Published in final edited form as:

Nat Immunol. 2009 July ; 10(7): 769–777. doi:10.1038/ni.1743.

S1P₁ receptor overrides regulatory T cell-mediated immune suppression through Akt-mTOR

Guangwei Liu¹, Samir Burns¹, Gonghua Huang¹, Kelli Boyd², Richard L. Proia³, Richard A. Flavell^{4,5}, and Hongbo Chi^{1,5}

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

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

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

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

⁵Department 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 (S1P₁) 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 S1P₁ blocked the differentiation of thymic T_{reg} precursors and function of mature Treg cells, and affected T_{reg}-mediated immune tolerance. S1P₁ induced the selective activation of the Akt-mTOR pathway to impede T_{reg} development and function. Dynamic regulation of S1P₁ contributed to lymphocyte priming and immune homeostasis. Thus, by antagonizing T_{reg}-mediated immune suppression, the lipid-activated S1P₁-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

Users may view, print, copy, and download text and data-mine the content in such documents, for the purposes of academic research, subject always to the full Conditions of use:http://www.nature.com/authors/editorial_policies/license.html#terms

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} cells^{6,7}. 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 activity^{9,10}. 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 (S1P₁-S1P₅). FTY720, a new class of immunosuppressants in clinical trials for transplantation tolerance and multiple sclerosis, sequesters T cells in lymphoid organs by acting on four of the five S1P receptors^{17, 18}. Genetic approaches to alter the function of S1P₁ (also known as Edg1, <http://www.signaling-gateway.org/molecule/query?afcsid=A000813>) indicate that S1P₁ is the main S1P receptor that regulates T cell trafficking. T cells from S1P₁-deficient mice fail to egress from thymus^{19, 20}, while S1P₁-transgenic T cells preferentially distribute to the blood rather than lymphoid organs^{21, 22}. Thus, S1P₁ is critical for the egress of T cells from lymphoid organs. S1P₁ primarily couples with pertussis toxin-sensitive G_i proteins. Major pathways downstream of S1P₁ 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.

S1P₁ 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 S1P₁ function selectively in T cells. Our results showed that loss of S1P₁ function resulted in enhanced thymic differentiation and suppressive activity of T_{reg} cells. Conversely, increased S1P₁ 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, S1P₁ negatively regulates both thymic generation and suppressive activity of T_{reg} cells. We further demonstrated that the function of S1P₁ in T_{reg} cells is mediated by the downstream Akt-mTOR pathway. Finally, S1P₁ expression was differentially regulated in T_{reg} cells as compared with conventional T cells (T_{conv} cells), suggesting that S1P₁ coordinates the responses of T_{reg} and T_{conv} cells to effect a productive and self-controlled immune response.

RESULTS

S1P₁ signaling reduces the thymic T_{reg} population

To investigate the intrinsic function of S1P₁ in T cells, we crossed mice carrying a conditional S1P₁ allele (*S1pr1*^{fl/fl})²⁰ with CD4-Cre transgenic mice to delete the floxed *S1pr1* allele specifically in T cells (S1P₁-KO mice). Compared with wild-type controls, S1P₁-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 S1P₁ in thymocyte egress^{19, 20}. To assess the requirement of S1P₁ 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, S1P₁-KO mice contained elevated numbers of the thymic T_{reg} population expressing Foxp3, CTLA4 and GITR (Fig. 1a and Supplementary Fig. 1c). Thus, S1P₁ deficiency causes expansion of the thymic T_{reg} cell population.

Is S1P₁ 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 S1P₁ in T cells (S1P₁-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 S1P₁, and implanted them into lymphoid *Rag1*^{-/-} mice. Following reconstitution, Foxp3⁺ thymocytes expressing S1P₁ were one-third the number of those which expressed the empty vector (Fig. 1c). Therefore, increased S1P₁ function reduces the thymic T_{reg} population.

