gene is expressed as the 'fold change' relative to that of wild-type unstimulated samples ($2^{-\Delta\Delta CT}$), as described49.

Statistical analysis

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

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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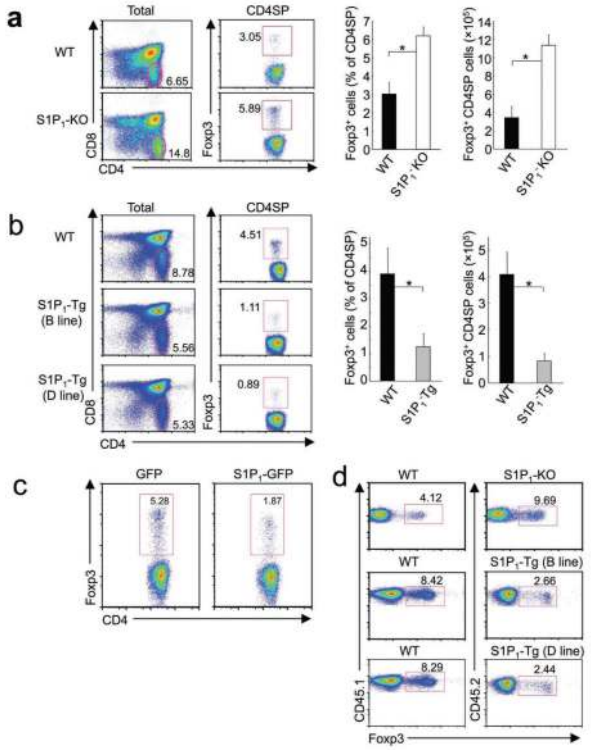

Figure 1. $S1P_1$ negatively regulates thymic $Foxp3^+$ T_{reg} population

(**a**,**b**) Flow cytometry of total and gated CD4SP thymocytes isolated from wild-type (WT) control, $S1P_1$-KO (**a**) and $S1P_1$-Tg mice (**b**). Panels on the right show the proportions and absolute numbers of $Foxp3^+$ CD4SP T_{reg} cells. Data are the mean (+s.d.) of 8-14 mice of each genotype from 7 experiments. (**c**) Foxp3 expression in bone marrow chimeras following retroviral transduction of $S1P_1$. Bone marrow stem cells from WT mice were transduced with retrovirus expressing $S1P_1$ ($S1P_1$-GFP) or empty vector (GFP), and transferred into sublethally irradiated *Rag1*$^{-/-}$ mice. At 6-8 weeks after reconstitution, Foxp3 expression was analyzed in gated CD4SP thymocytes. Data are representative of 2 independent experiments. (**d**) Expression of Foxp3 in mixed bone marrow chimeras. Bone marrow stem cells from WT ($CD45.1^+$) and $S1P_1$-KO or $S1P_1$-Tg mice ($CD45.2^+$) were mixed at 1:1, and transferred into *Rag1*$^{-/-}$ mice to generate mixed bone marrow chimeras. At 6-8 weeks after reconstitution, Foxp3 expression was analyzed in CD4SP thymocytes, and cells from different donors were distinguished by their CD45.1 and CD45.2 expression. Data are representative of 3 independent experiments. *, $P < 0.001$ (Student's *t*-test).

