Ssu72 phosphatase directly binds to ZAP-70, thereby providing fine-tuning of TCR signaling and preventing spontaneous inflammation

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ZAP-70 is required for the initiation of T cell receptor (TCR) signaling, and Ssu72 is a phosphatase that regulates RNA polymerase II activity in the nucleus. However, the mechanism by which ZAP-70 regulates the fine-tuning of TCR signaling remains elusive. Here, we found that Ssu72 contributed to the fine-tuning of TCR signaling by acting as tyrosine phosphatase for ZAP-70. Affinity purification–mass spectrometry and an in vitro assay demonstrated specific interaction between Ssu72 and ZAP-70 in T cells. Upon TCR stimulation, Ssu72-deficient T cells increased the phosphorylation of ZAP-70 and downstream molecules and exhibited hyperresponsiveness, which was restored by reducing ZAP-70 phosphorylation. In vitro assay demonstrated that recombinant Ssu72 reduced tyrosine phosphorylation of ZAP-70 via phosphatase activity. *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice showed a defect in the thymic development of invariant natural killer T cells and reductions in CD4^{+} and CD8^{+} T cell numbers in the periphery but more CD44^{hi}CD62L^{lo} memory T cells and fewer CD44^{lo}CD62L^{hi} naïve T cells, compared with wild-type mice. Furthermore, *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice developed spontaneous inflammation at 6 mo. In conclusion, Ssu72 phosphatase regulates the fine-tuning of TCR signaling by binding to ZAP-70 and regulating its tyrosine phosphorylation, thereby preventing spontaneous inflammation.

phosphatase Ssu72 | fine-tuning | ZAP-70 | tyrosine phosphorylation | autoimmunity

Ssu72 phosphatase regulates the recycling of RNA polymerase II by binding to the C-terminal domain (CTD) of RNA polymerase II and inhibiting the phosphorylation of serine and tyrosine residues in the CTDs in yeast and mammalian cells (1, 2). Recently, Ssu72 phosphatase was found to regulate the cell cycle by directly binding to Aurora B kinase in HeLa cells and retinoblastoma protein in hepatocytes (3). Moreover, Woo et al. demonstrated that Ssu72 bound to and reduced the phosphorylation of GM-CSF receptor (GM-CSFR) β-chain of alveolar macrophages, thereby providing fine-tuning of GM-CSFR signaling and being critical for the development and maturation of alveolar macrophages (4). These findings suggest that Ssu72 exerts RNA polymerase II–independent phosphatase activity in different cellular events, including immune cells. However, the function of Ssu72 in T cells has yet to be clearly reported.

T cells make up a major subset of the adaptive immune system that plays critical roles in the regulation of autoimmunity, defense against pathogens, and tumor surveillance. To establish efficient T cell–mediated adaptive immune responses in vivo, the initiation and maintenance of appropriate T cell receptor (TCR)–mediated activation in T cells are mandatory (5, 6). Under steady-state conditions, ζ-chain–associated protein kinase 70 (ZAP-70) is bound to immunoreceptor tyrosine-based activation motifs (ITAMs) but is not phosphorylated, thus remaining in an autoinhibited conformation during the response to self-peptides that are presented by a major histocompatibility complex class I or II molecule (5). In contrast, upon agonist peptide recognition, TCR complexes are clustered and lymphocyte-specific protein tyrosine kinase (Lck) phosphorylates tyrosine residues in the ITAMs of CD3 and ζ-chains. ZAP-70 is activated by binding to the phospho-tyrosine residues of the ζ-chains and by being phosphorylated itself (5, 6). In turn, activated ZAP-70 phosphorylates tyrosine residues on adaptor molecules such as linker for activation of T cells (LAT), thereby providing docking sites for cytosolic enzymes, including phospholipase C-γ1, and activating Ras and G proteins upstream of MAP kinases (5, 6). These findings indicate that ZAP-70 is an essential signaling molecule that regulates and propagates TCR signaling. In accordance, ZAP-70–deficient mice show an absolute defect in thymic development at the positive selection stage because of failure in TCR signaling (7).

To ensure the appropriate stimulation of T cells, this signaling cascade of intracellular molecules is tightly regulated by a variety of mechanisms, thereby fine-tuning TCR signaling (5, 6). Thus, the regulation of the TCR signaling cascade contributes to the determination of TCR signaling strength, which affects the responses of T cells during development and activation. Aberrant mutations of ZAP-70 trigger an altered transduction of TCR signaling in T cells, resulting in dysregulation of thymic selection

Significance

The appropriate T cell receptor (TCR)–mediated activation in T cells is mandatory for efficient adaptive immunity. However, the mechanism for maintaining appropriate TCR signaling remains elusive. Moreover, Ssu72 is a phosphatase that regulates RNA polymerase II activity in the nucleus, and its functions in the fine-tuning of TCR signaling has not been investigated. This study demonstrates that Ssu72 regulates the fine-tuning of TCR signaling by binding to ZAP-70 and regulating its tyrosine phosphorylation, thereby preventing spontaneous inflammation. These results provide an immunologic insight for the fine-tuning of TCR signaling during autoimmunity.

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The authors declare no competing interest.

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and autoimmune arthritis (8). Therefore, the fine-tuning of TCR signaling is critical for thymic development and the effector functions of T cells. For such fine-tuning, several mechanisms such as the progressive use of ITAM and modulation of signaling by coreceptors and inhibitory receptors have been suggested. The balancing of positive and negative regulation in critical signaling molecules, such as ZAP-70, also contributes to the fine-tuning of TCR signaling during T cell activation (5). However, less is known about the mechanism by which negative regulation of ZAP-70 determines TCR signaling strength than about that underlying positive regulation. ZAP-70 is dephosphorylated by several phosphatase, including phosphatase suppressor of TCR signaling (Sts)1, Sts2, low molecular weight phosphotyrosine phosphatase, and a vaccinia virus VH1-related, dual-specific protein phosphatase (5, 9–12). Ubiquitination and deubiquitination processes also regulate ZAP-70 activity by affecting interactions

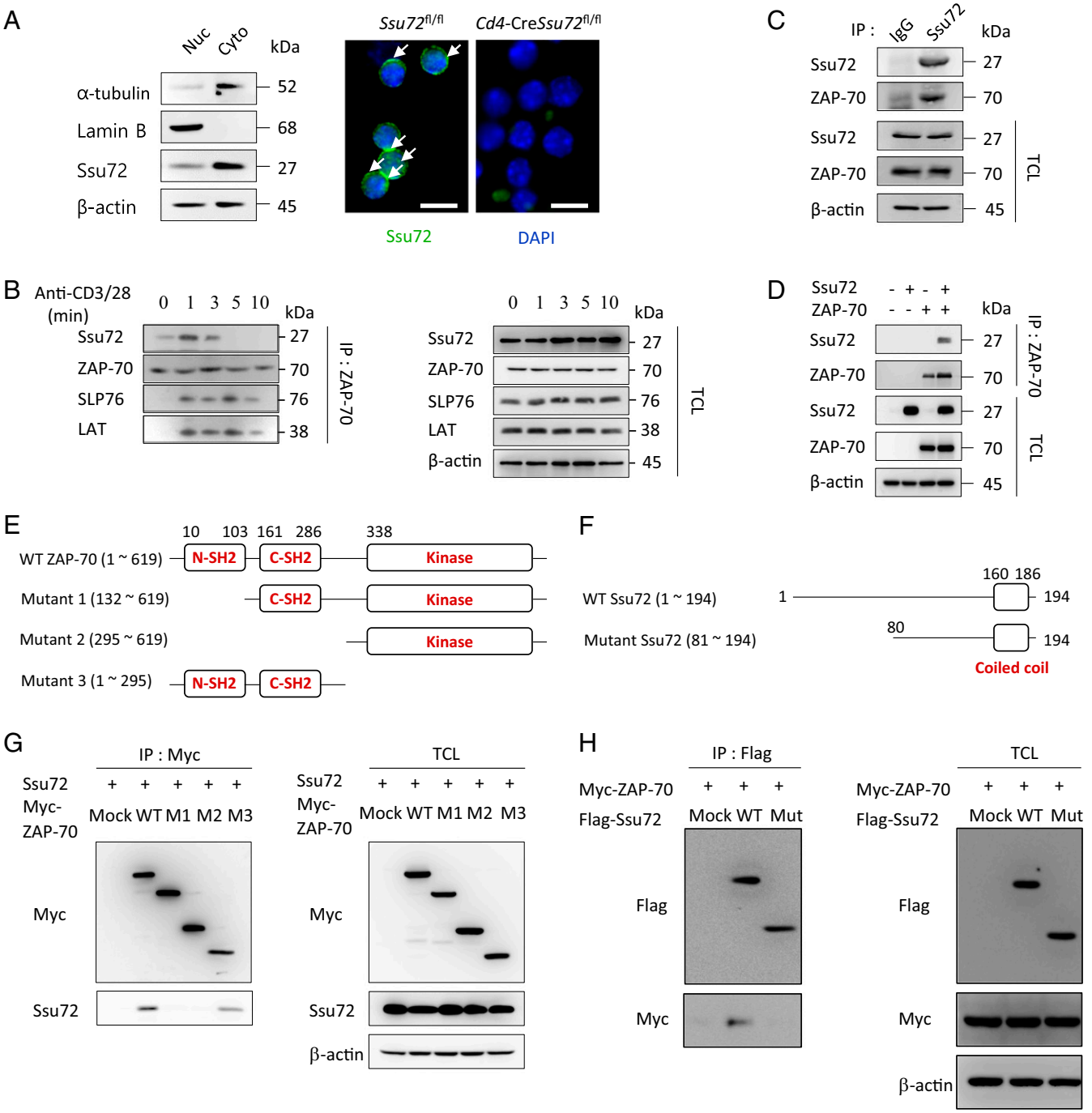

Fig. 1. The RNA polymerase II phosphatase Ssu72 binds to ZAP-70. (*A*) Expression patterns of Ssu72 in the nucleus and cytoplasm of $CD4^+$ T cells, revealed by blotting and immunofluorescence. (*B–D*) Lysates from $CD4^+$ T cells (*B* and *C*) and HEK 293T cells (*D*) that were cotransfected with expression plasmids encoding *ZAP70* or *SSU72* were immunoprecipitated and immunoblotted with the indicated antibodies. TCL represents total cell lysate. (*E* and *F*) Diagram of the WT and mutant (M) *ZAP70* and *SSU72* constructs. (*G* and *H*) HEK 293T cells were cotransfected with expression plasmids encoding Myc-*ZAP70* and Flag-*SSU72*, immunoprecipitated, and immunoblotted using the indicated antibodies. All experiments were performed three times independently and data are representative. IP, immunoprecipitation.

between ZAP-70 and phosphatases (13, 14). The ubiquitin E3 ligase Nrdp1 terminates $CD8^+$ T cell activation via K33-linked polyubiquitination of ZAP-70, whereas Usp9X and Otud7b promote T cell activation by removing inhibitory ubiquitin from ZAP-70 (13–15). Moreover, Nrdp1 and Otud7b regulate the association of ZAP-70 and Sts1/Sts2 during T cell activation (15). Thus, ubiquitination/deubiquitination and phosphorylation/dephosphorylation systems cross-talk and play critical roles in the regulation of ZAP-70 activation balance in T cells. Nevertheless, the mechanism by which ZAP-70 is regulated via phosphorylation–dephosphorylation during T cell activation remains unclear.