The defect in S1P₁-expressing cells in the BM chimeras suggests a cell-autonomous effect of S1P₁ 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 S1P₁-KO or S1P₁-Tg cells (CD45.2⁺). Compared with the co-transferred CD45.1⁺ cells, S1P₁-KO and S1P₁-Tg BM developed into elevated and reduced thymic Foxp3⁺ T_{reg} cells, respectively (Fig. 1d). We therefore conclude that S1P₁ has a cell-autonomous effect in inhibiting the thymic T_{reg} population.

S1P₁ inhibits thymic T_{reg} development

Altered thymic T_{reg} cells in S1P₁-KO and S1P₁-Tg mice might be due to the effects of S1P₁ on T_{reg} egress, differentiation or both. To distinguish these possibilities, we first analyzed the trafficking of S1P₁-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 S1P₁-KO T_{conv} cells were able to enter lymphoid organs but unable to exit into blood, as reported¹⁹. S1P₁-KO T_{reg} cells exhibited a similar defect as S1P₁-KO T_{conv} cells (Supplementary Fig. 2 online). Thus, S1P₁ 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 S1P₁-KO thymus. Further supporting this, CD4⁺CD8⁺

double-positive thymocytes, which do not egress into the periphery, contained altered Foxp3⁺ populations in S1P₁-KO and S1P₁-Tg mice (Supplementary Fig. 1d).

We used two strategies to directly assess an intrinsic role of S1P₁ 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 S1P₁-KO or S1P₁-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 S1P₁-KO thymus and a reciprocal decrease in S1P₁-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 S1P₁-KO cells and decreased in S1P₁-Tg cells (Fig. 2a). These findings collectively demonstrate that S1P₁ plays an intrinsic negative role in thymic T_{reg} development.

S1P₁ blocks differentiation of CD4⁺CD25⁺ Foxp3⁻ cells

We hypothesized that S1P₁ 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 stimulation^{26, 27}. Remarkably, the CD4⁺CD25⁺Foxp3⁻ population was greatly reduced in S1P₁-KO thymus but significantly increased in S1P₁-Tg thymus (Fig. 2b), suggesting that S1P₁ may act on these cells to restrain their further differentiation into mature Foxp3-expressing T_{reg} cells.

To directly test this, we crossed S1P₁-KO and S1P₁-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 S1P₁-KO cells, while a reciprocal change was observed in S1P₁-Tg cells, irrespective of the stimuli and doses used (Fig. 2c). Therefore, S1P₁ blocks the differentiation of CD4⁺CD25⁺Foxp3⁻ cells into mature T_{reg} cells.

Altered homeostasis and function of S1P₁-KO T_{reg} cells

Our results thus far have identified a negative role for S1P₁ in thymic differentiation of T_{reg} cells. We then examined whether S1P₁ affects homeostasis and suppressive activity of T_{reg} cells in the periphery. In S1P₁-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 S1P₁-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 S1P₁ deficiency resulted in increased numbers of Foxp3⁺ CD4SP cells, Foxp3 expression on a per cell basis was comparable between wild-type and S1P₁-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 S1P₁-deficient Foxp3⁺ thymic T_{reg} cells. S1P₁-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 S1P₁-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 S1P₁-KO thymus, it remains possible that the increased suppressive activity of S1P₁-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, S1P₁ deficiency directly potentiates T_{reg} suppressive activity.

Impaired suppressive activity of S1P₁-Tg T_{reg} cells

We next asked whether ectopic S1P₁ expression is sufficient to alter T_{reg} suppressive activity. In S1P₁-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 S1P₁-Tg BM cells, T_{reg} cells derived from S1P₁-Tg BM were selectively reduced as compared with co-transferred wild-type counterparts (Supplementary Fig. 5c). Thus, S1P₁-Tg T_{reg} cells have a competitive disadvantage in their peripheral maintenance. In T cell suppression assays, S1P₁-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 S1P₁-Tg T_{reg} cells as compared with wild-type T_{reg} cells (Fig. 4a and Supplementary Fig. 6a online). Further, S1P₁-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 S1P₁ function rather than secondary to defective thymic development, we transduced wild-type T_{reg} cells with S1P₁-expressing retrovirus. Expression of S1P₁ substantially impaired T_{reg} suppressive activity (Fig. 4b), highlighting an intrinsic inhibitory effect of S1P₁ on T_{reg} function.