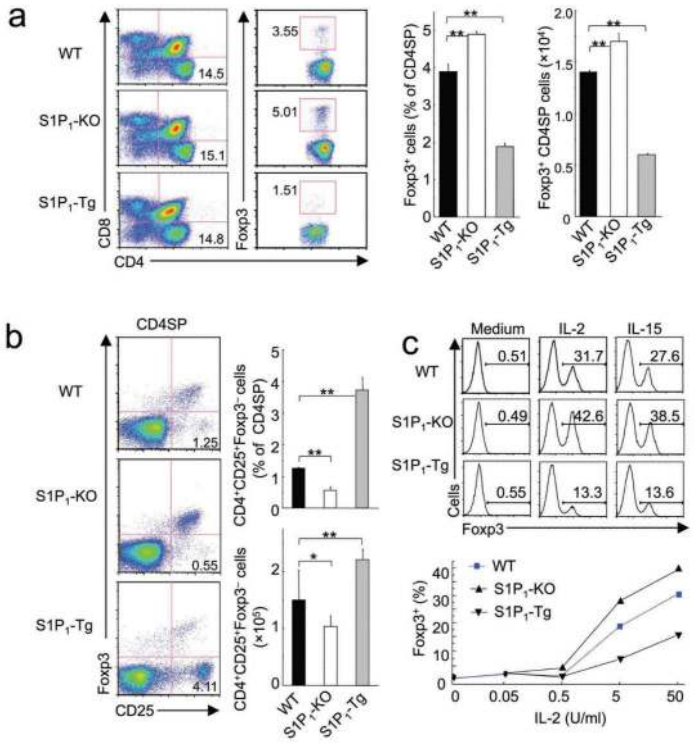

Figure 2. S1P$_1$ blocks thymic differentiation of T$_{reg}$ cells

(**a**) Flow cytometry of total and gated CD4SP thymocytes isolated from WT control, S1P$_1$-KO and S1P$_1$-Tg FTOC. Panels on the right show the proportions and absolute numbers of Foxp3$^+$ CD4SP T$_{reg}$ cells with the mean (+s.d.) calculated from ≥8 mice of each genotype. (**b**) Flow cytometry of gated CD4SP thymocytes from WT control, S1P$_1$-KO and S1P$_1$-Tg mice. Panels on the right show the proportions and absolute numbers of the CD4$^+$CD25$^+$Foxp3$^-$ precursor population, with the mean (+s.d.) calculated from ≥8 mice of each genotype. (**c**) Induction of Foxp3 expression in the CD4$^+$CD25$^+$Foxp3$^-$ population *in vitro*. CD4$^+$CD25$^+$Foxp3$^-$ cells were purified and stimulated with medium alone, IL-2 or IL-15 for 20 h, and induction of Foxp3 expression was measured by flow cytometry. The lower panel shows an IL-2 dependent dose response curve. Data are representative of 5 independent experiments. *, $P < 0.05$; **, $P < 0.01$ (Student's *t*-test).

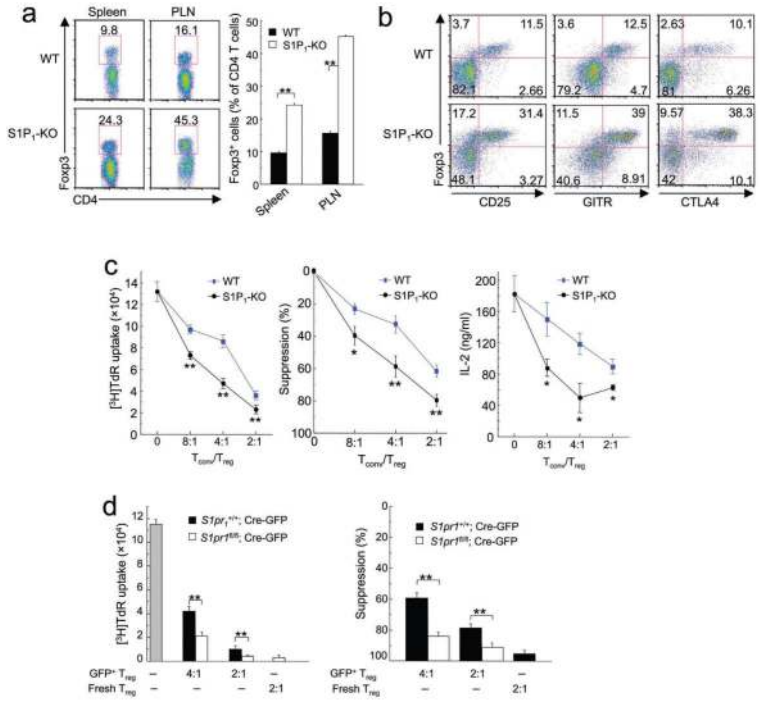

Figure 3. Enhanced peripheral population and suppressive activity of S1P$_1$-KO T$_{reg}$ cells

