Kobe J. Med. Sci., Vol. 63, No. 1, pp. E22-E29, 2017

Role of SIRPα in Homeostatic Regulation of T Cells and Fibroblastic Reticular Cells in the Spleen

DATU RESPATIKA[1], YASUYUKI SAITO[1], KEN WASHIO[1,2], SATOMI KOMORI[1], TAKENORI KOTANI[1], HIDEKI OKAZAWA[1], YOJI MURATA[1], and TAKASHI MATOZAKI[1,*]

[1]*Division of Molecular and Cellular Signaling, Department of Biochemistry and Molecular Biology, Kobe University Graduate School of Medicine, Kobe, Japan*

[2]*Division of Dermatology, Department of Internal Related, Kobe University Graduate School of Medicine, Kobe, Japan*

[*]*Corresponding author*

Received 23 January 2017/ Accepted 7 February 2017

Key words: Signal regulatory protein α, Dendritic cells, Fibroblastic reticular cells, T cells, Spleen

Signal regulatory protein α (SIRPα), is an immunoglobulin superfamily protein that is predominantly expressed in macrophages and dendritic cells (DCs), especially CD4$^+$ conventional DCs (cDCs). In this study, we demonstrated that, in addition to the reduced number of CD4$^+$ cDCs, the number of T cells was significantly decreased in the spleen of *Sirpa*$^{-/-}$ mice, in which full-length of SIRPα protein was systemically ablated. The size of the T cell zone was markedly reduced in the spleen of *Sirpa*$^{-/-}$ mice. In addition, *Sirpa*$^{-/-}$ mice revealed a marked reduction of CCL19, CCL21, and IL−7 expression, which are thought to be important for homeostasis of T cells in the spleen. Consistently, the abundance of fibroblastic reticular cells (FRCs), a subset of stromal cells in the T cell zone, was markedly reduced in the spleen of *Sirpa*$^{-/-}$ mice compared with *Sirpa*$^{f/f}$ mice. Moreover, we demonstrated that the mRNA expression of Lymphotoxin (LT) α, LTβ, and LIGHT was significantly reduced in the spleen of *Sirpa*$^{-/-}$ mice. These data thus suggest that SIRPα is essential for steady-state homeostasis of T cells and FRCs in the spleen.

The spleen and lymph nodes (LNs) are classified as secondary lymphoid organs (SLOs), where innate and adaptive immune responses take place [21]. Various types of immune cells including T cells, B cells, and dendritic cells (DCs), as well as non-hematopoietic stromal cells, are thought to provide microenvironments to initiate critical interactions between these cells in SLOs [21]. In the spleen, the white pulp consists of the T cell zone, B cell follicles, and their surrounding marginal zones [4,18]. Trafficking and positioning of lymphocytes are thought to be regulated by homeostatic chemokines and cytokines, which are produced by distinct stromal cells in the white pulp of the spleen. For instance, fibroblastic reticular cells (FRCs) extend throughout the T cell zone in the white pulp and secrete CCL19 and CCL21, which are ligands for CCR7$^+$ naive T cells [15]. FRCs also produce IL-7, which maintains survival and proliferation of naive T cells in the spleen [13]. By contrast, in the B cell follicles, follicular dendritic cells produce CXCL13 to attract CXCR5$^+$ B cells into the follicles [9,21]. Production of these homeostatic chemokines is largely regulated by lymphotoxin (LT) β receptor (LTβR) signaling, in which $LT\alpha_1\beta_2$-expressing hematopoietic cells interact with stromal cells, suggesting that the interaction between hematopoietic cells and non-hematopoietic cells is important for T cell homeostasis. However, the molecular basis for such homeostatic regulation of T cells, as well as of stromal cells, in the spleen remains unclear.

Signal regulatory protein α (SIRPα) is a transmembrane protein that comprises three Ig-like domains in its extracellular region and immunoreceptor tyrosine-based inhibition motifs that mediate the binding of protein tyrosine phosphatases Shp1 and Shp2 in its intracellular region [2,17]. The extracellular region of SIRPα interacts with its ligand CD47, and such interaction plays important roles in both hematological and immunological regulation [2,17,22]. Among immune cells, SIRPα is predominantly expressed on DCs, especially CD4$^+$ conventional DCs (cDCs) [25], macrophages and monocytes, while it is not detectable in T or B cells [17]. In contrast, CD47 is ubiquitously expressed in both hematopoietic cells as well as non-hematopoietic cells including T cells and stromal cells. We previously demonstrated that the mice that express a mutant form of SIRPα lacking most of the cytoplasmic region (SIRPα MT mice) manifested a significant reduction of CD4$^+$ cDCs in the spleen [25]. Moreover, the size of the T cell zone as well as the number of CD4$^+$ T cells were markedly reduced in the spleen of SIRPα MT mice [26]. Indeed, the expression of CCL19 and CCL21 was also

Phone: +81-78-382-5600 Fax: +81-78-382-5619 E-mail: matozaki@med.kobe-u.ac.jp

decreased in the mutant mice [26], suggesting that SIRPα is important for the development of the T cell zone as well as the expression of CCL19 and CCL21 in the spleen. However, the detailed mechanism by which SIRPα regulates the steady-state homeostasis of T cells, as well as of chemokine expression, in the spleen, has remained unclear. In addition, since SIRPα MT mice were not SIRPα-null mutant mice, their phenotypes might not be attributable to the simple ablation of SIRPα function.

Thus, we have generated the mice, in which full-length of SIRPα protein is systemically ablated (*Sirpa*$^{-/-}$ mice). By using *Sirpa*$^{-/-}$ mice, we here examined the role of SIRPα in the homeostatic regulation of T cells and FRCs in the spleen.