We next employed two approaches to investigate whether S1P₁ 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 S1P₁-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 S1P₁-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 S1P₁-Tg T_{reg} cell transfer groups (Supplementary Figs. 7b,c). Thus, S1P₁-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 S1P₁ 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 S1P₁-Tg or wild-type mice into *Rag1*^{-/-} recipients. In the resulting chimeras, only the S1P₁-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 S1P₁-Tg BM cells showed profound T cell activation and inflammatory diseases that were indistinguishable from *sf* alone chimeras (Supplementary Figs. 8b,c). Thus, S1P₁-Tg T_{reg} cells fail to mediate immune tolerance *in vivo*.

Defects in S1P₁-Tg T_{reg} cells cause autoimmunity

The impaired suppressive activity of T_{reg} cells from S1P₁-Tg mice prompted us to examine whether homeostasis of the immune system was altered in these mice. S1P₁-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 S1P₁-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 S1P₁-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 S1P₁-Tg mice were differentiated into a T_H1 phenotype. S1P₁-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 S1P₁-Tg mice (Fig. 5e). Together, increased S1P₁ 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 S1P₁-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 S1P₁-Tg BM cells alone or in combination into *Rag1*^{-/-} mice. As expected, T cells from mice given S1P₁-Tg BM alone

were spontaneously activated and hyper-responsive to TCR stimulation (data not shown). In contrast, in the mixed chimeras containing both S1P₁-Tg and wild-type cells, S1P₁-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 S1P₁-Tg mice was non-cell-autonomous and could be rescued by the presence of wild-type cells. Next we determined whether providing S1P₁-Tg mice with wild-type T_{reg} cells alone could prevent the T cell activation phenotypes. We injected 3-day old S1P₁-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 S1P₁-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 S1P₁-Tg mice accounts for the disrupted immune homeostasis.

S1P₁ signals through Akt-mTOR to affect T_{reg} cells

To investigate mechanisms mediating S1P₁ 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, S1P₁-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 injection^{32, 33}. Does increased Akt activity mediate S1P₁ functions in T_{reg} differentiation? To this end, we stimulated wild-type and S1P₁-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 S1P₁-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 S1P₁-Tg cells (Supplementary Fig. 10 online), indicating the specific involvement of the Akt-mTOR pathway in S1P₁ signaling. In contrast, treatment with U0126 (an inhibitor of Erk) did not affect T_{reg} differentiation (Fig. 6b). Therefore, the increased Akt activity in S1P₁-Tg T_{reg} precursors results in their defective differentiation.

Next, we determined the signaling pathways activated by increased S1P₁ expression in peripheral T_{reg} cells. Upon stimulation with IL-2, peripheral T_{reg} cells from S1P₁-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 S1P₁-Tg T_{reg} cells were stimulated with anti-CD3 plus anti-CD28 (Supplementary Fig. 11 online). We then treated peripheral S1P₁-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 S1P₁-Tg cells completely restored their suppressive activity (Fig. 6d). We conclude that increased S1P₁ function leads to excessive Akt activity which accounts for impaired T_{reg} differentiation and function.

S1P₁ is necessary for Akt activation in T_{reg} cells

Whereas the results above indicated that S1P₁ is sufficient to activate the Akt pathway to antagonize T_{reg} development and function, it remains unclear whether S1P₁ is necessary for Akt activation in T_{reg} cells. To address this issue, we stimulated T_{reg} precursors from wild-type or S1P₁-KO mice with IL-2. As compared with wild-type cells, S1P₁-KO cells exhibited substantially reduced Akt phosphorylation, but no change on STAT5 or Erk, following IL-2 stimulation (Fig. 7a). Similarly, S1P₁ deficiency decreased Akt activation in mature thymic T_{reg} cells (Fig. 7b). Moreover, after acute deletion of S1P₁ 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, S1P₁ is required to mediate IL-2 and TCR-induced Akt activation in T_{reg} cells.