In this study, we found that the phosphatase Ssu72 was bound to ZAP-70 and inhibited its tyrosine phosphorylation via phosphatase activity. Moreover, *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice developed

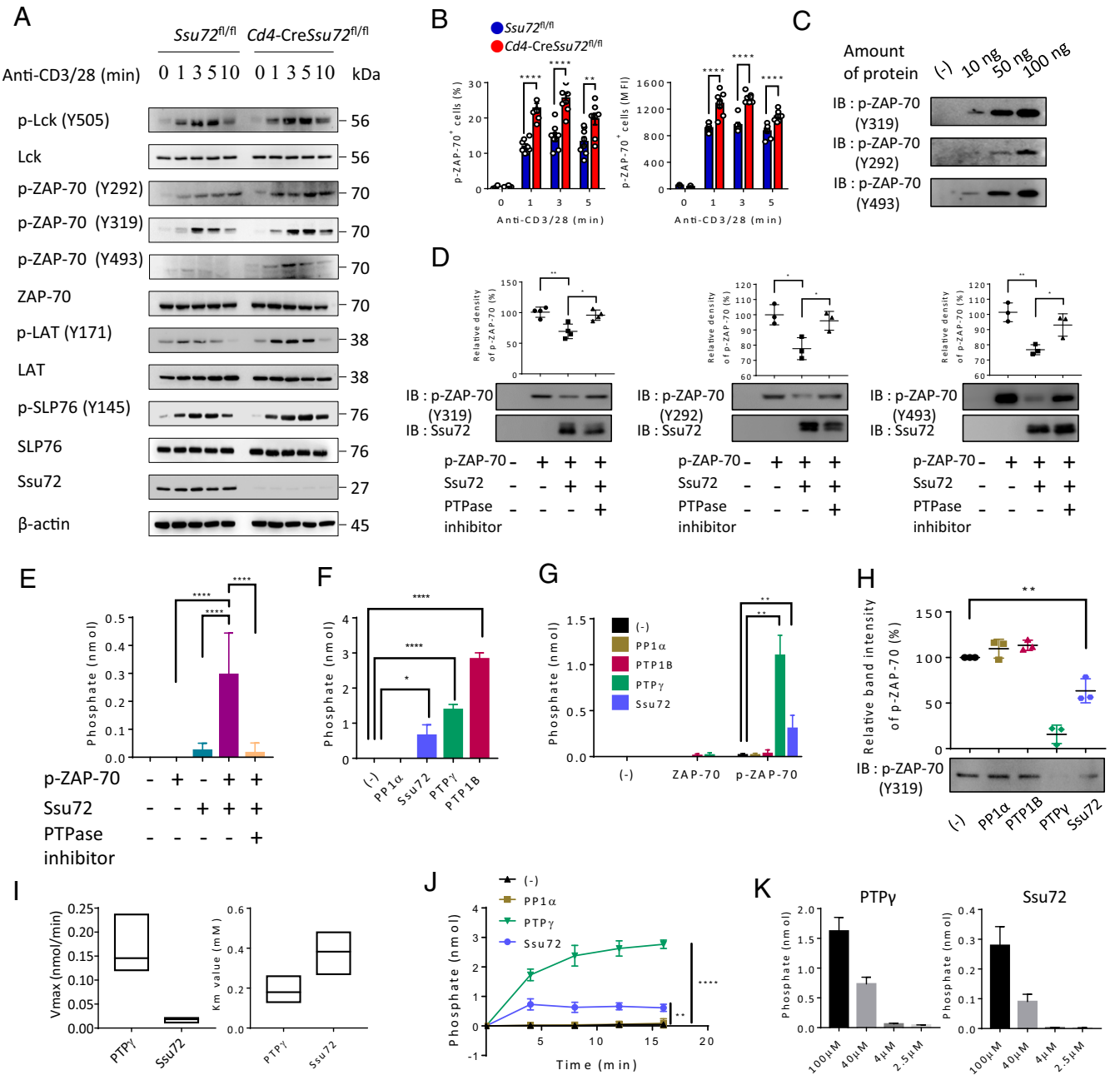

Fig. 2. Ssu72 negatively regulates tyrosine phosphorylation of ZAP-70 as tyrosine phosphatase. (*A*) Blotting assay for the phosphorylation of Lck, ZAP-70, LAT, and SLP 76 in T cells from *Cd4*-Cre*Ssu72*$^{fl/fl}$ and *Ssu72*$^{fl/fl}$ mice, in the presence or absence of plate-coated anti-CD3 (1 μg/mL) and soluble CD28 (1 μg/mL) mAbs for the indicated time. (*B*) Flow cytometric analysis of the expression of phosphorylated ZAP-70 on Y319 (p-ZAP-70) in sorted $CD4^+$ T cells upon stimulation with plate-coated anti-CD3 (1 μg/mL) and soluble anti-CD28 (1 μg/mL) mAbs for the indicated time ($n = 8$). Numbers in the diagrams indicate the percentages of p-ZAP-70^+ cells among $CD4^+$ T cells. (*C*) Phosphorylation status on tyrosine of purified active p-ZAP-70. During in vitro phosphatase assay, blotting (*D*) and the measurement of free phosphate (*E*) were performed using purified active p-ZAP-70, protein tyrosine phosphatase (PTPase) inhibitor and recombinant human Ssu72. (*F–H*) In vitro phosphatase assay and blotting (*H*) for various phosphatases using tyrosine phosphatase substrate I (*F*) and active p-ZAP-70 (*G* and *H*). (*I*) Measurement of V_{max} and K_m of Ssu72. (*J* and *K*) Kinetic analysis for Ssu72 in terms of time points and concentrations. All experiments were performed three times independently and data are representative (*A*, *C*, *E–G*, and *I–K*) or were pooled (*B*, *D*, and *H*). Data represent means ± SEMs and were analyzed using the two-tailed, unpaired, and parametric Student's *t* test (*B–F*, *H*, and *J*) or nonparametric Mann–Whitney *U* test (*G*). N.S., not significant. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

spontaneous inflammation via hyperactivation of T cells and the promotion of naïve T cell differentiation into effector and memory T cells.

Results

The RNA Polymerase II Phosphatase Ssu72 Binds to ZAP-70 and Negatively Regulates Tyrosine Phosphorylation of ZAP-70 by Acting as Tyrosine Phosphatase in T Cells. To identify a protein that binds to ZAP-70 and regulates its activity, we performed an affinity purification–mass spectrometry analysis using T cell–lineage EL4 cells in the absence of TCR stimulation. The analysis revealed several proteins. Among them, there was the phosphatase Ssu72, which is known to regulate RNA polymerase in the nucleus (1) (*SI Appendix*, Fig. S1*A* and Table S1). Ssu72 was expressed in the cytoplasm and nuclei of wild-type (WT), but not Ssu72-deficient, $CD4^+$ T cells (Fig. 1*A* and *SI Appendix*, Fig. S1 *B* and *C*). Immunoprecipitation using an anti–ZAP-70 antibody showed Ssu72 in EL4 cells and $CD4^+$ T cells from WT mice in the absence of TCR stimulation, and its binding increased, peaked, and decreased after stimulation with anti-CD3 and -CD28 monoclonal antibodies (mAbs) (Fig. 1*B* and *SI Appendix*, Fig. S1 *D* and *E*). ZAP-70 was also detected in the immunoprecipitate of $CD4^+$ T cells (Fig. 1*C*) and EL4 cell lysates using the anti-Ssu72 antibody. However, STAT1, STAT3, STAT6, and p38 were not detected (*SI Appendix*, Fig. S1*F*), which have been known to be critical molecules for effector $CD4^+$ T cell differentiation (16) and related to Ssu72-mediated T cell activation (17). To confirm specific binding between Ssu72 and ZAP-70, we cotransfected human embryonic kidney (HEK) 293T cells with *SSU72* and *ZAP-70* and immunoprecipitated cell lysates using the anti–ZAP-70 antibody; blotting revealed a Ssu72 band (Fig. 1*D*). Cotransfection of the HEK 293T cells with WT or mutated construct of *SSU72* and *ZAP-70* revealed that N-terminal amino acids (1 to 80) of Ssu72 and amino acids (1 to 131) of ZAP-70, containing the *N*-SH2 domain, are critically involved in the interaction of two proteins (Fig.1 *E–H*). Furthermore, the phosphorylation of ZAP-70 (Y292, Y319, and Y493), LAT (Y171), and SLP76 (Y145) was increased in Ssu72-deficient $CD4^+$ T cells, compared with WT $CD4^+$ T cells during T cell activation (Fig. 2*A* and *SI Appendix*, Fig. S2*A*). Flow cytometric analysis showed increases in the percentage of p-ZAP-70 (phosphorylated ZAP-70) (Y319)–positive $CD4^+$ T cells and the level of ZAP-70 (Y319) phosphorylation in Ssu72-deficient $CD4^+$ T cells, compared with WT $CD4^+$ T cells (Fig. 2*B*). During in vitro phosphatase assay, Ssu72 decreased phosphorylation status of p-ZAP-70 (p-ZAP-70 on Y292, Y319, and Y493), compared with p-ZAP-70 alone in terms of blotting (Fig. 2 *C* and *D*) and free phosphate generation (Fig. 2*E*), which was restored by adding protein tyrosine phosphatase inhibitor. Furthermore, Ssu72, but not serine/threonine phosphatase PP1-α, increased free phosphate from tyrosine phosphatase substrate I (Fig. 2*F*) and p-ZAP-70 (Fig. 2*G*) and decreased phosphorylation status on Y319 of ZAP-70 in blotting during in vitro phosphatase assay, which was lower compared with other tyrosine phosphatase such as PTP-γ and PTP1B (Fig. 2*H*). Consistently, the maximum reaction rate (V_{max}) and michaelis constant (K_m) of Ssu72 on ZAP-70 were lower and higher than those of PTP-γ, respectively (Fig. 2*I*), suggesting that binding affinity of Ssu72 to ZAP-70 might be lower compared to PTP-γ. In kinetic assay, Ssu72 increased free phosphate from tyrosine phosphatase substrate I on 5 min incubation and was sustained after then, while PTP-γ gradually increased free phosphate, whereas serine/threonine phosphatase PP1-α did not (Fig. 2*J*). Dephosphorylation of tyrosine phosphatase substrate I was dependent on concentration of Ssu72 and PTP-γ (Fig. 2*K*), and Ssu72 minimally regulated the phosphorylation of serine residues on ZAP-70 (*SI Appendix*, Fig. S2 *B* and *C*). Combined, these findings indicate that Ssu72 constitutively binds to and regulates the tyrosine phosphorylation of T cell ZAP-70 as tyrosine phosphatase. Furthermore, immunoprecipitation showed that Sts1 and Sts2 bound to ZAP-70 in T cells from *Cd4*-Cre*Ssu72*$^{fl/fl}$ or *Ssu72*$^{fl/fl}$ mice during TCR activation (*SI Appendix*, Fig. S3*A*), suggesting that Ssu72 might not critically contribute to binding between ZAP-70 and Sts1/Sts2. To confirm this, we transfected 293T cells with *ZAP-70*, *STS1*, *STS2*, and *SSU72* and performed immunoprecipitation. In overexpression system, binding between Ssu72 and Sts1 or Sts2 was not detected, while ZAP-70 bound to Sts1 and Sts2 (*SI Appendix*, Fig. S3 *B–E*). Thus, these experiments indicate that Ssu72 and Sts1/2 bind to ZAP-70 independently in T cells.