MATERIALS AND METHODS

Animals

Sirpa$^{f/f}$ mice were generated from C57BL/6J mice [32]. CMV-*Cre* mice from the Jackson Laboratory (Bar Harbor, ME, USA) were crossed with *Sirpa*$^{f/f}$ mice, and the resulting *Sirpa*$^{f/f}$; CMV-*Cre* (*Sirpa*$^{-/-}$) descendants were studied. Sex- and age-matched mice at 8 to 12 weeks of age were used for experiments in this study. Mice were bred and maintained at the Institute of Experimental Animal Research of Kobe University Graduate School of Medicine under specific pathogen–free conditions and all animal experiments were performed according to Kobe University Animal Experimentation Regulations.

Antibodies and reagents

An fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody (mAb) to B220 (RA3-682); a phycoerythrin (PE)-conjugated mAb to CD4 (RM4-5); and an allophycocyanin (APC)-conjugated mAb to CD11c (HL3) were obtained from BD Biosciences (San Jose, CA, USA). An FITC−conjugated mAb to Thy1.2 (53−2.1); a PE-conjugated mAb to F4/80 (BM8); purified mAbs to CD16/32 (93) and podoplanin (Pdpn) (eBio8.1.1); a biotin-conjugated mAb to Thy1.2 (30-H12) were obtained from eBioscience (San Diego, CA). An FITC−conjugated mAb to CD172a (P84); an Alexa-488-conjugated mAb to CD3ε (17A2); A PE-conjugated mAb to CD31 (MEC13.3); a peridinin chlorophyll protein complex (PerCP)-cyanine (Cy)5.5-conjugated mAb to CD45 (30-F11) and Ter119 (TER119); an APC-conjugated mAb to Pdpn (8.1.1); an APC-Cy7-conjugated mAb to B220 (RA3-6B2); a brilliant violet 421-conjugated CD11b (M1/70); a brilliant violet 510-conjugated mAb to CD8α (53-6.7); and Zombie Aqua Fixable Viability Kit were obtained from BioLegend (San Diego, CA). Cy3-conjugated donkey polyclonal antibodies (pAbs) to goat IgG and hamster IgG, and Cy3-conjugated streptavidin were from Jackson ImmunoResearch Laboratories (West Grove, PA). Goat anti-mouse CCL21 and CXCL13 pAbs were purchased from R&D Systems (Minneapolis, USA). Propidium iodide (PI) was obtained from Sigma-Aldrich (St. Louis, MO, USA).

Cell preparation and flow cytometry

Cell suspensions were prepared from the spleen as described previously with minor modifications [6,25]. For preparation of splenocytes, the spleen was minced and then digested with RPMI 1640 (Wako, Osaka, Japan) containing 1 mg/mL collagenase IV (Worthington Biochemical, Lakewood, NJ), 40 μg/mL DNaseI (Roche, Manheim, Germany), and 2% fetal bovine serum (FBS) for 20 minutes at 37°C. For preparation of stromal cells in the spleen, the spleen was cut into small pieces and digested for 30 minutes with RPMI 1640 (Wako) containing 0.2 mg/mL collagenase P (Roche, Manheim, Germany), 0.8 mg/mL Dispase II (Roche, Manheim, Germany), 100 μg/mL DNaseI (Roche), and 2% FBS for 20 minutes at 37°C. The undigested fibrous material was removed by filtration through a 70 μm nylon mesh, and red blood cells in the filtrate were lysed with pharm lyse buffer (BD Biosciences). The remaining cells were washed twice with FACS buffer containing 2% FBS and 2 mM EDTA in phosphate buffered saline (PBS). For flow cytometric analysis, cells were first incubated with a mAb specific for mouse CD16/32 to prevent nonspecific binding of labeled mAbs to Fcγ receptors and were thereafter labeled with specific mAbs, and then subjected to flow cytometric analysis with the use of an FACSVerse (BD Biosciences, San Jose, CA, USA). All data were analyzed with FlowJo X software (FlowJo. Inc).

Immunohistofluorescence analysis

For immunohistofluorescence analysis, the spleen was directly embedded in optimal cutting temperature compound (Sakura Fine Technical, Tokyo, Japan) and immediately frozen in isopentane cooled with liquid nitrogen, followed by cutting into 7 μm sections by a Cryostat (Leica, Wetzlar, Germany) and dried up by cold wind for 30 minutes. All sections were fixed with cold acetone (for Thy1.2, CCL21, and CXCL13 staining) or methanol (for Pdpn staining) for 10 minutes. Sections were then incubated for 1 h at room temperature in blocking solution (5% BSA in PBS) followed by staining with primary Abs diluted in blocking solution overnight at 4°C. They were then washed with PBS, stained with Cy3- or FITC- conjugated Abs diluted in

blocking solution for 1 h at room temperature before acquiring the image with a BX-51 microscope (Olympus, Tokyo, Japan). The images were then processed with Adobe Photoshop software (Adobe Systems, San Jose, USA). For measurement of areas for the Thy1.2$^+$, B220$^+$, CCL21$^+$, CXCL13$^+$, or Pdpn$^+$ area in the spleen, the values for positively stained regions from each section were obtained and averaged by the use of ImageJ software (National Institute of Health, Bethesda, MD, USA).