In addition to S1P₁, T_{reg} cells also express all of the other four receptors for S1P₂₋₄. Exposure of T cells to S1P results in the activation of Akt35, although whether this is mediated by S1P₁ is not known. We treated wild-type and S1P₁-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 S1P₁, activation of Akt, but not Erk, was substantially reduced as compared with wild-type counterparts (Supplementary Fig. 13c). Thus, among the S1P receptors, S1P₁ 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 S1P₁-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 S1P₁-deficient T_{reg} cells (Fig. 7c). Therefore, the reduced Akt activity is responsible for the enhanced suppressive activity of S1P₁-KO T_{reg} cells.

Function and signaling of S1P₁ in T_{conv} cells

Our data above have delineated that S1P₁, by activating Akt, negatively regulates development and function of T_{reg} cells. What are the function and signaling mechanisms of S1P₁ 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 S1P₁-Tg mice. To further extend this observation, we used retroviral systems to ectopically express S1P₁ 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 S1P₁-KO mice (Supplementary Fig. 14c). Thus, S1P₁ does not affect the proliferative response of T_{conv} cells.

To address the signaling mechanisms of S1P₁ in T_{conv} cells, we activated wild-type and S1P₁-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 S1P₁-deficient T_{conv} cells, IL-2 and TCR induced normal activation of Akt (Supplementary Figs. 16a,b online). Thus, unlike in T_{reg} cells, S1P₁ 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 S1P₁ signaling, we stimulated S1P₁-sufficient and deficient T_{conv} cells with S1P. S1P₁ deficiency resulted in reduced S1P-induced Akt activation (Supplementary Fig. 16c). Therefore, whereas T_{conv} cells use S1P₁ to mediate Akt activation in response to S1P, the activation of Akt in response to TCR or IL-2 occurs independently of S1P₁ in these cells.

The differential function of S1P₁ in T_{reg} and T_{conv} cells prompted us to examine whether S1P₁ 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 reported^{21, 22}, whereas it was downregulated gradually in T_{reg} cells (Fig. 8a). Expression of KLF2, a transcription factor essential for S1P₁ expression in thymocytes³⁶, exhibited a similar pattern as that of S1P₁ (Supplementary Fig. 18 online), suggesting that KLF2 contributes to the differential regulation of S1P₁ between peripheral T_{reg} and T_{conv} cells. To examine whether this differential regulation of S1P₁ 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 S1P₁ function and expression are differentially regulated between T_{reg} and T_{conv} cells. We propose that dynamic regulation of S1P₁ expression contributes to lymphocyte priming and the maintenance of immune homeostasis (Supplementary Fig. 19 online).

DISCUSSION

Recent work on T_{reg} cell biology has mainly focused on mechanisms of T_{reg}-mediated immune suppression^{4, 8}. How the development and function of T_{reg} cells are regulated remains poorly understood. Here we report that S1P₁ 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, S1P₁ 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 S1P₁ 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 S1P₁ functions, we identified an inhibitory role for S1P₁ on the thymic T_{reg} population. Although S1P₁ facilitates thymic egress of T_{reg} cells, an intrinsic function for S1P₁ 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 S1P₁-KO and S1P₁-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 S1P₁ delivers a crucial negative signal for thymic development of T_{reg} cells. Previous studies have shown that S1P₁ 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 S1P₁ is a critical negative regulator of T_{reg} function. S1P₁ is highly expressed in both naïve T_{reg} and T_{conv} cells. At an early stage of immune activation, S1P₁ 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, S1P₁ 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 cells^{19, 21}. The overall function of S1P₁ in T_{reg} and T_{conv} cells at this stage is to promote a productive immune response. At a late stage of immune activation, S1P₁ 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, S1P₁ is slowly recovered in T_{conv} cells to allow their egress into peripheral tissues^{19, 38}. It remains unclear how T_{reg}-mediated suppression is attenuated during acute infection to establish protective immunity^{9, 11, 39, 40}, and our findings suggest that coordination of T_{reg} and T_{conv} responses by S1P₁ is crucial for a productive and self-controlled immune response.

