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ARTICLE

Ssu72 is a T-cell receptor-responsive modifier that is indispensable for regulatory T cells

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The homeostatic balance between effector T cells and regulatory T cells (Tregs) is crucial for adaptive immunity; however, epigenetic programs that inhibit phosphorylation to regulate Treg development, peripheral expression, and suppressive activity are elusive. Here, we found that the Ssu72 phosphatase is activated by various T-cell receptor signaling pathways, including the T-cell receptor and IL-2R pathways, and localizes at the cell membrane. Deletion of Ssu72 in T cells disrupts $CD4^+$ T-cell differentiation into Tregs in the periphery via the production of high levels of the effector cytokines IL-2 and IFNγ, which induce $CD4^+$ T-cell activation and differentiation into effector cell lineages. We also found a close correlation between downregulation of Ssu72 and severe defects in mucosal tolerance in patients. Interestingly, Ssu72 forms a complex with PLCγ1, which is an essential effector molecule for T-cell receptor signaling as well as Treg development and function. Ssu72 deficiency impairs PLCγ1 downstream signaling and results in failure of Foxp3 induction. Thus, our studies show that the Ssu72-mediated cytokine response coordinates the differentiation and function of Treg cells in the periphery.

Keywords: Ssu72; Regulatory T cells; FoxP3; T cell receptor; Autoimmunity

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INTRODUCTION

Forkhead box P3 (Foxp3)-expressing regulatory T cells (Tregs) are a suppressive subset of $CD4^+$ T cells that control autoimmune, allergic, infection, and tumor responses.[1–3] $Foxp3^+$ Tregs can be divided into two main subgroups: thymus-derived Tregs (tTregs), which are generated in the thymus, and peripherally induced Tregs (pTregs), which are induced from naive $CD4^+$ T cells in the periphery.[4,5] The T-cell receptor (TCR) repertoires of tTregs and pTregs are different. Interestingly, tTregs develop from precursors expressing TCRs with high affinity for self antigen–MHC complexes present on thymic antigen-presenting cells.[6] Thus, most of the $Foxp3^+$ Tregs recirculating in the lymphoid organs of healthy mice originate in the thymus.[7] However, a large proportion of pTregs derived from conventional T cells that have not been negatively selected in the thymus are found in the gut (particularly in the lamina propria and gut-associated lymphoid tissues) under tolerogenic conditions.[8,9] Therefore, pTregs are thought to be mainly responsible for tolerance to nonself antigens, such as allergens, ingested dietary components, and fetal antigens,[10] whereas tTregs are preferentially involved in the regulation of autospecific responses.[11]

Thymic and mature T-cell development occur in response to TCR signaling. Upon recognition of an antigen, naive T cells in peripheral lymphoid tissues differentiate into distinct T-cell lineages, such as tumor antigen-specific effector T cells (Teffs) and pTregs, by clonal deletion.[12,13] However, it is unclear whether these distinct T-cell fates are determined by a unique TCR signaling mechanism and additional cell fate-specifying signals. Numerous Treg-cell-specific factors, including the IL-2 receptor (CD25), CTLA4, IKZF4 (Eos), Helios, and Neuropilin 1 (Nrp-1), are expressed independently of the Foxp3 protein.[9] Because both pTregs and tTregs express CD25 and Foxp3, distinguishing between the two populations in peripheral organs remains a challenge. Currently, Nrp-1 and Helios are two candidates utilized for this differentiation. However, recent studies strongly suggest that it is inappropriate to limit the selection of Teff, pTreg or tTreg populations based on a simple comparison between Nrp-1 and Helios expression.[14,15] Moreover, the relative contribution of tTregs and pTregs to disease pathophysiology also remains elusive. Recent progress suggests developmental and functional differences between tTreg and pTreg cells,[13] although the precise mechanisms underlying the differences remain to be clarified. In particular, specific epigenetic modifications involved in transcriptional regulation, including acetylation, methylation and phosphorylation, regulate signaling mechanisms to promote homeostasis of either tTregs or pTregs.[16–18] However, the specific epigenetic modifications involved remain to be elucidated. Unlike tTreg cells, induced Treg cells (iTregs) generated in vitro have

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been shown to be unstable. However, epigenetic programs facilitate the conversion of naive and effector or memory T cells and even unstable iTreg cells into stable and persistent iTreg cells.[18]

In addition to kinases, phosphatases play a critical role in integrating intracellular signaling in Treg cells during autoimmunity. For example, phosphatase and tensin homolog (PTEN) is a dual protein phosphatase that negatively regulates the PI3K-AKT axis by blocking the conversion of phosphatidylinositol to PIP3.[19] It is highly expressed in Treg cells, regulating their differentiation.[20] Defective PTEN expression in humans and mice leads to autoimmune disorders, lymphoid hyperplasia, colitis, and lymphopenia.[21,22] Protein phosphatase 2A (PP2A) signaling was also shown to be crucial for Treg function, and mice deficient in PP2A-expressing Tregs developed spontaneous, severe, and progressive multiorgan autoimmune disease.[23] A recent study reported that the phosphorylation status of Foxp3 determined by protein phosphatase 1-mediated dephosphorylation is important in controlling Treg cell function in rheumatoid arthritis.[17] Therefore, dephosphorylation is a pivotal process for Treg cell development and homeostasis.

Ssu72 is a dual-specificity protein phosphatase and is expressed in a tissue-specific manner.[24] Recently, we showed that Ssu72 acts as a cohesin-binding phosphatase and interacts with Aurora B kinase to regulate duplicated sister chromatid separation.[25,26] We also found that deletion of hepatocyte-specific Ssu72 increased the incidence of fatty liver disease.[24] Notably, Ssu72 was activated by signaling from various receptors, including IL-2R and TCRs, after stimulation with PMA and ionomycin (in this study) and by signaling from TLRs (our unpublished observation). Thus, Ssu72 may act as a receptor signaling-responsive phosphatase in T cells. It is widely known that interactions of Foxp3 with other regulatory proteins are able to induce a common Treg-type gene expression pattern that cannot be achieved solely by Foxp3, indicating that other factors require interaction of Foxp3 with other regulatory proteins. Considering these results collectively, it appears likely that the Ssu72 phosphatase acts as a critical regulator in orchestrating various tissue-specific homeostatic functions. Thus, we present the first in vivo evidence suggesting that Ssu72 positively regulates IL-2 and TGFβ signaling at the initiation of Foxp3 expression. Moreover, Ssu72 plays an essential role in peripheral Treg differentiation and in the pathway leading to homeostasis of mucosal tolerance by balancing the numbers of pTreg cells and inflammatory Teffs.

RESULTS

Ssu72 is a TCR signaling-responsive factor involved in the differentiation of effector and regulatory T cells

To gain insight into the function of Ssu72, we examined its subcellular distribution in vivo. Immunohistochemical analysis of tissues from patients with renal disease revealed that Ssu72 was expressed in the nucleus and cytoplasm (Supplementary Fig. 1). In addition, we found clear plasma membrane localization of Ssu72 (Supplementary Fig. 1a). We also observed similar results with immunofluorescence staining of T cells isolated from mouse spleens (Fig. 1a; Supplementary Fig. 1b). While naive $CD4^+$ T cells isolated from naive wild-type mice had low basal expression of Ssu72 in the cytoplasm and nucleus, Ssu72 was strongly activated by anti-CD3/28 and observed to be abundantly expressed in the cytoplasm and nucleus. Interestingly, naive $CD4^+$ T cells, which are activated by anti-CD3/28 in combination with IL-2 and TGFβ, frequently exhibited recruitment of Ssu72 to the cytoplasmic face of the cell membrane (Fig. 1a), indicating that Ssu72 can traffic from the nucleus and cytoplasm to the cytoplasmic face of the plasma membrane in response to TCR signaling mechanisms.

Notably, single-cell RNA-seq analysis using IL-17-producing T_H17 cells isolated from a mouse model of autoimmune encephalomyelitis (EAE) revealed that Ssu72 was downregulated in T_H1/T_H17-like memory subpopulations.[27] Furthermore, we previously reported that Ssu72 overexpression attenuated collagen-induced arthritis (CIA) by potentially regulating the T_H17/Treg balance.[28] IL-6 and TGFβ are required for the differentiation of $CD4^+$ T cells into T_H17 cells. Stimulation with IL-2 and TGFβ promotes Treg differentiation. IL-2 controls the balance between T_H17 cells and Tregs by reducing IL-6 receptor expression and inducing Stat5 activation, which promotes Treg differentiation but suppresses T_H17 cell generation.[29,30] To investigate the association of Ssu72 with the differentiation of T_H17 cells or Tregs, we incubated T_H17- or Treg-polarizing cytokines with naive $CD4^+$ T cells (Fig. 1a). Interestingly, T cells stimulated with PMA plus ionomycin (PMA/Io), αCD3/28 (TCR), or IL-2 showed marked activation of Ssu72 expression compared with that in unstimulated naive $CD4^+$ T cells (Fig. 1b, c; Supplementary Fig. 1c). In contrast, the protein level of Ssu72 was reduced when naive $CD4^+$ T cells were treated with IL-6. The level of Ssu72 showed no significant change upon TGFβ stimulation, which induced apoptotic cell death of naive $CD4^+$ T cells. The level of PTEN, which has been well characterized with regard to Treg stability,[31] was slightly increased in response to TCR stimuli but was constant during IL-2 stimulation. These results indicate that Ssu72 may act as a receptor signaling-responsive phosphatase with a nonredundant role in regulating TCR- and/or IL-2-mediated receptor signaling.

To elucidate the role of Ssu72 in T-cell differentiation and function, we conditionally deleted the *Ssu72* gene in T cells. Mice carrying a floxed *Ssu72* gene were crossed with mice expressing CD4-Cre to delete the *Ssu72* gene in double-positive ($CD4^+CD8^+$) and CD4/CD8 single-positive T lymphocytes. In this study, $Ssu72^{flox/flox}$ mice are referred to as "wild-type (WT)", whereas $Ssu72^{flox/flox}$; CD4-Cre mice are designated "cKO" (Fig. 1d). In cKO mice, the *Ssu72* gene was successfully deleted in $CD4^+$ and $CD8^+$ cells (Fig. 1e, f). Although Ssu72 depletion started at the transitional $CD4^+CD8^+$ stage under double-positive conditions, the differentiation and maturation of T cells in the thymus otherwise progressed normally in cKO mice (Supplementary Fig. 2). We further isolated naive $CD4^+$ T cells from WT and cKO mice and compared the efficiency of their differentiation into various effector T-cell lineages. While polarization of cKO T cells into T_H17 populations appeared to be normal, Ssu72 deficiency enhanced the production of IFN-γ and IL-4 under T_H1- and T_H2-polarizing conditions, respectively (Fig. 1g, h). In addition, markedly fewer iTregs characterized by Foxp3 expression were detected among cKO $CD4^+$ T cells than among WT $CD4^+$ T cells. Taken together, these results suggest that Ssu72 deficiency increases $CD4^+$ T-cell differentiation into the T_H1 or T_H2 lineage while restricting $CD4^+$ T-cell differentiation into Treg cells.