Preparation of cDNA and quantitative real-time PCR

Total RNA was extracted from the freshly isolated spleen using Sepasol (Nacalai, Kyoto, Japan) and RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The first-strand cDNA was synthesized from 1 μg total RNA using the QuantiTect Reverse Transcription kit (Qiagen) according to the manufacturer's instructions. cDNA fragments of interest were amplified using the QuantiTect SYBR Green PCR kit (Qiagen) on LightCycler 480 (Roche Applied Science, Penzberg, Germany) in 96-well or 384-well plates (Roche Diagnostics). The amplification results were analyzed by the use of LightCycler 480 software (Roche Applied Science) and then normalized to *Gapdh* levels for each sample. Primer sequences for quantitative real-time PCR were as follows: *Gapdh*, forward: 5'-AGGTCGGTGTGAACGGATTTG-3', reverse: 5'-TGTAGACCATGTAGTTGAGGTCA-3'; *Ccl21*, forward: 5'-ATCCCGGCAATCCTGTTCTC-3', reverse: 5'-GGGGCTTTGTTTCCCTGGG-3'; *Ccl19*, forward: 5'-GGGGTGCTAATGATGCGGAA-3', reverse: 5'-CCTTAGTGTGGTGAACACAACA-3'; *Cxcl13*, forward: 5'-GGCCACGGTATTCTGGAAGC-3', reverse: 5'-GGGCGTAACTTGAATCCGATCTA-3'; *Il7*, forward: 5'-GATAGTAATTGCCCGAATAATGAACCA-3', reverse: 5'-GTTTGTGTGCCTTGTGATACTGTTAG-3'; *Lta*, forward: 5'-TCCACTCCCTCAGAAGCACT-3', reverse: 5'-AGAGAAGCCATGTCGGAGAA-3'; *Ltb*, forward: 5'-TGCGGATTCTACACCAGATCC-3', reverse: 5'-ACTCATCCAAGCGCCTATGA-3'; *Tnfsf14* (LT-like, exhibits inducible expression and competes with HSV glycoprotein D for herpes virus entry mediator, a receptor expressed by T lymphocytes [LIGHT]), forward: 5'-CAACCCAGCAGCACATCTTA-3', reverse: 5'-ATACGTCAAGCCCCTCAAGA-3'.

Statistical analysis

Data are presented as means ±SE and were analyzed by Student's *t* test with the use of Prism6 software (GraphPad Software, Inc. USA). A P value of <0.05 was considered statistically significant.

RESULTS

Marked reduction of CD4$^+$ cDCs and T cells in the spleen of *Sirpa*$^{-/-}$ mice

To examine the impact of SIRPα ablation on hematopoietic cells in the spleen, we examined the population of hematopoietic cells in the spleen of *Sirpa*$^{-/-}$ mice, in which full-length SIRPα protein was systemically ablated from the mice. Similar to the result from SIRPα MT mice [11], the weight of the spleen at 9 weeks of age was significantly increased in *Sirpa*$^{-/-}$ mice compared with control *Sirpa*$^{f/f}$ mice (**Figure 1A**). In the spleen, DCs are classified into two major populations, namely plasmacytoid DCs (pDCs) and cDCs. pDCs play an important role in type I interferon production upon viral infection, whereas cDCs are thought to be professional antigen-presenting cells that interact with T cells during immune responses [19]. cDCs are further subdivided into CD4$^+$ (CD4$^+$CD8α$^-$), CD8$^+$ (CD4$^-$CD8α$^+$), and double-negative (DN, CD4$^-$CD8α$^-$) cDCs, based on the expression of CD4 and CD8α [25]. As we have recently demonstrated [32], flow cytometric analysis showed that the frequency, as well as the absolute number, of cDCs in the spleen of *Sirpa*$^{-/-}$ mice were significantly reduced compared with those in control *Sirpa*$^{f/f}$ mice (**Figure 1B**). In contrast, the frequency and the absolute number of pDCs subsets in the spleen of *Sirpa*$^{-/-}$ mice were similar to those of *Sirpa*$^{f/f}$ mice (**Figure 1B**). Among cDCs subsets, the proportion and the absolute number of CD4$^+$ cDCs were markedly reduced in the spleen of *Sirpa*$^{-/-}$ mice compared with *Sirpa*$^{f/f}$ mice, while those of CD8$^+$ cDCs and DN cDCs subsets in the spleen of *Sirpa*$^{-/-}$ mice were comparable to *Sirpa*$^{f/f}$ mice (**Figure 1C**). These phenotypes are identical to those of SIRPα MT mice [25]. Among mature hematopoietic cell lineages in the spleen, SIRPα is also expressed in macrophages and monocytes, defined as CD11b$^+$F4/80$^+$ and CD11b$^+$F4/80$^-$ cells within B220$^-$CD11c$^-$ cells, respectively [32]. We found that the frequency and the absolute number of these cells in the spleen were similar between *Sirpa*$^{-/-}$ and *Sirpa*$^{f/f}$ mice (**Figure 1D**).

We next examined the T cell population in the spleen of *Sirpa*$^{-/-}$ mice. Quantitative immunohistofluorescence analyses of T cells (Thy1.2$^+$) and B cells (B220$^+$) in *Sirpa*$^{-/-}$ mice revealed that the area of the Thy1.2$^+$ T cell zone was significantly reduced in the spleen of *Sirpa*$^{-/-}$ mice (**Figure 1E**). Consistently, the absolute number of CD4$^+$ T cells or CD8$^+$ T cells was significantly decreased in the spleen of *Sirpa*$^{-/-}$ mice compared with that apparent for *Sirpa*$^{f/f}$ mice (**Figure 1F**). In contrast, the size of the B cell zone as well as the absolute number of B cells in the spleen of *Sirpa*$^{-/-}$ mice were similar to those of *Sirpa*$^{f/f}$ mice (**Figures 1, E and F**).