Ssu72 Regulates Homeostasis of $CD4^+$ and $CD8^+$ T Cells in the Periphery. To investigate the function of Ssu72 in T cells in vivo, we studied T cell subsets in the thymus and peripheral lymphoid organs from *Cd4*-Cre*Ssu72*$^{fl/fl}$ and *Ssu72*$^{fl/fl}$ mice. During thymic development, *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice showed similar percentages, numbers, and apoptosis of $CD4^-CD8^-$ (double negative: DN), $CD4^+CD8^+$ (double positive: DP), $CD4^+CD8^-$ ($CD4^+$ single positive: $CD4^+$ SP), and $CD4^-CD8^+$ ($CD8^+$ SP) thymocytes to those of *Ssu72*$^{fl/fl}$ mice, whereas the percentages of $CD3^+$ T cells and numbers of $CD3^+$, $CD4^+$, and $CD8^+$ T cells were reduced in the spleens and lymph nodes from *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice compared with *Ssu72*$^{fl/fl}$ mice. In contrast to T cells, the percentages and numbers of $CD19^+$ B cells were increased in the periphery of *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice (Fig. 3 *A–C*, and *SI Appendix*, Fig. S4 *A* and *B*), which might be attributable to cytokine production of hyperactivated T cells and altered the peripheral niche in *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice (18). Moreover, the number of $CD8^+$ T cells was more reduced than that of $CD4^+$ T cells in *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice, resulting in a low $CD8^+/CD4^+$ T cell ratio (Fig. 3*C*). *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice exhibited similar percentages of T_{reg} cells among $CD4^+$ T cells but low cell numbers in peripheral organs compared with *Ssu72*$^{fl/fl}$ mice, which showed a similar suppressive effect on activated T cells (*SI Appendix*, Fig. S5 *A* and *B*). However, the body, thymus, and spleen weights; size of spleen and lymph; and cell numbers of thymus, spleen, and lymph nodes were similar in *Cd4*-Cre*Ssu72*$^{fl/fl}$ and *Ssu72*$^{fl/fl}$ mice at 7 to 8 wk of age (Fig. 3 *D* and *E*). In mixed bone marrow (BM) chimera mice using lethally irradiated WT recipient mice with a 1:1 ratio of $CD45.1^+$ C57BL/6 and $CD45.2^+$ *Cd4*-Cre*Ssu72*$^{fl/fl}$ BM cells, Ssu72-deficient BM cells showed similar thymic development, but decreased $CD4^+$ and $CD8^+$ T cell numbers, in the periphery compared with WT BM cells (Fig. 3 *F* and *G*), indicating that the intrinsic expression of Ssu72 regulates the homeostasis of $CD4^+$ and $CD8^+$ T cells in the periphery but not in the thymus.

Ssu72 Regulates Thymic Development of *i*NKT Cells in an Extrinsic Manner. In real-time PCR analysis, thymic invariant natural killer T (*i*NKT) cells showed the expression of *Ssu72* similar to that in conventional thymocytes (*SI Appendix*, Fig. S1*B*). In contrast to conventional T cells, *i*NKT cells were found in minimal numbers in the thymi, spleens, and livers from *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice (Fig. 4 *A* and *B*). To analyze the *i*NKT cell population during various developmental stages, we enriched *i*NKT cells using the CD1d/PBS57 tetramer (*SI Appendix*, Fig. S6 *A–D*). Among the thymic developmental stages, stage 0 ($CD24^+$) and 1 ($NK1.1^-CD24^-CD44^-$) *i*NKT cells were characterized by the increased numbers of *i*NKT cells in *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice compared with *Ssu72*$^{fl/fl}$ mice, whereas stage 3 ($NK1.1^+CD24^-CD44^+$) was characterized by a decreased number of thymic *i*NKT cells in *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice (*SI Appendix*, Fig. S6*A*). The percentage of NKT1 cells, among *i*NKT cell subsets, was substantially decreased in *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice compared with *Ssu72*$^{fl/fl}$ mice, whereas that of NKT17 cells was increased (*SI Appendix*, Fig. S6*B*). The level of apoptosis of Ssu72-deficient *i*NKT cells was higher than that of WT *i*NKT cells in the thymus (*SI Appendix*, Fig. S6*C*). Mixed BM-chimeric mice exhibited similar thymic development and peripheral homeostasis of *i*NKT cells from *Ssu72*$^{fl/fl}$ mice and *Cd4*-Cre*Ssu72*$^{fl/fl}$ BM