Ssu72 controls pTreg development

Tregs differentiating from $Foxp3^-CD4^+$ T cells via TGFβ stimulation are termed iTreg cells when generated in vitro or pTreg cells when generated in vivo. Ssu72 deficiency resulted in a strong reduction in the iTreg population. Therefore, we investigated whether Ssu72 depletion affected the development of Treg cells in vivo. Both the thymus and spleen were isolated from WT and cKO mice, and the proportion of $Foxp3^+CD4^+$ T cells was analyzed (Fig. 2a, b). We found a lower proportion of Treg cells among thymocytes derived from cKO mice than among those derived from WT mice, whereas the frequency of Tregs was similar in both populations of splenocytes. However, compared to WT mice, cKO mice had an increased population of proliferating $BrdU^+$ Tregs and conventional $CD4^+$ T (T_{CONV}) cells in the spleen but not in the thymus. Given the suppression of iTreg differentiation in cKO mice, these results suggest that Ssu72-deficient tTregs proliferate in the peripheral lymphoid organs to compensate for the

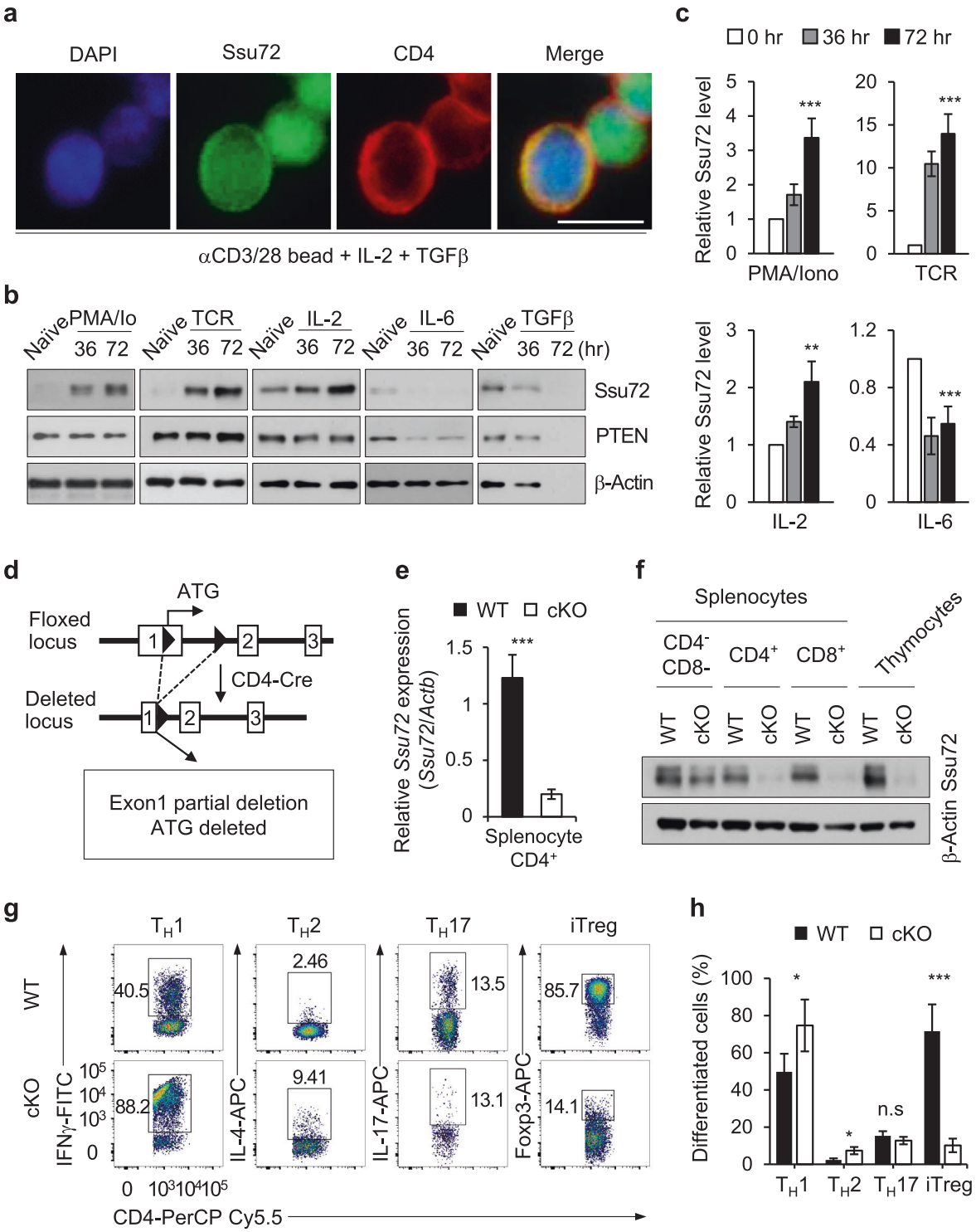

Fig. 1 Ssu72 is responsive to TCR signaling and regulates effector T-cell differentiation. **a** Sorted CD25^{-}CD44^{lo}CD62L^{hi} wild-type naive CD4^{+} T cells were stimulated by anti-CD3/28 beads in combination with IL-2 and/or TGFβ for 3 days. Cells were fixed, stained with anti-Ssu72 (green) and anti-CD4 (red) antibodies, and analyzed by fluorescence microscopy. Nuclei were stained with DAPI (blue). The scale bars represent 10 μM. **b** Sorted CD25^{-}CD44^{lo}CD62L^{hi} naive CD4^{+} T cells were treated with PMA/Io (PMA, 50 ng/mL; ionomycin, 500 ng/mL), TCR (plate-coated anti-CD3 antibody, 2 μg/mL; soluble anti-CD28 antibody, 1 μg/mL), IL-2 (20 ng/mL), IL-6 (40 ng/mL) or TGFβ (2 ng/mL) for the indicated time. The cells were harvested and subjected to immunoblotting. **c** Graph showing the relative intensity of Ssu72 normalized to that of β-Actin. **d** The Ssu72 floxed allele contains two loxP sites (black triangles) flanking exon 1 and including the ATG codon. Mice with floxed Ssu72 alleles (Ssu72$^{f/f}$, WT) were crossed with CD4-Cre mice to generate mice with T-cell-specific deletion of the Ssu72 allele (Ssu72$^{f/f}$;CD4-Cre, cKO). **e** The expression level of *Ssu72* mRNA in both WT and cKO sorted CD4^{+} T cells was measured and normalized to that of *Actb*. **f** Ssu72 expression was analyzed by immunoblotting. **g**, **h** Flow cytometric analysis to determine the differentiation efficiency by staining for IFNγ, IL-4, IL-17 or Foxp3 in WT and cKO naive T cells under various polarization conditions for 3 days. The data are presented as the mean ± SEM values. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; n.s not significant. *t* test

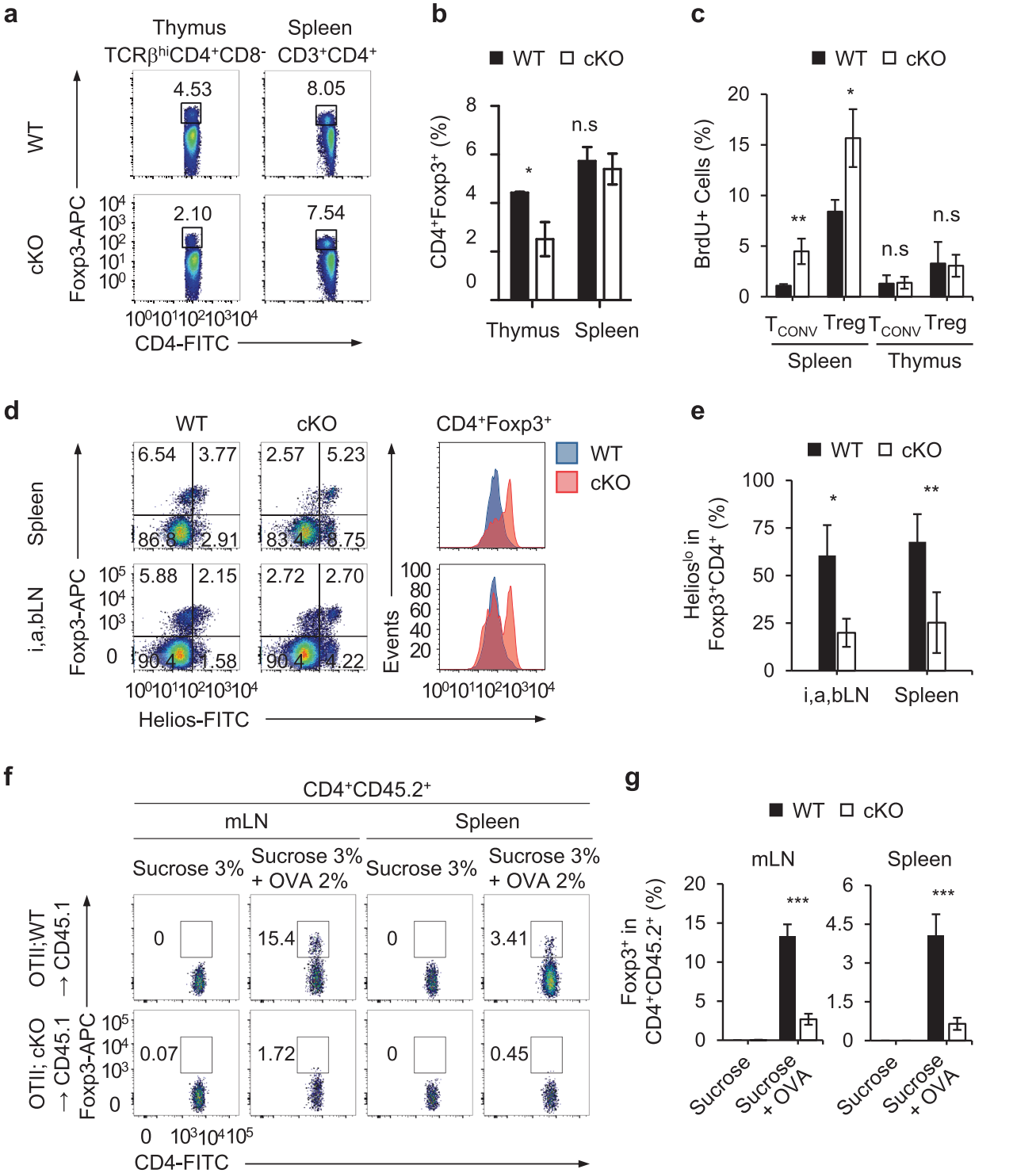

Fig. 2 Ssu72 is regulated during homeostasis maintenance of peripheral Treg cells. **a**, **b** Frequency of Foxp3$^+$ cells among TCRβ^{hi}CD4$^+$CD8$^-$ thymocytes and CD3$^+$CD4$^+$ splenocytes obtained from 11-week-old WT and cKO mice. The numbers indicate the proportions of gated Foxp3$^+$ cells (**a**). Graph summarizing the frequency of CD4$^+$Foxp3$^+$ T cells derived from the thymus and spleen (**b**) ($n = 4$ per group). **c** Analysis of BrdU incorporation (18 h pulse) in T_{CONV} (CD4$^+$Foxp3$^-$) and Treg (CD4$^+$Foxp3$^+$) T cells in spleens and thymuses obtained from WT and cKO mice. **d** Spleens and i.a.bLNs (inguinal, axillary, and brachial lymph nodes) obtained from 11-week-old WT and cKO mice were analyzed ($n = 4$ per group). **e** Graph showing the percentage of Helios^{lo} cells among Foxp3$^+$CD4$^+$ T cells from spleens and i.a.bLNs (inguinal, axillary, and brachial lymph nodes). **f**, **g** CD45.1$^+$ recipient mice were transferred with 10^6 naive CD4$^+$ T cells (CD4$^+$CD25$^-$CD44^{lo}CD62L^{hi}) sorted from OTII;WT or OTII;cKO mice (CD45.2$^+$). Recipient mice were fed either 3% sucrose or 3% sucrose containing 2% ovalbumin (OVA) in the drinking water for 5 days. The frequency of Foxp3$^+$ cells among CD45.2$^+$CD4$^+$ T cells was analyzed in mesenteric lymph nodes (mLNs) and spleens from recipient mice (**f**). Graph summarizing the frequency of CD45.2$^+$CD4$^+$Foxp3$^+$ T cells (**g**) ($n = 3$ per group). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; n.s not significant. *t* test

reduction in the Treg frequency, although the number of Tregs derived from the thymus was reduced (Fig. 2c).

We further compared pTreg development between WT and cKO T cells since Ssu72 depletion contributed to defective Foxp3 induction (Fig. 1g, h). Interestingly, the vast majority of Tregs derived from cKO peripheral lymphoid tissue expressed a high level of Helios, which has been shown to be constitutively expressed in thymus-derived tTregs but not in pTregs or in vitro-generated iTregs,[14] indicating that cKO mice exhibited a significant decrease in the proportion of pTregs (Fig. 2d, e). To further investigate this finding, we used a well-established mouse model of oral tolerance induced by ovalbumin (OVA) feeding. Naive $CD4^+CD45.2^+$ T cells isolated from OTII;WT and OTII;cKO mice were transferred into $CD45.1^+$ congenic mice, which were subsequently fed OVA for 5 days. The frequency of $Foxp3^+CD4^+$ T cells was significantly decreased in the mesenteric lymph nodes (mLNs) and spleens of OTII;cKO mice compared with OTII;WT mice (Fig. 2f, g). Taken together, these results indicate that Ssu72 is required for pTreg development in vivo and is essential for maintaining pTreg homeostasis.