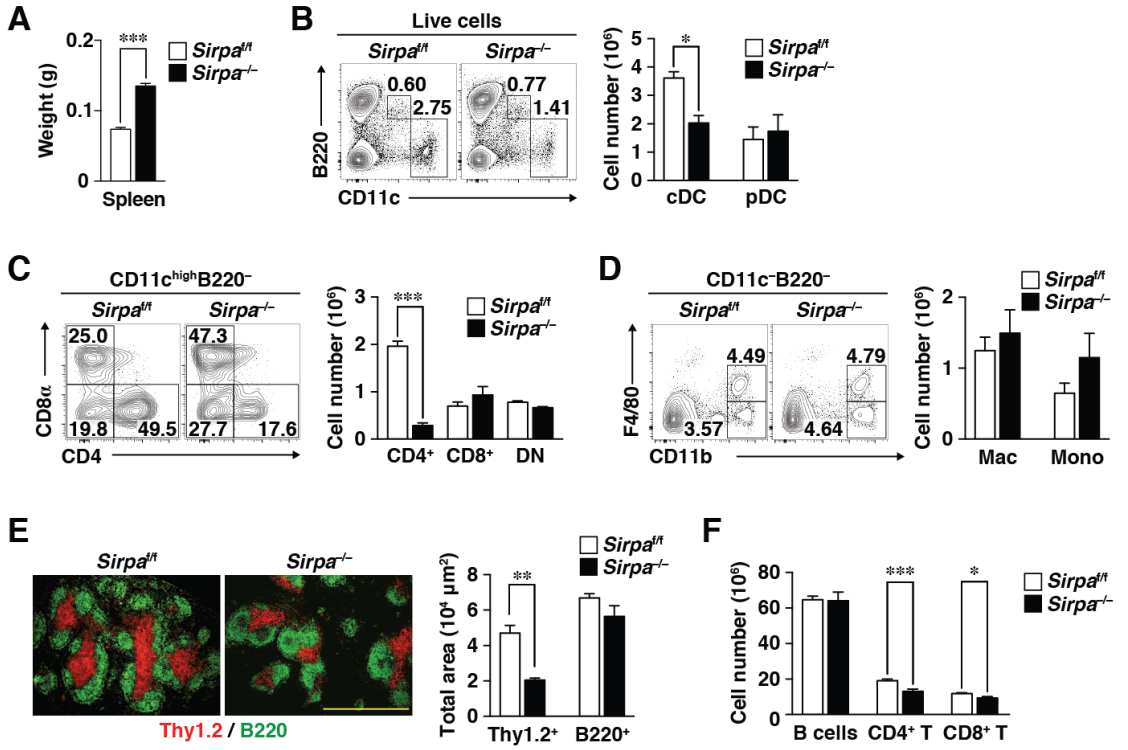

Figure 1. Marked reduction of $CD4^+$ cDCs and T cells in the spleen of *Sirpa*$^{-/-}$ mice. (**A**) Weight of the spleen of *Sirpa*$^{f/f}$ or *Sirpa*$^{-/-}$ mice at 9 weeks of age. Data are means ±SE from six mice per group. *** $p < 0.001$ (Student's *t* test). (**B-D**) Splenocytes isolated from *Sirpa*$^{f/f}$ or *Sirpa*$^{-/-}$ mice were stained for CD4, CD8α, CD11c, CD11b, F4/80 and B220 as well as with PI and were then analyzed by flow cytometry. (**B**) Frequency of cDCs ($CD11c^{high}B220^-$) and pDCs ($CD11c^{int}B220^+$) among PI^- live cells from the spleen of *Sirpa*$^{f/f}$ or *Sirpa*$^{-/-}$ mice (left panel). The absolute number of total cDCs and pDCs were shown (right panel). Data are means ±SE of values from total three mice per group in three independent experiments. * $p < 0.05$ (Student's *t* test). (**C**) Frequency of $CD4^+$ ($CD4^+CD8\alpha^-$), $CD8^+$ ($CD4^-CD8\alpha^+$), and DN ($CD4^-CD8\alpha^-$) cDC subsets among $CD11c^{high}B220^-$ cells (left panel). The absolute number of total $CD4^+$, $CD8^+$, and DN cDCs were shown (right panel). Data are means ±SE of values from total three mice per group in three independent experiments. *** $p < 0.001$ (Student's *t* test). (**D**) Frequency of macrophages (Mac: $CD11b^+F4/80^+$) and monocytes (Mono; $CD11b^+F4/80^-$) among $CD11c^-B220^-$ cells from the spleen of *Sirpa*$^{f/f}$ or *Sirpa*$^{-/-}$ mice (left panel). The absolute number of total macrophages and monocytes were shown (right panel). Data are means ±SE of values from total three mice per group in three independent experiments. (Student's *t* test). (**E**) Frozen sections of the spleen from *Sirpa*$^{f/f}$ or *Sirpa*$^{-/-}$ mice were stained with mAbs to Thy1.2 (red) and B220 (green). Images are representative of three mice per group. Scale bar, 1 mm. The area for $Thy1.2^+$ area and $B220^+$ area were measured per each image (right panel). Data are means ±SE of values from three mice per group with three fields of view for each sample. ** $p < 0.01$ (Student's *t* test). (**F**) Splenocytes isolated from *Sirpa*$^{f/f}$ or *Sirpa*$^{-/-}$ mice were stained for CD4, CD8α, CD3ε, and B220 as well as with PI and were then analyzed by flow cytometry. The absolute number of $CD3\varepsilon^-B220^+$ (B cells), $CD4^+CD8\alpha^-CD3\varepsilon^+$ ($CD4^+$ T cells), or $CD4^-CD8\alpha^+CD3\varepsilon^+$ ($CD8^+$ T cells) among PI^- cells from the spleen of *Sirpa*$^{f/f}$ or *Sirpa*$^{-/-}$ mice was shown. Data are means ±SE of values from three mice per group in three independent experiments. * $p < 0.05$; *** $p < 0.001$ (Student's *t* test).