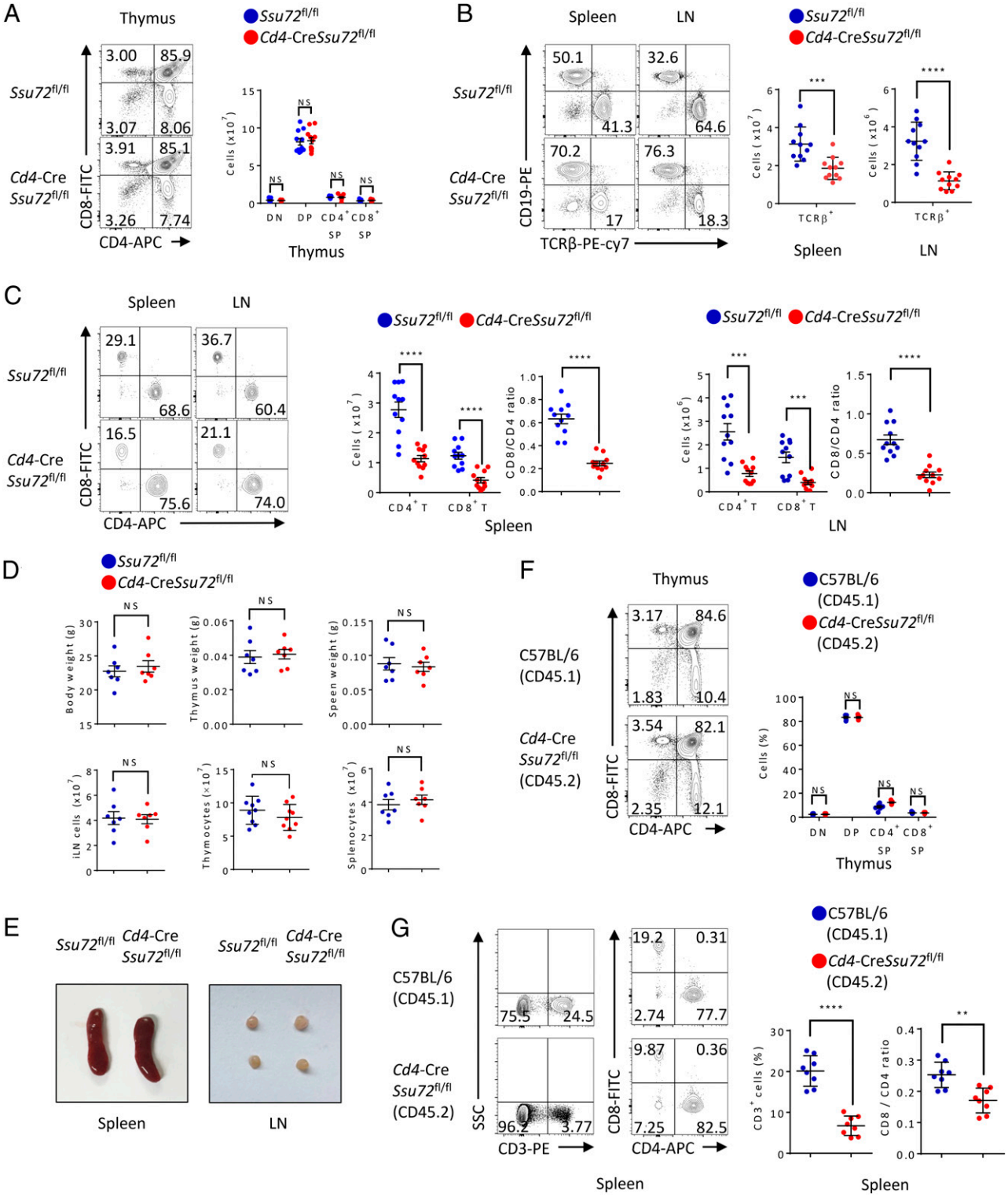

Fig. 3. Ssu72 regulates the homeostasis of conventional CD4$^+$ and CD8$^+$ T cells in the periphery. (*A* and *B*) The percentages of CD4$^+$ and CD8$^+$ cells among thymocytes (*A*) and the percentages of TCRβ$^+$ T cells and CD19$^+$ B cells among forward scatter low (FSC^{lo}) small side scatter low (SSC^{lo}) lymphocytes in spleens and lymph nodes from *Ssu72*$^{fl/fl}$ and *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice at 7 to 8 wk of age (*B*). (*C*) The percentages of CD4$^+$ and CD8$^+$ T cells among TCR-β$^+$ T cells and the absolute numbers and ratios of CD4$^+$ and CD8$^+$ T cells in spleens and lymph nodes from *Ssu72*$^{fl/fl}$ and *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice at 7 to 8 wk of age. (*D*) Measurement of body, thymus, and spleen weights, and cell numbers of lymph nodes, thymus, and spleens from mice at 7 to 8 wk of age. (*E*) Photographs of spleen and inguinal lymph nodes from mice at 7 to 8 wk of age. (*F* and *G*) To generate BM-chimeric mice, CD45.1$^+$ WT BM and CD45.2$^+$ BM cells from *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice were adoptively transferred into irradiated CD45.2$^+$ WT mice. (*F*) The percentages of CD45.1$^+$ WT or CD45.2$^+$ Ssu72–deficient CD4$^+$ and CD8$^+$ cells in the thymus. (*G*) The percentages of CD3$^+$ T cells (among FSC^{lo} SSC^{lo} lymphocytes) and CD4$^+$ T or CD8$^+$ T cells (among TCR-β$^+$ T cells) and ratios of CD4$^+$ T and CD8$^+$ T cells in the spleen. Data from three independent experiments were pooled (*A*–*D*, *F*, and *G*) or are representative (*E*) and presented as the means ± SEMs (*A*–*C*, n =11; *D* and *E*, n = 7; and *F* and *G*, n = 8 per group). Statistical analysis was performed using the two-tailed, unpaired, and parametric Student's t test (*B*–*D* and *G*) or non-parametric Mann–Whitney U test (*A* and *F*). LN, lymph node; N.S., not significant. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

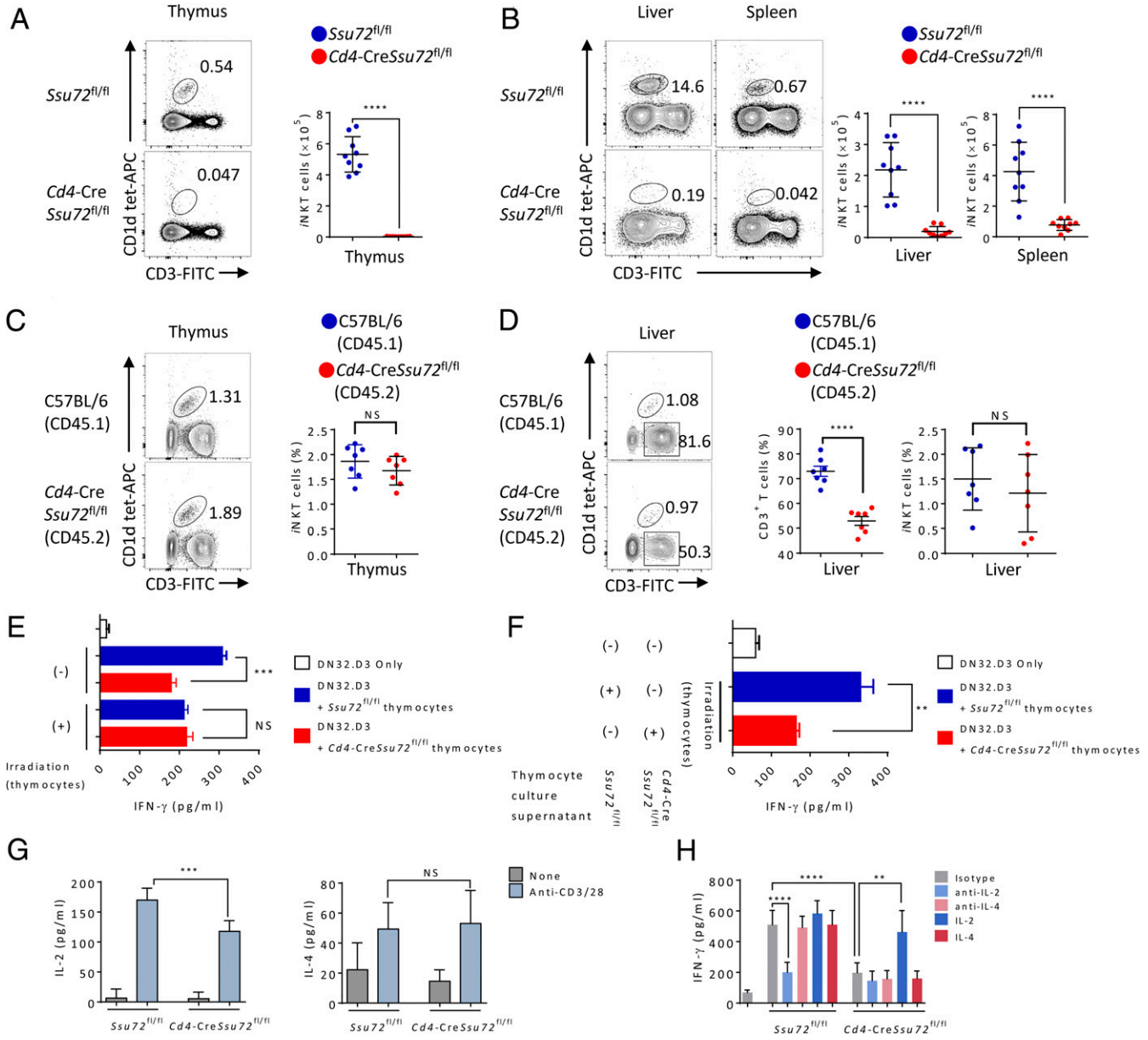

Fig. 4. Ssu72 is required for the thymic development of *i*NKT cells in an extrinsic manner. (*A* and *B*) Percentages and absolute numbers of *i*NKT cells in the thymus (*A*), liver, and spleen (*B*) from *Ssu72*$^{fl/fl}$ ($n = 9$) and *Cd4*-Cre*Ssu72*$^{fl/fl}$ ($n = 9$) mice. The percentages of CD3^{int} CD1d/PBS57 tetramer^{+} *i*NKT cells among total thymocytes (thymus) and forward scatter low (FSC^{lo}) small side scatter low (SSC^{lo}) lymphocytes (spleen and lymph nodes) were calculated. (*C* and *D*) Percentages of CD45.1^{+} WT or CD45.2^{+} Ssu72–deficient *i*NKT cells and CD3^{+} T cells among FSC^{lo} SSC^{lo} lymphocytes in the thymi and livers of BM-chimeric mice generated using CD45.1^{+} WT BM and CD45.2^{+} BM cells from *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice ($n = 7$). (*E*) NKT cell hybridoma (DN32.D3) cells were cocultured with unirradiated or irradiated thymocytes from *Ssu72*$^{fl/fl}$ and *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice in the presence of α-GalCer (200 ng/mL). The amount of IFN-γ was measured in culture supernatants. (*F*) IFN-γ levels in culture supernatants of DN32.D3 cells and irradiated thymocytes, measured using ELISA after the addition of culture media of unirradiated thymocytes from *Ssu72*$^{fl/fl}$ and *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice upon stimulation with plate-coated anti-CD3 (5 μg/mL) and soluble anti-CD28 (1 μg/mL) mAb for 24 h. (*G*) IL-2 and IL-4 levels were measured in culture supernatants of thymocytes from *Ssu72*$^{fl/fl}$ and *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice after stimulation with plate-coated anti-CD3 (5 μg/mL) and soluble anti-CD28 (1 μg/mL) mAb for 24 h. (*H*) The amount of IFN-γ was estimated in culture supernatants of DN32.D3 cells and irradiated thymocytes using ELISA after the addition of culture media from unirradiated thymocytes from *Ssu72*$^{fl/fl}$ and *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice upon stimulation with plate-coated anti-CD3 (5 μg/mL) and soluble anti-CD28 (1 μg/mL) mAb for 24 h in the presence of treatment with blocking mAb or recombinant cytokine. All experiments were performed three times independently, and data were pooled (*A–D*) or are representative (*E–H*). Data are presented as the means ± SEMs. Statistical analysis was performed using the two-tailed, unpaired, and parametric Student's *t* test (*C–H*) or nonparametric Mann–Whitney *U* test (*A* and *B*). N.S., not significant. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$.

cells (Fig. 4 *C* and *D*, and *SI Appendix*, Fig. S6*D*), in contrast to conventional T cells, indicating that Ssu72 deficiency perturbs the thymic development of *i*NKT cells in an extrinsic rather than intrinsic manner. Transcriptome analysis demonstrated that increased expression of various genes that related to TCR signaling and the inflammatory response in conventional DP thymocytes from *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice compared with those from *Ssu72*$^{fl/fl}$ mice (*SI Appendix*, Fig. S6 *E* and *F*), suggesting that cellular function differs between WT and Ssu72-deficient thymocytes. In accordance, NKT hybridoma cells produced less IFN-γ during the coculture with Ssu72-deficient thymocytes than with WT thymocytes in the presence of α-GalCer stimulation (Fig. 4*E* and

SI Appendix, Fig. S6*G*). However, irradiated thymocytes from *Ssu72*$^{fl/fl}$ and *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice similarly activated NKT hybridoma cells upon α-GalCer stimulation, which was restored by the addition of culture supernatant from WT thymocytes but not that from Ssu72-deficient thymocytes simulated with anti-CD3 and -CD28 mAbs (Fig. 4 *E* and *F*, and *SI Appendix*, Fig. S6*G*). These findings suggest that the Ssu72-dependent function of conventional thymocytes affects the activation of *i*NKT cells in a soluble manner, rather than a membrane protein-dependent manner. In addition, thymocytes from *Ssu72*$^{fl/fl}$ and *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice showed similar expression of CD1d but different cytokine production upon TCR stimulation (Fig. 4*G* and *SI Appendix*, Fig. S6 *H* and *I*). Among these cytokines, recombinant IL-2 restored IFN-γ production by DN32.D3 cells during coculture with irradiated thymocytes from *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice (Fig. 4*H* and *SI Appendix*, Fig. S6*J*). Taken together, these results suggest that Ssu72 in conventional thymocytes critically contributes to *i*NKT cell development in the thymus by regulating cytokine production.