Ssu72 is required for T-cell homeostasis

Since Treg cells are critical mediators of peripheral self-tolerance and immune homeostasis, we examined whether Ssu72 deletion in T cells alters the function of Tregs in systemic immune tolerance and homeostasis. In 11-week-old cKO mice, there was a significant reduction in the splenic $CD3^+$ T-cell population, while the absolute number and proportion of lymphocytes were similar to those in littermate WT controls (Supplementary Fig. 3a–c), indicating that Ssu72-deficient mice had smaller T-cell populations. In addition, no significant differences were observed in the size of the $CD4^+$ T-cell population among $CD3^+$ T cells between WT and cKO mice (Supplementary Fig. 3a). However, cKO mice had fewer $CD3^+CD8^+$ T cells but more $CD3^+CD4^-CD8^-$ cells, such as γδT cells and invariant natural killer T cells (Supplementary Fig. 3a, b). Ssu72-deficient T cells showed normal T-cell development and maturation in the thymus (Supplementary Fig. 2); therefore, it is likely that Ssu72 is required for the survival of naive T cells and peripheral T-cell homeostasis. We therefore analyzed the population of T cells expressing an activated T-cell phenotype by measuring the expression of surface markers such as CD44 and CD62L. In WT mice, most $CD4^+$ and $CD8^+$ T cells isolated from the spleen were in a naive state ($CD44^{lo}CD62L^{hi}$) (Fig. 3a). However, in cKO mice, 30% of $CD4^+$ T cells and 65% of $CD8^+$ T cells were classified as $CD44^{hi}$ (Fig. 3a, b). cKO mice had nearly 5-fold more $CD4^+CD44^{hi}CD62L^{lo}$ cells and 15-fold more $CD8^+CD44^{hi}CD62L^{lo}$ cells than WT mice. In addition, the signature markers of active/memory T cells, including CD122, CD69, KLRG1, and CXCR5, were increased in Ssu72-deficient T cells (Supplementary Fig. 3d). Consistent with these results, compared to WT Tregs, cKO Tregs ($CD4^+CD25^+$) exhibited decreased suppressive activity toward effector cells in vitro (Supplementary Fig. 4a, b). We also found that after αCD3/CD28 stimulation, most of the Ssu72-deficient T cells were highly susceptible to activation-induced cell death (AICD) compared to WT T cells (Supplementary Fig. 3e, f). These results indicate that T cells in cKO mice were hyperactivated in vivo and showed enhanced susceptibility to AICD. In vitro (Supplementary Fig. 4a, b), suppressive activity was examined by coculturing Tregs and effector cells under αCD3/CD28 stimulation. Even though the suppressive activity of cKO Tregs was reduced, given that these cells are susceptible to AICD, it is possible that they do not lose their suppressive activity but instead undergo AICD.

To confirm the function of Ssu72-deficient T cells expressing an active/memory-like phenotype, we further analyzed cytokine production by these cells. Splenic $CD4^+$ and $CD8^+$ T cells in cKO mice produced large amounts of IFNγ. In particular, more than 30% of IL-2^+CD4^+ T cells also produced IFNγ (Fig. 3c, d). Increased IFNγ production by cKO $CD4^+$ T cells was observed in 5-week-old mice and increased further in subsequent weeks. The proportion of $IFN\gamma^+CD8^+$ T cells was particularly elevated in cKO mice and was age dependent (Fig. 3e). Furthermore, analysis of mRNA expression revealed increased levels of *Il2* and *Ifng* in Ssu72-deficient $CD4^+$ T cells in 11-week-old mice (Fig. 3f). These results suggest that deficiency in Ssu72 independently leads to the activation of T cells.

Ssu72 controls Foxp3 induction

Ssu72 deficiency inhibited the differentiation of naive $CD4^+$ T cells into iTregs. This finding raised the possibility that Ssu72 is required for the induction of Foxp3 expression by a receptor-mediated signaling cascade. To test this hypothesis, we compared Foxp3 induction in WT and cKO T cells (Fig. 4a). Under iTreg-polarizing conditions that increased the population of $CD25^+Foxp3^+$ cells among WT naive $CD4^+$ T cells, the relative proportion of the $CD25^+Foxp3^+$ population generated from cKO naive $CD4^+$ T cells was substantially lower than that generated from WT naive $CD4^+$ T cells. Induction of Foxp3 and CD25 is a hallmark of IL-2-mediated iTreg polarization.[32] While analysis of Foxp3 mRNA expression in WT $CD4^+$ T cells eventually showed marked induction of Foxp3 under iTreg-polarizing conditions, Ssu72-deficient T cells displayed marked abrogation of Foxp3 expression. Taken together, these data indicate that Ssu72 deficiency in $CD4^+$ T cells leads to severe defects in iTreg generation due to the absence of Foxp3 (Fig. 4b).

To further determine whether impaired iTreg generation correlates with loss of Ssu72 phosphatase activity, we compared the ability of Ssu72 WT with that of the Ssu72 C12S mutant (with loss of phosphatase activity[26]) to rescue Foxp3 induction (Supplementary Fig. 5a). Based on evaluation of WT $CD4^+$ T cells transfected with empty vector, 3 days was sufficient to induce iTreg generation. Although Foxp3 induction was not observed in transfected cKO CD4+ T cells, only cKO $CD4^+$ T cells overexpressing wild-type Ssu72 survived for 3 days (Supplementary Fig. 5b, c). After an additional 3 days, overexpression of Ssu72 WT in cKO T cells partially rescued Foxp3 induction compared to that in cells expressing the Ssu72 C12S mutant (Supplementary Fig. 5d, e), indicating that the catalytic phosphatase activity of Ssu72 is essential in the prevention of AICD and induction of Foxp3 expression for iTreg differentiation.

To characterize the status of cKO $CD4^+$ T cells after iTreg stimulation, we determined the transcription levels of effector cytokines for helper T cells. In WT T cells, the expression of *Il2*, *Ifng*, and *Il17* was increased in the early stages of iTreg differentiation and was eventually downregulated. However, cKO T cells showed impaired regulation of *Il2*, *Ifng*, and *Il17* expression, as the expression of all increased dramatically, and the increased expression was sustained (Fig. 4c). Moreover, cKO T cells exhibited upregulation of genes encoding signature helper T-cell molecules, including T_H1-, T_H2-, and T_H17-related cytokines, during the 72 h post conditioning of iTreg polarization (*Ifng*, T_H1; *Il4* and *Il10*, T_H2; *Il17*, T_H17) (Fig. 4d). Consistent with these results, the levels of the mRNAs encoding the T_H1- and T_H2-related transcription factors *Tbx21* and *Gata3*, respectively, were also increased in cKO T cells. cKO T cells exhibited increased potential to undergo T_H1 and T_H2 polarization (Fig. 1e). This finding raised the possibility that Ssu72 prevents the differentiation of naive T cells into T_H1 and T_H2 cells via inhibition of *Tbx21* and *Gata3* induction. However, *Il17* expression was also upregulated in cKO T cells, although the expression of *Rorc*, which is the master transcription factor for IL-17 expression, was comparable between WT and cKO T cells. The mRNA level of *Tgfb1*, which is expressed and secreted by Treg cells, remained unchanged. These results indicate that naive $CD4^+$ T cells derived from cKO mice do not acquire the characteristics of Treg cells in response to iTreg-polarizing stimulation. To support this finding, we characterized the identity of cKO T cells at 72 h post iTreg stimulation using the defined Treg signature[33] (Fig. 4e).

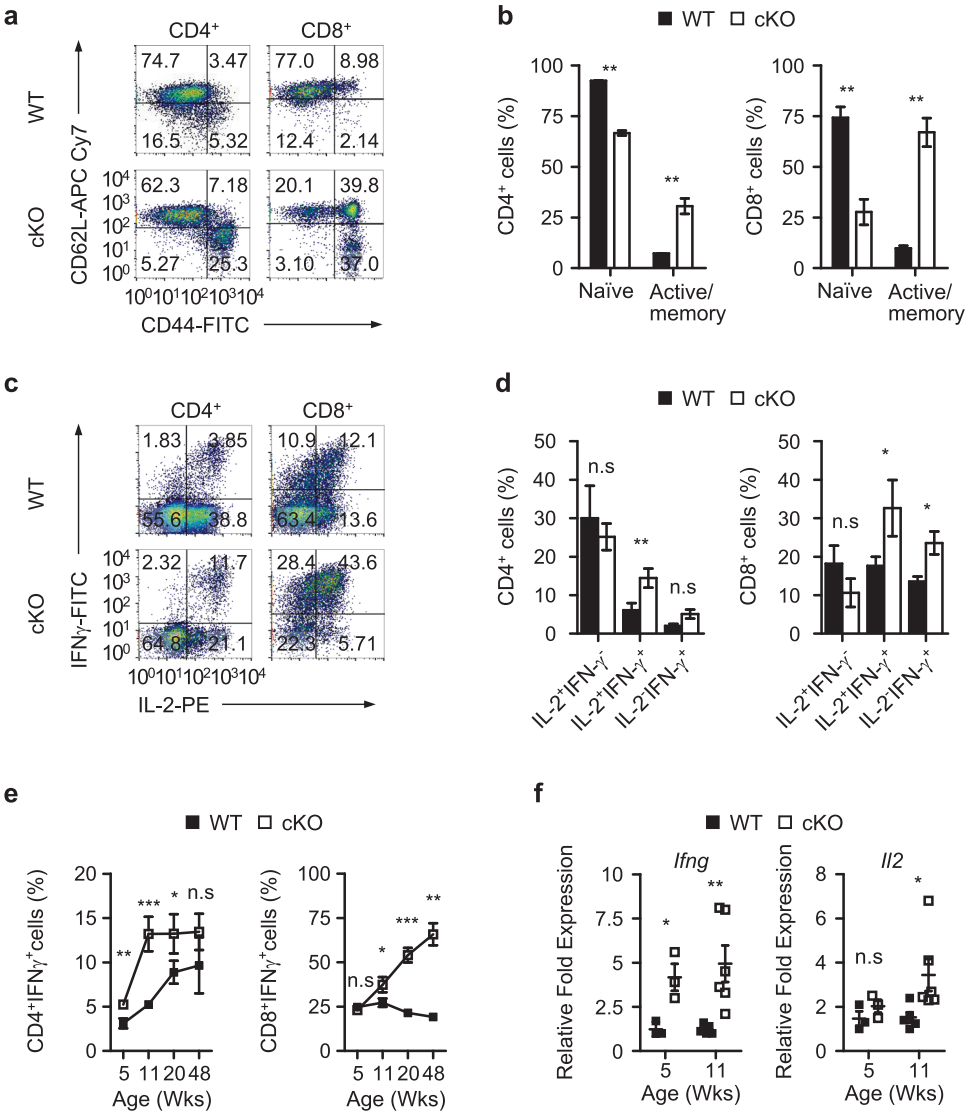

Fig. 3 Ssu72 deficiency causes activation of T cells in vivo. **a** Frequencies of $CD44^{lo}CD62L^{hi}$ (naive) and $CD44^{hi}$ (active/memory) $CD4^+$ or $CD8^+$ T cells among $CD3^+$ splenocytes derived from 11-week-old WT and cKO mice. **b** Graph showing the proportions of naive and active/memory $CD4^+$ or $CD8^+$ T cells assessed as in **a** ($n = 4$ per group). **c**, **d** Total splenocytes isolated from 11-week-old WT and cKO mice were incubated with PMA (50 ng/mL), ionomycin (500 ng/mL), and brefeldin A (BFA) for 5 h. The cells were analyzed by flow cytometry **c**. The results from three independent experiments are summarized in **d** ($n = 4$ per group). **e** Splenocytes from WT or cKO mice of different age groups were stimulated with PMA, ionomycin and BFA as shown in **c**. The graph shows the frequencies of $CD4^+IFN\gamma^+$ and $CD8^+IFN\gamma^+$ T cells among CD3-expressing cells obtained from WT and cKO mice in different age groups ($n = 4$ per group). **f** Levels of *Ifng* and *Il2* mRNA were measured in $CD4^+$ T cells obtained from 5- and 11-week-old WT and cKO mice. mRNA expression was assessed by qRT-PCR and normalized to *Actb* expression ($n = 4$ per group). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; n.s not significant. *t* test

qRT-PCR analysis revealed that in cKO cells, several Treg signature genes upregulated in freshly isolated Treg cells compared with freshly isolated $CD25^-CD4^+$ T cells (Fig. 4e; upper panel) were downregulated; in addition, the effector signature genes, which were downregulated in Treg cells compared with $CD25^-CD4^+$ T cells after in vitro activation (Fig. 4e; lower panel), were significantly upregulated. These results demonstrate that Ssu72-deficient $CD4^+$ T cells fail to differentiate into iTreg cells. Taken together, our results show that Ssu72 regulates iTreg cell lineages by potentially suppressing proinflammatory effector cells.

The Ssu72-PLCγ1 interaction is essential for the development of regulatory T cells in the periphery

The essential cytokines involved in the differentiation of iTregs appear to be TGFβ and IL-2. Although TGFβ is the most potent cytokine in iTreg differentiation, IL-2-deficient T cells lack the capability for TGFβ-induced iTreg differentiation.[34] In addition, it has been reported that stimulation with IL-2 alone induces Foxp3 expression.[35] Thus, to examine the effect of Ssu72 on the IL-2 and TGFβ signaling cascades, we compared IL-2- and TGFβ-dependent signaling pathways between WT and cKO $CD4^+$ T cells (Fig. 4f). Stat5 and Smad2/3 were phosphorylated by IL-2 and TGFβ stimulation in WT $CD4^+$ T cells, whereas cKO T cells showed limited phosphorylation of Stat5 and Smad2/3 under iTreg-polarizing conditions. This pattern raises the possibility that Ssu72-deficient T cells do not respond to upstream signals from iTreg-inducing cytokines via hypophosphorylation of components of the Stat5-Smad2/3 signaling axis. However, we were unable to observe a direct interaction of Ssu72 with Stat5 or Smad2/3 (data not shown).