(**a**) Flow cytometry of gated CD4 T cells from the spleen and peripheral lymph nodes (PLN) of WT and S1P$_1$-KO mice. The panel on the right shows the proportions of Foxp3$^+$ T$_{reg}$ cells among total CD4$^+$ T cell population, with the mean (+s.d.) calculated from 4 mice of each genotype. (**b**) Flow cytometry analysis of T$_{reg}$ markers (Foxp3, CD25, GITR and CTLA4) in PLN of WT and S1P$_1$-KO mice. Data are representative of 2 independent experiments. Similar findings were observed in other peripheral lymphoid organs (not shown). (**c**) *In vitro* T-cell suppression assays using Foxp3$^+$ CD4SP cells from WT and S1P$_1$-KO mice. The left panel shows a representative proliferative assay of 4 independent experiments, the middle panel is the percentage of suppression with the mean (±s.d.) calculated from 4 experiments, and the right panel shows a representative of 2 independent experiments measuring IL-2 production. (**d**) *In vitro* T-cell suppression assays using S1P$_1$-deleted peripheral T$_{reg}$ cells. Foxp3$^+$ T$_{reg}$ cells from the periphery of *S1pr1*$^{+/+}$ and *S1pr1*$^{fl/fl}$ mice were transduced with Cre-expressing retrovirus (Cre-GFP), and sorted GFP$^+$ T$_{reg}$ cells were used in the T-cell suppression assays with different T$_{conv}$ and T$_{reg}$ ratios; freshly isolated T$_{reg}$ cells were used as a comparison. The left panel is a representative of 3 independent experiments, and the right panel shows the percentage of suppression with the mean (+s.d.) calculated from 3 experiments. *, $P < 0.01$; **, $P < 0.001$ (Student's *t*-test).

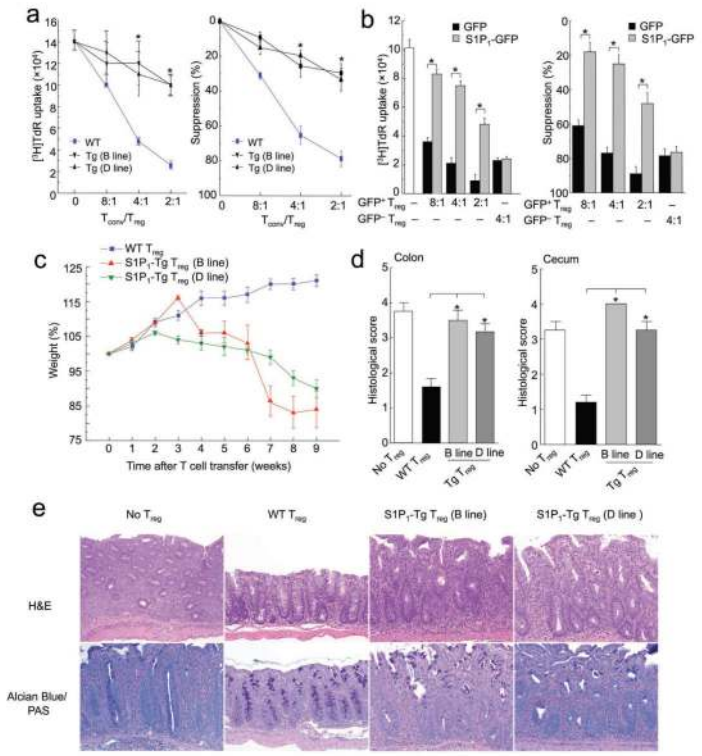

Figure 4. Reduced suppressive activity of S1P$_1$-Tg T$_{reg}$ cells *in vitro* and *in vivo*