Ssu72 Negatively Regulates T Cell Activation. To investigate the function of Ssu72 in conventional T cells, we performed a microarray assay using CD4$^+$ T cells. A multidimensional scaling plot, heat map, and gene ontology enrichment analysis of microarray data showed distinct populations, global gene expression, and the immune response-related gene profile of Ssu72-deficient CD4$^+$ T cell, which differed from those of WT CD4$^+$ T cells (Fig. 5 *A* and *B* and *SI Appendix*, Fig. S7*A*). Consistently, Ssu72-deficient total CD4$^+$ T cells and naïve CD4$^+$ and CD8$^+$ T cells produced IL-2, proliferated, and induced apoptosis to significantly higher levels than observed in WT total CD4$^+$ T cells and naïve CD4$^+$ and CD8$^+$ T cells; all of these effects were restored upon the reduction of ZAP-70 phosphorylation using an Lck inhibitor in total CD4$^+$ T cells (Fig. 5 *C*–*H* and *SI Appendix*, Fig. S7 *B*–*I*). Blotting results suggested that concentration (20 nM) of Lck inhibitor might be appropriate to differentially induce functional restoration in WT and Ssu72-deficient T cells, based on the phosphorylation status of ZAP-70 in T cells during TCR stimulation. However, cell proliferation of Ssu72-deficient T cells was restored at 10 nM Lck inhibitor, whereas cytokine production and cell death in Ssu72-deficient T cells were restored at 20 nM Lck inhibitor during TCR stimulation. These findings suggest that some functional events might be altered earlier than change in the blotting-based phosphorylation status of ZAP-70 in T cells (Fig. 5 *F*–*H* and *SI Appendix*, Fig. S7 *D*–*F*).

T cell activation with phorbol 12-myristate 13-acetate (PMA)/ionomycin activates the distal signal pathway of TCR via activation of protein kinase C and calcineurin by bypassing proximal signals such as TCR-associated molecules (19–21). Thus, to rule out effect on RNA polymerase II, naïve CD4$^+$ and CD8$^+$ T cells were stimulated using PMA + ionomycin. Upon PMA + ionomycin-mediated activation, there was no difference in cell death, proliferation, and cytokine production between WT and Ssu72-defieicnt naïve CD4$^+$ and CD8$^+$ T cells, which was in contrast to anti-CD3 and -CD28 mAb stimulation (*SI Appendix*, Fig. S7 *G*–*I*). These results support that the effects associated with the deficiency of Ssu72 might be attributable to TCR proximal signaling rather than to distal signaling events such as RNA synthesis.

To estimate proliferation status in vivo, we adoptively transferred T cells from *Cd4*-Cre*Ssu72*$^{fl/fl}$ or *Ssu72*$^{fl/fl}$ mice into *Rag1* knockout (KO) mice and measured proliferation status of CD4$^+$ and CD8$^+$ T cells using Cell Trace Violet (CTV) labeling. CD4$^+$ and CD8$^+$ T cells from *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice were more proliferated than those from *Ssu72*$^{fl/fl}$ mice (Fig. 5 *I* and *J*). To confirm this further, a mixture of T cells from CD45.2$^+$ *Cd4*-Cre*Ssu72*$^{fl/fl}$ and CD45.1$^+$ WT C57BL/6mice were also adoptively transferred into *Rag1* KO mice. Consistently, CD45.2$^+$CD4$^+$ and CD45.2$^+$CD8$^+$ T cells from *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice were more proliferated than CD45.1$^+$CD4$^+$ and CD45.1$^+$CD8$^+$ T cells from WT C57BL/6 mice (Fig. 5 *K* and *L*). Moreover, the phosphorylation of MEK, JNK, ERK, IκBα, and p65 and nuclear translocation of p65 and NFAT1 were increased in Ssu72-deficient CD4$^+$ T cells, compared with that in WT CD4$^+$ T cells during T cell activation (Fig. 5 *M*–*O* and *SI Appendix*, Fig. S8 *A*–*C*). The strength of TCR signaling has been noted to regulate the target T cells for T_{reg}-mediated suppression (22). To examine this effect, CD4$^+$ T cells from *Ssu72*$^{fl/fl}$ and *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice were activated in the presence of WT T_{reg} cells. The proliferation of WT CD4$^+$ T cells was inhibited by WT T_{reg} cells in a dose-dependent manner, whereas Ssu72-deficient CD4$^+$ T cells were suppressed less by WT T_{reg} cells, indicating that Ssu72-deficient CD4$^+$ T cells are less sensitive to T_{reg}-mediated suppression (*SI Appendix*, Fig. S8*D*). These findings indicate that Ssu72 is involved in the fine-tuning of TCR signaling via the regulation of proximal TCR molecules.

Ssu72 Inhibits the Differentiation of Naïve T Cells into Effector and Memory T Cells. In a microarray analysis, Ssu72-deficient CD4$^+$ T cells showed a greater expression of various cytokines for helper T (Th) cell differentiation than did WT CD4$^+$ T cells (Fig. 6 *A* and *B*). In accordance with this finding, TCR stimulation increased the expression of IFN-γ, IL-4, IL-17, GM-CSF, and transcription factors, including *Tbx21*, *Gata3*, and *Rorc* in Ssu72-deficient CD4$^+$ T cells, compared with that in WT CD4$^+$ T cells (Fig. 6 *C*–*E*). Moreover, in vitro experiments demonstrated a greater differentiation of Ssu72-deficient CD4$^+$ T cells into Th1, Th2, and Th17 cells compared to WT CD4$^+$ T cells (Fig. 6*F* and *SI Appendix*, Fig. S8*E*). At 7 to 8 wk of age, *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice showed greater percentages of effector–memory CD44^{hi}CD62L^{lo} CD4$^+$ and CD8$^+$ T cells and fewer naïve CD44^{lo}CD62L^{hi} CD4$^+$ and CD8$^+$ T cells in the spleen and lymph nodes than did *Ssu72*$^{fl/fl}$ mice (Fig. 6*G*). This inverse correlation of CD44^{hi}CD62L^{lo} effector–memory cells with CD44^{lo}CD62L^{hi} naïve CD4$^+$ and CD8$^+$ T cells in *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice was stronger in aged mice (6 mo) than in young adult mice (Fig. 6*H*). These findings indicate that Ssu72 negatively regulates the differentiation of naïve T cells into effector and memory T cells in the periphery.

***Aged Cd4*-Cre*Ssu72*$^{fl/fl}$ Mice Spontaneously Develop Spontaneous Inflammation.** Hyperactivation of T cells and large numbers of effector and memory T cells are associated with autoimmunity. Thus, we assessed autoimmunity phenotypes in *Cd4*-Cre*Ssu72*$^{fl/fl}$ and *Ssu72*$^{fl/fl}$ mice at 24 wk. *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice exhibited reduced body weights and intestine lengths but increased lymph node and spleen sizes and numbers of total and TCRβ$^+$ T cells (Fig. 7 *A*–*C*). Among T cells, the numbers of CD4$^+$ T cells were higher in the spleen and lymph nodes of *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice than those in *Ssu72*$^{fl/fl}$ mice, while CD8$^+$ T cell numbers were similar in two groups of mice, resulting in low CD8$^+$/CD4$^+$ T cell ratio in *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice (Fig. 7*D*). The levels of serum IgG and IgG1 were also increased in *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice, compared with those in *Ssu72*$^{fl/fl}$ mice (Fig. 7*E*). Histological examination revealed the infiltration of considerable numbers of inflammatory cells in various organs, including the esophagus, stomach, colon, lung, and liver (Fig. 7*F*), but not other organs (*SI Appendix*, Fig. S9*A*) from *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice. Flow cytometric analysis demonstrated that inflammatory cells consisted of neutrophils, eosinophils, monocytes, macrophages, and T cells (*SI Appendix*, Fig. S9*B*). The percentages of CD69$^+$ T, effector–memory T, and T_{reg} cells were higher in *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice than in *Ssu72*$^{fl/fl}$ mice, whereas those of naïve T cells were lower (*SI Appendix*, Fig. S9*B*). The percentages of IFN-γ or IL-17–producing CD4$^+$ T cells were increased in these organs from *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice compared with those from *Ssu72*$^{fl/fl}$ mice (Fig. 7 *G* and *H*). Ratio of T_{conv}/T_{reg} were higher in *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice than those in *Ssu72*$^{fl/fl}$ mice at 24 wk, whereas the ratio of T_{conv}/T_{reg} were similar between *Ssu72*$^{fl/fl}$ and *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice at 8 wk (*SI Appendix*, Fig. S9*C*). It