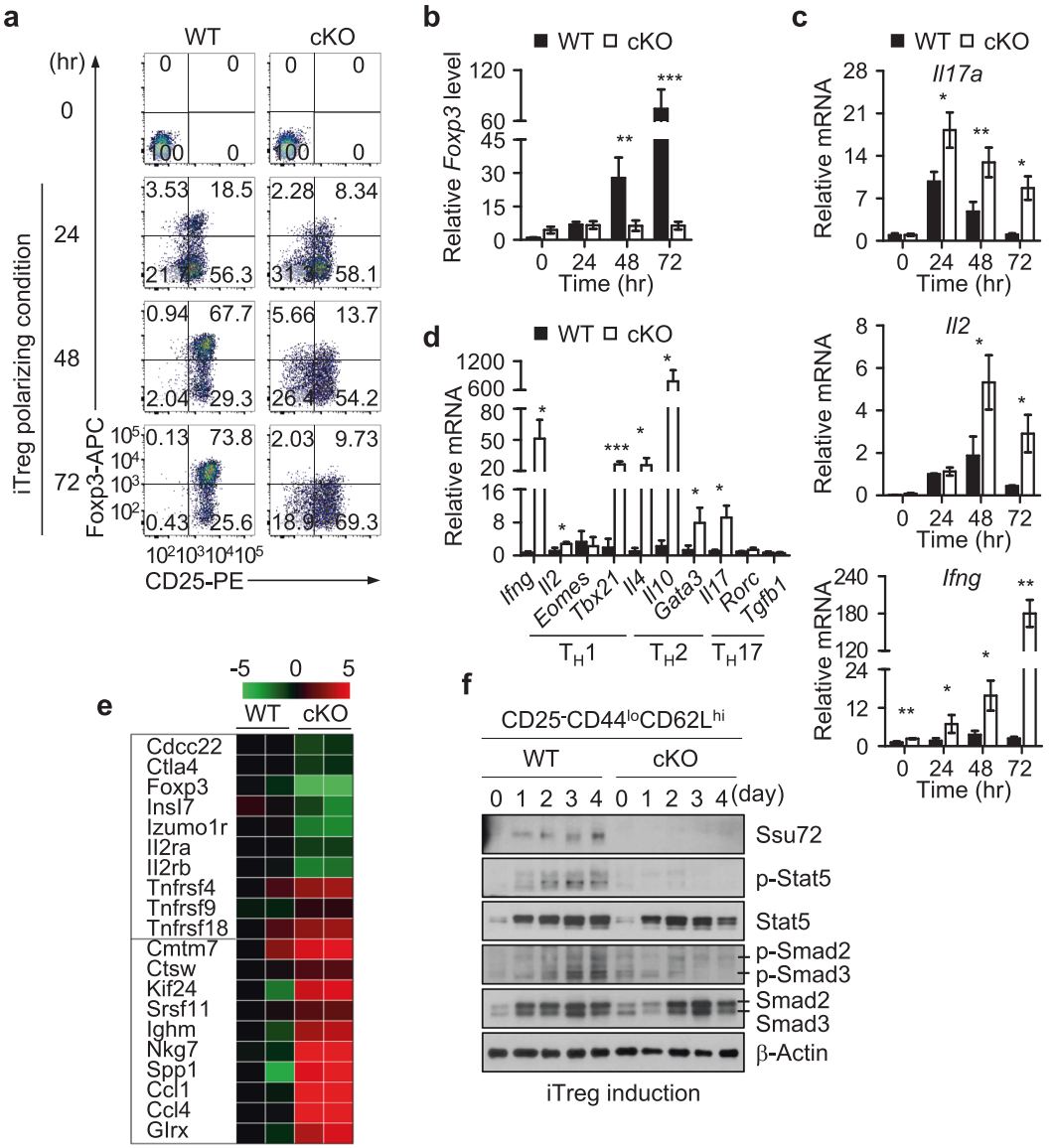

Fig. 4 Ssu72 controls iTreg lineage commitment. **a** Sorted CD25^{-}CD44^{lo}CD62L^{hi} naive WT and cKO CD4^{+} T cells were stimulated with anti-CD3 and CD28 antibodies in the presence of IL-2 and TGF-β for the indicated times and analyzed by flow cytometry ($n = 4$ per group). **b** Cells stimulated as in **a** were lysed at the indicated time points, and the expression of *Foxp3* was analyzed by qRT-PCR ($n = 3$ per group). **c** Time course of *Il2*, *Ifng*, and *Il17a* mRNA expression in WT and cKO naive CD4^{+} T cells differentiated for the indicated times under iTreg-polarizing conditions ($n = 3$ per group). **d** Cells stimulated as in **a** were lysed, and the mRNA expression of the indicated genes was measured by qRT-PCR and normalized to that of *Actb* ($n = 3$ per group). **e** Naive CD4^{+} T cells sorted from WT and cKO mice were differentiated into iTreg cells. The mRNA expression of the indicated genes was analyzed by qRT-PCR. The expression levels of genes are presented as Log2 transformed fold change values relative to WT ($n = 2$ per group). RNA was obtained from two independent experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; n.s not significant. *t* test. **f** Sorted CD25^{-}CD44^{lo}CD62L^{hi} naive WT and cKO CD4^{+} T cells were stimulated with anti-CD3 and CD28 antibodies in the presence of IL-2 and TGFβ for the indicated time and were harvested for immunoblot analyses

We next determined the interaction of Ssu72 with a series of signaling molecules downstream of the TCR, CD28, and IL-2 receptors. Jurkat T cells were stimulated with anti-CD3 and CD28 antibodies, which are sufficient to activate proximal TCR signaling (Supplementary Fig. 6a). Our binding assay using cellular extracts isolated from activated Jurkat T cells revealed that the interaction between GST-Ssu72 and endogenous PLCγ1 was distinctly detectable in both untreated and anti-CD3/anti-CD28 (αCD3/28)-treated Jurkat T cells and that higher PLCγ1 levels were observed in Ssu72-bound complexes in αCD3/28-treated Jurkat T cells (Supplementary Fig. 6b). Under the same experimental conditions, we were unable to detect physical interactions between Ssu72 and other signaling molecules downstream of the TCR, CD28, and IL-2 receptors, including mTOR, PI3K, LAT, LCK, SLP76, and AKT (Supplementary Fig. 6b and data not shown). Cellular extracts were also immunoprecipitated with an anti-Ssu72 antibody and immunoblotted with an anti-PLCγ1 antibody (Fig. 5a). To determine whether the interaction between Ssu72 and PLCγ1 is direct or indirect, recombinant His-PLCγ1 was incubated with WT and C12S mutant GST-Ssu72. We found a direct interaction of Ssu72 with PLCγ1 that was independent of its catalytic activity (Supplementary Fig. 6c). To further examine whether Ssu72 can dephosphorylate PLCγ1, we employed Phos-tag gel electrophoresis, in which the movement of phosphorylated proteins is retarded. As shown in Fig. 5b, overexpression of WT Ssu72 increased PLCγ1 hypophosphorylation, whereas overexpression of

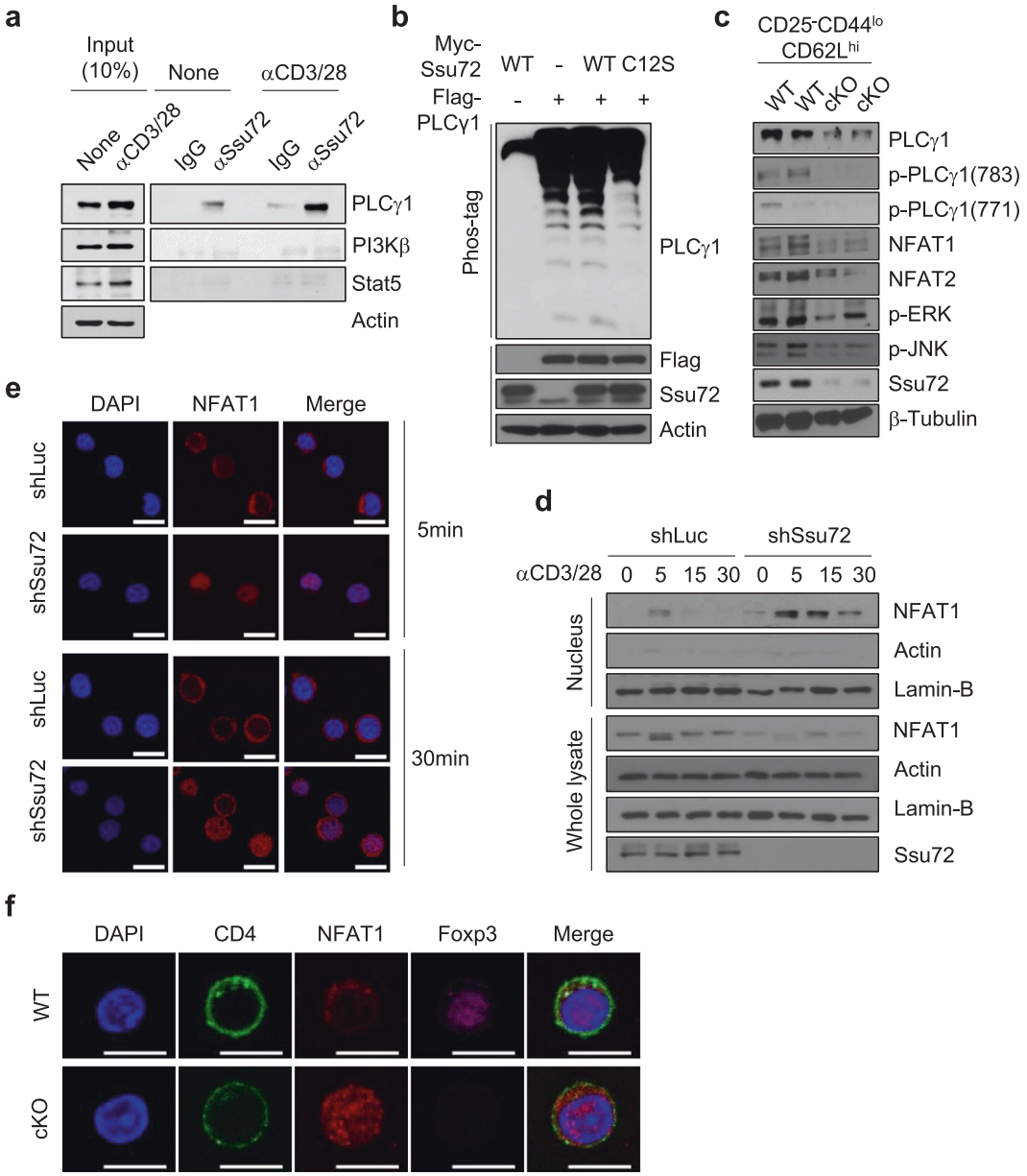

Fig. 5 The Ssu72-PLCγ1 interaction is essential for TCR-mediated signaling. **a** Cellular extracts as described in Fig. 4b were further immunoprecipitated with an anti-Ssu72 antibody or normal IgG and detected by immunoblotting with the indicated antibodies. **b** Phos-tag gel electrophoresis analyses and immunoblots of PLCγ1 in 293T cells transfected with the indicated plasmids. **c** Sorted CD25$^-$CD44^{lo}CD62L^{hi} naive T cells were isolated from 7-week-old WT and Ssu72 cKO mice, and cell extracts were immunoblotted with the indicated antibody. **d** Immunoblot analysis of nuclear NFAT1 in control and Ssu72-depleted Jurkat T cells during anti-CD3/28 stimulation for the indicated times. **e** The localization of NFAT1 (red) was examined by immunofluorescence staining of Jurkat T cells from **d**. Nuclei were stained with DAPI (blue). The scale bar represents 20 μm. **f** Isolated naive T cells from WT and Ssu72 cKO mice were incubated with anti-CD3/28 antibodies, IL-2, and TGFβ for 3 days and were then harvested. The localization of NFAT1 (red) was examined by immunofluorescence staining. Cells were costained with DAPI (blue) and for CD4 (green) and Foxp3 (magenta). The scale bar represents 10 μM

the Ssu72 C12S mutant enhanced PLCγ1 hyperphosphorylation. Notably, the Ssu72 C12S mutant appeared to act as a dominant negative mutant by inhibiting the activity of endogenous Ssu72. Taken together, these results indicate that the phosphorylation of PLCγ1 is regulated by the Ssu72 phosphatase.