To identify the molecular mechanisms mediating S1P₁ functions in T_{reg} cells, we examined signaling pathways activated by S1P₁. We found a specific role for S1P₁ 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} differentiation^{32, 33}, but the cellular factors responsible for activating Akt have not been established. Our studies identified that S1P₁ is a key receptor that activates Akt-mTOR. Importantly, restoration of proper Akt-mTOR activity corrected the defects caused by S1P₁ loss and gain of function. Therefore, activation of Akt-mTOR by S1P₁ mediates the negative effects of S1P₁ on T_{reg} development and function.

We further identified a fundamental difference in S1P₁ signaling between T_{reg} and T_{conv} cells. S1P₁ 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, S1P₁ is required for Akt activation in response to S1P stimulation in both T_{reg} and T_{conv} cells. It appears that S1P₁ 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 S1P₁ 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 stimulation^{42, 43}, and our studies on S1P₁ further highlight the distinct mechanisms in Akt signaling between these two T-cell subsets.

In addition to S1P₁, T_{reg} cells also express all of the other four receptors for S1P₂₋₅, the expression of which was not substantially altered in S1P₁-KO and S1P₁-Tg T_{reg} cells (data not shown). Whether and how S1P₁ 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 S1P₁, has been shown to enhance T_{reg} activity^{24, 44, 45}. These effects caused by FTY720 appear to be similar as S1P₁ deficiency, suggesting that FTY720 may act as an antagonist to inactivate S1P₁ 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 receptors^{17, 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 S1P₁ pathway in T cells, and unequivocally revealed an intrinsic negative role for S1P₁ in T_{reg} development and function.

In summary, S1P₁ delivers a key negative signal for the development, maintenance and function of T_{reg} cells. The function of S1P₁ 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 S1P₁ and Akt-mTOR, respectively. Our studies suggest that a shared mechanism may contribute to immunomodulatory functions of these drugs. The S1P₁-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, S1P₁-Tg 21 and Foxp3^{gfp} knockin 28 have been described previously, and have been backcrossed to the C57BL/6 background extensively. WT controls for S1P₁-KO (CD4-Cre; *S1pr1*^{fl/fl}) included Cre⁺ mice (CD4-Cre; *S1pr1*^{+/+}) to account for Cre effects; controls for S1P₁-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 × 10⁷ 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 μ g/ml anti-CD3 (145-2C11) and irradiated splenocytes for 72 h. T cell proliferation was determined by pulsing with [3 H]thymidine at 1 μ Ci 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 μ M U0126, 10 μ M Ly294002 or 100 nM rapamycin (all from Calbiochem) for 1 h before stimulation.

Retroviral transduction

Slpr1 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 *Slpr1* gene by Cre expression in *Slpr1^{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 S1P₁-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 S1P₁-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 S1P₁-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- μ m 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 (*Slpr1*, 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 (CT). The relative expression of each target gene is expressed as the 'fold change' relative to that of wild-type unstimulated samples (2^{-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.

ACKNOWLEDGEMENTS

The authors acknowledge A. Rudensky (U. of Washington) for Foxp3^{gfp} knockin mice, S. Shrestha for help with genotyping, M. McGargill for help with the FTOC procedure, R. Cross and G. Lennon for cell sorting, and D. Green and Dario Vignali for scientific discussions and sharing important reagents. This work was supported in part

by National Institutes of Health, the Arthritis Foundation and Arthritis National Research Foundation, and ALSAC (H.C.), and the Intramural Research Program of the National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Diseases (R.L.P.).