(**a**) *In vitro* T-cell suppression assays using Foxp3$^+$ T$_{reg}$ cells from WT and S1P$_1$-Tg mice. The left panel shows a representative proliferative assay of 8 independent experiments, and the right panel is the percentage of suppression with the mean (±s.d.) calculated from 8 experiments. (**b**) *In vitro* T-cell suppression assays using Foxp3$^+$ T$_{reg}$ cells transduced with S1P$_1$-expressing retrovirus. WT T$_{reg}$ cells were transduced with S1P$_1$-expressing (S1P$_1$-GFP) and empty vector (GFP) retroviruses, and sorted GFP$^+$ T$_{reg}$ cells were used in the T-cell suppression assays with different T$_{conv}$ and T$_{reg}$ ratios. The left panel shows a representative proliferative assay of 5 independent experiments, and the right panel is the percentage of suppression with the mean (+s.d.) calculated from 5 experiments. (**c**-**e**) Failure of S1P$_1$-Tg T$_{reg}$ cells to control colitis *in vivo*. T$_{conv}$ cells were transferred alone or in combination with WT or S1P$_1$-Tg T$_{reg}$ cells into *Rag1*$^{-/-}$ mice. (**c**) Changes in body weight after transfer. (**d**) Histology scores of experimental mice. (**e**) Representative colon histology. Data are the mean (+s.d.) of 5 mice of each genotype and are representative of 2 independent experiments. *, $P < 0.001$ (Student's *t*-test).

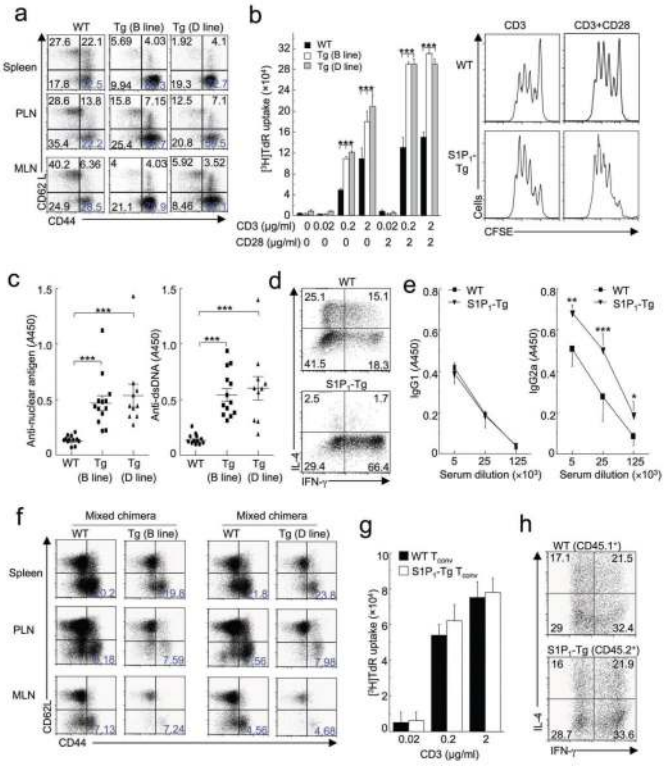

Figure 5. S1P$_1$-Tg mice show disrupted immune homeostasis and develop age-related autoimmunity due to defects in the T$_{reg}$ compartment

(**a**-**e**) Analysis of WT and S1P$_1$-Tg mice. (**a**) Flow cytometry of T cell activation markers from peripheral lymphoid organs of aged mice (10 months). MLN, mesenteric lymph nodes. Data are representative of 6 independent experiments. (**b**) Proliferative response to TCR stimulation of T$_{conv}$ cells from WT and S1P$_1$-Tg mice (2 months). Data are representative of 6 independent experiments. (**c**) Titers of anti-nuclear antigen and anti-ds DNA antibodies of aged mice (10 months). Data are the mean (±s.d.) of >10 mice of each genotype and are representative of 4 independent experiments. (**d**) Effector cytokine production of activated T cells from WT and S1P$_1$-Tg mice (5-6 months). Data are representative of 2 independent experiments. (**e**) Serum titers of IgG1 and IgG2a (5-6 months). Data are the mean of 5 mice of each genotype and are representative of 3 independent experiments. (**f**-**h**) Analysis of WT and S1P$_1$-Tg T cells in the mixed BM chimeras (6-9 months after reconstitution), including expression of activation markers (**f**), proliferation (**g**), and effector cytokine production (**h**). Data are representative of 3 independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (Student's *t*-test).