PLCγ1 is an essential effector molecule in TCR signaling.[36] PLCγ1 deficiency affects positive and negative selection, significantly reduces the populations of single-positive thymocytes and peripheral T cells and impairs TCR-induced proliferation and cytokine production.[37] PLCγ1 deficiency also impairs the development and function of FoxP3$^+$ regulatory T cells, causing inflammatory/autoimmune symptoms. LAT (linker for activation of T cells) is an important scaffold that coordinates PLCγ1 signaling downstream of TCR stimulation.[38,39] Phosphorylation of LAT at Y132 leads to recruitment of PLCγ1 after its phosphorylation by ZAP-70. Importantly, conditional inhibition of the LAT-PLCγ1 interaction impairs TCR-mediated signaling, proliferation, and IL-2 production.[40] Furthermore, Foxp3$^+$ nTreg cells from mice with LAT-PLCγ1 interaction deficiency were unable to suppress the proliferation of conventional T cells, indicating that the LAT-PLCγ1 interaction is essential for the suppressive function of CD4$^+$CD25$^+$ regulatory T cells.

Subsequent immunoblot analysis using CD3+ T-cell (CD25$^-$CD44^{lo}CD62L^{hi} cells) extracts isolated from WT and cKO

mice revealed that T cells from cKO mice displayed marked reductions in PLCγ1 expression and PLCγ1 phosphorylation at tyrosine 783 (p-PLCγ1 Y783) (Fig. 5c). Notably, mutation of PLCγ1 Y783 was shown to abolish PLCγ1 signaling.[41] Although the basal activities of ERK and JNK were high in T cells from WT mice, activation of these kinases was reduced in cKO mice (Fig. 5c). Moreover, regardless of αCD3/28 stimulation, TCR-induced activation of the translocation of certain transcription factors—nuclear factor of activated T cells (NFAT)1, NFAT2—and activation of phospho-ERK and phospho-JNK was severely impaired in T cells from cKO mice compared with those from WT mice (Fig. 5c). Collectively, these data demonstrate that PLCγ1 plays a central role in TCR-mediated activation of multiple signaling pathways. Importantly, PLCγ1 deficiency deregulates TCR-induced proliferation and cytokine production, as well as activation of ERK, JNK, and NFAT, and thus impairs the development and function of FoxP3$^+$ regulatory T cells.[37]

Considering that the expression levels of IFNγ and IL-2 were increased in cKO T cells (Fig. 3), we further investigated PLCγ1 downstream signaling. Activation of PLCγ1 by TCR engagement is followed by the Ca2+ release from stores, which causes the translocation of NFAT into the nucleus to activate its target genes, such as Ifng and *Il2*.[42,43] To determine how the downregulated but hyperphosphorylated PLCγ1 resulting from Ssu72 depletion affects NFAT activity, we generated Jurkat cells expressing short hairpin RNAs targeting luciferase (shLuc) and Ssu72 (shSsu72). Similar to the results shown in Fig. 5c, the level of NFAT1 was lower in in the total lysate from cells transfected with shSsu72 than in the total lysate from cells transfected with shLuc. After TCR stimulation, NFAT1 was translocated to the nucleus, but prolonged TCR stimulation induced its return to the cytoplasm (Fig. 5d, e; Supplementary Fig. 6d). However, depletion of Ssu72 increased the levels of nuclear NFAT1 after TCR stimulation. Interestingly, we observed that the localization of NFAT1 to the nucleus was sustained in cells with Ssu72 depletion, despite prolonged TCR stimulation (Fig. 5d, e; Supplementary Fig. 6d). Given that Ssu72 directly dephosphorylates PLCγ1 (Fig. 5a), even though the levels of both PLCγ1 and NFAT1 were diminished, the residual hyperphosphorylated and activated PLCγ1 still induced NFAT translocation into the nucleus and resulted in the expression of target genes such as IL-2 and INFγ.

Since NFAT1 plays a crucial role in the function of Treg cells,[42,44,45] we examined the localization of NFAT1 under iTreg-polarizing conditions. In WT Foxp3$^+$CD4$^+$ iTreg cells, NFAT1 was largely localized to the cytoplasm (Fig. 5f), consistent with a previous study.[45] However, NFAT1 was concentrated in the cytoplasm of Foxp3$^+$CD4$^+$ cKO cells after iTreg polarization (Fig. 5f), indicating that loss of Ssu72 dysregulates the subcellular distribution of NFAT1. In summary, the Ssu72-PLCγ1 interaction appears to be an important signaling event for the development of regulatory T cells in the periphery, and the defective Foxp3 induction in cKO T cells may be attributed to unresponsive TCR signaling.

Ssu72 in CD4$^+$ T cells is indispensable for peripheral tolerance

The expansion of pTregs that can recognize nonself antigens appears to be essential for the maintenance of tolerance at mucosal sites, such as the intestine and lung.[46] Because Ssu72 deficiency affected the differentiation of pTregs (Fig. 2), we reasoned that Ssu72 depletion may affect mucosal homeostasis. Therefore, we analyzed T-cell activation in the small intestinal lamina propria, lungs, and Peyer's patches. The populations of naive CD4$^+$ and CD8$^+$ T cells (CD44^{lo}CD62L^{hi}) were decreased by 20% and 65%, respectively, in Peyer's patches of cKO mice compared to those of WT mice (Fig. 6a). These findings were consistent with observations in the spleen (Fig. 3). Notably, the vast majority of T cells isolated from the intestinal lamina propria and lungs of cKO mice were in an active state (CD44^{hi}), with few naive CD4$^+$ and CD8$^+$ T cells in either tissue (Fig. 6a).

Recent reports have also suggested that pTregs (but not tTregs) play a suppressive role that manifests mainly in the intestine.[47] Based on our findings of defective pTreg differentiation in cKO mice, we assumed that cKO mice might exhibit impaired mucosal immunity associated with the gut and lung. Compared with WT mice, cKO mice had substantially longer small intestines, and the length increased with age (Fig. 6b, c). We also observed that cKO mice had a thickened smooth muscle layer and enlarged goblet cells, suggesting enteritis (Fig. 6d). In adult mice, we rarely observed severe inflammation in the peripheral tissues of cKO mice, with the exception of the intestine (data not shown). However, histological examination revealed mild mononuclear cell infiltration in the lungs of 1-year-old cKO mice (Fig. 6e; left panel). The lungs of cKO mice also had significantly higher numbers of CD3$^+$ cells (Fig. 6e; right panel and Supplementary Fig. 7). To determine the associations between Ssu72 deficiency and both defective pTregs and exacerbation of inflammation, we isolated lymphocytes from the spleen, small intestinal lamina propria and lung and analyzed IFNγ and IL-17 expression in these cells. CD4$^+$ T cells from cKO mice displayed an enhanced inflammatory response, with an increased proportion of IL-17$^+$ and IFNγ$^+$ cells compared with that in their WT littermates (Fig. 6f). Interestingly, cKO mice had a significantly larger proportion of IFNγ/IL-17 double-positive cells, which exhibited pathogenic potential for autoimmune disease, than of single-positive cells.[48] These results suggest that Ssu72 is required for appropriate pTreg differentiation to control mucosal immunity.

Ssu72 deficiency in T cells is associated with susceptibility to inflammatory bowel disease

To further understand the abovementioned findings, we used the dextran sulfate sodium (DSS)-induced colitis mouse model, which is a well-established system for analyzing the T-cell response to mucosal damage. We induced acute colitis in mice via oral administration of 2.5% DSS in the drinking water. Ssu72 is essential for maintaining pTreg homeostasis and mucosal tolerance; therefore, we hypothesized that cKO mice would exhibit an exacerbated disease phenotype of DSS-induced colitis. The disease activity index of DSS-induced colitis in WT and cKO mice was calculated based on substantial diarrhea, loss of fecal consistency, and appearance of visible blood in the stool, as well as weight loss. As expected, acute colitis was exacerbated in cKO mice compared with WT mice. Strikingly, Ssu72 depletion in T cells was associated with decreased survival and a drastic reduction in body weight (Fig. 7a–c). Moreover, gross tissue morphology revealed that cKO mice had significant mucosal hyperemia and colon ulceration. Colon lengths on day 8 were reduced in cKO mice compared with WT mice (Fig. 7d, e). Histological analysis showed increased mononuclear cell infiltration, significant mucosal damage, and epithelial necrosis in the colons of DSS-treated cKO mice (Fig. 7f). Although there was no significant difference in the frequency of IFNγ-expressing cells within the splenic CD4$^+$ T-cell population between DSS-treated WT and cKO mice, the proportions of IFNγ$^+$CD4$^+$ T cells in mLNs and IL-17$^+$CD4$^+$ T cells in mLNs and the spleen were significantly increased in cKO mice compared with WT mice (Fig. 7g), indicating that Ssu72 deficiency was associated with enhanced susceptibility to inflammatory bowel disease (IBD).

Based on our finding that cKO mice exhibit enhanced susceptibility to DSS-induced colitis, we compared Ssu72 levels between human colon tissues. We examined tissues from 11 healthy donors and 30 patients with IBD (ten with inactive ulcerative colitis [iUC], ten with active ulcerative colitis [aUC], and ten with active Crohn's disease [aCD]; Table E3). Immunohistochemical staining for Ssu72 in tissues from healthy controls showed strong staining in lymphocytes and monocytes (Fig. 8a; left panel). In IBD tissues, we observed that although the number of infiltrating cells in the lamina propria was significantly increased