Reduced expression of CCL19, CCL21, and IL-7 in the spleen of *Sirpa*$^{-/-}$ mice

The smaller size of the T cell zone and the reduced number of T cells in the spleen of *Sirpa*$^{-/-}$ mice suggested that homing or survival of T cells is impaired. The amounts of CCL19 and CCL21 are necessary for attraction and retaining of naive T cells into T cell area of the spleen [27]. On the other hand, the production of CXCL13 is crucial for homing of B cells into the B cell zone of the spleen [8]. We found that the expression levels of *Ccl19* and *Ccl21* mRNAs were significantly reduced in the spleen of *Sirpa*$^{-/-}$ mice compared with *Sirpa*$^{f/f}$ mice (**Figure 2A**). IL-7 is required for the proliferation and survival of naive T cells [13,28]. We also found that the expression level of *Il7* mRNA was markedly reduced in the spleen of *Sirpa*$^{-/-}$ mice compared with that of *Sirpa*$^{f/f}$ mice (**Figure 2A**). Immunohistofluorescence analysis showed that a significant reduction of CCL21 staining in the spleen of *Sirpa*$^{-/-}$ mice compared with *Sirpa*$^{f/f}$ mice (**Figure 2B**). By contrast, the intensity of CXCL13 staining in the spleen was comparable between *Sirpa*$^{-/-}$ and *Sirpa*$^{f/f}$ mice (**Figure 2C**).

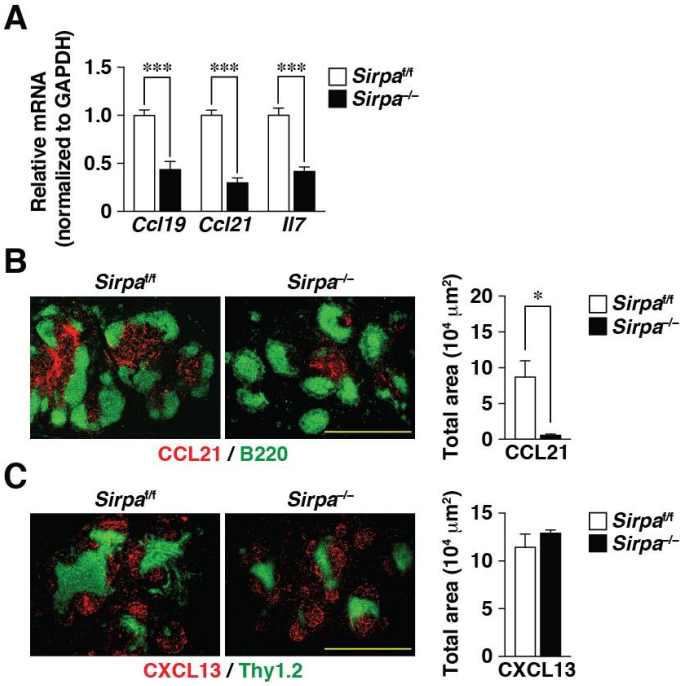

Figure 2. Reduced expression of CCL19, CCL21, and IL-7 in the spleen of *Sirpa*$^{-/-}$ mice. (**A**) Quantitative real time−PCR analysis of CCL19 (*Ccl19*), CCL21 (*Ccl21*), or IL−7 (*Il7*) mRNA in the spleen of *Sirpa*$^{f/f}$ or *Sirpa*$^{-/-}$ mice. The amount of each mRNA was normalized by that of GAPDH mRNA and expressed as fold increase relative to the value for *Sirpa*$^{f/f}$ mice. Data are means ±SE of values from total three mice per group. *** $p < 0.001$ (Student's *t* test). (**B**) Frozen sections of the spleen from *Sirpa*$^{f/f}$ or *Sirpa*$^{-/-}$ mice were stained with pAb to CCL21 (red) and a mAb to B220 (green). Images are representative of three mice per group (left panel). Scale bar, 1 mm. The area for CCL21 was measured per each image (right panel). Data are means ±SE of values from three mice per group with three fields of view for each sample. * $p < 0.05$ (Student's *t* test). (**C**) Frozen sections of the spleen from *Sirpa*$^{f/f}$ or *Sirpa*$^{-/-}$ mice were stained with pAb to CXCL13 (red) and a mAb to Thy1.2 (green). Images are representative of three mice per group (left panel). Scale bar, 1 mm. The area for CXCL13 was measured per each image (right panel). Data are means ±SE of values from three mice per group with three fields of view for each sample. (Student's *t* test).

Reduction of FRCs in the spleen of *Sirpa*$^{-/-}$ mice

In SLOs, stromal cells are known to be essential for the maintenance of hematopoietic cells. In particular, FRCs express CCL19, CCL21, and IL-7 for attraction of naive T cells to the white pulp [28], implicating that FRCs are indispensable for the maintenance of T cells in the spleen. By use of flow cytometry, CD45$^-$ Ter119$^-$ non-hematopoietic cells in the spleen were classified into three subsets of stromal cells on the basis of the surface expression of Pdpn and CD31 [5], namely CD31$^-$Pdpn$^+$ (FRCs), CD31$^+$Pdpn$^-$ (blood endothelial cells, BECs), and CD31$^-$Pdpn$^-$ (double-negative cells, DNCs), respectively (**Figure 3A**). We found that the expression of SIRPα was negative in all splenic stromal cell subsets in the spleen (**Figure 3A**). We next examined the size of FRCs area in the spleen of *Sirpa*$^{-/-}$ mice. Immunohistofluorescence analyses revealed that the staining for Pdpn, a mucin-type transmembrane glycoprotein and a marker for FRCs [21], was markedly reduced in the spleen of *Sirpa*$^{-/-}$ mice compared with that of *Sirpa*$^{f/f}$ mice (**Figure 3B**).