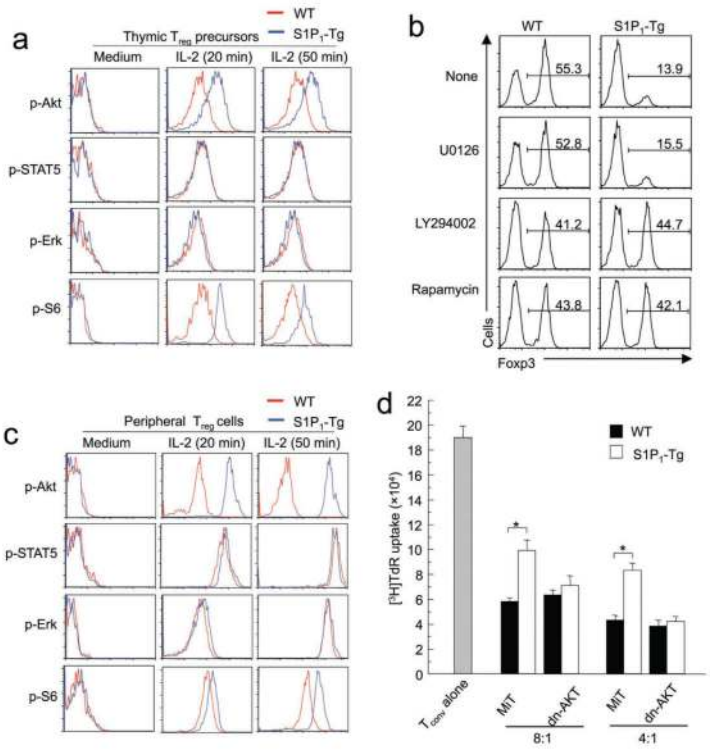

Figure 6. $S1P_1$ induces activation of Akt-mTOR to inhibit T_{reg} development and function

(**a**) IL-2 activated signaling pathways in thymic T_{reg} precursors from WT and $S1P_1$-Tg mice. $CD4^+CD25^+Foxp3^-$ cells were purified and stimulated with medium alone or IL-2, and activation of Akt, STAT5, Erk and S6 ribosomal protein (S6) were examined by flow cytometry using phospho-specific antibodies. Data are representative of 4 independent experiments. (**b**) Effects of drug treatments on IL-2 induced Foxp3 expression in T_{reg} precursors. $CD4^+CD25^+Foxp3^-$ cells were treated with U0126, LY294002 and Rapamycin for 30 minutes, followed by IL-2 stimulation. Data are representative of 3 independent experiments. (**c**) IL-2 activated signaling pathways in peripheral T_{reg} cells from WT and $S1P_1$-Tg mice. T_{reg} cells were stimulated with medium alone or IL-2, and activation of Akt, STAT5, Erk and S6 ribosomal protein (S6) were examined by flow cytometry using phospho-specific antibodies. Data are representative of 5 independent experiments. (**d**) Suppressive activity of T_{reg} cells transduced with dn-Akt retrovirus. WT and $S1P_1$-Tg T_{reg} cells were transduced with control (MiT) and dn-Akt expressing (dn-Akt) retroviruses (non-transduced cells are shown on the right as a comparison), and transduced cells were sorted and used in the T-cell suppression assays with different T_{conv} and T_{reg} ratios. Data are representative of 3 independent experiments. *, $P < 0.001$ (Student's *t*-test).