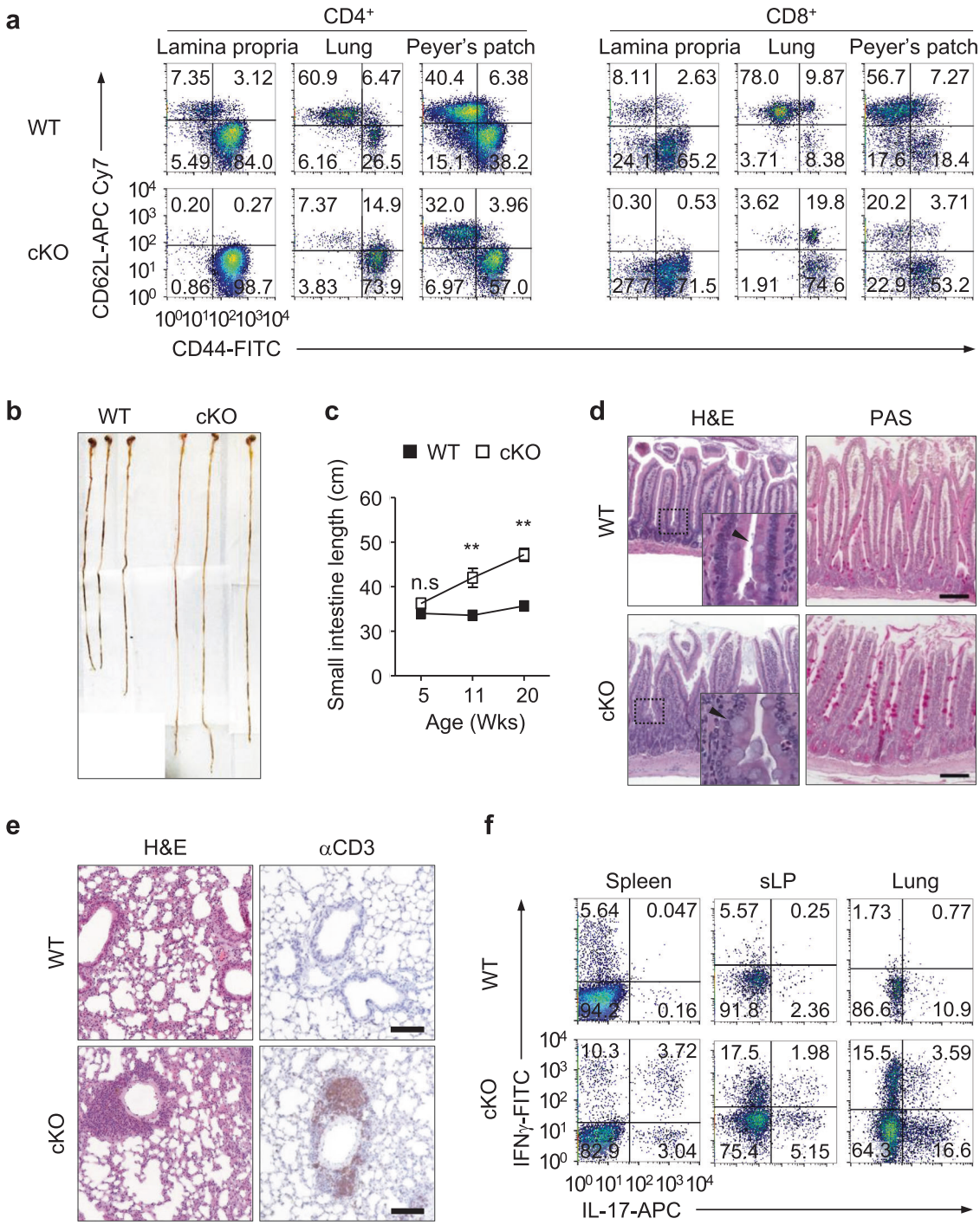

Fig. 6 Ssu72 is required for maintenance of peripheral tolerance. **a** Representative FACS plots of CD44 versus CD62L on gated $CD4^+CD3^+$ or $CD8^+CD3^+$ T cells in the small intestinal lamina propria, lungs, and Peyer's patches obtained from 11-week-old WT and cKO mice. The numbers indicate the percentages of cells in the respective quadrants ($n = 4$ per group). **b** Gross morphology of the small intestine of WT and cKO mice aged 11 weeks ($n = 3$ per group). **c** The age-dependent length of the small intestine from WT and cKO mice was calculated ($n = 3$ per group). **d** H&E and periodic acid Schiff (PAS) staining of small intestines harvested from WT and cKO mice aged 11 weeks. The insets show higher magnification images of the regions enclosed in rectangles. The black arrows indicate goblet cells ($n = 4$ per group). **e** H&E staining (left) and immunohistochemical analysis with anti-CD3 antibodies (right) of lungs from WT and cKO mice aged 1 year ($n = 3$ per group). **f** Total lymphocytes were isolated from the spleen, small intestinal lamina propria (sLP), and lung of 6-month-old WT and cKO mice and incubated with PMA (50 ng/mL), ionomycin (500 ng/mL), and brefeldin A (BFA) for 5 h. The cells were analyzed by flow cytometry. $CD3^+CD4^+$ T cells isolated from WT or cKO mice were further sorted according to the expression of IFN-γ and IL-17 ($n = 4$ per group). The scale bar represents 100 μm. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; n.s not significant. *t* test

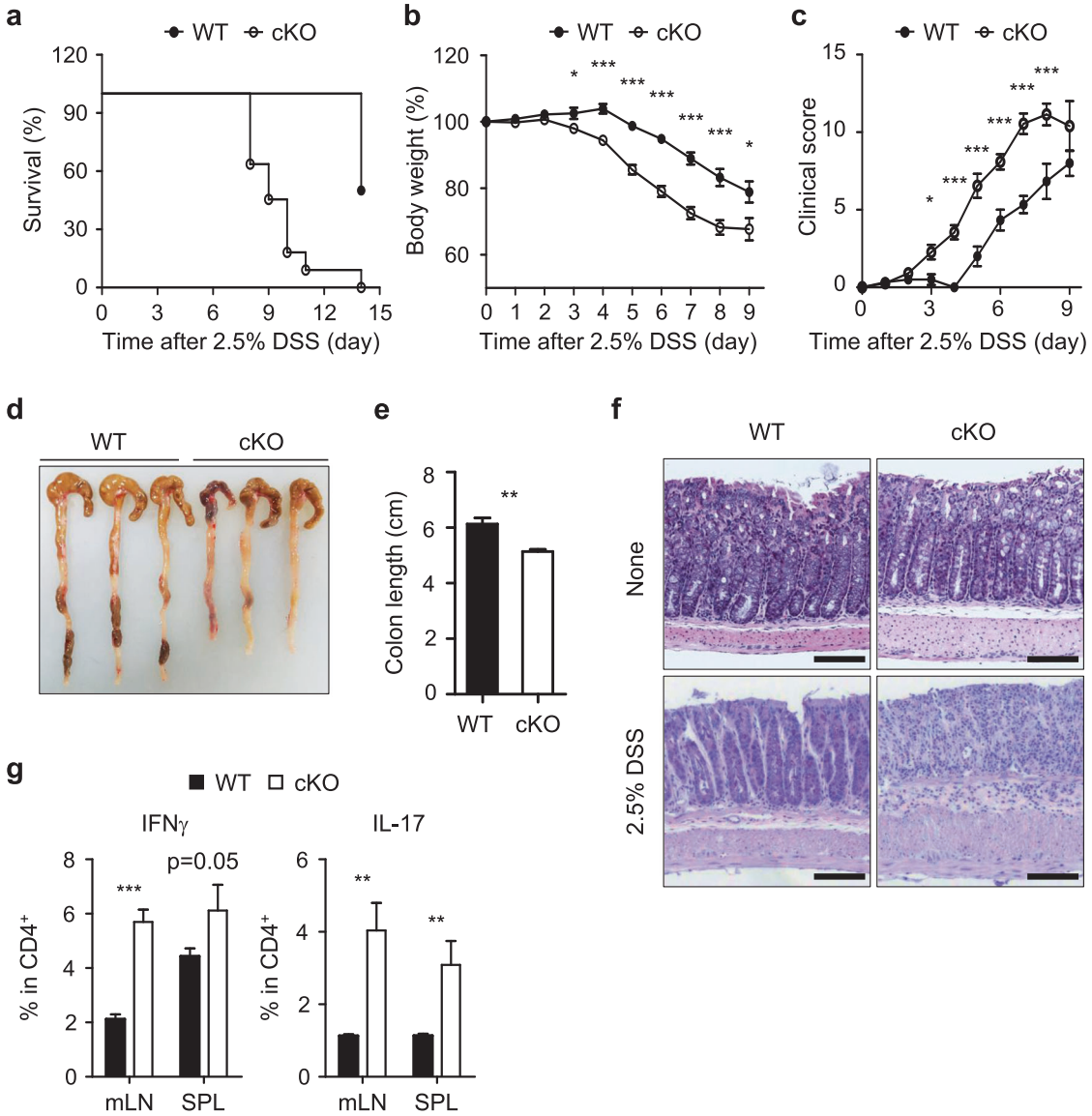

Fig. 7 Ssu72 deficiency in T cells enhanced susceptibility to colitis. **a** Survival rate of WT and cKO mice after treatment with 2.5% DSS ($n = 11$ per group). **b**, **c** WT and cKO mice were treated with 2.5% DSS for 9 days. The percentage body weight change (**b**) and total clinical score (**c**) were measured during DSS treatment ($n = 11$ per group). **d** Gross morphology of the colon in 2.5% DSS-treated WT and cKO mice on day 8 after treatment ($n = 3$ per group). **e** The length of colons isolated from 2.5% DSS-treated WT and cKO mice was assessed on day 8 after treatment ($n = 3$ per group). **f** Representative H&E-stained colon sections from 2.5% DSS-treated WT and cKO mice on day 8 after treatment ($n = 2$ per group). **g** Total lymphocytes in mLNs and SPLs harvested from 11-week-old WT and cKO mice were incubated with PMA (50 ng/mL), ionomycin (500 ng/mL), and brefeldin A (BFA) for 5 h. The cells were stained and analyzed by flow cytometry. The frequency of IFNγ- or IL-17-expressing cells among $CD4^+$ T cells was measured ($n = 5$ per group). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; n.s not significant. *t* test

compared to that in healthy controls, the expression of Ssu72 in those cells was downregulated in aUC and aCD tissues (Fig. 8b). However, Ssu72 was clearly expressed in those cells in healthy control and iUC tissues. In addition, the expression of Ssu72 in epithelial cells was downregulated in aUC and aCD patients.

We next examined the correlation between Ssu72 expression in Treg cells and disease activity of IBD. Immunofluorescence (Fig. 8a; right panel) and immunohistochemical studies (Fig. 8a; left panel) using anti-Ssu72 antibodies in combination with anti-Foxp3 (Treg cells) or anti-RORγt (T_H17 cells) antibodies revealed that the numbers of infiltrating $ROR\gamma t^+$ cells and $Foxp3^+$ cells in the lamina propria were increased in patients with aUC and aCD compared with patients with iUC (Fig. 8b, c). Interestingly, the number of infiltrating $Foxp3^+$ cells was also increased in patients with iUC compared to controls. In addition, tissues from UC patients exhibited more infiltration of $Foxp3^+$ cells than $ROR\gamma t^+$ cells, but this pattern was not seen in CD patients. Although the numbers of $ROR\gamma t^+$ cells and $Foxp3^+$ cells in the lamina propria were increased in patients with aUC and aCD, there was a significant reduction in Ssu72 expression in infiltrating $Foxp3^+$ cells in tissues from IBD patients compared to those from healthy controls (Fig. 8d). Interestingly, lower expression levels of Ssu72 in $Foxp3^+$ cells were also observed in patients with aUC and aCD than in patients with iUC. However, infiltrating $ROR\gamma t^+$ cells had consistent Ssu72 expression levels across all groups. The ratio of Ssu72 expression in $ROR\gamma t^+$ cells to that in $Foxp3^+$ cells was calculated for each patient (Fig. 8e). There was a significant increase in the expression ratio in aUC and aCD tissues compared to iUC and healthy control tissues, indicating that Ssu72 expression in $Foxp3^+$ cells was down-regulated in active IBD patients. These results indicate a close

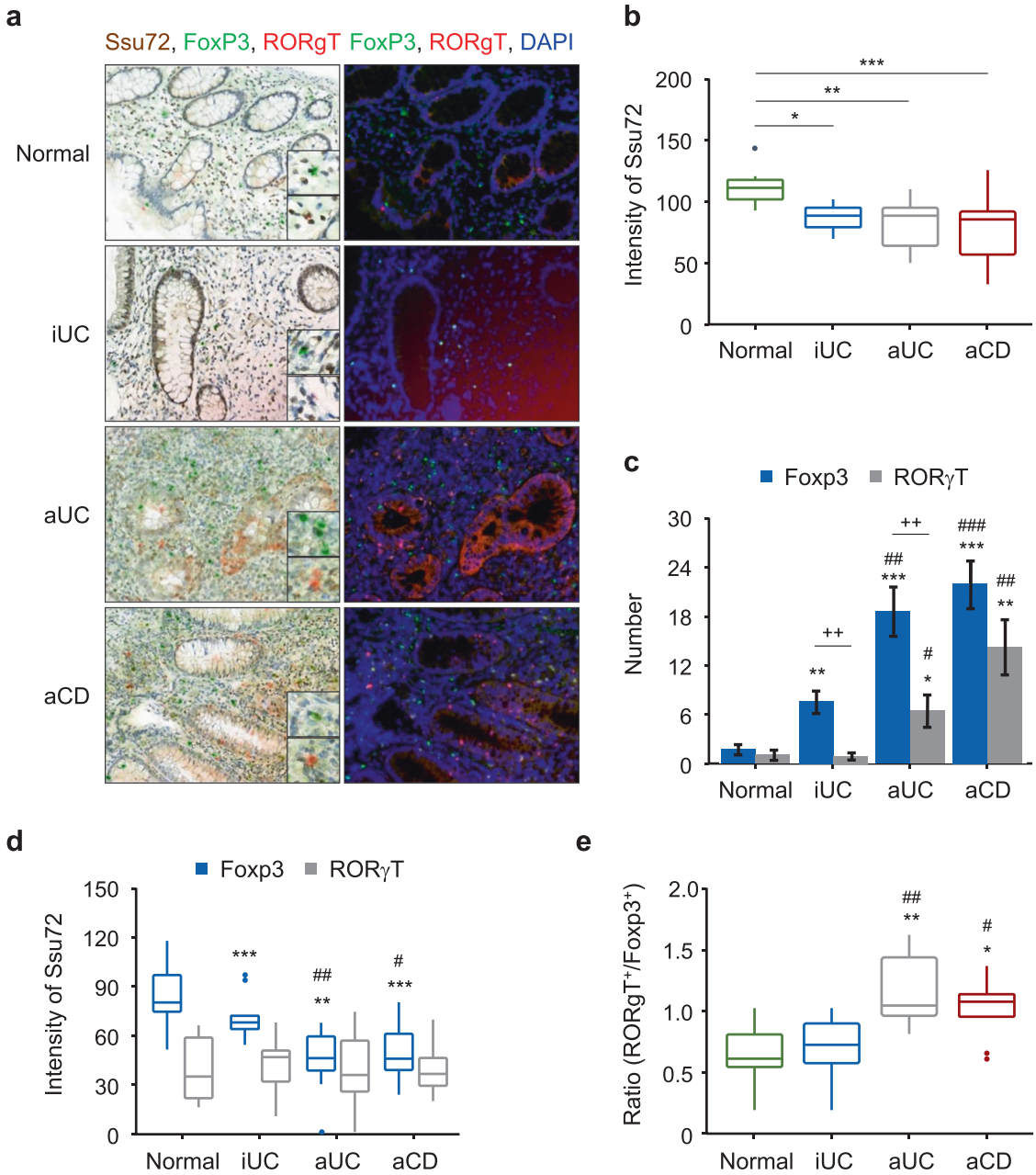

Fig. 8 Ssu72 expression in FoxP3 and RORγT cells in the colonic mucosa of humans with inflammatory bowel disease. **a** Ssu72 (brown), Foxp3 (green), and RORγT (red) immunohistochemical staining (IHC) in tissue from normal control individuals ($n = 11$) and patients with inactive ulcerative colitis (iUC) ($n = 10$), active ulcerative colitis (aUC) ($n = 10$), and active Crohn's disease (aCD) ($n = 10$). Ssu72 IHC was performed on serial sections of colon tissue. The slides were then subjected to OPAL staining and DAPI staining for nuclei, and images of each stain were superimposed using the Photoshop program. (IHC: Ssu72, brown; OPAL: FoxP3, green; DAPI: RORγT, red) (×400). **b** Comparison of the relative Ssu72 mean expression intensity in inflammatory cells per patient between groups. **c** Comparison of the average numbers of FoxP3-positive and RORγT-positive cells per patient in each group. **d** Comparison of the relative Ssu72 mean expression intensity in FoxP3-positive and RORγT-positive cells per patient between groups. **e** The ratio of Ssu72 intensity in RORγT-positive cells to that in FoxP3-positive cells in each group. Symbol (*), significantly different from the normal group; symbol (#), significantly different from the iUC group; symbol (+), significant difference between the $Foxp3^+$ and $ROR\gamma T^+$ groups (post hoc analysis with Tukey's HSD test; *, $^{\#}P < 0.05$; **, $^{\#\#}$, $^{++}P < 0.01$; ***, $^{\#\#\#}P < 0.001$)