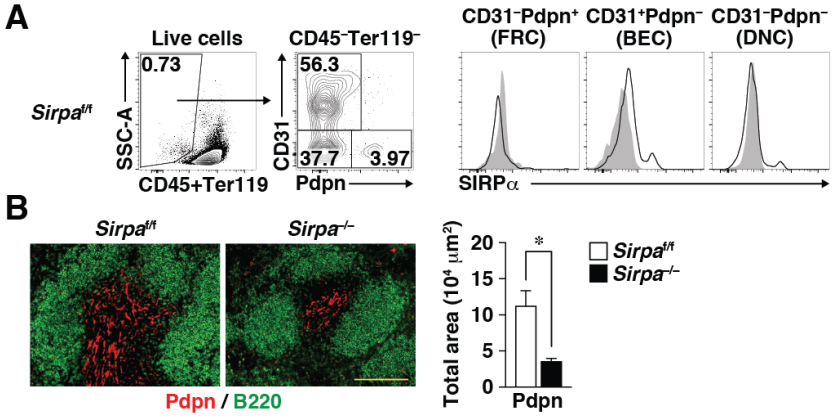

Figure 3. Reduction of FRCs in the spleen of *Sirpa*$^{-/-}$ mice. (**A**) Splenocytes isolated from *Sirpa*$^{f/f}$ mice were stained for CD45, Ter119, CD31, Pdpn, and Aqua, as well as SIRPα or with isotype control antibody, and were then analyzed by flow cytometry. Aqua$^-$ live cells were gated on Ter119$^-$CD45$^-$ cells and were further divided into FRC (CD31$^-$Pdpn$^+$), BEC (CD31$^+$Pdpn$^-$), and DNC (CD31$^-$Pdpn$^-$) (left panel). The expression of SIRPα (open traces) or isotype control (filled traces) within FRC, BEC, and DNC was shown (right panel). Numbers indicate the frequency of cells in each gate. Data are representative of two independent experiments. (**B**) Frozen sections of the spleen from *Sirpa*$^{f/f}$ or *Sirpa*$^{-/-}$ mice were stained with mAbs to Pdpn (red) and to B220 (green). Images are representative of three mice per group (left panel). Scale bar, 200 μm. The area for Pdpn was measured per each image (right panel). Data are means ±SE of values from three mice per group with three fields of view for each sample. * $p < 0.05$ (Student's *t* test).

Reduced expression of TNFR and LTβR ligands in the spleen of *Sirpa*$^{-/-}$ mice

Tumor necrosis factor receptor (TNFR) and LTβR signaling are known to regulate the development of the T cell zone in the spleen [23]. Thus, we next examined the expression of TNFR or LTβR ligands in the spleen of *Sirpa*$^{-/-}$ mice. LT consists of either a soluble form ($LT\alpha_3$) or a membrane-anchored form ($LT\alpha_1\beta_2$), which binds TNFR or LTβR, respectively [14]. LIGHT (TNFSF14) also binds to LTβR with high affinity. LT and LIGHT are thought to be important for CCL19 or CCL21 production from FRCs [24,31]. We found that mRNA expression levels of LTα, LTβ, and LIGHT were markedly decreased in the spleen of *Sirpa*$^{-/-}$ mice compared with that of *Sirpa*$^{f/f}$ mice (**Figure 4**).

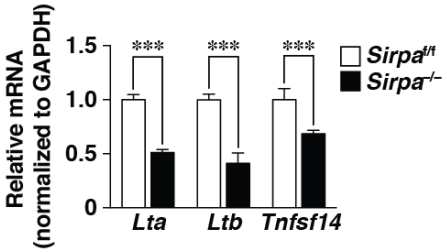

Figure 4. Reduced expression of LTα, LTβ, and LIGHT in the spleen of *Sirpa*$^{-/-}$ mice. Quantitative RT−PCR analysis of lymphotoxin α (*Lta*), lymphotoxin β (*Ltb*), or LIGHT (*Tnfsf14*) mRNA in the spleen of *Sirpa*$^{f/f}$ or *Sirpa*$^{-/-}$ mice. The amount of each mRNA was normalized by that of GAPDH mRNA and expressed as fold increase relative to the value for *Sirpa*$^{f/f}$ mice. Data are means ±SE of values from total six mice per group examined in three independent experiments. *** $p < 0.001$ (Student's *t* test).

DISCUSSION

In the present study, we demonstrated that SIRPα null-mutant (*Sirpa*$^{-/-}$) mice manifested marked reduction of $CD4^+$ or $CD8^+$ T cells in the spleen. Consistently, the $Thy1.2^+$ T cell zone was also reduced in the spleen of *Sirpa*$^{-/-}$ mice. Such phenotype was identical to that observed in SIRPα MT mice [26], in which only cytoplasmic region of SIRPα protein was ablated [10]. Thus, as we previously described [26], we here confirm that SIRPα is indeed indispensable for homeostasis of T cells in the spleen. Moreover, it is now suggested that phenotypes of SIRPα MT mice are indeed attributable to loss of SIRPα function, particularly signaling downstream of SIRPα mediated by Shp1 or Shp2.

We also demonstrated that the expression of CCL19, CCL21, and IL-7, all of which are produced by FRCs and thought to be essential for the attraction and survival of naive T cells, was significantly reduced in the spleen of *Sirpa*$^{-/-}$ mice. Furthermore, the size of the $Pdpn^+$ FRC area was markedly reduced in the spleen of *Sirpa*$^{-/-}$ mice. Therefore, impaired homeostasis of T cells in the spleen of *Sirpa*$^{-/-}$ mice is likely attributable to reduced population of FRCs that produce these chemokines. By contrast, we here showed that the expression of SIRPα is minimal in splenic FRCs. Given that the expression of SIRPα is also minimal in T cells [17], SIRPα is unlikely required in a cell autonomous manner for homeostatic regulation of T cells or FRCs in the spleen.