REFERENCES

1. Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. *Cell*. 2008; 133:775–787. [PubMed: 18510923]
2. Zheng Y, Rudensky AY. Foxp3 in control of the regulatory T cell lineage. *Nat Immunol*. 2007; 8:457–462. [PubMed: 17440451]
3. Campbell DJ, Ziegler SF. FOXP3 modifies the phenotypic and functional properties of regulatory T cells. *Nat Rev Immunol*. 2007; 7:305–310. [PubMed: 17380159]
4. Tang Q, Bluestone JA. The Foxp3+ regulatory T cell: a jack of all trades, master of regulation. *Nat Immunol*. 2008; 9:239–244. [PubMed: 18285775]
5. Shevach EM, et al. The lifestyle of naturally occurring CD4+ CD25+ Foxp3+ regulatory T cells. *Immunol Rev*. 2006; 212:60–73. [PubMed: 16903906]
6. Fontenot JD, Rasmussen JP, Gavin MA, Rudensky AY. A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat Immunol*. 2005; 6:1142–1151. [PubMed: 16227984]
7. Liu Y, et al. A critical function for TGF-beta signaling in the development of natural CD4+CD25+Foxp3+ regulatory T cells. *Nat Immunol*. 2008; 9:632–640. [PubMed: 18438410]
8. Vignali DA, Collison LW, Workman CJ. How regulatory T cells work. *Nat Rev Immunol*. 2008; 8:523–532. [PubMed: 18566595]
9. Pasare C, Medzhitov R. Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells. *Science*. 2003; 299:1033–1036. [PubMed: 12532024]
10. Yang Y, Huang CT, Huang X, Pardoll DM. Persistent Toll-like receptor signals are required for reversal of regulatory T cell-mediated CD8 tolerance. *Nat Immunol*. 2004; 5:508–515. [PubMed: 15064759]
11. Peng G, et al. Toll-like receptor 8-mediated reversal of CD4+ regulatory T cell function. *Science*. 2005; 309:1380–1384. [PubMed: 16123302]
12. Suttmoller RP, et al. Toll-like receptor 2 controls expansion and function of regulatory T cells. *J Clin Invest*. 2006; 116:485–494. [PubMed: 16424940]
13. Liu H, Komai-Koma M, Xu D, Liew FY. Toll-like receptor 2 signaling modulates the functions of CD4+ CD25+ regulatory T cells. *Proc Natl Acad Sci U S A*. 2006; 103:7048–7053. [PubMed: 16632602]
14. Schwab SR, Cyster JG. Finding a way out: lymphocyte egress from lymphoid organs. *Nat Immunol*. 2007; 8:1295–1301. [PubMed: 18026082]
15. Rosen H, et al. Modulating tone: the overture of S1P receptor immunotherapeutics. *Immunol Rev*. 2008; 223:221–235. [PubMed: 18613839]
16. Rivera J, Proia RL, Olivera A. The alliance of sphingosine-1-phosphate and its receptors in immunity. *Nat Rev Immunol*. 2008; 8:753–763. [PubMed: 18787560]
17. Mandala S, et al. Alteration of lymphocyte trafficking by sphingosine-1-phosphate receptor agonists. *Science*. 2002; 296:346–349. [PubMed: 11923495]
18. Brinkmann V, et al. The immune modulator FTY720 targets sphingosine 1-phosphate receptors. *J. Biol. Chem*. 2002; 277:21453–21457. [PubMed: 11967257]
19. Matloubian M, et al. Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. *Nature*. 2004; 427:355–360. [PubMed: 14737169]
20. Allende ML, Dreier JL, Mandala S, Proia RL. Expression of the sphingosine 1-phosphate receptor, S1P1, on T-cells controls thymic emigration. *J. Biol. Chem*. 2004; 279:15396–15401. [PubMed: 14732704]
21. Chi H, Flavell RA. Cutting edge: regulation of T cell trafficking and primary immune responses by sphingosine 1-phosphate receptor 1. *J Immunol*. 2005; 174:2485–2488. [PubMed: 15728452]
22. Graler MH, Huang MC, Watson S, Goetzl EJ. Immunological effects of transgenic constitutive expression of the type 1 sphingosine 1-phosphate receptor by mouse lymphocytes. *J Immunol*. 2005; 174:1997–2003. [PubMed: 15699128]