correlation between loss of Ssu72 expression in $Foxp3^+$ Treg cells and the activity and severity of IBD. Considering these findings collectively, we concluded that Ssu72 is essential for pTreg generation and maintenance of mucosal tolerance.

DISCUSSION

The importance of the Ssu72 phosphatase has been unappreciated to date. However, our gene disruption studies clearly demonstrate the lack of redundancy between Ssu72 and other phosphatases in adaptive immunity. This study revealed a critical role for the Ssu72 phosphatase in controlling the homeostatic balance between effector T-cell and regulatory T-cell differentiation in the periphery. Although the relative amount of Ssu72 in the cell membrane is much lower than that in the cytoplasm or nucleus, it was clearly observed that Ssu72 translocates to the cell membrane in response to TCR and IL-2R activation, suggesting a role for Ssu72 as a TCR signaling-responsive phosphatase (Fig. 9).

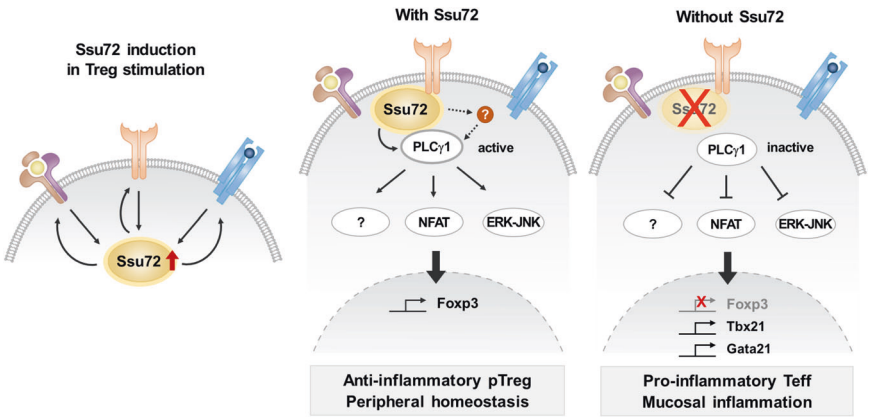

Fig. 9 A model for the functions of Ssu72 in pTreg differentiation. A detailed discussion of this model is provided in the text

During T-cell development, thymic selection leads to the development of FoxP3$^+$ T regulatory (T reg) cells, which play a critical role in maintaining self-tolerance.[49,50] A major stimulus of thymic and mature T-cell development is TCR signaling. PLCγ1 is an essential effector molecule in TCR signal transduction; after activation, this protein hydrolyzes the membrane lipid phosphatidylinositol 4,5-bisphosphate to generate diacylglycerol and inositol 1,4,5-trisphosphate.[51] The capacity of PLCγ1 to regulate multiple signaling pathways and transcription factors has attracted considerable interest to its biological role. Indeed, PLCγ1 deficiency deregulates TCR-induced proliferation and cytokine production.[37] Moreover, PLCγ1 deficiency impairs the development and function of FoxP3$^+$ regulatory T cells, causing inflammatory/autoimmune symptoms. Interestingly, mice with T-cell lineage-specific Ssu72 deficiency were found to have phenotypes and pathological lesions that were very similar to those of mice with T-cell lineage-specific PLCγ1 deficiency. Similarly, global knockout of the Ssu72 gene was embryonic lethal, as was the case for the PLCγ1 gene.[24,52] Our findings provide strong evidence that the interaction between Ssu72 and PLCγ1 is important for TCR signaling (Fig. 9). The consistent phenotypes resulting from inhibition of the interaction between Ssu72 and PLCγ1 in mice implies a breakdown of peripheral tolerance. In addition, conditional knockout of Ssu72 demonstrated an important role for Ssu72 in TCR-mediated signaling, cell proliferation, and stimulation of IL-2 and IFNγ production. FoxP3$^+$ Treg cells from Ssu72-defective mice were unable to suppress the proliferation of conventional T cells, indicating that the Ssu72-PLCγ1 interaction is essential for the suppressive function of CD4$^+$CD25$^+$ regulatory T cells in the periphery (Fig. 9). However, we do not exclude the possibility that signal transduction mediated by PLCγ1 is also be regulated through currently unidentified Ssu72-binding proteins. Therefore, the underlying molecular mechanisms by which the PLCγ1 signaling network is finely tuned by Ssu72-mediated hypophosphorylation still require further elucidation.

TCR signals trigger the Ssu72-PLCγ1 signaling cascade in a phosphorylation-dependent manner. LAT also coordinates TCR signals in a phosphorylation-dependent manner after receptor stimulation.[38,39,53] LAT phosphorylation at Y132 is important for recruitment of PLCγ1 after its phosphorylation by ZAP-70. Binding of Ssu72 to PLCγ1 induces its dephosphorylation. Phosphorylation is important for the activation and degradation of proteins. For instance, active MARK phosphorylated by LKB1 is targeted for degradation by the SCF complex.[54] Although further studies are needed to identify the precise modification site and the effects of Ssu72 on PLCγ1, we found that loss of Ssu72 leads to hyperphosphorylation and downregulation of PLCγ1 (Fig. 5b, c). These results indicated that Ssu72 expression was increased by TCR engagement, maintained PLCγ1 levels to convert TCR stimulation signals into appropriate cellular responses and further modulated the activity of PLCγ1 to prevent its uncontrollable activation. Thus, Ssu72-mediated PLCγ1 signaling eventually leads to cellular effector and transcriptional responses in the periphery.

Although we were unable to observe a direct interaction between Ssu72 and TGFβ signaling, Ssu72 deficiency significantly reduced cellular responsiveness to IL-2 and TGFβ signaling. IL-2 signaling is negatively regulated by Socs1, which is stabilized and phosphorylated by Pim1 kinase.[55,56] In addition, phosphorylation of TGFβ receptor 2 at Ser416 inhibits its kinase activity.[57] These observations support the possibility that Ssu72 dephosphorylates the IL-2 and/or TGFβ receptor or negatively regulates the IL-2 and TGFβ signaling axes, which is important for maintaining IL-2 and TGFβ signal transduction and inducing Foxp3 expression in peripheral Treg cells. Naive T cells stimulated by high concentrations of TGFβ are capable of differentiating into Foxp3$^+$ regulatory T cells. However, these high levels of TGFβ may be rare under physiological conditions. Interestingly, the level of Ssu72 expression is controlled by receptor-mediated signaling. This observation suggests that TCR or IL-2 stimulation increases Ssu72 expression and that elevated Ssu72 facilitates the differentiation of activated T cells into Treg cells. Autocrine TGFβ secreted by Tregs further augments the expansion of Treg cells. This observation is also supported by the phenomenon of CIA attenuation in response to exogenous Ssu72 expression, resulting in an increased proportion of Tregs.[28] Thus, the status of the Ssu72 level and activity under various physiological conditions is important for the differentiation of regulatory T cells.

Loss of Ssu72 led to a dramatic reduction in the pTreg population and almost complete inhibition of iTreg differentiation under iTreg-polarizing conditions; however, Ssu72-deficient T cells showed unexpectedly high levels of the effector cytokines IL-2 and IFNγ. These results apparently conflict with the general roles of IL-2 and IFNγ in Treg cells. However, the results in Ssu72-deficient mice suggested that T cells remain undifferentiated and maintain effector CD4$^+$ T-cell function rather than exhibiting functional defects of differentiated Treg cells after Ssu72 loss. Our evidence seems to support this hypothesis because under iTreg-polarizing conditions, NFAT1 in cKO CD4$^+$ T cells was largely localized to the nucleus compared to NFAT1 in WT iTreg cells. NFAT1 concentrated in the nucleus of cKO T cells could greatly facilitate the induction of target genes, such as *Ifng* and *Il2*. This observation raises the possibility that the hyperphosphorylated residual PLCγ1 was activated and was sufficient to stimulate NFAT1 translocation into the nucleus, even though the level of PLCγ1 was decreased by Ssu72 depletion (Fig. 3 and Fig. 5c). These results strongly suggest that Ssu72 is indispensable for the differentiation of naive CD4$^+$ T cells into pTreg and iTreg cells.

Moreover, Ssu72-deficient mice, independent of their IL-2 expression status, exhibited spontaneous intestinal inflammation resembling IBD in humans. However, in mice, Ssu72 deficiency did not lead to massive lymphoproliferation or multiorgan inflammation. Dysfunction of tTregs has been implicated in the pathophysiology of systemic autoimmune disease. While loss of Ssu72 led to spontaneous intestinal enteritis, pulmonary inflammation, and increased susceptibility to colitis, it did not promote other systemic autoimmune diseases. This finding may be attributed to impairment of pTreg and iTreg development but not tTreg development.

Loss of Ssu72 reduced the proportion of tTregs in the thymus by half, but this reduction was not as great as the reduction in the proportion of pTregs. The stage at which Foxp3 induction occurs during the multistep process that characterizes Treg development in the thymus remains unclear. While some studies reported that Foxp3 expression may be observed in the double-positive (DP) stage,[58] others showed that Treg cells isolated from the double-negative (DN) stages had high Foxp3 expression.[59] Since we crossed $Ssu72^{f/f}$ mice with transgenic mice expressing Cre under the control of the CD4 promoter, the possibility was raised that specific Tregs exhibit Foxp3 expression before Ssu72 deletion. $Foxp3^{+}CD4^{+}$ Tregs in which Ssu72 was not completely knocked out that were generated in cKO mice circulated and expanded in the peripheral tissue to compensate for Treg insufficiency. This assumption was further supported by the increased frequency of $BrdU^{+}$ Tregs and the absence of systemic autoimmune disease, indicating that Ssu72 is involved in Foxp3 induction in pTregs but not in tTreg proliferation and suppression. Importantly, our current observation further supports the finding that compared to $Ssu72^{f/f}$; CD4-Cre mice, mice with Treg-cell-specific deletion of the Ssu72 gene showed similar phenotypes but more severe systemic autoimmune diseases. However, to differentiate the precise role of Ssu72 between pTreg and tTreg populations, further studies are needed to elucidate the role of Foxp3-YFP/iCre.[60]

T_H17 differentiation requires TGFβ and IL-6 to induce the expression of RORγt as well as IL-17.[61] However, in response to TGFβ, Ssu72-deficient naive $CD4^{+}$ T cells differentiated into T_H17 cells, while Smad2/3 was not efficiently phosphorylated. Moreover, the population of cKO $CD4^{+}$ T cells showed an increased proportion of $IL\text{-}17^{+}$ cells in DSS-treated mice. According to a previous report, $IL\text{-}17^{+}$ T cells differentiate via stimulation with IL-6, IL-23, and IL-1β in the absence of TGFβ.[62] In addition, unlike TGFβ-dependent differentiation, TGFβ-independent generation of T_H17 cells triggered severe experimental allergic EAE and was associated with significantly enhanced IFNγ and IL-17 double-positive T cells.[62] Importantly, $IFN\gamma^{+}IL\text{-}17^{+}$ T_H17 cells were observed in tissue lesions in the setting of autoimmune disease.[63] Thus, it is possible that Ssu72 normally regulates the IL-6 and/or IL-23 receptor-mediated signaling cascade to inhibit Tbx21 expression and that Ssu72 deficiency converts T_H17 cells into more pathogenic $IFN\gamma^{+}IL\text{-}17^{+}$ T_H17 cells. This hypothesis is supported by our results demonstrating that Ssu72 deficiency increased susceptibility to DSS-induced colitis and the proportion of $IFN\gamma^{+}IL\text{-}17^{+}$ T_H17 cells in mucosal tissue.