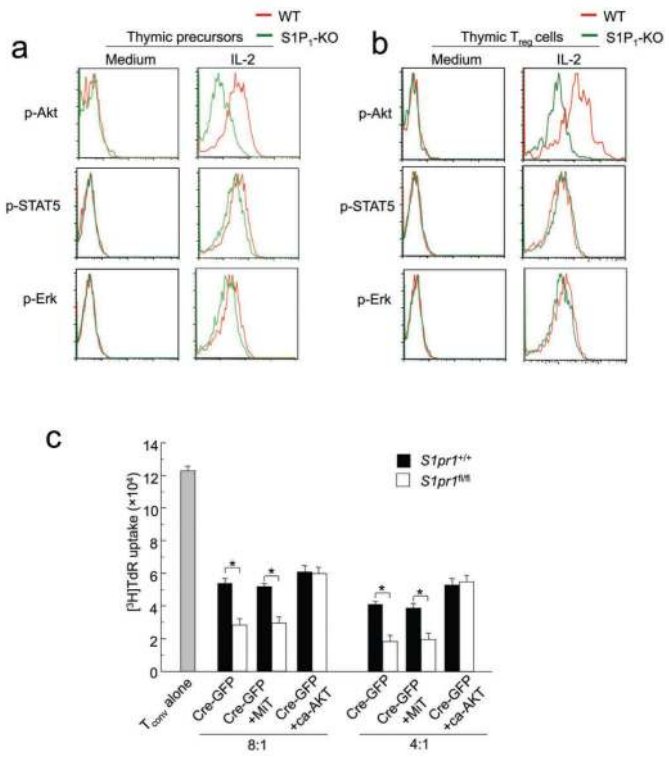

Figure 7. $S1P_1$ is necessary for Akt activation in T_{reg} cells

(**a**,**b**) IL-2 activated signaling pathways in thymic T_{reg} precursors (**a**) and $Foxp3^+$ reg T_{reg} cells (**b**) from WT and $S1P_1$-KO mice. Purified $CD4^+CD25^+Foxp3^-$ cells (**a**) or $CD4^+Foxp3^+$ cells (**b**) were stimulated with medium alone or IL-2, and activation of Akt, STAT5 and Erk were examined by flow cytometry using phospho-specific antibodies. Data are representative of 3 independent experiments. (**c**) Suppressive activity of T_{reg} cells transduced with Cre-GFP retrovirus alone or in combination with constitutively active Akt. $Foxp3^+$ T_{reg} cells from the periphery of *S1pr1*$^{+/+}$ and *S1pr1*$^{fl/fl}$ mice were transduced with Cre-expressing retrovirus (Cre-GFP) alone or in combination with empty control (MiT) or constitutively active Akt (ca-Akt) retroviruses, and transduced cells were sorted and used in the T-cell suppression assays with different T_{conv} and T_{reg} ratios. Data are representative of 2 independent experiments. *, $P < 0.001$ (Student's *t*-test).

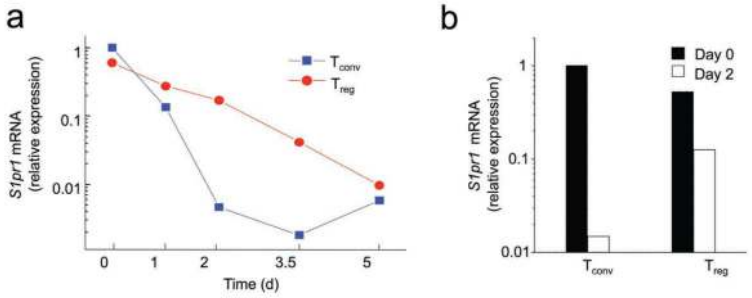

Figure 8. Differential regulation of $S1P_1$ expression in T_{reg} and T_{conv} cells

(**a**) T_{reg} and T_{conv} cells from WT mice were stimulated with anti-CD3, anti-CD28 and IL-2, and *S1pr1* mRNA expression was analyzed by quantitative PCR. Data are representative of 3 independent experiments. (**b**) OT-II TCR-transgenic mice were crossed with $Foxp3^{gfp}$ knockin mice, and sorted T_{reg} and T_{conv} cells from these mice were transferred into C57BL/6 mice, followed by s.c. immunization with ovalbumin emulsified in CFA. Two days after immunization, T_{reg} and T_{conv} cells from the draining lymph nodes were purified based on GFP expression, and *S1pr1* mRNA expression was analyzed in each T-cell subset. Data are representative of 2 independent experiments.