23. Ishii I, Fukushima N, Ye X, Chun J. Lysophospholipid receptors: signaling and biology. *Annu Rev Biochem.* 2004; 73:321–354. [PubMed: 15189145]
24. Sawicka E, et al. The sphingosine 1-phosphate receptor agonist FTY720 differentially affects the sequestration of CD4+/CD25+ T-regulatory cells and enhances their functional activity. *J Immunol.* 2005; 175:7973–7980. [PubMed: 16339533]
25. Fontenot JD, Dooley JL, Farr AG, Rudensky AY. Developmental regulation of Foxp3 expression during ontogeny. *J Exp Med.* 2005; 202:901–906. [PubMed: 16203863]
26. Lio CW, Hsieh CS. A two-step process for thymic regulatory T cell development. *Immunity.* 2008; 28:100–111. [PubMed: 18199417]
27. Burchill MA, et al. Linked T cell receptor and cytokine signaling govern the development of the regulatory T cell repertoire. *Immunity.* 2008; 28:112–121. [PubMed: 18199418]
28. Fontenot JD, et al. Regulatory T cell lineage specification by the forkhead transcription factor foxp3. *Immunity.* 2005; 22:329–341. [PubMed: 15780990]
29. Powrie F, Correa-Oliveira R, Mauze S, Coffman RL. Regulatory interactions between CD45RB^{high} and CD45RB^{low} CD4+ T cells are important for the balance between protective and pathogenic cell-mediated immunity. *J Exp Med.* 1994; 179:589–600. [PubMed: 7905019]
30. Theofilopoulos AN, Koundouris S, Kono DH, Lawson BR. The role of IFN-gamma in systemic lupus erythematosus: a challenge to the Th1/Th2 paradigm in autoimmunity. *Arthritis Res.* 2001; 3:136–141. [PubMed: 11299053]
31. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol.* 2003; 4:330–336. [PubMed: 12612578]
32. Sauer S, et al. T cell receptor signaling controls Foxp3 expression via PI3K, Akt, and mTOR. *Proc Natl Acad Sci U S A.* 2008; 105:7797–7802. [PubMed: 18509048]
33. Haxhinasto S, Mathis D, Benoist C. The AKT-mTOR axis regulates de novo differentiation of CD4+Foxp3+ cells. *J Exp Med.* 2008; 205:565–574. [PubMed: 18283119]
34. Knight ZA, et al. A pharmacological map of the PI3-K family defines a role for p110alpha in insulin signaling. *Cell.* 2006; 125:733–747. [PubMed: 16647110]
35. Nombela-Arrieta C, et al. A central role for DOCK2 during interstitial lymphocyte motility and sphingosine-1-phosphate-mediated egress. *J Exp Med.* 2007; 204:497–510. [PubMed: 17325199]
36. Carlson CM, et al. Kruppel-like factor 2 regulates thymocyte and T-cell migration. *Nature.* 2006; 442:299–302. [PubMed: 16855590]
37. Miyara M, Sakaguchi S. Natural regulatory T cells: mechanisms of suppression. *Trends Mol Med.* 2007; 13:108–116. [PubMed: 17257897]
38. Ledgerwood LG, et al. The sphingosine 1-phosphate receptor 1 causes tissue retention by inhibiting the entry of peripheral tissue T lymphocytes into afferent lymphatics. *Nat Immunol.* 2008; 9:42–53. [PubMed: 18037890]
39. Belkaid Y, Piccirillo CA, Mendez S, Shevach EM, Sacks DL. CD4+CD25+ regulatory T cells control *Leishmania* major persistence and immunity. *Nature.* 2002; 420:502–507. [PubMed: 12466842]
40. Lund JM, Hsing L, Pham TT, Rudensky AY. Coordination of early protective immunity to viral infection by regulatory T cells. *Science.* 2008; 320:1220–1224. [PubMed: 18436744]
41. Takabe K, Paugh SW, Milstien S, Spiegel S. “Inside-out” signaling of sphingosine-1-phosphate: therapeutic targets. *Pharmacol Rev.* 2008; 60:181–195. [PubMed: 18552276]
42. Bensinger SJ, et al. Distinct IL-2 receptor signaling pattern in CD4+CD25+ regulatory T cells. *J Immunol.* 2004; 172:5287–5296. [PubMed: 15100267]
43. Crellin NK, Garcia RV, Levings MK. Altered activation of AKT is required for the suppressive function of human CD4+CD25+ T regulatory cells. *Blood.* 2007; 109:2014–2022. [PubMed: 17062729]
44. Sehrawat S, Rouse BT. Anti-Inflammatory Effects of FTY720 against Viral-Induced Immunopathology: Role of Drug-Induced Conversion of T Cells to Become Foxp3+ Regulators. *J Immunol.* 2008; 180:7636–7647. [PubMed: 18490766]