The enhanced susceptibility to DSS-induced colitis was attributed to decreased Ssu72 expression and associated severe pTreg cell deficiency. Moreover, Ssu72 deficiency induced a profound inflammatory response in mucosal tissues such as lung and intestinal tissues. Treg cells have been implicated in the pathophysiology of systemic autoimmune disease, and the varied numbers and functional roles of Tregs reflect the heterogeneity of these diseases.[12] Changes in Treg cell numbers might be attributed to the drastic increase in the levels of circulating IL-2 and IFNγ, which are required not only for T-cell expansion and survival but also for AICD.[64] In this study, the high levels of the effector cytokines IL-2 and IFNγ in mice with Ssu72 deficiency may have led to Fas-dependent AICD to modulate T-cell homeostasis. Thus, the inhibition of Treg cell development in Ssu72-deficient mice was likely caused not only by decreased Foxp3 expression in $CD4^{+}CD25^{+}$ T cells but also by increased AICD due to the secretion of high levels of IL-2 and IFNγ by $CD4^{+}$ T cells.

The PI3K/Akt/mTOR cascade is a major signaling pathway downstream of IL-2 and is closely tied to cellular metabolism. Recent evidence has shown that Tregs utilize aerobic glycolysis to manifest their full suppressor function.[65] These metabolic mechanisms are controlled largely by the PI3K/Akt/mTOR signaling axis.[65] Recent evidence increasingly suggests that the interplay between IL-2 signaling and Treg metabolism is important for immunomodulation. Although further studies are required to understand the possible role of Ssu72 in IL-2 production and Treg metabolism via PI3K/Akt/mTOR signaling, our unpublished observations show that adipose Ssu72 is associated with the signaling mechanism related to β-oxidation during lipolysis and potentially regulates AMPK activity. Fatty acid oxidation is generally associated with an anti-inflammatory phenotype and maintenance of Treg lineage stability. Tregs induce high levels of AMPK, which simultaneously promotes fatty acid oxidation while inhibiting mTOR signaling and subsequent glycolysis.[66] In the gut, short-chain fatty acids are also known to inhibit mTOR.[67] In addition, tTreg and pTreg/iTreg cells differ considerably in their mechanisms of fatty acid oxidation: pTreg/iTreg cells generally rely upon exogenous fatty acids for their metabolic needs.[68] However, it is uncertain whether tTreg cells import exogenous fatty acids in vivo.[69] Considering these observations collectively, it is possible that Ssu72 also affects metabolism, particularly via pTreg/iTreg lineage stability and suppressor function. In summary, epigenetic modification via hypophosphorylation for the conversion of unstable iTreg cells to stable iTreg cells is a key breakthrough in clinical applications, and genetic and epigenetic approaches aimed at enhancing Ssu72 activity thus constitute an important strategy for the induction of Treg cell development, peripheral expansion, and suppression.

MATERIALS AND METHODS

Mice

The $Ssu72^{flox/flox}$ mouse strain was described previously.[24] CD4-Cre mice were purchased from The Jackson Laboratory and crossed in house with $Ssu72^{flox/flox}$ mice to generate T-cell-specific Ssu72 knockout ($Ssu72^{flox/flox}$;CD4-Cre, cKO) mice. All mice were maintained on a C57BL/6 background. All animal experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of Sungkyunkwan University School of Medicine (SUSM). SUSM is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International) and complies with the Institute of Laboratory Animal Resources (ILAR) guidelines.

Antibodies

A list of all antibodies used in this study can be found in Supplementary Table 1. For immunohistochemical analysis of Ssu72 in kidney tissue, an affinity-purified mouse monoclonal antibody was used.[25]

Flow cytometry

Thymuses, spleens, and lymph nodes were aseptically removed from WT and cKO mice and crushed by being passed through 70-mm strainers (Sartorius) with a syringe plunger. Cells were suspended in complete medium [RPMI-1640 (CM059-050, GenDEPOT) supplemented with 10% FBS (16000044, Gibco) and 50 μM β-mercaptoethanol (Sigma-Aldrich)] and centrifuged for 5 min at $300 \times g$, and red blood cells were removed using red blood cell lysis buffer (eBioscience). Cells were suspended in staining buffer [1× phosphate-buffered saline, 0.09% NaN_3, and 3% fetal bovine serum]. Surface marker expression was assessed by FACS analysis.

For intracellular cytokine staining, splenocytes/lymphocytes were stimulated with phorbol 12-myristate 13-acetate (PMA) (50 ng/mL, Sigma-Aldrich) and ionomycin (500 ng/mL, Sigma-Aldrich) with BFA (eBioscience). After 5 h of stimulation, cells were stained with antibodies. Stained cells were fixed and permeabilized with an Intracellular Fixation & Permeabilization Buffer Set (eBioscience) as recommended by the manufacturer. Intracellular cytokines were stained. For Foxp3 and Helios staining, cells were fixed and permeabilized with a Foxp3/Transcription Factor Staining Buffer Set (eBioscience) prior to staining for Foxp3 and Helios. Stained cells were acquired on a BD FACS Canto II (BD Biosciences) and analyzed with FlowJo software (Tree Star). All flow cytometry results were obtained by live cell gating and cell doublet exclusion based on FSC/SSC.

Immunofluorescence microscopy

Round coverslips were coated with poly-L-lysine (Sigma) for 1 h at room temperature and placed in 12-well plates. Jurkat T cells were resuspended at 1×10^6 cells/mL in complete medium in the presence or absence of anti-CD3 [145-2C11] (5 μg/mL, eBioscience) and anti-CD28 [37.51] (2 μg/mL, BD Biosciences) antibodies and incubated on ice for 30 min. Goat anti-mouse IgG (secondary crosslinking Ab) was added, and cells were transferred to 37 °C for the indicated times. Stimulation was terminated by two washes with cold PBS. Naive T cells from mice were differentiated as indicated. A total of 1×10^5 cells were transferred onto poly-L-lysine-coated coverslips and centrifuged for 15 min at 4 °C. After centrifugation, cells were fixed with 4% PFA for 10 min at room temperature. After washing with PBS, 0.3% PBST (PBS + 0.3% Tween 20) was added for permeabilization. Cells were washed with 0.1% PBST (PBS + 0.1% Tween 20) and blocked with 3% skim milk in 0.1% PBST. Cells were incubated with primary antibodies for 1 h at room temperature, washed with 0.1% PBST three times and incubated with secondary antibodies for 1 h at room temperature. After washing with 0.1% PBST three times, cells were mounted with mounting medium containing DAPI (Vector Laboratories, H-1200). Images of stained cells were acquired using an LSM 710 confocal microscope (ZEISS), and the images were processed with ZEISS ZEN microscope software.

Phos-tag analysis

Sequences from the Ssu72 and Ssu72 C12S plasmids[26] were subcloned into the pMyc vector, and sequences from the PLCγ1 plasmid (SinoBiological, HG17529-UT) were subcloned into the pCMV-Flag vector. 293T cells were transfected with Myc-Ssu72, Myc-Ssu72 C12S, and Flag-PLCγ1 fusion constructs with Lipofectamine (Invitrogen) according to the manufacturer's instructions. Phos-tag gels containing 6% acrylamide solution, 50 μM Phos-tag acrylamide (Waco Pure Chemicals, 304-93521) and 100 μM $MnCl_2$ were prepared. After SDS-PAGE, gels were washed twice with transfer buffer containing 1 mM EDTA and once with transfer buffer. Proteins were transferred using transfer buffer containing 10% methanol.

Isolation of lamina propria cells

The small intestine and colon were isolated, and Peyer's patches were removed. Tissues were cut longitudinally and washed in Ca^{2+}/Mg^{2+}-free Hank's balanced salt solution (HBSS). Tissues were incubated in ice-cold 5 mM EDTA/HBSS on ice. After 5 min, tissues were transferred into HBSS, and the wash step was repeated at least four times. After washing, tissues were cut into small pieces and incubated for 30 min at 37 °C in RPMI medium containing fetal bovine serum (FBS, 10% vol/vol), collagenase D (Sigma, 0.5 mg/mL) and DNase I (Sigma, 0.5 mg/mL). Cell suspensions were filtered through a 70 μM strainer and centrifuged at 1800 rpm and 4 °C. Pellets were resuspended in 44% Percoll (Sigma), layered on top of 67% Percoll, and centrifuged at 2200 rpm for 20 min at room temperature. Interphase cells were collected, washed with PBS twice, and stained with antibodies for flow cytometric analysis.

In vitro $CD4^+$ T-cell differentiation

For in vitro $CD4^+$ T-cell differentiation, single-cell suspensions were prepared as described above and washed with cell separation buffer [1× phosphate-buffered saline, 3% fetal bovine serum and 10 mM EDTA]. $CD4^+$ T cells were pre-enriched using a MagniSort™ Mouse CD4 T-cell Enrichment Kit (eBioscience prior to staining with CD4 [GK1.5], CD25 [PC61.5], CD44 [IM7], and CD62L [MEL-14]. Naive $CD4^+CD25^-CD44^{lo}CD62L^{hi}$ cells were sorted from WT and cKO mice to >95% purity with a BD FACS Aria II (BD Biosciences). Sorted cells were resuspended at 1×10^6 cells/mL in complete medium, and 100 μL of the cell suspension was added to 96-well U-bottom plates coated with an anti-CD3 [145-2C11] antibody (5 μg/mL, eBioscience) in complete medium supplemented with an anti-CD28 [37.51] antibody (2 μg/mL, BD Biosciences). For Th1 polarization, IL-12 (20 ng/mL, R&D) and an anti-IL-4 [11b11] antibody (10 μg/mL, BioLegend) were used. For Th2 polarization, IL-4 (40 ng/mL, R&D) and an anti-IFNγ [XMG1.2] antibody (10 μg/mL, BioLegend) were used. For Th17 polarization, TGFβ (2 ng/mL, R&D), IL-6 (40 ng/mL, R&D), an anti-IL-4 antibody (10 μg/mL), and an anti-IFNγ antibody (10 μg/mL) were used. For iTreg cell differentiation, TGFβ (2 ng/mL) and IL-2 (20 ng/mL, R&D) with an anti-IFNγ antibody (10 μg/mL) were used. After 5 days of incubation, cells were analyzed using flow cytometry prior to RNA extraction and western blot analysis.

In vivo BrdU incorporation assay

Eleven-week-old mice were intraperitoneally (i.p.) injected with a single dose of 200 μg BrdU (10 mg/mL) (Sigma-Aldrich) for 18 h prior to tissue isolation. Mice were sacrificed, and lymphocytes were isolated from various tissues prior to assessment of BrdU incorporation using FITC BrdU Flow Kits (BD).

RNA isolation and quantitative real-time PCR analysis

RNA from each tissue or from $CD4^+$ T cells was isolated with an RNeasy Plus Mini kit (Qiagen), and cDNA templates were synthesized using an EasyScript cDNA synthesis kit (Abm) with random primers. Quantitative real-time PCR analyses were performed with SYBR Green Chemistry (Applied Biosystems) in an ABI Prism 7900 detection system using previously described primer sets. All procedures followed the manufacturer's protocols. The average level of expression of each gene was normalized to that of Actb, and relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method. A list of all primers used in this study can be found in Supplementary Table 2.

Histology

For histological analyses, intestines were obtained from WT and cKO mice and fixed in 10% neutral buffered formalin (Sigma-Aldrich). All samples were paraffin embedded, sectioned (5–8 μm), and stained with hematoxylin and eosin (H&E). All procedures were performed using standard histological techniques.

Statistical analysis

P values were calculated using Student's *t* test (GraphPad Prism). *P* values less than 0.05 were considered significant. All error bars indicate the mean and S.E.M values.

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AUTHOR CONTRIBUTIONS

J.-K.L. and S.Y.K. designed the studies, analyzed the data, and prepared and wrote specific portions of the manuscript. H.-M.N., J.-B.L., J.K., E.-J.P., and K.-M.K. participated in data generation and analysis. T.J.K. provided materials and participated in data generation. H.L. and H.G. designed the studies and wrote part of the manuscript. C.-W.L. designed the studies, supervised the overall project, wrote the manuscript, and performed the final manuscript preparation. All authors provided feedback and agreed on the final manuscript.

ADDITIONAL INFORMATION

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