We previously demonstrated that, by use of bone marrow chimera mice, hematopoietic SIRPα is likely important for maintenance of T cells in the spleen [26]. Indeed, the generation of stromal cells is thought to require interaction of hematopoietic cells with mesenchymal cells [3]. For instance, during the fetal development of the SLOs, $CD3^-CD4^+$ lymphoid tissue-inducer (LTi) cells interact with mesenchymal precursors to generate stromal cells [3]. LTi cells are also present in the adulthood SLOs and are implicated to be important for maintenance of the SLO organization [12]. Thus, loss or dysfunction of a certain type of hematopoietic cells, such as SIRPα-expressing DCs or LTi cells, might be a cause for the reduction of FRCs and T cells in the spleen of *Sirpa*$^{-/-}$ mice.

We here showed that *Sirpa*$^{-/-}$ mice displayed reduced expression of LTα, LTβ, and LIGHT in the spleen. Mice lacking LTα, LTβ, and LTβR revealed the small size of the white pulp of the spleen [1,7,30]. In addition, the expression of CCL21 was significantly decreased after treatment with antagonists for LTβR and LTβ in the spleen [23]. Thus, reduced expression levels of LTα, LTβ, and LIGHT were likely a cause for reduction of FRCs in the spleen of *Sirpa*$^{-/-}$ mice. LTα and LTβ are expressed on T cells and B cells, as well as on cDCs or LTi cells [12,16]. Of note, we previously showed that the mRNA expression levels of LTα and LTβ in isolated T cells or B cells isolated from SIRPα MT spleen did not differ from those of wild-type spleen [26]. Given that the number of T cells, but not B cells, was reduced in the spleen of *Sirpa*$^{-/-}$ mice, the reduction of LTα and LTβ expression was most likely attributable to the reduced number of T cells in the spleen of *Sirpa*$^{-/-}$ mice. Moreover, given the expression of LIGHT on T cells and DCs [20,29], the reduction of T cells and DCs may also reduce its expression in the spleen of *Sirpa*$^{-/-}$ mice. In addition, the reduced expression of LTα and LTβ in LTi cells might result in the generation of stromal cells such as FRCs in the spleen of these mutant mice. Further investigation is obviously required to determine the precise mechanism by which SIRPα regulates homeostasis of T cells and FRCs in the spleen.

ACKNOWLEDGEMENTS

We thank Y. Takase and D. Tanaka for technical assistance. This work was supported in part by a Grant-in-Aid for Scientific Research for Scientific Research (B) and (C) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan. None of the authors has any conflicts of interest or any financial ties to disclose.

REFERENCES

1. **Banks, T.A., Rouse, B.T., Kerley, M.K., Blair, P.J., Godfrey, V.L., Kuklin, N.A., Bouley, D.M., Thomas, J., Kanangat, S., and Mucenski, M.L.** 1995. Lymphotoxin-α-deficient mice. Effects on secondary lymphoid organ development and humoral immune responsiveness. J Immunol **155**: 1685–1693.
2. **Barclay, A.N., and van den Berg, T.K.** 2014. The interaction between signal regulatory protein alpha (SIRPα) and CD47: structure, function, and therapeutic target. Annu Rev Immunol **32**: 25–50.
3. **Brendolan, A., and Caamano, J.H.** 2012. Mesenchymal cell differentiation during lymph node organogenesis. Front Immunol **3**: 381.
4. **Bronte, V., and Pittet, M.J.** 2013. The spleen in local and systemic regulation of immunity. Immunity **39**: 806–818.
5. **Fasnacht, N., Huang, H.-Y., Koch, U., Favre, S., Auderset, F., Chai, Q., Onder, L., Kallert, S., Pinschewer, D.D., MacDonald, H.R., Tacchini-Cottier, F., Ludewig, B., Luther, S.A., and Radtke, F.** 2014. Specific fibroblastic niches in secondary lymphoid organs orchestrate distinct Notch-regulated immune responses. J Exp Med **211**: 2265–2279.
6. **Fletcher, A.L., Malhotra, D., Acton, S.E., Lukacs-Kornek, V., Bellemare-Pelletier, A., Curry, M., Armant, M., and Turley, S.J.** 2011. Reproducible isolation of lymph node stromal cells reveals site-dependent differences in fibroblastic reticular cells. Front Immunol **2**: 35.
7. **Fütterer, A., Mink, K., Luz, A., Kosco-Vilbois, M.H., and Pfeffer, K.** 1998. The lymphotoxin β receptor controls organogenesis and affinity maturation in peripheral lymphoid tissues. Immunity **9**: 59–70.
8. **Gunn, M.D., Ngo, V.N., Ansel, K.M., Ekland, E.H., Cyster, J.G., and Williams, L.T.** 1998. A B-cell-homing chemokine made in lymphoid follicles activates Burkitt's lymphoma receptor-1. Nature **391**: 799–803.
9. **den Haan, JM., Mebius, R.E., and Kraal, G.** 2012. Stromal cells of the mouse spleen. Front Immunol **3**: 201.
10. **Inagaki, K., Yamao, T., Noguchi, T., Matozaki, T., Fukunaga, K., Takada, T., Hosooka, T., Akira, S., and Kasuga, M.** 2000. SHPS-1 regulates integrin-mediated cytoskeletal reorganization and cell motility. EMBO J **19**: 6721–6731.
11. **Ishikawa-Sekigami, T., Kaneko, Y., Okazawa, H., Tomizawa, T., Okajo, J., Saito, Y., Okuzawa, C., Sugawara-Yokoo, M., Nishiyama, U., Ohnishi, H., Matozaki, T., and Nojima, Y.** 2006. SHPS-1 promotes the survival of circulating erythrocytes through inhibition of phagocytosis by splenic macrophages. Blood **107**: 341–348.
12. **Kim, M.-Y., McConnell, F.M., Gaspal, F.M.C., White, A., Glanville, S.H., Bekiaris, V., Walker, L.S.K., Caamano, J., Jenkinson, E., Anderson, G., and Lane, P.J.L.** 2007. Function of $CD4^+CD3^-$ cells in relation to B- and T-zone stroma in spleen. Blood **109**: 1602–1610.
13. **Link, A., Vogt, T.K., Favre, S., Britschgi, M.R., Acha-Orbea, H., Hinz, B., Cyster, J.G., and Luther, S.A.** 2007. Fibroblastic reticular cells in lymph nodes regulate the homeostasis of naive T cells. Nat Immunol **8**: 1255–1265.
14. **Lu, T.T., and Browning, J.L.** 2014. Role of the lymphotoxin/LIGHT system in the development and maintenance of reticular networks and vasculature in lymphoid tissues. Front Immunol **5**: 47.
15. **Luther, S.A., Tang, H.L., Hyman, P.L., Farr, A.G., and Cyster, J.G.** 2000. Coexpression of the chemokines ELC and SLC by T zone stromal cells and deletion of the ELC gene in the plt/plt mouse. Proc Natl Acad Sci U S A **97**: 12694–12699.
16. **Malhotra, D., Fletcher, A.L., Astarita, J., Lukacs-Kornek, V., Tayalia, P., Gonzalez, S.F., Elpek, K.G., Chang, S.K., Knoblich, K., Hemler, M.E., Brenner, M.B., Carroll, M.C., Mooney, D.J., Turley, S.J., and the immunological genome project consortium** 2012. Transcriptional profiling of stroma from inflamed and resting lymph nodes defines immunological hallmarks. Nat Immunol **13**: 499–510.
17. **Matozaki, T., Murata, Y., Okazawa, H., and Ohnishi, H.** 2009. Functions and molecular mechanisms of the CD47-SIRPα signalling pathway. Trends Cell Biol **19**: 72–80.
18. **Mebius, R.E., and Kraal, G.** 2005. Structure and function of the spleen. Nat Rev Immunol **5**: 606–616.
19. **Mildner, A., and Jung, S.** 2014. Development and function of dendritic cell subsets. Immunity **40**: 642–656.
20. **Morel, Y., Truneh, A., Sweet, R.W., Olive, D., and Costello, R.T.** 2001. The TNF superfamily members LIGHT and CD154 (CD40 Ligand) costimulate induction of dendritic cell maturation and elicit specific