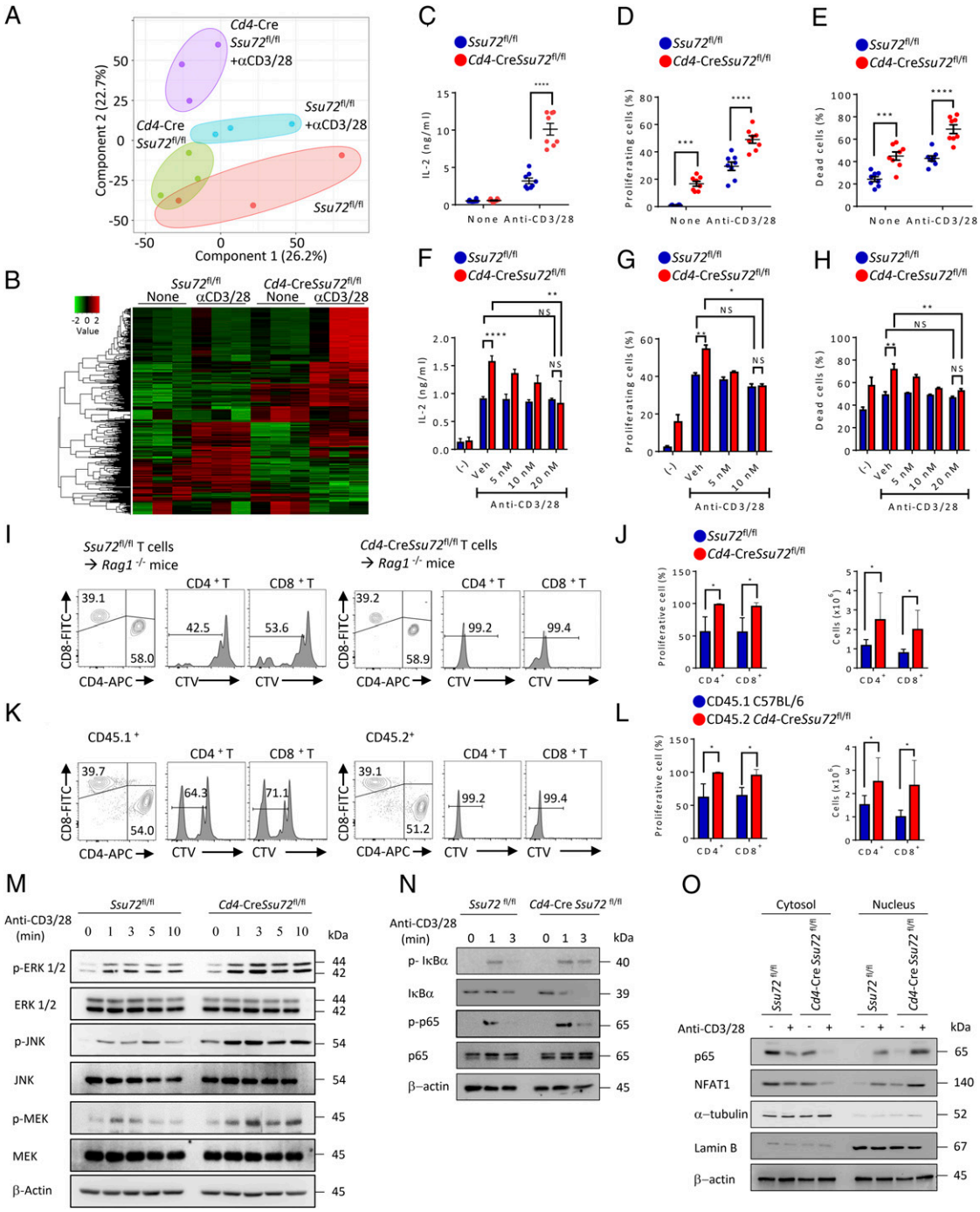

Fig. 5. Ssu72 negatively regulates T cell activation. Multidimensional scaling plot (*A*) and heat map (*B*) of microarray data of three replicates using sorted naïve CD4$^+$ T cells from *Ssu72*$^{fl/fl}$ and *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice via flow cytometry. (*C*) IL-2 amounts in culture supernatants of naïve CD4$^+$ T cells from *Ssu72*$^{fl/fl}$ (n = 8) and *Cd4*-Cre*Ssu72*$^{fl/fl}$ (n = 8) mice upon stimulation with plate-coated anti-CD3 (1 μg/mL) and soluble anti-CD28 (1 μg/mL) mAbs for 24 h. (*D* and *E*) The percentage of proliferating (*D*) or dead cells among naïve CD4$^+$ T cells (*E*) in the presence or absence of plate-coated anti-CD3 (0.5 μg/mL for CTV and 1 μg/mL for zombie violet) and soluble CD28 (2.5 μg/mL for CTV and 1 μg/mL for zombie violet) mAbs, determined using CTV and zombie violet, respectively (n = 8 per group). Cells were incubated for 24 h and 2 to 3 d for zombie violet and CTV staining, respectively. (*F*–*H*) The amounts of IL-2 in culture supernatants and percentages of proliferating or dead cells among naïve CD4$^+$ T cells, determined using ELISA, CTV, and zombie violet staining, upon treatment with the Lck inhibitor (at the indicated concentration) during incubation with plate-coated anti-CD3 (1 μg/mL for zombie violet and ELISA and 0.5 μg/mL for CTV) and soluble anti-CD28 (1 μg/mL for zombie violet and ELISA, 2.5 μg/mL for CTV) mAbs for 24 h (n = 7 per group). (*I*–*L*) Measurement of proliferation status of CD4$^+$ and CD8$^+$ T cells using CTV labeling in *Rag1* KO mice adoptively transferred with naïve T cells from *Cd4*-Cre*Ssu72*$^{fl/fl}$ or *Ssu72*$^{fl/fl}$ mice (*I* and *J*) and a mixture of naïve T cells from CD45.2$^+$ *Cd4*-Cre*Ssu72*$^{fl/fl}$ and CD45.1$^+$ WT C57BL/6 mice (*K* and *L*). (*M*–*O*) Blotting assay for the phosphorylation of downstream molecules of TCR signaling and nuclear translocation of NFκB p65 and NFAT1 in naïve CD4$^+$ T cells from *Cd4*-Cre*Ssu72*$^{fl/fl}$ and *Ssu72*$^{fl/fl}$ mice in the presence or absence of plate-coated anti-CD3 (1 μg/mL) and soluble anti-CD28 (1 μg/mL) mAbs for the indicated time (*N*) and 4 h (*O*). All experiments were performed independently three times, and data were pooled (*A*–*H*, *J*, and *L*) or are representative (*I*, *K*, and *M*–*O*). TCR-β$^+$ T cells were sorted using flow cytometry (*A* and *B*) or magnetic beads (*C*–*O*). Data are presented as the means ± SEMs. Statistical analysis was performed using the two-tailed, unpaired, and parametric Student's *t* test. N.S., not significant. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

might be attributable to the higher number of T_{conv} cells in *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice compared with *Ssu72*$^{fl/fl}$ mice. The TCR repertoires of CD4$^+$ T cells in the spleen from *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice were similar to those from *Ssu72*$^{fl/fl}$ mice (*SI Appendix*, Fig. S9*D*). Moreover, Ssu72-deficient T cells did not show a morphological transformation in the thymus, spleens, and lymph nodes (*SI Appendix*, Fig. S9*E*), although survival percentages of *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice were lower than those of *Ssu72*$^{fl/fl}$ mice (Fig. 7*I*). The expression of chemokines was increased in various organs of *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice compared with those of *Ssu72*$^{fl/fl}$ mice (*SI Appendix*, Fig. S9*F*). Taken together, these results indicate that aged *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice developed spontaneous inflammation.

Around 24 wk after generation of BM chimera mice using lethally irradiated recipient WT C57BL/6 mice with a 1:1 ratio of CD45.1$^+$ WT C57BL/6 and (CD45.2$^+$*Cd4*-Cre*Ssu72*$^{fl/fl}$ or *Ssu72*$^{fl/fl}$ BM cells), flow cytometric analysis demonstrated that mixed BM chimera mice exhibited similar thymic development and peripheral homeostasis of *i*NKT cells from *Ssu72*$^{fl/fl}$ mice and *Cd4*-Cre*Ssu72*$^{fl/fl}$ BM cells (*SI Appendix*, Fig. S10*A*). However, *i*NKT cell numbers were profoundly reduced in the liver of both mice reconstituted with BM from CD45.1$^+$ WT C57BL/6 + CD45.2$^+$*Cd4*-Cre*Ssu72*$^{fl/fl}$ mice (WT/Ssu72-deficient BM mice) and mice reconstituted with BM from CD45.1$^+$WT C57BL/6 + CD45.2$^+$*Ssu72*$^{fl/fl}$ mice (WT/control BM mice), whereas WT/Ssu72-deficient BM mice showed higher effector T cells but lower naïve T cells than WT/control BM mice did (*SI Appendix*, Fig. S10*A*). WT/Ssu72-deficient BM mice exhibited reduced body weights and intestine lengths but an increase in sizes and cell numbers of lymph node and spleen and high levels of serum IgG and IgG1, compared with WT/control BM mice (*SI Appendix*, Fig. S10 *B–D*). Considerable numbers of inflammatory cells were found in various organs, including the esophagus, stomach, colon, lung, and liver (*SI Appendix*, Fig. S11*A*), but not other organs (*SI Appendix*, Fig. S11*B*) from WT/Ssu72-deficient BM mice. The percentages of IFN-γ or IL-17–producing CD4$^+$ T cells, neutrophils, eosinophils, monocytes, macrophages, and chemokine expression were increased in these organs from WT/Ssu72-deficient BM mice, compared with those of WT/control BM mice (*SI Appendix*, Fig. S11 *C–E*). These results indicate that spontaneous inflammation in *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice might be attributable to Ssu72-deficient T cells rather than the reduction of *i*NKT cell numbers.