45. Daniel C, et al. FTY720 ameliorates Th1-mediated colitis in mice by directly affecting the functional activity of CD4+CD25+ regulatory T cells. *J Immunol.* 2007; 178:2458–2468. [PubMed: 17277153]
46. Payne SG, et al. The immunosuppressant drug FTY720 inhibits cytosolic phospholipase A2 independently of sphingosine-1-phosphate receptors. *Blood.* 2007; 109:1077–1085. [PubMed: 17008548]
47. Wan YY, Chi H, Xie M, Schneider MD, Flavell RA. The kinase TAK1 integrates antigen and cytokine receptor signaling for T cell development, survival and function. *Nat Immunol.* 2006; 7:851–858. [PubMed: 16799562]
48. Mitchell TC, et al. Immunological adjuvants promote activated T cell survival via induction of Bcl-3. *Nat Immunol.* 2001; 2:397–402. [PubMed: 11323692]
49. Menon S, et al. COP9 signalosome subunit 8 is essential for peripheral T cell homeostasis and antigen receptor-induced entry into the cell cycle from quiescence. *Nat Immunol.* 2007; 8:1236–1245. [PubMed: 17906629]

Figure 1. S1P₁ negatively regulates thymic Foxp3⁺ T_{reg} population

(a,b) Flow cytometry of total and gated CD4SP thymocytes isolated from wild-type (WT) control, S1P₁-KO (a) and S1P₁-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 S1P₁. Bone marrow stem cells from WT mice were transduced with retrovirus expressing S1P₁ (S1P₁-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 S1P₁-KO or S1P₁-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. S1P₁ blocks thymic differentiation of T_{reg} cells

(a) Flow cytometry of total and gated CD4SP thymocytes isolated from WT control, S1P₁-KO and S1P₁-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, S1P₁-KO and S1P₁-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 S1P₁-KO T_{reg} cells
(a) Flow cytometry of gated CD4 T cells from the spleen and peripheral lymph nodes (PLN) of WT and S1P₁-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 S1P₁-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 S1P₁-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 S1P₁-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 S1P₁-Tg T_{reg} cells *in vitro* and *in vivo*

(a) *In vitro* T-cell suppression assays using Foxp3⁺ T_{reg} cells from WT and S1P₁-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 S1P₁-expressing retrovirus. WT T_{reg} cells were transduced with S1P₁-expressing (S1P₁-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 S1P₁-Tg T_{reg} cells to control colitis *in vivo*. T_{conv} cells were transferred alone or in combination with WT or S1P₁-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. S1P₁-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 S1P₁-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 S1P₁-Tg mice (2 months). Data are representative of 6 independent experiments. (c) Titers of anti-nuclear antigen and anti-dsDNA 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 S1P₁-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 S1P₁-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. S1P₁ 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 S1P₁-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 S1P₁-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 S1P₁-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. S1P₁ 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 S1P₁-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 S1P₁ 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.