CTL activity. J Immunol **167**: 2479–2486.

21. **Mueller, S.N., and Germain, R.N.** 2009. Stromal cell contributions to the homeostasis and functionality of the immune system. Nat Rev Immunol **9**: 618–629.
22. **Murata, Y., Kotani, T., Ohnishi, H., and Matozaki, T.** 2014. The CD47-SIRPα signalling system: Its physiological roles and therapeutic application. J Biochem **155**: 335–344.
23. **Ngo, V.N., Korner, H., Gunn, M.D., Schmidt, K.N., Riminton, D.S., Cooper, M.D., Browning, J.L., Sedgwick, J.D., and Cyster, J.G.** 1999. Lymphotoxin alpha/beta and tumor necrosis factor are required for stromal cell expression of homing chemokines in B and T cell areas of the spleen. J Exp Med **189**: 403–412
24. **Randall, T.D., Carragher, D.M., and Rangel-Moreno, J.** 2009. Development of secondary lymphoid organs. Annu Rev Immunol **26**: 627–650.
25. **Saito, Y., Iwamura, H., Kaneko, T., Ohnishi, H., Murata, Y., Okazawa, H., Kanazawa, Y., Sato-Hashimoto, M., Kobayashi, H., Oldenborg, P-A., Naito, M., Kaneko, Y., Nojima, Y., and Matozaki, T.** 2010. Regulation by SIRPα of dendritic cell homeostasis in lymphoid tissues. Blood **116**: 3517–3525.
26. **Sato-Hashimoto, M., Saito, Y., Ohnishi, H., Iwamura, H., Kanazawa, Y., Kaneko, T., Kusakari, S., Kotani, T., Mori, M., Murata, Y., Okazawa, H., Ware, CF., Oldenborg, P-A., Nojima, Y., and Matozaki, T.** 2011. Signal regulatory protein α regulates the homeostasis of T lymphocytes in the spleen. J Immunol **187**: 291–297.
27. **Siegert, S., and Luther, S.A.** 2012. Positive and negative regulation of T cell responses by fibroblastic reticular cells within paracortical regions of lymph nodes. Front Immunol **3**: 285.
28. **Surh, C.D., and Sprent, J.** 2008. Homeostasis of naive and memory T cells. Immunity **29**: 848–862.
29. **Tamada, K., Shimozaki, K., Chapoval, A.I., Zhai, Y., Su, J., Chen, S-F., Hsieh, S-L., Nagata, S., Ni, J., and Chen, L.** 2000. LIGHT, a TNF-like molecule, costimulates T cell proliferation and is required for dendritic cell-mediated allogeneic T cell response. J Immunol **164**: 4105–4110.
30. **De Togni, P., Goellner, J., Ruddle, N.H., Streeter, P.R., Fick, A., Mariathasan, S., Smith, S.C., Carlson, R., Shornick, L.P., and Strauss-Schoenberger, J.** 1994. Abnormal development of peripheral lymphoid organs in mice deficient in lymphotoxin. Science **264**: 703-707.
31. **Ware, CF.** 2008. Targeting lymphocyte activation through the lymphotoxin and LIGHT pathways. Immunol Rev **223**: 186–201.
32. **Washio, K., Kotani, T., Saito, Y., Respatika, D., Murata, Y., Kaneko, Y., Okazawa, H., Ohnishi, H., Fukunaga, A., Nishigori, C., and Matozaki, T.** 2015. Dendritic cell SIRPα regulates homeostasis of dendritic cells in lymphoid organs. Genes Cells **20**: 451–463.