Discussion

In this study, it was demonstrated that Ssu72 bound to ZAP-70 in EL4 and CD4$^+$ T cells, even in the absence of TCR stimulation, suggesting that Ssu72 constitutively binds to ZAP-70 in T cells. The binding of the two proteins increased, peaked, and gradually decreased in CD4$^+$ T and EL4 cells after TCR stimulation, which was different from the dephosphorylation status of ZAP-70 in terms of timing (Figs. 1*B* and 2*A*). This phenomenon might be attributable to various factors, including the unique biology of protein interaction and the discrepancy in binding and enzyme activity of Ssu72. Moreover, in vitro assay demonstrated that recombinant Ssu72 reduced tyrosine (Y292, Y319, and Y493) phosphorylation of ZAP-70 via phosphatase activity. These findings indicate that Ssu72 acts as tyrosine phosphatase on ZAP-70 in T cells, although dephosphorylation activity and binding affinity of Ssu72 for p-ZAP-70 was lower than a ZAP-70 phosphatase PTP-γ (23–25). Consistent with our results, Meinhart et al. have reported that Ssu72 is a potential tyrosine phosphatase (26). Combined, these findings indicate that Ssu72 binds to ZAP-70 in naïve and activated T cells in different manners, thereby regulating ZAP-70 phosphorylation via tyrosine phosphatase activity. Thus, it is conceivable that Ssu72 might act as serine/threonine or tyrosine phosphatase, depending on cell types and/or target proteins.

In accordance, Ssu72-deficient CD4$^+$ T cells showed the greater phosphorylation of ZAP-70 (Y292, Y319, and Y493) and TCR downstream molecules upon TCR stimulation compared with WT CD4$^+$ T cells. The phosphorylation of Y319, located in interdomain B, is required for the kinase activity of ZAP-70 and TCR signaling via the promotion of the transition of ZAP-70 from an inactive to an active conformation, which provides a docking site for the SH2 domain of Lck. Along with Y493 phosphorylation in the activation loop, this process leads to the full activation of ZAP-70 (5, 6, 27, 28). Based on these findings, Ssu72 most likely contributes to maintenance of inactive ZAP-70 in naïve T cells and the balancing of ZAP-70 activation status during TCR-mediated activation. Moreover, the greater phosphorylation of TCR signaling molecules, IL-2 production, transcription factor expression, proliferation, and apoptosis were observed in Ssu72-deficient than WT CD4$^+$ T cells. These alterations were restored by the reduction of ZAP-70 phosphorylation using an Lck inhibitor. The differentiation of naïve T cells into Th1, Th2, and Th17 cells in vitro and in vivo was increased in Ssu72-deficient CD4$^+$ T cells, compared with WT CD4$^+$ T cells. These findings suggest that the Ssu72-mediated fine-tuning of TCR signaling regulates T cell activation and the differentiation of effector T cells by reducing ZAP-70 phosphorylation as tyrosine phosphatase, although whether the phosphatase activity of Ssu72 for RNA polymerase II also contributes to the function of T cells remains unclear. Nevertheless, our experiments demonstrated that PMA/ionomycin did not alter cellular events in Ssu72-deficient T cells, whereas TCR stimulation increased the activation of them, supporting that the Ssu72 deficiency effects in T cells is attributable to TCR proximal signaling rather than to more distal events, such as RNA synthesis. Thus, it is more likely that Ssu72-mediated RNA polymerase activity minimally affect T cell activation.

Consistent with Ssu72-deficient CD4$^+$ T cells, ZAP-70–binding phosphatase Sts1- and Sts2-deficient (Sts1/2 double KO) T cells had the increased phosphorylation of ZAP-70, LAT, SLP-76, and downstream signaling molecules during TCR-induced stimulation (29). In our experiments, the numbers and percentages of T cells at various developmental stages, as well as those of T_{reg} cells, were similar in the thymi from *Cd4*-Cre*Ssu72*$^{fl/fl}$ and *Ssu72*$^{fl/fl}$ mice. Thymic development of conventional T cells from *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice was similar to that of Sts1- and Sts2-deficient (Sts1/2 double KO) mice (29). In contrast, *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice demonstrated reductions in CD4$^+$ and CD8$^+$ T cells and dysregulation of CD8/CD4 T cell ratios in the periphery, whereas comparable proportions of peripheral T cells and ratios of CD4$^+$ and CD8$^+$ T cells were found in Sts1/2 double-KO mice (29). Furthermore, our experiments demonstrated that Ssu72 did not bind to Sts1 and Sts2 during T cell activation. These findings suggest that Ssu72 and Sts1/Sts2 bind to ZAP-70 in T cells, in the absence of direct interaction between Ssu72 and Sts1/Sts2, and differentially regulate the homeostasis of peripheral T cells, although these proteins bind to ZAP-70 and regulate its phosphorylation.

In contrast to conventional T and T_{reg} cells, *i*NKT cells were detected in minimal numbers in the thymi of *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice; such a finding has not been described in Sts1/2 double-KO mice (29). ZAP-70 mutation-mediated reduction of TCR signaling strength decreased *i*NKT cell numbers but induced a predominance of NKT1 cells in the thymus, indicating that altered TCR signaling strength affects the thymic development of *i*NKT cells (30). However, our experiments demonstrated that the intrinsic, Ssu72-mediated regulation of TCR signaling in *i*NKT cells was dispensable for their thymic development. Instead, the alteration of Ssu72-mediated, functional regulation of conventional thymocytes affected the thymic development of *i*NKT cells. Co-culture experiments using NKT hybridoma cells and thymocytes suggest that Ssu72 expression in conventional thymocytes might contribute to the thymic development of *i*NKT cells in a soluble

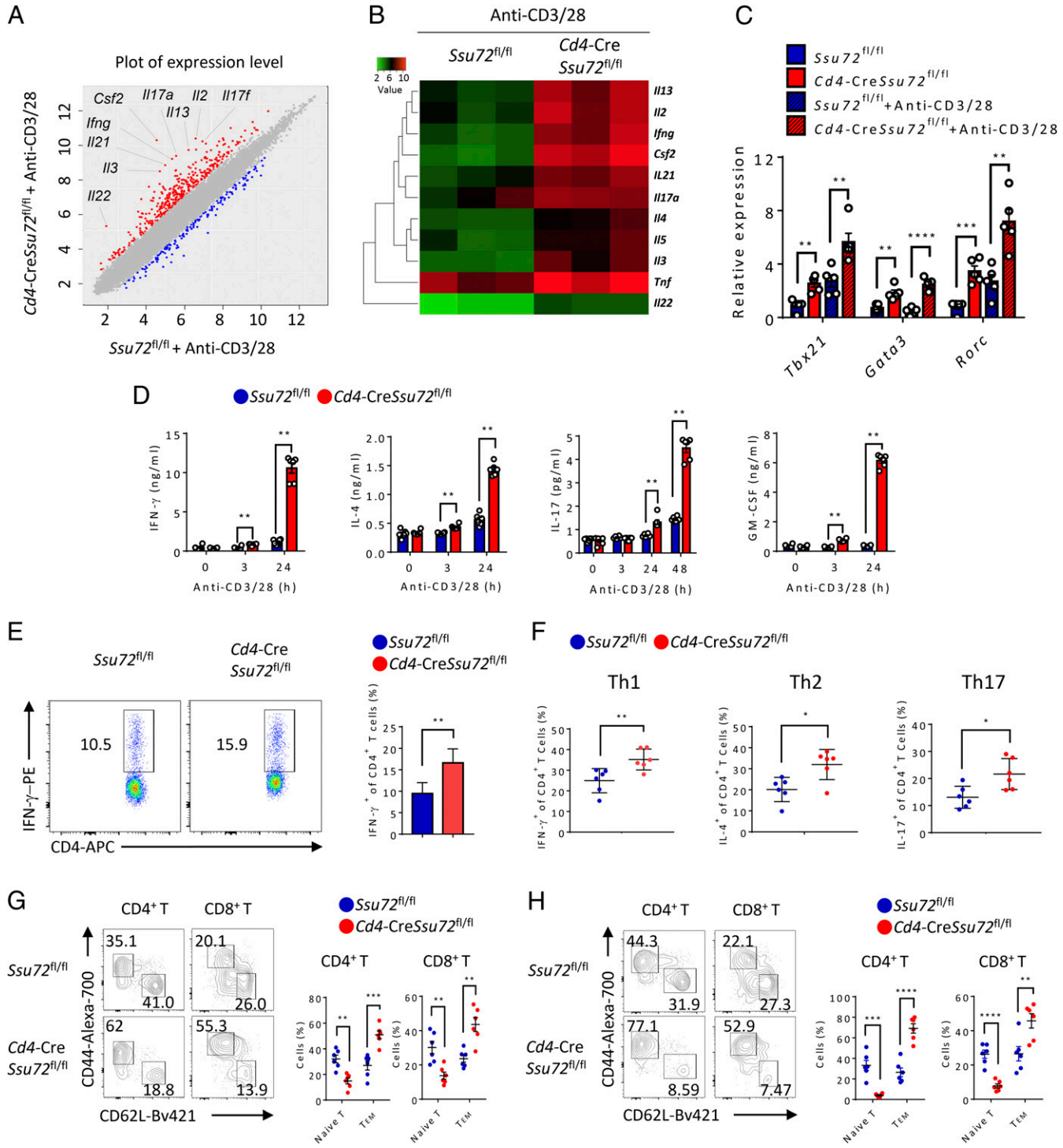

Fig. 6. Ssu72 regulates the differentiation of naïve T cells into effector and memory T cells. Scatter plot (*A*), heat map from microarray analysis (*B*), and expression levels of transcripts of various transcriptional factors (*C*) using sorted naïve CD4$^+$ T cells from *Ssu72*$^{fl/fl}$ and *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice using flow cytometry (*A* and *B*) or magnetic beads (*C*) in the presence and absence of plate-coated anti-CD3 (1 μg/mL) and soluble anti-CD28 (1 μg/mL) mAbs for 24 h. (*C*) $n = 5$ in *Ssu72*$^{fl/fl}$ and *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice. (*D* and *E*) The amounts of various cytokines in the culture supernatants (*D*) and intracytosol of naïve CD4$^+$ T cells (*E*) from *Ssu72*$^{fl/fl}$ ($n = 6$) and *Cd4*-Cre*Ssu72*$^{fl/fl}$ ($n = 6$) mice using magnetic beads in the presence and absence of plate-coated anti-CD3 (1 μg/mL) and soluble anti-CD28 (1 μg/mL) mAbs for the indicated time (*D*) or 24 h (*E*), determined using ELISA and intracellular staining, respectively. (*F*) The percentages of IFN-γ$^+$, IL-4$^+$, and IL-17$^+$ cells among CD4$^+$ TCRβ$^+$ T cells, measured by flow cytometric analysis after the culturing of sorted CD44^{lo}CD62L^{hi} naïve CD4$^+$ T cells from *Ssu72*$^{fl/fl}$ ($n = 6$) and *Cd4*-Cre*Ssu72*$^{fl/fl}$ ($n = 6$) mice using flow cytometry for in vitro differentiation of Th1, Th2, and Th17 cells. (*G* and *H*) Percentages of effector memory CD44^{hi}CD62L^{lo} CD4$^+$ and CD8$^+$ T cells and naïve CD44^{lo}CD62L^{hi} CD4$^+$ and CD8$^+$ T cells among TCR-β$^+$ T cells, measured by flow cytometric analysis, in spleens from *Ssu72*$^{fl/fl}$ ($n = 6$) and *Cd4*-Cre*Ssu72*$^{fl/fl}$ ($n = 6$) mice at 7 to 8 wk of age (young adult) (*G*) and 6 mo (*H*). Data from three independent experiments were pooled (*B*–*H*) and are representative (*A*) and presented as the means ± SEMs. Statistical analysis was performed using the two-tailed, unpaired, and parametric Student's *t* test (*C* and *E*–*H*) or nonparametric Mann–Whitney *U* test (*D*). N.S., not significant. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$.

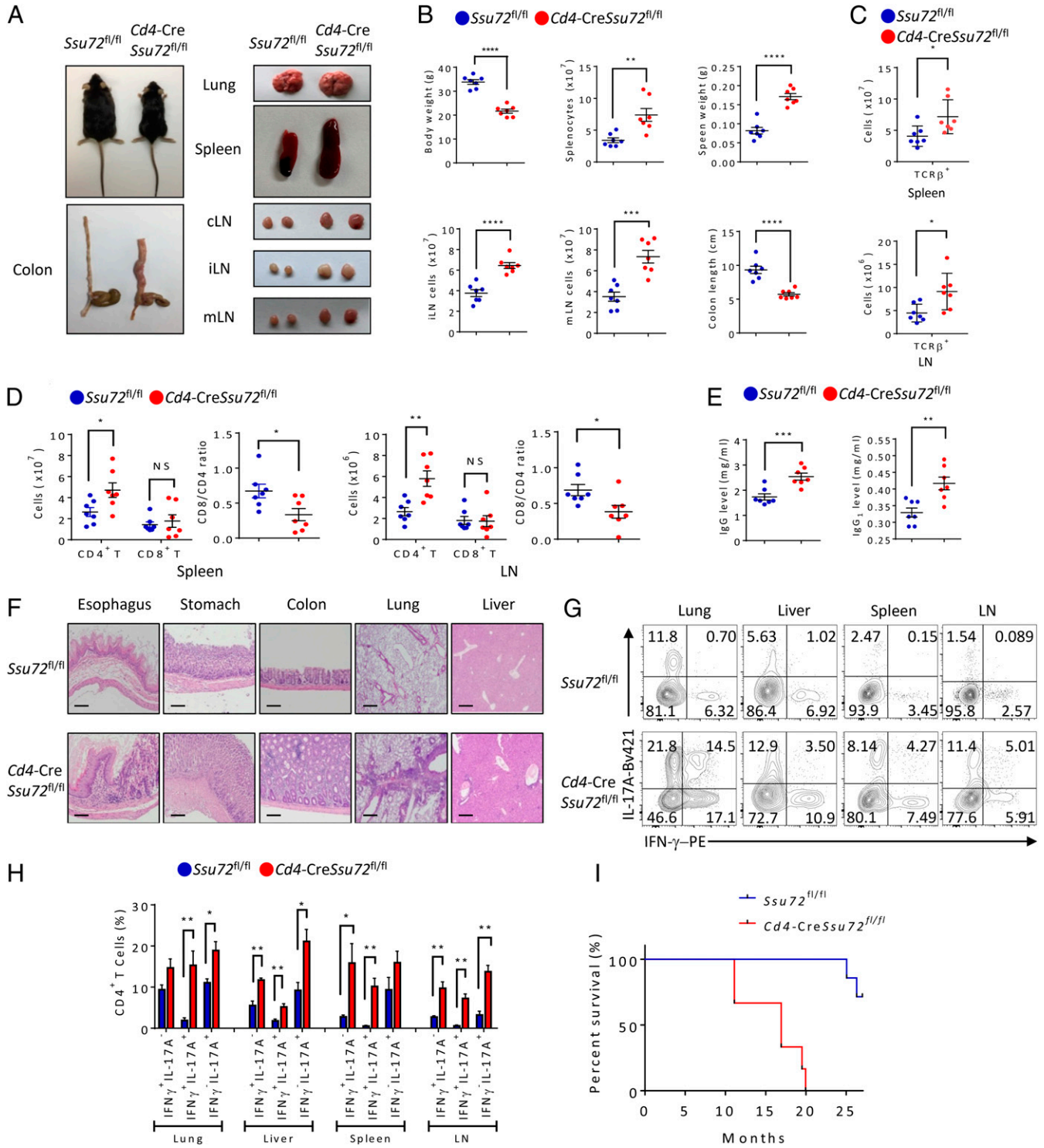

Fig. 7. *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice develop spontaneous inflammation. (*A*) Photographs of *Ssu72*$^{fl/fl}$ and *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice and their various organs at 6 mo of age. (cLN, cervical; iLN, inguinal; and mLN, mesenteric lymph node). (*B–E*) Body and spleen weights, colon length, spleen and lymph node cell numbers (total, TCR-β$^+$, CD4$^+$, and CD8$^+$ T cells), and serum IgG and IgG1 levels in *Ssu72*$^{fl/fl}$ and *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice at 6 mo of age. (*F*) Microscopic photographs of various organs from *Ssu72*$^{fl/fl}$ and *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice at 6 mo of age. The bars indicate 100 μm. (*G* and *H*) Upon stimulation with PMA (200 ng/mL)/ionomycin (1 μg/mL) the percentage of IL-17A- and/or IFN-γ–producing CD4$^+$ T cells was estimated in various organs from *Ssu72*$^{fl/fl}$ and *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice at 6 mo of age (*A–H*) ($n = 7$ per group). (*I*) The percentages of survival of *Ssu72*$^{fl/fl}$ ($n = 4$) and *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice ($n = 6$). All experiments were performed independently three times, and data are representative (*A*, *F*, and *G*) or were pooled (*B–E*, *H*, and *I*). Data are presented as the means ± SEMs. Statistical analysis was performed using the two-tailed, unpaired, and parametric Student's *t* test. LN, lymph node; N.S., not significant. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$.

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factor-dependent manner. Several studies have shown that GM-CSF and IL-15 play critical roles in the differentiation of pre-NKT cells into mature *i*NKT cells in the thymus (31, 32). However, our experiments suggest that Ssu72-mediated IL-2 (rather than GM-CSF and IL-15) production by conventional thymocytes regulates *i*NKT cell development in the thymus, although it has not been reported whether IL-2 regulates the development of *i*NKT cells in the thymus.

In the current study, aged *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice developed spontaneous inflammation spontaneously in various organs. Consistent with our findings in *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice, ZAP-70–binding phosphatase Sts1- and Sts2-deficient mice showed the enhancement of TCR-induced proliferation of T cells and the aggravation of spontaneous inflammation (29). The greater activation of T cells and larger numbers of effector (Th1 and Th17) and memory T cells, but smaller numbers of naïve CD4$^+$ and CD8$^+$ T cells in the periphery, were found in *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice than in young adult *Ssu72*$^{fl/fl}$ mice, and these effects were stronger in aged mice. These findings suggest that the accumulation of these effector and memory T cells in the periphery contributes to the development of spontaneous inflammation in *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice. Consistent with our results, a recent study demonstrated that Ssu72 overexpression inhibited Th17 cell responses in vitro and in vivo, resulting in the amelioration of experimental autoimmune arthritis (17). Furthermore, our experiments demonstrated that spontaneous inflammation developed in aged BM chimera mice that had a minimal number of *i*NKT cells and dysfunctional, Ssu72-deficient conventional T cells but not in BM chimera mice that had minimal *i*NKT cell number alone. These findings suggest that the minimal number of *i*NKT cells alone is not sufficient for the development of spontaneous inflammation in the absence of dysregulated conventional T cells in *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice. In addition, young adult *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice showed similar percentages and the suppressive effect of T_{reg} cells but larger percentages of effector–memory T cells compared with *Ssu72*$^{fl/fl}$ mice, whereas the percentages of T_{reg} and effector–memory T cells were increased in aged *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice. These results suggest that the ratios of effector–memory T cells versus T_{reg} cells in the periphery differ among young adult and aged *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice and *Ssu72*$^{fl/fl}$ mice. However, the contributions of T_{reg} cells to spontaneous inflammation in aged *Cd4*-Cre*Ssu72*$^{fl/fl}$ mice remain elusive.

In conclusion, our results suggest that Ssu72 regulates the fine-tuning of TCR signaling by binding to ZAP-70 and regulating its tyrosine phosphorylation in T cells via phosphatase activity, thereby inhibiting T cell activation and preventing spontaneous inflammation.

Materials and Methods

Mice. C57BL/6 mice (7 to 8 wk of age) were purchased from KoaTech (Pyeongtaek, Gyeongki-do, Korea). Congenic C57BL/6 (45.1$^+$), *Rag1* knockout (KO) and Foxp3$^{YFP\text{-}Cre}$ mice were purchased from The Jackson Laboratory. Age- and sex-matched littermate mice were used for all experiments. Young adult (7 to 8 wk of age) mice were used in most experiments, while aged mice (24 wk of age) were also used for investigating spontaneous inflammation. Details for mouse information are described in *SI Appendix*.

Detailed information for the materials and methods used in this study is provided in *SI Appendix, Materials and Methods*.

Data Availability. All study data are included in the article and/or *SI Appendix*.

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